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edited by

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To DR. CHARLES A. JANEWAY

Dr. Janeway, who was for many years Physician-in-Chief of the Children's Hospital Medical Center in Boston, and father of one of the editors of this book, died on May 28, 1981. His early studies on immunodeficiency diseases greatly expanded our understanding both of the physiological importance of an intact antibody response, and of the nature and complexity of the immunoglobulins. For his scientific contributions and for the personal inspiration he provided, his son would like to take this opportunity to give public thanks.

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PREFACE

In the early 1960s, Kunkel and Oudin independently described antigenic determinants unique to particular immunoglobulin molecules. These individually specific antigenic determinants have been termed idiotopes, and the collection of idiotopes found on a single immunoglobulin molecule the *idiotype*. Immunoglobulin idiotypes have interested immunologists for a variety of reasons. Since their initial description, they have been a principal probe for the structure of immunoglobulin variable or *V* regions where idiotypic determinants are located. As such, they have played a crucial role in the development of our ideas on immunoglobulin variability. They have also been used to map variable region genes of both heavy and light immunoglobulin chains and to determine their linkage to constant region genes. Idiotypic determinants found on a antigen-specific T cells or their secreted products that cross react with those present on Ig molecules have strongly suggested that T and B cells employ the same set of genes to make up their antigen-combining sites. In the early 1970s, Niels Jerne proposed that the immune system might employ idiotypes as targets for recognition by anti-idiotypic regulatory cells or molecules, thus using specific signals generated within the system for endogenous regulation. This idiotypic network hypothesis has greatly stimulated experimentation on the regulation of idiotype expression over the past decade. Thus, immunoglobulin idiotypes can serve as a focus for the study of immunoglobulin genes, their structure and their products, the nature of T cell receptors, and the regulation of the immune response. To examine these questions, an ICN-UCLA symposium on *Immunoglobulin Idiotypes and Their Expression* was held February 8–15, 1981. The proceedings of the symposium form the contents of this book.

Our understanding of the immune system has grown tremendously in the past twenty years since the clear enunciation of the principle that the information for an immune response was stored in a clonally distributed fashion

in individual small lymphocytes. The deciphering of the structure of immunoglobulins demonstrated clearly that each molecule had a unique structure, allowing it to bind to particular antigenic determinants and also to express particular idiotopes. Thus, the stage was set for the determination of the structure of the unique genes responsible for the generation of such a diverse family of molecules as well as for the understanding of the cellular organization of the immune system.

In early 1976, five years before the Salt Lake City symposium, Niels Jerne convened a small group of immunologists to discuss idiotypes. The transcript of this meeting has recently been published, and it presents a fascinating historical backdrop to our state of knowledge in 1981. At the outset of that meeting, Jerne enumerated fourteen questions to which he felt answers were needed in order to understand both the nature and the functional significance of immunoglobulin idiotypes. We feel that it would be worthwhile to reiterate those questions and then to present the questions that were relevant for consideration at the present symposium. In brief, the following were Jerne's fourteen questions:

1. What is the relationship of V domain primary structure, antigen binding specificity and idiotype? Do all antibody moleucles of a given specificity have the same idiotype, and do all idiotypically related molecules bind the same antigen?

2. How many V domains exist in one animal? Are there as many idiotypes as V domains?

3. Do two genetically identical individuals have the same set of idiotypes?

4. Do inherited differences in idiotype expression reflect the operation of regulatory or structural genes?

5. Are all antibody molecules also anti-idiotypes? If so, then all antigens should thus already be present in an animal as an internal idiotypic image.

6. If there is an idiotypic network, is it of functional significance to the animal, that is, is it essential to the functioning of the immune system?

7. Do T cells recognize idiotypes?

8. Do T cells bear endogenously produced idiotypes? Do T cells use VH but not VL genes to make their receptors?

9. What does anti-idiotype antibody do to the immune system, and by what mechanism?

10. If anti-idiotype affects the immune response, what concentrations are effective? Can autologous anti-idiotype antibodies affect the response?

11. What happens to the immune response when idiotype is injected?

12. Are the inherited, dominant idiotypes studied by many investigators special cases, or are they typical? Can we draw general conclusions from results obtained in such systems?

13. Are some cases of idiotype suppression due to suppressor T cells specific for the idiotype? Are all cases due to this?

14. If the T cell can recognize idiotype, does it do so in association with MHC gene products, and of which type?

The discussion at this 1976 meeting is fascinating to read in that little information was available to the participants with which to answer these difficult questions. In the next five years, answers to some of them have emerged quite clearly, while others remain essentially unresolved. For instance, it is now clear that antigen binding specificity and idiotype are *not* synonymous. A clear example of this is shown graphically by Michael Potter in his introductory chapter on the structure of idiotypes. We have the advantage of hindsight in reading this discussion of five years ago, and perhaps from our perspective, some of the questions seem naive. At the time, they were merely issues to be discussed and later tested in the laboratory. The fruits of such discussions are reflected in many instances in the papers forming the subsequent chapters of this book.

During the intervening five years, three similarly named technological advances in three distinct areas have greatly increased both the experimental possibilities of immunology and our concept of the functioning of the genes and cells of the immune system. All three advances involve cloning: the cloning in plasmids and phages of cDNA and genomic DNA, allowing a detailed characterization of the organization of immunoglobulin genes; the cloning of B cells, first as plasmacytomas induced experimentally by Potter and, more recently, as specific hybrids between nonsecreting myeloma cells and antibody forming cells (hybridomas) by Milstein and Kohler; and the cloning of T lymphocytes, either as long-term nontransformed lines or as hybrids formed by fusion with T lymphoma cells. These technologies have had an immense impact on the level of resolution of both the questions that can be asked and the type of answers obtainable as is clearly evident in the pages that follow.

In this book, certain questions regarding the immune system in general and idiotypes in particular are addressed and in some cases given at least partial answers. The book is organized into three main substantive sections:

Immunoglobulin Genes and Their Products: The Molecular Bases of Idiotypy; T Cell Receptors; Regulation of the Immune Response: Selective Influences on Idiotype Expression. A fourth part consists of summary chapters by four rapporteurs who attended all the working sessions, and a historical perspective on changing paradigms in immunology. Finally, an appendix records summaries of workshops on particular idiotypic systems which are frequently employed experimentally.

In particular, these articles deal with a new but overlapping set of fourteen questions:

1. What is the structure of the genes that encode immunoglobulin molecules?

2. Can all the diversity observed at the protein level be accounted for by information encoded in the germ line, or does somatic diversification account for a substantial portion of the observed diversity?

3. What is the structure of the related genes encoding major histocompatibility antigens?

4. What regulates the rearrangement of immunoglobulin genes leading to the phenomena of allelic exclusion and isotype switching?

5. What is the precise structure of an idiotype?

6. What is the genetic origin of both the variable and constant portions of antigen-specific receptors expressed by T cells?

7. What is the structure of the T cell receptor, and are T cell receptors and T cell factors related to one another?

8. Do different types of T cells express distinct classes of receptors akin to immunoglobulin isotypes?

9. What are the sets of cells and their secreted products that can be defined as regulating immune responses? Are certain T cell interactions restricted by Ig gene products and others by MHC gene products?

10. What is the relative importance of each of the various regulatory cells in a particular response?

11. Are certain idiotope-recognizing cells MHC-restricted and others not MHC-restricted? What are their respective roles?

12. Is idiotype expression regulated independently of the total, antigen specific response, and what mechanisms are involved in such regulation?

13. Is isotype regulation, in broad outline, comparable to predominant idiotype regulation?

14. Are all idiotopes regulated within the idiotypic network, and how important are these regulatory events in the regulation of the response as a whole?

Many other questions were raised during the course of this symposium, and it is probably safe to say that none of these questions has been conclusively answered. Particularly resistant to answer are the questions on allelic exclusion, the nature of the T cell receptor, and questions about the relative importance of the individual, defined, regulatory pathways. Nevertheless, a great deal of new information is presented in this volume, and it represents a thorough treatment of the problem of idiotypy at the present time. We believe that it will serve as a useful introduction to idiotypes and to many other areas of basic immunological research for those with some knowledge of the field.

We would like to thank all the symposium participants for their contributions to this book. In addition to the authors of the following articles, many other participants gave freely of their time, energy, and ideas, which are contained in summaries of the various working sessions.

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C. A. Janeway, Jr.

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Structure of Idiotypes and Idiotopes

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History and Definitions

Slater, Ward and Kunkel (1) in 1955 first described individual antigenic specificities (IAS) of human myeloma proteins. In 1963 Kunkel *et al.* (2) and Oudin and Michel (3) showed that human blood group and rabbit *S. typhi* antibodies respectively also had IAS. In both situations the antigenic specificities were unique to the antibodies of the individuals from which they were derived. Kunkel *et al.* (2) examined a number of blood group antibodies from different individuals with the hope of finding IAS in homologous antibodies of other individuals but none were found. In 1968 Williams *et al.* (4) described for the first time cross specific antigenic specificities in human monoclonal cold agglutinins using heterologous antisera. Oudin and Michel (5,6) extended their studies on rabbit anti-rabbit antibodies and coined the term idiotypy to describe unique antigenic properties of antibodies of an individual rabbit. The *S. typhi* antibodies in one rabbit could be distinguished serologically from *S. typhi* antibodies induced in another rabbit. Further the population of antibodies with its unique antigenic properties could even disappear from that rabbit and be replaced by new antibodies with similar binding activity that lacked the IAS.

Individual antigenic specificites were found in mouse myeloma proteins (7). Iverson (8) showed that the individual specificity of the C3H X5563 myeloma protein was also in normal CBA serum, but this signal observation was not immediately appreciated because no function had been associated with X5563. With the exception of the human cold agglutinins individual antigenic specificities or

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idiotypes were peculiarities of homogeneous immunoglobulins. With the availability of antigen binding myeloma proteins in inbred mice (see 9 for refs) antigenic relationships were established for the first time between antigen binding myeloma proteins and antibodies with the same binding specificity. Five phosphorylcholine binding myeloma proteins were found to share a common IAS (10). Then Blomberg et al. (11), Sher and Cohn (12) and Cosenza and Kohler (13) independently showed that idiotypes associated with α 1,3 dextran (11) phosphorylcholine (12,13) binding myeloma proteins respectively could also be found in antibodies of the same binding specificity. Cross-specific idiotypes (IdX) were also found in association with induced antibodies to azophenyl arsonate (ARS) (14) streptococcal A polysaccharide A5A (15) and NIP (16) 3-nitro-4-hydroxy-5-nitrophenyl acetyl-substituted proteins. These immunizations were characterized by the emergence of dominant clonotypes. IdX idiotypes became highly useful genetic markers when it was discovered that genes linked to the polymorphic Igh complex locus determined the expression of IdX (see 17, 18 for refs.) in different strains of mice.

An important aspect of the IdX systems in the mouse was the availability of antigen binding myeloma proteins that carried the cross-reactive idiotypes. These homogeneous immunoglobulins were available in sufficient quantity for amino acid sequencing (19-23) and have provided the basis for determining the structures of idiotopes. The hybridoma technology of Kohler and Milstein (24) has now provided an unlimited source of monoclonal antibodies for determining the structural basis of idiotypes. Structural studies of monoclonal immunoglobulins associated with IdX idiotypes has revealed that immunoglobulins bearing IdX idiotypes are closely related to each other, i.e., their respective V_L and V_H regions are derived from the same V_L and V_H subgroups.

These sequences then show that in most responses where characterisic IdX's are expressed, only a few clonotypes participate in many of the responses, or in the more heterogenous responses, dominant clonotypes appear with regularity. In the mouse then, specific clonotypes are generated with physiologic regularity implying that these immune responses depend upon the exclusive activation of specific V_L and V_H regions in the same cell.

The term idiotype has been increasingly associated with cross-specific idiotypes or IdX, however, the individual antigenic specificities (IAS or IdI) of homogenous immunoglobulins are still called idotypes. Since IAS or IdI may be located anywhere on the Fv, including non antigen binding site regions, the term idiotype becomes difficult to define. One might be tempted to define an idiotype as any antigenic specificity recognized by a homolgous antiserum that can be assigned to the Fv - but there are exceptions. Most immunologists do not consider the "a" allotypes of rabbits which are on Fv, as idiotypes because these antigenic determinants are found on many different kinds of antibodies. Idiotypes are antigenic specificities that are associated with a single immunoglobulin or a group of very closely related immunoglobulins that have a defined antigen or hapten binding specificity. The term idiotype should usually be preceded by a qualifying prefix, e.g., ARS-CRI (or IdX), T15IdX, T601-X24 IdX, X24 IdI (or X24 IAS) to indicate the monoclonal source of the idiotype, i.e., the name antigen binding myeloma protein or monoclonal antibody, or the nature of the binding specificity. Thus each idiotype is defined by a set of immunochemical criteria. The idiotypic specificity may or may not be involved with the antigen binding function. It need only be found in association with an antigen binding activity. This preserves Oudin's original definition.

Relationship of Idiotypes to Immunoglobulin Structure

The great sensitivity and specificity of serological methods for detecting small amounts of protein carrying an idiotype has made anti-idiotypic antibodies of extraordinary value in detecting the expression of specific immunoglobulin V-regions. The detection of a reactive substance with an anti-idiotypic antibody has sometimes been offered as evidence that a specific Ig-V-region is present. In this context it is probably important to draw such conclusions from a more detailed characterization of the anti-idiotypic antibody and its epitope. The term idiotope is relevant to this discussion. An idiotope can be generally defined as the topographical part of the immunoglobulin molecule that is contacted by an anti-idiotypic antibody. Hypothetically some idiotopes could be confined to V_L or V_H or to framework or to Wu-Kabat antigen complementarity determining regions (CDR), but others may span V_L and V_H structures or require the presence of both for structural stability. Further idiotopes may be derived from different exons (V_L, J_L, V_H, D, or J_H). The demarcation zones of antigen binding site associated structures from framework are not clearly defined, rather they blend into each other. Thus it could not be expected an anti-idiotypic antibody would bind to only antigen binding site structures. Very little is known about the structure of idiotopes, but the availability of groups of closely related immunoglobulins with common antigen or hapten binding specificities provides a basis for more precise definition. As will be discussed, models of immunoglobulin Fv that permit visualization of the surface topography are an invaluable aid to the study of idiotopes.

Models

The three dimensional structure of immunoglobulins has been elucidated from x-ray crystallographic studies (for refs. see 25). To date only 2 Fab fragments have been determined. One of these, the McPC603 is from a

BALB/c mouse phosphorylcholine binding myeloma protein (26) the other from a human myeloma protein (27). Padlan and Davies (28) demonstrated that the mouse (McPC603) and the human (REI) V_K domains had an extraordinarily close domain architecture despite the fact that the two genes controlling these domains have been separated in different species for over 80 million years. A similar conformational homology has been found in the α-carbon skeletons of a human and mouse V_H domain as well. In both cases when the two domains are superimposed in space most of the framework portions of the domain closely coincide. Domains from different immunoglobulins may differ, from each other in one or more of three secondary structures that interface with solvent, (the Wu-Kabat complementarity determining regions, CDR) (29). For the most part CDR's are bends or loops that connect beta strands. They vary in length and secondary structure. Immunologists faced with the problems of describing thousands of immunoglobulins have a need for constructing hypothetical models for purposes of examining the spatial relationships of amino acid side chains in the antigen binding sites. We describe here a method of model building that is largely carried out on computers. These models use the basic framework of McPC603, but fabricate parts of some of the CDR regions. The models are displayed on the Evans and Sutherland picture system in either the classic Kendrew stick form, or in a space filled form, devised by one of us (30). In the space filled model the atoms were represented by spheres that corresponded to the respective Van der Waals radii (30). Both can be photographed and viewed in stereo. These models are hypothetical and their chief value is derived from establishing spatial relationships of amino acid side chains to each other, and gross contour topology of the molecular surface. They can also be used with some reservations in locating hapten binding sites when sufficient supportive immunochemical data is available.

Topology of space filled McPC603 Fv

The McPC603 spacefilled model is based on X-ray data, and coordinates kindly supplied to us by Dr. David R. Davies. These coordinates are currently being further refined. The space filled model of McPC603 closely represents relationships in the McPC603 X-ray structure as no changes have been intentionally made.

The Fv is formed by the intimate union of the V_L and V_H domains, and is connected to C_L and C_H1 through its J_L and J_H segments. Five exons code for Fv, in the V_L domain: V_L, J_L,; in the V_H domain: V_H, D and J_H. The Fv which is a somewhat elliptical globular mass, will be viewed and described from different perspectives, frontal (surface facing the solvent, most distal from the C_L and C_H); top surface when the Fv is rotated 90° down; and bottom surface when the Fv is rotated 90° up. The front, top and bottom perspectives reveal 3 respective surfaces. Most antigen binding studies thus far indicate antigens or hapten binding sites are associated with the frontal surface. A cleavage zone formed along the V_L-V_H interface traverses the frontal surface at an angle of about 45%. The Wu-Kabat CDR-regions occupy a large portion of the frontal surface and smaller parts of the top and bottom surfaces. The surfaces present their own contoured planes with cavitary and groovelike depressions. These are most pronounced on the frontal surface, but do not exclude possibilities for antigen binding on other surfaces, even though supportive data is not available. A set of pictures of McPC603 Fv have been submitted for publication (31), a black and white version is shown in Fig. 1.

J and D Segments

The J_L joins the V_L in the interior of the Fv. The junction amino acids are barely visible from the frontal surface. The J_K segment courses towards the C_L on the bottom surface forming tight interactions with neighboring β -structures. In McPC603 the V_H joins D at the top surface, then loops forward, about in the middle, and then turns downward and under, twists, and courses toward C_H1 along the top surface. In McPC603, the D

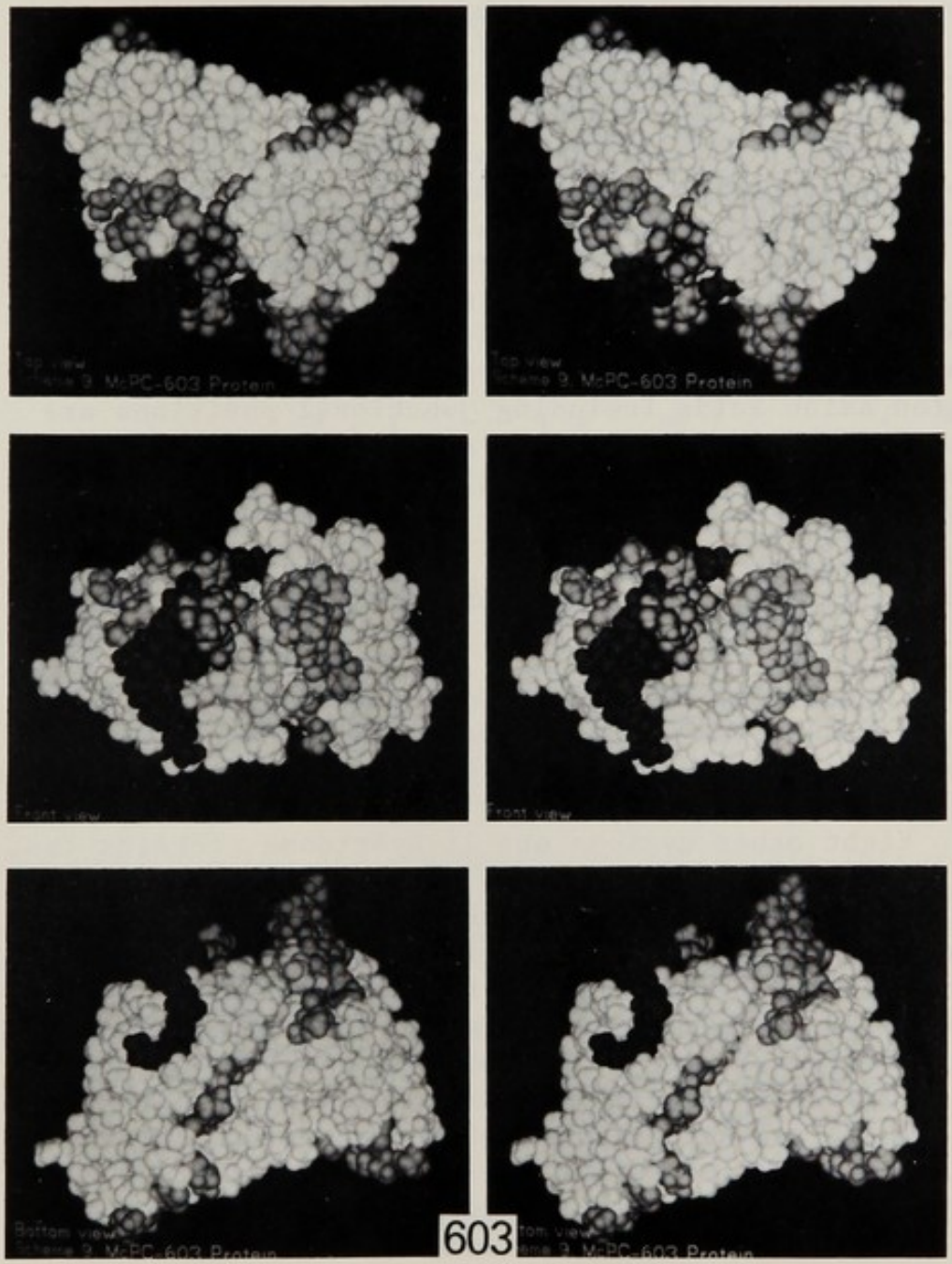


Fig. 1. Stereopictures of McPC603, show three views from top to bottom: top, front and bottom. Shaded amino acids are Wu-Kabat CDR regions: CDR1V_L=L_1 CDR1,2,3 V_H = H1, H2 and H3, and J and D-regions.

region is a twisted loop that has close interactions with CDR-1 V_H. The J_HD and D-V_H junctions are visible on the top and frontal surfaces respectively. The joining of D with CDR-1 V_L forms a cavity on the front surface. This cavity lined externally by V_H amino acids contains most of the amino acids that interact with phosphorylcholine. It may be inferred that the V_L participates in forming and stabilizing the cavitary phosphorylcholine antigen binding site. Amino acid side chains of the J_H and J_L regions are for the most part exposed to the top and bottom surfaces. D-region amino acids including junctional positions are located on the frontal and top surfaces. Thus both J and D can contribute to the antigenicity (idiotypy) of an Fv.

Hypothetical Model Building of Galactan Binding Myeloma Proteins

Our laboratory has been studying for some time the β-1,6-D-galactan binding myeloma proteins GALBMP (32-38). Although one of these proteins (J539) has been crystallized, a high resolution structure has not yet been determined (39). Eight other myeloma and 11 hybridoma proteins that bind β1,6 D-galactan containing antigens are currently in our collection. All of these proteins so far studied by complete or partial amino acid sequence analysis have V_K4 or VH1B regions indicating they are controlled by the same or very closely related V-genes (21,23) (Table 1). The association constants for a number of β 1,6 D-galactans e.g., β-methyl D-galactoside, Gal_2, Gal_3 and others have been determined for most of the proteins (35-36). All bind these haptens, but the binding constants range from 5×10^4 to 1×10^5 Mol^{-1}/L (34,35,38).

Model Building of J539

Essentially in the method to be outlined, the J539 Fv or any of the structurally similar GALBMP is made from a primary structure by modifying the X-ray determined McPC603 structure (31). Each new Fv to be made requires special assumptions and steps. The steps used in making the J539 are:

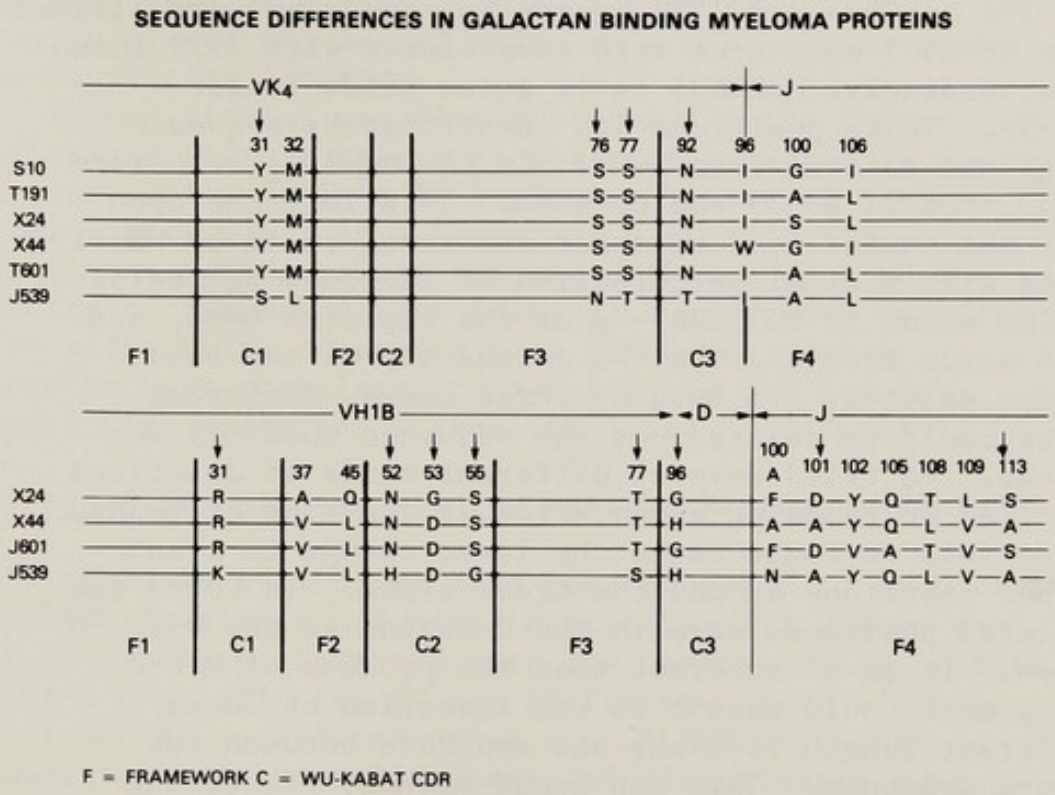


TABLE 1. Amino acids are designated by the single letter code. Arrows indicate amino acids that are located on the surface of the molecule, the sequences were determined by Dr. S. Rudikoff and N. Rao (21,23).

1) The McPC603 and J539 V_L and V_H sequences were aligned and the McPC603 sequences were substituted with J539 amino acids accordingly. McPC603 is 11 amino acids longer than J539. These positions are identified by sequence homology and deleted from M603. In the M603 V_L structure the loop from 31 to 39 was deleted. This left the open ends of the chain close together and these could be easily rejoined with minimal perturbation of surrounding residues. Two amino acids in 603 CDR2-V_H at the tip of a bend, and 2 amino acids from 603 CDR3-V_H at the tip of the bend, were also deleted. In both of these cases, different residues could be deleted, as the sequence homology was not clear. We tried several different kinds of deletions but settled on removing amino acids at the tips of bends. By doing this we could cause the least change in α and β carbon positions around the break sites. In CDR-3 the two deleted positions were in the D-region at the D-J junction. It is of interest that the process of V-D-J rearrangement could result in the insertion of loops of different length (1-14aa) and sructure between two framework segments. This can occur without disrupting the folding of V_H or its ability to interact with V_L to form the Fv.

2) A 15 Å to 20 Å radius contained around the deletion site was visualized in 3-dimensions on the Evans and Sutherland picture system. The amino acids including side chains were in the Kendrew stick form. The amide planes of amino acids contiguous to the break points were differentially rotated by moving separate controls, one for each plane. When the phi angle was rotated positively the psi angle was rotated negatively. This movement allowed minimal perturbation of the adjoining structure and permitted rejoining of the broken ends. The joining required a series of trials moving usually one to three amide planes on one or both sides of the break. The changes in all positions were recorded. The open ends were placed 1.4 Å apart in the same plane.

When all rejoinings were complete the new structure was refined by a computer program that one by one searched the space around each amino acid side chain, and placed the respective side chain in a location where the least

contacts were made with adjoining side chains (31). This program placed all but 12 amino acid side chains in a non-contacting location.

Hapten fitting was also performed in the Evans-Sutherland picture system by moving the hapten in various positions in a 20 Å global display of the binding site (31). In a number of hapten fitting experiments evidence was presented that supported the fitting of oxygens of the terminal sugar close to amino acid side chains emanating from CDR-2 V_H. Other contacts were postulated to involve the 3rd and 4th sugars with amino acids from CDR-1 and 3 of V_L (41).

Idiotypes of Galactan Binding Myeloma Proteins (GALBMP)

Idiotypic antibodies have been prepared to each of the 8 GALBMP in strain A/J mice. A specific RIA system was set up for each antiserum with its corresponding protein, using a high dilution of the antiserum. Each of the other GALBMP as well as other non GALBMP were used as competitors in the solid phase radioimmunoassay system. As may be seen (Table 2) many of the sera were absolutely specific for the immunizing protein and none of the other proteins competed at 1 µg/20 λ . The A/J anti T601 was not specific and cross-reacted with S10, X24 and X44. Some of these antisera were used in cross-specific assays where the target was a GALBMP not used in the immunization. One IdX was identified in which S10, X24, X44 and T601 competed.

X24 Individual Idiotope

Strain A/J mice immunized with the X24 BALB/c myeloma protein produce antibodies that are specific for X24 (23,24). These specific antibodies are detected by an RIA system using X24 as the target (37). X24 at 10 ng/20 λ gave 50% control binding (Table 2), while all the other GALBMP tested failed to compete in this system at over 1000 x this concentration (37). This indicates the extraordinary specificity of the antiserum raised by conventional methods. The only other source of X24 IdI that has been found is in galactan antibodies raised to β 1,6 D-galactan containing polysaccharides, e.g., gum ghatti,

ANTIGENIC SPECIFICITIES OF GALACTAN BINDING HOMOGENEOUS IMMUNOGLOBINS

Idiotypic antibody to:/target	ng of homogeneous galactan binding immunoglobulin that gives 50% control binding in RIA							
	J1	C4	S10	X24	X44	T191	J539	T601
J1/J1	(5)	–	–	–	–	–	–	–
C4/C4	–	(20)	–	–	–	–	–	–
S10/S10	–	–	(6)	–	–	–	–	[410]
X24/X24	–	–	–	(10)	–	–	–	–
X44/X44	–	–	–	–	(7)	–	–	–
T191/T191	–	–	–	–	–	(25)	–	–
J539/J539	–	–	–	–	–	–	(3)	–
T601/T601	–	–	[100]	[50]	[150]	–	–	(6)
T601/24	–	–	[800]	[14]	[40]	–	–	[3]

TABLE 2. The dashed line indicates no competition with 1000 ng of indicated competitor.

Larchwood arabinogalactan (37). The X24 idiotope can be localized by comparing the amino acid sequences of X24 with the other GALBMP that fail to compete in the α X24:X24 system (Table 2). The closest related protein by sequence with which the anti X24 does not react is T601 (Table 3) which differs from X24 at 5 positions, one in J_K, 3 in the J_H and one (position 53) in CDR2-V_H. The J region amino acids are not exposed to the surface of the molecule, strongly suggesting that position 53 in CDR2 V_H, which is Asp in T601 and Gly in X24 is a major structural influence in the X24 individual epitope. In the 3-dimensional space filled model, the section of CDR2 position 53 is a protruding pyramidal structure that juts out of the frontal surface (Figure 2). The The interdomain cleft is on the V_K side of this structure. The anti X24 IdI probably has a complementarity cavity-like structure that fits over CDR2-V_H. A roughly similarly shaped structure is found in T601 that differs only by having a negatively charged aspartic acid. Thus anti X24 antibody probably physically interacts with both X24 and T601 molecules but is repelled by the prominent negative charge in T601. It could be argued though that the glycine in X24 is associated with a considerable modification in the secondary structure of this region and that this modifies the shape of CDR2 so that only the fit with α X24 and X24 is possible.

It is imagined that the anti idiotypic antibody interacts more extensively over the peripheral idiotope environment. Data supporting this contention is given by Pawlita et al. (41) in an analysis of a monoclonal anti X24 antibody. presented at this meeting. The data indicates the monoclonal anti X24 has a second set of interactions with a sub-region that is common to both X24, X44 and T601. When the RIA system is set up using X24 as the target with the monoclonal antibody X44 can compete at a concentration around 1000 ng/20 λ . The binding to X44 is strong enough to permit the formation of an αX24:X44 system. Now X44 and X24 strongly compete, T601 less so and J539 not at all. The evidence suggests the monoclonal anti X24 interacts not only with the CDR-2 structure but additional amino acids that are common to X24, X44 and T601 (possibly the region around Arg 31 that is about 16 Å distant from Ser 54). This evidence suggests the idiotope of X24 is much larger than the CDR-2 structure.

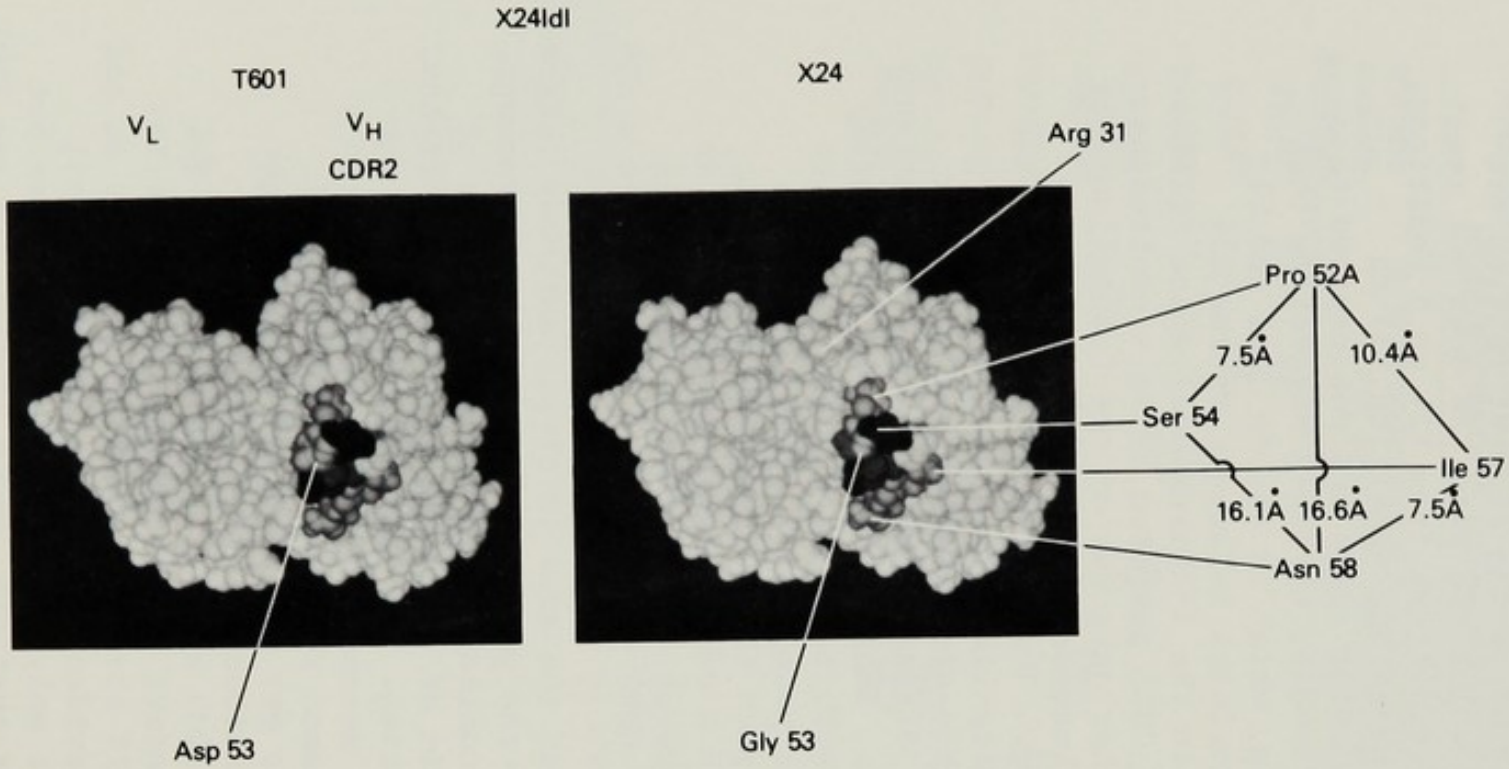
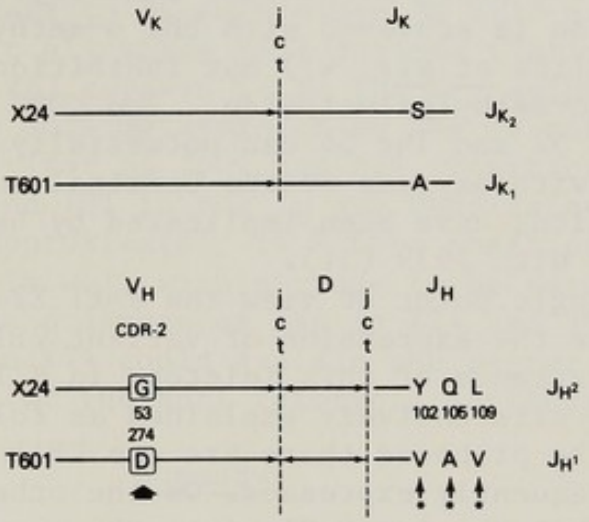


Fig. 2. Stereopictures showing the idiotope (shaded) defined by A/J anti X24 serum. The same views of T601 (left) and X24 (right) are shown. When viewed with a stereoscope such as the model CF-8 Abrams Instrument Corp., Lansing, MI, all atoms will be the same in the two proteins except position 53. By moving the eyes back and forth from right to left with the stereoscope stationary the additional Asp atoms will appear and disappear. Arg 31 is away from Ser 34, other distances are shown on the right.

AMINO ACID SEQUENCE DIFFERENCES BETWEEN X24 AND T601 GALBMP



α X24:X24 inhibited by hapten.
X24 Id I found in normal galactan antibodies.
* = Internal

TABLE 3

X44 Id I

	V_H CDR-1	V_H CDR-2	D	J_H		X44 Id I
T601	R	N D S	G Y Y G	Y F D V A T S	J_{H^1}	–
X44	R	N D S	H Y Y G	Y A A Y Q L A	J_{H^3}	+
J539	K	H D G	H Y Y G	Y N A Y Q L A	J_{H^3}	–
	31	52 55	96	101		

α X44:X44 is hapten inhibitable.
X44 Id I is not found in normal α galactanantibodies.

TABLE 4

Both the conventional and monoclonal X24 IdI antibody interactions with X24 can be inhibited by haptens, and can be quantitated using monoclonal antibody. The most potent inhibition is achieved with the o-methyl trisaccharide (Pawlita et al., 41) but inhibition can be obtained with βo-methylgalactoside. Two CDR2 V_H residues on Asn 52 and Thr 56 can potentially make hydrogen bonds with oxygens of the terminal sugar. These two positions have been implicated by hapten binding studies with J539 (31).

From a biologic point of view the anti X24 idiotopic antibody detects the expression of variant VH1B structure. The regular occurrence of this idiotope in β 1,6 D-galactan antibody can be alternatively explained as follows: first it could be proposed there are two VH1B genes, the T601 is more frequently expressed. On the other hand it may be argued that the Asp → Gly interchange at position 53 V_H in X24 results from a somatic mutation, that is effectively selected for in immune responses to galactan.

X44 IdI

The X44 IdI is more complex than the X24 IdI. It has not yet been detected in conventionally induced galactan antibodies. It does appear to be associated with the binding site as hapten inhibits the conventional αX44:X44 IdI system (37). The complexity of this idiotype is encountered when the X44 sequence is compared with the 3 other sequences (X24, T601 and J539) that do not compete in the anti X44:X44 RIA system. First no external amino acid distinguished X44 from the others and thus it must be assumed that the X44 IdI is determined by combinations of amino acids (Table 4). These amino acids appear to be located in CDR2 V_H, CDR1 V_H and the D and J regions. The specific amino acids implicated are Arg 31 (of VH1B origin), His 96 (of D-region origin) and Ala 101 (of J_H3 origin). Possibly Asn 52 and Ser 55 of VH1B origin are also involved. Ala 101, His 96 and Arg 31 are close enough to each other (from 8-14 Å) to form the main part of the idiotope. The CDR-2 amino acids could comprise a second subsite.

T601-X24-X44 IdX

This IdX appears to be determined by VH1B amino acids that are common to all three VH1B regions (Table 1). Arg 31 (in CDR-2 V_H) appears to be the key amino acid but cannot be the sole determinant as X44 is not as effective a competitor as T601 and X24. Positions 96 in the D region and 101 in the J_H are candidates. Position 101 is approximately 14 Å from position 31 and therefore is well within range to be part of the idiotope. The presence of Asp in X24 and T601 contrast to Ala in X44 could explain the better affinity of X24 and T601, for the anti idiotypic antibody.

Summary

This paper describes topographical space filled models of variable fragments (Fv) of the phosphorylcholine binding myeloma protein McPC603 and hypothetical models of two galactan binding myeloma proteins X24 and T601. These were used to locate idiotopes associated with galactan binding myeloma proteins. Although this work is preliminary it does indicate the localization of idiotopes is complex and requires a series of closely related protein structures as well as topographical models.

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REFERENCES

1. Slater, R.J., Ward, S.M., and Kunkel, H.G. (1955) J. Exp. Med. 101, 85.

2. Kunkel, H.G., Mannik, M., and Williams, R.C. (1963) Science 140, 1218.

3. Oudin, J., and Michel, M. (1963) C.R. Acad. Sci. Paris 257, 805.

4. Williams, R.C., Kunkel, H.G., and Capra, J.D. (1968) Science (Wash. DC) 161, 379.

5. Oudin, J., and Michel, M. (1969) J. Exp. Med. 130, 595.

6. Oudin, J., and Michel, M. (1969) J. Exp. Med. 130, 619.

7. Potter, M., Lieberman, R., and Dray, S. (1966) J. Mol. Biol. 16, 334.

8. Iverson, G.M. (1970) Nature 227, 273.

9. Potter, M. (1977) Adv. Immunol. 25, 141.

10. Potter, M., and Lieberman, R. (1970) J. Exp. Med. 132, 737.

11. Blomberg, B., Geckler, W.R., and Weigert, M. (1972) Science 177, 178.

12. Sher, A. and Cohn, (1972) Eur. J. Immunol. 2, 319.

13. Cosenza, H., and Kohler, H. (1972) Science 176, 1027.

14. Kuettner, M.E., Wang, A.L., and Nisonoff, A. (1972) J. Exp. Med. 135, 579.

15. Eichmann, K. (1973) J. Exp. Med. 137, 603.

16. Imanishi, T., and Makela, O. (1974) J. Exp. Med. 140, 1498.

17. Eichmann, K. (1975) Immunogenetics 2, 491.

18. Weigert, M., and Riblet, R. (1978) Springer Semin. Immunopathol. 1, 133.

19. Rudikoff, S., and Potter, M. (1976) Proc. Natl. Acad. Sci. USA 73, 2109.

20. Vrana, M., Rudikoff, S., and Potter, M. (1978) Proc. Natl. Acad. Sci. USA 75, 1957.

21. Rao, D.N., Rudikoff, S., Krutzsch, H., and Potter, M. (1979) Proc. Natl. Acad. Sci. USA 76, 2890.

22. Vrana, M., Rudikoff, S., and Potter, M. (1979) J. Immunol. 122, 1905.

23. Rudikoff, S., Rao, D.N., Glaudemans, C.P.J., and Potter, M. (1980) Proc. Natl. Acad. Sci. USA 77, 4270.

24. Kohler, G., and Milstein, C. (1975) Nature (London) 256, 495.

25. Davies, D.R., Padlan, E.A., and Segal, D.M., (1975) Ann. Rev. Biochem. 44, 639.

26. Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M., and Davies, D.R. (1974) Proc. Natl. Acad. Sci. USA 71, 4298.

27. Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerly, R.P., and Saul, F. (1973) Proc. Natl. Acad. Sci. USA 70, 3305.

28. Padlan, E.A., and Davies, D.R. (1975) Proc. Natl. Acad. Sci. USA 72, 819.

29. Kabat, E.A., Wu, T.T., and Bilofsky, H. (1979) Sequences of Immunoglobulin Chains, NIH Publication No. 80-2008.

30. Feldmann, R.J., Bing, D.H., Furie, B.C., and Furie, B. (1978) Proc. Natl. Acad. Sci. USA 75, 5409.

31. Feldmann, R.J., Potter, M., and Glaudemans, C.P.J. (1981) Mol. Immunol., in press.

32. Potter, M., Mushinski, E.B., and Glaudemans, C.P.J. (1972) J. Immunol. 108, 295.

33. Rudikoff, S., Mushinski, E.B., Potter, M., Glaudemans, C.P.J., and Jolley, M.E. (1973) J. Exp. Med. 138, 1095.

34. Jolley, M.E., Rudikoff, S., Potter, M., and Glaudemans, C.P.J. (1973) Biochemistry 12, 3039.

35. Jolley, M.E., Glaudemans, C.P.J., Rudikoff, S., and Potter, M. (1974) Biochemistry 13, 3179.

36. Mushinski, E.B., and Potter, M. (1977) J. Immunol. 119, 1888.

37. Potter, M., Mushinski, E.B., Rudikoff, S., Glaudemans, C.P.J., Padlan, E.A., and Davies, D.R. (1979) Ann. Immunol. (Inst. Pasteur) 130C, 163.

38. Manjula, B.N., Glaudemans, C.P.J., Mushinski, E.B., and Potter, M. (1976) Proc. Natl. Acad. Sci. USA 73, 932.

39. Navia, M.A., Segal, D.M., Padlan, E.A., Davies, D.R., Rao, N., Rudikoff, S., and Potter, M. (1979) Proc. Natl. Acad. Sci. USA 76, 4071.

40. Pawlita, M., Mushinski, E.B., Potter, M., unpublished observations.

GAPS IN OUR UNDERSTANDING OF HOW ANTIBODY SPECIFICITY AND COMPLEMENTARITY ARE GENERATED

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Understanding antibody specificity at the structural and the genetic level is one of the major problems of this era. From the immunochemical viewpoint, one would like to know at sufficiently high resolution how various antigenic determinants fit into their antibody combining sites; the goodness of fit; the shapes that antibody combining sites may have; how cross reactive determinants fit; the contributions of L and H chains to the binding, whether conformational changes elsewhere alter site complementarity and whether grossly unrelated structures can fit. Unfortunately, despite the existence of six X-ray crystallographic structures (1-7, see 8) on Fab fragments of myeloma proteins and on V_L and Fv dimers of Bence Jones proteins, in no instance has a ligand which fills the site completely been found. This is due largely to the difficulty of determining the precise specificity of myeloma proteins with antibody activity. However, the X-ray crystallographic data have clearly confirmed our prediction (9, 10) from a statistical analysis of the variability of amino acid sequence at each position, that antibody combining sites would be formed by the three hypervariable regions of the light and heavy chains, now called complementarity determining regions (CDR) (8, 11), with the remaining segments forming a framework (FR), and the possibility indicated (9) "that binding could be influenced to some extent by different residues adjacent to a site but not in themselves complementarity determining". To the extent that ligands are available which partially fill the site, the X-ray data show that they interact with residues in the CDR of H and of L chains (1-8) and affinity labeling studies support this (see 12). These findings have led to model building studies in which attempts were made to construct antibody combining sites by replacing the CDR's of antibodies of known specificity on the FR's of those proteins whose X-ray structure had been determined (13-16). In one instance (13), introduction of the sequence of

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CDR's of type III anti-pneumococcal antibody on to the FR of McPC603, a myeloma protein with a cavity-type site binding phosphorylcholine (2, 3) converted it into a groove-type site complementary to a hexasaccharide, type III pneumococcal polysaccharide, in good agreement with earlier immunochemical mapping of the site (17). Two attempts were made to construct a site specific for ε-DNP lysine in a similar manner (15, 16) one also incorporating NMR data; both are very informative in that noncovalent bonding to various CDR residues makes for reasonable fitting; nevertheless, the orientation of the ε-DNP lysine in the two models is essentially opposite (cf. 8). Similar efforts are being made to construct models of the myeloma antibodies with specificity for β1→6 galactans and two models have been proposed (18). In all these instances substitution of each CDR on the framework has been limited to using CDR segments of equal length and to preserving the secondary structure. This is necessary at the present level of understanding but such modeling studies have a severe limitation in that substantial length variations in CDR3 of V_H and of CDR1 of V_L occur frequently and contribute importantly to the generation of antibody complementarity; variations in length of other CDR and FR segments are also seen which again may influence site complementarity and greatly increase the size of the repertoire of antibody combining sites.

In the absence of X-ray crystallographic or NMR data, insight into the sizes and shapes of the combining sites of antibodies and myeloma proteins has come from quantitative immunochemistry especially as applied to anti-carbohydrate antibodies (19-21, see 8, 22, 23). The dextran α1→6 antidextran system is the prototype for such studies because dextran is a largely linear homopolymer of α1→6 linked glucoses and the series of isomaltosyl oligosaccharides from the di- to the octasaccharide (IM2 to IM8) and synthetic linear dextrans of high molecular weight (24) were available. The system, using precipitin inhibition or competitive binding assays, thus provided a molecular ruler for measuring the sizes and shapes of antidextran combining sites and using hybridomas of anti α1→6 specificity provided an approach to evaluating the repertoire of α1→6 antidextran sites (25). Summarizing several decades of work on this system (19-21, see 8, 22-26), α1→6 antidextran combining sites may be grooves or cavities (26), may vary in size from those showing a lower limit, complementary to between one and two α1→6 linked glucoses to an upper limit of seven α1→6 linked glucoses and are strictly α1→6 specific in that oligosaccharides with substitutions of other than α1→6 linked glucoses at either end or in the middle reduced the

activity as compared with the comparable unsubstituted isomaltosyl oligosaccharide. Jacqueline Sharon, a graduate student with Sherie Morrison and with me has studied twelve α1→6 antidextran hybridoma proteins and compared them with the myeloma antidextrans QUPC52 and W3129 studied earlier (25, 27). All had groove type sites, six most complementary to the hexasaccharide; IM6, IM7, and IM8 giving identical inhibition and the others most complementary to the heptasaccharide; IM7 and IM8 giving equal inhibition all on a molar basis. Nevertheless, all sites were not identical in binding constants; in the relative contribution of each glucose to the total binding energy (27); in their precipitin curves with various dextrans and in their expression of the QUPC50 idiotype (28).

A most important finding was made by Peabody, Ely and Edmundson (29) who devised a method for making crystalline hybrid L chain dimers in good yield from pairs of Bence Jones proteins. One of the pair, Mcg, had been studied crystallographically. It thus became possible by binding studies to evaluate the contribution of each chain of the hybrid to the binding. When the hybrids are studied crystallographically, the contacting residues will be defined more precisely. Should this method of making hybrids prove extendable to obtaining LH hybrids in crystalline form, study of such antibody combining sites may make possible an evaluation of the assumption that random association of L and H chains contributes significantly to generating the repertoire of different antibody combining sites. Obviously without random L and H association, 1,000 L and 1,000 H chains would not make 10^6 specificties. From a statistical analysis Perelsen and Oster (30) concluded that, even if such random association of L and H chains occurs, it could at most increase the degree of diversity by a factor of ten. Studies on the specificity of hybridoma antibodies (31-34) are revealing hitherto unsuspected differences in antibody combining sites and are greatly increasing the size of the repertoire. Thus all twelve anti-α1→6 dextran hybridoma proteins studied by Sharon *et al.* (25, 27), despite their falling into two groups with respect to site size, show differences in their combining sites both in association constants and with respect to the relative contribution of each of the α1→6 linked glucoses in the determinant to the total binding energy. These differences in site specificity must ultimately be explainable in terms of amino acid sequence differences and in site structure. Moreover, although these hybridomas were formed with a parent secreting kappa chain, the hybrid molecule had negligible binding for α1→6 dextran

and there was a strong preference for formation of the specific HL association. Since both the specific and parent light chains were synthesized de novo by the same hybridoma this would eliminate the objection that hysteresis, e.g., that all in vitro LH recombinations were done with previously assembled chains and thus that they had built in specificity, was responsible for the preferential recombination.

Another serious gap in our understanding has arisen from a collaborative effort in which Barbara Newman, Shunji Sugii, and I, and Mitsuo Torii in Osaka, are studying with Brian Clevinger and Joseph M. Davie their α1→3 antidextran hybridomas whose heavy chains have been sequenced by Schilling et al. (34). We find several types of quantitative precipitin curves with dextrans of different structures. Two hybridomas H12 and H31 gave identical quantitative precipitin curves with three class II dextrans having the highest proportions of α1→3 linkages, and gave no reaction whatever with 20 other dextrans thus resembling CAL20 TEPC1035 (35). They differ in their CDR3 (D) and J segments in all amino acid residues but one and are identical in sequence for the rest of the V region. Other hybridomas, however, also with V_H sequences identical to these through FR3 react best with the three class II dextrans but cross react in quantitative precipitin studies with other dextrans of class I giving curves resembling those published for MOPC104E (37), J558 (36, 38) and UPC102 (26). These findings strongly suggest that the light chains, although λ, are probably contributing to site complementarity and one hopes that V_L sequencing will be done to make a definitive interpretation possible. Moreover, the D and J segments although very different in sequence, may actually form part of sites which block access to the cross reacting dextrans. We will also have to carry out inhibition studies to characterize the sites but larger oligosaccharides than are available as well as larger amounts of oligosaccharides will be needed and are being prepared. The two α1→3 antidextrans although giving precipitin curves like CAL20 TEPC1035 differ in their inhibition patterns in that panose, DGlcα1→6DGlcα1→4DGlc, is the best inhibitor with CAL20 TEPC1035 (35) but no inhibition with panose was found with H31; panose also did not inhibit one of the cross reacting hybridomas, nor the α1→3 myeloma antidextrans. It is obvious that immunochemical characterization of antibody combining sites is indispensable to a true understanding of antibody complementarity and the principles established must be verified by X-ray crystallography.

If we turn now to the genetic aspects of the antibody complementarity problem, it is clear that at the DNA level J minigenes coding for FR4 plus several residues of CDR3 in both L and H chains (39-43) and D minigenes coding for CDR3 (44, 45) exist as proposed from our assortment (46-50) of FR segments of identical amino sequence in human, mouse, and rabbit V_L and of human and mouse V_H chains - and that somatic assembly at the DNA level is an essential step in antibody synthesis (46). The assortment principle has been used by Weigert et al. (51) to define J minigenes in the V_K21 group. The data on nucleic acid sequences of the J_L (43, 52) and J_H (44, 45) minigene segments have indicated that diversity in amino acid sequence could occur as a consequence of V_K-J_K recombination within a single codon for the four J segments which are considered to be functional and Weigert et al. (51, 53) have generated even more diversity at the V-J junctions because of the existence of an extra amino acid, position 95A (11) in one V_K21 protein. Similarly, although the D minigenes were defined by the absence of coding sequences on the 3' side of V_H and the 5' side of J_H, intracodon recombination in somatic assembly of the V-J-D nucleotide segments could also generate substantial diversity especially since CDR3, the D segments, can vary substantially in length, nucleotides coding for five (44) and for 14 (45) amino acids having been missing from the two matched V and J segments studied.

It becomes crucial to evaluate how much of this diversity is contributing directly to the generation of antibody complementarity and how much is essentially recombinational noise involving the generation of non functional sites. Max et al. (55) have found a V_L-J_L recombinant clone with a missense reading frame so that this was a non functional gene; it is of interest that this V_L gene also coded for Leu at position 35 although to date all of 231 light chains sequenced have Trp at this position (11).

In the light chains position 96, one of the two amino acid residues in CDR3 as originally defined (9), shows very high variability. How much of this variability, if intracodon recombination occurs, involves site complementarity and how much is recombinational noise. Position 96 in V_L is at the bottom of the antibody combining site; in only one of the six X-ray structures, McPC603 (55, 2) has it been shown clearly to be a contacting residue for the hapten, phosphorylcholine; it is also important for non covalent binding of V_L-V_H and of V_L dimers (56). By sequence comparison (58) with McPC603, position 96 of another phosphorylcholine myeloma protein MOPC167 was also inferred to contact hapten. However,

Rudikoff et al. (58) have found that, at position 96, five of six anti-βI→6 galactans contain an amino acid, Ile, which could not be generated by the hypothesized intracodon recombination. The sixth has a Trp at this position. Several of the five with Ile differ in binding constant whereas the substitutions of Trp for Ile did not involve a significant change in binding constants or specificity; J539 with Ile and XRPC24 with Trp had binding constants of $1.45X10^5$ and $1.75X10^5$ respectively and showed the same specificity for 30 different haptens. We must conclude that the postulated diversity generated by V-J joining, and position 96 will not account for much antibody complementarity and specificity especially since the crystallographic structures of those sites of myeloma antibodies already studied show the complementarity-determining regions to be much larger than any ligand which has been found to bind in the site.

It is evident from examining the X-ray crystallographic structures, that in the heavy chains the D minigene which comprises most of CDR3 (44, 45) could contribute substantially to the generation of antibody complementarity, especially in view of the variations in length of D_H. Until clones coding for the D minigenes are located and sequenced and intracodon recombination in V-D-J joining established, one will not be able to evaluate its relative contribution to recombinational noise and to the generation of antibody complementarity.

Turning now to the role of the rest of the V-region, of the light and heavy chains in the generation of antibody complementarity, it is clear both from X-ray crystallogrpahy (1-7, see 8) from model building (12-18) and from affinity labeling (see 12) that CDR1 and CDR2 of the both chains and CDR3 minus residues 96 and 97 of V_L contribute a substantial portion and perhaps even most of the site complementarity. Cloning data on sperm, on 13 day mouse embryo and in fetal liver DNA indicate that nucleotides coding for residues 1-95 of V_L (41, 59) and 1 through the end of FR3, residue 94, of V_H (44, 45) exist as contiguous gene segments. Indeed several investigators (60, 61) have reported pairs of V_H genes separated by 12, 14, 15 and 16 kilobases in 13 day mouse embryo and from fetal liver DNA. Moreover, by cross hybridization with mouse probes, human V-region genes of similar length have been isolated (62).

Antibody complementarity due to the CDR of V_H excluding D and J and of V_L excluding J is extremely important and it has become necessary to evaluate proposed genetic mechanisms as to how these gene segments contribute to the generation of complementarity. Estimates of 300 mouse and

40-50 human V_H genes have been made by multiplying bands on Southern blots by the proposed number of subgroups; numbers of V_L genes computed similarly are are of the same order. These numbers appear far too low to account for diversity on a germ line basis even when V_L-J_L assortment is taken into account without random V_L-V_H association, an assumption whose weaknesses have been noted above, so that somatic mutation in the CDR's has been proposed (63, 64) to increase the variability, based predominantly on the findings with mouse V_λ chains (64, 65) and more recently on sequences of V 21 (65) and on the variation in IgG as compared wih IgM sequences of phosphorylcholine binding hybridomas (33). Subgroup differences in V_κ chains are also ascribed to somatic mutations (66); for evidence from population studies against somatic mutation in the CDR (see 67, 68).

In contrast we have assembled a considerable body of evidence favoring independent assortment of minigene segments which successfully predicted the J and D minigenes (46-50) but which also indicated that assortment by recombination was occurring in the rest of the V_L and V_H segments; matches of nucleotide segments identical in V_κ and V_λ chains were seen bounding the CDR segments which could serve as sites for such assortment (69). Moreover, it was possible to demonstrate assortment of all of the FR, CDR, and a J segment of rabbit V_κ chains (48). Of especial note was the finding of an identical FR2 segment, positions 35-44, in one human V_κIV, 22 mouse V_κ and 13 rabbit V_κ chains indicating that this segment had been preserved for over 80 million years (46, 48). At least nine alternative sequences exist in the rabbit and twelve in the mouse which may differ from the preserved segment at 13 of the 15 positions and in from one to five amino acid residues, several of which involve two base changes. Indeed, the only alternative sequence which occurs in both mouse and rabbit involves a Phe substitution for Tyr at position 36 showing that the evolution of this segment differed in the two species (67). These alternative forms occur much less frequently; of 14 NZB sequences, 6 BALB/c and 13 rabbit sequences, the preserved FR2 set occurred 10, 5, and 12 times. Thus if all V_κ genes minus J occur sequentially in DNA a substantial number of copies of this preserved segment must exist (47). If some assortment of CDR and FR segments by recombination can take place, the number of copies of this preserved gene segment could be substantially reduced. Since it is clear that V_κ regions minus J do exist in substantial numbers in the genome, other hypotheses are needed; among these would be a cassette model in which

clusters of FR and CDR segments exist elsewhere in the genome; this could be tested by hybridizing with small probes coding for the FR and CDR segments and such experiments would also provide an estimate of the number of copies of the preserved segment. Another problem which arises is that the numbers of CDR's are substantially greater than the numbers of FR's (48); thus in the genome there would have to be replications of FR segments if each CDR is to be represented in an intact V gene.

Both cloning data and assortment led independently to the recognition of J and D minigenes; they are continuing to tell us something important about the generation of diversity in the rest of the V-region. What has got to be learned is whether some additional recombinational mechanism is involved in the generation of antibody complementarity and how to distinguish diversity due to recombinational noise from antibody complementarity.

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REFERENCES

1. Amzel, L.M., and Poljak, R.J. (1979). Ann. Rev. Biochem. 48, 961.
2. Davies, D.R., Padlan, E.A., and Segal, D. (1975). Ann. Rev. Biochem. 44, 639.
3. Davies, D.R., Padlan, E.A., and Segal, D. (1975). Contemporary Topics in Molecular Immunology 4, 127.
4. Dwek, R.A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S.J., and Givol, D. (1977). Nature (London) 266, 31.
5. Wang, B.-C., Yoo, C.S., and Sax, M. (1979). J. Mol. Biol. 129, 657.
6. Edmundson, A.B., Ely, K.R., Girling, R.L., Abola, E.E., Schiffer, M., Westholm, F.A., Fausch, M.D., and Deutsch, H.F. (1974). Biochemistry 71, 3816.
7. Marquart, M., Deisenhoffer, J., and Huber, R. (1980). J. Mol. Biol. 141, 369.
8. Kabat, E.A. (1978). Adv. Protein Chem. 32, 1.
9. Wu, T.T., and Kabat, E.A. (1970). J. Exp. Med. 132, 211.

10. Kabat, E.A., and Wu, T.T. (1971). Ann. N. Y. Acad. Sci. 190, 382.
11. Kabat, E.A., Wu, T.T., and Bilofsky, H. (1979). Sequences of Immunoglobulin Chains. Tabulation and analysis of amino acid sequences of precursors, V-regions, C-regions, J-chain, and β_2-microglobulins. U.S. Dept. Health, Education and Welfare, NIH Publication No. 80-2008, Bethesda, Maryland.
12. Givol, D. (1974). Essays in Biochemistry 10, 73.
13. Davies, D.R., and Padlan, E.A. (1977). In Haber, E., and Krause, R.M., (eds.): "Antibodies in Human Diagnosis and Therapy," New York: Raven Press, p. 119.
14. Dwek, R.A. (1977). In Porter, R.R., and Ada, G.L. (eds.): "Contemporary Topics in Molecular Immunology," New York: Plenum Press, Vol. 6, p. 1.
15. Padlan, E.A., Davies, D.R., Pecht, I., Givol, D., and Wright, C. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 627.
16. Dwek, R.A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S.J., and Givol, D. (1977). Nature (London) 266, 31.
17. Mage, R.G., and Kabat, E.A. (1963). Biochemistry 22, 1278.
18. Feldman, R.J., Potter, M., and Glaudemans, C.P.J. (1981). Mol. Immunol. (in press)
19. Kabat, E.A. (1956). J. Immunol. 77, 377.
20. Kabat, E.A. (1957). J. Cell. Comp. Physiol. 50, 79.
21. Kabat, E.A. (1960). J. Immunol. 84, 82.
22. Kabat, E.A. (1961). "Kabat and Mayer's Experimental Immunochemistry" Second Ed., Illinois, Chas. C. Thomas.
23. Kabat, E.A. (1976). "Structural Concepts in Immunology and Immunochemistry" Second Ed., New York: Holt, Rinehart, and Winston.
24. Ruckel, E.R., and Schuerch, C. (1967). Biopolymers 5:515.
25. Sharon, J., Morrison, S.L., and Kabat, E.A. (1981). in preparation. see Sharon J., 1981 Ph.D. Thesis Columbia University Part III.
26. Cisar, J., Kabat, E.A., Liao, J, and Potter, M. (1974). J. Exp. Med. 142, 435.
27. Sharon, J., Morrison, S.L., and Kabat, E. A. (1981). in preparation. See Sharon, J. (1981) Ph.D. Thesis, Columbia University, Part IV.
28. Sharon, J., D'Hoostelaire, L., Potter, M., Kabat, E.A., and Morrison, S.L. in preparation.
29. Peabody, D.S., Ely, K.R., and Edmundson, A.B. (1980). Biochemistry 19, 2827.

30. Perelson, A.S., and Oster, G.F. (1979). J. Theor. Biol. 81, 645.
31. Estess, P., Lamoyi, E., Nisonoff, A., and Capra, J.D. (1980). J. Exp. Med. 151, 863.
32. Marshak-Rothstein, A., Benedetto, J.D., Kirsch, R.L., and Gefter, M.L. (1980). J. Immunology 125, 1987.
33. Gearhart, P.J., Nelson, N.D., and Hood, L.E. (1981). Nature (London) (in press).
34. Schilling, J., Clevinger, B., Davie, J.M., and Hood, L. (1980). Nature (London) 283, 35.
35. Sugii, S., Kabat, E.A., Shapiro, M. and Potter, M. (1981). J. Exp. Med. (in press).
36. Cisar, J., Kabat, E.A., Dorner,M., and Liao, J. (1975). J. Exp. Med. 142, 435.
37. Leon, M.A., Young, N.M., and McIntire, K.H. (1970). Biochemistry 9, 1023.
38. Lundblad, A., Steller, R., Kabat, E.A., Hirst, J., Weigert, M.G., and Cohn, M. (1972). Immunochemistry 9, 535.
39. Tonegawa, S., Maxam, A.M., Tizard, R., Bernard, O., and Gilbert, W. (1977). Proc. Natl. Acad. Sci. U.S.A. 75, 1485.
40. Brack, C., Hirama, M., Lenhard-Schuller, R., and tonegawa, S. (1978). Cell 15, 1.
41. Bernard, O., Hozumi, N., and Tongawa, S. (1978). Cell 15, 1133.
42. Max, E.E., Seidman, J.G., and Leder, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 3450.
43. Sakano, H., Huppi, K., Heinrich, G., and Tonegawa, S. (1979). Nature (London) 280, 288.
44. Early, P., Huang, H., Davis,H., Calame, K., andHood, L. (1980). Cell 19, 981.
45. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature (London) 286, 676.
46. Kabat, E.A., Wu, T.T., and Bilfosky, H. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 2429.
47. Kabat, E.A., wu, T.T., and Bilofsky, H. (1979). J. Exp. Med. 149, 1299.
48. Kabat, E.A., Wu, T.T., and Bilfosky, H. (1980). J. Exp. Med. 152, 72.
49. Kabat, E.A. (1980). J. Immunol. 125, 961.
50. Kabat, E.A. (1980). In Cohen, E.P., and Köhler, H., "Membranes, Receptors and the Immune Response", Prog. Clin. and Bio. Res. Vol. 42, pp. 1, Alan R. Liss, Inc., New York.
51. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978). Nature (London) 276, 785.

52. Max, E.E., Seidman, J.G., and Leder, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 3450.
53. Weigert, M., Perry, R., Kelley, D., Hunkapiller, T, Schilling, J., and Hood, L. (1980). Nature (London) 283, 497.
54. Max, E.E., Seidman, J.G., Miller, H., and Leder, P. (1980). Cell 21, 793.
55. Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M., and Davies, D.R. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 4298.
56. Stevens, F.J., Westholm, F.A, Solomon, A., and Schiffer, M. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 1144.
57. Rudikoff, S., Satow, Y., Padlan, E., Davies, D., and Potter, M. (1981). Molec. Immunol. (submitted).
58. Rudikoff, S., Rao, D.N., Glaudemans, C.P.J., and Potter, M. (1980). Proc. Natl. Acad. Sci. U.S.A. 77, 4270.
59. Nishioka, Y., and Leder, P. (1980). J. Biol. Chem. 255, 3691.
60. Kemp, D.J., Cory, S., and Adams, J.M. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4627.
61. Matthyssens, G., and Rabbitts, T.H. (1981). Proc. Natl. Acad. Sci. U.S.A. 77, 3625.
62. Rabbitts, T.H., Hamlyn, P.H., and Matthyssens, G. (1980). Natl. Research Council of Canada p. 176.
63. Milstein, C., and Munro, A.J. (1970). Ann. Rev. Microbiol. 24, 335.
64. Cohn, M., Blomberg, B., Geckeler, W., Raschke, W., Riblet, R., and Weigert, M. (1974). In Sercarz, E.E. Williamson, A.R., and Fox, C.F. "The Immune System, Genes, Receptors, and Signals; p. 89, Academic Press, New York.
65. Weigert, M., and Riblet, R. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 837.
66. Potter, M. (1977). Adv. Immunol. 25, 141.
67. Ohta, T. (1980). J. Mol. Evol. 15, 29.
68. Ohta, T. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 5108.
69. Wu, T.T., Kabat, E.A., and Bilofsky, H. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4617.

IMMUNOGLOBULIN LIGHT CHAIN GENES OF MOUSE AND MAN

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Kappa light chain diversity arises in large part from a site specific DNA recombination event that joins one of several hundred germline V-region genes to one of four active J segments. The combinational power of this system is increased by a recombination mechanism which allows variation in the crossover point. Although the flexible nature of this recombination event generates increased diversity in a coding region critical to the formation of the antigen combining site, it also leads to aberrantly rearranged genes which cannot produce a functional light chain product. In order to study the evolution and development of this process, we have turned to the human immunoglobulin light chain genes. The κ and λ light chain genes of man have been cloned using corresponding mouse gene segments as probes under low stringency hybridization conditions. The mouse and human genes have selectively conserved sequences which are thought to be involved in DNA and RNA splicing reactions. The human λ light chain genes constitute a complex locus that includes at least six non-allelic constant region genes that are clustered on the chromosome. In addition, we have identified several polymorphisms in restriction sites surrounding these genes that provide useful human chromosomal gene markers. The arrangement of light chain genes has been analyzed in a variety B cell lines and lymphocytic leukemias that represent discrete stages in the development of an immunocyte. The results suggest that the process of light chain gene rearrangements, which appears to involve the frequent deletion of the κ constant region gene, may be ordered so as usually to progress from the κ to the λ genes.

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INTRODUCTION

The study of the structural organization and expression of the kappa light chain gene system in the mouse has provided insight into the nature of the site-specific DNA recombination event which leads to the formation of an active immunoglobulin light chain gene and the contribution that this somatic recombination event can make in generating antibody diversity. The early hypothesis of Dreyer and Bennett (1), that variable and constant portions of the immunoglobulin molecule are separately encoded in the genome, is essentially correct, although there are additional somatic mechanisms which contribute to the generation of variable region diversity. This tremendous diversity is accounted for in large part by a combinational process in which presumably any one of several hundred germline variable region genes (2,3) can be joined to one of four active J segment genes located a few kilobases to the 5' side of a single kappa constant region gene (4-8). In addition, the precise crossover point of this recombination event can vary. This flexibility allows the organism to generate increased diversity around the site of DNA recombination (6,8,9). Although this flexible recombination serves to generate enormous diversity, it also poses a problem to the organism in terms of frequently generating aberrantly rearranged genes which are non-functional (10-14).

We have turned our attention to the study of the human kappa and lambda light chain genes in order to establish the structural basis for the diversity seen in human light chains and to ask specific questions about isotypic and allelic exclusion. In so doing, we have been able to compare gene systems which have evolved during the 70 million years or so in which the mouse and human species have diverged and to identify to what extent particular elements have been conserved during evolution. More importantly, there exist in man a wide variety of B cell lines and lymphocytic leukemias which are thought to represent clonal expansions of cells frozen at discrete stages in lymphoid development. Since kappa and lambda light chains are expressed in comparable amounts in human immunoglobulin molecules, we have asked whether the arrangement of the kappa and lambda light chain genes in these cells offers insight into the mechanism or order of their selection during B cell development. The results have implications for allelic and isotypic exclusion and suggest an ordered hierarchy of light chain gene rearrangements in which kappa genes are rearranged or deleted prior to lambda gene rearrangement.

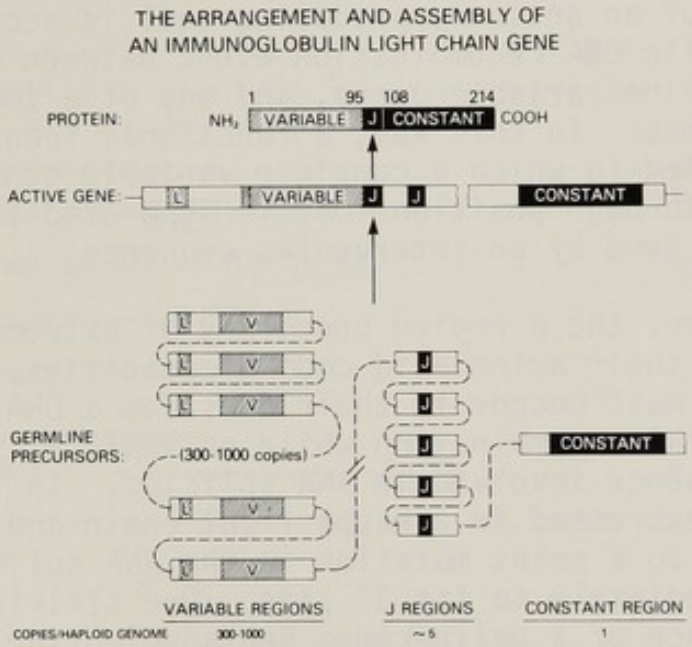

FIGURE 1. A representation of the arrangement and assembly of an immunoglobulin light chain gene. The sequence of events is described in the text. L, hydrophic leader, V, variable region; J, J segment.

RESULTS AND DISCUSSION

V-J Recombination Creates an Active Light Chain Gene

The general structural features for the arrangement and assembly of a kappa immunoglobulin light chain gene are shown in Figure 1. A major approach in much of this work has involved the isolation and detailed structural determination of active recombinant genes from mouse myeloma DNA and a comparison of these structures to the germline precursor genes from which they were derived in embryonic DNA. Direct cloning and sequencing of germline variable region genes in the lambda system (15) and in the kappa system (2) showed that the amino acid coding capacity fell short by some 13 amino acids (encoding positions 96-108 of the light chain variable region). In the kappa system, these 13 amino acids are encoded in each of five distinct coding segments called "J" or

joining segments, which are situated between 2 and 4 kb to the 5' side of a single kappa constant gene (6,8). The formation of an active kappa light chain gene during the somatic development of an antibody producing cell is accomplished by a site-specific DNA recombination event between one of several hundred germline variable genes, and one of a smaller set of J segment genes. In this way, a functional transcription unit is created in which a complete variable gene (encoding amino acids through position 108) is separated from the constant region gene by an intervening sequence.

Of course, the J region genes are of extreme interest not only for their amino acid coding capacities, but also because each must encode to their 5' sides a DNA sequence involved in DNA recombination while on their 3' sides must encode a sequence involved in RNA splicing. In fact, J3 is never found expressed in a kappa light chain and this is probably due to a point mutation in the RNA splice donor sequence immediately to its 3' side. One striking feature is the presence of a palindromic heptanucleotide and a T rich sequence 20 basepairs away, to the 5' side of each of these J segment genes. In fact, when the 3' flanking sequence of a number of variable region genes were compared, this same heptanucleotide $CAC^{A}/_{T}GTG$ was also found immediately flanking the variable genes. These sequences suggested that a base paired stem structure between this inverted repeat 3' to variable genes and 5' to J genes might be involved in a recombination intermediate, bringing the variable region and J segment genes into proximity (6-8). A possible implication of such an intermediate would be the deletion of DNA which lies between the particular V region and J segment which have undergone recombination. Using appropriate J and V region probes in definitive in situ hybridization experiments, we have shown this indeed to be the case (16).

Recombinational Flexibility Generates Diversity But Also Creates Non-Functional Genes.

Although a simple model of V-J joining is an attractive one and can account for a great deal of diversity, all of the known kappa light chain protein sequences in positions 96-108 could not be accounted for by the germline coding sequences of the four active J region segments. These discrepancies were all in position 96 (the first amino acid encoded by the J regions) and could be accounted for by assuming that the crossover point of DNA recombination may vary and in so doing can generate diversity at position 96 (6,8).

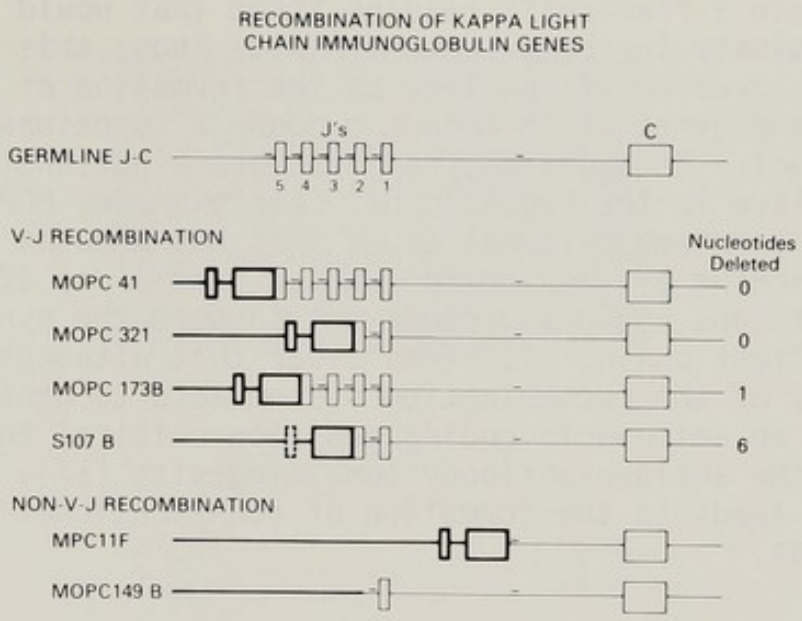

FIGURE 2. Diagrammatic representation of rearranged kappa genes. Germline DNA is represented by thin lines. DNA which has recombined into this region is represented by thick lines. Coding signals are boxed. The heptanucleotide CACTGTG is represented by a dash above each line. MOPC-321 was characterized by Sakano et al. (8).

This notion has been confirmed by cloning and sequencing several rearranged light chain genes (7,8,10,13) which are shown diagrammatically in Figure 2. For example, in the case of MOPC41, recombination has taken place between the second and third nucleotides of codon 95 forming an active kappa gene and retaining the germline coding sequence of J5 (7). On the other hand, V-J recombination in S107B has taken place in such a way as to delete six nucleotides corresponding to codons 96 and 97, leaving the amino acid reading frame in phase. Clearly, the crossover site of recombination is different for these recombinant kappa genes supporting the hypothesis that there can be variability in the V-J joining site.

The question immediately is raised as to what mechanism might exist which would assure that the translational reading frame would be maintained. In fact, we have shown that

the phase is not always maintained. For example, in the case of the MOPC173B gene, the recombination event has occurred so as to produce a frameshift reading frame that would prematurely terminate in the J segment (10). Thus, this variation in the crossover point can lead to the formation of aberrantly rearranged genes which cannot produce a functional light chain product. In addition, non-V/J recombinational events can take place in the kappa locus. For example, MOPC149B represents a recombinational event that has brought in a DNA sequence lacking a V region to a point just 3' to J2. Such a recombinant gene obviously could not support the synthesis of an active light chain. It seems clear that although the flexibility of the recombination system acts to generate diversity in an amino acid coding position critical to the formation of the antigen-antibody combining site (17), it also frequently leads to the formation of non-functional recombinant genes.

Evolution of the Human Kappa Constant and J Region Locus

Although a great deal of human protein sequence is available, the molecular cloning of the human immunoglobulin genes has been made difficult by the fact that human myeloma lines that produce large quantities of immunoglobulin mRNA are not available. To overcome this difficulty, we have used techniques that allow the isolation of human immunoglobulin genes using the corresponding cloned mouse gene segments as probes under relaxed hybridization conditions (18). We have compared the structural organization of the human kappa constant and J region genes to the kappa constant locus of the mouse to determine to what extent the general features, outlined above, which serve to generate diversity in the mouse kappa system, have been conserved in humans. Although we have sequenced extensive regions in the human kappa constant locus, the simplest way of comparing the human and mouse genes is by heteroduplex analysis (Figure 3). This allows the visualization in the electron microscope of regions in the DNA which have been conserved during evolution. Six regions of homology can be seen. The most extensive homology maps to a region which corresponds to the kappa constant region gene and includes regions which flank the coding block. This entire region has been sequenced at the DNA level in the cloned human segment and shown to correspond to the InV3 kappa constant allele (18). In addition, four short regions of homology, separated by bubbles of non-homology can be seen a few kilobases to the 5' side of the kappa constant genes.

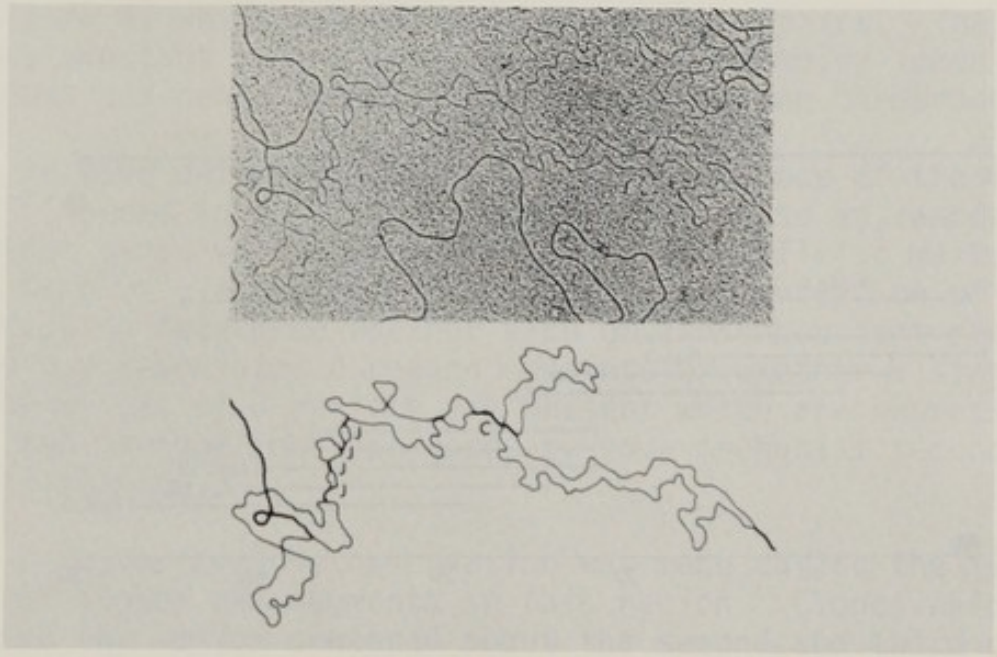

FIGURE 3. Electron micrograph of heteroduplex between mouse and human germline kappa J/C loci. The orientation is 5' to 3' left to right. The J region and constant region homologies are indicated by J and C respectively. The cloned mouse fragment is the one described by Seidman and Leder (19); the human fragment by Hieter et al. (18).

The most plausible interpretation of this structure was that these regions correspond to four human J region genes which were showing homology to four of five mouse J region genes. This has been substantiated by direct nucleotide sequence analysis. In fact, a fifth functional human J region gene is present immediately 3' to the cluster of four J segments identified in the heteroduplex. Finally, a sixth region of homology is observed which lies between the J and constant regions. The functional significance of this conserved sequence within the intervening sequence is as yet unclear.

The Human Lambda Constant Genes: A Complex Locus

In contrast to the mouse which has an extremely limited repertoire in the lambda light chain system, human immuno-

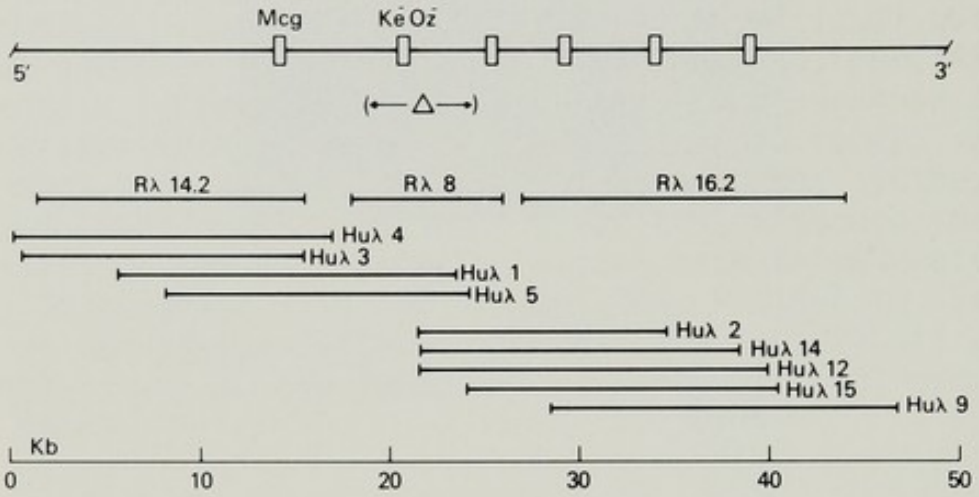

FIGURE 4. Overlapping human lambda constant gene containing library clones and a physical map derived from them. Clones were obtained from a human DNA library (20) using a mouse lambda cDNA fragment as probe. Their orientation was deduced by restriction endonuclease site mapping. Six regions, which are represented by boxes on the composite map (top line), demonstrated strong homology to the mouse lambda constant probe.

globulins are composed of one-third lambda light chain bearing molecules and, therefore, the human lambda gene system is likely to contribute significantly to antibody diversity in man. In addition, the human lambda constant region consists of at least four non-allelic forms representing limited amino acid substitutions for the non-allelic markers Kern, Oz, and Mcg. We were, therefore, interested in determining the structural organization of these genes and ultimately to direct experiments toward an understanding of the mechanisms involved in the selection of either kappa or lambda expression in a developing B lymphocyte in man.

To accomplish this, we have screened a human DNA library (20) for the human lambda genes utilizing a cDNA probe derived from a mouse lambda light chain sequence (provided by

Dr. A. Bothwell). By ordering the overlapping clones we have constructed a linkage map of the lambda constant genes, shown in Figure 4, which extends some 50,000 base pairs. The human lambda constant region genes constitute a complex locus of at least six genes tandemly arranged along the chromosome.

We have determined the nucleotide sequence of the three most 5' genes in the cluster identifying these as lambda constant genes which correspond to the non-allelic markers Mcg, Kern^{-}Oz^{-}, and Kern^{-}Oz^{+}. It will, of course, be of great interest to determine whether each of these constant region genes has associated J region segments or whether a single J region or set of J regions are present which are expressed with the various constant genes by some mechanism secondary to V-J joining.

A rather curious observation was made during the isolation of cloned DNA segments in this region. Clones which spanned the region centered about the second and third genes invariably deleted extensive regions during propagation in the bacterial host. Thus, there appeared to be a highly unstable or recombinogenic sequence associated with this region (designated by the Δ symbol) at least as recognized by the bacterial host. This recombinogenic sequence maps to the central 8 kilobase gene containing Eco R1 fragment and it was of interest to know whether this sequence might be unstable in vivo, i.e., subject to gross changes during evolution. To test this, we prepared DNA from fifteen individuals and asked whether the 8 kilobase Eco R1 fragment was unstable in the population. The results of this experiment are shown in Figure 5. Even in this small sampling we have identified three different genomic arrangements of the lambda constant genes. These polymorphisms represent differences in the lengths of the restriction fragments on which the lambda constant genes reside. Case 1 represents the most common genotype which is characterized by 8, 14, and 16 kb Eco R1 restriction fragments. Case 2 has a different gene containing restriction fragment which is 18 kb in length and, in fact, represents a homozygous condition for the 8/18 kb polymorphic allele since no 8 kb fragment is present. Case 5 represents an individual who is heterozygous for these alleles. A third polymorphism was demonstrated in an individual heterozygous for a 21 kb allele (Case 10). It is interesting to note that this polymorphism, which may be the result of a gross insertion/deletion type evolutionary event, has occurred in the region which is highly unstable during cloning in bacteria. In any event, these polymorphisms provide useful chromosomal markers for future genetic studies.

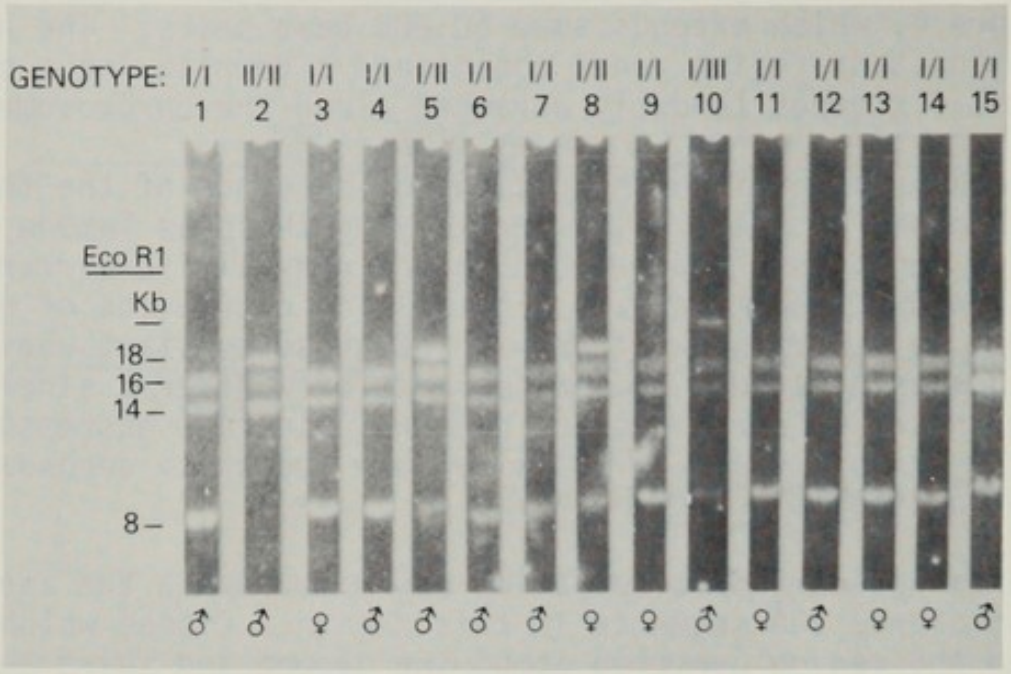

FIGURE 5. Genomic arrangement of lambda constant containing restriction fragments in fifteen human individuals. DNA was prepared from white cells isolated from a few cc of blood, digested with Eco Rl restriction endonuclease, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose, hybridized to a human lambda constant region probe (DNA fragments corresponding to the genes Mcg and Kern Oz) and autoradiographed.

Kappa Constant Genes are Deleted or Rearranged in Lambda Producing B Cells: Implications for Allelic and Isotypic Exclusion

Having cloned the human kappa and human lambda constant region genes, we were in a position to use these gene segments as hybridization probes to study gene rearrangements that occur in human immunoglobulin producing cells. Since a given lymphocyte expresses either kappa or lambda, but never both, there must be some mechanism by which the cell ensures the selection of a single light chain class. In addition, after commitment to the expression of a particular light chain allele, the excluded class and excluded allele remain silent throughout the functional life of the lymphocyte. These phenomena are termed isotypic and allelic exclusion.

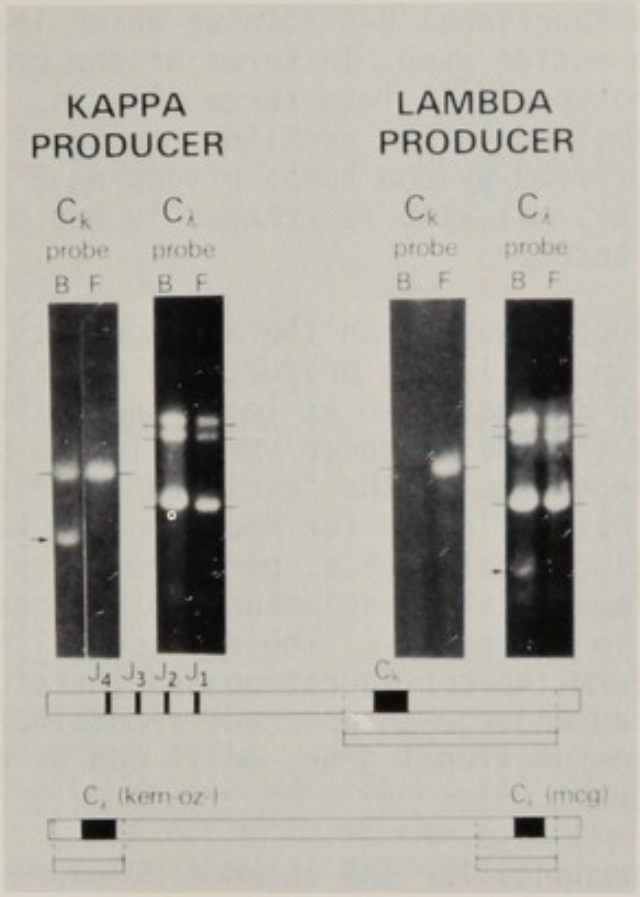

FIGURE 6. Detection of rearranged kappa and lambda genes in human kappa and lambda producing B cells. Shown are representative cases. B cells (B) were freshly obtained from patients with high count chronic lymphocytic leukemia. A fibroblast line (F) established from the same individuals served as germline control. The probes used are diagrammed and have been described elsewhere (21). DNA was extracted and analyzed as described in the legend to Figure 5.

To gain some insight into these processes, we have analyzed the arrangement of the kappa and lambda light chain genes in human lymphocytes at the B cell stage of differentiation, i.e., cells expressing surface immunoglobulin containing either kappa or lambda light chains (Figure 6). Our results, analyzing 8 kappa producing and 10 lambda producing human B lymphocytes, have led us to speculate as to a model concerning an order of gene rearrangements during development which takes into account allelic and isotypic exclusion (21).

As expected, all eight kappa producing B cells demonstrated at least one rearrangement of a kappa gene when compared to the germline configuration. These are probably

the result of a functional V-J joining which leads to the formation of an active gene. In terms of the other "excluded" allele, the genotypes fall into three classes. The second kappa allele remained in the germline configuration in four cases (as exemplified by the kappa producing B cell genotype in Figure 6), was also rearranged in two cases, and was deleted in two cases.

In an analogous way, when the arrangements of the lambda constant genes in ten lambda producing B cells were examined, as expected each demonstrated at least one rearranged lambda constant allele (lambda producer versus lambda probe, Fig. 6). Again, the arrangements of the "excluded" alleles fell into the same three categories as for the opposite kappa alleles in kappa producing B cells (i.e. germline, also rearranged, or deleted). Thus allelic exclusion can be accounted for in these cells in three ways. The excluded allele can either remain in the germline configuration and therefore cannot be expressed, it can undergo an aberrant recombination event leading to a non-functional gene, or it can be entirely lost by a deletion event.

But what accounts for the absence of expression of the excluded light chain class? To gain insight into this question we have analyzed the arrangement of kappa genes in lambda producing B cells and the arrangement of lambda genes in kappa producing B cells and obtained a rather surprising result. As might be expected, all eight kappa expressing B cells retained their lambda genes in the germline configuration (example shown in Figure 6). However, when the lambda producing B cells were analyzed for the arrangement of their kappa genes, a striking and unanticipated result was obtained (example Fig. 6). In each of the lambda producing B cells, both kappa constant alleles were entirely lost, except for a single exception and even in this case one kappa allele was deleted while the other was retained but in a rearranged configuration. Thus the kappa genes exhibit an extraordinary lability in lambda expressing cells.

An Order of Light Chain Gene Rearrangements During B Lymphocyte Development.

The fact that lambda genes remain in the germline configuration in kappa producing B cells, while kappa genes are deleted or rearranged in lambda producing B cells suggests a hierarchy of gene rearrangements during B cell development. If we accept the proposal of Alt and Baltimore (22) that the expression of a functional light chain shuts down the light

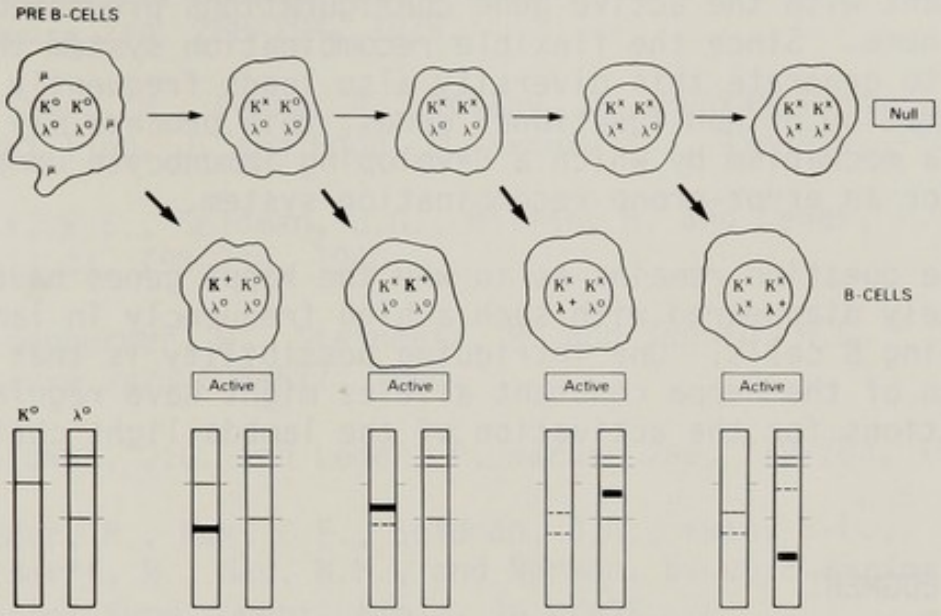

FIGURE 7. Developmental scheme of ordered light chain gene rearrangements in pre-B cells. The scheme is described in the text. The genotypes and representative light chain gene fragment configurations for each of the four light chain producing cell types are shown. Bold faced lines represent the active allele; a dotted line represents a deleted or aberrantly arranged allele. Genotype superscripts are as follows: 0, germline; +, viable V-J joining event; X, aberrant rearrangement or deletion.

chain recombination system and if we assume that kappa genes rearrange before lambda genes, the genotypes of the human B cells examined can be accounted for by the developmental scheme presented in Figure 7.

A pre-B cell would first make an attempt at a functional gene rearrangement at one of its kappa alleles. If this rearrangement were valid, producing a functional kappa light chain product, no further gene rearrangements would take place and the cell would be committed to kappa expression. If the initial kappa rearrangement were non-functional (either as an aberrant V-J joining or deletional event) a second kappa rearrangement would take place on the opposite allele. If functional, the cell would become committed to kappa expression and subsequent gene rearrangements would not take place. If both kappa rearrangements were non-functional, the pre-B cell would then move to its lambda system, attempting functional rearrangement of one of its lambda alleles. Again, a productive rearrangement would commit the cell to lambda expression, and so on down the cascade shown in the figure. The genotypes of the B cells we have examined are

consistent with the active gene configurations predicted by this scheme. Since the flexible recombination system that serves to generate this diversity also leads frequently to the formation of non-functional genes, this process may constitute a mechanism by which a developing immunocyte compensates for an error-prone recombination system.

The question remains as to why the kappa genes have completely diappeared with such a high frequencly in lambda expressing B cells. One intriguing possibility is that the deletion of the kappa constant alleles might have regulatory implications for the activation of the lambda light chain locus.

ACKNOWLEDGMENT

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REFERENCES

1. Dreyer, W.J. and Bennett, J.C. *Proc. Natl. Acad. Sci. U.S.A. 54,* 864-869, 1965.

2. Seidman, J.G., Leder, A., Edgell, M.H., Polsky, F., Tilghman, S., Teimeier, D. and Leder, P. *Proc. Natl. Acad. Sci. U.S.A. 75,* 3881-3885, 1978.

3. Valbuena, O., Marcu, K.B., Weigert, M. and Perry, R.P. *Nature 276,* 780-784, 1978.

4. Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M. and Tonegawa, S. *Proc. Natl. Acad. Sci. U.S.A. 75,* 4709-4713, 1978.

5. Seidman, J.B., Leder, A., Nau, M.M., Norman, B. and Leder, P. *Science 202,* 11-17, 1978.

6. Max, E.E., Seidman, J.G. and Leder, P. *Proc. Natl. Acad. Sci. U.S.A. 76,* 3450-3454, 1979.

7. Seidman, J.G., Max, E.E. and Leder, P. *Nature 280,* 370-375, 1980.

8. Sakano, H., Huppi, K., Heinrich, G., and Tonegawa, S. *Nature 280,* 288-294, 1979.

9. Weigert, M., Perry, R., Kelley, D., Hunkapiller, T., Schilling, J. and Hood, L. *Nature 283,* 497-499, 1980.

10. Max, E.E., Seidman, J.G., Miller, H. and Leder, P. *Cell 21,* 793-799, 1980.

11. Altenburger, W., Steinmetz, M. and Zachau, H.G. *Nature 287,* 608-617, 1980.

12. Seidman, J.G. and Leder, P. *Nature 286,* 779-783, 1980.

13. Leder, P., Max, E.E., Seidman, J.G., Kwan, S-P., Scharff, M., Nau, M.M., and Norman, B. *Cold Spring Harbor Symp. Quant. Biol.*, in press.

14. Bothwell, A.L.M., Paskind, M., Schwartz, R.C., Sonenshien, G.E., Gefter, M.L. and Baltimore, D. *Nature 290,* 65-67, 1981.

15. Tonegawa, S., Maxam, A., Tizard, R., Bernard, O. and Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A. 75,* 1485-1489, 1978.

16. Seidman, J.G., Nau, M.M., Norman, B., Kwan, S.-P., Scharff, M. and Leder, P. *Proc. Natl. Acad. Sci. U.S.A. 77,* 6022-6026, 1980.

17. Padlan, E.A., Davies, D.R., Pecht, I., Givol, D., and Wright, C. *Cold Spring Harbor Symp. Quant. Biol. 41,* 627-637, 1976.

18. Hieter, P.A., Max, E.E., Seidman, J.G., Maizel, J.V., Jr. and Leder, P. *Cell 22,* 197-207, 1980.

19. Seidman, J.G. and Leder, P. *Nature 276,* 790-795, 1978.

20. Maniatis, T., Hardison, R.C., Lacy, R.E., Lauer, J., O'Connell, C., Quon, D., Sin, G.K., Efstradiatis, A. *Cell 15,* 687-702, 1978.

21. Hieter, P.A., Korsmeyer, S.J., Waldmann, T. and Leder, P. *Nature 290,* 368-372, 1981.

22. Alt, F.W., Enea, V., Bothwell, A.L. and Baltimore, D. *Cell 21,* 1-12, 1980.

NUMBER AND ORGANISATION OF IMMUNOGLOBULIN VARIABLE REGION GENES IN THE MOUSE

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ABSTRACT To examine the germline Vκ repertoire, 'Southern blot' hybridisation analysis of mouse embryo DNA was performed with nine different cloned κ cDNA probes. Assessment of the sets of homologous germline Vκ genes revealed by these probes permitted two independent estimates for the total number of germline genes. Taken together, the results suggest a value of 90-320 genes and favor an important contribution by somatic mutation to the generation of diversity. Similar hybridisation analysis with four VH probes demonstrated the existence of three distinct germline VH gene families, one of which, 'the VH76 family', includes V region sequences of inulin-binding heavy chains. Sequences flanking VH genes were found to be conserved for the different members of one family but not of another. Analysis of clones bearing pairs of related VH genes argues against a random assortment of VH genes and favors the clustering of related genes, a typical spacing being 14-16 kb. Deletion mapping ordered the three gene families with respect to the CH locus.

INTRODUCTION

The question of the origin of antibody diversity is still not resolved despite significant advances in our understanding of immunoglobulin variable region genes. A specific V region amino acid sequence is now known to be encoded as several distinct germline elements which must be fused by recombination prior to expression (reviewed in ref. 1). Most of a light or heavy chain V sequence is encoded by a V gene proper, but the last 13-17 amino acids are encoded by a distant joining (J) gene, and a few amino acids immediately preceding the heavy chain J segment are encoded by a diversity (D) gene. While combinatorial join-

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ing of V, (D) and J genes augments germline diversity, particularly within the third hypervariable region of heavy chains, most variability can only be accounted for by the sequences of the V genes themselves. Are there sufficient Vκ and VH genes within the mouse germline to account for the vast array of expressed amino acid sequences, or do somatic mechanisms acting on relatively few germline sequences account for diversity, as appears to be the case for the limited number of λ sequences (2,3)? While the multiplicity of germline V genes has been established (4,5,6), the limited number of sequences examined made it difficult to assess the total repertoire. In this paper we summarise our recent studies bearing on the number of mouse Vκ and VH genes and the organisation of the VH locus.

RESULTS AND DISCUSSION

Vκ Repertoire

Sets of Germline Vκ Genes. To examine the germline Vκ repertoire, we have used filter hybridisation to size-fractionated restriction digests of embryo DNA (7). A 'Southern blot' permits counting of the number of fragments that bear Vκ genes homologous to particular Vκ sequences. Since the probes comprised nine different cloned κ cDNA sequences (8), the results provide a more substantial data base than hitherto available for extrapolating to the total number of germline Vκ genes (Cory, Tyler and Adams, submitted).

As shown in Fig. 1 for the κ sequence expressed in BFPC 61, each Vκ probe hybridised to several different fragments and different types of digest yielded comparable numbers of fragments. Certain fragments were labeled more strongly than others, presumably because they bear genes more closely related to the probe. Particular probes labeled up to 17 fragments, of which up to 8 were strongly labeled. The results favor the notion that the germline contains sets or families of related Vκ genes.

Extent of the Vκ Repertoire. A minimal estimate of the number of germline Vκ genes is the total number of different fragments observed, which was 64. We have made two independent estimates of the total number of germline genes. The first uses a statistical analysis of the hybridisation pattern repetitions to assess the number of independent gene sets. Our surprising observation that nine probes gave only four non-overlapping patterns suggests that the repertoire contains only about 5 gene sets (with 95% confidence limits of 4 to 10) that behave independently under our conditions. The average number of total bands detected per set was 16,

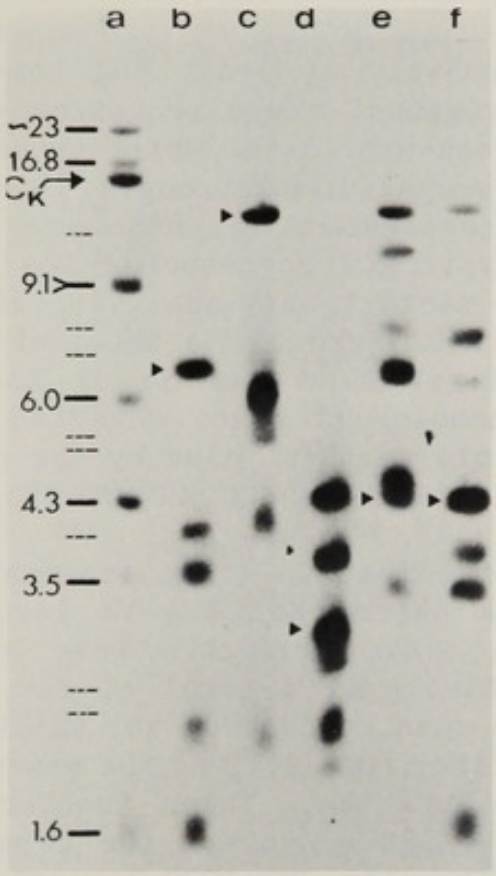

Figure 1. Vκ genes in BALB/c embryo DNA hybridising to κ cDNA clone B61κ16. The following digests were fractionated by electrophoresis on a 0.7% horizontal agarose gel and hybridised as described previously (18): a, EcoRI; b, EcoRI + Bam H1; c, Bam H1; d, Bam H1 + Hind III; e, Hind III; f, Hind III + EcoRI. Cκ fragments are arrowed.

and the actual number is unlikely to be more than 22. Assuming only one gene per fragment, this calculation suggests a total of (16-22) x (4-10) = 64-220 genes, the most probable number being 90. If Vκ genes prove to be less than 10 kb apart, which is considerably closer than the known VH genes (see below), certain fragments would bear more than one gene, and these values would be increased: for example, by a factor of 1.6 for a spacing of 5 kb.

The second estimate entailed relating our results to data on κ N-terminal amino acid sequences. Potter (9) assigned the 64 known BALB/c amino acid sequences to 26 "isotypes", each isotype having no more than two amino acid differences within the first 23 residues (FR1). If one considers only strong bands, six probes representing different isotypes each hybridised to a distinct set, and two different probes from the Vκ-21 isotype gave the same strong band set, as did two from Vκ-9. While these data indicate that strong band sets are similar to isotypes, they fit best with a somewhat wider κ grouping. Under our conditions,

strong bands are probably at least 86% homologous to the probe in framework regions, since sequences <80% homologous do not detectably cross-hybridise, while sequences which are 90% homologous cross-hybridise strongly. Consideration of known sequences suggests that 86% homology over all three framework regions typically corresponds to 3 or 4 differences in FR1. A statistical analysis of published κ sequences suggests that there are a total of about 39 amino acid sets of the first kind and 34 of the second. The number of closely homologous genes detected by our probes averaged 8.1. Multiplying this value by the probable number of groups gives estimates of about 320 and 275 genes respectively.

Summing the sizes of restriction fragments in the non-overlapping sets indicates that the Vκ locus is at least 419 kb long. Since these fragments bear at least 64 Vκ genes, our upper estimate of a total of 320 genes suggests that the locus could span up to 2000 kb, which is very large but still amounts to less than 0.1% of the mouse genome.

Need for Somatic Mutation. Taken together, our results suggest a value of 90-320 genes for the germline Vκ repertoire, rather lower than the 200-1000 suggested earlier (5). The extent of somatic mutation for the whole locus cannot yet be determined, because there is no direct measure of the total number of expressed Vκ sequences. Nevertheless, there is strong evidence that somatic mutation augments diversity within the Vκ-21 isotype. A Vκ-21 probe gave 8 strong bands in digests of both NZB and BALB/c DNA. Judging by their intensities relative to the Cκ band, these bands probably represent a maximum of 12 genes. Moreover, two probes from distinct Vκ-21 subgroups gave the same pattern, so both recognise the entire group rather than merely a subset. Since NZB mice express at least 22 different Vκ-21 amino acid sequences (10), somatic mutation must increase diversity at least two-fold. A similar conclusion was reached by Valbuena et al. (11), using saturation hybridisation.

VH Repertoire

Three Different VH Gene Families. Three cloned VH cDNA sequences from HPC 76, S107 and HOPC 1 (12) each revealed a different family of germline VH genes while the rearranged VH gene from ABLS-8.1 lymphoma cells (13) hybridised to the same genes as the HOPC 1 probe. Fig. 2 compares the three patterns for replicate EcoRI digests. The number of genes detected under these relatively stringent hybridisation conditions varies from a minimum of 4 in the 'VHS107 family' to 14 in the 'VH1/A8 family'. The total number and size of the fragments detected by the three probes indicates that the

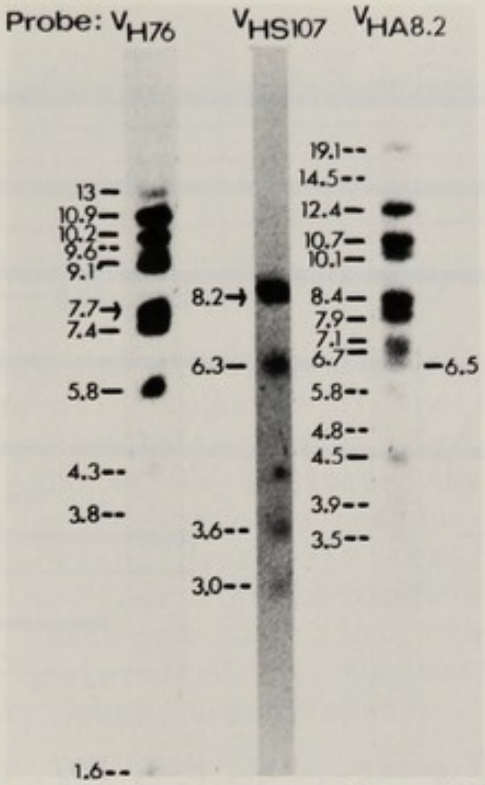

Figure 2. Three distinct germline VH gene families. Replicate fractionated EcoRI digests of BALB/c embryo DNA were hybridised to VH-specific fragments of HPC 76 and S107 cDNA clones (12) and with a fragment of a genomic clone bearing the rearranged VH gene in ABLS-8.1 (d in Fig. 3). Sizes are indicated in kb and weak bands by broken lines. The VH76 and VHS107 patterns have been described previously (18). The VH76 gene expressed in HPC 76 is arrowed. The spot between the second and third VHS107 bands is an artefact, seen only in this experiment.

VH locus consists of at least 29 VH genes within 346 kb of DNA.

Cloned VH Genes. To investigate the organisation of VH genes, we have cloned a number of genes from the VH76 family as well as two from the VHS107 family and one from the VH1/A8 family (6,13; Kemp et al., in preparation). Fig. 3 depicts selected clones, three of which bear a pair of VH genes. Hybridisation to restriction digests of the clones at high stringency with the three cDNA probes established that V1 and V2 are members of the VHS107 family and V3-V7 are members of the VH76 family. VH76 in clone M11 is the germline equivalent of the VH gene expressed in HPC 76 and preliminary evidence suggests that V2 is the germline equivalent of the expressed S107 VH gene. To investigate whether the clones might bear VH genes other than those shown in Fig. 3, we hybridised with the same three cDNA probes at low stringency and also examined heteroduplexes formed at low stringency. No other VH gene was detected.

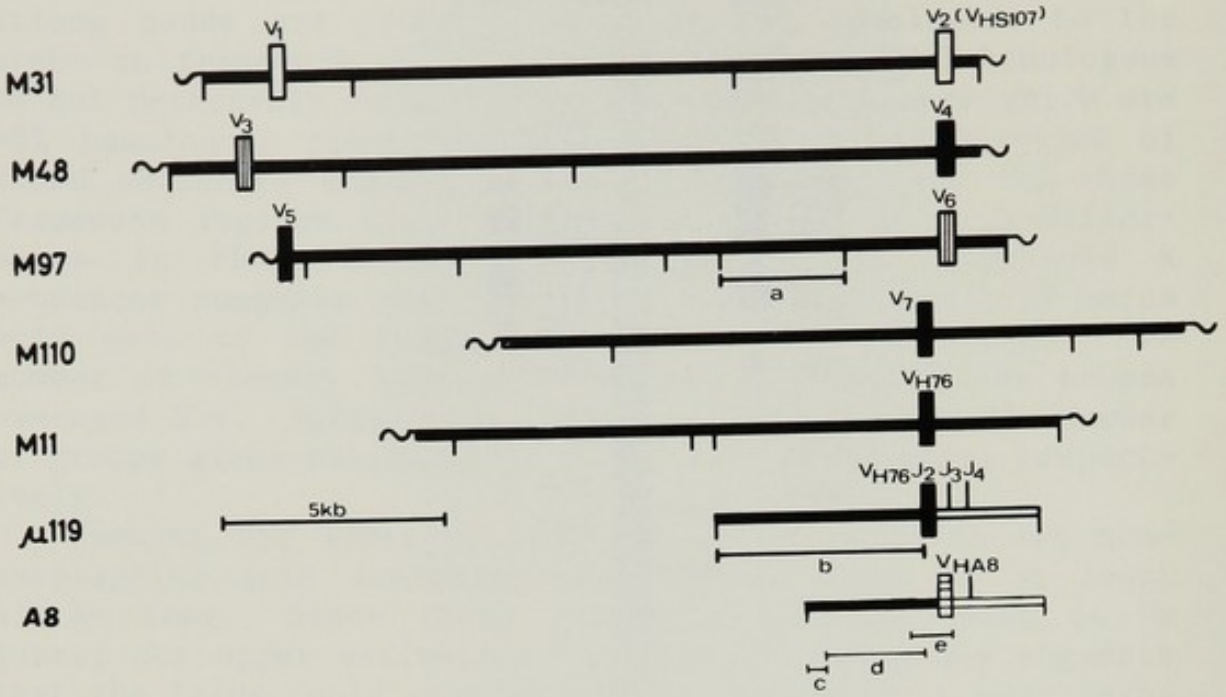

Figure 3. Cloned VH genes. M31, M48, M97, M110 and M11 are VH clones isolated from our EcoRI* library of large fragments of BALB/c mouse embryo DNA (6). μ119 bears the EcoRI fragment of HPC 76 DNA containing the expressed VH gene (13,14). A8 bears the EcoRI fragment of ABLS-8.1 DNA containing the rearranged VH gene (S. Gerondakis, unpublished). EcoRI sites are indicated by vertical lines. a-e indicate fragments used for hybridisation studies (see text). DNA derived from the VH locus is indicated as a solid bar, that from the JH locus by an open bar.

The two VHS107-related genes V1 and V2 are separated by 14 ± 2 kb while the VH76-related genes are separated by ~15 (V3 and V4) and ~14 kb (V5 and V6). Taken together, these results favor the notion that related VH genes are clustered and that a typical spacing of these VH genes is 14-16 kb (6).

The VH76 Gene Family. We have determined nucleotide sequences from three members of the VH76 family. The VH76 cDNA sequence (14) is identical to its germline counterpart in M11 over the region compared so far (Gly 16 to His 58), which includes the first complementarity determining region (CDR1) and 63% of CDR2. VH76 may therefore represent a gene which has undergone no somatic mutation. Significantly, the amino acid sequence encoded by the VH76 gene is extremely homologous to that of the VH sequences of four inulin-binding immunoglobulins characterised by Vrana, Rudikoff and Potter (15), as shown in Fig. 4. The very limited differences between the four inulin-binding heavy chains are confined to framework regions (Fig. 4) and can be accounted for

Figure 4. Comparison of inulin-binding heavy chains with VH76. The amino acid sequence of the ABPC 4A VH region is indicated in the one letter code. For the other sequences, identity with ABPC 4A is indicated by a horizontal line. Dots indicate the absence of a residue found in other heavy chains. The numbering system is that of Kabat (19). The sequences of the four inulin-binding heavy chains were determined by Vrana et al. (15); the VH76, V3 and V6 sequences were determined by nucleotide sequencing (14; O. Bernard and N. Gough, unpublished).

by single base substitutions (15). It is not known whether or not HPC 76 IgM binds inulin, but the HPC 76 amino acid sequence encoded by the VH gene proper is identical to that of ABPC 4A except where a glycine residue (GGT) replaces the sequence His 53 - Asn 54 (CAPyAAPy) within CDR2. The sequence change requires at least two base substitutions and a deletion of 3 bases. It does not result from somatic mutation of the germline gene because the VH76 gene in M11 also bears the GGT sequence. The HPC 76 amino acid sequence differs in both the D and J region from the ABPC 4A and UPC 61 sequences, which themselves differ at a single residue in the J region, presumably due to a somatic mutation (16). From the divergence in the D region, it seems likely that all these VH genes end with codon 93 rather than 95 as found for the VHS107 gene (17).

It is apparent from this comparison that the VH76 sequence would strongly cross-hybridise with the VH gene(s) encoding the inulin-binding sequences. Fig. 2 shows that there are at least five embryo DNA fragments labeled to about the same extent as the 7.7 kb fragment bearing the VH76 gene. It thus seems likely that inulin-binding VH sequences are encoded by several germline genes.

Fig. 4 shows that the V3 and V6 genes are extremely homologous to each other; only three bases differ in the 179 sequenced. V3 and V6 are only 78% homologous to VH76, the differences occurring in both framework and complementarity determining regions. Surprisingly, however, V3 and V6 map (at an unknown distance) 3' and 5' respectively to the VH76 gene (Kemp et al., in preparation). Thus the most closely

related VH sequences are not always adjacent.

VH Gene Flanking Sequences Are Conserved Within One VH Family But Not Another. Seidman et al. (5) found strong conservation of flanking sequences for the MOPC 149 Vκ gene 'family' and suggested that this was a characteristic of V genes to facilitate recombination between members of a group. We have found that the VH1/A8 family exhibits highly conserved flanking sequences, but that the VH76 family shows minimal conservation (Kemp et al., in preparation). For the VH1/A8 family, all flanking sequences tested (fragments c, d and e in Fig. 3) hybridised to all genes of the VH1/A8 family. In contrast, probes from the regions 5' to V6 and to VH76 (fragments a and b in Fig. 3) labeled different unique DNA sequences, indicating that neither region is detectably conserved within that family.

It seems likely that the evolution of the VH locus has involved numerous duplication events. Presumably each gene family represents amplification of a specific region of DNA bearing VH gene(s). We infer from our results that the VH76 family resulted from much earlier duplications than the VH1/A8 family and that the sequence of DNA between VH genes diverges much more rapidly than that of the V genes themselves.

The DNA Sequences Flanking VH Genes Varies Between Different Mouse Strains. If unequal crossing-over operates

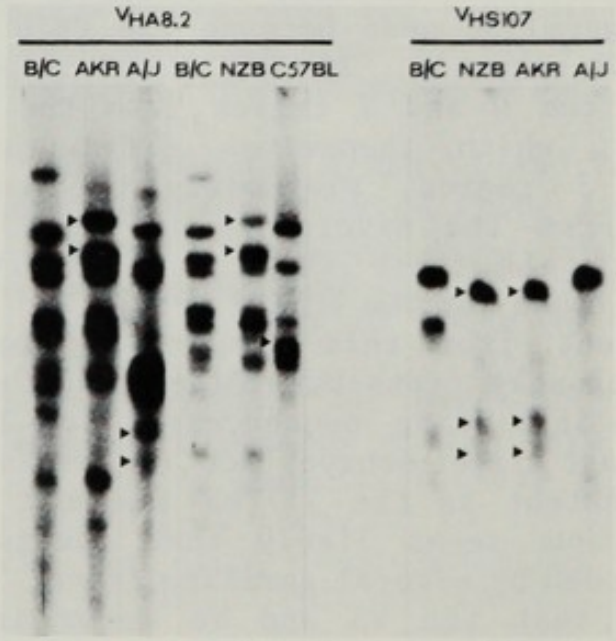

Figure 5. Strain variation in organisation of VH genes. The VHA8 genomic probe (fragment d in Fig. 3) and the VHS107 cDNA probe were hybridised to EcoRI digests of DNA from BALB/c embryos (12-14d), livers of NZ/B, AKR and A/J mice and a C57BL lymphoma (EL4). For VHA8.2, the first three tracks are from one gel and the last three from another.

on the tandem array of VH genes, the organisation of VH genes may vary within a species, for example, by expansion or contraction of different gene sets. We therefore compared the VH1/A8 and VHS107 gene sets within several strains (Fig. 5). The results indicate that the number of genes within each set does not vary greatly, but there is some variation in fragment sizes, those which clearly differ from BALB/c being indicated by arrows. We conclude that the sequence of flanking DNA has diverged in the different mouse strains.

Mapping VH Genes. Deletion mapping has enabled us to place the three gene families we have studied in the order VH76-VHS107-VH1/A8, with respect to the CH locus (Fig. 6). The mapping took advantage of the large deletions associated with VH-DH-JH joining. Convincing results can be obtained only with tumor cell lines having deletions which span a similar region on the active and inactive alleles. This is illustrated in Fig. 6 for HPC 76 DNA. Since all copies of certain members of the VH76 family are deleted from HPC 76 DNA (18), we infer that they map downstream from the expressed VH76 gene and that deletions span a similar region on the HPC 76 active and inactive alleles. Since no copies of any of the VHS107 genes remain in HPC 76 DNA, the VHS107 family maps closer to Cμ than the VH76 gene (18). Moreover, all members of the VH1/A8 family are totally absent from HPC 76 DNA and S107 DNA and all members of the VHS107 family are present in A8 DNA, consistent with the order shown (Kemp et al., in preparation).

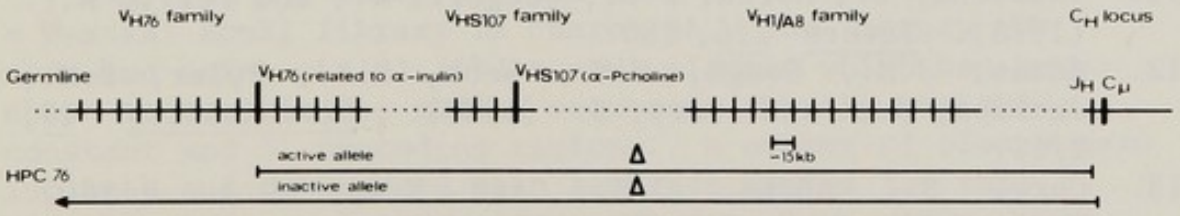

Figure 6. Ordering VH gene families by deletion mapping. The VH76 family includes sequences conferring specificity for β2→1-linked fructans (Gβ1 Fructan; ref. 9) and therefore able to bind inulin. The VHS107 sequence confers phosphorylcholine binding. The VHA8 family has no identified specificity. The deletions in HPC 76 for the active and inactive alleles extend respectively from VH76 to JH2, and from an unknown position 5' to the entire VH76 family to a site between the JH locus and Cμ gene (13).

ACKNOWLEDGMENTS

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REFERENCES

1. Adams, J.M. (1980). Immunology Today 1, 10.
2. Weigert, M.G., Cesari, I.M., Yankovich, S.J., and Cohn, M. (1970). Nature 228, 1045.
3. Bernard, O., Hozumi, N., and Tonegawa, S. (1978). Cell 15, 1133.
4. Lenhard-Schuller, R., Hohn, B., Brack, C., Hirama, M., and Tonegawa, S. (1978). Proc. Natl. Acad. Sci. USA 75, 4709.
5. Seidman, J.G., Leder, A., Nau, M., Norman, B., and Leder, P. (1978). Science 202, 11.
6. Kemp, D.J., Cory, S., and Adams, J.M. (1979). Proc. Natl. Acad. Sci. USA 76, 4627.
7. Southern, E.M. (1975). J. Mol. Biol. 98, 503.
8. Gough, N.M., Webb, E.A., Cory, S., and Adams, J.M. (1980). Biochemistry 19, 2702.
9. Potter, M. (1977). Adv. Immunol. 25, 141.
10. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978). Nature 276, 785.
11. Valbuena, O., Marcu, K.B., Weigert, M., and Perry, R.P. (1978). Nature 276, 780.
12. Adams, J.M., Gough, N.M., Webb, E.A., Tyler, B.M., Jackson, J., and Cory, S. (1980). Biochemistry 19, 2711.
13. Cory, S., Adams, J.M., and Kemp, D.J. (1980). Proc. Natl. Acad. Sci. USA 77, 4943.
14. Bernard, O., and Gough, N.M. (1980). Proc. Natl. Acad. Sci. USA 77, 3630.
15. Vrana, M., Rudikoff, S., and Potter, M. (1978). Proc. Natl. Acad. Sci. USA 75, 1957.
16. Gough, N.M., and Bernard, O. (1981). Proc. Natl. Acad. Sci. USA 76, in press.
17. Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). Cell 19, 981.
18. Cory, S. and Adams, J.M. (1980). Cell 19, 37.
19. Kabat, E.A., Wu, T.T., and Bilofsky, H. (1979). U.S. Dept. Health, Education and Welfare, NIH Publication No. 80-2008.

ORGANIZATION OF LIGHT CHAIN VARIABLE REGION GENES

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INTRODUCTION

The mouse κ light chain immunoglobulin variable region genes comprise a large multigene family. This family may be divided into multiple groups, each of which has been proposed to constitute a subgroup based on relatedness of amino acid sequences. When a Southern analysis is done of the mouse genomic DNA using a variable region probe, multiple bands are seen. This is consistent with the proposal that a subgroup in the genome consists of perhaps six to ten cross-hybridizing genes. The origin of these sets of variable region genes is open to conjecture. Proposals include recombination between variable region genes, somatic mutation, and assembly of the genes from a set of minigenes representing variable region gene segments. Here we present a preliminary report of work which may, when complete, provide resolution of the different possibilities.

RESULTS

DNA from the mouse myeloma MOPC 21 was used to construct a partial EcoRI library in Charon 4A. The library was screened using pL21-1 (1), a cDNA plasmid which has been shown to contain the coding sequences for the variable, constant and 3' noncoding regions. A number of clones were isolated and rescreened with separate probes for the variable and constant regions. Twenty-eight clones were isolated which contained variable region sequences and did not hybridize to constant or J-region probes. We assume that these contain variable regions that are not rearranged with respect to the embryonic pattern.

Further analysis of the twenty-eight variable region clones was done using restriction enzyme fragments of pL21-1. As seen in Fig. 1, this may be cleaved to yield probes which roughly correspond to the different framework regions (FRs) of the variable region. When these were used in a dot-blot screening, they showed different patterns of hybridization for the clones. The results of these experiments can be seen in Fig. 2 and are summarized in Table 1.

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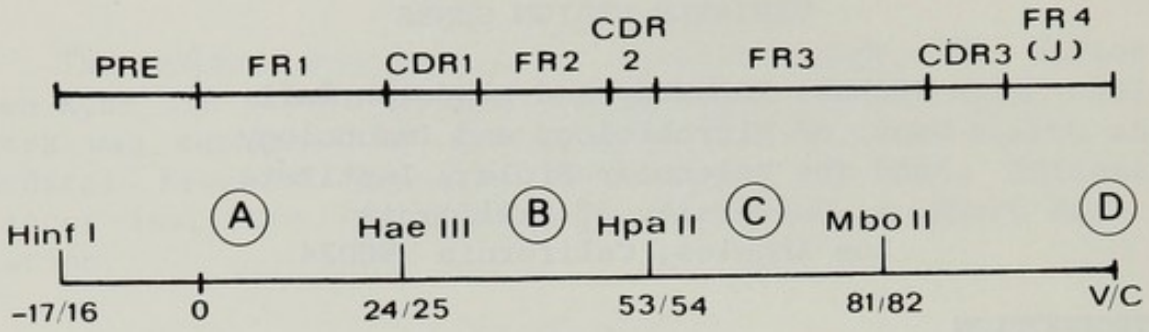

Figure 1. Hybridization Probes from MOPC 21 κ light chain cDNA clone, pL21-1 (1). A partial restriction map of the pL21-1 variable region is shown on the lower line. The numbers below the enzyme sites indicate amino acid positions. The upper line shows an alignment of the positions of the framework and complementarity-determining regions. The encircled letters above the lower line show the positions of the probe fragments used in the dot-blot hybridizations. A is thus an FRI probe, B is FR 2+ CDR1, C is FR3 and D is FR4(J) and C_K. Not shown is E, which is from the 3' untranslated region of the κ light chain mRNA.

One group of six clones hybridized to all of the framework region probes. Three clones hybridized to only the FR 1 probe, ten hybridized to only the FR 2 probe and nine hybridized to only the FR 3 probe. None of the clones hybridized to J, C_K or 3' untranslated region probes. In order to determine if the different groups of clones represented multiple isolates of identical genome segments, restriction enzyme mapping was done. The restriction maps of the clones show no obvious similarities and indicate these to be different DNA segments (results not shown). Southern blot analysis of the mouse genomic DNA showed eight bands when the entire variable region from pL21-1 was used (results not shown). When Southern blots were done with the six clones which hybridized to all FR probes, five of the clones contained bands which could be matched to genomic DNA bands. It therefore seems likely that these five clones are isolates of the bands seen on the genomic DNA blots. These have been proposed to represent the variable region subgroup for a particular probe (2).

DISCUSSION

It appears that a given set of framework regions associated in a variable region may also exist in other forms in the genome. We consider three possibilities to account for

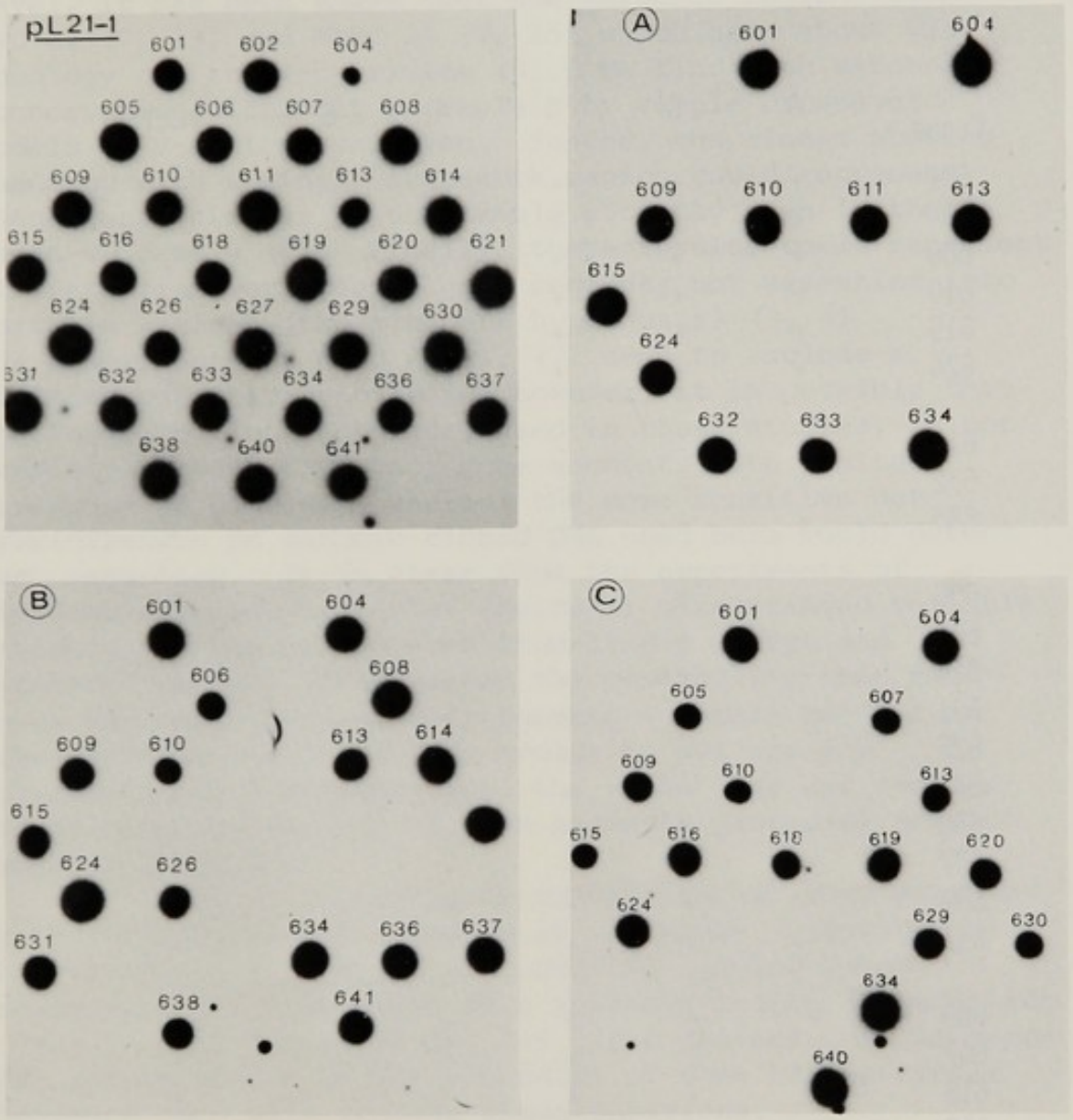

Figure 2. Results of Dot-Blot Hybridizations. Large plaques were made by spotting phage stocks of recombinant DNA clones on lawns of DP50-supF (it is important to use the minimum amount of phage necessary to obtain total lysis), making filter blots, hybridizing these to the appropriate probe and exposing an autoradiograph of the hybridized filter. The probes used are described in the text and Fig. 1.

these findings. First, the assortments observed could represent hybridization of the probe with parts of other variable regions, in conjunction with different framework regions. Comparative protein sequence analysis makes it clear that V-regions from different subgroups can contain identical framework regions (3, 4). At the nucleotide

Clone	A	B	C	D	E	pL21-1
601	+	+	+	-	-	+
604	+	+	+	-	-	+
609	+	+	+	-	-	+
610	+	+	+	-	-	+
615	+	+	+	-	-	+
624	+	+	+	-	-	+
611	+	-	-	-	-	+
632	+	-	-	-	-	+
633	+	-	-	-	-	+
606	-	+	-	-	-	+
608	-	+	-	-	-	+
614	-	+	-	-	-	+
621	-	+	-	-	-	+
626	-	+	-	-	-	+
631	-	+	-	-	-	+
636	-	+	-	-	-	+
637	-	+	-	-	-	+
638	-	+	-	-	-	+
641	-	+	-	-	-	+
605	-	-	+	-	-	+
607	-	-	+	-	-	+
616	-	-	+	-	-	+
618	-	-	+	-	-	+
619	-	-	+	-	-	+
620	-	-	+	-	-	+
629	-	-	+	-	-	+
630	-	-	+	-	-	+
640	-	-	+	-	-	+

Table 1. Summary of Dot-Blot Hybridizations. A, B, C, D, and E refer to probes described in Fig. 1. pL21-1 is described in the text. A (+) sign denotes hybridization and a (-) sign denotes no hybridization. Clones 602 and 627 are C_K embryo clones and are included as controls. Clones 613 and 634 are isolates identical to, respectively, clones 611 and 624, and are not included in this summary.

level, it has been shown that the variable regions from MPC 11 (V_K 19) and MOPC 21 (V_K 15) which have about 82% homology can cross-hybridize (5). We find such extensive conservation difficult to explain by simple crossover models of V-region evolution. Second, the clones showing reaction with a single framework region could represent pseudogenes similar to an example recently seen in the human V_K family (6). Finally, these results could represent discrete framework region gene segments not assembled into variable regions (the minigene hypothesis) (3, 4) The experiments of Joho et al, (7) seem to exclude a minigene assembly hypothesis; however, it is possible that the Southern blot technique, used in those studies, is not sensitive enough to detect gene segments with limited stretches of homology, whereas the more sensitive dot hybridization on genomic cloned DNA used here would detect such sequences. It is clear from the experiments of Nishioka and Leder (8) that identical unrearranged variable region genes can be isolated from 12-day embryo and plasmacytoma DNA. This raises the possibility that minigenes may have been actively forming variable regions at some point in immunoglobulin evolution but are now quiescent. If this were true, the mechanisms and reasons for the discontinuation of such assembly processes are not immediately obvious.

The appeal of the minigene hypothesis has been enhanced by the discovery of D segments in the mouse V_H system (9) thus providing an example of a somatic joining event necessary for construction of a variable region (in addition to the V-J joining necessary for light chains). It is hoped that determination of the sequences of some of the clones discussed here will resolve these questions.

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REFERENCES

1. Wall, R., Gilmore-Hebert, M., Higuchi, R., Komaromy, M., Paddock, G., Strommer, J. and Salser, W. *Nucleic Acids Res.* 5, 3113-3128 (1978).

2. Seidman, J.G., Leder- A., Edgell, M.H., Polsky, T., Tilghman, S.M., Tiemeier, D.C. and Leder, P. *Proc. Nat. Acad. Sci. U.S.A.* 75, 3881-3885 (1978).

3. Kabat, E.A. *J. Immunol.* 125, 961-964 (1980).

4. Kabat, E.A., Wu, T.T. and Bilofsky, H. *Proc. Nat. Acad. Sci. U.S.A.* 75, 2429-2433 (1978).

5. Rabbitts, T.H., Matthyssens, G. and Hamlyn, P.H. *Nature* 284, 238-243 (1980).

6. Bentley, D.L. and Rabbitts, T.H. *Nature* 288, 730-733 (1980).

7. Joho, R., Weissman, I.L., Early, P., Cole, J. and Hood, L. *Proc. Nat. Acad. Sci. U.S.A.* 77, 1106-1110 (1980).

8. Nishioka, Y. and Leder, P. *J. Biol. Chem.* 255, 3691-3694 (1980).

9. Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. *Cell* 19, 981-992 (1980).

THE NUMBERS AND REARRANGEMENTS OF GENES IN THE T15 κ GROUP

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ABSTRACT. Cloned cDNAs for two κ chains which are in different κ groups, those expressed in the T15 and M21 myelomas, anneal to a completely overlapping set of restriction fragments from mouse DNA. Although the amino acid sequences of these κ chains have an overall difference of 31%, these differences are mainly located in FR1 and CDR1(which differ by 57%), whereas FR2, CDR2, and FR3 are only 15% different. The results suggest that the M21 Vκ sequence may have evolved from the T15 group. A further implication of the results is that the definition of κ groups useful for estimating the total number of Vκ genes by DNA annealing should be broader than the definition derived from amino acid sequences, thus reducing the total number of Vκ genes. The rearrangements of the T15 and M21 Vκ genes in cells expressing these genes were studied by Southern blotting. The restriction fragments in germ line DNA bearing the Vκ genes expressed in T15 and M21 cells are tentatively identified. Finally, an attempt to order a few Vκ gene families by deletion mapping led to results in conflict with published results (32).

INTRODUCTION. The current estimates of the total number of genes for immunoglobulin (Ig) variable (V) regions present in the genome have been derived by a combination of the analysis of DNA and of amino acid sequences of Igs. Cloned Ig V genes have been used to detect related Ig V genes, and then the total number of V genes has been derived by estimating what fraction of the V genes would be related enough to anneal with a given V DNA sequence. Often it has been assumed that one V DNA sequence will anneal with all the V genes coding for one V region group, as defined by amino acid sequencing (1,2). The total number of groups estimated to exist (50-100 for Vκ in the mouse (2-5)) is multiplied by the number of V genes detected in one group, which frequently is 6-10 (2,4,6). The estimated number of Vκ genes generally cited is between 300 and 1000 (2,4,5).

The definition of groups by amino acid sequencing and by DNA annealing are, however, not necessarily the same. We report here that two cloned cDNAs coding for two Vκ sequences which

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differ greatly in their overall amino acid sequence, those expressed in the myelomas TEPC15 (T15) (κ22 group), and in MOPC 21 (M21) (κ15 group) (1) appear to detect a completely congruent set of bands on blots of restriction fragments of genomic DNA.

RESULTS AND DISCUSSION. Fig. 1 (left side) shows the restriction maps of the T15κcDNA clones used in these experiments: pT15κ which contains DNA sequences coding for the amino acids in positions 25-203 (consecutive numbering) of the T15κ chain, and two subclones coding for T15Vκ sequences alone, pT15Vκ, or Cκ sequences alone, pCκ. The restriction map of the V region, and the partial nucleotide sequence (Legend to Fig. 1) of the region encoding amino acids in positions 36-46 both agree with the T15 amino acid sequence (7).

Number of genes in the T15 Vκ group. When the pT15Vκ subclone was annealed to DNA blots (14) of fragments produced by digestion of Balb/c liver DNA with BamHI or EcoRI, 6-8 relatively intense bands, and 4 or more faint bands were detected (Fig. 2). Liver cells contain Ig genes in the germ line context, as in sperm or embryo cells (15-18). The number of T15 Vκ genes detected is similar to the numbers of genes detected in other κ groups (2,4,6), except in the MOPC167 group, which appears to contain only one Vκ gene (19).

When a probe coding for the entire M21κ chain, pL21-1 (given by W. Salser) (20), was annealed with BamHI and EcoRI fragments of genomic DNAs, the same bands were detected as with pT15κ, which codes for both the T15Vκ and Cκ regions (Fig. 3). The relative intensity of the bands detected by the two probes was quite similar, although there are some differences (see below).

The congruency of the bands detected was very surprising because the amino acid sequences of the M21 and T15 Vκ regions differ by 31% (7,22). Furthermore, the framework 1 (FR 1) sequences of the T15 and M21 κ chains differ at 13 amino acid residues, i.e. by 57%, and therefore, these κ chains are in different κ groups (1). However, these κ chains share extensive sequence homology in FR2, CDR2, and FR3. In these regions these two proteins are only 15% different, and contain three stretches of identical sequences of 11-14 amino acids long.

To ensure that the T15 and M21 probes are indeed different, the restriction enzyme maps of these two clones were compared.

The cloned T15Vκ sequence does not have a MspI site, whereas the M21Vκ sequence does; and (2) the size of the HaeIII fragments obtained from the two plasmids differ from each other (data not shown). The restriction map of pT15Vκ is similar to that predicted by the DNA sequence of the S107 κ cDNA (7). (The amino acid sequences of the T15 and S107 κ chains appear to be identical (7)). Furthermore, the partial DNA sequence determined for amino acid residues 36-46 agrees with the T15, but not with the M21 protein sequence.

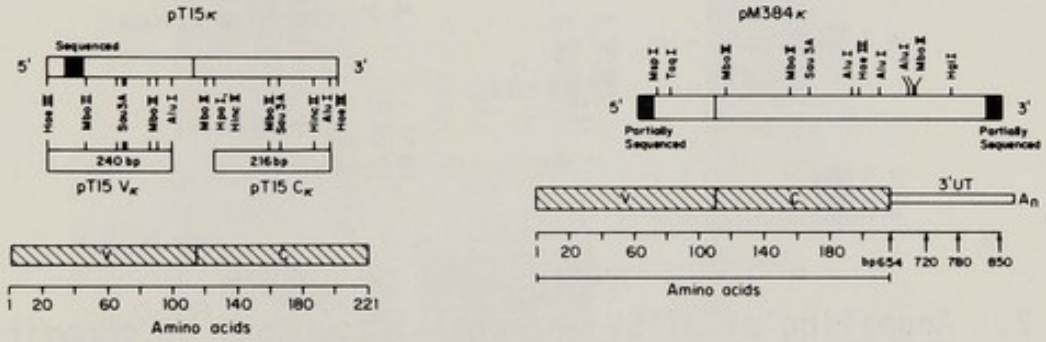

Fig. 1. Restriction maps of cDNAS for κ chains synthesized in the myelomas T15 and MOPC384(M384). Double-stranded cDNAs were transcribed from mRNAs by reverse transcriptase and DNA polymerase I (given by M. Modak) (8). T15 κcDNA was digested with HaeIII, which cuts approximately at the nucleotide sequences coding for the alanines at positions 25 and 203 (7,9), ligated to oligonucleotides containing the recognition sequence for HindIII, and ligated into the HindIII site of pBR322 (10). M384κ cDNA was digested with Hinf, which cuts approximately at the nucleotide sequence coding for the serine at position 62 (11), and in the 3' untranslated region 14 nucleotides 5' to the poly(A) sequence (9). The M384 cDNA was made blunt-ended by treatment with S_1 nuclease and DNA Pol I (12), ligated to HindIII linkers, and inserted into the HindII site of pBR322. The DNA sequence of the T15 cDNA was partially determined (13) by sequencing one strand of the region coding for amino acid positions 36-46. The nucleotide sequence determined and the corresponding amino acid sequence is:

```
     val his tyr leu ala trp tyr gln lys lys pro
     GTG CAC TAC TTG GCT TGG TAC CAG AAG AAG CCG
       A           A                       A
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Subclones of pT15 κ were produced by digestion with AluI and HpaI, ligation to HindIII linkers, and insertion into the HindIII site of pBR322.

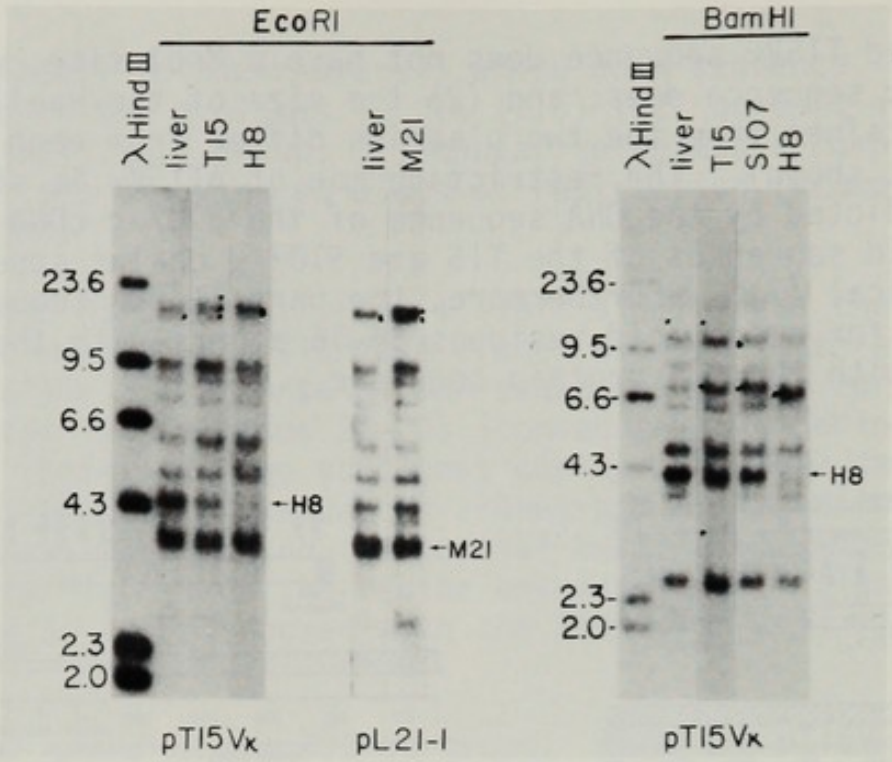

Fig. 2. Annealing of pT15V and pL21-1 to blots of genomic DNAs. Genomic DNAs from the indicated cells were digested with BamHI or EcoRI, electrophoresed in 0.7% agarose gels, blotted to nitrocellulose (14), and annealed (21) in 6XSSC, 50% formamide at 42°C with a nick-translated DNA insert, prepared from pT15Vκ (the V region subclone) by digestion with BamHI and EcoRI. The blots were washed in 0.1XSSPE (1 X SSPE=0.15MNaCl/10mMNaPO$_4$/1mMNa$_3$EDTApH7.0) at 52°C. After autoradiography, the annealed probe was removed by heating at 68°C in annealing mix, and the blot was annealed with nick-translated pL21-1 (containing M21 Vκ and Cκ sequences). The probes used are indicated at the bottom of the lanes. The positions of the fragments that anneal with a Cκ probe are indicated with small dots drawn in the lanes. The position of the Vκ fragment in liver DNA that appears to contain the Vκ gene expressed in H8, S107, and T15 cells (see text below) is indicated by:←H8; and the position of the fragment in liver DNA that appears to contain the Vκ gene expressed in M21 cells by:←M21. The sizes of the marker DNA fragments (in kb) produced by digestion of λ DNA with HindIII are indicated. Although T15, S107, and H8 κ chains appear to have the identical amino acid sequence (7,26), the expressed Vκ gene in T15 and S107 cells is on a 7.0 kb Bam fragment, whereas the expressed Vκ gene in H8 is on a 6.7 kb Bam fragment. This result has been reproduced 5 times (though not in the blot shown in Fig. 3 , apparently because of variations in the amount of DNA loaded onto this gel). It is possible that a new Bam site was introduced by mutation at a site 2 kb 5' to the T15 Vκ gene expressed in H8 cells, or

that a different, but closely related Vκ gene is expressed in H8 cells. The latter possibility appears less likely since the same germ line Vκ fragment has decreased in intensity in T15, S107, and in H8 cells (see text below). This 300 base pair difference in size cannot be caused by joining of the T15Vκ gene to a different Jκ segment in H8 cells than in S107 cells, since in S107 cells the Jκ segment closest to Cκ ($J\kappa_5$) is expressed (7).

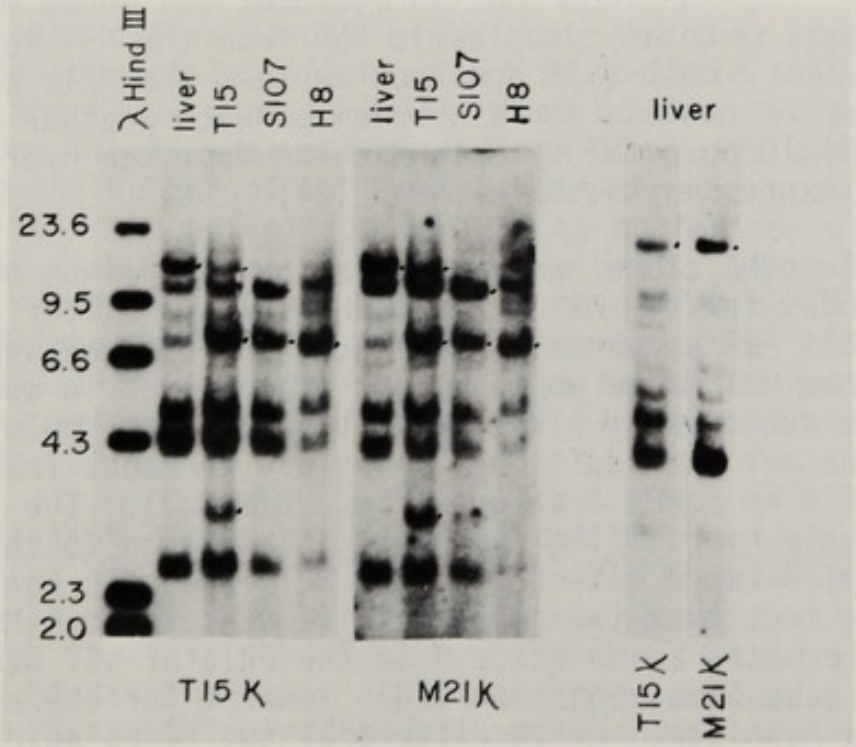

Fig. 3. Annealing of pT15κ and pL21-1 to blots of genomic DNAs. The HindIII insert from pT15κ (containing both V and C regions), and the plasmid, pL21-1 were annealed sequentially (as in Fig. 2) to DNA blots containing BamHI fragments of genomic DNAs (two blots on left) and EcoRI fragments of liver DNAs (two lanes on right). The fragments that anneal with the Cκ probe are indicated with black dots on the right side of each lane. The probes used are indicated at the bottom of the lanes. M21κ=pL21-1.

To further examine the question of whether the T15Vκ and the M21Vκ sequences anneal with each other, pT15Vκ was hybridized to a RNA blot containing RNA from a hybridoma (Id 43) expressing the M21κ gene contributed by the fusing cell, P3-N-1-Ag (NS1) (23,24), and also containing poly(A)+ RNA from T15 cells. The T15Vκ probe hybridized well with both mRNAκs (Fig. 4).

Fig. 4. Hybridization of pT15Vκ with ctyoplasmic RNAs from cells expressing T15 and M21 κ chains. RNA was denatured with glyoxal and DMSO, and electrophoresed on an agarose gel in 10 mM phosphate pH 7 (25). RNA was blotted to diazophenylthioether paper (B. Seed, unpublished), and hybridized with an insert prepared from pT15Vκ, as in Fig. 2. The lanes contained (from left to right): (1) one μg poly(A)+ RNA from the T15 myeloma; (2) 20 μg of total cytoplasmic RNA from the NS1 fusing cell (NS1 does not express the M21κ chain unless it is hybridized with another cell (23)); and (3) 10 μg total cytoplasmic RNA from the hybridoma Id 43, which expresses the M21κ chain (24).

Since the M21κ cDNA clone extends past the N-terminus of the κ chain, it codes for the M21 FR1 sequences which differ greatly from the T15 FR1 sequences (by 57%); therefore, we would expect that the M21 probe would detect other genes in addition to those detected by pT15κ. But the M21 probe only appeared to detect one additional faint 6.6 kb EcoRI fragment and a faint 6.4 kb BamHI fragment (Figs. 2 and 3). The fact that no strongly hybridizing fragments in addition to those detected by pT15κ were detected by the M21κ probe is consistent with the fact that the amino acid sequence of the M21 FR1 differs by 8 or more amino acids from the FR1s of all other κ chains which have been sequenced (26) (except for one synthesized in a hybridoma, which differs by two amino acids (27)), and suggests that there may be no identifiable κ15 group, but rather that the M21 κ chain is related to and evolved from the κ22 group.

One can only speculate about how the FR1 of the M21 κ chain became so different from the FR1 of the T15 κ chain. Perhaps the M21Vκ gene underwent unequal recombination, as has been inferred to have occurred between two tandem human globin genes (28). Komaromy and Wall (in this volume) have isolated a large number of Vκ genes which they detected with pL21-1. Presumably among these Vκ genes are the genes for the κ22 group. It will be very interesting to determine if the M21 and K22 V genes are located in one cluster on the chromosome, and also to compare the nucleotide sequence of the M21 and T15 FR1 and 5' flanking sequences to attempt to understand the evolution of the M21 Vκ sequence.

We conclude that the definition of κ groups, and therefore

the estimated number of κ groups, which has been derived from amino acid sequencing data, is not always in agreement with the κ groups defined by DNA annealing, i.e. sequences which can anneal with each other. It appears that DNA annealing can readily detect sequences that code for κ chains whose amino acid sequences differ by 31%, depending on the location of the differences, whereas amino acid sequences that differ by 5 or more amino acids, i.e. by 5%, are placed into different κ groups (1). Furthermore, κ groups are defined by the FR1 sequence. Any two sequences which differ by three or more amino acids in FR1, i.e. by 13% or more, are placed into different groups. The T15 and M21 κ chains differ by 57% in FR1, and yet they appear to cross hybridize completely. Although it is possible that the evolution of the M21Vκ gene is unlike that of other Vκ genes, it seems more likely that the definition of groups useful for calculating the number of Vκ genes by DNA annealing should be broader than the definition derived by comparison of amino acid sequences (see Cory et al. in this volume). Since DNA annealing can readily detect sequences which differ by 15%, the number of Vκ groups useful for estimating the total number of Vκ genes should probably be reduced by a factor of about two, to 25-50 and hence, the total number of Vκ genes is probably no more than 200-400.

Identification of the germline T15 and M21 Vκ fragments; Rearrangement of T15 and M21 Vκ genes. The T15 Vκ sequence appears to be the predominant light chain sequence expressed in antibodies specific for phosphorylcholine (PC) in Balb/c mice (1,29). Since some other strains of mice produce a different κ chain in PC-specific antibodies, and also contain restriction fragments detected with pT15Vκ that differ in size from those found in Balb/c (29), we attempted to determine which germ line DNA fragment contained the T15 and M21 Vκ genes. We first compared the intensity of the bands detected in liver DNA with the two probes. The 4.5 kb EcoRI and the 4.2 kb Bam fragments (Fig. 2), usually appear more intense when detected with the T15 probe than when detected with the M21κ probe. The 10 kb Bam and the 3.7 kb EcoRI fragments often appear more intense when detected by the M21 probe than with the T15 probe (Fig. 2 and 3)

If the fragments in liver DNA that anneal most strongly with the T15 and M21 probes correspond to the Vκ genes expressed in the T15 and M21 myelomas, respectively, it may be possible to detect a decrease in the intensity of these bands in DNA from cells which express these genes, since in these cells these Vκ genes would be rearranged. T15, S107, and H8 are three myelomas which probably express the germ line T15Vκ gene, since their amino acid sequences are identical as far as has been determined (1,7). DNA from H8 cells contains only one Cκ BamHI or EcoRI

fragment (data not shown), which is rearranged relative to germ line DNA, and also anneals with the probe containing only T15Vκ (Fig. 2). Tentatively, we hypothesized that H8 cells may be haploid for the chromosome which bears the κ chain genes (chromosome 6) (30), or perhaps, haploid for only a portion of it. Figs. 2 and 3 show that the DNA fragments, hypothesized above to contain the germ line equivalent of the expressed T15Vκ gene are absent or much reduced in intensity in the H8 DNA pattern. Generally, these fragments also appear to be less intense in T15 and S107 DNA (Fig. 2). These results are consistent with the hypothesis that these fragments contain the germ line T15Vκ gene. These fragments, which bear the germ line T15Vκ genes, are present in strains of mice which do not express the T15Vκ sequence (see ref. 29).

The myeloma M21 has at least two Cκ genes (data not shown), so we would not expect an absence of the fragment containing the putative germ line M21 Vκ gene, although by comparing the intensity of the 3.7kb EcoRI fragment (unpublished data and Fig. 2; indicated with ←M21) detected in liver with that detected in M21 DNA, it appears that the 3.7kb fragment may be less intense in M21 DNA.

When the karyotype of H8 cells was analyzed to determine if these cells do indeed contain only one chromosome 6, three copies of chromosome 6 were detected. In addition, these myeloma cells contain a number of small pieces of unidentified chromosomes. Since H8 cells contain only one Cκ fragment, and since deletion of one T15Vκ gene was observed, these cells may contain three copies of the chromosome 6 bearing the expressed T15Vκ gene. However, in addition, they may contain pieces of the homologous chromosome 6 bearing some Vκ genes, but no Cκ gene.

The joining of V and J genes is believed to occur by deletion of the DNA lying between these genes (31,32), hence some Vκ genes should be deleted from myeloma cells containing only one κ chain chromosome. In H8 DNA the only κ22 gene fragments that appeared to be deleted were the intensely annealing fragments which appear to contain the expressed Vκ gene. Hence, the Vκ gene expressed in H8, and presumably also in T15 and S107 cells, may be located in a position 3' position relative to other members of the T15 group of Vκ genes. We also attempted to detect deletion of other groups of Vκ genes from the H8 cells. Cloned cDNAs for κ chains expressed in the myelomas: M321 (κ21 group) (given by S. Tonegawa), M41 (κ9) (given by J. Seidman and P. Leder), M384 (κ8) (Fig. 1), and M167 (κ24) (given by P. Early and L. Hood) were annealed with restriction fragments from H8

and liver cells. Only one of these groups (the M384 κ group) was deleted from H8 cells. Therefore, we tentatively hypothesized that the κ22 genes were located 3' to these other Vκ genes, except for the M384Vκ genes. But this result disagrees with the finding that NP2 cells (which also contain only one rearranged Cκ gene, and express a member of the M321 κ group) also have not deleted the T15 κ group (32; J.S. and K.J.S., unpublished). This latter result predicts that the T15Vκ genes are located on the 5' side rather than the 3' side of the M321 Vκ genes, i.e. in the order T15...M321...Cκ. Again, however, when the karyotype of NP2 cells was examined, two or three copies of chromosome 6 were found per cell, in addition to small pieces of unidentified chromosomes. It appears that the use of myeloma cells to order the Vκ genes by deletion mapping will be unreliable, or alternatively, not all V genes located between the expressed V and J-C genes are deleted during V-J joining.

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REFERENCES

1. Potter, M. (1977) Adv. Immunol. 25, 141.
2. Seidman, J.G., Leder, A., Nau, M., Norman, B. and Leder, P. (1978) Science 202, 11.
3. Weigert M. and Riblet, R. (1976) Cold Spring Harbor Symp. Quant. Biol. 41, 873.
4. Valbuena, O., Marcu, K.B., Weigert, M. and Perry, R.P. (1978) Nature 276, 780.
5. Gottlieb, P.D. (1980) Mol. Immunol. 17, 1423.
6. Lenhard-Schuller, R., Hohn, B., Brack, C. Hirama, M. and Tonegawa, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4709.
7. Kwan, S.P., Rudikoff, S., Seidman, J.G., Leder, P. and Scharff, M.D. Manuscript submitted.
8. Wickens, M.P. Buell, G.N. and Schimke, R.T. (1978) J. Biol. Chem. 253, 2483.
9. Hamlyn, P.H., Brownlee, G.G., Cheng, C.C., Gait, M.J. and Milstein, C. (1978) Cell 15, 1067.

MOUSE KAPPA CHAIN POLYMORPHISM

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ABSTRACT: Differences in kappa chain expression have been demonstrated by several methods and many of these have singled out the strains AKR, RF, PL and C58 as having a distinctly different repertoire of Vk genes (or Vk regulatory apparatus). More recently, additional Vk polymorphisms have subdivided the inbred strains into a total of five haplotypes. Work in the authors' laboratory has concentrated on the nature of the Ef2 marker affecting the expression of characteristic bands in normal light chain IF-profiles. Complete V-region sequences of 4 Ef2-related light chains indicate they could potentially be coded by a single Vk gene if allowance were made for somatic mutation of single framework residues. Alternatively, the light chains could be coded by a cluster of virtually identical Vk genes which must segregate as a unit in genetic studies.

INTRODUCTION

Mouse kappa chains provide an excellent model for studying the evolution and regulation of a multigene family. These chains constitute over 90% of the light chains of mouse immunoglobulin and they are believed to be encoded by several hundred to a thousand germ-line V-genes (1). Amino acid sequence analysis has indicated that kappa chains may be grouped into subsets (groups) of closely related proteins on the basis of shared amino acid substitutions and gaps (2). Proteins belonging to a group possess fewer than three differences over the first 23 residues (3). On the basis of available N-terminal sequence data from myeloma light chains the number of groups has been estimated to be around 50 (4). Each group of kappa chains in turn may be divisible into a small number of subgroups on the basis of shared substitutions throughout the V-region. The most well-studied group to date is the Vk-21 group. Complete V-region sequences of 31 members of the Vk-21 group has revealed the existence of at least 6 subgroups (5,6). The difference between subgroups may be as little as two framework substitutions (eg. Vk-21E and Vk-21F) or as great as 22 residues (eg. Vk-21A and Vk-21F). Each subgroup may be coded by a single Vk gene or a cluster of virtually identical Vk genes. Studies on germ-line DNA suggest that the number of restriction fragments containing sequences coding a Vk group correspond approximately to the number of subgroups (7). This

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would support the notion that each Vk subgroup may be coded by a single Vk gene.

Studies on polymorphism of kappa chains at the level of expression and at the DNA level provide a unique opportunity to gain insight into the mode of regulation of V-gene expression. Differences in the content of Vk genes, long predicted from expansion-contraction models of light chain evolution, would be expected to result in the disappearance of certain subgroups from the immunoglobulin pools of the animal. Such differences provide a means of identifying light chains coded by given V-genes and this in turn could help in establishing the coding capacity of each Vk gene. Differences in the context of Vk genes, demonstrable by restriction mapping could also be reflected at the phenotypic level. For example, the order of expression of individual Vk genes in ontogeny could be affected by their location within the Vk complex. This could have an important effect on the representation of given Vk subgroups in the adult B-cell repertoire.

Vk Markers

To date, with one exception, all reported genetic markers affecting mouse kappa chains have been closely linked to the chromosome 6 locus Lyt-3. These include the I_B peptide marker (Trp) (10), the PC-8 marker, (PC-) (11) the isoelectric focusing markers Ef1 and Ef2, (12,13) markers affecting expression of idiotypes to ARS (14) and to DNP (MOPC 460 idiotype) (15) and the kappa chain marker determining the response to α-1,3-dextran (16). A summary of the known Vk markers is given in table 1. Since the Vk complex of the mouse also resides on chromosome 6 (17,18), it is presumed that these markers involve differences in either content or arrangement of genes within the Vk complex or in closely linked genes controlling the expression of Vk genes.

VH Effects: In some respects it is surprising that relatively few reports have demonstrated IgVH-influences on light chain expression and vice versa. Perlmutter et al. (19) have reported differences in light chain spectrotypes of anti-group A streptoccocal carbohydrate antibodies in IgCH and IgVH congenic strains. It may be that such differences will be demonstrable only in relatively homogeneous responses involving a few VH and VL combinations. No VH effects have been observed at the level of normal serum light chain expression. This would suggest that combinatorial association must to a large extent obscure such effects.

Vk Effects on Idiotype: The relatively few instances in which idiotypic markers have been correlated with Vk loci would suggest either that idiotype may not always require

Table 1. Summary of Vk Markers

Allo-Group	Strain Distribution	Lyt3	Trp	Ef1	PC	ARS	Ef2	M460 DNP	α-1,3-DEX
a	BALB/c etc.	b	b	b	b	*	a	Id+	non-resp.
	CB20	b	b	b	b		a		non-resp.
b	AKR/J, RF/J	a	a	a	a		a		non-resp.
	PL/J	a	a	a	a	Id(-)	a		
	C.AKR	a	a	a	a		a	Id+	
	B6.PL/Cy	a	a	a	a		a		non-resp.
	M16/Boy	a	a	a			a		non-resp.
c	C58/J, C.C58	a	a	a	a		b	Id-	
d	NZB/BlnJ, BDP/J	b	b	b			b		
	P/J, I/LnJ	b		b			b		
	CE/J	b	b	b	b		b		non-resp.
e	C57BL/6J	b	b	b	b	*	a		resp.
Ref.		9	10	13	11	14	13	15	16

*These strains possess the necessary Vk genes to produce Id(+) anti-ARS antibodies in the presence of the IgCH allogroup of strain A/J.

given light chains or that the majority of strains used in these studies possess identical light chain repertoires. In the case of the K-PC8 marker, idiotypically identical anti-PC antibodies were found to possess different light chains only after the light chains were isolated and analysed by IEF (11). V_K-effects on expression of idiotype have been demonstrated in two instances (14,15). In both cases studies on the inheritance of idiotypic markers were carried out in strains known to differ with respect to V_K. In view of the extensive V_K differences found in strains such as AKR, it will not be surprising if many more examples of V_K associated idiotype restriction will be found.

Normal Light Chain IF-Markers

The Ef1 Marker: Normal light chain IF-markers were detected by isoelectric focusing analysis of pooled normal light chains from various mouse strains. While the majority of inbred strains showed a virtually identical normal light chain banding pattern, strains AKR, RF, PL and C58 were found to express a modified pattern (Ef-1^a) characterized by multiple differences in band positions and intensities. These differences are expressed co-dominantly in F1 hybrids and segregate as a Mendelian trait in genetic studies. The characteristic differences are not obliterated by polyclonal stimulation of B cells by LPS indicating they exist at the level of B cells as well as in the mature immunoglobulin secreting cell population. Many of the characteristic Ef1-related differences are also evident in light chains produced in heterogeneous responses to BSA and to DNP. In fact, Ef1-related differences are even more evident in DNP-light chains than in normal serum light chain profiles (Gibson et al., in preparation) and one has the impression that the light chain repertoires of $Ef1^a$ and $Ef1^b$ strains must differ extensively. In view of the multiplicity of differences associated with the Ef1 marker, it is evident that the polymorphism is not one of a simple nature. One possibility which remains to be excluded is that the polymorphism involved the J region or even the Ck region. Differences in J or Ck would create multiple differences in normal light chain IF-profiles. We have compared the IF-behavior of C-region fragments of mice differing at Ef1 and found no detectable difference (Fig. 1). In addition, Gottlieb has found no evidence for C_k-region differences in tryptic peptide maps of normal light chains from strains carrying the I_B and $Ef1^a$ markers (10). On the contrary, structural work has indicated that these light chains differ in the V-region and recent work has demonstrated the presence of at least one unique subgroup of kappa chains in $Ef1^a$ strains (20).

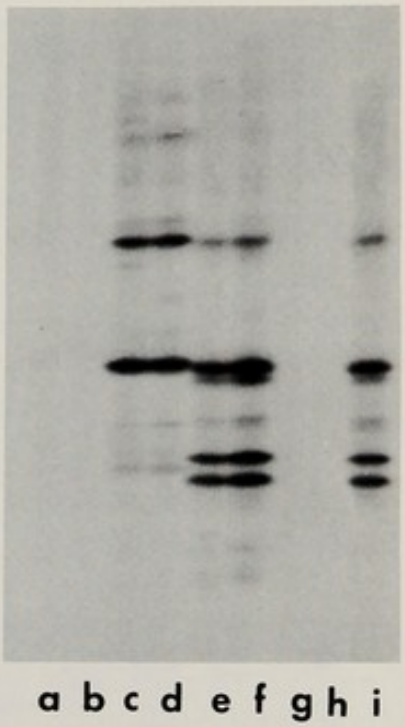

FIGURE 1. Analysis of C-region fragments of normal light chains from SWR ($Ef1^b$,$Ef2^a$), C58 ($Ef1^a$, $Ef2^b$) and AKR ($Ef1^a$, $Ef2^a$) mice. Fragments of approximately 11K were generated by digestion of intact light chains with trypsin (c,d) or chymotrypsin (e,f,i) at a ratio of 1/100 for 15 minutes at 37°. Lanes a,b,g and h represent 0 time controls. Following digestion samples were completely reduced and alkylated and fractionated on a urea formate gel at pH 3. Fragments migrating ahead of the undigested light chain were transferred to an isoelectric focusing gel (13). Lanes (c) SWR; (d) C58; (e) SWR; (f) C58 and (i) AKR.

The Ef2 Marker: A second IF-marker designated Ef2 was also detected in normal mouse light chain IF-profiles (13). $Ef2^b$ strains of mice, which include NZB, C58, P, CE, I/1n and BDP are characterized by light chain patterns lacking certain characteristic bands. C58 is the only strain so far which has been typed $Ef1^a$, $Ef2^b$ and would thus appear to represent a recombinant haplotype. Of particular interest was the fact that one of the major differences controlled by Ef2 involved two distinct bands that were separated by the typical one-charge spacing observed with myeloma light chains. This suggested to us that the two bands might represent the same light chain or light chain subgroup. We have since identified a total of 8 light chains co-focusing with the Ef2-marker bands in a screening of 275 BALB/c myelomas. Several of these light chains are illustrated in Figure 2, which also shows the normal light chain focusing profiles of BALB/c ($Ef2^a$) and NZB ($Ef2^b$) mice. In order to define the nature of this group of light chains we have determined complete V-region sequences of four of the proteins and we are currently working on two more. The sequences revealed that the light chains indeed represented a single Vk subgroup (Table 2). Two of the light chains, T-105 (CAL20) and T-817 (BALB/c) were identical throughout the V-region except for the deletion of residue 95 in the T-817 sequence. It seems likely that these two sequences are coded by the same Vk gene and that residue 95 was deleted at the time of V-J joining (21). Light chains F-1 and T-821 differed from the prototype sequence by 3 (1crd, 2fr) and 1 (fr) residues, respectively. Partial sequence of T-602 has so far revealed only one substitution, compared to T-105, a valine for alanine at position 19. These data indicate that we have defined an extremely closely related set of

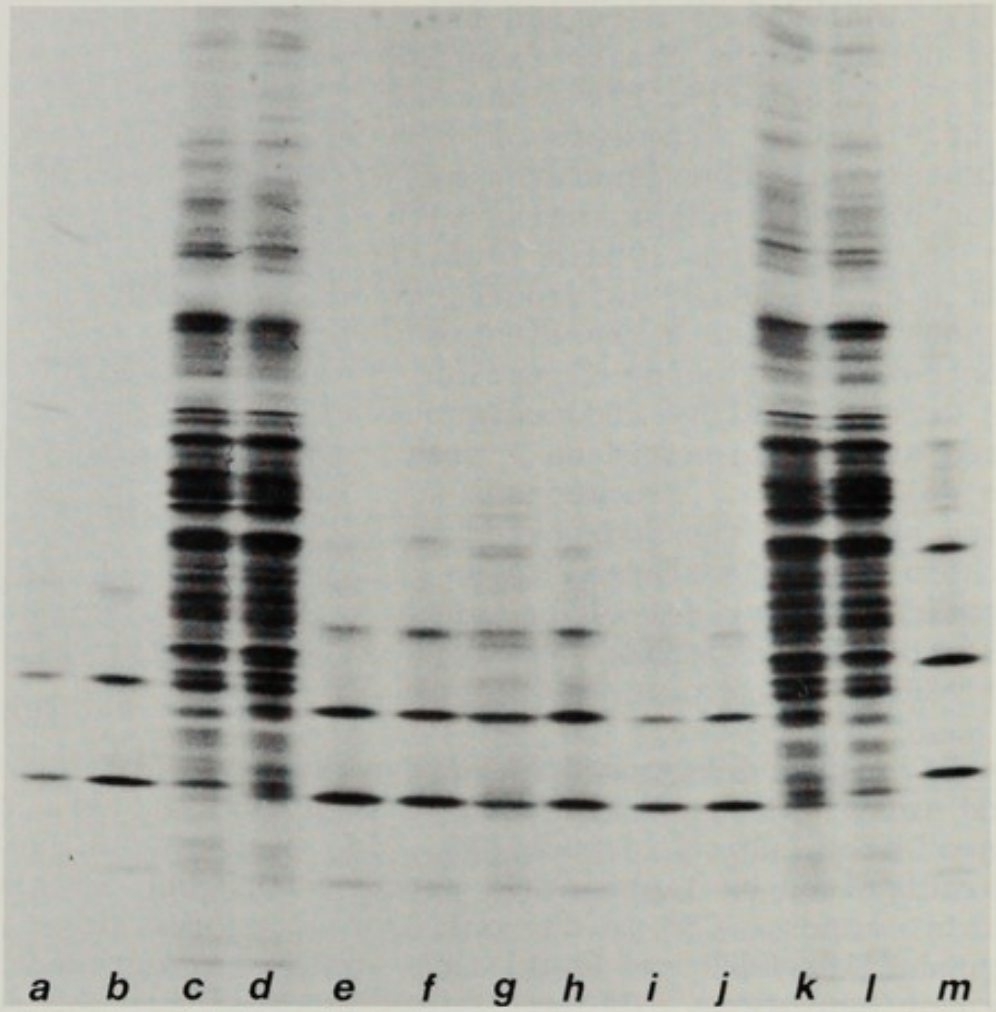

FIGURE 2. Isoelectric focusing of normal NZB and BALB/c light chains and myeloma light chains related to the Ef2 marker bands. Lane (a) T-989; (b) PC2205; (c) normal NZB; (d) normal BALB/c; (e) T-821; (f) T-105; (g) T-817; (h) F-1; (i) T-119; (j) T-602; (k) normal BALB/c; (l) normal NZB; (m) PC2567.

light chains. This has been designated Vk-1A after Potter (4). The question that arises is whether all the sequences may be coded by a single Vk gene. The fact that the pair of bands corresponding to this subgroup behaves as a Mendelian character in normal serum light chains would argue in favor of the single gene hypothesis. If this were true it would mean that the framework substitutions found in 3 of the proteins must have occurred somatically either prior to or during the myeloma induction. If they occurred prior to myeloma induction, it would necessitate postulating that selection for somatic mutations may operate on framework as well as complementarity determining regions. Although this is contrary to the classical somatic mutation-selection model (22), there is no compelling evidence to exclude framework regions from having an influence on the precise geometry of the combining site. Indeed, the fact that a great variety of framework regions are found in mouse kappa chains would indicate

```
T-105  -----A---HS--H-----K--------K---Y-S-STVP.-J5-
T-817  ---------------------------------------*.-J4-
F-1    ----------N-----------------R---------I-.-J5-   Vk-1A
T-821  --------------------------------F-------.-J5- (BALB/c)
T-602  -----V------------                ----------.-J1-


2205   -------------Y-----R--------------F-Q---.-J5-   Vk-1B
2567   ---------Y---Y-----R--------------F-QV--.-J4-   (NZB)

Bases       1   11  1     1         1    1 1 2211
* = Deletion
```

Table II. V-Region Sequences of Ef2-Related Light Chains. Complete sequence data to be presented elsewhere (24).

that selection for framework diversity must have occurred at some level. An alternative to proposing an antigen driven selective mechanism to explain framework substitutions would be that they occurred at the time of myeloma induction. One could imagine that escape from network regulation could provide a selective force leading to the induction of mutants. The fact that at least two of the proteins (T-105 and T-817) are essentially identical and must therefore represent the germ-line sequence however would indicate that mutation away from the germ-line sequence cannot be an absolute requirement for myeloma induction. A more conventional interpretation of the data would be that the subgroup is coded by a cluster of virtually identical Vk genes which behaves as a unit in genetic studies. Models invoking Vk-linked, subgroup-specific regulation can also be considered.

When partial sequences of the Ef2-related light chains became available, it was evident that closely related light chains (PC 2205 and PC 2567) also existed in the NZB myelomas (23). Since the Ef2-marker bands are not expressed in NZB serum light chains and no light chains co-focusing with the Ef2 marker bands have been identified in a screening of over 100 NZB myelomas (8), it was of interest to establish the relationship between the two NZB proteins and the polymorphic Vk-1A subgroup. Complete V-region sequencing of the two NZB light chains, (Table 2) revealed that they constituted a distinct Vk subgroup (Vk-1B) differing by 4 residues from Vk-1A. The fact that the two subgroups differ by 4 residues would indicate that V1-1A and Vk-1B are probably not allelic but represent different, possibly adjacent Vk genes.

It is more than an interesting coincidence that Dzierzak et al. (15) have recently found that a Vk locus necessary for the expression of the MOPC 460 idiotype in the response to DNP correlates with the expression of the Ef2 marker. The relationship between the two observations became evident when it was realized that Ef2 controls a subgroup of light chains of which the MOPC460 light chain appears to be a member (8). The MOPC460 light chain differs from the Ef2-related light chains on IF-however, suggesting that it may represent a somatic mutant of the Vk-1A gene or the product of another member of the Vk-1A cluster.

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REFERENCES

1. Valbuena, O., Marcu, K.B., Weigert, M. and Perry, R.P. (1978) *Nature (Lond.)* 276, 780.

2. Hood, L., Barstad, P., Loh, E. and Nottenburg, C. (1974) in The Immune System: Genes, Receptors, Signals, eds. Sercarz, E.E., Williamson, A.R. and Fox, C.F. Academic Press, New York, pp. 119-139.

3. Potter, M. (1977) *Adv. Immunol.* 25, 141.

4. Weigert, M. and Riblet, R. (1976) *Cold Spring Harbor Symp. Quant. Biol.* 41(2), 837.

5. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. and Hood, L. (1978) *Nature (Lond.)* 276, 785.

6. McKean, D.J., Bell, M. and Potter, M. (1978) *Proc. Nat. Acad. Sci. USA* 75. 3913.

7. Joho, R., Weissman, I.L., Early, P., Cole, J. and Hood, L. (1980) *Proc. Nat. Acad. Sci. USA* 77, 1106.

8. Lazure, C., Hum, W.-T. and Gibson, D.M. (1980) *J. Exp. Med.* 152, 555.

9. Staats, J. (1980) *Cancer Res.* 40, 2083.

10. Gottlieb, P.D. (1974) *J. Exp. Med.* 140, 1432.

11. Claflin, J.L., Taylor, B.A., Cherry, M. and Cubberley, M. (1978) *Immunogenet.* 6, 379.

12. Gibson, D.M., Taylor, B.A. and Cherry, M. (1978) *J. Immunol.* 121, 1585.

13. Gibson, D.M. and MacLean, S.J. (1979) *J. Exp. Med.* 149, 1477.

14. Laskin, J.A., Gray, A., Nisonoff, A., Klinman, N.R. and Gottlieb, P.D. (1977) *Proc. Nat. Acad. Sci. USA* 74, 4600.

15. Dzierzak, E.A., Janeway, C.A. Jr., Rosenstein, R.W. and Gottlieb, P.D. (1980) *J. Exp. Med.* 152, 720.

16. de Preval, C., Blomberg, B. and Cohn, M. (1979) *J. Exp. Med.* 149, 1265.

17. Hengartner, H., Meo, T. and Muller, E. (1978) *Proc. Nat. Acad. Sci. USA* 75, 4494.

18. Swan, D., D'Eustachio, P., Lienwand, L., Seidman, J., Kiethley, D. and Ruddle, F.H. (1979) *Proc. Nat. Acad. Sci. USA* 76, 2735.

19. Perlmutter, R.M., Briles, D.E., Greve, J.M. and Davie, J.M. (1978) *J. Immunol.* 121, 149.

20. Gottlieb, P.D., Tsang, H. C-W., Gibson, D.M. and Cannon, L.E. (1981) *Proc. Nat. Acad. Sci. USA* 78, 559.

21. Weigert, M., Perry, R., Kelly, D., Hunkapiller, T., Schilling, J. and Hood, L. (1980) *Nature* 283, 497.

22. Cohn, M., Blomberg, B., Geckeler, W., Raschke, W., Riblet, R. and Weigert, M. (1974) in The Immune System: Genes, Receptors, Signals. eds. Sercarz, E.E., Williamson, A.R. and Fox, C.F. Academic Press, New York.

23. Loh, E., Black, B., Riblet, R., Weigert, M., Hood, J.M. and Hood, L. (1979) *Proc. Nat. Acad. Sci. USA* 76, 1395.

24. Gibson, D.M., Lazure, C., Hum, W.-T., *J. Exp. Med.* in press.

Vκ(SER): A UNIQUE Vκ GROUP ASSOCIATED WITH TWO MOUSE LIGHT CHAIN GENETIC MARKERS

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The C.C58 and C.AKR congenic strains of mice differ from Balb/c at loci on chromosome 6 which govern immunoglobulin Vκ light (L) chain polymorphisms and the Lyt-2 and Lyt-3 alloantigens. Amino acid sequence analysis of L chains from myelomas induced in these strains revealed one, C.C58 M75, which had an amino-terminal serine and which differed sufficiently from published Vκ sequences to define a new group, Vκ(Ser), apparently not expressed by Balb/c mice. Peptide map analysis indicated that the M75 L chain contained the I_B-peptide marker, a Vκ polymorphism expressed by C.C58 but not by Balb/c mice, which is determined by the IgK-Trp^a allele on chromosome 6. This same L chain was found by isoelectric focussing (IF) to correspond to IgK-Ef1^a, another Vκ genetic marker of C.C58 and C.AKR mice. Analysis by IF of nearly 200 C.C58 and C.AKR L chains revealed seven additional IgK-Ef1^a-specific L chains. The three additional C.C58 L chains belonged to the Vκ(Ser) group, contained the I_B-peptide marker, and had the same sequence as M75 over the 49 residues examined. The sequence of the one C.AKR L chain studied, F17, showed that it belonged to the Vκ(Ser) group but differed from the others at one position. Thus the differences in Vκ repertoires represented by the I_B-peptide and IgK-Ef1^a markers appear to reflect expression (or failure of expression) of a distinct group of Vκ regions.

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INTRODUCTION

Genetic studies have revealed differences in the repertoire of immunoglobulin (Ig) kappa L chain variable regions expressed by inbred strains of mice. Peptide maps of normal serum Ig L chains have shown that unique peptides containing the first V_K cysteine (Cys I) are expressed by C58/J, AKR/J, RF/J and PL/J but not by other strains (1,2). This "I_B-peptide marker" is codominantly expressed in F_1 mice, and its segregation is consistent with a simple Mendelian trait. Its expression is controlled by the *IgK-Trp* locus which is closely linked to the *Lyt-2* and *Lyt-3* loci (3,4) on chromosome 6 (5) which code for T cell surface antigens. Other loci on this chromosome control additional L chain polymorphisms: the *IgK-Ef1* and *IgK-Ef2* loci control V_K variants detected by isoelectric focussing (IF) of normal serum Ig L chains (6-8); the *IgK-Pc1* locus governs V_K IF variants associated with anti-phosphorylcholine antibodies which bear the H8 idiotype (9,10); the *IgK-ARS* locus determines V_K regions employed in the major cross-reactive idiotype of anti-azobenzene-arsenate antibodies of A/J mice (11); the *IgK-Id460* locus governs κ chains needed for expression of the MOPC 460 idiotype (12). Thus polymorphic loci on chromosome 6 determine the expression of certain V_K regions and thereby affect the phenotype of immune responses.

Studies by Swan and coworkers (13) employing somatic cell genetics and hybridization with nucleic acid probes have shown that V_K and C_K germ line structural genes reside on chromosome 6. Thus the V_K polymorphisms described above may reflect differences among inbred strains in V_K coding information. Alternatively, these traits could reflect polymorphism at closely linked regulatory loci which result in strain-specific expression of V_K genes common to all mice. One approach to resolving this issue is to obtain monoclonal examples of each strain-specific V_K region and to clone suitable DNA probes to test whether the corresponding V_K gene is present in the germ line of all strains or only those which express that L chain.

RESULTS AND DISCUSSION

To distinguish between the above alternatives, we have constructed the C.C58 and C.AKR strains of mice, congenic with the myeloma-inducible Balb/cAn strain but which express V_K polymorphisms of C58/J and AKR/J, respectively (14). They also bear the *Lyt-2*^{a}, *Lyt-3*^{a} genotype characteristic of these strains. Approximately 100 myelomas were induced in both the C.C58 and C.AKR strains by intraperitoneal injection of

pristane as described by Potter (15). Myeloma proteins from the C.C58 strain were purified from ascites fluids by starch zone electrophoresis and gel filtration of Sephadex G-200. L chains were then isolated at random and subjected to automated amino acid sequence analysis as previously described (16) to obtain a random sample of the C.C58 myeloma repertoire for comparison with that of Balb/c. The underlying assumption in this approach was that known differences in the L chain repertoire observed with normal serum Ig L chains and antibodies would be reflected in the myeloma repertoire, and this was found to be the case. The L chain from the C.C58 M75 IgA myeloma protein was found to have a sequence unlike that of any Vκ region previously described (Figure 1). The amino-terminal serine has never been observed in κ chains of mice or any other species. Comparison with a large number of NZB and Balb/c Vκ sequences (17-20) revealed that with the exception of the MOPC 35 L chain, which differs from M75 by at least three and possibly five of the first 23 residues, all other Vκ regions differ by at least eight of the first 23 positions. Thus the C.C58 M75 L chain and others like it (see Figure 1 and below) constitute a new Vκ group[1], designated Vκ(Ser), expressed by C.C58 but not by Balb/c or NZB strains.

The C.C58 M75 κ Chain Contains the I_B-Peptide Marker

Since the M75 L chain contains arginine and lysine at positions 18 and 24, respectively, tryptic digestion of the fully-reduced and ^{14}C-iodoacetic acid-alkylated L chain yielded a radiolabeled hexapeptide containing Cys I (residue 23). As shown previously (1), the I_B-peptide marker is a group of tryptic hexapeptides derived from Cys I of κ chains. Since the sequence of the M75 Cys I hexapeptide was unlike any myeloma L chain previously observed and was consistent with the composite sequence of I_B-hexapeptides from normal AKR/J serum L chains (1,16), the M75 L chain was peptide mapped (Figure 2). A tryptic digest of fully reduced and radioalkylated M75 L chains was peptide mapped alone and as a mixed map with a digest of similarly labeled AKR/J normal serum L chains (Figure 2, panels d and c, respectively). Similar peptide maps of normal serum L chains from AKR/J (I_B-positive) and C57BL/6J (I_B-negative) were included for comparison (Figure 2, panels b and a, respectively). Results showed that the M75 Cys I hexapeptide coincides with that portion of the AKR/J peptide map which corresponds to the I_B-peptide marker (16).

[1]*The definition of Vκ groups suggested by Potter (17) is used: Vκ regions differing at more than three of the amino-terminal 23 positions belong to different Vκ groups.*

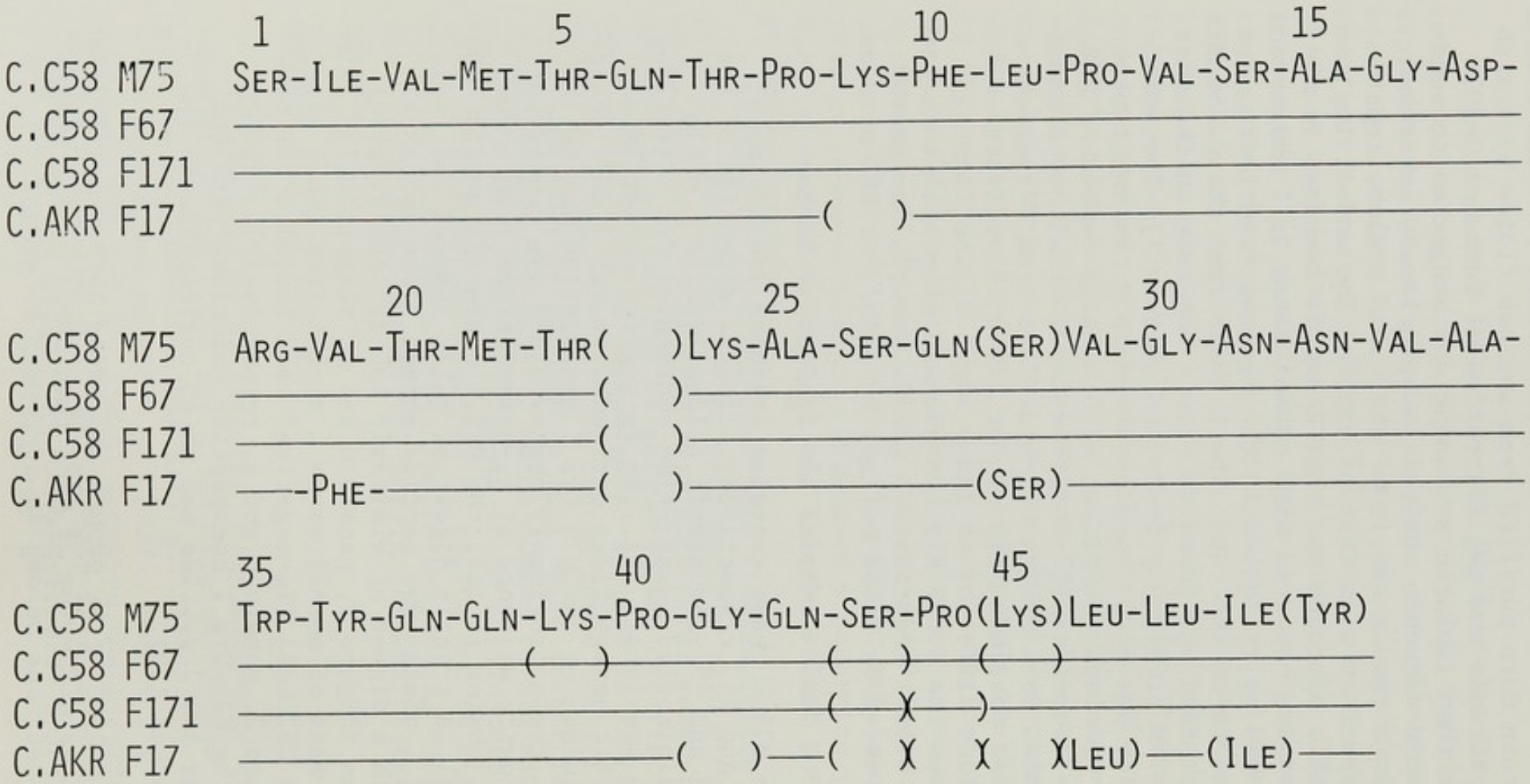

FIGURE 1. Amino acid sequences of C.C58 L chains M75, F67 and F171, and C.AKR L chain F17. Position number is according to Kabat et al. (18). A line denotes identity with the M75 sequence. Residues within parentheses were identified by one method only. All others were identified by two or three independent methods (see ref. 16). A blank within parentheses denotes failure to identify a Pth-amino acid.

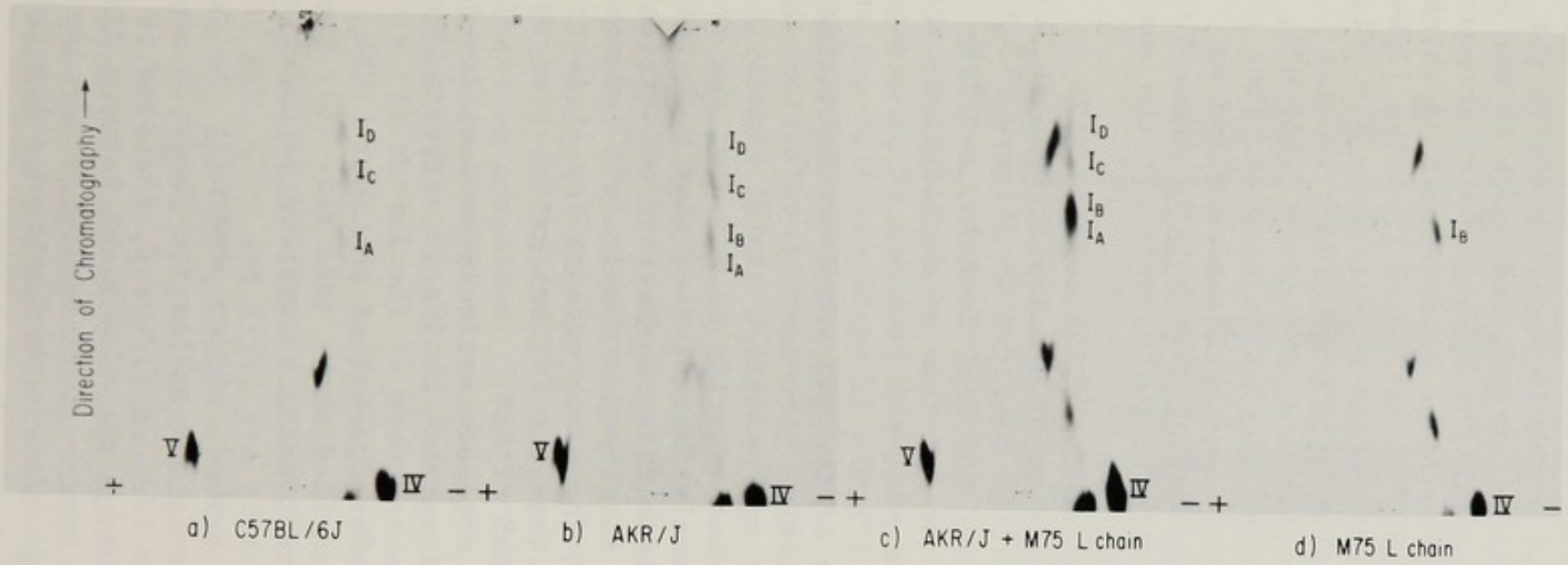

FIGURE 2. Autoradiograms of tryptic peptide maps of ^{14}C-iodoacetic acid-alkylated normal serum L chains and the C.C58 M75 myeloma κ chain. a) C57BL/6J normal serum L chains (I_B-negative) b) AKR/J normal serum L chains (I_B-positive); c) mixture of AKR/J normal serum L chains and the C.C58 M75 L chain; d) the C.C58 M75 L chain alone. Electrophoresis was in the horizontal dimension and chromatography from bottom to top as previously described (1). Positions of Cκ-region Cys IV and Cys V and the Vκ Cys I hexapeptides I_A, I_B, I_C and I_D are indicated. The absence of the Cys V peptide in panel d reflects the fact that isolation of the C.C58 M75 L chain was preceded by partial reduction and alkylation of the intact myeloma protein with non-radioactive iodoacetic acid. (Figure taken from ref. 16).

The M75 L Chain has the Properties of the IgK-Ef1^a Marker

The C.C58 and C.AKR myeloma collection was also tested by IF to determine whether it contained L chains characteristic of the IgK-Ef1^a phenotype (6) which is associated with the *Lyt-2^a*, *Lyt-3^a* genotype of these strains and which is not expressed by Balb/c. In particular, the L chains were screened for correspondence with the prominent bands 66 and 58 which are present in IF gels of AKR/J normal serum L chains (IgK-Ef1^a) but not of Balb/c L chains (IgK-Ef1^b)(Figure 3)[2]. Six myeloma L chains focussed with their predominant species aligned with band 66, and two others, including C.C58 M75, focussed with their predominant species at band 58. Thus the M75 L chain represents both the I_B-peptide marker and an IgK-Ef1^a-specific marker, indicating that both of these V_K markers reflect, at least in part, polymorphism in the same gene or group of genes.

The amino acid sequences of the C.C58 F67 and F171 L chains, which focus with their major species at band 66, are identical to that of M75 through position 49 (Figure 1). During sequence analysis of the C.C58 M170 L chain, two Pth-amino acids were identified at a number of degradation cycles (data not shown), consistent with the presence of at least two myeloma L chains by IF (Figure 3, track c). Although the data is consistent with one of the two sequences being identical to that of M75, definitive sequence determination will require purification of the appropriate L chain. Finally, consistent with their sequence identity to M75 over the region studied, peptide map analysis indicated that both the M170 and F171 L chains contained the I_B-peptide marker (data not shown). The finding of three and probably four independent C.C58 myeloma L chains with the same sequence for at least 49 residues strongly suggests that the V_K(Ser) group is encoded in the germ line of the C.C58 strain.

That the V_K(Ser) group does not entirely account for the I_B-peptide marker of normal serum L chains is suggested by comparison of the composite sequence of the latter (1) with the I_B-peptide Val-Thr-Met-Thr-Cys-Lys of the four C.C58 V_K(Ser) representatives described above (see ref. 16). The predominant amino acid corresponding to residue 21 in normal

[2]*IF of IgK-Ef1^a normal serum L chains generally yields a doublet near band 58, and only one of the two bands is present in IgK-Ef1^b normal L chains (6). In Figure 3, the band 58 doublet is not resolved, making the difference between AKR/J and Balb/c normal L chains appear quantitative rather than qualitative. This doublet is clearly resolved in other gels.*

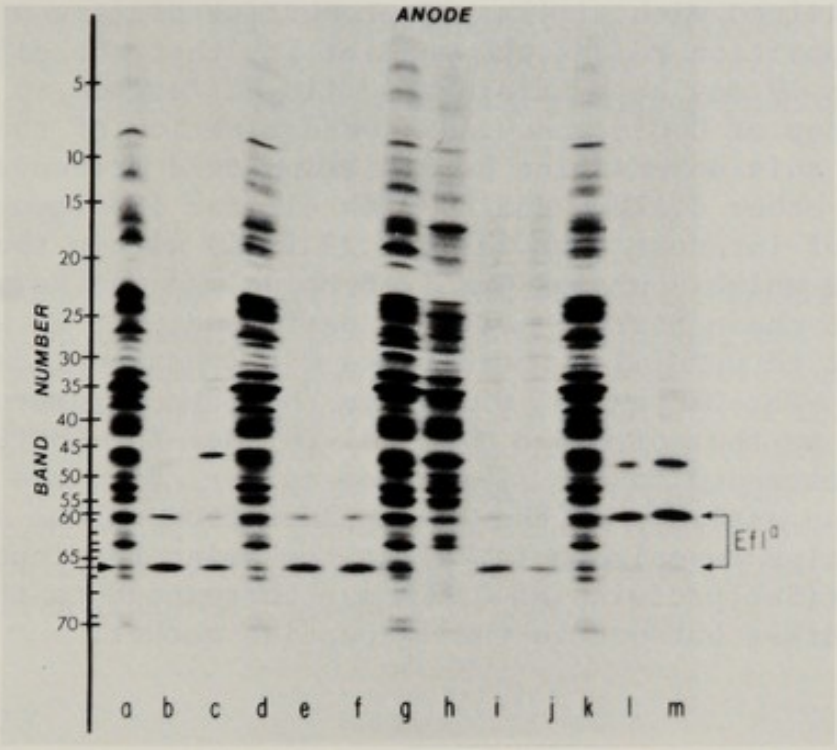

FIGURE 3. Autoradiogram of IF analysis of ^{14}C-iodoacetamide-alkylated normal serum L chains and C.C58 and C.AKR myeloma L chains. a,d,g,k) AKR/J normal serum L chains; b)C.C58 F67 L; c) C.C58 M170 L; e) C.C58 F171 L; f) C.AKR F17 L; h) Balb/c normal serum L; i) C.AKR F59 L; j) C.AKR M45 L; l) C.C58 M75 L; m) C.AKR F46 L. The positions of IgK-Ef1^a bands 58 and 66 are indicated[2].

AKR/J serum I_B-peptides was Ile and not the Met found in the Vκ(Ser) L chains analyzed here (1). The I_B-peptides of normal C58/J or C.C58 serum L chains have not been similarly analyzed and it is possible that Met may be the predominant (or only) amino acid at residue 21 of I_B-peptides in these strains. Analysis of additional C.C58 Vκ regions and of the C.AKR myeloma κ chains which align with the IgK-Ef1^a markers (Figure 3) may shed further light on this question.

Amino Acid Sequence Analysis of the C.AKR F17 L Chain

IF analysis of the C.AKR F17 L chain showed that like C.C58 F67, M170 and F171, its predominant species corresponded to the IgK-Ef1^a-specific band 66 (Figure 3). However, as shown in Figure 1, the amino acid sequence of the F17 L chain was identical to these L chains over the region studied with the exception of residue 19, which was Phe instead of Val. The finding that three and probably four C.C58 L chains corresponding to the IgK-Ef1^a polymorphism have identical sequences for approximately 49 residues, and that the only C.AKR

protein examined with similar IF properties differs at one framework position raises the possibility that the difference at position 19 may be a strain-specific difference in the V_K(Ser) group of C.C58 and C.AKR. Determination of the significance of this observation awaits amino acid sequence analysis of the other C.AKR L chains with similar IF properties.

It is of interest that residue 19 falls within the tryptic hexapeptide which contains Cys I. Peptide map analysis of the C.AKR F17 L chain has not yet been performed, but it will be of interest to determine whether its Cys I hexapeptide, Phe-Thr-Met-Thr-CMCys-Lys, will have the same chromatographic properties as that of C.C58 M75, Val-Thr-Met-Thr-CMCys-Lys, which corresponded to the I_B-peptide marker. Previous composite sequence analysis of the I_B-peptides from normal AKR/J serum L chains revealed only Val at the amino terminus (1). Thus the V_K(Ser)proteins of C.AKR may correspond to the IgK-Ef1^a marker but not to the I_B-peptide marker.

Conclusion

Examination of myeloma L chains from the C.C58 and C.AKR strains, congenic with Balb/c but differing at loci determining L chain polymorphisms linked to the *Lyt-2*^{a}, *Lyt-3*^{a} genotype, has revealed repertoire differences consistent with those seen in normal serum L chains. In particular, a new V_K group, designated V_K(Ser), has been found which gives rise, at least in part, to both the I_B-peptide marker and the IgK-Ef1^a polymorphism (16). The unusual Ser amino terminus, whose codons differ form the usual Asp or Asn amino terminus by at least two base changes, and the finding of three and probably four independent C.C58 myeloma V_K(Ser) L chains with identical amino acid sequences through position 49 indicate that the V_K(Ser) group must be determined by germ line genes different from those of any other known V_K group. Lazure and coworkers (21) have provided another example of inherited differences in L chain repertoire reflecting expression (or failure of expression) of a particular V_K group. In that instance, the V_K1 group was found to give rise to the IgK-Ef2^a IF marker (7). Expression of the V_K1 group is also involved in the IgK-Id460 polymorphism (12) since the MOPC 460 L chain belongs to the V_K1 group. Whether these differences in expression of V_K groups among congenic strains reflect differences in the germ line V_K structural gene repertoire encoded on chromosome 6, or in linked regulatory genes or flanking sequences required for rearrangement, transcription and/or post-transcriptional processing (22,23) of particular V_K groups is not known. However, since the V_K(Ser) group differs substantially in amino acid sequence from other

mouse Vκ groups, use of cloned Vκ(Ser) DNA to directly analyze the genomes of mice which do or do not express this group should permit definitive determination as to the presence or absence of Vκ(Ser) coding information. These studies are in progress.

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REFERENCES

1. Edelman, G.M. and Gottlieb, P.D. (1970). *Proc. Natl. Acad. Sci. USA 67*, 1192.

2. Gottlieb, P.D. and Durda, P.J. (1977). *Cold Spring Harbor Symp. Quant. Biol. 41*, 805.

3. Gottlieb, P.D. (1974). *J. Exp. Med. 140*, 1432.

4. Green, M.C. (1979). *Immunogenetics 8*, 89.

5. Itakura, K., Hutton, J.J., Boyse, E.A. and Old, L.J. (1972). *Transplantation 13*, 239.

6. Gibson, D. (1976). *J. Exp. Med. 144*, 298.

7. Gibson, D.M. and MacLean, S.J. (1979). *J. Exp. Med. 149*, 1477.

8. Gibson, D.M., Taylor, B.A. and Cherry, M. (1978). *J. Immunol. 121*, 1585.

9. Claflin, J.L. (1976). *Eur. J. Immunol. 6*, 666.

10. Claflin, J.L., Taylor, B.A., Cherry, M. and Cubberley, M. (1978). *Immunogenetics 6*, 379.

11. Laskin, J.A., Gray, A., Nisonoff, A., Klinman, N. and Gottlieb, P.D. (1977). *Proc. Natl. Acad. Sci. USA 74*, 4600.

12. Dzierzak, E.A., Janeway, C.A., Jr., Rosenstein, R.W. and Gottlieb, P.D. (1980). *J. Exp. Med. 152*, 720.

13. Swan, D., D'Eustachio, P., Leinwand, L., Seidman, J., Keithley, D. and Ruddle, F.H. (1979). *Proc. Natl. Acad. Sci. USA 76*, 2735.

14. Gottlieb, P.D., Marshak-Rothstein, A., Auditore-Hargreaves, K., Berkoben, D.B., August, D.A., Rosche, R.M. and Benedetto, J.D. (1980). *Immunogenetics 10*, 545.

15. Potter, M. (1972). *Physiolgical Revs. 52*, 631.

16. Gottlieb, P.D., Tsang, H. C-W., Gibson, D.M. and Cannon, L.E. (1981). *Proc. Natl. Acad. Sci. USA 78*, 559.

17. Potter, M. (1978). *Adv. Immunol. 25*, 141.

18. Kabat, E.A., Wu, T.T. and Bilofsky, H. (1979). *Sequences of Immunoglobulin Chains*, Bolt, Beranek and Newman, Inc., Cambridge, MA.

19. Loh, E., Black, B., Riblet, R., Weigert, M., Hood, J.M. and Hood, L. (1979). *Proc. Natl. Acad. Sci. USA 76*, 1395.

20. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. and Hood, L. (1978). *Nature (Lond.) 276*, 785.

21. Lazure, C., Hum, W-T. and Gibson, D.M. (1980). *J. Exp. Med. 152*, 555.

22. Sakano, H., Hüppi, K., Heinrich, G. and Tonegawa, S. (1979). *Nature (Lond.) 280*, 288.

23. Max, E.E., Seidman, J.G. and Leder, P. (1979). *Proc. Natl. Acad. Sci. USA 76*, 3450.

T15 LIGHT CHAIN V REGION MARKERS

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Inbred strains of mice vary in their expression of L chain found in anti-phosphocholine antibodies carrying the T15 idiotype (Id). Genetic studies have demonstrated two phenotypes, Igk-Pc-A (AKR, C58, RF, and PL) and Igk-Pc-B (BALB/c, I, C57BL/6), which behave as co-dominant alleles controlled at a single locus on chr. 6. T15 Id$^+$ hybridoma antibodies (HP) from AKR and BALB/c exhibit the expected phenotypic variation, except for a single hybridoma of AKR origin which unexpectedly secretes a "Pc-B" L chain. The protein sequences of the Pc-A and Pc-B are compared and are essentially identical through residue 36. Binding specificity studies with different choline analogs show no detectable differences between the polymorphic forms. Southern blot analysis with DNA from T15 idiotype positive myelomas, hybridomas, and liver from different strains of mice, using a T15 V-region probe, reveals the size of the expressed Pc-B T15 L chain gene, and shows a conservation of the strongly hybridizing bands which carry V-genes belonging to the T15 (VK-22) gene family. Our current working model is that the two phenotypes arose from either allelic genes or pseudo-alleles, rather than major compositional differences in the genes between the two strains.

INTRODUCTION

One of the most interesting questions confronting immunologists today is how the response of the immune system to a specific antigen is determined, and how this translates into specificity. Is it solely a function of the coding sequences within the structural genes which code for the heavy (H) and light (L) chains of immunoglobulin, and the different

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combinations in which these sequences can combine, or is the response also controlled by linked regulatory genes? Clearly, a complete explanation of immunoglobulin gene expression must include two kinds of genetic mechanisms, one intrinsic and the other extrinsic to the structural gene coding sequences.

Intrinsic to the structural genes are the coding elements for H and L polypeptide chains that undergo specialized recombination in somatic cells during lymphocyte differentiation. This mechanism involves combinatorial joining of one of a hundred or more V_L or V_H gene segments to one of four J_K (L) or to an undefined number of D and then to one of four J_H. Flexibility in the exact position of recombination, combinatorial pairing of H and L chains, and somatic variability within coding sequences can account for further structural diversity.

Extrinsic to the structural gene sequences and critical to the expression of immunoglobulin genes, is the mechanism of quantitative regulation by immune response genes. The Ir-1 genes, which are linked to the major histocompatibility locus and unlinked to the structural genes, control the immune response to one or more antigens and operate at the level of antigen presentation. A second major set of genes, which control the expression of individual antibody molecules and appear to be distinct from structural genes, are either suspected or have been identified in mice, rats, and rabbits (1-5). Genes controlling different V_K genetic markers are linked to the Ly-3 (6-8). Whether the genetic markers defined are the loci of structural genes or of linked regulatory genes is a crucial question.

MURINE FAMILIES OF ANTIBODIES TO PHOSPHOCHOLINE

Through extensive analysis of serum anti-PC antibodies and anti-PC HP, our laboratory has identified three major families of antibodies in the murine response to PC (Table 1) (9-11). These families are related to three different PC-binding MP, TEPC 15, MOPC 167, and McPC 603. The H chains of these are strikingly similar. All belong to the VH-4 group of proteins (12) and appear to be derived from the same gene (Hood, this symposium). The L chains related to each are distinct and belong to 3 different groups: T15, VK-22; M167, VK-24; M603, VK-8; protein sequences differ from each other by 44% to 60% in the first 23 residues (13). Interestingly, all protein sequences completed so far are encoded by J5 and have a leucine residue coded at the V-J junction (residue 96) (14, M. Scharff and S. Rudikoff, personal communication).

X-ray crystallographic studies of McPC 603 have confirmed that the contact residues for interaction with antigen are entirely within the binding site and that the majority of these are provided by H chain CDRs (12). Residue 96 on the L chain appears to be a contact residue. In addition, the first CDR of the L chain interacts intimately with the D region of the H chain, stabilizing it (Potter, this symposium). Thus, the L chain may be crucial in determining the shape and size of the binding site, even though neither the first CDR of the L chain nor the H chain D region is involved in direct binding.

Table 1. PC-BINDING MYELOMA PROTEINS, HYBRIDOMA PROTEINS AND ANTIBODIES

	V-Region				Anti-PC serum and hybridoma antibodies		
	V_H		V_L				
Myeloma Prototypes	V_S	*J*	V_S	*J*	*Family*	V_H	V_K
TEPC15, HOPC8, S107	*4A*	*1*	*22*	*5*	*T15*	*4*	*22*
MOPC167, MOPC511	*4D*	*1*	*24*	*5*	*M167*	*4*	*24*
McPC603	*4C*	*1*	*8*	*5*	*M603*	*4*	*8*

GENETIC POLYMORPHISM IN VK-22 L CHAINS

In our laboratory, a subject of considerable interest is the variability in response to immunization with PC seen between two groups of inbred mice. A polymorphism in the VK-22 group exists at the Igk-Pc locus, which controls the expression of two phenotypic variants of the L chain, designated Igk-Pc-A and Igk-Pc-B (8). The expression of the two phenotypes is coded by genes on chromosome 6 which behave as autosomal, codominant alleles at a single locus: Pc^a, the prototype strain being AKR, and Pc^b, with BALB/c as prototype.

The protein sequence of a Pc-A L chain (HP22.1A4) up to residue 35 has recently been determined by S. Rudikoff (Table 2). This L chain was obtained from an AKR T15 Id^+ hybridoma and displayed the electrophoretic pattern of Pc-A molecules. Although the sequence is incomplete, it is clear that Pc-A and Pc-B L chains are strikingly homologous. The only relatively certain difference between the two occurs at residue 31. However, this single residue cannot account for the electrophoretic difference between the molecules, indicating that the distinguishing residues must be carboxyterminal to position 36. This data could indicate allelism of the two genes.

Table 2. N-TERMINAL SEQUENCES OF Pc-A AND Pc-B T15 L CHAINS

		V_L-Region
		CDR1
Protein	*Igk*	*1 10 20 a f 30*
T15	*Pc-B*	*DIVMTQSPTFLAVTASKKVTISCTASESLYSSKHKVHYLAWY*
HP22.1A4	*Pc-A*	———————————————— — —(N)———

However, our finding of an apparent "Pc-B" L chain in a hybridoma of AKR origin (15), raises the possibility that these VK-22 genes may not be true alleles but pseudo-alleles whose expression is encoded by a regulatory locus.

ANALYSIS OF VK-22 GENES

We have recently begun to directly explore this question of allelism in VK-22 gene segments. A cDNA probe for VK-22 sequences was prepared from T15 mRNA and used in hybridization studies of genomic DNA from (a) liver of different strains of mice, (b) Pc-B myeloma prototypes (S107 and H8), (c) hybridomas which express the Pc-A and Pc-B phenotypic variants and (d) the hybridoma of AKR origin which expresses the unexpected "Pc-B" L chain, (HP10.1.3).

The autoradiograms from experiments where DNA samples were digested with Eco RI, or with Bam HI, and analyzed by the Southern method are shown in Figure 1. The germline C_K gene is found on a single fragment and the V_K genes of the VK-22 group are found on several (6-8) fragments. In contrast to similar experiments using probes for VK-19 and VK-21 genes (Konrad Huppi, *et al.*, this volume), we see no gross differences between different mouse strains in the size of the bands which hybridize most strongly to the T15 V-region probe. Some differences are evident between the $\underline{Pc}^{a}$ and the $\underline{Pc}^{b}$ mice in T15 V-region minor hybridizing bands, however. A comparison of the blotting patterns of the two Pc-B myeloma prototypes

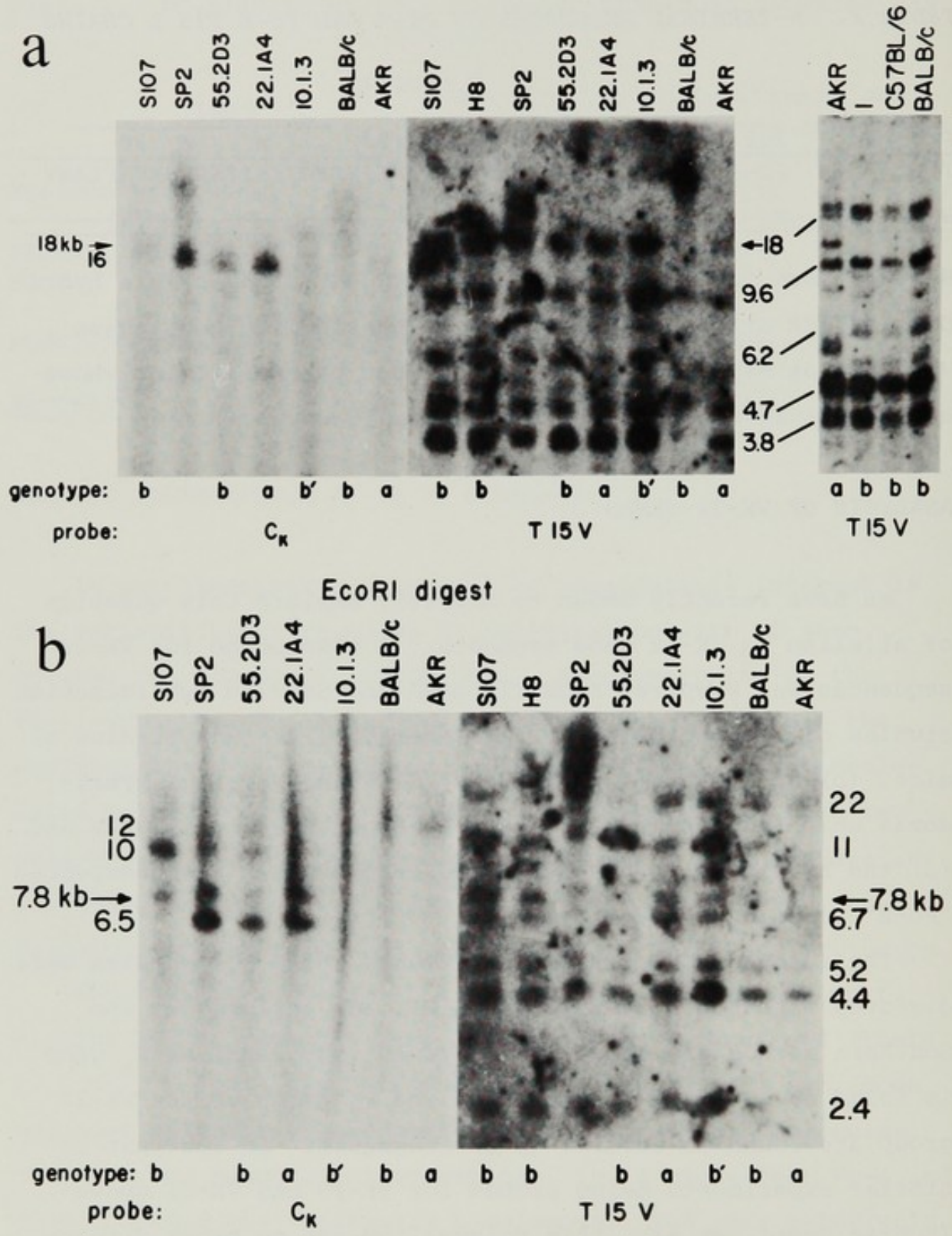

FIGURE 1. Hybridization (17) of radioactive C_K (pL21-5, R. Wall) and T15 V probes to electrophoretically separated (a) Eco RI or (b) Bam HI digests of DNA. S107 and H8, BALB/c plasmacytomas; HP55.2D3, BALB/c hybridoma; HP22.1A4, AKR (Pc-A) hybridoma; HP10.1.3, AKR hybridoma expressing Pc-'B'. Sizes of fragments in kb are indicated along vertical axes.

(S107 and H8) shows no real evidence of differences.

As Seidman, et al. (16) have pointed out, the interpretation of Southern blotting experiments of restriction endonuclease-digested DNA from myelomas and hybridomas is complicated by the fact that most myeloma cell lines, and certainly hybridoma lines, are at least diploid and that they contain one or more inactive chromosomes which are sometimes in the germline configuration, and sometimes rearranged. Moreover, the in situ hybridization pattern of V gene sequences is very complex because V genes are encoded in homologous families of 6-10 members detected by the same V gene probe, and thus there may be a significant background of germline V genes obscuring the presence of the rearranged gene on the active chromosome.

Nevertheless, it is possible to derive some conclusions concerning expressed VK-22 genes. Figure 1a shows that a germline-sized Eco RI C region fragment of 16 kb is present in SP2 DNA, the cell line used in hybridoma fusions, as well as in the hybridomas. We conclude that the rearranged Eco RI gene fragment in S107 and H8 DNA is slightly larger, about 18 kb, and that the germline T15 V gene fragment is probably in the 4.7 kb fragment. From the Bam HI digests (Figure 1b), we conclude that the germline C region fragment is 12 kb, and that the rearranged Bam HI fragment is 7.8 kb in S107 cells. The autoradiogram in Figure 1b suggests to us that the germline T15 V-region gene is on a 4.4 kb Bam HI fragment.

Work is in progress on cloning and sequencing the VK-22 gene that is expressed in the AKR hybridoma of the expected Pc-A phenotype. A complete sequence for this gene should establish how the V region differs from the BALB/c T15 V region.

BINDING SITE STUDIES

Functional properties of polymorphic MP and HP were compared by fine specificity analysis of antigen binding. Testing a panel of 15 analogues of PC as competitive inhibitors of radiolabeled PC-protein conjugates, inhibition curves were generated for 13 members of the T15 family, plus several representatives of the M511 and M603 families. As shown by three typical examples in Figure 2, the profiles of the T15 proteins were always similar and often superimposable, although together they included four H chain isotypes and arose from six different strains. Included in Figure 2 are examples of both L chain phenotypes: T15 (Pc-B), versus HP22.1A4 and HP103.3C3.2 (Pc-A). The characteristic T15 pattern can also be generated using labeled PC as it occurs in the streptococcus (R36A) cell wall. Proteins of the M603 and M511 families never resembled the T15 pattern (Ms. in preparation).

SUMMARY AND CONCLUSIONS

Our experiments using in situ hybridization of DNA fragments using a T15 V-region probe show that there are no gross differences in the major hybridizing bands between strains which express the two different phenotypic T15 L chains. The fact that the VK-22 major hybridizing bands from different strains co-migrate suggests that the flanking sequences of these V-region genes are similar in these mice. Moreover, it also suggests that the T15 genes are highly conserved or that the gene family arose by duplication recently. Resolution of the problem will come from comparison of flanking sequences of expressed V-region genes from mice of different phenotypes as well as the structural gene coding sequences.

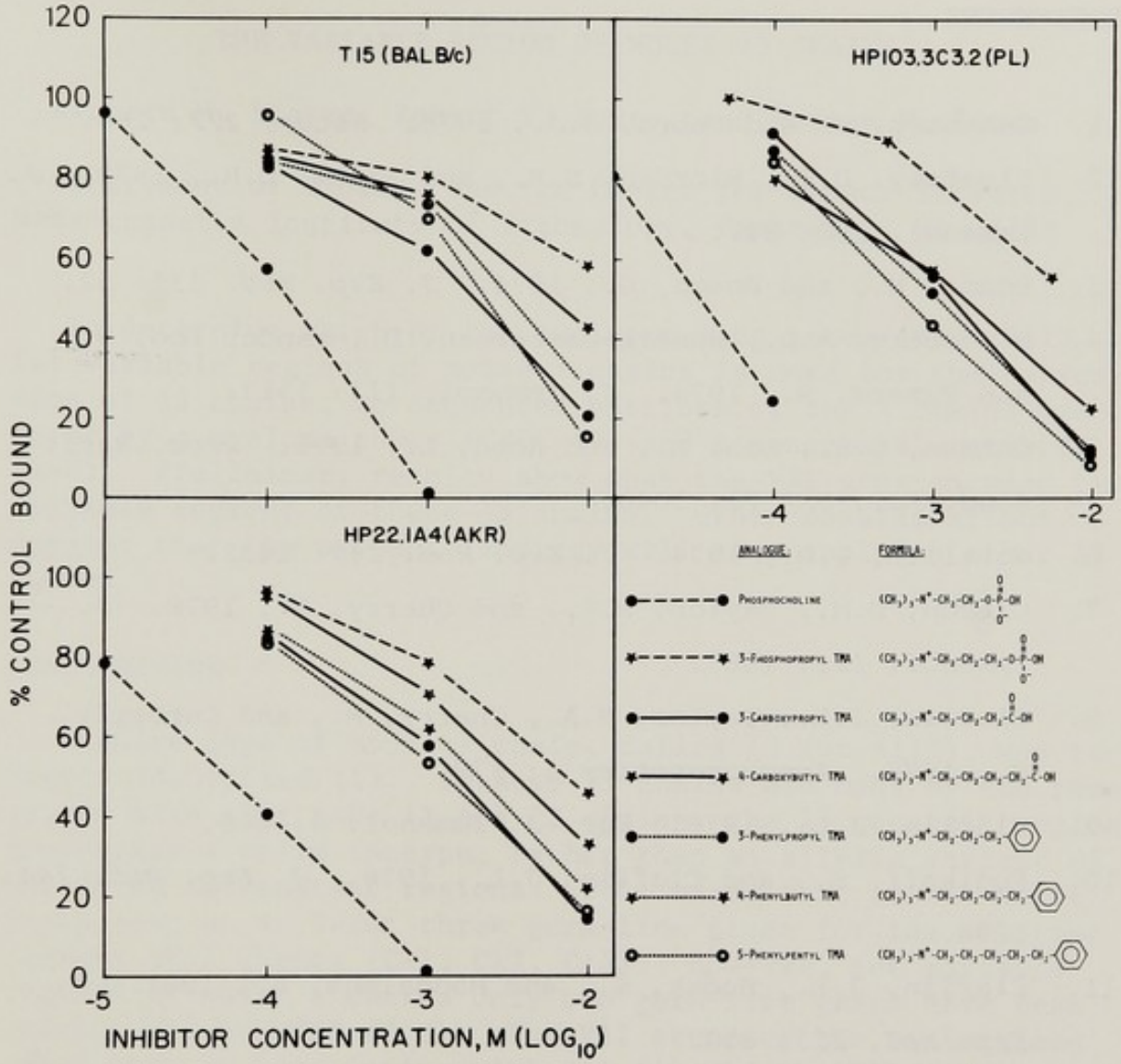

FIGURE 2. Binding specificity profiles of T15 Id$^+$ MP and HP. Inhibition of binding of ^{125}I-PC-bovine gamma globulin by PC and analogues in an RIA.

Proteins of the T15 idiotype family, with the VK (and VH) polymorphisms characteristic of the various strains of origin, demonstrate identical fine specificity profiles for PC and its analogues. The failure of structural diversity to translate into detectable functional differences also suggests conservation of this biologically important gene family. Thus, if Pc-A and Pc-B are pseudo-alleles, it will become important to analyze the allelic genes. This information should provide a clue to the kind of mechanism selectively controlling expression of one of two apparently equivalent molecules.

REFERENCES

1. Gearhart, P. and Cebra, J.J., 1978. *Nature 272*: 264.
2. Pisetsky, D.S., Riordan, S.E., and Sachs, D.H., 1979. *J. Immunol. 122*: 842.
3. Bosma, M., and Bosma, G., 1974. *J. Exp. Med. 139*: 52.
4. Strosberg, A.D., Hamers-Casterman, D., vander Loo, W., and Humers, R., 1974. *J. Immunol. 113*: 1313.
5. Gutman, G.A., Loh, E., and Hood, L., 1975. *Proc. Natl. Acad. Sci. USA 72*: 5046.
6. Gottlieb, P.D., 1974. *J. Exp. Med. 140*: 1432.
7. Gibson, D.M., Taylor, B.A., and Cherry, M., 1978. *J. Immunol. 121*: 1585.
8. Claflin, J.L., Taylor, B.A., Cherry, M., and Cubberly, M., 1978. *Immunogenetics 6*: 379.
9. Claflin, J.L., 1976. *Eur. J. Immunol. 6*: 669.
10. Rudikoff, S., and Claflin, J.L., 1976. *J. Exp. Med. 144*: 1294.
11. Claflin, J.L., Hudak, S., and Maddalena, A., 1981. *J. Exp. Med. 153*: 352.
12. Potter, M., 1977. *J. Immunol. 25*: 141.
13. Barsted, P., Rudikoff, S., Potter, M., Cohn, M., Konigsberg, W., and Hood, L., 1974. *Science 183*: 962.
14. Rudikoff, S., Satow, Y., Padlan, E., Davies, D., and Potter, M. (Submitted).
15. Claflin, J.L., and Wei, S.P., 1978. *J. Immunol. 122*: 1051.
16. Seidman, J.G., Nau, M.M., Norman, B., Kwan, S.-P., Scharff, M., and Leder, P., 1980. *Proc. Natl. Acad. Sci. USA 77*: 6022.
17. Southern, E.M., 1975. *J. MOl. Biol. 98*: 503.

THE VARIABLE REGION OF MOUSE λ3 CHAINS[1]

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To determine which of the two germ-line genes (Vλ1, Vλ2) for variable regions of mouse λ chains is used for the expression of λ3 chains, we sequenced portions of the λ chains produced by a myeloma tumor (CBPC-49) and a monoclonal antibody (5-8). Preliminary results show that the Vλ1 gene encodes the variable segment of these λ3 chains. Other considerations suggest that the present findings will also apply to other λ3 chains.

INTRODUCTION

A third type of mouse λ chain, called λ3 (or λIII), was recently identified (1). Because λ3 chains are made by the same inbred mice that make λ1 and λ2 chains, the λ3 constant region represents a third isotype, rather than an allelic variant of the λ1 or λ2 constant regions. This means that in the mouse there must be at least three germ-line genes for the constant regions of λ chains (Cλ1, Cλ2, Cλ3). However, for variable regions of mouse λ chains only two germ-line genes have been found: Vλ1 and Vλ2 (2,3). The Vλ1 sequence has so far been found only in association with Cλ1 (4) and the Vλ2 sequence has so far been detected only in chains with the Cλ2 sequence (5-7). These chains can be designated as V1-C1 and V2-C2.

Which V region is associated with the Cλ3 sequence? Is it Vλ1 (V1-C3), Vλ2 (V2-C3), or both (V1-C3 chains and V2-C3 chains)? Alternatively there could be a third Vλ gene (V3-C3), though this seems unlikely (2,3). To answer these questions we have been studying the amino acid sequences of λ3 chains. The evidence presented here indicates that the variable regions of λ3 chains derive from the Vλ1 gene; that is, λ3 chains are V1-C3.

METHODS

Light chains L^{C49} and L^{5-8} were obtained from myeloma protein CBPC-49 (α,λ3) and monoclonal antibody 5-8 (μ,λ3), respec-

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tively. The latter was prepared by fusion of Sp-2 myeloma cells with spleen cells of Balb/c mice that had been immunized with DNP-Ficoll (8). The procedures used to purify these immunoglobulins and to isolate their light chains, in mildly reduced and alkylated form, have been described (9). These chains have pyrollidone carboxylic acid (PCA) at their N-terminus and are therefore blocked to the Edman reaction. The N-terminal sequence of L^{C49} was determined after the PCA residue was removed by digestion with calf liver PCAse (10); the L^{C49} was then extensively reduced and alkylated with ^{14}C-iodoacetate and subjected to automated Edman degradation(see below).

Intact L^{C49} was cleaved at methionine residues with CNBr and chromatographed on Sephadex G-100 to yield fragment 1-120 (1). This fragment was extensively reduced and alkylated with ^{14}C-iodoacetate. One portion of this material was digested with PCAse and subjected to automated Edman degradation (see below). Another portion was reacted with citraconic anhydride, to block the ε-amino group of lysine residues, and was then cleaved at arginine residues with trypsin (E:S::1:50, 6 hr at 37°C in 50mM NH_4HCO_3). The digest was lyophilized and then chromatographed on Sephadex G-50 in 1M HAc. A major peak (CTα), detected by ^{14}C and absorption at 280nm, had the amino acid composition expected of tryptic peptides 1-23 plus 23-56 of λ1 (Table 1). Because fragment 1-23 has a blocked N-terminus, the sequence obtained by Edman degradation of the mixture could be assigned to residues 24-56.

Monoclonal antibody 5-8 was purified by adsorption on ε-DNP-lysine-Sepharose and elution with 25mM DNP-ε-aminocaproate. After mild reduction and alkylation with iodoacetate, heavy and light chains were separated by gel filtration on Sephadex G-100. Isolated L^{5-8} was extensively reduced and alkylated with ^{14}C-iodoacetate and then digested with trypsin (E:S::1:100, 20hr at 37°C). Peptides were separated by reverse phase chromatography on a Waters μBondpak C_{18} column employing a linear gradient from 0.25M ammonium acetate (pH 6.0) to 0.12M ammonium acetate (pH 6.0), 97% propanol. Fractions were collected and analysed by a) absorbance at 280nm, b) ^{14}C content, c) amino acid analysis, and d) manual Edman degradation. Manual Edman degradations were performed by a modification of the three-step procedure of Edman (11) as described by Sauer *et al.* (12). Automated degradations were performed with a Beckman 890C Sequencer, using 0.1M Quadrol (13) and polybrene (14). PTH-amino acid derivatives were identified and quantified by gas-liquid chromatography and high pressure liquid chromatography. Peptides and proteins were hydrolyzed in HCl and amino acids were analyzed on a Durham D500 Amino Acid Analyzer.

RESULTS

Figure 1 shows the partial λ3 variable region amino acid sequence derived from tryptic peptides from L^{C49} and L^{5-8}, and from an amino terminal degradation of L^{C49}. Known sequences of λ1 and λ2 chains were used to align our data. Since we have not yet directly overlapped our tryptic peptide sequences, we cannot now rule out that there may be small insertions or deletions in the λ3 sequence relative to the λ1 and λ2 sequences.

The six residues at which Vλ1 and Vλ2 germ line sequences differ are of particular interest. At each of these positions where the λ3 sequence is known, λ3 corresponds to Vλ1:Glu at position 16, Asn at 54, Ala at 62, Glu at 85, and Ile at 87. Other differences are not diagnostic because of uncertainties in amide assignments or because they occur in hypervariable regions. The composite sequence of the N-terminal 97 residues of the two λ3 chains is identical with the Vλ1 germ-line sequence.

In contrast, for residues 98-110, which are encoded by the J sequence (15), λ3 differs at three positions from λ1 (Ile-Val at 99, Ser-Gly at 102, and Val-Leu at 106) and at one position from λ2 (Ser-Gly at 102). At position 106, λ1 chains have Leu while λ2 chains have Val (16,4,5), indicating that they have separate J sequences (Jλ1 and Jλ2). Our findings suggest that there is a separate J sequence (Jλ3) for λ3 chains as well.

DISCUSSION

Each light chain is specified by three linked encoding sequences: a V gene for positions 1-97, a J sequence for 98-110 and a C gene for 111 to the C-terminus, usually 214. For mouse κ chains there are (on chromosome 6) probably several hundred Vκ genes, 4-5 Jκ sequences and one Cκ gene. There appears to be no restriction in the joining of any Vκ to a particular Jκ sequence in the formation of a complete gene for an expressed κ light chain. For mouse λ chains, located on chromosome 16 (17), the number and organization of genes is evidently different. There are at least three Cλ genes, each with its own Jλ sequence, but only two Vλ genes. The present results suggest that Vλ1 can combine with either Jλ1-Cλ1 or with Jλ3-Cλ3.

The idea that V1 is associated with J1-C1 or J3-C3, whereas V2 is expressed only with J2-C2, is based on the sequences of twenty λ1 chains (4), three λ2 chains (5 - 7), and the current study on λ3. If divergence from these V-C restrictions exist, they may be revealed by amino acid analyses. There is one methionine in Vλ2 and none in Vλ1. Cλ2 has one methionine, while Cλ1 and Cλ3 each have two methionines. Hence a V1-C2 chain would have one methionine, and a V2-C3 chain would have

TABLE 1

AMINO ACID ANALYSIS OF CTα FROM L^{C49}

AMINO ACID	FOUND	EXPECTED[a] FOR 1-23 + 24-56
Asx	4.8	5
Thr	10.6	11
Ser	5.0	5
Glx	6.1	6
Pro	2.0	2
Gly	5.8	5
Ala	4.2	4
Val	4.6	5
Ile	1.0	1
Leu	4.3	4
Tyr	1.0	1
Phe	1.0	1
His	0.9	1
Lys	1.0	1
Arg	2.0	2
Trp	ND	1
CM-Cys	0.1	1

[a]From the known sequence of MOPC-104F, a λ_1 chain (16).

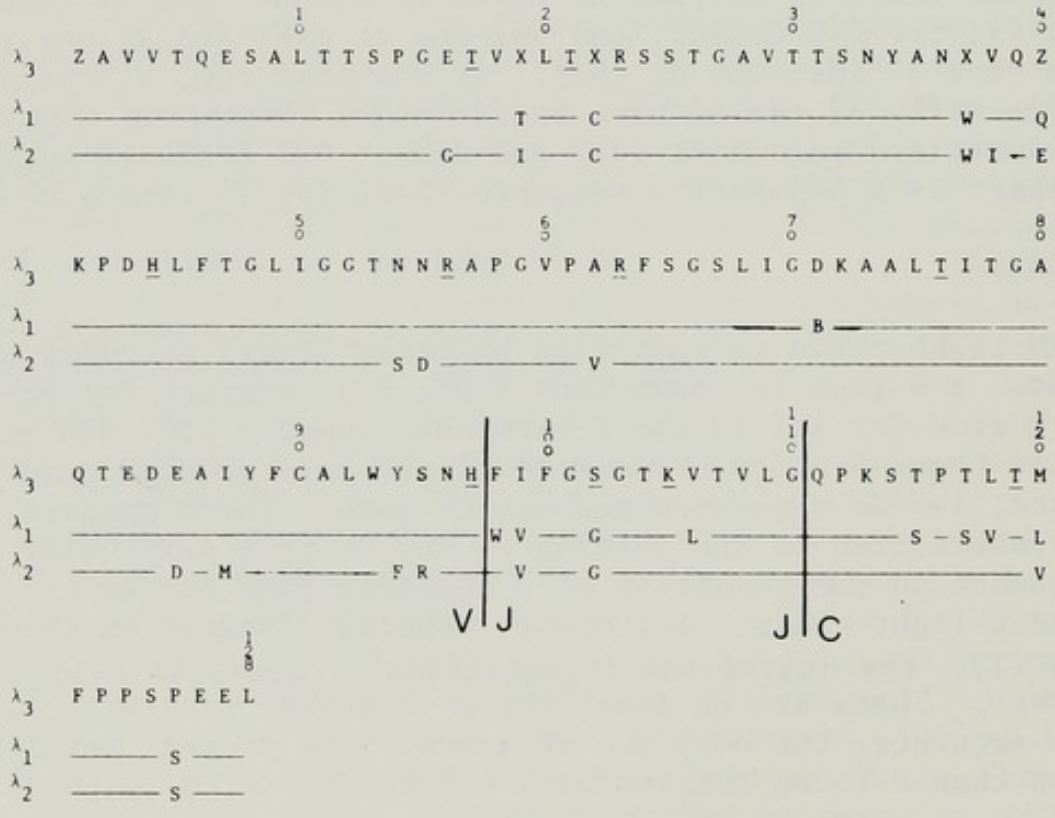

Fig. 1. The N-terminal sequence of λ3 chains (1-56 from L^{C49} and 57-128 from L^{5-8}). Underlined residues are still ambiguous. The λ1 and λ2 sequences are from MOPC-104E (16) and MOPC-315 (5). Position 71 is Asn in MOPC-104E and Asp in λ1 germ line DNA (15). The L^{5-8} sequence has been extended through most of the constant region (shown here only to 128) and agrees with the published sequence for Cλ3 (1). The L^{5-8} sequence from 111 to 119 extends the basis for comparing λ constant regions: in this region λ3 is identical to λ2 and differs from λ1 at three positions.

The standard one letter code is used; note B = Asx, Z = Glx or PCA, X = undetermined residue.

three. Thus far, all λ chains analyzed have two methionines, as expected for V1-C1, V2-C2, and V1-C3 chains.

The validity of these V-C constraints is supported by recent studies on the organization of λ genes by Miller et al. (18), who have shown that Cλ3 and Cλ1 are on the same EcoR1 restriction fragment, while Cλ2 is on a different fragment. Blomberg and Tonegawa et al (19) have shown that there are in fact two discrete gene clusters, 5'J3-C3...J1-C1 3' and 5'J2-C2...J4-C4 3', where J4-C4, a fourth λ isotype (λ4), homologous to Cλ1, is as yet unidentified in a secreted Ig. From these data it is postulated that C3 and C1 would associate with V1, and C2 and C4 with V2, if C4 is indeed active.

Why in certain antibodies (e.g., to dextran, NP, and TNP) do λ1 levels increase (20,21,8) while λ3 levels remain low (8)? Since λ1 and λ3 chains probably share the same sequences from positions 1 to 97, it seems unlikely that the difference is due to antigen-selection, though the differences between J3 and J1 might come into play here. Other possibilities are that different heavy chains are associated with λ1 and λ3 chains (in which case differences due to antigen-selection are still possible) or that there is coordinate modulation of λ-producing B cells by T cells that recognize λ constant region determinants. In the latter case, it would be expected that λ1 and the hypothetical λ4 chains would be regulated in tandem, while λ2 and λ3 would also exhibit coordinate expression. Still another possibility is that the recombination mechanism that rearranges immunoglobulin genes favors the joining of Vλ1 to Jλ1-Cλ1 over the joining of Vλ1 to Jλ3-Cλ3, leading to fewer λ3-than λ1-bearing B cell precursors. It should be possible with currently available procedures to distinguish among these possibilities.

REFERENCES

1. Azuma, T., Steiner, L.A., Eisen, H.N. (1981). Proc. Natl. Acad. Sci. USA 78,569.
2. Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. (1978). Cell 15,1.
3. Tonegawa, S., Maxam, A.M., Tizard, R., Bernard, O., and Gilbert, M. (1978). Proc. Natl. Acad. Sci. USA 75,1485.
4. Weigert, M. and Riblet, R. (1976). Cold Spring Harbor Symp. Quant. Biol. 41,837.
5. Dugan, E.S., Bradshaw, R.A., Simms, E.S., and Eisen, H.N. (1973). Biochemistry 12,5400.
6. Azuma, T., Steiner, L.A., and Eisen, H.N. (1980). Fourth International Congress of Immunology, Paris 1.1.01 (abstr).
7. Elliott, B.W.,Jr., Steiner, L.R., and Eisen, H.N. (1981). Federation Proceedings, Vol. 40, No. 3, in press (abstr).

8. Zaugg, R., Igras, V., and Eisen, H.N. In preparation.
9. Cotner, T. (1979). Dissertation (Massachusetts Institute of Technology, Cambridge, MA).
10. Podell, D.N., and Abraham, G.N. (1978). Biochemical and Biophysical Research Communications 81,176.
11. Edman, P. (1960). Ann. N.Y. Acad. Sci. 88,602.
12. Sauer, R.T., Niall, H.D., Hogan, M.L., Keutmann, H.T., O'Riordan, J.L.H., and Potts, J.T., Jr. (1974). Biochemistry 13,1994.
13. Brauer, A.W., Margolies, M.N., and Haber, E. (1975). Biochemistry 14,3029.
14. Tarr, G.E., Beecher, J.F., Bell, M., and McKean, D.J. (1978). Anal. Biochem. 84,622.
15. Bernard, O., Hozumi, N. and Tonegawa, S. (1978). Cell 15, 1133.
16. Appella, E. (1971). Proc. Natl. Acad. Sci. 68,590.
17. D'Eustachio, P., Bothwell, A.L.M., Takaro, T.T., Baltimore, D. and Ruddle, F.H. (1981). J. Exp. Med. In press.
18. Miller, J., Bothwell, A. and Storb, U. This volume and Proc. Natl. Acad. Sci., in press.
19. Blomberg, B., Traunecker, A., Eisen, H.N. and Tonegawa, S., Proc. Natl. Acad. Sci., in press.
20. Imanishi-Kari, T., Rajnavolgyi, E., Takemori, T., Jack, R.S., and Rajewsky, K. (1979). Eur. J. Immunol. 9,324.
21. Geckeler, W.R., (1977). Dissertation (University of California, San Diego, CA).

THE ORIGIN OF THE NP^b ANTIBODY FAMILY: SOMATIC MUTATION EVIDENT IN A HEAVY CHAIN VARIABLE REGION[1]

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To probe the molecular origin and idiotypic determinants characteristic of the NP^b antibody family, we have derived heavy chain cDNA clones from two members of this family. The B1-8 hybridoma (IgM) was derived during a primary response and the S43 hybridoma (IgG2a) was derived during a hyperimmune response. A comparison of the nucleotide sequences revealed the V_H regions were very similar and differed at only 10 bp. The sequence encoding the leader and J segments (J_2) were identical while the D sequences were very different. The chromosomal DNA containing cross-hybridizing germ line V_H genes was cloned from a partial Eco RI library of C57BL/6 DNA. Seven out of 15 V_H regions were found to be the best candidates for regions that could have encoded the cDNA derived variable regions and their nucleotide sequences were determined.

One, V(186-2), was found to contain precisely the DNA sequence contained in the μ cDNA clone. The remaining six germ line V_H genes were very similar in DNA sequence yet none contained any of the 10 bp found as differences between the S43 γ2a clone and the B1-8 derived μ clone. We assume these 10 bp are the result of somatic mutation of the V(186-2) gene and that most of the V_H regions that contribute to the NP^b antibody family are derived from this germ line gene.

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INTRODUCTION

The response to certain immunogens can result in the expression of a family of related antibody molecules. An extreme example of this phenomenon is the response of C57BL/6 mice to the hapten NP (4-hydroxy-3-nitrophenyl)acetyl. This family of NP binding antibodies has the following characteristics. Virtually all of the antibodies have a $\lambda 1$ light chain (1). They are also "heteroclitic" which describes the property that they have a higher affinity for an iodo derivative of NP (NIP) than for NP (2, 3). When either pooled or individual sera are analyzed by isoelectric focusing a characteristic and limited number of forms are seen (4). Finally, xenogenic antisera produced in response to the anti-NP antibodies of C57BL/6 define an idiotypically related set of antibodies (1, 4). It is designated the NP^b family because it is defined in a strain having the Igh^b haplotype. This family was one of the first to be characterized by analyzing hybridoma products. It contains a large number of unique antibodies having a number of common idiotypic determinants expressed in different combinations (5, 6).

From a comparative study of such a related set of antibodies and the DNA which encodes them it might be possible to study the development of antibody diversity. Since this immune response has been so well characterized it also offers a system to combine detailed biological and structural information.

The heavy chain variable regions contain the main source of the idiotypic diversity in these antibodies since little sequence variability has been found in λ light chains. To characterize the V_H regions we began by deriving molecular cDNA clones from hybridoma mRNAs. These clones were characterized and then used as probes to purify cross-hybridizing germ line V_H regions. These have been characterized extensively by DNA sequence analysis. The results presented here provide some conclusions concerning the origin of the idiotype in the germ line and the role of somatic mutations in the development of the response in B cells.

RESULTS

Hybridoma sequences

To characterize the NP^b family of antibodies we chose two hybridomas which produced proteins that possessed idio-

typic determinants yet were serologically as distant as any pair of hybridomas. One was the IgM-producing hybridoma, B1-8, which was derived during a primary response; the other was the IgG2a-producing hybridoma, S43, which was isolated during a hyperimmune response (5; Reth et al., this volume).

The variable regions encoding the μ and γ2a heavy chains were cloned from cDNA. A comparison of the restriction enzyme sites present in the variable regions suggested they were related (data not shown). Although there were several sites that appeared common to both regions, the μ clone contained at least two sites, Pst I and Acc I, which were not present in the γ2a clone. A V region probe derived from the γ2a clone, pVγ2a-1, showed extensive cross-hybridization with the V region present in the μ cDNA clone (data not shown).

To determine the precise relationship between the μ and γ2a clones the nucleotide sequences of the variable regions were determined (Fig. 1). The two sequences were nearly identical, differing in only 10 base pairs in the variable and leader regions. The D regions (defined later by analysis of germ line clones) were very different in both nucleotide sequence and length. The germ line J_2 sequence was used in both cases.

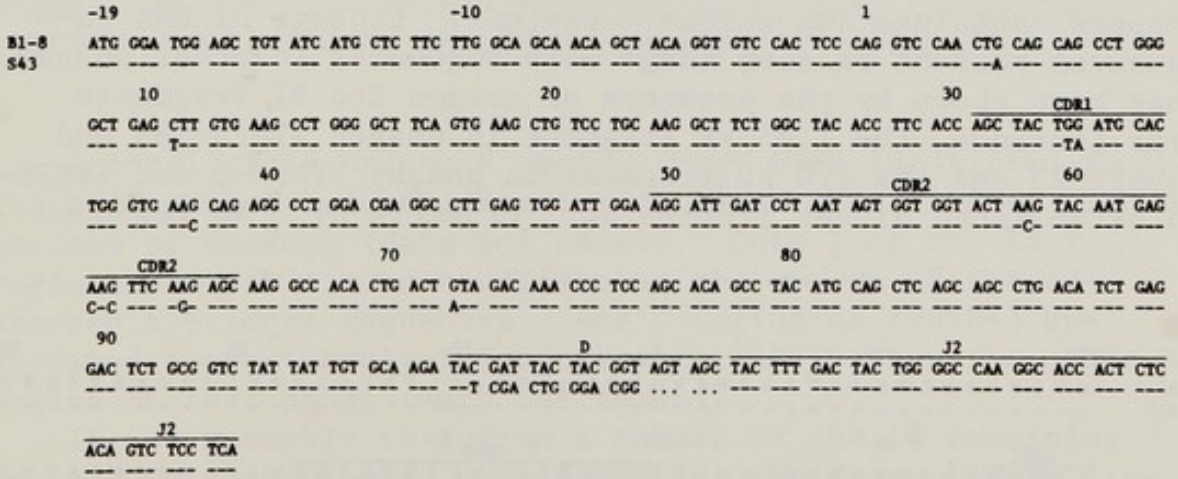

Figure 1. Nucleotide sequences of the μ and γ2a heavy chain variable regions. The B1-8 sequence was used as a reference sequence. A dash (-) indicates the S43 sequence is identical at that position to the B1-8 sequence and a period (.) indicates a gap used only for sequence comparison.

The nucleotide sequence has been used to predict the amino acid sequences of the μ and γ2a proteins (Fig. 2). Seven codon differences were evident, 3 occurred in framework residues (11, 38 and 72) and 4 in CDR regions (33, 59, 63 and 65). In addition, the D region amino acid sequence was completely different except for the occurrence of a tyrosine residue at the V-D junction. These proteins have been extensively characterized by monoclonal anti-idiotope reagents. The predicted amino acid sequence comparison now enables a preliminary localization of some of these idiotopes (Reth et al., this volume).

Germ line sequences

The relationship of the expressed hybridoma DNA sequences to germ line sequences was studied by an analysis of cross-hybridizing variable regions. The DNA from the C57BL/6 strain contained 10 cross-hybridizing Eco RI DNA bands (data not shown). Other mouse strains contained a similar pattern of bands yet only SJL and C57BL/6 DNAs gave identical patterns. These were the only two strains analyzed that had the Igh-1^b allotype.

Cross-hybridizing V_H regions were isolated from a library of partially Eco RI-digested C57BL/6 DNA constructed in λ Charon 4A. Ten recombinant phages were chosen for detailed study. The complete Eco RI digestion products present as inserts in these phages are listed in Table 1. Half of these phages contained two unique V regions. Linkage of DNA sequences present on some phage with sequences in other phages has been shown by the presence of common Eco RI fragments. For instance, the 1.85 kb fragment in phages λVNPB-186 and λVNPB-23 and the 1.0 kb fragment in phages λVNPB-6 and λVNPB-3 established direct linkage of 2 clusters of 3 V_H regions.

```
          -19              -10               1               10              20              30
B1-8      MGWSCIMLFLAATATGVHSQVQLQQPGAELVKPGASVKLSCKASGYTFT
S43       ------------------------------F--------------------

                CDR1          40          50          CDR2  60          70
          SYWMHWVKQRPGRGLEWIGRIDPNSGGTKYNEKFKSKATLTVDKPSSTA
          --L-----N-------------------T---H-R-------I--------

          80              90              D               J2
          YMQLSSLTSEDSAVYYCARYDYYGSSYFDYWGQGTTLTVSS
          ----------------RLGR..----------------
```

Figure 2. Amino acid sequences of the V_H regions. The amino acid sequences were predicted from the DNA sequences presented in Figure 1. Dashes and periods are used as described in the legend to Figure 1.

TABLE 1

ECO RI DNA FRAGMENTS IN λVNPB PHAGE

Phage	*Eco RI DNA Fragments*
λVNPB-102	*5.5, 5, 2.2, 2.3, 0.25*
λVNPB-23	*6.5, 6.5, 1.85, 1.6*
λVNPB-186	*5.5, 5.0, 1.85, 1.7, 1.0, 0.38*
λVNPB-145	*6.5, 6.5, 0.38*
λVNPB-6	*8.0, 5.5, 1.0*
λVNPB-3	*21, 1.0*
λVNPB-147	*16.0, 2.0*
λVNPB-104	*12.0, 1.0, 0.5, 0.1*
λVNPB-168	*6.5, 5.5, 2.2, 0.35, 0.24*
λVNPB-130	*8.0, 5.0, 0.46*

The phages which hybridized to pVγ2a-1 were designated λVNPB-n, where VNPB refers to a variable region gene derived from C57BL/6 DNA which hybridized to the probe for the NP^b idiotype and n refers to the isolate number. The Eco RI fragments contained in inserts in the Charon 4A vector are listed and the fragments which hybridized to the V probe are underlined. Note that in λVNPB-186 there is only one fragment of 5.0 kb listed. The subclone analysis and DNA sequence studies discussed later show that there are 2 V genes on 2 different 5.0 kb Eco RI fragments contained on this phage. The single Eco RI fragments containing V regions in λVNPB-3 and λVNPB-104 actually contain 2 V regions.

A total of 15 unique V regions have been identified in these 10 phages by hybridization to phage DNAs cleaved with a variety of enzymes (data not shown). Only 1 of the 15 V regions was not a good candidate as the source of the expressed hybridoma sequences: one V region on λVNPB-3 hybridized approximately 5-fold less intensely than the other V region on that phage (data not shown).

It was possible to define a subset of the 14 remaining V regions which were good candidates for the correct germ line genes by two complementary methods. First, a probe which showed greater homology to some V regions was made by preparing the pure V_H region insert from pVγ2a-1 instead of the entire plasmid DNA. A hybridization signal could only be generated by a very homologous sequence since the probe was small, probably 50-300 bp after nick translation. When the ten phage DNAs were cleaved with Eco RI and analyzed with this probe intense hybridization was apparent with the V

regions on λVNPB-6, -23, -145 and -186 (Fig. 3). One V region on λVNPB-102 was more homologous than the other. The V

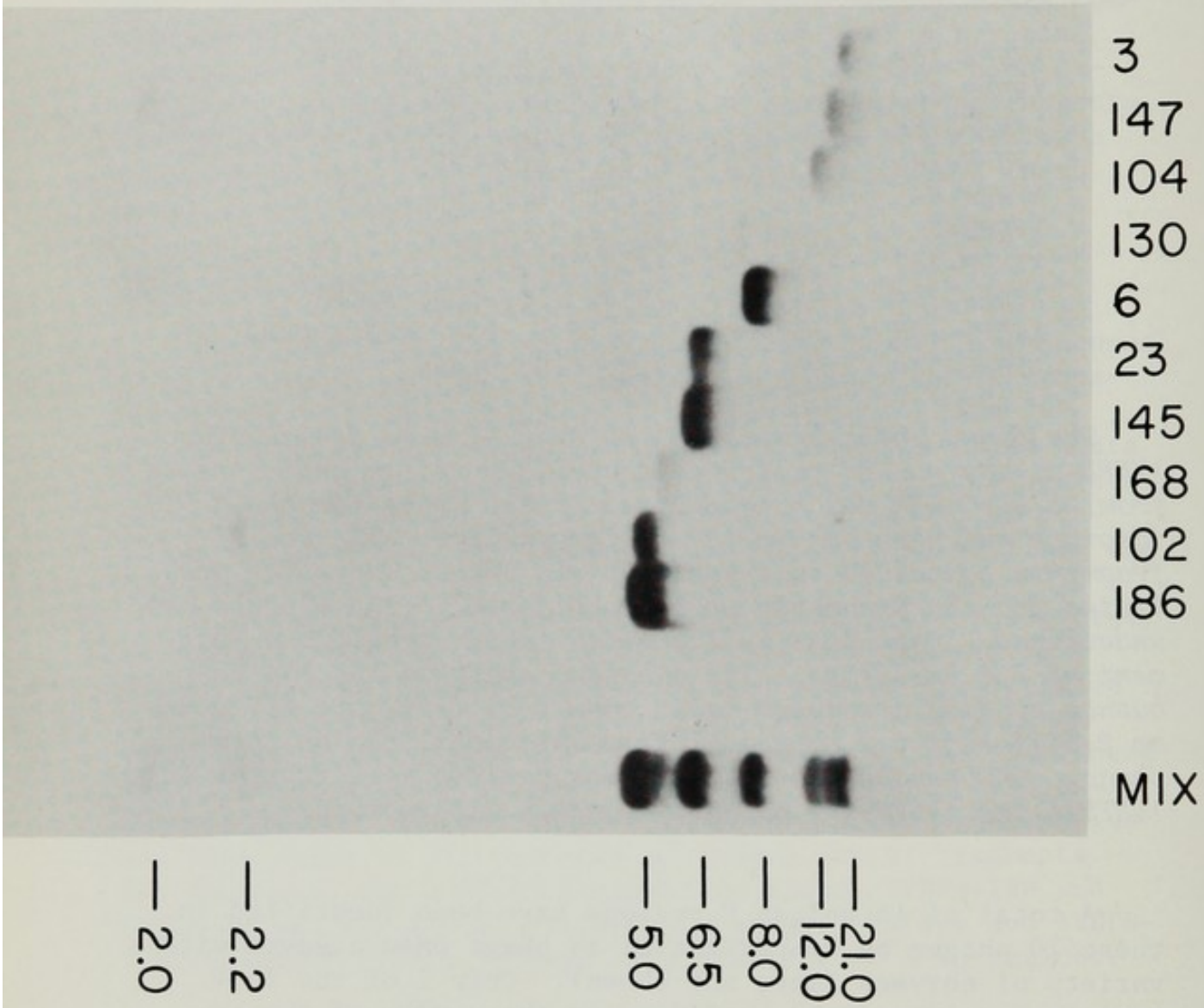

Figure 3. Hybridization of cloned genomic V_H genes. Approximately an equal amount of each phage DNA was cleaved with Eco RI and analyzed by Southern blot analysis with the nick translated V region insert from pVγ2a-1. In the lane designated MIX, a mixture containing equal amounts of each phage DNA was analyzed. The sizes in kb of the fragments hybridizing with the probes are indicated. Table 1 lists all of the Eco RI fragments contained as inserts in these phages.

regions that are most homologous to the probe may form a cluster of linked genes. The V regions that are more homologous to the probe on λVNBP-102 and -3 may occupy the terminal positions in the cluster but complete linkage is required to prove this.

The second means of comparing V region homology was to compare restriction enzyme sites in the cloned germ line genes with the cDNA clone restriction maps. It was found that only the seven genes which showed greater homology to the more specific V region probe gave restriction patterns consistent with the μ cDNA restriction pattern (data not shown). All seven genes contained both the Pst I and Acc I sites found in the μ clone. Since the γ2a clone lacked these sites, no germ line V region was consistent in both its hybridization properties and restriction map with the γ2a clone.

To identify the genes which could have produced the cDNA sequences all seven V regions were sequenced (Fig. 4). One V region, V(186-2), was chosen as a reference sequence because it was identical in sequence to the coding region of the μ cDNA clone. Basic features of germ line V regions were observed: the presence of an intron between the leader sequence and the V region and the prototypic sequences at the 3' end of the V region. The germ line sequences were all very similar yet could be separated into 2 groups. Three genes -- V(186-2), V(186-1) and V(145) -- were very closely related as were the remaining 4 V regions. In addition there were several positions at which both V(6) and V(23) were more closely related than V(3) and V(102) and vice versa. This group of four genes showed coincidental variation from the other 3 genes most obviously in the region encoding the leader sequence and the region 5' to the leader. In addition, V(145) and V(6) appear to be non-functional. The codon for residue 22 in V(145) was serine instead of cysteine -- the cysteine in position 22 is presumably essential to the formation of an intra-V region disulfide linkage -- and in V(6) the translational reading frame was altered.

DISCUSSION

Since we chose hybridomas which were so different serologically, it was anticipated that the chances of detecting products of more than one germ line gene would be maximized. Comparison of the C57BL/6 germ line sequences from the most likely candidate for the B1-8 or S43 V_H region revealed that only one was identical to the B1-8 V_H region. None of the 10

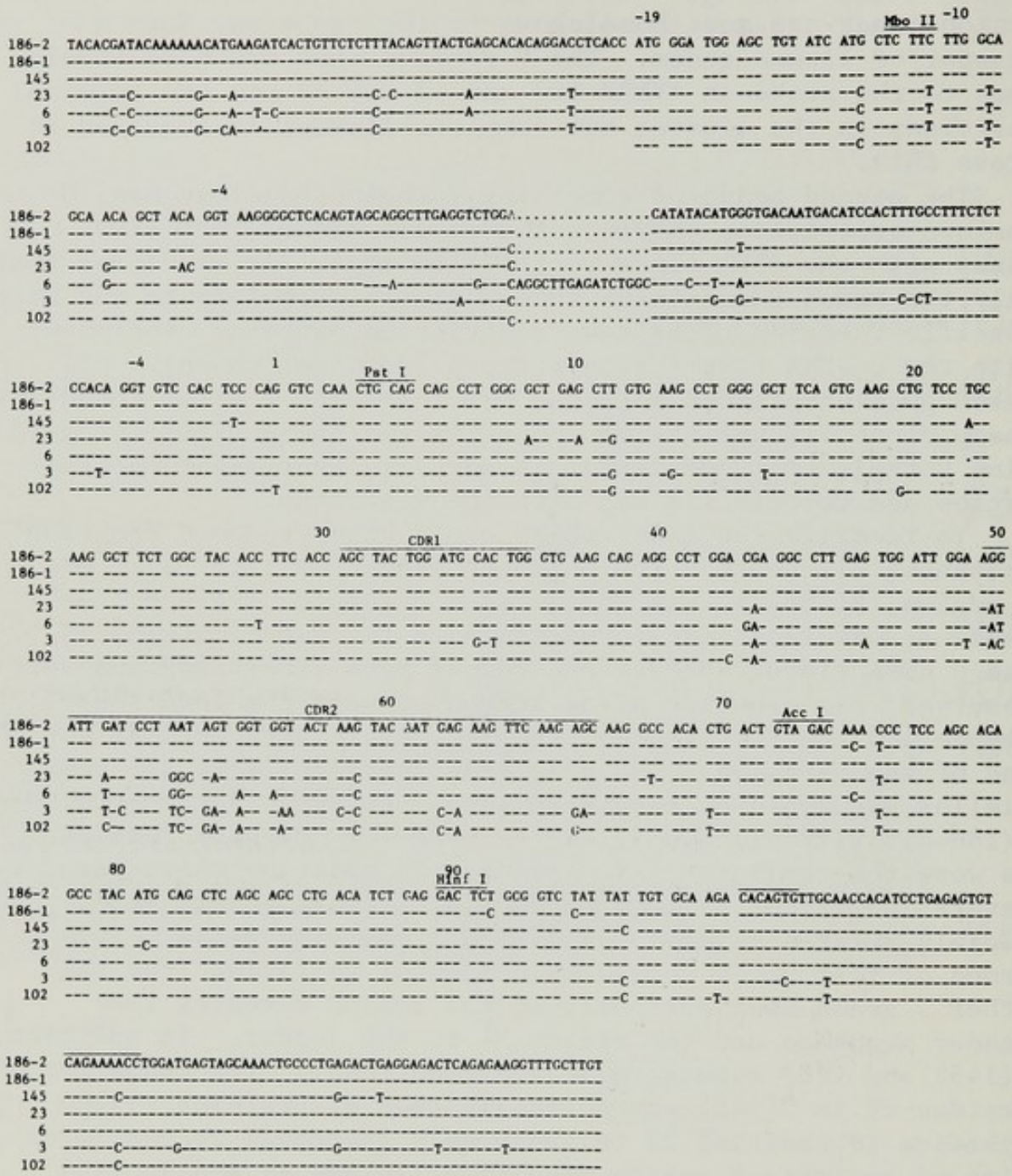

Figure 4. Nucleotide sequence of germ line V_H regions. The sequences are presented in the manner described in the legend to Figure 1. The coding sequence is presented in triplets to facilitate identification of codons. Above the sequence the amino acid codons are numbered, certain restriction sites are indicated for reference and the recognition sequences involved in DNA rearrangement are indicated with a line. The numbers used to identify V_H gene sequences are derived from Table 1. The DNA sequence reported for V(6) shows that the codon for the residue 22 contains a deletion

of 1 base pair. We are repeating the sequence of this region to verify this result. Aside from the insertion of 16 bp in the intron of the V(6) sequence, which is almost a direct repeat of the preceding (5') 16 bp, there is no other insertion or deletion that is necessary when comparing the DNA sequences.

bases which differed between B1-8 and S43 were found in any of the other 6 germ line sequences. The S43 V_H sequence is more closely related to the V(186-2) sequence than any other. We now assume that the 10 bases that are different in S43 are the result of somatic mutation. Recently, we have cloned the functional V_H gene from the B1-48 hybridoma and by restriction enzyme analysis it must also be derived from the V(186-2) gene.

Two observations can be made concerning the residues at which somatic mutations occurred in the V(186-2) gene. First, 6 of 10 changes occurred at G residues while 3 others were at A residues. This preference for purine residues and more specifically G residues as targets for somatic mutation has also been predicted from the protein sequence data of somatic variants of the λ_1 light chains (7). These observations might argue for a common mechanism and specificity in the generation of such mutations. However, the second observation is that half of the mutations are transitional mutations and the other half are transversions. Since we have no understanding of the mutational mechanisms it is impossible to predict whether this might indicate that more than one mechanism was involved.

The sequences of the germ line V_H regions show some remarkable features. Although there are differences over the entire region, they occur more frequently in the second CDR. There is also a selection for replacement mutations versus silent mutations in this region. This gene family has likely been generated by a process of gene duplication possibly as a consequence of unequal cross-over events. Currently we are characterizing regions flanking V regions and deriving more clones to prove the physical linkage of this entire V gene family. This may permit a detailed model of the expansion of the V gene cluster. The DNA sequences suggest that gene conversion may have occurred in the evolution of the cluster. The coincidental variation of nucleotides in leader and 5' flanking regions and the second CDR is suggestive.

The observation that the μ cDNA clone had no differences from a germ line sequence and the γ2a clone contained 10 variant bases is remarkable. This finding may be more general in that μ chains may utilize unmodified V_H regions

and other isotypes may contain somatic mutations in their V regions. This suggestion is in agreement with the observation that idiotypic specificities and affinities of IgM antibodies do not change over long periods of time yet the affinity of IgG antibodies increases considerably (8, 9). A model can now be proposed in which combinatorial processes occurring in the bone marrow generate functional rearrangements that could generate μ chains. Once such a cell emerges from the bone marrow it may be subjected to antigen stimulation and clonal expansion. In this process both μ secreting plasma cells and memory cells would be generated. In these cell types, both somatic mutation and/or isotype switching may occur. Somatic mutations may occur simultaneously with the switching event and/or as these cells undergo their ensuing cell divisions. A subsequent exposure to antigen by hyperimmunization would then result in the selection of clones with potentially higher affinity and altered idiotypic specificities.

This model could account for some of the observations concerning NP^b antibodies. During the hyperimmune response there is a loss of idiotypic determinants and an increase in the level of κ chains (5). This could occur if somatic mutation was now generating higher affinity antibodies from previously underrepresented V regions.

Presently we have evidence that only one V_H region and one V_L region ($V\lambda_I$) is used to generate the NP^b antibody family. It has been shown that the interaction of hapten with antibody results in a UV spectral shift that is likely due to the interaction of hapten with tryptophan residues (especially Trp-97) in the combining site (5). Therefore the hapten NP may have a precise chemical interaction with the tryptophan residues of the λ_1 chains and perhaps the tyrosine residue at the V-D junction in the heavy chain that results in clonal expansion. A more extensive characterization of members of this family will provide structural information and hopefully insight into the mechanisms of antigen selection and clonal expansion of resting B-lymphocyres.

A molecular definition of the characteristic NP^b idiotopes is essential to an understanding of the roles of the various types of T-cells and idiotope regulation in the primary and hyperimmune responses. The present definition of germ line and somatically generated idiotopes will certainly refine experimental approaches. It is important to characterize other idiotypically related families since the ability of a given mouse strain to mount an immune response will vary according to its inherited V gene repertoire. The response in C57BL/6 mice to NP may be less complex because the particular V region can be efficiently selected. Other mouse strains

or responses to other haptens may require different levels of T-cell help and somatic mutation.

REFERENCES

1. Jack, R.S., Imanishi-Kari, T. and Rajewsky, K. (1977). *Eur. J. Immunol.* 8, 559.
2. Imanishi, T. and Makela, O. (1974). *J. Exp. Med.* 140, 1498.
3. Imanishi, T. and Makela, O. (1975). *J. Exp. Med*, 141, 840.
4. Makela, O. and Karjalainen, K. (1977). *Immunol. Rev.* 34, 119.
5. Reth, M., Hammerling, G.J. and Rajewsky, K. (1978). *Eur. J. Immunol.* 8, 393.
6. Reth, M., Imanishi-Kari, T. and Rajewsky, K. (1979). *Eur. J. Immunol.* 9, 1004.
7. Weigert, M. and Riblet, R. (1976). *Cold Spring Harbor Symp. Quant. Biol.* 41, 837.
8. Rodwell, J.D. and Karush, F. (1980). *Molecular Immunology* 17, 1553.
9. Eisen, H.N. and Siskind, G.W. (1964). *Biochemistry* 3, 996.

ORGANIZATION OF IMMUNOGLOBULIN HEAVY CHAIN GENES AND GENETIC MECHANISM OF CLASS SWITCH[1]

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INTRODUCTION

During differentiation of a given B lymphocyte, a single variable (V) gene is first expressed as a part of the μ chain and at a later stage, the constant (C) region of the expressed heavy (H) chain switches from μ to γ or to α without alteration of the V region sequence. Genetic and molecular bases for such a phenomenon called H chain class switch is one of the most fascinating biological questions.

Comparison of rearranged and germline H chain genes lead us to propose a molecular mechanism to explain H chain class switch (1-3). A complete H chain gene is formed by two types of recombinational events as shown in Figure 1. The first type of recombination takes place between a given V_H, D and a J_H segments, which completes a V region sequence. This recombination is referred to as V-D-J recombination. After such recombination the V region sequence is expressed as a part of the μ chain. The second type of recombination takes place between S_μ and S_γ (or S_α) regions. The S region was defined as a functional region responsible for the class switch and assumed to be located in the 5' flanking region of each C_H gene. The S_μ region is located between J_H and the μ gene and the S_γ (or S_α) is present at the 5' side of each γ gene (or α gene). The second type of recombination, which is called S-S recombination, can replace the C part of the H chain without affecting the V region sequence. Other groups (4,5) also reached similar conclusion.

Both types of recombination result in the deletion of the intervening DNA segment from the chromosome (6-12). The order of C_H genes was previously proposed to be 5'-μ-γ3-γ1-γ2b-γ2a-α-3' (6). This is based on the deletion

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profile of C_H genes in myelomas which produce different classes of the H chain proteins.

In this paper we will present the direct evidence for the proposed organization of the C_H gene cluster as described in Figure 1. We will also describe the structural basis of the S region which was originally defined as a functional region and discuss the molecular mechanism of the deletion. Finally, we will present evidence that there are conserved segments at the 3' side of each C_γ gene.

Linkage of Mouse C_H Genes

Most of the mouse C_H gene fragments including C_μ, C_δ, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, $C_{\gamma 3}$ and C_α have been cloned. We have already reported complete nucleotide sequences of the C_μ, $C_{\gamma 1}$, $C_{\gamma 2a}$ and $C_{\gamma 2b}$ genes (13-16). Recently, we have suceeded in cloning the ε gene from an IgE-producing hybridoma (17). Partial nucleotide sequences of the cloned ε gene were determined and the amino acid sequences deduced from the nucleotide sequences were similar to that of the human ε chain (Figure 2). Using these cloned DNA segments as probes, we have isolated overlapping chromosomal segments of the C_H gene cluster from phage libraries containting embryonic mouse DNA (17-19). We have isolated a number of clones containing C_H genes and their flanking regions and aligned them by characterization with restriction enzyme cleavage and

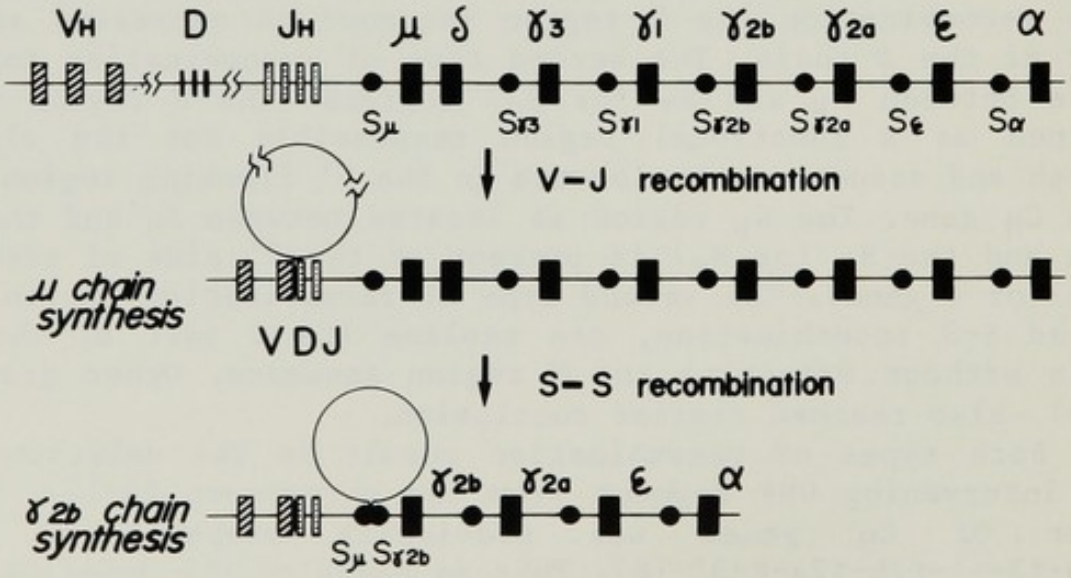

FIGURE 1. A genetic mechamism of heavy chain class switch. Recombination events, which take place to form a complete $\gamma 2b$ gene during differentiation of a B lymphocyte, are schematically represented.

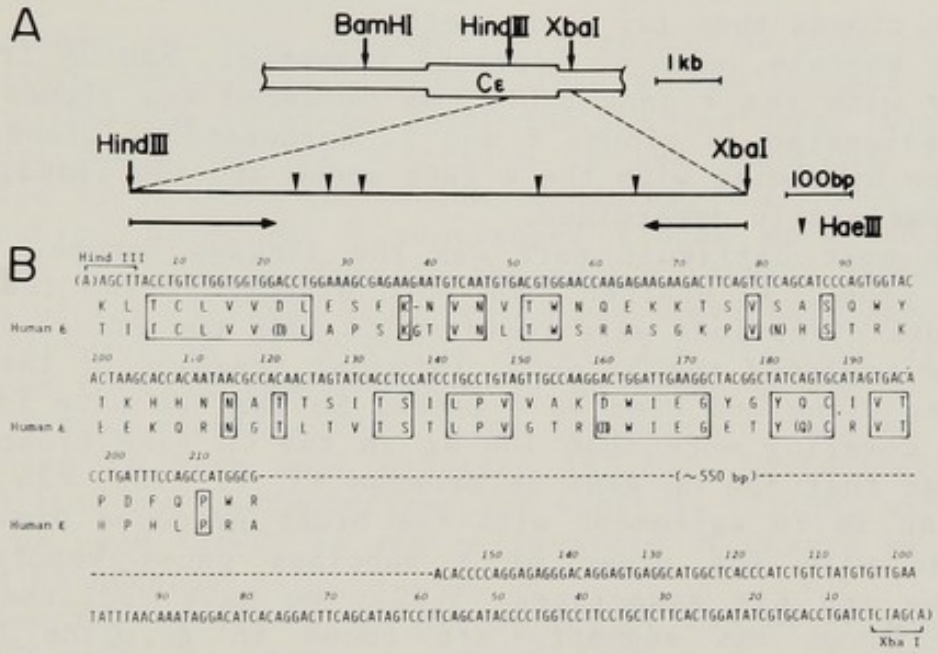

FIGURE 2. Partial nucleotide sequence of the ε gene. (A) Strategy for nucleotide sequence determination. The 0.95 kb Hind III-Xba I fragment was isolated and sequenced according to Maxam and Gilbert (20). Ranges and direction of sequences read are shown by horizontal arrows. (▼), Hae III sites. (B) Nucleotide sequences at both termini. The amino acids predicted by the nucleotide sequence are shown under the coding sequences. The amino acid sequence corresponding to the C_{H3} domain (residue 345-418) of human ε chain (21) is presented at the bottom row and the homologous amino acids are boxed. Amino acids are expressed by one letter code. Reproduced from Nishida et al. (17).

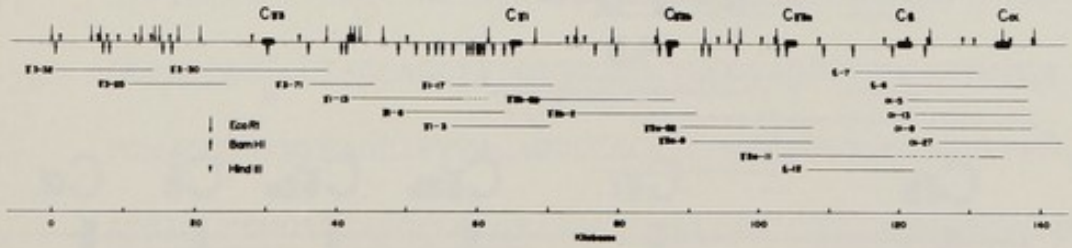

FIGURE 3. Restriction endonuclease cleavage maps of overlapping cloned fragments in the C_H gene cluster. The chromosomal segment containing γ3, γ1, γ2b, γ2a, ε and α genes is shown schematically. Mouse C_H gene clones were isolated from phage libraries of embryonic mouse (Balb/c) DNA and characterized by restriction enzyme cleavage, Southern blot hybridizaton and R-loop formation. All the clones were aligned with use of overlapping regions (3,17-19). Closed boxes indicate structural genes.

Southern blot hybridization experiments. Figure 3 summarizes all the clones thus far characterized.

For example, when we screened a partial Sau 3A fragment library with the ε gene probe, we isolated two clones which were designated as clones 6 and 7. Fortunately, clone 6 was shown to hybridize with the α gene probe and the linkage of ε and α was easily determined.

The most difficult part was the linkage between the γ3 and γ1 genes. We started from both directions and two steps from both directions were required to join two genes. In each step we isolated the 5' or 3' terminal fragment of the newly isolated clone and used as a probe. So far, we have isolated clones covering more than 150 kp in the C_H gene cluster. It is clear that the C_H gene is ordered γ3, γ1, γ2b, γ2a, ε and α, which is in agreement with the order proposed previously (6) and with the order of γ subclass genes reported by Tonegawa and his associates (5,22). We have confirmed that these cloned DNA segments are found in germline DNA by Southern blot to exclude the possibility of cloning artefact.

Several groups reported that J segments are present about 6.5 kb 5' of the μ gene. (23-25). Liu et al. (26) reported that the δ gene is only 4.5 kb 3' to the μ gene. These results taken together, the general organization of the immunoglobulin C_H gene is now elucidated except that we do not know the distance between the δ and γ3 gene (Figure 4). It is worth noting that all the C_H genes ordered have the same orientation and thus are transcribed from the same strand of DNA.

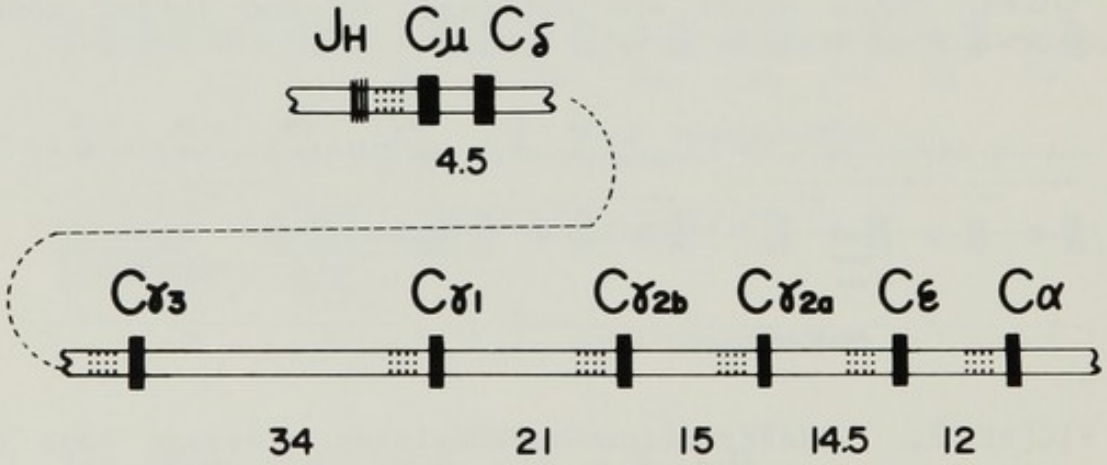

FIGURE. 4 Summary of the C_H gene organization. Closed boxes indicate structural genes. Dotted areas show regions containing repetitive sequences. Numbers in kb.

Structure of the S Region

Originally we have defined the S region as such that is functionally responsible for the class switch recombination. Recent nucleotide sequence determination indicated that the structural basis of the S region is the beautiful tandem repetition of related nucleotide sequences. The class switch recombination sites of 7 rearranged clones were thus far determined and they cluster in the region located about 5 kb upstream of the C_μ gene. This region was previously sequenced and characteristic of a simple repetitive sequence (2,25). Downstream to this region there is a region which is often deleted during cloning. The μ gene fragments cloned by other groups seem to have such deletion (23,25). Fortunately, we have isolated and characterized the whole S_μ region, probably because we used λgtWES as vector in stead of Charon 4A which was used by others.

Nucleotide sequences of the S_μ region can be represented by (GAGCT)n(GGGGT) where n ranges from 1 to 17 with the highest frequency at 3 (27). The core part of the S_μ region was very difficult to be sequenced because there were not appropriate restriction sites. When we digested it with Alu I and analyze the digests by polyacrylamide gel

Table I Nucleotide Sequences of Repeat Units of S Regions

S Region	Unit Sequences
S_μ	GAGCTGAGCTGGGGTGAGCT
$S_{\gamma 1}$	GPPTCCAGGCTGAGCAGCTACAGGGGAGCTGGGGYAPPTGGGAPTPTPG
$S_{\gamma 2b}$	GGGACCAG$^{T}_{A}$CCTAGCAGCTPTGGGGGAGCTGGGGA$^{A}_{T}$GGTPGGAPTPTGA
$S_{\gamma 3}$	PGNACC$^{A}_{T}$GPNTPAGCAPYYACAGGGGAGC$^{T}_{A}$GGGG$^{A}_{T}$PGGTGGGAGTATPP
S_α	ATGAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTGGT
	GTGAGCTGGGTTAGGCTGAGCTGAGCTGGA
Common Sequences	GAGCTG, TGGGG

Common sequences are underlined. Sources of sequences are S_μ (27), S_γ (28) and S_α (29).

electrophoresis, major bands of 5 and 10 bp and minor bands of 20 and 15 bp were found. The results indicate that the core sequence also contains a tremendous number of Alu I sites (AGCT) probably one site every five or ten bases. This is consistent the sequence determined at the region adjacent to the core fragment. There are regions which contain precise tandem repeat of 20 bp unit exactly where deletion occurrs.

We have also determined partial nucleotide sequences surrounding the class switch recombination site in the $S_{\gamma 1}$, $S_{\gamma 2b}$ and $S_{\gamma 3}$ regions (28). All of them comprise tandem repetition of 49 base-pair units. The nucleotide sequence of the $S_{\gamma 2b}$ region shows that each repeating unit is similar to each other. Such tandem repetitive region lies between 1.8 to 5.2 kb 5' to the structural gene. We have also determined a partial nucleotide sequence of the S_{α} region, which comprises tandem repetition of 80 bp unit (29,30). Table 1 summarizes nuclotide sequences of the repeat units of S regions. The S_{μ} region shares short common sequences (GAGCTG and TGGGG) with other S regions. GAGCTG and TGGGG appear 50 to 100 times in all the S regions.

A combined structure of tandem repetition and short common sequences provides a large number of the possible recombination sites in the S region and increase the chance of S-S recombination. The S-S recombination does not have to be highly specific to the nucleotide joined together because it takes place in the intervening sequence. Instead, the class switch recombination is expected to be efficient since it takes place during relatively short period of time after stimulation with an antigen. The above structure satisfies these biological features required for the class switch recombination. We think that these repetitive sequence is the structural basis for the S region. We find the repetitive sequence in the 5' flanking region of each C_H gene except for the δ gene which we have not tested as shown in Figure 4.

We have cloned human μ gene from human DNA library and compared its structure with mouse μ gene (31). Studies using heteroduplex analysis and Southern blot hybridization clearly show that not only the coding region but also the S_{μ} region is homologous between human and mouse μ genes. The results suggest that the nucleotide sequence in the S_{μ} region plays an essential biological function and thereby it has been conserved in these organisms for decades of million years (31,32).

In addition, we found that the S_{μ}-related sequences are found among a wide variety of organisms. It is remarkable that DNA of sea urchin, which obviously does not have immunoglobulin genes, also contains clear bands hybridizing with mouse S_{μ} sequence. The results indicate that the immunoglobulin gene seems to have used, as S region sequences, some pre-

existing sequence which may or may not have other biological functions. We have cloned these S_μ-related sequence from sea urchin, Drosophila and Xenopus (33). We are going to test that these sequences are linked to a gene expressed in these organisms or not.

Sister Chromatid Exchange Model

It is established that deletion of C_H genes accompanies the S-S recombination. Two alternative models can be proposed to explain the mechanism of the C_H gene deletion in B-lymphocytes as shown in Figure 5 (3,29). The first model postulates that the S-S recombination takes place on a single chromosome by mutual recognition of two S regions. The intervening DNA segment if looped out and lost from the chromosome. This model is referred to as a looping-out model. Such recombination can occur at any stage of the cell cycle in principle. The other model, called a sister chromatid exchange model, explains the deletion of DNA segment by an unequal crossing-over event between sister chromatids. According to this model one of the daughter cells contains an additional copy of the C_H gene that is lost in the other daughter cell. Sister chromatid exchange is unlikely to occur at any other stage of the cell cycle except for the mitotic phase.

In either case we think the basic mechanism of the S-S recombination is mediated by repeated short common sequences. It is likely that a putative recombinase or a recombinase complex catalyze excision and ligation of two DNAs. But actual recombination sites do not seem to be highly specific to the nucleotide joined together.

Structural analyses of an expressed $\gamma 1$ gene clone lead us to conclude that the sister chromatid exchange model may be more favorable. The structure of the expressed $\gamma 1$ gene of myeloma MC101 can be represented as follows; 5'-V-D-J-S_μ-S_α-$S_{\gamma 1}$-$C_{\gamma 1}$-3' (29,34). This $\gamma 1$ gene contains a short S_α segment(490 bp) between S_μ and $S_{\gamma 1}$ regions. This fact appears to contradict the linear arrangement of C_H gene (5'-μ-$\gamma 3$-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-ε-α-3') and the stepwise looping-out mechanism. Such $\gamma 1$ gene, however, can be created by two or three successive unequal crossing-over events.

There are various possible pathways to create MC101 $\gamma 1$ gene, several examples of which are illustrated in Figure 6. In one pathway, the first recombination produces a chromosome with a duplicated segment containing the μ and γ genes. The second crossing-over occurs between the S_μ and S_α regions, resulting in the expression of the μ gene that is linked to a V gene, the S_μ and S_α regions at its 5' side. The third crossing-over takes place between the S_α and S_γ regions

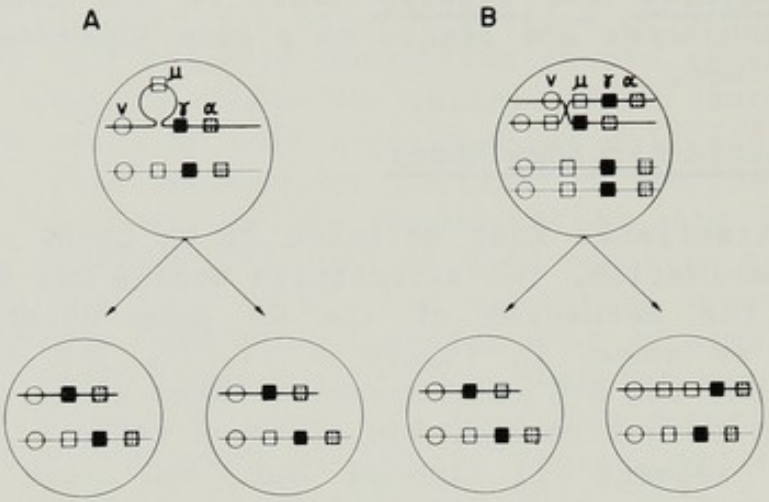

FIGURE 5. Possible models for deletion of C_H genes in class switch. A. looping-out model. B. sister chromatid exchange model. Reproduced from Obata et al. (29).

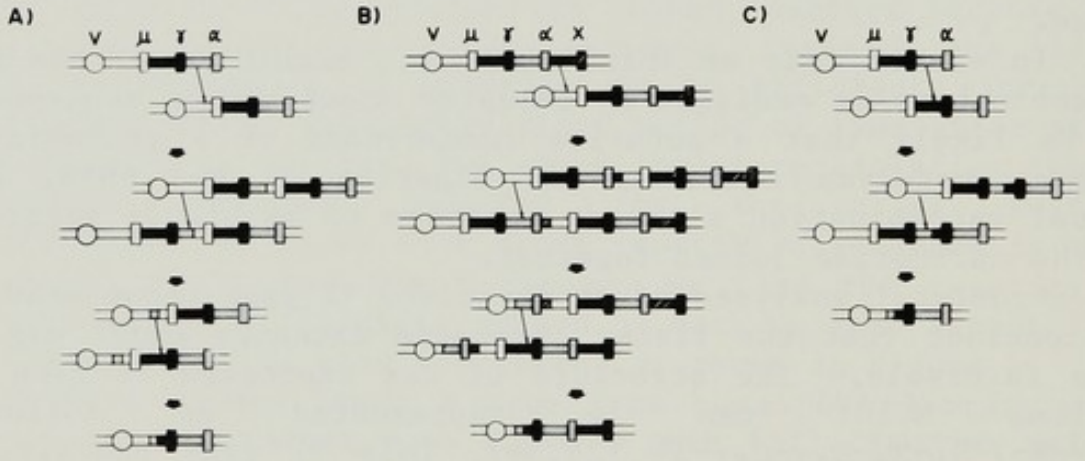

FIGURE 6. Possible pathways to create the expressed γ1 gene of MC101 myeloma. Reproduced form Obata et al. (29).

giving rise to a γ gene that is linked to a V gene, the S_μ and the S_α region at its 5' side. In a second pathway we postulated another C_H gene at the 3' side to the α gene.

In a third we allowed a recombination event that does not result in the class switch. In contrast to the looping-out model, the sister chromatid exchange model allows some lymphocytes to switch in a reverse direction of the C_H gene order. The number of clones switching in the reverse direc-

tion may be lower than that switching in the forward direction because the number of the recombination required for the reverse switch is larger than that for the forward switch. In addition, one of such recombination products could be inviable, and therefore could not be established among progenies. Nonetheless, Radbruck et al. (35) recently reported that a variant of myeloma X63 can switch from γ2b to γ1. Reverse switch can be easily explained by sister chromatid exchange as shown Figure 6 B.

The sister chromatid exchange model can be directly tested by analyzing the content and context of C_H genes in the progeny of a single B lymphocyte because asymmetric segregation of C_H genes innevitably produces progeny clones with duplicated as well as deleted C_H genes.

Membrane Domain Exons in γ Genes

We have compared the flanking regions of different γ genes by heteroduplex analyses. Such study revealed interesting homology regions in the 3' flanking region of all the γ genes (36). Heteroduplex molecules formed between γ2a and γ3 genes showed four separate homology regions as shown in Figure 7. Comparison of these pictures with the restric-

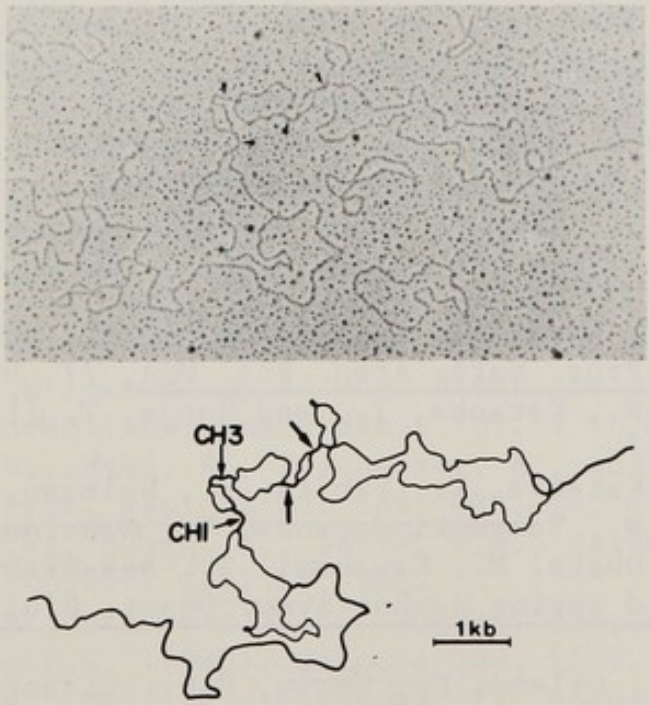

FIGURE 7. Heteroduplex formed between γ2a and γ3 genes. Upper picture shows electronmicrograph of the heteroduplex formed between Igγ2a-11 and Igγ3-31 (clone of J606) and its interpretation is illustrated below. The homology regions in the 3' flanking region are indicated by arrows.

tion maps of the γ2a and γ3 genes indicated that two homology regions are located at the 5' side of the clones correspond correspond to the CH1 and CH3 domains. In the 3' flanking region there are two homology regions of 270 and 250 bp in length which are separated by a 550 to 600 bp long intervening sequence. These 3' homology regions are located about 1.4 to 1.7 kb 3' to the CH3 domain. Similar analyses carried out with all the γ genes showed that all the γ genes have two homology regions of similar size at similar location. It is likely that these conserved segments are membrane domains similar to those found in the μ gene (37,38). We have tested this possibility by hybridizing a DNA segment of the γ2a-gene homology region to 2PK3 mRNA (prepared by Drs. V. Oi and L. Herzenberg) which contains mRNA encoding membrane-form γ2a chain (39). The homology segment of the 3' flanking region of the γ2a gene hybridized to 4kb mRNA while the DNA segment of the CH1 and CH2 domains hybridized to both 1.8 and 4 kb mRNAs. We have also demonstrated R-loop formation between 2PK3 mRNA and γ2a gene with the size and location similar to the homology regions in the 3' flanking region.

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REFERENCES

1. Kataoka, T., Kawakami, T., Takahashi, N., and Honjo, T. (1980). Proc. Natl. Acad. Sci. USA, 77, 919.
2. Takahashi, N., Kataoka, T., and Honjo, T. (1980). Gene 11, 117.
3. Honjo, T., Kataoka, T., Yaoita, Y., Shimizu, A., Takahashi, N., Yamawaki-Kataoka, Y., Nikaido, T., Nakai, S., Obata, M., Kawakami, T., and Nishida, Y. (1981). Cold Spring Harbor Symp. Quant. Biol. (in press).
4. Davis, M.M., Calame, K., Early, P.W., Livant, L., Joho, R. Weissman, I.L., and Hood, L. (1980). Nature 283, 733.
5. Maki, R., Traunecker, A., Sakano, H., Roeder, W., and Tonegawa, S. (1980). Proc. Natl. Acad. Sci. USA, 77, 2138.

6. Honjo, T. and Kataoka, T. (1978). Proc. Natl. Acad. Sci. USA, 75, 2140.
7. Yaoita, Y. and Honjo, T. (1980). Nature 286, 850.
8. Yaoita, Y. and Honjo, T. (1980). Biomed. Res. 1, 164.
9. Coleclough, C., Cooper, D., and Perry, R.P. (1980). Proc. Natl. Acad. Sci. USA, 77, 1422.
10. Cory, S. and Adams, J.M. (1980). Cell 19, 37.
11. Cory, S., Jackson, J., and Adams, J.M. (1980). Nature 285, 450.
12. Rabbitts, T.H., Forster, A., Dunnick, W., and Bentley, D.L. (1980). Nature 283, 351.
13. Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N., and Mano, Y. (1979). Cell 18, 559.
14. Kawakami, T., Takahashi, N., and Honjo, T. (1980). Nucleic Acids Res. 8, 3933.
15. Yamawaki-Kataoka, Y., Kataoka, T., Takahashi, N., Obata, M., and Honjo, T. (1980). Nature 283, 786.
16. Yamawaki-Kataoka, Y., Miyata, T., and Honjo, T. (1981). Nucleic acids Res. (in press).
17. Nishida, Y., Kataoka, T., Ishida, N., Nakai, S., Kishimoto, T., Böttcher, I., and Honjo, T. (1981). Proc. Natl, Acad. Sci. USA (in press).
18. Shimizu, A., Takahashi, N., Yamawaki-Kataoka, Y., Nishida, Y., Kataoka, T., and Honjo, T. (1981). Nature 289, 149.
19. Honjo, T., Nishida, Y., Shimizu, A., Takahashi, N., Kataoka, T., Obata, M., Yamawaki-Kataoka, Y., Nikaido, T., Nakai, S., Yaoita, Y., and Ishida, N., (1981). Proc. Symp. on Mechanismin Mucosal Immunity, Raven Press, New York (in press).
20. Maxam, A.M. and Gilber, W. (1977). Proc. Natl. Acad. Sci. USA, 74, 560.
21. Bennich, H., and von Bahr-Lindstrom, H. (1974). Progree in Immunology II eds. Brent, L. and Holborow, J. (North-Holland, Amsterdam), Vol. I p.49.
22. Roeder, W., Maki, R., Traunecker, A., and Tonegawa, S. (1981). Proc. Natl. Acad. Sci, USA, 78, 474.
23. Early, P.W., Huang, H., Davis, M.M., Calame, K., and Hood, L. (1980). Cell 19, 981.
24. Newell, N., Richards, J.E., Tucker, P.W., and Blattner, F.R. (1980). Science 209, 1128.
25. Sakano, H., Maki, R., Kurosowa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
26. Liu, C.-P., Tucker, P.W., Mushinski, J.F., and Blattner, F.R. (1980). Science 209, 1348.
27. Nikaido, T., Nakai, S., and Honjo, T. in preparation.
28. Kataoka, T., Miyata, T., and Honjo, T. (1981). Cell 23, 357.

29. Obata, M., Kataoka, T., Nakai, S., Yamagishi, H., Takahashi, N., Yamawaki-Kataoka, Y., Nikaido, T., Shimizu, A., and Honjo, T. (1981). Proc. Natl. Acad. Sci. USA (in press).
30. Davis, M.M., Kim, S.K., and Hood, L. (1980). Science 209, 1360.
31. Takahashi, N., Nakai, S., and Honjo, T. (1980). Nucleic Acids Res. 8, 5983.
32. Ravetch, J.V., Kirsch, and Leder, P. (1980). Proc. Natl. Acad. Sci. USA, 77, 6734.
33. Sakoyama, T., Yamawaki-Kataoka, Y. Yaoita, Y., and Honjo, T. in preparation.
34. Kataoka, T. Nikaido, T., and Honjo, T. in preparation.
35. Radbruck, A., Liesegang, B., and Rajewsky, K. (1980). Proc. Natl. Acad. Sci. USA, 77, 2909.
36. Nakai, S., and Honjo, T. in preparation.
37. Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980). Cell 20, 293.
38. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). Cell 20, 313.
39. Oi, V.T., Bryan, V.M., Herzenberg, L.A., and Herzenberg, L.A. (1980). J. Exp. Med. 151, 1260.

PRIMARY STRUCTURAL STUDIES OF MONOCLONAL A/J ANTI-ARSONATE ANTIBODIES DIFFERING WITH RESPECT TO A CROSS-REACTIVE IDIOTYPE

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INTRODUCTION

While subserving its primary role of recognizing antigen, the antibody molecule can also display to an immune system its own array of antigens in immunogenic form. Those determinants which are both unique to that antibody molecule and provoke an immune response define an idiotype (1,2). When, as first reported for cold agglutinins (3) and anti-gamma globulins (4), these unique determinants are found on antibody populations other than those produced by the individual which defined them, the anti-idiotype utilized defines a "cross-idiotypic specificity" (3). A considerable body of data now supports the notion originally proposed by Jerne (5) that these idiotypic determinants may serve as pivotal targets for the regulation of the immune response.

In the A/J mouse, the induced antibody response to the hapten p-azophenylarsonate (Ar) on haptenated KLH has a predominant homogeneous component (20-70% of the serum response) bearing the serological determinants comprising the cross-reactive idiotype (CRI) as defined by an appropriately absorbed rabbit antiserum (6). This homogeneity has lent itself particularly well to both genetic and structural analysis. Using conventional inbred strains of mice, the transmission of the Ar idiotype behaves as a single gene inherited in simple Mendelian fashion and is linked, as other variable region markers, to the heavy chain constant region locus (7). Mapping to the heavy chain alone presented a paradox in light of our current understanding that the

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light chain is also required for the expression of the idiotypic determinants. However, recent genetic studies employing the relatively few mouse strains carrying a defined kappa light chain polymorphism (8,9) demonstrate the requirement for an appropriate kappa chain gene complement for the formation of antibodies bearing the cross-reactive idiotype (10,11). Thus, a complete understanding of the nature of the inherited cross-reactive idiotype in the arsonate system will require detailed analysis of both heavy and light chain structures.

The definition of the structural correlate(s) of the CRI has been a major goal of this laboratory for several years. While initial studies centered on the serum molecules (12-16), a more detailed analysis of the range of the anti-Ar response in both CRI+ and CRI- sets required clonal analysis, made feasible by somatic cell fusion techniques. Amino-terminal studies on the resulting monoclonal anti-Ar antibodies revealed a surprising degree of heterogeneity among CRI+ molecules in both H and L chains, providing a striking contrast to the homogeneity of the serum sequences (17-20). This heterogeneity was corroborated by serological analyses demonstrating that although each idiotype positive hybridoma antibody expressed the CRI, albeit variably, each was also serologically unique (21). Accordingly, the interaction between a rabbit anti-idiotype to a monoclonal antibody, prepared in an analogous fashion to the serum anti-CRI (6), and the monoclonal antibody against which it was prepared, generally could not be inhibited extensively by any other hybridoma antibody, whether CRI+ in character or not, indicating the individuality of each of these molecules. In a heterologous system, which measured the interaction between one CRI+ hybridoma product and an anti-idiotype prepared against another, any monoclonal antibody defined as CRI positive could inhibit (22). Thus, all CRI positive antibodies share idiotypic determinants. Therefore, the capability of an inbred strain to produce different antibodies, constrained by the sharing of both specificity for antigen and idiotype, appears immense. The mechanism through which this capability is manifested remains a major dilemma.

In an effort to confer a structural basis to the CRI and determine the degree of variability within which this entity can fluctuate, the commonality among these heterogeneous molecules can be sought in the primary structure. To this end, the entire amino acid sequence of the variable regions of heavy and light chains derived from four CRI positive hybridomas and one CRI negative hybridoma was undertaken.

MATERIALS AND METHODS

Preparation and Isolation of Hybridoma Antibodies

Hybridomas secreting anti-Ar antibodies were prepared as previously described (16,17). Ascites containing large amounts of monoclonal anti-Ar antibodies (2-5 mg/ml) were generated by injecting 5 x 10^6 hybrid cells into the peritoneal cavity of Pristane-primed (Balb/c x A/J)F_1 mice. Ascites fluids were drained and the antibody specifically purified by passage through an affinity column of arsonate-derivatized human gamma globulin conjugated to Sepharose 4B (23). Anti-Ar antibodies were eluted with 0.2 M arsanilate, dialyzed exhaustively *vs* distilled water, and lyophilized.

Idiotypic Characterization

The idiotypic characterization of these molecules has been extensively described (18,21,22). Serological analysis of idiotypic determinants was performed as described (6) in a competition assay utilizing ^{125}I labeled A/J serum anti-Ar antibodies as ligand for the rabbit anti-CRI, and inhibiting with hybridoma supernatants. Those hybrid cells producing antibodies capable of inhibiting this interaction by at least 50% are classified as CRI positive. The amount of hybridoma required for this inhibition varied dramatically from 10 ng to 2900 ng. Subsequent characterization of these hybridoma antibodies included more extensive serologic studies (22,23). Briefly, anti-idiotypic reagents were prepared in rabbits against several of the CRI positive hybridoma antibodies. Absorption of these was performed as in the production of serum anti-idiotype (6). A competition assay was designed as outlined above, but using labeled monoclonal antibody as ligand. Inhibition of the association of the ligand with anti-idiotype reagents prepared against any CRI+ hybridoma was then measured using hybridoma antibodies as competitors (18,21,22). In a representative experiment, CRI+ monoclonal antibodies were added to inhibit the 93G7 anti-93G7 interaction. Only the homologous 93G7 appreciably inhibited, highlighting the individuality of 93G7. However, in an assay system using a heterologous combination, e.g., 93G7 as ligand but anti-123E6 as indicator, any CRI+ hybridoma inhibited equally well, emphasizing the commonality among these molecules. Thus, these serological studies demonstrated features that were *shared* and features which were *unique* to this set of antibodies.

Preparation of Isolated Chains and Peptides

Light Chains. Purified anti-Ar antibodies were completely reduced, alkylated and ^{3}H-carboxymethylated in the presence of 7M guanidine-HCl. Heavy and light chains were then separated by Sephadex G-100 gel filtration in 5M guanidine-HCl.

Light chain peptides were generated by tryptic digestion of citraconylated material or by chymotryptic digestion. Peptides were subsequently purified by a combination of gel filtration chromatography over Sephadex G-50 or G-75 followed by high voltage paper electrophoresis when appropriate. Aliquots of purified peptides were hydrolyzed and their compositions determined on a Durrum D500 amino acid analyzer as previously described (24).

Heavy Chains. Isolation of relevant heavy chain V region peptides was effected employing an initial CNBr fragmentation of the entire IgG1 molecule without reduction, followed by gel filtration on G-100 Sephadex in 5M Guanidine·HCl. This strategy allows a segregation of the V region fragments away from the bulk of the rest of the molecule, including most of the H chain constant region and the light chain, which run together as CNBr fragments interlinked by disulfide bonds in the excluded volume of the column. Pools containing V region CNBr fragments are completely reduced and alkylated, then separated by ion exchange chromatography on SP Sephadex in 8M urea, pH 3.0. Resulting peptides were subjected either to amino acid sequence analysis or further digested with trypsin. Tryptic peptides were subsequently separated by HPLC utilizing a C18 column, and 0.2% TFA/acetonitrile buffers.

Automated Amino Acid Sequencing

Automated sequencing was performed using a Beckman 890C amino acid sequencer, modified by the addition of a cold trap (25), utilizing dimethylallylamine or 0.1 M Quadrol programs and the non-protein carrier polybrene (26) as described by this laboratory previously (12). The phenylthiohydantoin amino acid derivatives were identified by gas chromatography (27), high pressure liquid chromatography (26) and amino acid analysis after back hydrolysis with hydriodic acid (28).

RESULTS AND DISCUSSION

Amino acid sequence studies of serum antibodies induced in A/J mice with KLH-Ar revealed that, while the CRI+ heavy

chain variable region was entirely homogeneous, the CRI+ light chain variable region was entirely homogeneous, the CRI+ light chain contained positions within framework segments at which minor heterogeneity of amino acids was evident, indicating that multiple variable region frameworks could be associated with identical hypervariable regions (14). This apparent sharp restriction in the serum response implied that one or a very few antibodies bore the entire set of determinants making up the CRI. Therefore, the degree of heterogeneity found subsequently among monoclonal antibodies was unexpected.

Anti-arsonate Hybridomas are Serologically Distinct

The serological heterogeneity of the antibodies for which primary sequences are presented here is shown in Table 1 (17). The amount of each hybridoma required to inhibit the interaction between radiolabeled A/J CRI-containing anti-Ar serum and its rabbit anti-CRI ranges from about 10 ng for R16.7 and 93G7, comparable to the inhibition obtained by A/J anti-Ar serum, to 2900 ng for 124E1. Hybridoma product 91A3 did not appreciably inhibit even in quantities in excess of 20,000 ng. A comparison of molecules at the extremes of scoring in the idiotype assay should provide maximum information toward the structural definition of these idiotypic determinants.

TABLE 1. Inhibition of Binding of Radiolabeled A/J Anti-Arsonate to its Rabbit Anti-idiotype by Purified Hybridoma Products†

Unlabeled Inhibitor	*Nanograms Required for 50% Inhibition*	*Percent Inhibition by 2000 ng*
A/J Anti-Ar	*15*	*101*
HP R16.7	*9*	*94*
HP 93G7	*12*	*90*
HP 123E6	*50*	*58*
HP 124E1	*2900*	*48*
HP 91A3	‡	*4*

†*Assays utilized 10 ng of* ^{125}*I-labeled specifically purified A/J anti-arsonate antibodies and slightly less than an equivalent amount of anti-idiotype.*

‡ *50% inhibition not achieved with 20,000 ng.*

Studies using anti-idiotypic antiserum prepared against hybridoma products themselves reenforced the notion of the serologic individuality of these hybridoma molecules. The results of experiments measuring the capacity of monoclonal antibodies to inhibit the binding of HP 93G7 to its autologous rabbit anti-idiotypic antiserum are shown in Table 2. The test system utilized 10 ng of ^{125}I-labeled, specifically purified HP 93G7, sufficient rabbit anti-id to bind 66% of the labeled HP, and a slight excess of goat anti-rabbit Fc to precipitate complexes of id and anti-id. Though all of these monoclonal antibodies inhibited by at least 50% in the conventional assay described in the previous paragraph, 2,000 ng failed to cause 50% inhibition in this test system. Maximum inhibition obtained by all proteins by 93G7 ranged from 20-40%. Pooled A/J anti-Ar antibodies did inhibit the binding of labeled 93G7 to its anti-id, but 650 ng were required for 50% inhibition. Only the autologous protein, 93G7, using 30 ng, caused 50% inhibition. These results suggest that, though these monoclonal antibodies contain determinants of the major CRI, 93G7 possesses unique determinants not found on the others.

*TABLE 2. Inhibition of Binding of Radiolabeled HP93G7 to Anti-Id(93G7)**

Unlabeled Inhibitor	*Nanograms Required for 50% Inhibition*	*Percent Inhibition by 2000 ng*
A/J anti-Ar	*650*	*71*
HP 93G7	*30*	*97*
HP 121D7	*>2000*	*40*
HP 123E6	*>2000*	*7*
HP 124E1	**	*10*
HP 91A3	**	*7*
Ars	**	*NA****

* *Assays utilized 10 ng ^{125}I-specifically purified HP93G7 and sufficient anti-Id (93G7) to bind 66% of this ligand.*

** *50% inhibition not achieved with 20 μg of antibody or 20 mM arsanilic acid.*

*** *Not applicable.*

Anti-Ar Hybridomas Share Serologic Features

To analyze the extent of sharing of idiotypic determinants, a test system was designed which minimized the role played by unique idiotypic determinants. This was achieved in an assay using anti-idiotypic antibodies prepared against one HP, another HP as radiolabeled ligand and inhibiting this interaction with monoclonal antibodies bearing or lacking the CRI. Since the anti-idiotypic reagent would recognize only those determinants shared by the ligand and the antibody against which the anti-id was made, private idiotypic determinants would not be measured. These inhibition data (Table 3) are quite different from those obtained in the autologous system. Each of the CRI+ hybridoma products tested, as well as A/J anti-Ar serum was capable of causing 50% inhibition of binding, and were often nearly as effective as the unlabeled ligand in displacement of the labeled ligand. These results indicate sharing of idiotypic determinants in this system, and emphasize that while marked heterogeneity exists among these CRI+ HPs, the basic tenet that they vary around determinants comprising a major CRI in common with induced serum A/J anti-Ar antibodies is still apparent.

*Table 3. Inhibition of Binding of Radiolabeled HP93G7 to Anti-Id(123E6)**

Unlabeled Inhibitor	*Nanograms Required for 50% Inhibition*	*Percent Inhibition by 2000 ng*
A/J anti-Ar	*200*	*89*
HP 93G7	*32*	*96*
HP 121D7	*85*	*68*
HP 123E6	*87*	*97*
HP 124E1	*48*	*79*
HP 91A3	**	*0*
Ars	*2 mM*	*NA****

* *Assays utilized 10 of ^{125}I-labeled HP93G7 and sufficient anti-id(123E6) to bind 66% of this ligand.*

** *50% inhibition never achieved.*

*** *Not applicable.*

Light Chain Sequences

Anti-arsonate Hybridomas Differ Dramatically from the Pooled Serum Sequence. The sequences of the light chain V regions derived from CRI+ and one CRI- monoclonal antibody are presented in Figure 1, shown in comparison with the major sequence of the CRI+ serum light chain. The most striking feature of this comparison is the marked differences between the CRI+ monoclonal light chains and their serum counterpart. These differences occur in hypervariable as well as framework regions. The total number of positions at which any differences from the serum sequence are found is 30 out of 108 V region residues. While this accounting scores variation at every position where any monoclonal varies from the serum sequence, it is not an exaggeration of the extent of difference between individual monoclonals and the serum sequence. For example, the two light chains derived from CRI+ monoclonal antibodies most similar to the serum sequence, R16.7 and 93G7, have 26 differences from the serum sequence, while the most divergent from that sequence, 123E6 and 124E1, differ at 27 positions. The light chain derived from the CRI- molecule 91A3 differs at 28 positions from the serum sequence. Furthermore, of the 9 positions previously found to vary in the serum sequence (14) only position 76, containing a Ser in the minor sequence, corresponds to that found in the monoclonal chains. Thus, the sampling of light chains derived from hybridoma cell lines differs substantially from the light chains selected in response to antigen challenge in vivo. These apparent differences may be attributable to the varying immunization regimens used to obtain the two sets of antibodies or to a selectivity in the fusion process itself.

Anti-arsonate Light Chains from CRI+ Serum Antibodies and from Anti-arsonate Hybridomas Possess Identical J Regions Including a A/J Junctional Position Possibly Generated as a Result of Somatic Recombination. An important aspect of the similarities between the monoclonal and serum molecules is that while a consideration of the distribution of the differences between the two sets of molecules shows variation in frameworks 1, 2 and 3 and hypervariable regions 1, 2 and 3, framework 4, or the J region, is completely identical among all anti-Ar light chains sequenced to date, including the light chain of the CRI- 91A3. In addition, there is no other continuous 14 residue variable region stretch shared by both serum and monoclonal light chains. In this regard, it is relevant to note that the J region is encoded as a separate genetic element in the DNA (29,30).

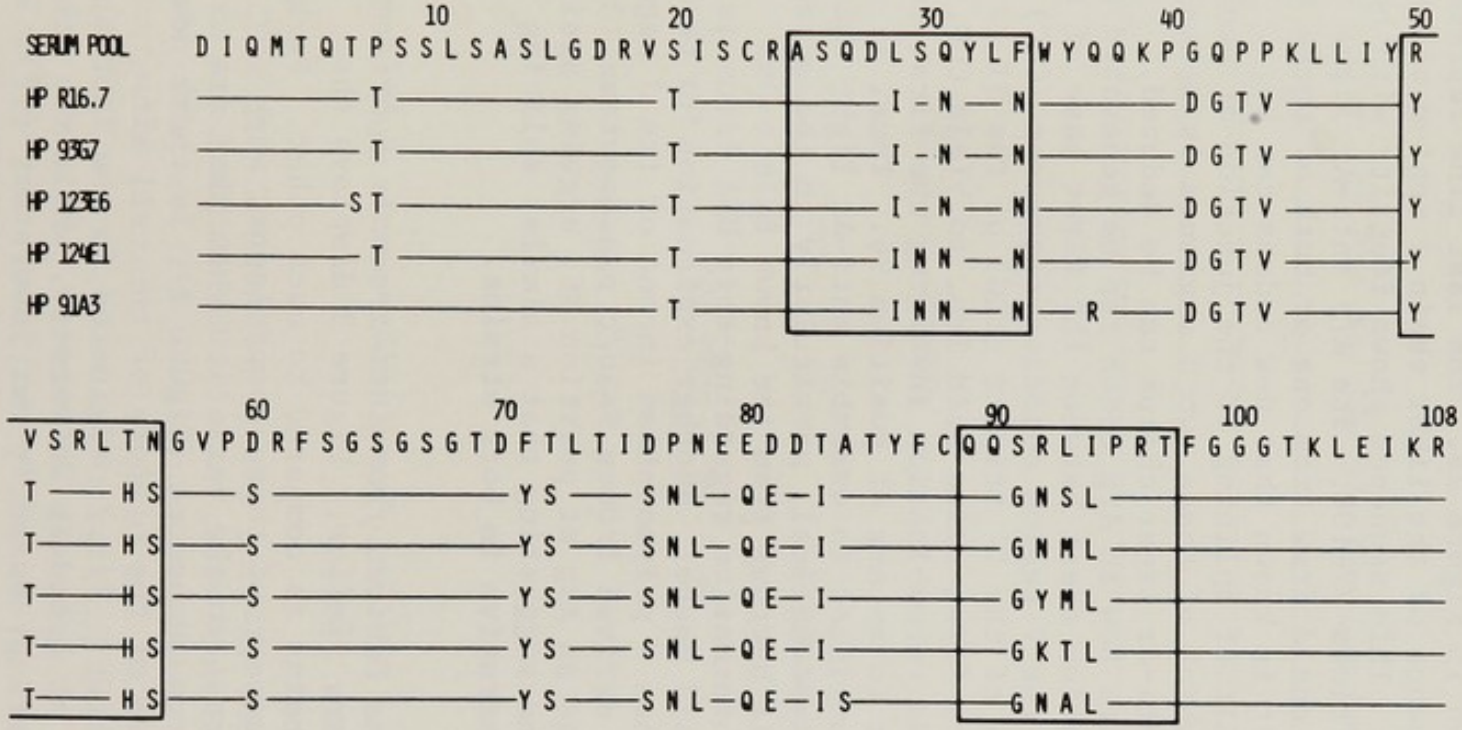

FIGURE 1. Anti-arsonate hybridoma light chains. Comparison of the amino acid sequences of light chains derived from CRI positive and CRI negative hybridomas with the sequence of light chains derived from CRI positive serum antibodies. Identical residues are indicated by a line and only differences are noted. Hybridoma light chains are listed in order of the relative capacity of their parent molecules to inhibit the reaction between A/J serum anti-arsonate and its rabbit anti-idiotype (see Table 1). Hypervariable regions are outlined.

Evidence from DNA sequencing indicates that there are only four J_K segments which can be utilized to assemble productive light chains (31). The four J_K sequences found in Balb/c DNA and known to be represented in Balb/c antibodies are shown in Figure 2a. The last line represents the amino acid sequence of the light chain J region of the anti-Ar antibodies. This sequence shows identity with both J1 and J2 from positions 97-108. The A/J anti-Ar light chains, therefore, could draw from one or both of these two J regions. Since it is known that new codons can be generated as a result of splicing at the junction of gene segments (31-34), which of these two J segments is actually utilized to form anti-Ar light chains can be deduced. Figure 2b illustrates how an Arg codon can be generated from a somatic recombination event between the first base of the triplet just 3' to the V region encoding sequences (CCC) and the second base of the 5' triplet (TGG) of the J1 segment encoding its first amino acid Trp, to yield CGG, a triplet for Arg. No recombination involving the first codon of J2 can give rise to an Arg at position 96. Thus, it is likely that J1 is utilized to assemble anti-Ar light chains. This mechanism is hypothetical, particularly to the extent that of necessity it borrows from the known Balb/c DNA sequences, as no information regarding this DNA locus is available for the A/J mouse. Another explanation for the Arg at 96 is that A/J has a polymorphism in one of its J segments. However, MOPC 173, derived from a Balb/c plasmacytoma (35) (Figure 4), also has an Arg at position 96, arguing against this alternative and suggesting that a similar splicing mechanism may be operative in both strains.

The Light Chains Derived from Anti-arsonate Hybridomas are Structurally Very Similar. Figure 3 displays the monoclonal light chains as compared to each other. Perhaps the most imposing aspect of these comparisons, when considered in contrast to the variation when they are compared to the serum sequences (Figure 2), is their near identity, regardless of the history of the cell line producing the antibody. R16.7 originated in the laboratory of Dr. A. Nisonoff at Brandeis University, while the other three antibodies were produced in our laboratory (17), 93G7 and 91A3 the result of one fusion, 123E6 and 124E1 the result of another. A cross-comparison of the differences between the light chains is rendered in Table 4. This shows that among four independently derived Ar-bidning CRI+ light chains, the V regions vary by as many as four amino

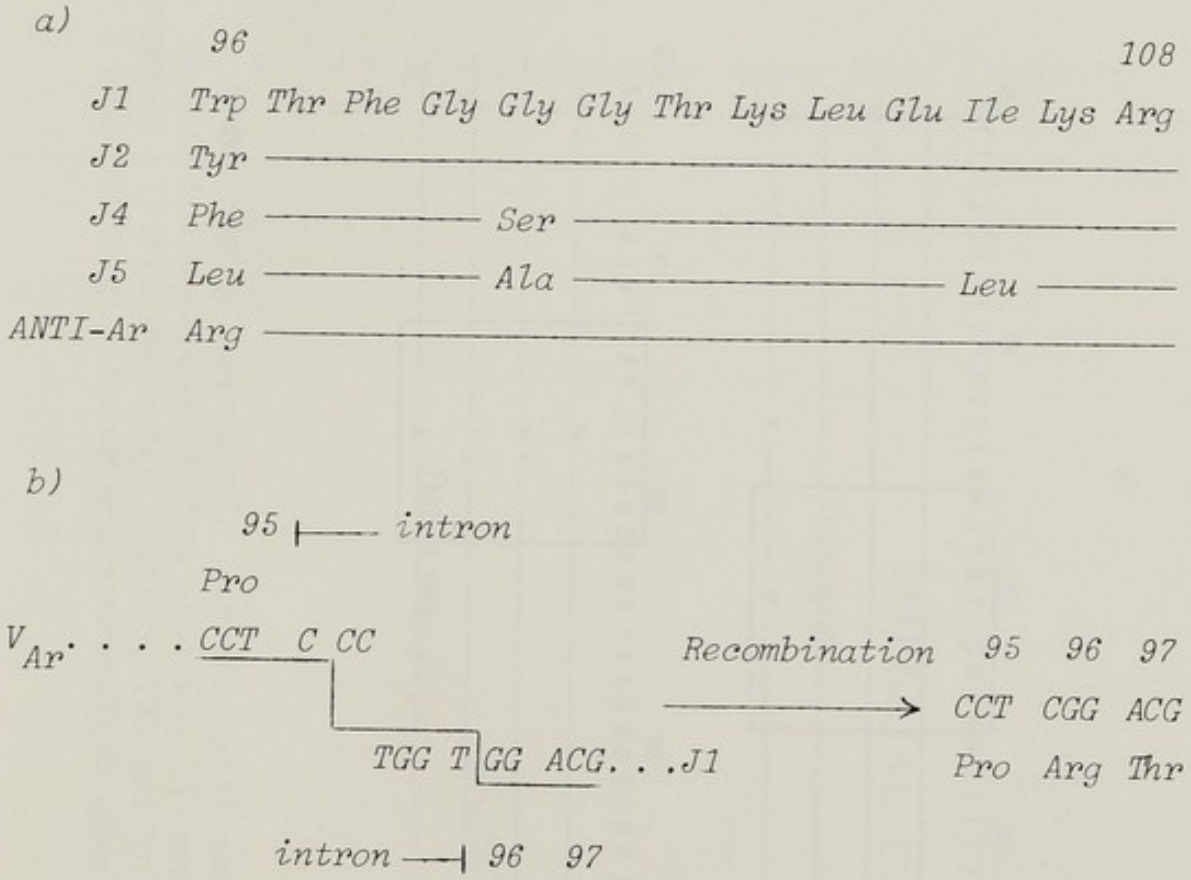

FIGURE 2. Generation of the anti-Ar light chain region. a) Amino acid sequences of four germ-line Balb/c J_K gene segments obtained from DNA sequences (29-31). The bottom line represents the protein sequence of all anti-Ar J_K regions. A straight line indicates identity with the topmost J1 sequence. Only differences are indicated. b) Suggested mechanism for the formation of Arg at position 96 from a splicing even tduring V/J joining. Hypothetical V_{Ar} is taken from a MOPC 41 DNA sequence (31). Numbers correspond to amino acid positions in the mature light chains. See text for further explanation.

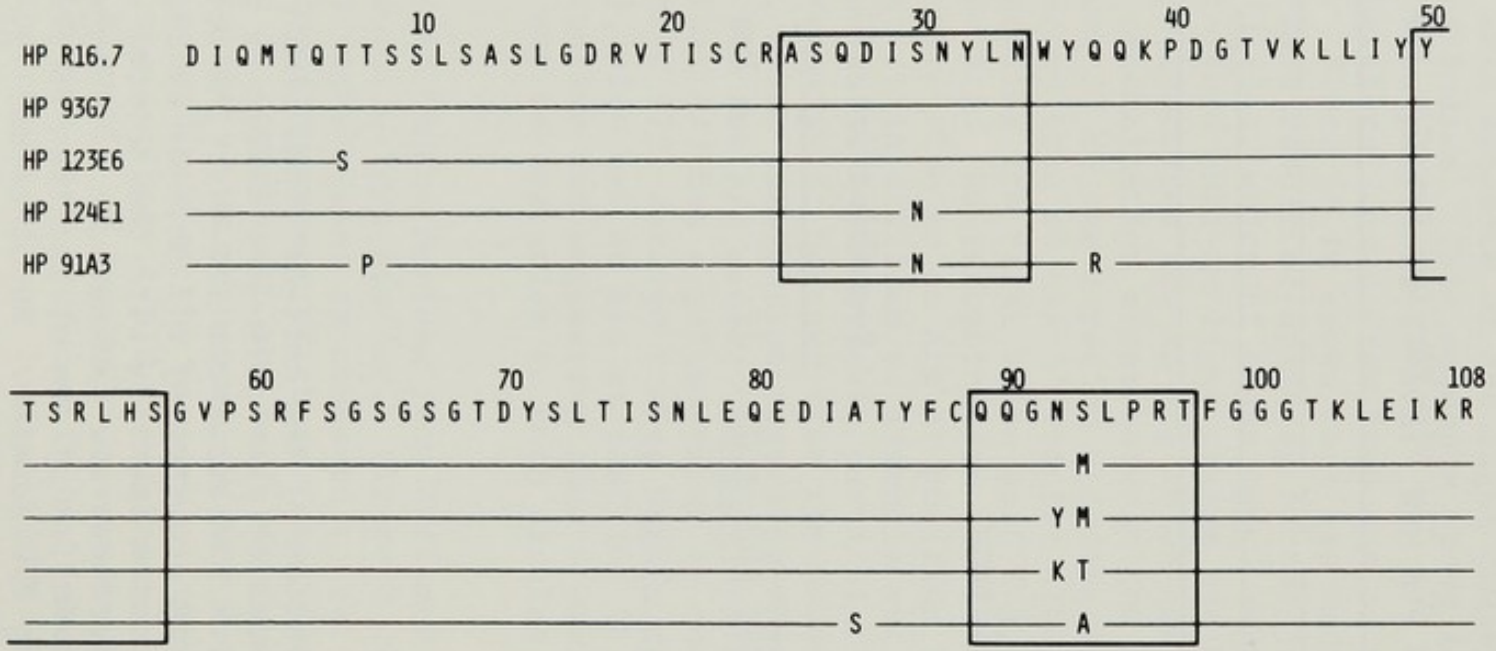

FIGURE 3. Anti-arsonate hybridoma light chains. Comparison of the amino acid sequences of the light chains from CRI positive and CRI negative antibodies with the sequence of the light chain of the CRI positive hybridoma R16.7. Identical residues are indicated by a line and only differences are noted. Hypervariable regions are outlined.

acids (123E6 vs 124E1), and as few as one amino acid (93G7 vs R16.7). None of the sequences show complete identity, in spite of their strict restriction to a single kappa subgroup, V_K10 (36).

Table 4. V Region Differences Among Light Chains Derived from Ar-Binding Hybridomas

	R16.7	*93G7*	*123E6*	*124E1*
R16.7				
93G7	*1*			
123E6	*3*	*2*		
124E1	*4*	*3*	*4*	
91A3	*5*	*5*	*7*	*5*

There is a Correlation Between Light Chain Structure and Serological Characteristics of the Anti-Arsonate Hybridomas. It is noteworthy that the light chains originating from CRI+ hybridomas rank roughly in their order of positivity for idiotype according to the number of amino acid differences found between them. The two most idiotype positive molecules, 93G7 and R16.7 (Table 1), both inhibit to the same extent in the idiotype assay at about 10 ng. These variable regions differ at only a single position, 93. 123E6, intermediate in CRI activity, requires 50 ng to inhibit the idiotype assay by 50% and differs from R16.7 and 93G7 at 2 and 3 positions, respectively. The CRI+ 124E1 requires 2900 ng to inhibit the assay, and differs from the other three light chains at 3 or 4 sites in the V region. Thus, the ordering of these light chains by the extent of amino acid differences follows precisely the ordering of idiotypic character of their parent molecules, i.e., R16.7, 93G7, 123E6, and 124E1. 91A3, a CRI- molecule also conforms to this hierarchy in that it varies from R16.7 at five positions.

The distribution of these differences is dispersed throughout the V region. There is one variant in the first framework region at position 7 where 123E6 has a Ser interchange while the other molecules contain a Thr. This

requires only a single base change at the nucleotide level. Another interchange is found in the first hypervariable region at position 30, where 124E1 possesses an Asn in place of the Ser found in the other three molecules, the amino acid also found in the serum sequence, the interchange again requiring only a single base change.

The most intriguing differences are found in the third hypervariable region. Positions 92 and 93 are the two successive sites in the V regions where variability is most striking. Three alternative amino acids are present at these two positions, contrasting with the other two variant positions, 7 and 30, where one molecule had a variant, the other 3 being identical. Furthermore, two of the possible interchanges, Tyr to Lys, and Met to Ser, are two base change substitutions, though this holds only in the case that they derive from each other and not from a separate parent sequence which would require only a single base change. Additionally, the argument made for the correlation of idiotype positivity and overall sequence variation holds for these two positions alone. That is, 93G7 and R16.7 have a single difference at position 93, 93G7 and 123E6 have a single difference at position 92, and 124E1 differs from the other three chains at both sites. This suggests further that position 92 makes the most critical contribution to the idiotypic character in the light chain. The proteins 93G7 and R16.7 behave nearly identically in the idiotype assay, and share in common an Asn at 92. 123E6 and 93G7 have a Tyr-Met exchange at 92 but are identical at 93, yet, they vary substantially in their degree of idiotype reactivity. 124E1 differs from all other chains at both positions, but at 92, Lys is distinctive from Asn or Tyr in that it has a longer side group and is positively charged. These observations can be construed to account for the more marginal idiotypic behavior of the 124E1 molecule. Thus, this condition is entirely consistent with a vital role for the third hypervariable region, particularly position 92, in the modulation of the idiotype in this system. It should be stressed, however, that much more structural data remains to be gathered for the heavy chains and for the CRI- molecules. It is of related interest, though, that the D segment, which contributes to the third hypervariable region of the heavy chain, is thought to control the private idiotype in dextran-binding antibodies (37).

These data imply that the 91A3 light chain, though derived from a CRI- molecule, is itself CRI+ in nature. At the proposed crucial positions, 92 and 93, 91A3 contains an Asn-Ala, which is quite similar to the strongly idiotype positive R16.7, whose light chain sequence is Asn-Ser, or

93G7 which possesses the sequence Asn-Met at these positions. Since 91A3 contains an Asn at position 92, characteristic of the light chains derived from the most CRI+ molecules, the 91A3 light chain may very well be idiotype positive. This interpretation is consistent with recent experiments suggesting serologic similarity of light chains from CRI+ and CRI- molecules (38).

When compared overall to the CRI+ light chains, 91A3 differs from R16.7, 93G7 and 124E1 at five positions, and from 123E6 by seven. Three of the 91A3 variations are framework changes which were invariant in the other monoclonal light chains: position 8, a Pro-Thr substitution, Pro being the residue found at this position in the serum CRI+ light chains; position 37, an Arg-Gln interchange; and position 83, containing a Ser rather than the Ala found in the other chains. These all involve single nucleotide base changes. Though these cannot be entirely dismissed to account for the lack of idiotype reactivity of 91A3, it is unlikely that they could effect a total abrogation of this serologic property, nor is the Asn-Ser interchange at position 30, which is shared with the CRI+ 124E1, likely to be significant. The absence of a proper CRI+ heavy chain is, therefore, the most probable explanation for the lack of the Ar cross-reactive idiotype on the 91A3 molecule, particularly if residues 92 and 93 are essential for the light chain contribution to that entity. The increased number of framework differences in 91A3 may indicate that these substitutions favor association of this light chain with a heavy chain with which it more readily combines, i.e., a CRI- heavy chain. Obvious predictions of these conclusions can be readily tested in chain recombination experiments.

A Novel Mechanism May Generate the Diversity in the Third Hypervariable Region of the Light Chain. In any case, it is clear that the third hypervariable region of the light chain manifests a high degree of variability. Junctional diversity generated at the actual splice site of V/J joining, the mechanism generally invoked to explain variation in this region of the light chain, cannot adequately account for the high degree of variation at positions 92 and 93, which are quite removed from that site. The situation is reminiscent of heavy chains, where the D segment is encoded separately in the germ-line (39). However, at least in the Balb/c kappa chain locus, there is no evidence for a separately encoded D segment analogue. Unless the A/J mouse actually has a configuration in its light chain DNA similar to that found for heavy chains in Balb/c, or the DNA sequenced in the

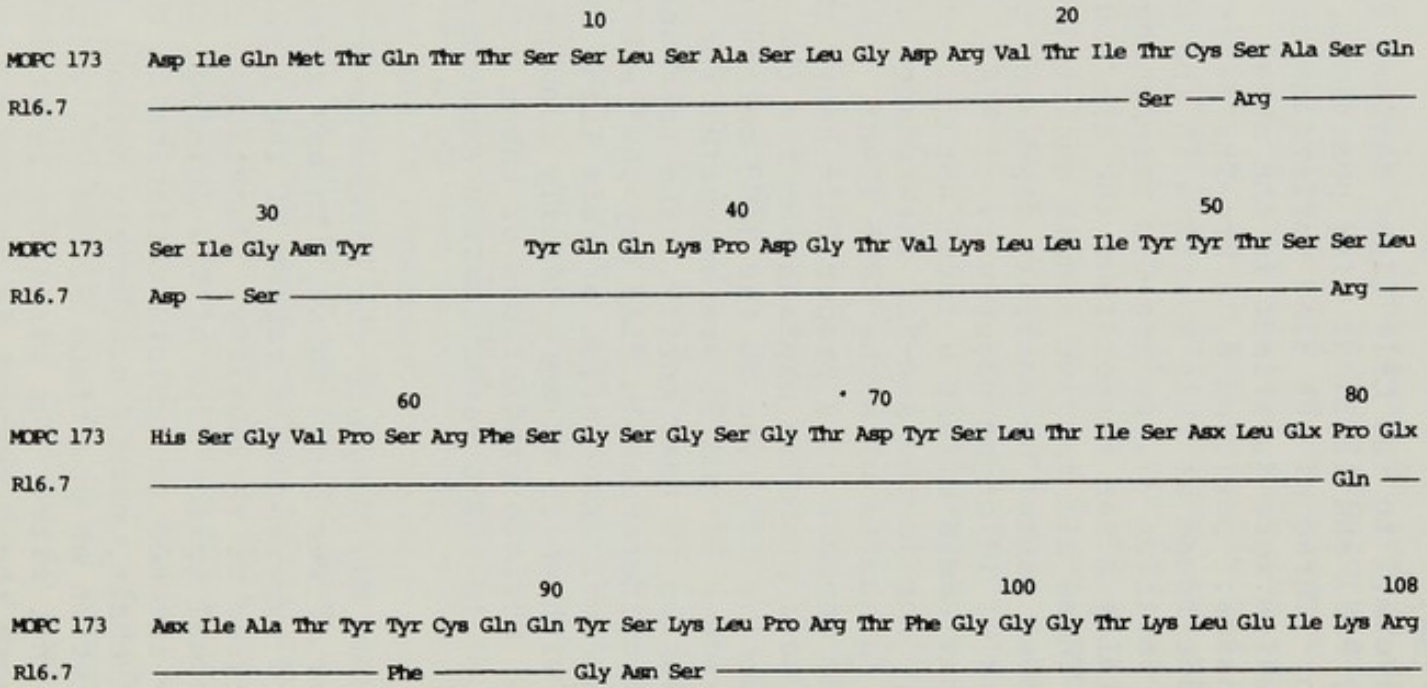

FIGURE 4. Similarity of the MOPC 173 light chain with the light chain of the CRI positive hybridoma R16.7. Shared sequences are indicated by a line and only differences are noted.

Balb/c represents a sequence that was preceded by a V/D rearrangement, another mechanism must generate this diversity. An alternate possibility is that this variation is generated as a consequence of joining V and J, and that during the splicing event, nicks in the DNA occur in the vicinity of the junctional event 5' to that site. These may be subsequently repaired in an altered form. Finally, this area may simply be subject to a higher rate of somatic mutation. The concentration of variability in a complementarity determining region may be suggestive of a role for somatic mutation in the generation of diversity among these light chains.

The Anti-Arsonate Hybridoma Light Chains in A/J Mice May Derive from a Germ-line Gene Similar to the MOPC 173 Balb/c Gene. Another feature of the monoclonal light chains comes from comparisons to previously sequenced light chains. The CRI+ monoclonals were found to share remarkable homology with one particular mouse kappa chain, MOPC 173 (35) derived from a Balb/c plasmacytoma which has no known antibody activity. The sequences of R16.7 and MOPC 173 are shown in Figure 4. These sequences differ at only 9 positions throughout the V region discounting a three residue gap in MOPC 173. In comparison, the next most similar light chain sequence, MOPC 41, has 31 differences from R16.7. Most kappa chains for which the V region sequence is available differ from the monoclonal sequences at 4-50 positions. The similarity of the anti-arsonate light chains with MOPC 173 becomes even more apparent when characteristic regions are compared. The clusters of amino acids between 41-44 and 55-60 are unique to MOPC 173 among published kappa sequences, are distinct from those found in the anti-Ar serum sequences, and are shared among all of the monoclonal light chains. This pattern is most evocative of a Balb/c germ-line V kappa gene and its A/J allelic counterpart in the CRI+ monoclonals. Considering the apparent extent of sharing of light chain repertoires between these two strains (8,10),this suggestion seems particularly viable. A CRI+ monoclonal antibody from a CAL.20 mouse, which is now available, should aide in clarifying whether these light chains are derived from a gene allelic to the MOPC 173 gene. The light chain in the CAL.20 molecule is provided by the Balb/c background in this congenic strain. Of additional interest is that, with the exception of one NZB derived kappa chain, PC6684 (40), which is quite different from the light chains sequenced here, the

MOPC 173 light chain is the only other sequenced mouse light chain containing an Arg at 96. This implies that if this position is generated as a junctional even in V/J joining as previously discussed, the mechanism is very selective for the type of V region on which it operates.

Thus, light chains associated with arsonate specificity and originating as somatic cell fusion products show heterogeneity but in a sharply delineated fashion. The light chain V regions so far examined, whether belonging to molecules bearing or lacking the cross-reactive idiotypic determinants, are from 93 to 99% homologous. Much of the identity observed among these light chains, particularly the stringent conservation in complementarity determining regions, may be driven in large part by the original selection of these antibodies by their ability to bind arsonate. It is nevertheless evident that additional selective forces act to generate these molecules. They are restricted to a single V region subgroup, V 10, suggesting derivation from an A/J counterpart to the Balb/c light chain V region gene which gives rise to the MOPC 173 light chain. They vary from that germ-line sequence by a few scattered substitutions in the V gene segment in a manner suggestive of somatic mutation. In addition, positions 92 and 93 manifest an exceptional focus of variability which could be attributable to hypermutability or to a novel mechanism of DNA nicking and repair as a secondary consequence of V/J joining. Finally, the Arg at position 96 consistently found in these light chains may be formed as a reproducible somatic recombination between the germ-line V gene segment and a specific J region segment.

These data are most consistent with the light chains from these Ar-binding hybridoma antibodies originating from a single germ-line V gene segment and one J segment. The heterogeneity found among them is more likely the result of the previously discussed array of somatic mechanisms than a selection of very similar germ-line genes.

Heavy Chain Sequences

A comparision of the H chain sequences of the monoclonal antibodies with that of the serum sequence is presented in Figure 5. As was true for the light chains, the monoclonal heavy chain V regions differ considerably from the serum sequence at a total of 50 of 121 positions, comprising 41.3% variation. In spite of this striking variation, the monoclonal sequences remain closer to the serum sequence than to any other mouse V region sequenced to date. The

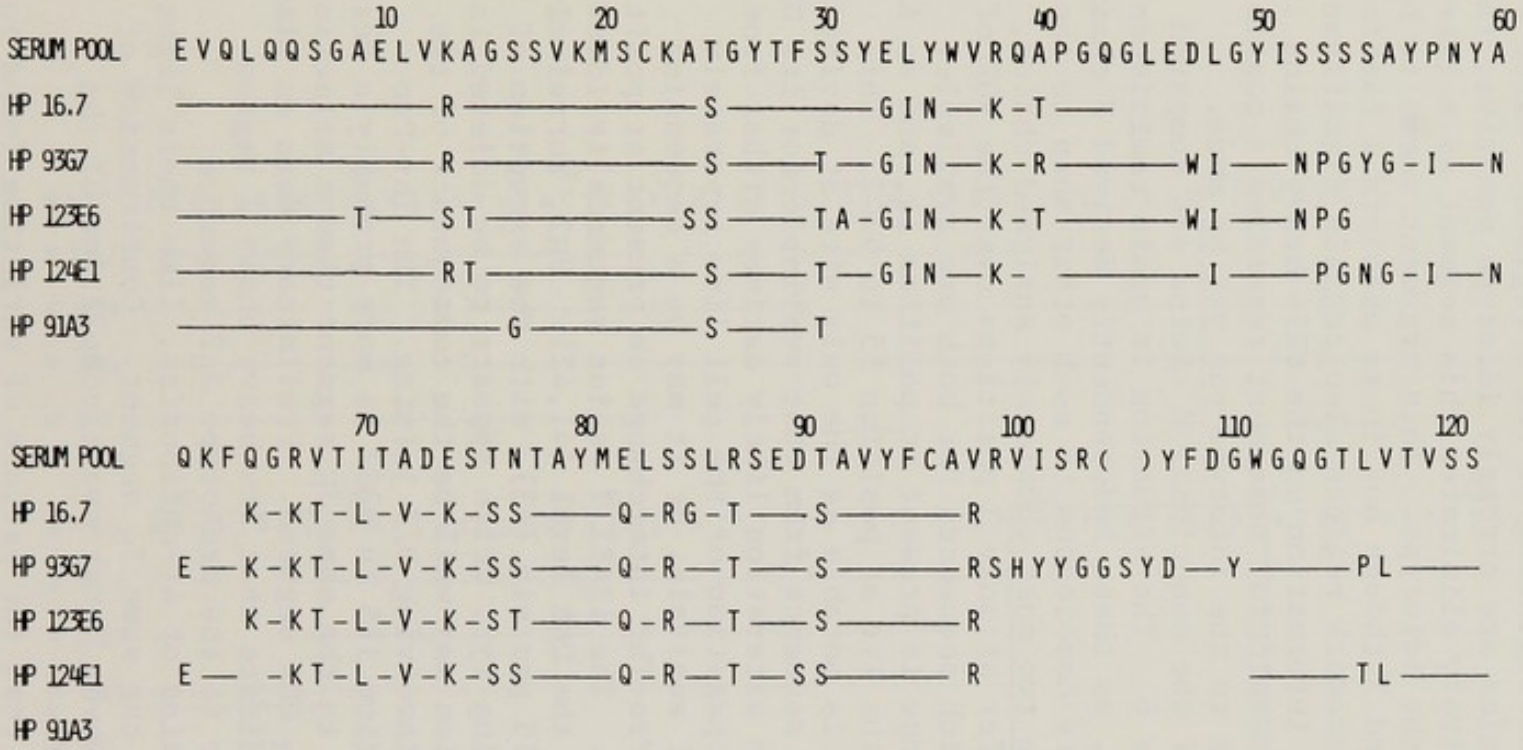

FIGURE 5. Anti-arsonate hybridoma heavy chains. Comparison of the amino acid sequences of heavy chains derived from CRI positive and CRI negative hybridomas with the sequence of heavy chains derived from CRI positive serum antibodies. Identical residues are indicated by a line and only differences are noted. Hypervariable regions are outlined.

differences are distributed throughout the entire V region including all HVR, framework regions and, in contrast to the light chains, a few J region positions as well. The third HVR is particularly distinctive in that the 93G7 sequence does not share a single position in common with its serum counterpart up to the beginning of the J regions. Since both of these molecules are strongly idiotype positive, this limited data effectively eliminates this segment as a likely structural seat of the idiotype. This argument seems to hold as well for HVR 1 and 2 though not in so absolute a fashion. In addition, all framework regions also contain significant differences. Since information on the CRI- heavy chains is so rudimentary, no meaningful statement regarding a possible structural correlate to the idiotype can yet be made.

The sequences of the monoclonal H chains are compared to each other in Figure 6. The situation is again reminiscent of the light chains, as these independently derived H chains are considerably more homologous to each other than to the serum sequence. The two most completely analyzed chains, 93G7 and 124E1, differ at only 3 positions over the 90% of the V region for which sequences for both are available. An Ala-Thr interchange is present at position 14, and an Asn for 124E1 replaces the Tyr at position 55 in 93G7. This latter substitution is notable as the only site of deviation of the 93G7 protein sequence from that available for its DNA sequence (44). This alteration likely derives either from a mutation after propagation of the cell line separately for the two types of analysis, or it may be a sequencing error. Finally, a Pro-Thr interchange is present at position 115 in the J region. The 124E1 J region sequence is identical to one sequenced at the DNA level (41,42). J_H2, correlating with that of the M315 protein (43), with the exception of the first triplet encoding Tyr, which appears to be deleted in 124E1. This deletion may well be the consequence of the recombination even involving D/J joining. The Thr-Pro interchange at position 115 in 93G7 is more probably a result of a point mutation in the same J_H segment than a selection of an alternative J_H DNA segment. Preliminary data on 91A3, a CRI- molecule, indicate that its heavy chain J region is also most similar to the prototype J_H2 sequence. Therefore, the H chains of Ar-antibodies, like their light chains, may draw on the same J_H segment. Confirmation of this suggestion, however, must await completion of the remaining heavy chains.

It appears then that the pattern of variation found in the monoclonal light chains seems generally borne out by the monoclonal heavy chains thus far as well. Both are strikingly different from but still related to the serum

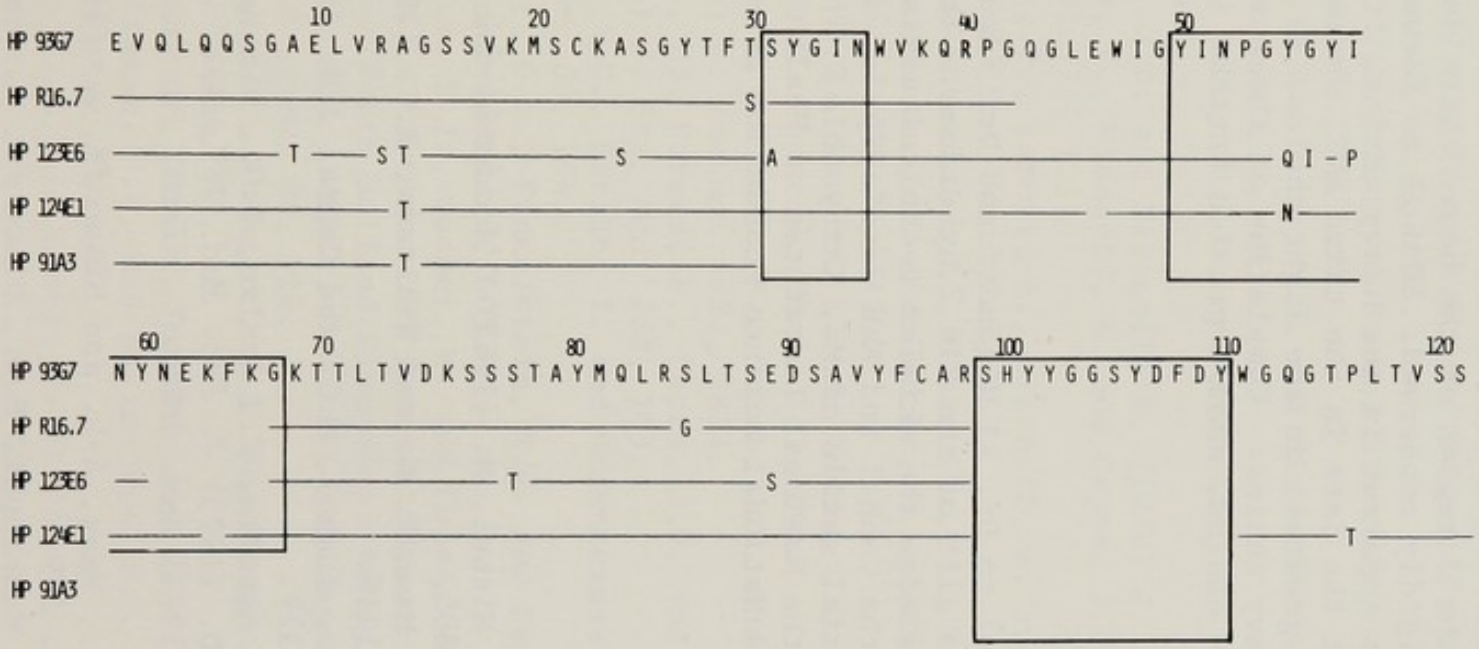

FIGURE 6. Anti-arsonate hybridoma heavy chains. Comparison of the amino acid sequences of the heavy chains from CRI positive and CRI negative antibodies with the sequence of the heavy chain of the CRI positive hybridoma R16.7.

sequences. They are much more homologous to each other than to the serum sequence, varying by only a few scattered positions throughout the variable region. Though the data is incomplete for the heavy chains, both heavy and light chains seem to select a single J region to form their mature polypeptides. In contradistinction to the light chains, however, the J region of the heavy chain does not appear to be so rigidly conserved. Though no focus of variability is yet apparent in the heavy chains, it should be emphasized that the data in the third HVR, where variability seems greatest in the light chains, is most scanty for the heavy chains. Completion of these chains, including the CRI- chains, should provide further insights.

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REFERENCES

1. Oudin, J. and Michel, M. (1963) *L'Academie des Sciences (Paris) 257*, 805.
2. Kunkel, H.G., Mannik, M. and Williams, R.C. (1963) *Science 140*, 1218.
3. Williams, R.C., Kunkel, H.G. and Capra, J.D. (1968) *Science 161*, 379.
4. Kunkel, H.G., Agnello, V., Joslin, F.G., Winchester, R.J. and Capra, J.D. (1973) *J. Exp. Med. 137*, 331.
5. Jerne, N.K. (1974) *Ann. Immunol. (Inst. Pasteur) 125C*, 373.
6. Kuettner, M.G., Wang, A-L. and Nisonoff, A. (1972) *J. Exp. Med. 135*, 579.
7. Pawlak, L.L., Mushinski, E.B., Nisonoff, A. and Potter, M. (1973) *J. Exp. Med. 137*, 22.
8. Edelman, G.M. and Gottlieb, P.D. (1970) *Proc. Natl. Acad. Sci. USA 67*, 1192.
9. Gibson, D. (1976) *J. Exp. Med. 144*, 298.

10. Laskin, J.A., Gray, A., Nisonoff, A., Klinman, N.R. and Gottlieb, P.D. (1977) *Proc. Natl. Acad. Sci. USA 74*, 4600.
11. Brown, A.R., Estess, P., Lamoyi, E., Gill-Pazaris, L., Gottlieb, P.D., Capra, J.D. and Nisonoff, A. (1980) *Progress in Clinical and Biological Research 42*, 231.
12. Capra, J.D., Tung, A.S. and Nisonoff, A. (1975a) *J. Immunol. 114*, 1548.
13. Capra, J.D., Tung, A.S. and Nisonoff, A. (1975b) *J. Immunol. 115*, 414.
14. Capra, J.D., Tung, A.S. and Nisonoff, A. (1977) *J. Immunol. 119*, 993.
15. Capra, J.D., Ju,S-T. and Nisonoff, A. (1978) *J. Immunol. 121*, 953.
16. Capra, J.D. and Nisonoff, A. (1979) *J. Immunol. 123*, 279.
17. Estess, P., Nisonoff, A. and Capra, J.D. (1979) *Mol. Immunol. 16*, 1111.
18. Estess, P., Lamoyi, E., Nisonoff, A. and Capra, J.D. (1980) *J. Exp. Med. 151*, 863.
19. Marshak-Rothstein, A., Siekevitz, M., Margolies, M.N., Mudgett-Hunter, M. and Gefter, M.L. (1980) *Proc. Natl. Acad. Sci. USA 77*, 1120.
20. Alkan, S.S., Knecht, R. and Braun, D.G. (1980) *Hoppe-Seylers Z. Physiol. Chem. 361*, 191.
21. Lamoyi, E., Estess, P., Capra, J.D. and Nisonoff, A. (1980a) *J. Immunol. 124*, 2834.
22. Lamoyi, E., Estess, P., Capra, J.D. and Nisonoff, A. (1980b) *J. Exp. Med. 152*, 703.
23. March, S.C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 194.
24. Klapper, D.G., Finkelstein, R.A. and Capra, J.D. (1975) *Immunochemistry 13*, 605.
25. McCumber, L.J., Qadeer, M. and Capra, J.D. (1980) *Methods in Peptides and Protein Sequence Analysis*, Elsevier/North Holland Biomedical Press, pp. 165-172.
26. Klapper, D.G., Wilde, C.W. III and Capra, J.D. (1978) *Anal. Biochem. 85*, 126.
27. Pisano, J.J., Bronzert, T.J. and Brewer, H.B. Jr. (1972) *Anal. Biochem. 68*, 47.
28. Smithies, O., Gibson, D., Fanning, E.M., Goodfleisch, R.M., Gilman, J.G. and Ballantyne, D.L. (1971) *Biochemistry 10*, 4912.
29. Weidman, J.G. and Leder, P. (1978) *Nature 277*, 790.
30. Bernard, O., Hozumi, N. and Tonegawa, S. (1978) *Cell 15*, 1133.
31. Max, E., Seidman, J.G. and Leder, P. (1979) *Proc. Natl. Acad. Sci. USA 76*, 3450.

32. Seidman, J.G., Max, E.E. and Leder, P. (1979) *Nature 280*, 370.
33. Sakano, H., Hüppi, K., Henrich, G. and Tonegawa, S. (1979) *Nature 280*, 288.
34. Rudikoff, S., Rao, D.N., Glaudemans, C.P.J. and Potter, M. (1980) *Proc. Natl. Acad. Sci. USA 77*, 4270.
35. Schiff, C. and Fougereau, M. (1975) *Eur. J. Biochem. 59*, 525.
36. Potter, M. (1977) *Adv. Immunol. 25*, 141.
37. Schilling, J., Clevinger, B., Davie, J.M. and Hood, L. (1980) *Nature 283*, 35.
38. Gill-Pazaris, L.A., Lamoyi, E., Brown, A.R. and Nisonoff, A. (1981) *J. Immunol. 126*, 75.
39. Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. (1980) *Cell 19*, 981.
40. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. and Hood, L. (1978) *Nature 276*, 785.
41. Bernard, O. and Gough, N.M. (1980) *Proc. Natl. Acad. Sci. USA 77*, 3630.
42. Sukano, H., Maki, R., Kurosowa, Y., Roeder, W. and Tonegawa, S. (1980) *Nature 286*, 676.
43. Francis, S.H., Leslie, R.G.Q., Hood, L. and Eisen, H.N. (1974) *Proc. Natl. Acad. Sci. USA 71*, 1123.

ANTI-DEXTRAN ANTIBODIES: SEQUENCES AND IDIOTYPES*

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The V_H amino acid sequences of sixteen dextran-binding hybridoma and myeloma proteins have been analyzed. Sequence patterns in these proteins suggest that random association of a relatively few germline V, D, and J gene segments generates a large part of the anti-dextran repertoire. In addition, some variants suggest a contribution by somatic mutation. These proteins have also allowed us to compare the fine specificity of heterologous and monoclonal anti-dextran idiotypic reagents. We have found that monoclonal anti-idiotype reagents can have significantly different fine specificity patterns as compared to heterologous reagents.

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INTRODUCTION

It has now been demonstrated that a V_H region is encoded by three genes, V_H, D and J_H (1,2,3,4). In the DNA of uncommitted cells, clusters of V_H, D and J_H genes are separated by introns. These introns are removed during immunological commitment to produce a contiguous V, D and J DNA sequence (1). It was an analysis of the amino acid sequences reviewed here that first suggested the three gene structure of V_H and encouraged the search for D gene sequences (2). Here we will review the sequences of several dextran binding myeloma and hybridoma proteins and discuss how somatic mechanisms contribute to antibody diversity. Secondly, we will demonstrate the use of the anti-dextran proteins to determine and compare the fine specificity of heterologous and monoclonal anti-idiotypic reagents.

SEQUENCES OF ANTI-DEXTRAN BINDING PROTEINS

Figure 1 shows the V_H regions of 14 dextran binding proteins and two myelomas M104 and J558. All the sequences shown are from $\mu\lambda$ proteins except for J558 which is an $\alpha\lambda$ myeloma. Although no two V_H regions are identical, the sequences do follow a clear pattern consistent with their genetic origin. Homology is found in the first 99 residues of these sequences followed by two positions, 100-101, of great heterogeneity. Another region, 102-117, of relative homogeneity completes the sequences. Clearly, this pattern reflects the three-gene basis of these sequences. However, the division of these sequences into V, D and J segments is based solely on the amino acid sequence data. Thus the precise genetic origin of each residue especially at the V-D and D-J junctions is unknown.

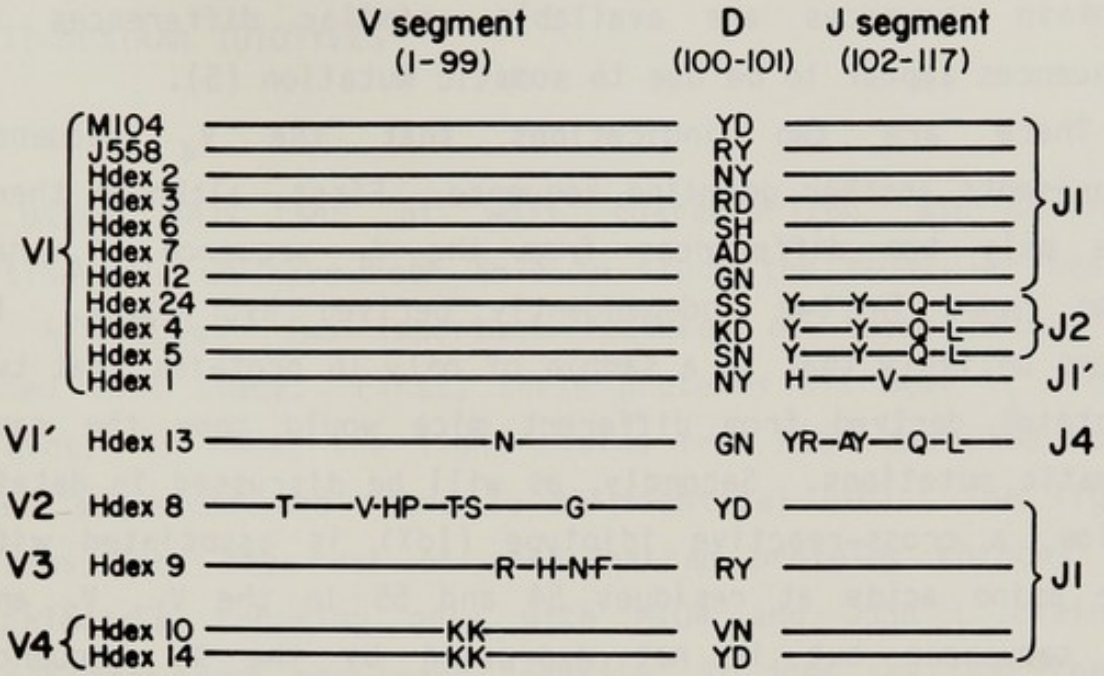

FIGURE 1. Diversity patterns of V_H regions of anti α(1→3) dextran-binding myeloma and hybridoma proteins. The V_H sequences of M104 is used as the prototype sequence. Solid lines in the V and J segment denotes identity with M104. Differences are indicated by the one-letter code of Dayhoff. The D-segment sequences are given for each protein.

Inspection of the V segment sequences demonstrates that the prototype M104 sequence, termed V_1, is found in 11 of the 16 sequences and thus must certainly represent a germline sequence. The V_1 has a single asparagine substitution that can be derived from the V_1 nucleotide sequence by a single base change and may be a somatic mutation. The V_2 and V_3 sequences have 7 and 4 substitutions respectively and all but one can be accounted for by single base changes. Whether these represent germline sequences or more extensive somatic mutation is unknown and awaits gene sequencing. However, in studies with anti-phosphocholine antibodies where both gene and

protein sequences are available, similar differences in sequences appear to be due to somatic mutation (5).

There are two indications that the V_4 sequence represents another germline sequence. First, although there are only two differences from the V_1 sequence, V_4 has been found in two independently derived hybridomas. It seems unlikely that in a sample of only 16 proteins that two proteins derived from different mice would show the same somatic mutations. Secondly, as will be discussed in detail below, a cross-reactive idiotype (IdX) is associated with the amino acids at residues 54 and 55 in the V_1, V_2 and V_3 sequences but is not expressed by the V_4 sequence (6). Therefore, the lysine substitutions correlate with the absence of IdX. While the majority of anti-dextran antibodies are IdX positive, there is a significant quantity of serum IdX negative antibodies (7). Thus, either these substitutions represent a germline sequence or an unusually high rate of somatic mutation yielding the V_4 sequence. Therefore, the V segments of the anti-dextran proteins show five V segment sequences which probably represent a minimum of two germline genes and perhaps as many as four.

The germline heavy chain J genes have been sequenced recently (8,9). Three of the four germline genes are found in the anti-dextran proteins. Similar to the V1 segment, the J1 segment predominates in 11 of the 16 sequences. The J2 sequence is represented three times and J4 has been found once. The sequence labeled J1' has a single amino acid substitution that is not consistent with the J1 sequence. This may be an example of somatic mutation in the J segment. It is also possible that this is an allelic substitution since this is one of two proteins (Hdex 1 and 2) which was derived from a single BRVR/BAB14 mouse. The J1' sequence may have been derived from the BRVR chromosome.

ANTI-DEXTRAN IDIOTYPES

Using this set of well characterized anti-dextran antibodies, we have been able to study the molecular basis of idiotypes. Two important features of these molecules aided this study. First, these proteins all bear λ_1 light chains. In fact, the light chains from M104 and J558 have been sequenced and found to be identical (10). The light chains from the rest of anti-dextran proteins cofocus on isoelectric focusing gels with M104 and J558 L chains. While this does not prove identity, it does say that these chains are at least very similar. A second feature of these proteins was the fact that in several cases, idiotype positive and idiotype negative proteins only differed by two adjacent amino acids.

The anti-dextran idiotypes are based on the dextran binding myelomas M104 and J558. The cross-reactive idiotype IdX, first described by Blomberg and coworkers, is expressed equally by M104 and J558, and a large portion of serum anti-dextran antibodies in the BALB/c mouse (11). Hansburg and coworkers characterized individual or private idiotypes, IdI(M104) and IdI(J558), which are unique to their respective myelomas (7). These idiotypes are also found in immune sera but on only a few percent of the anti-dextran antibodies.

As already mentioned, IdX is associated with two asparagines at positions 54 and 55 and a carbohydrate moiety attached to residue 55. Figure 2 summarizes the V segment sequences along with their relative IdX expressions. The V_1 and V_3 segments have both asparagines and the carbohydrate and fully express the IdX. The V_2 segment

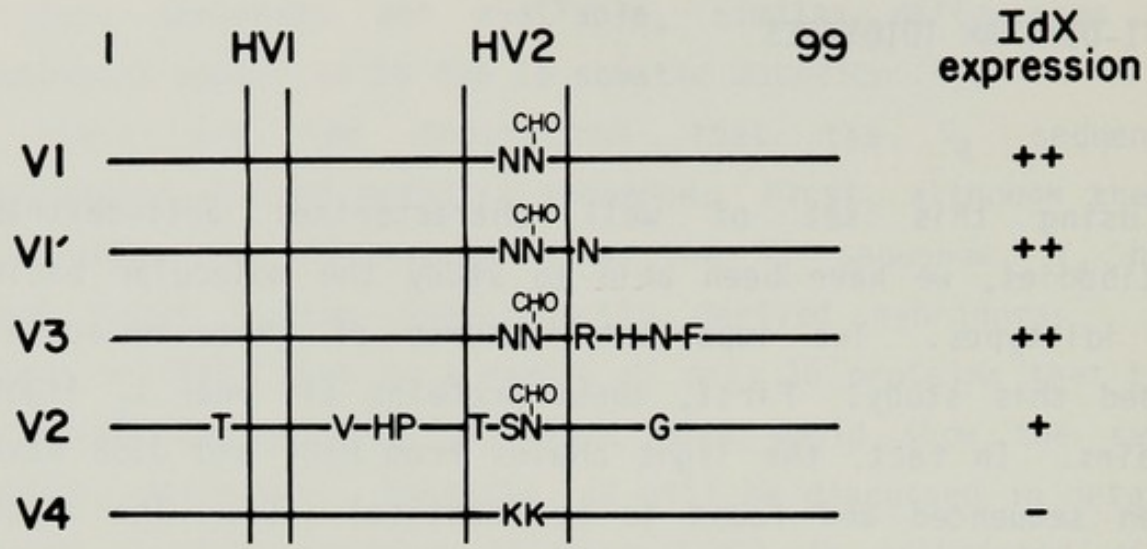

FIGURE 2. Localization of amino acid important for IdX expression. The five different V segments from dextran-binding proteins are shown along with their relative IdX expression. M104 is used for comparison; solid lines denote identity with M104. Differences are indicated by the one-letter code of Dayhoff. CHO represents a carbohydrate moiety attached to residue 5S in all V segments except V_4. IdX expression is measured by a competitive type radioimmune assay described in reference 7.

has a serine substitution at residue 54 and the IdX expression has been significantly depressed. The V_4 segment has two lysines, no carbohydrate and does not express IdX.

Since the variable regions of M104 and J558 differ only in the D segment residues, it is not surprising to find IdI determinants associated with these residues. Figure 3 shows that other proteins that have the D segments of M104 and J558 express the IdI idiotypes regardless of the V and J segments. Thus, in anti-dextran antibodies, the IdX is associated with the V segment while the IdI determinants are associated with D segments.

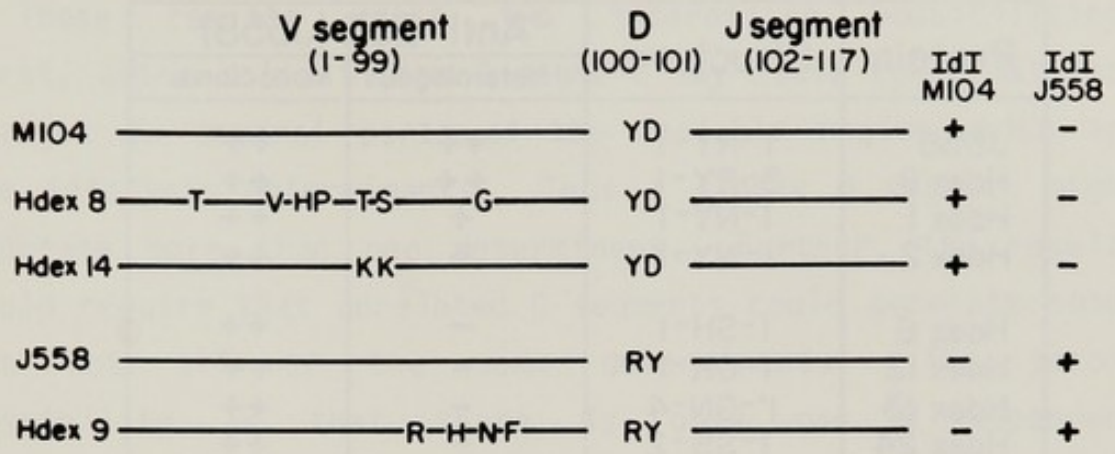

FIGURE 3. Localization of amino acids important for IdI M104 and IdI J558 expression. The V_H region sequences of M104, J558 and 3 dextran binding proteins are shown along with their expression of IdI M104 and IdI J558. M104 is used for comparison; solid lines for the V and J segments denote identity. Differences are indicated by the one-letter code of Dayhoff. The D-segment sequences are given for each protein. Expression of IdI is measured as described in reference 7.

These associations were made using appropriately absorbed goat and rabbit antisera. However, we have also analyzed the fine specificity of several monoclonal anti-idiotypic reagents produced in A/J and SJL mice. Figure 4 summarizes the reactivity of the heterologous and monoclonal anti-IdI(J558) reagents using the anti-dextran proteins as probes for their fine specificity. The heterologous reagent recognizes those proteins bearing the arginine-tyrosine (RY) D segment. In addition the two proteins with asparagine-tyrosine (NY) give a reduced but significant reaction. All other proteins with different D segments are negative. The monoclonal anti-IdI(J558) reagent, however, gives a different pattern. While it also recognizes all of

Protein	Structure	Anti-IdI (J558)	
		Heterologous	Monoclonal
J558	I-RY-I	++	++
Hdex 9	3-RY-I	++	++
Hdex I	I-NY-I′	+	++
Hdex 2	I-NY-I	+	++
Hdex 6	I-SH-I	-	++
Hdex I2	I-GN-I	-	++
Hdex I3	I′-GN-4	-	++
Hdex 24	I-SS-2	-	++
Hdex 3	I-RD-I	-	-
Hdex 4	I-KD-2	-	-
Hdex 5	I-SN-2	-	-
Hdex 7	I-AD-I	-	-
Hdex 8	2-YD-I	-	-
Hdex I0	4-VN-I	-	-
Hdex I4	4-YD-I	-	-
MI04	I-YD-I	-	-

FIGURE 4. Comparison of the fine specificity of heterologous and monoclonal IdI J558 reagents. The structure of sixteen dextran-binding proteins are shown along with their reactivity in idiotype assays using the rabbit heterologous reagent or the monoclonal reagent. The V-D-J structure is indicated by the V and J segment numbers shown in Figure 1. The D segment amino acids are indicated by the one-letter code of Dayhoff. The expression of idiotype was determined as described in reference 7.

the proteins reactive with the heterologous reagent, it differs by recognizing a group of proteins not carrying the RY or NY sequence. These D segments include serine-histidine, glycine-asparagine and serine-serine, all forming very different D segments. In fact, there appears to be no obvious correlation of structure and reactivity with the monoclonal reagent.

These results raise two interesting possibilities. First, amino acids in the D segment may cause conformational changes in several parts of the variable region which are the idiotypic determinants. Thus a single D segment might generate more than one determinant. Further, the results would require that unrelated D segments could generate some, but not all of the same determinants. The second possibility is that there is only one D dependent determinant, but that the monoclonal reagent is polyfunctional; that is, it has subsites within the combining site that bind to different antigenic determinants. Currently, we are trying to resolve this issue by identifying and characterizing the determinants that actually are being bound by these reagents. However, regardless of which explanation proves correct, the monoclonal reagent shows a broader specificity range than does the heterologous reagent.

To summarize, idiotypes can be associated with V, D and possibly J region segments. In anti-dextran antibodies, a cross-reactive idiotype is associated with amino acids located in the V segment while individual idiotypes associate with D segment residues. Secondly, monoclonal reagents may show a broader range of fine specificities as compared to their heterologous counterparts. It is, therefore, important to carefully analyze the fine specificity of monoclonal reagents before using them in place of heterologous reagents.

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REFERENCES

1. Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). Cell 19, 981.
2. Schilling, J., Clevinger, B., Davie, J., and Hood, L. (1980). Nature 283, 35.
3. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
4. Sakano, H., Kurosawa, Y., Weigert, M., and Tonegawa, S. (1981). Nature 290, 562.
5. Gearhart, P., Johnson, N., Douglas, R., and Hood, L. (1981). Nature, in press.
6. Clevinger, B., Schilling, J., Hood, L., and Davie, J. (1980). J. Exp. Med. 151, 109.
7. Hansburg, D., Briles, D., and Davie, J. (1977). J. Immunol. 119, 1406.
8. Newell, N., Richards, J., Tucker, P., and Blattner, F. (1980). Nature 209, 1128.
9. Gough, N., and Bernard, O. (1981). Proc. Natl. Acad. Sci. (USA) 78, 509.
10. Weigert, M., Cesari, I., Yonkovich, S., and Cohn, M.(1970). Nature 228, 1045.
11. Blomberg, B., Geckeler, W., and Weigert, M. (1972). Science 177, 178.
12. Sakano, H., Kurosawa, Y., Weigert, M., and Tonegawa, S. (1981). Nature 290, 562.

STRUCTURAL PROPERTIES OF THE HAPTEN BINDING SITE AND OF IDIOTOPES IN THE NP^b ANTIBODY FAMILY

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ABSTRACT. The λ1-bearing, dextran-binding myeloma protein MOPC104E and two λ1-bearing, NIP-binding monoclonal antibodies of the NP^b antibody family (B1-8 and S43) carry heavy chain variable regions with very similar amino acid sequences. Replacement of five amino acids in the variable region of MOPC104E appears sufficient to change binding specificity from dextran to NIP. Two of these amino acids are located in the first hypervariable region, two in the second and one in the D region. A striking similarity between the NIP binding site of B1-8 and S43 and of the DNP binding site of MOPC315 (ref. 24, 25) emerges and a model of the NIP binding site can thus be constructed. The sequence comparison of the MOPC104E, B1-8 and S43 heavy chain variable regions also allows us to tentatively assign heavy chain sequences to idiotopes defined on the B1-8 variable region by monoclonal anti-idiotope antibodies, since these idiotopes are not expressed by MOPC104E and S43. The idiotopes fall into two groups, group 1 idiotopes being closer to the hapten binding site than group 2 idiotopes. The results suggest that certain amino acids in the second hypervariable region of the heavy chain participate in the construction of group 2 idiotopes whereas in the case of group 1 idiotopes D region sequences appear to be involved.

INTRODUCTION

The NP^b antibody family. The humoral immune response of C57BL/6 mice against the hapten 4-hydroxy-3-nitro-phenylacetyl (NP) is dominated by λ chain-bearing antibodies which have a characteristic fine specificity of hapten binding, show restricted isolelectric focussing spectra and express a complex inheritable idiotype called NP^b (1-4). An analysis of this response at the level of monoclonal antibodies (5) revealed that it consists of a large family of similar but distinct antibodies which we called the NP^b antibody family (6-9). Other restricted antibody responses such as those against α(1-3)dextran, arsonate

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and phosphorylcholine also reflect the expression of families of closely related antibodies (10-14, and P. Gearhart et al., this volume), and microheterogeneity of this type thus appears to represent a general phenomenon. What is its molecular and genetic basis?

In the NP system, the nucleotide sequences encoding the heavy chain variable regions of two λ1-bearing anti-NP antibodies of C57BL/6 origin are now available (15, see also Bothwell et al., this volume). One of these antibodies is called B1-8, belongs to the IgM class and was isolated from the primary anti-NP response; the other, S43, is an IgG2a from the hyperimmune response (7). The genes encoding the variable regions of the B1-8 and S43 heavy chains differ from each other by 10 base pairs in V and have very different D but identical J segments (J2). In the C57BL/6 germ line, seven V_H genes were found with a S43 V_H probe. They all differ in sequence significantly from S43 V_H, but one of the genes is identical to the V_H gene expressed in antibody B1-8. The V_H gene expressed in S43 is closely related to B1-8 V_H, much more so than to the other isolated germ line V_H genes. Taken together, the sequence data show that the microheterogeneity in the NP^b antibody family is due to combination of different genetic elements in the construction of VDJ genes, and, presumably, somatic mutation.

The microheterogeneity of the anti-NP response is seen also by the immune system itself and can thus also be analyzed at the level of immunological recognition. We have isolated a set of monoclonal anti-idiotope antibodies which define a series of idiotopes on the anti-NP antibody B1-8 (9, 16). Idiotopes constructed by any of the first 98 amino acids of the B1-8 heavy chain are parts of the phenotype of the B1-8 germ line V_H gene as it is seen by the immune system. Others may represent D region determinants. Thus, potentially, the anti-B1-8-idiotope antibodies enable us to study the expression and modification of a germ line V_H and of a D region gene in a specific immune response. The anti-idiotopes also turn out to be potent immunological regulators (17-19) in the sense of the network hypothesis (20) and may thus themselves influence antibody microheterogeneity as expressed in the NP^b antibody family.

It is thus essential to learn on which segments of the variable region of the B1-8 heavy chain the various idiotopes as defined by our anti-idiotope antibodies are located and what their relation is to the B1-8 hapten binding site. We approach this problem below, by a comparison of the amino acid sequences of the heavy chain variable region of B1-8, S43 and a related, dextran-binding and λ-chain-bearing myeloma, namely MOPC104E (10, 21). The latter is useful for our analysis, since of all published amino acid sequences of murine V_H regions that of the MOPC104E protein stands out as closely related to the B1-8 and

S43 V_H sequences (see Fig. 1). It is striking that MOPC104E as well as B1-8 and S43 express λ1 light chains. Lambda chains may thus be expressed more readily in combination with certain V_H isotypes than with others. The λ1 chain of MOPC104E carries the germ line-encoded $V_{\lambda O}$ sequence. This appears also to be the case for the λ1 chain of B1-8, as suggested by peptide mapping (T. Imanishi-Kari, personal communication). The λ1 chain of S43 has not yet been analyzed.

RESULTS

The hapten binding site of antibody B1-8. Despite their similar V region sequences MOPC104E on the one side and B1-8 and S43 on the other express different binding specificities. B1-8 and S43 have a high affinity for the aromatic NP-hapten and an even higher one (10^{-7} and 2.4×10^{-8}M) for the related hapten 4-hydroxy-5-iodo-3-nitro-phenylacetyl (NIP) (7). Since our binding studies were usually done with NIP- rather than NP-caproic acid we shall refer to the hapten binding sites of B1-8 and S43 as bearing specificity for NIP. MOPC104E, on the other hand, reacts with a carbohydrate, α(1-3)dextran. Binding of MOPC104E to NIP or of B1-8 to α(1-3)dextran was not detectable in a solid phase radioimmunoassay which picks up antibodies even when their affinity is low. A more quantitative analysis of crossreactivity remains to be done. Since the V_Ls of MOPC104E and B1-8 are probably identical some of the amino acids by which their V_H regions differ should be of key importance for their distinct antigen binding specificities. Furthermore, since B1-8 and S43 both bind the hapten with a similar fine specificity (7) and the V_H region of S43 appears to be derived from that expressed in B1-8 by somatic mutation, we expect that amino acid residues essential for NIP-binding specificity should be shared between the two proteins. In Fig. 1 we compare the amino acid sequences of the heavy chain variable regions of MOPC104E, B1-8 and S43 and determine in which positions of the first (HV1) and second (HV2) hypervariable regions and of the D region the two anti-NP antibodies are identical to each other but different from MOPC104E (We consider framework substitutions as isofunctional or compensatory in terms of overall structure in a first approximation.). Two positions in HV1 (pos. 31, 35), five in HV2 (50, 52, 55, 62, 66) and one at the V-D boundary (99) fulfill these criteria. On the basis of an argument developed below positions 55, 62 and 66 are unlikely to be part of the hapten binding site. We therefore propose that the replacement of amino acids in the remaining five positions (boxed in Fig. 1) which are highly conserved in anti-dextran antibodies (22) changes binding specificity from α(1-3)dextran to NIP-caproic acid.

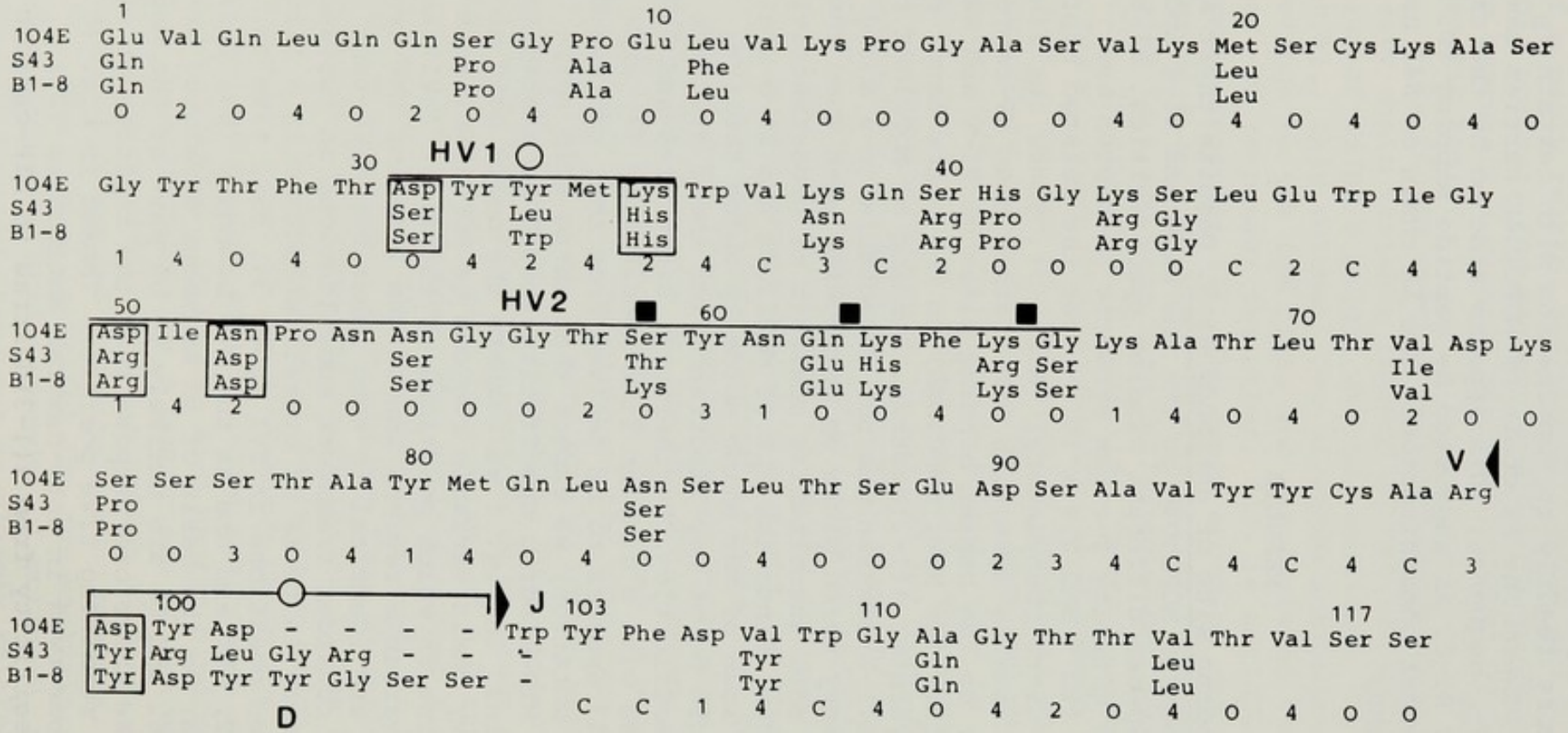

Fig. 1. Sequence comparison of the heavy chain variable regions of MOPC104E, S43 and B1-8. The amino acid sequence data are from Kehry et al. (23) and Bothwell et al. (15). V, D and J encoded regions and the first and second hypervariable region (HV1 and HV2) are marked. The numbers under each position are taken from Padlan et al. (24) and indicate the structural location of the corresponding amino acids in the heavy chain variable region of the MCPC603 myeloma protein: O (completely exposed to solvent) - 4 (completely burried in domain interior), C (in contact with homologous domain). The five positions considered important for the change from dextran to NIP-binding specificity are boxed. Symbols indicate positions potentially involved in B1-8 idiotope formation: ○, group 1 idiotopes; ■, group 2 idiotopes.

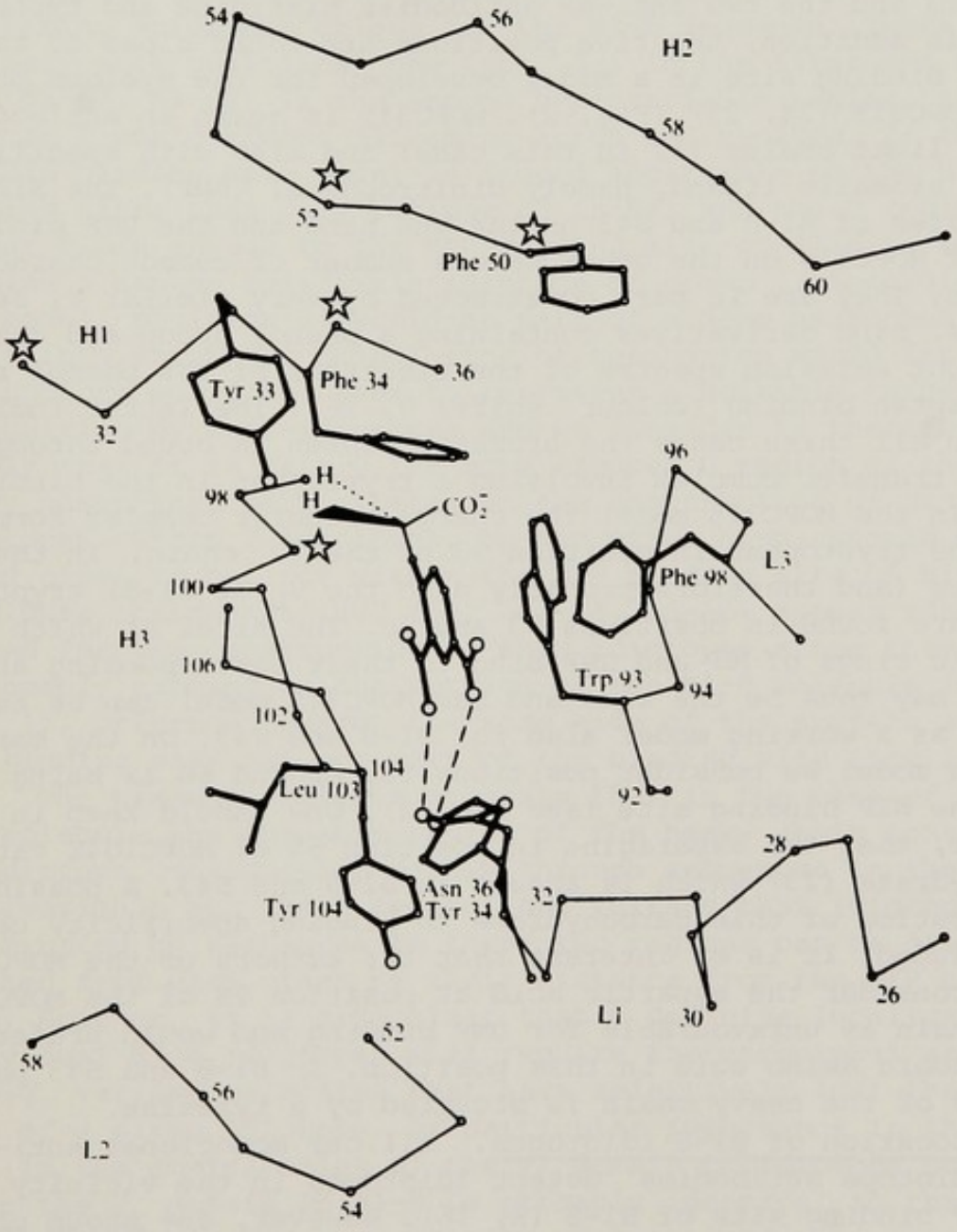

Fig. 2. The model developed by Dwek et al. for the DNP binding site of MOPC315. The hapten in the center is surrounded by the hypervariable regions of the light (L1, L2, L3) and the heavy chain (H1, H2, H3). The figure is taken from ref. 25. The positions identified by the sequence comparison in Fig. 1 as being of importance for NIP binding are marked with an asterisk.

Independent arguments can be made in support of this notion. Thus, the change from dextran- to NIP-binding specificity involves an exchange of hydrophilic by more hydrophobic and aromatic amino acids in some of the five positions. This is best seen at positions 35 and 99 where MOPC104E has lysine and aspartic acid and the two anti-NP antibodies histidine and tyrosine.

In addition, the five positions are found close to the hapten binding site in a model developed for the myeloma protein MOPC315 (24, 25) (Fig. 2). MOPC315 is again an antibody with λ light chains ($\lambda 2$ in this case) and also with specificity for an aromatic ligand, namely dinitrophenol (DNP). The NIP binding sites of B1-8 and S43 on the one hand and the DNP binding site of MOPC315 on the other have a number of common characteristics. They are in part constructed by very similar V_λ sequences, bind derivatives containing a phenol group and change the light emission spectra of the electrons of the phenol ring upon hapten binding (colour shift; 9, 26). The latter indicates that in all three cases the aromatic hapten is bound through a charge transfer complex involving a tryptophan in the binding site. In the MOPC315 model the charge transfer complex forms with the tryptophan in position 93 of the $\lambda 2$ chain. In the λ_o sequence (and therefore probably also the V_L of B1-8) tryptophans are found in positions 93 and 98. The sites at which the aromatic rings of NIP and DNP bind to their corresponding antibodies may thus be the same and the MOPC315 model can be regarded as a working model also for B1-8 and S43. On the basis of this model we consider positions 55, 62 and 66 as being outside the NIP binding site (see Fig. 2). One should keep in mind, however, that the asparagine in position 55 of MOPC104E carries carbohydrate (23) which is absent in B1-8 and S43. A possible contribution of this carbohydrate to binding specificity cannot be excluded. It is of interest that the authors of the MOPC315 model consider the aspartic acid at position 99 of the MOPC315 heavy chain as unfavourable for DNP binding and would prefer a hydrophobic amino acid in this position. In B1-8 and S43 position 99 of the heavy chain is occupied by a tyrosine.

Location of B1-8 idiotopes. All our monoclonal anti-B1-8 idiotope antibodies detect idiotopes in the vicinity of the NIP binding site of B1-8 (9, 16). However, one group of idiotopes (group 1) appears more intimately associated with the NIP binding site than the other(group 2): The binding to B1-8 of anti-idiotopes recognizing group 1 idiotopes is inhibited by free hapten whereas in the case of group 2 idiotopes free hapten has no effect and multivalent hapten-carrier conjugates are required for inhibition (9, 16).

How can we map group 1 and group 2 idiotopes in more detail? We know that both types of idiotopes are expressed on B1-8 but are absent from S43 and MOPC104E (9, and unpublished data). By comparing the amino acid sequences of the heavy chain

variable regions of the three proteins, we should be able to identify in the variable region of the B1-8 heavy chain amino acids potentially involved in idiotope construction (Fig. 1). Whereas in the preceeding section we searched for positions in which the same amino acids occurred in B1-8 and S43 but not MOPC104E, we now look for positions in which one or two adjacent amino acids are unique for the B1-8 sequence. There are not many such positions, one (pos. 33) in the first and three (59, 62-63, 65-66) in the second hypervariable region, and only in D could almost all amino acids be involved. If the MOPC315 binding site model is indeed applicable to the B1-8 binding site, then only residue 33 in HV1 and the D region appear close enough to the NIP binding site of B1-8 as to determine a hapten modifiable idiotope of group 1. According to the model, position 33 would be part of a deep hydrophobic cleft into which the phenol ring of the hapten would fit. Such a structure would not seem to represent a suitable target for recognition by anti-idiotopes and we consider it therefore likely that it is mainly the more exposed D region of the B1-8 heavy chain that participates in the construction of group 1 idiotopes. Group 2 idiotopes, on the other hand, should be located close to but not within the NIP binding site of B1-8. The candidate residues in HV2 (59, 62-63, 65-66) appear ideally suited for the construction of such idiotopes since they are presumably exposed on the surface of the B1-8 molecule (see Fig. 1), oriented toward the same side of the surface as the NIP binding site but not part of the binding site itself.

The present analysis suggests that in the case of the B1-8 molecule the variable region of the heavy chain contributes to idiotope construction mainly through sequences in the second hypervariable and the D region, and further that idiotopes constructed by sequences in these two regions can be distinguished from each other by their distance from the NIP binding site. However, it is also clear that a definite localization of individual B1-8 idiotopes will require the structural analysis of e.g. variant molecules that have selectively lost the expression of a given idiotope. Of particular importance in this context is the contribution of the V_L domain to idiotope structure. While the V_L domain in B1-8 is probably identical to that of MOPC104E and other $V_{\lambda O}$-expressing myelomas and thus can not give rise to B1-8 specific idiotopes by itself, it is possible and in fact likely, that sequences of V_L are part of B1-8 idiotopes. The absence of such idiotopes from S43 could therefore be caused by sequence differences between the two proteins in the variable regions not only of the heavy but also the light chains.

CONCLUDING REMARKS

Our sequence comparison shows that the binding specificity of the dextran-specific binding site of MOPC104E can be changed drastically by only a few amino acid replacements in the hypervariable regions of the heavy chain. The analysis enables us to define amino acid replacements in 5 positions some or all of which convert the dextran-specific binding site into a binding site specific for the aromatic hapten NIP. The latter binding site has many features in common with the DNP-specific binding site of the myeloma protein MOPC315 for which a detailed molecular model has been developed (24, 25). A satisfactory working model for the binding sites of two monoclonal anti-NP antibodies is thus at hand. One of these antibodies (B1-8) expresses germ line-encoded V regions.

The idiotopes defined on the B1-8 variable region by monoclonal anti-idiotope antibodies appear all to be located in the vicinity of the NIP binding site, yet their expression can change without much effect on hapten binding specificity. The best example for this is represented by antibody S43 which has lost all B1-8 idiotopes but binds the hapten with a similar fine specificity as B1-8 and with a four-fold higher affinity (9). Thus, extensive idiotypic microheterogeneity is found in antibody families whose members may possess similar antigen binding properties - a situation which is now amply documented in many systems (7-14). If idiotope expression and antigen binding specificity are indeed largely independent of each other then we would postulate that B1-8 idiotopes should also be expressed in antibodies lacking specificity for NIP. Indeed, experiments reported elsewhere have shown that such antibodies appear to be even more frequent than NIP-specific ones (27).

Our analysis suggests that B1-8 idiotopes as recognized by homologous and isologous anti-idiotopes are preferentially constructed by amino acid sequences in the second hypervariable and the D region of the heavy chain. We do not know whether this represents a special case or a general phenomenon, but it is of interest to note that in murine dextran binding antibodies idiotypic determinants have been assigned to the very same heavy chain regions (22). The D regions of heavy chains are encoded in the germ line separately, as a set of diverse genetic elements (Tonewaga et al., this volume). The second hypervariable region of heavy chains is of unusual length and well exposed on the surface of the V domain. In the cluster of B1-8-related germ line V_H genes HV2 exhibits more sequence diversity than any other region of V_H (15). It is tempting to speculate that the second hypervariable regions of heavy chains and possibly also the D regions might have been

selectively diversified in evolution in order to provide targets for network regulation.

Be this as it may, it is clear that anti-idiotope antibodies will eventually serve as sensitive reagents by which the expression of structurally defined elements of antibody variable regions can be monitored in the immune system. We already know that all B1-8 idiotopes are regularly expressed in anti-NP responses and that group 1 idiotopes are less frequent than idiotopes of group 2 (17-19, and unpublished results). The latter finding is easily compatible with our suggestion that the former are constructed by the D and the latter by the V segment of the heavy chain. We have also found that B1-8 idiotopes are preferentially expressed on IgM as compared to IgG antibodies (18, 19, and unpublished data) and that idiotopes usually expressed in association on the same antibody molecule can in rare individuals be expressed independently of each other (16). We are engaged in further investigations along these lines which should lead us to a better understanding of the control of antibody expression and diversification.

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REFERENCES

1. Imanishi, T., and Mäkelä, O. (1973). Eur. J. Immunol. 3, 232.
2. McMichael, A.J., Phillips, I.M., Williamson, A.R., Imanishi, T., and Mäkelä, O. (1975). Immunogenetics 2, 161.
3. Jack, R.S., Imanishi-Kari, T., and Rajewsky, K. (1977). Eur. J. Immunol. 7, 559.
4. Karjalainen, K., and Mäkelä, O. (1978). Eur. J. Immunol. 8, 105.
5. Köhler, G., and Milstein, C. (1975). Nature 256, 495.
6. Reth, M., Imanishi-Kari, T., Jack, R.S., Cramer, M., Krawinkel, U., Hämmerling, G.J., and Rajewsky, K. In Regulatory Genetics of the Immune System: ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. VI. E.E. Sercarz, L.A. Herzenberg and C.F. Fox (eds.), Academic Press Inc., New York, 1977, p. 139.
7. Reth, M., Hämmerling, G.J., and Rajewsky, K. (1978). Eur. J. Immunol. 8, 393.

8. Imanishi-Kari, T., Reth, M., Hämmerling, G.J., and Rajewsky, K. In Current Topics in Microbiology and Immunology, Vol. 81 Lymphocyte Hybridomas. F. Melchers, M. Potter and N. Warner (eds.), Springer-Verlag, Berlin, Heidelberg, 1978, p. 20.
9. Reth, M., Imanishi-Kari, T., and Rajewsky, K. (1979). Eur. J. Immunol. 9, 1004.
10. Potter, M. (1977). Adv. Immunol. 25, 141.
11. Schilling, J., Clevinger, B., Davie, J.M., and Hood, L. (1980). Nature 283, 35.
12. Berek, C., Schreier, M.H., Sidman, C.L., Jaton, J.C., Kocher, H.P., and Cosenza, H. (1980). Eur. J. Immunol. 10, 258.
13. Estess, O., Nisonoff, A., and Capra, J.D. (1979). Mol. Immunol. 16, 1111,
14. Marshak-Rothstein, A., Siekevitz, M., Mudgett-Hunter, M., Margolies, M.N., and Gefter, M.L. (1980). Proc. Nat. Acad. Sci. US 77, 1120.
15. Bothwell, A.L.M., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., and Baltimore, D. (1981). Cell, in press.
16. Rajewsky, K., Takemori, T., and Reth, M. (1981). In Monoclonal Antibodies and T Cell Hybridomas, J.F. Kearney, U. Hämmerling and G.J. Hämmerling (eds.), Elsevier North-Holland Inc., in press.
17. Kelsoe, G., Reth, M., and Rajewsky, K. (1980). Immunol. Rev. 52, 15.
18. Reth, M., Kelsoe, G., and Rajewsky, K. (1981) Nature 290, 257.
19. Kelsoe, G., Reth, M., and Rajewsky, (1981). Eur. J. Immunol., in press.
20. Jerne, N.K. (1974). Ann. Immunol. (Inst. Pasteur) 125C 373.
21. McIntire, K.R., Asofsky, R.M., Potter, M., and Kuff, E.L., (1965). Science 150, 361.
22. Clevinger, B., Schilling, J., Hood, L., and Davie, J.M. (1980). J. Exp. Med. 151, 1059.
23. Kehry, M., Sibley, C., Fuhrman, J., Schilling, J., and Hood, L. (1979). Proc. Nat. Acad. Sci. US 76, 2932.
24. Padlan, E.A., Davies, D.R., Pecht, T., Givol, D., and Wright, C. (1976). Cold Spring Harb. Symp. Quant. Biol. 41, 627.
25. Dwek, R.A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S.J., and Givol, D. (1977). Nature 266, 31
26. Eisen, H.N., Simms, E.S., and Potter, M. (1968). Biochemistry 7, 4126.
27. Rajewsky, K., Reth, M., Takemori, T., and Kelsoe, G. (1981). In The Immune System - A Festschrift in Honour of N.K. Jerne, I. Lefkovits and Ch. Steinberg (eds.), S. Karger AG, Basel, in press.

ALLELIC FORMS OF ANTI-PHOSPHORYLCHOLINE ANTIBODIES: THEIR EXPRESSION IN INBRED AND WILD MICE

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ABSTRACT Wild mice were tested for the allelic forms of phosphorylcholine (PC) antibodies previously described in inbred strains (1). Genetic and structural studies showed that C3Id associated with C3 phosphorylcholine binding myeloma protein (PBMP) and anti-PC antibodies of C57BL mice and T15Id associated with T15PBMP and anti-PC antibodies of BALB/c mice behaved like allelic markers. Four phenotypes were found among the inbred strains: $C3^{+}T15^{-}$ associated with *Igh-*$C^{b,j}$, $C3^{-}T15^{+}$ with *Igh-*C^{a}, $C3^{-}T15^{-}$ with *Igh-*$C^{c,d,e,f,g}$ and $C3^{+}T15^{+}$ with BAB14 (*Igh-*C^{b}) a V_H recombinant strain derived from a C57BL-BALB/c cross. Species of wild mice that were more distantly related to *Mus musculus* including Coelomys *M. pahari* and *Mus caroli, cookii, cervicolor popaeus* and *spretus* were $C3^{-}T15^{-}$ or $C3^{-}T15^{+}$. A rare *cervicolor popaeus* mouse expressed $C3^{+}T15^{+}$. *M. musculus molossinus, castaneus, brevirostris, praetextus, musculus* and *domesticus* subspecies exhibited $C3^{+}T15^{+}$ and $C3^{-}T15^{+}$ and infrequently $C3^{-}T15^{-}$ phenotypes. A large group of *M. m. domesticus* (41 mice) from Eastern Shore, Md. failed to produce anti-PC antibodies and were $C3^{-}T15^{-}$. $C3^{+}T15^{-}$ which was frequently seen in inbred mice was not found in the wild mice. There was no concordance of allotype and the allelic forms of PC antibodies observed in inbred strains.

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INTRODUCTION

Many idiotypic determinants that are shared by homogeneous (myeloma) immunoglobulins and antibodies of the same binding specificity in inbred mice have proven to be invaluable genetic markers (2-5). It has been found that many of these are expressed in the antibodies from some inbred strains but not others. Of particular genetic value are different idiotypes that identify homologous V-regions. Two such markers are the C3Id and T15Id that are associated with phosphorylcholine (PC) binding myeloma proteins (PBMP) and antibodies of C57BL/6 and BALB/c, respectively. Amino acid sequence studies have shown that the C3 and T15 myeloma proteins have V_H regions that differ by only four amino acids (6).

Genetic analysis of T15 and C3Id markers in F_1, BC_1 progeny, B.C8, C.B20 and BAB14 congenic strains and CxBD, E, G, H, I, J and K recombinant inbred (RI) strains derived from crosses of BALB/c and C57BL mice indicate that these behave like allelic markers linked to the *Igh-C* locus (1). T15Id is associated with $Igh\text{-}C^a$ and C3Id with $Igh\text{-}C^b$ and *j* haplotypes. The one exeeption is BAB14 which expresses $T15^+C3+$. BAB14 ($Igh\text{-}C^b$) is congenic to BALB/c and has been shown to have some BALB/c V_H *genes*.

To provide further insight into the genetics of these two markers, we have examined the PC antibodies of: 1) several species in the genus *Mus* some of which are distantly related to *Mus musculus* and cannot hybridize with *M. musculus (M. pahari; M. cookii; M. caroli; M. cervicolor);* 2) less distantly related species that hybridize with *M. musculus* but produce sterile males (*M. spretus*) and 3) a number of subspecies of *M. musculus* that have recently been isolated from widely distant geographic locations. The T15Id was found in distantly related species. In the subspecies of *M. musculus* different combinations of T15Id and C3Id were expressed, including mice that carried both C3 and T15.

Although at present these wild mice are not inbred, we show evidence that indicates that the genes controlling C3 and T15Id that appear to be separate in most inbred strains (except BAB14) are probably linked on the same chromosome.

We have also attempted to identify several of the common allotypic markers found in inbred mice. Allotypes were found only in *M. spretus*, and the *M. musculus* subspecies. No obvious relationship of allotype and the *C3* and *T15* markers was found.

MATERIALS AND METHODS

Mice

Inbred Strains. BALB/C AnN (C), C57BL/6N (B), DBA/2N, AKR/N, CBA/N and A/HeN are from Animal Production, National Institutes of Health. CE/J and RIII/J are from Jackson Laboratories, Bar Harbor, Maine. Ig congenic strains C.B20, BAB14 and B.C8 and recombinant inbred (RI) strains CxBD, CxBE, CxBG, CxBH, CxBI, CxBJ and CxBK were bred at the NCI, NIH, under Contract No. NO1-CB-94326 with Litton Bionetics, Kensington, Md.

Wild Mice. Origin and source of wild mice are shown in Table 1.

TABLE 1

Sources of Recently Isolated Stocks of Mice

	Geographic Origin	Isolated by
M. pahari	Thailand	Joe T. Marshall, Jr.
M. cervicolor cervicolor	Thailand	Joe T. Marshall, Jr.
M. cervicolor popaeus	Thailand	Joe T. Marshall, Jr.
M. caroli	Thailand	Joe T. Marshall, Jr.
M. cookii	Thailand	Joe T. Marshall, Jr.
M. spretus	Spain	Richard Sage
M. spretus	Morocco	Richard Sage
M. musculus (m.) molossinus	Kyushu, Japan	Prof. Fusanori Hamajima
M. m. castaneus	Thailand	Joe T. Marshall, Jr.
M. m. brevirostris	Morocco	Richard Sage
M. m. praetextus	Morocco	Richard Sage
M. m. musculus	Czechoslovakia	Richard Sage
M. m. musculus	Czechoslovakia	Richard Sage
M. m. musculus	Skive, Denmark	Verne Chapman
M. m. musculus	Vegrumbro, Denmark	Verne Chapman
M. m. domesticus	Centerville, Queen Annes Co., Md.	M. Potter

Myeloma Proteins

BALB/C PBMP TEPC15(T15), HOPC8 (H8), MOPC167 (M167), MCPC603 (M603), and CBPC3 (C3) of C57BL origin were purified on Sepharose-PC columns as previously described (7).

R36a-Pneumococci Immunization

R36a (*S. pneumoniae*) which elicits PC antibodies was kindly given to us by Dr. B. Prescott (BRL Inc., Rockville, Md). The immunization was the same as previously reported (1). A single i.p. injection of heat killed $0.5x10^9$ organisms was given and the mice were bled on 7, 10 and 14 days after the injection and their sera tested for anti-PC antibodies. In some of the wild mice, 2 injections at weekly intervals were given and the same procedure followed. In some Maryland Eastern Shore mice a conjugate of keyhole limpet hemocyanin(KLH)-PC kindly given to us by Dr. Latham Claflin (University of Michigan Medical School, Ann Arbor, Michigan) was also used. For immunization, 100 µg KLH-PC in complete Freund's adjuvant was injected into 2 foot pads and lymph node draining regions, followed by a similar injection 1 week later in incomplete Freund's adjuvant. Mice were bled 1 week later and at weekly intervals for several weeks.

Anti-PC Antibody Assay

Bovine serum albumin(BSA)-PC conjugate was kindly given to us by Stephen Clark, NCI, NIH. SRBC were coated with 50 µg of BSA-PC by the chromic chloride method and used in a hemagglutination system to titer anti-PC antibodies (1). All mouse sera were preabsorbed with SRBC prior to testing.

Anti-C3 and T15Id Serum

Homologous anti-C3 and anti-T15Id sera used in these studies have previously been described (1).

Selection, however, of antisera of the appropriate specificity is essential since most anti-T15 and anti-C3Id sera exhibit different patterns of cross reactivity among PBMP. We selected antiserum 6733, which was prepared in A/HeN mice, immunized with the T15 PBMP. This antiserum was specific for T15 at a dilution of 1/2000. Antiserum 13997 made in AL immunized with C3, which was specific for C3 at a dilution of 1/4000, was also used.

Detection of C3 and T15Id

Hemagglutination inhibition (HI) assay which was used to detect C3 and T15Id has been described (1).

Igh-C Allotypes of Wild Mice

Allotyping of wild mice was done by HI with antisera that have been previously described (8). Serum of wild mice were tested for allotypic determinants expressed by IgG_{2a} of the *a*, *b*, *c*, *d*, *e*, *f* and *g* allelic forms in inbred mice (Table 2). As indicated 1, 2 and 5 are "private" allotypic determinants which distinquish the *a*, *b* and *f* allelic forms. G2a.3 is a "public" allotypic determinant shared by allelic forms *c* and *g*. The expression on IgG_{2b} of G2b.22 allotypic determinant distinquish the allelic form *c* from *g*.

TABLE 2

TYPING OF WILD MICE FOR Igh-C ALLOTYPES

Anti Allotype Sera	Absorbed with	Allotype Identified	Haplotype Assoc. with allotype	Prototype Strain
A/He anti-BALB/c Ig	M.195	G2a.1	a	BALB/c
BALB/c anti-CBPC 101	---	G2a.2	b	C57BL
BALB/c anti-DBA/2 Ig	---	G2a.3	c,g	DBA/2, RIII
BALB/c anti-ALPC3	---	G2a.4	d,e	AKR, A
BALBc/C57BL anti-CEIg	---	G2a.5	f	CE
RIII anti-M195	---	G2b.22	c	DBA/2
Allotype antisera used when G2a 1,2,3,4,5 allotypes are not present				
C57BL anti-M173	---	G2a.6,7,8	a,c,d,e,f,g	
A anti-M195	---	G2b.9,11	a,b,c,f,g	
A anti-E109	---	A.12,14	a,f	
CE anti-M467	---	A.12,13	a,d,e	
BALB/c anti-M320	---	A.15	b	

Sera of wild mice were tested for allotype by inhibition of hemagglutination of the antiallotype serum with SRBC coated with the appropriate Ig class (8).
BALB/c M.P: M173 (G2a), M195 (G2b), E109 and M467 (IgA)
AL/N M.P: ALPC3 (G2a)
C.B20 M.P: CBPC 101 (G2a)
BALB/CxC57BL M.P: M320 (IgA)

If wild mice did not express G2a.1,2,3,4,5 determinants, they were tested for additional "public" allotypic determinants indicated in the table expressed by IgG_{2a}, IgG_{2b} and IgA immunoglobulins.

RESULTS

Anti-C3 and T15Id Sera Specificity

C3Id was identified by an anti-C3Id antisera which agglutinated C3 PBMP-coated SRBC. Agglutination was inhibited by C3 PBMP, PC antibodies of C57BL, congenic C.B20 and BAB14 and recombinant strains CxBD, E, H, I and K (Tables 3 and 4) but not by T15, or BALB/c PC antibodies. T15Id was identified by an anti-T15Id serum that agglutinated T15 PBMP-coated SRBC and was inhibited by T15 and H8 PBMP and anti-PC antibodies of BALB/c, congenic B.C8 and BAB14 and recombinant strains CxBJ and CxBG, but not by C3 PBMP or C57BL and related PC antibodies.

TABLE 3

Specificity of Anti-C3 and T15Id Antiserum

PBMP 1 mg/ml	AL anti-C3: C3 SRBC	A anti-T15: T15 SRBC
	HI titer ($\log^2$)	
T15	0	11
H8	0	10
C3	11	0
M167	0	0
M603	0	0
M511	0	0
BALB/c anti-PC	0	6
C57BL anti-PC	7	0

PBMP T15, H8, M167, M603 and M511 are of BALB/c origin

C3 is of C.B20 origin

TABLE 4

Genetics of C3 and T15Id Markers

	Igh-C	C3Id	T15Id
BALB/C	a	-	+
C57BL(B)	b	+	-
(CxB)xC			
C/C	a	-	+
C/B	ab	+	+
C.B20	b	+	-
BAB14	b	+	+
B.C8	a	-	+
CxBD	b	+	-
CxBE	b	+	-
CxBG	a	-	+
CxBH	b	+	-
CxBI	b	+	-
CxBJ	a	-	+
CxBK	b	+	-

Anti-PC Titers of Wild Mice Immunized with R36 Pneumococci

Wild mice species of the genus *Mus* and *M. musculus* were immunized with R36 pneumococci and tested for anti-PC antibodies by hemagglutination (HA) of BSA-PC coated SRBC (Tables 5 and 6). Most of the mice in the more distantly related mice, responded to PC except for *M. pahari* where only 25% showed anti-PC antibodies. All of the *M. stretus* and *M. musculus* subspecies with the exception of the Md. Eastern Shore mice gave anti-PC responses. The Md. Eastern Shore mice which comprise a very large group failed to show any

anti-PC antibodies. Eastern Shore, Md. mice immunized with KLH-PC also failed to respond to PC. These mice appear to be non-responders to PC antigens.

C3 and T15Id in the Genus Mus and M. musculus Subspecies Immunized with R36 Pneumococci

Different stocks of *Mus* and *M. musculus* subspecies of mice immunized with R36 pneumococci were examined for the expression of C3 and T15Id.

Of those which responded to PC, T15 but not C3Id was expressed by *M. caroli, cookii, cervicolor* (2 subspecies) and *spretus* (Morocco) (Table 5). A rare *M. cervicolor popaeus* expressed both C3 and T15Id. *M. spretus* (Spain) comprising 14 mice, all produced anti-PC antibodies but none expressed T15 or C3Id. In summary: the mice distantly related to *M. musculus* for the most part responded modestly

TABLE 5

C3 and T15 Id in Sera of *Mus* Species of Wild Mice Immunized with R36a Pneumococci

Species		Total	HI titer ($\log_2$)									
			0	1	2	3	4	5	6	7	8	>8
	PC	12	9	-	-	-	-	2	-	1	-	-
M. pahari	C3	12	12	-	-	-	-	-	-	-	-	-
	T15	12	12	-	-	-	-	-	-	-	-	-
	PC	13	1	2	3	1	3	1	2	-	-	-
M. caroli	C3	13	13	-	-	-	-	-	-	-	-	-
	T15	13	2	1	5	2	2	-	-	1	-	-
	PC	3	-	-	-	-	-	2	-	1	-	-
M. cookii	C3	3	3	-	-	-	-	-	-	-	-	-
	T15	3	-	-	2	1	-	-	-	-	-	-
	PC	9	1	1	1	3	1	-	2	-	-	-
M. cervicolor	C3	9	9	-	-	-	-	-	-	-	-	-
cervicolor	T15	9	2	-	4	1	1	-	-	1	-	-
	PC	10	4	1	-	1	1	-	1	-	-	2
M. cervicolor	C3	10	8	1	-	1	-	-	-	-	-	-
popaeus	T15	10	7	-	-	1	2	-	-	-	-	-
	PC	14	2	-	2	4	2	3	1	-	-	-
M. spretus	C3	14	14	-	-	-	-	-	-	-	-	-
(Spain)	T15	14	14	-	-	-	-	-	-	-	-	-
	PC	9	-	-	-	-	-	4	3	-	-	2
M. spretus	C3	9	9	-	-	-	-	-	-	-	-	-
(Morocco)	T15	9	3	-	-	1	1	2	2	-	-	-

to PC and most expressed T15 except *M. pahari* and *M. spretus* (Morocco). C3Id was not expressed except for a rare *M. cervicolor popaeus* mouse.

Most wild mice more closely related to *M. musculus* gave good anti-PC responses and expressed T15Id alone or in combination with C3Id (Table 6). As shown, 4/4 *M. m. molossinus*, 9/9 *M. m. m. Czech I* and 7/7 *M. m. m. Czech II* were phenotypically $C3^{+}T15^{+}$. A notable exception among the *musculus* subspecies was the 41 Md. Eastern Shore mice which failed to respond to PC and were phenotypically $C3^{-}T15^{-}$. *M. musculus Czech I* also expressed both C3 and T15. Among

TABLE 6

C3 and T15 Id in Sera of *Mus musculus* Species of Wild Mice Immunized with R36a Pneumococci

Species		Total	HI titers ($\log_2$)									
			0	1	2	3	4	5	6	7	8	>8
M. m. praetextus	PC	10	3	-	-	-	1	-	1	-	-	5
	C3	11	8	-	3	-	-	-	-	-	-	-
	T15	11	6	-	3	-	2	-	-	-	-	-
M. m. brevirostris	PC	10	-	-	-	-	2	1	1	-	-	6
	C3	10	5	-	2	2	1	-	-	-	-	-
	T15	10	-	-	1	2	1	-	1	2	3	-
M. m. castaneus	PC	9	1	-	3	-	1	-	1	-	-	3
	C3	9	3	-	1	5	-	-	-	-	-	-
	T15	9	2	-	2	1	-	-	4	-	-	-
M. m. molossinus	PC	4	-	-	-	1	-	-	2	1	-	-
	C3	4	-	-	-	-	4	-	-	-	-	-
	T15	4	-	-	-	2	1	-	1	-	-	-
M. m. czech I	PC	9	-	-	-	1	1	2	-	-	-	5
	C3	9	-	-	-	1	3	2	1	1	1	-
	T15	9	-	-	2	-	-	1	3	2	1	-
M. m. czech II	PC	7	-	-	-	-	-	1	1	1	-	4
	C3	7	-	-	3	-	-	2	-	2	-	-
	T15	7	-	-	-	2	1	1	1	2	-	-
M. m. skive	PC	11	-	1	-	1	1	-	3	1	2	2
	C3	11	5	1	4	1	-	-	-	-	-	-
	T15	11	2	-	1	1	3	2	2	-	-	-
M. m. vegrumbro	PC	5	2	-	-	-	2	1	-	-	-	-
	C3	5	5	-	-	-	-	-	-	-	-	-
	T15	5	2	1	1	1	-	-	-	-	-	-
M. m. domesticus	PC	41	41	-	-	-	-	-	-	-	-	-
	C3	41	41	-	-	-	-	-	-	-	-	-
	T15	41	41	-	-	-	-	-	-	-	-	-

the 9 mice tested, all were phenotypically $C3^{+}T15^{+}$. Eight out of 9 mice carried the $G2a^{c,e}$ and 1/9 the $G2a^{a}$ allotypes. These mice are presently being mated to BALB/c to determine the segregation pattern of the V-region markers and also the linkage to allotype.

In summary there was a difference in the expression of the *C3* and *T15Id* V-region markers in *Mus* and *M. musculus* subspecies of wild mice. The latter gave a better anti-PC response and was usually phenotypically $C3^{-}T15^{+}$ or $C3^{+}T15^{+}$. The one exception was the Md. Eastern Shore stocks which gave no anti-PC responses and were $C3^{-}T15^{-}$. None of the *M. musculus* expressed C3Id alone.

V_H Region (*C3* and *T15*) and C_H Region Phenotypes of Wild and Inbred Mice Immunized with R36 Pneumococci

In previous studies of PC antibodies of inbred strains of mice, we showed that $C3^{+}T15^{-}$ phenotype is associated with *Igh-*C^{b} and *j haplotypes;* $C3^{-}T15^{+}$ with *a;* and $C3^{-}T15^{-}$ with *c, d, e, f* and *g haplotypes*. BAB14 expressed $C3^{+}T15^{+}$ phenotypes suggesting the presence of both *BALB/c* and *C57BL genes* in the V_H region (1). Most *Mus* subspecies showed none of the allotypic determinants found in inbred strains, except for *M. spretus* (Spain) and *M. spretus* (Morocco) (Table 7). The *M. musculus* stocks all expressed different combinations of allotypic markers and for the most part appeared heterozygous. One notable exception was the *M. m. castaneus* which appeared to be homozygous for IgG_{2a}.4 allotypic specificity associated

TABLE 7

PHENOTYPIC EXPRESSION OF PC IDIOTYPES AND ALLOTYPES IN WILD MICE

Subgenus	Species	PC-Idiotypes	IgG2a Alleles*
Pyromys	M. shortridgei	ND	None
Coelomys	M. pahari	0	None
Nannoyms	-	-	-
Mus	M. c. cervicolor	T15	None
	M. c. popaeus	T15+C3, 0	None
Infertile	M. caroli	T15	None
	M. cookii	T15	None
Semi-fertile	M. spretus	0	a, f
	spretus	T15, 0	
	M. musculus		
	molossinus	T15+C3	ab
	castaneus	T15+C3	e
	brevirostris	T15+C3, T15	e,f,e+f
	praetextus	T15+C3, T15	a,f,a+f
Fertile	musculus		
	czech I	T15+C3	a,c,e,a+e
	czech II	T15+C3	b,b+e
	skive	T15+C3, T15, 0	a,a+e, a+f, c+e, e+f
	vegrumbro	T15	a
	domesticus	0(PC^-)	a,e,f,a+e, e+f, a+f
	inbred	T15, C3, 0, T15+C3	a,b,c,d,e,f

0 indicates absence of C3 and T15Id
PC^- indicates absence of anti-PC antibodies
* See Table 2 for identification of allotypes

with Ig^e haplotype and of these 6/9 expressed $C3^+T15^+$ phenotypes. The concordant expression of the V_H markers with the allotype observed in inbred mice was not seen in *M. musculus* stocks of wild mice.

DISCUSSION

Idiotypic determinants have proven to be extremely valuable markers in the study of immunoglobulin structure and genetics. One set of such markers which have been extensively studied are those associated with the BALB/c PBMP binding myeloma protein T15 (1, 6, 9, 10). Evidence from a variety of studies has indicated that the V_L and V_H regions of T15 are encoded in germ line genes which are highly conserved in the inbred mouse strains (6, 11). We have recently described a T15- like molecule (C3) from C57BL which is highly homologous to T15 (6). These 2 proteins have an identical light chain amino acid sequence through the first complementarity determining region (11). Their heavy chains differ at 4 positions in the V_H framework and we have shown that 2 of these differences are present in all anti-PC antibodies elicited in C57BL. This result led to the suggestion that the BALB/c and C57BL forms of the T15 heavy chain might represent allelic products of this germ line V_H region. We have now prepared idiotypic antibodies specific for T15 and C3 and have begun to explore the expression of these markers in both inbred and wild mouse strains.

Antisera specific for C3 can be raised quite easily by immunization of AL mice with the C3 myeloma protein. Conversely, it is quite difficult to prepare antisera to T15 which do not cross react with C3. We have found that most antisera prepared to T15 also react with C3, making it essential to carefully screen antisera used in idiotypic assays attempting to distinguish these 2 proteins. This cross reaction may explain the differences reported in the expression of T15 in C57BL mice. We have never found the T15Id idiotype in serum antibody from R36 immunized C57BL, although Gearhart and Cebra have found this marker on pre-B cells from this strain (9). It is possible that the T15Id found on pre-B cells is indeed identical to that of the BALB/c marker and that the absence of this marker in serum antibody is a reflection of phenotypic regulation. When these T15 and C3I specific antisera were used to assess the distribution of T15 and C3Id's in backcross progeny, congenic and recombinant strains derived from BALB/c-C57BL crosses these markers were found to segregate as simple Mendelian alleles (Table 4). In the limited backcross progeny tested and the congenic and recombinant strains the idiotypes were found to be linked to the *Igh-C* locus. One exception noted to the simple segregation pattern of V-region alleles was found in BAB14 which expressed both idiotypes. BAB14 is a congenic strain derived at the fourteenth backcross generation of the C57BL $Igh\text{-}C^b$ haplotype onto a BALB/c

background and has previously been shown to express C57BL C_H and BALB/c V_H genes presumably resulting from a crossover between BALB/c V_H and C57BL C_H. The presence of the T15 and C3Id's suggests that both BALB/c and C57BL V_H regions are expressed in this strain. We have described several possible mechanisms to explain this phenotype including unequal crossing over, crossing over between pseudo alleles within the V_H PC locus, and the regulatory expression of pseudo alleles in the V_H locus (1). The expression of both markers is of further interest in terms of results that we obtained in the wild mouse stocks of worldwide origin.

We have recently established a colony of several species of mice from the genus *Mus* that are distantly related to *M. musculus*, but are clearly more closely related to *M. musculus* than to rats. These include *M. pahari*, *M. cervicolor cervicolor*, *M. cervicolor popaeus*, *M. caroli*, *M. cookii and M. spretus*. Further, we have a number of *M. musculus* subspecies with origins in Europe, Asia, Africa and North America. These mice have now been analyzed for their anti-PC response as well as the expression of T15 and C3Id's. All species tested responded to PC immunization with the exception of *M. m. domesticus* mice from the Md. Eastern Shore. *M. pahari*, one of the most ancient and furthest removed species from the laboratory mouse, was also somewhat atypical in that only 3 of 12 animals produced anti-PC antibodies, yet these three had quite high titers (Table 5).

Examination of the R36 immunized wild mice for expression of T15 and C3Id's produced a variety of phenotypes. Most of the *Mus* species (i.e., *cookii*, *caroli*, *cervicolor)* which are the most distant in evolution from the laboratory mouse expressed some T15, demonstrating the persistence of these *V* genes over long evolutionary periods. C3 was found at significant levels in only one *M. cervicolor popaeus* mouse. Among the subspecies of *M. musculus* both idiotypes were frequently expressed in the same animal. Subspecies from Japan (*molossinus*), Thailand (*castaneus*), Morocco *(brevirostris, praetextus)* and Europe *(czech I & II, skive, vegrumbro)* expressed both markers in addition to having members which usually expressed the T15Id alone (Table 6). The frequency of the phenotype $C3^+T15^+$ is somewhat surprising in that the only inbred laboratory strain expressing the same phenotype is BAB14. Thus, if *C3* and *T15* were true alleles, which in BAB14 are both expressed presumably as a result of unequal crossing over, this event must be relatively frequent as judged by the wild mouse strains. Alternatively and probably more likely, *T15* and *C3* may be pseudo-alleles which

have arisen by gene duplication in one of the older *M.* species and then undergone various recombinations during evolution. The phenotype $C3^{+}T15^{-}$ has been found only in inbred laboratory strains and could indicate either a loss of the T15 gene or regulatory control of these two structural genes.

Species and subspecies in the genus *Mus* recently isolated from the wild are maintained as outbred colonies in order to preserve gene pools and prevent loss of fertility. It, therefore, becomes somewhat of a problem to determine if the *Ig genes* in these colonies are polymorphic. We are engaged in examining wild mice for allotypes found in inbred mice but until such time as appropriate (breeding studies are undertaken), we can only determine the phenotypes. Each mouse requires extensive testing to determine the genotype and depending on the allotypes, or lack of allotypes present, some are studied in more detail than others. The more distant *Mus* species *(pahari, cookii, cervicolor)* with the exception of *Mus spretus* exhibited none of the allotypes associated with *IgG2a*, *IgG2b*, and *IgA* loci (See Table 2) that we tested. *M. spretus* is the most closely related of the *Mus* species to the *M. musculus*. All mice of the *Mus musculus* species showed allotypes similar to the inbred strains. The combination of allotypic markers or phenotypes found suggested in some cases homozygosity but more often heterozygosity. The close association or linkage of PC alleles and specific allotypes in inbred mice was not observed in wild mice. There was no concordance of the PC Id markers with allotype. For example $C3^{+}T15^{+}$ was found in mice with $IgG2a^{e}$ and *f* allotypes *(M. m. castaneus)*. Matings of wild mice with the appropriate inbred strains are in progress to determine the segregation patterns of *C3*, *T15* and *Igh-C* genes.

REFERENCES

1. Lieberman, R., Rudikoff, S., Humphrey, W., Jr., and Potter, M. (1981). *J. Immunol.* 126, 172.
2. Blomberg, B., Geckeler, W. R., and Weigert, M. (1972). *Science* 177, 178.
3. Lieberman, R., Potter, M., Mushinski, E. B., Humphrey, W., Jr., and Rudikoff, S. (1974). *J. Exp. Med.* 139, 983.
4. Lieberman, R., Potter, M., Humphrey, W., Jr., and Chen, C. C. (1976). *J. Immunol.* 117, 2105.
5. Potter, M. (1977). *Adv. Immunol.* 25, 141.
6. Rudikoff, S., and Potter, M. (1980). *J. Immunol.* 124, 2089.

7. Rudikoff, S., and Potter, M. (1976). *Proc. Natl. Acad. Sci.* 73, 2109.
8. Lieberman, R. (1978). *Springer Semin. Immunopathol.* 1, 7.
9. Gearhart, P.J., and Cebra, J.J. (1978). *Nature* 272, 264.
10. Weigert, M., and Riblet, R. (1978). *Springer Semin. Immunopathol.* 1, 133.
11. Claflin, J. L., and Rudikoff, S. (1977). *Cold Spring Harbor Symp. Quant. Biol.* 41, 725.

CONSTANT REGION GENE PRODUCTS: ISOTYPES, ISOALLOTYPES AND ALLOTYPES[1]

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Our present knowledge of the structural basis for immunoglobulin (Ig) constant region variants raises questions about how Ig constant regions have evolved to their present state of diversity. The nature of some protein structural variants and their evolutionary origins are addressed in the presentations that follow. Interpretation of the results of structural studies of C-region gene products requires consideration of Ig structural gene organization. In addition, molecular biological approaches and DNA sequences are providing new information that may help us to begin answering questions about evolutionary origins of some of the structural diversity. Some of these studies are also presented in the section on "Ig Structural Gene Mapping."[2]

It had been concluded very early that substitutions in human λ-type light chains such as the Oz (Lys/Arg at 190), Kern (Gly/Ser at 152) and Mcg (Asn & Thr/Ala & Ser at 112 & 114) were due to isotypes rather than allelic allotypes of human λ-type light chain constant regions (1-4). At this symposium it was reported that there are at least six copies of the λ-type light chain constant region gene in man (5). The small differences in sequence at positions 112, 114, 143, 152, 163 and 190 of human lambda chain C-regions represent groups of substitutions occurring in the isotypic forms. On the other hand, alternative amino acid substitutions also occur at positions 153 and 191 of human kappa chain constant regions. The Inv allotypes (Km1:Val, Leu, Km1,2:Ala, Leu, and Km3:Ala, Val) behave as allelic aternatives in the human population (6). The recent cloning of the human kappa chain constant region gene (7) and analyses by Southern blotting

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[1] *Introduction to the topical review session on "Structural Studies of Ig C-Region Gene Products."*

[2] *The themes of "Structural Gene Mapping" and "Structural Studies of Constant Region Gene Products" are so interrelated, that although separate sessions on these topics were originally scheduled, a combined session was held.*

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techniques (5, 7) support the notion that the Inv types are true allelic allotypes since only one copy of the human kappa constant region gene is found per haploid genome.

In BALB/c mice, the λ1 and λ2 types are known to be isotypes although the constant regions of λ1 and λ2 differ at a much larger proportion of positions (about 36% differ) (8-10). This is similar to the percentage difference (20-38%) observed between the constant region sequences of rabbit kappa type light chains of b4, b5, b6 and b9 types (11-14).

Table 1. Protein Sequence Homologies: Allotypes and Isotypes

	Approximate Protein Sequence Homology	*References*
LIGHT CHAIN ALLOTYPES		
Rabbit kappa b4 b5 b6 b9	*62-80*	*(11-14)*
Rabbit kappa b4 b4 var	*98*	*(15)*
LIGHT CHAIN ISOTYPES		
Mouse λ1 λ2	*64*	*(8-10)*
Mouse λ2 λ3	*95*	*(10)*
HEAVY CHAIN ALLOTYPES		
Mouse IgG2a 1^a 1^b		
CH2 domain	*93*	*(16)*
CH3 domain	*73*	
HEAVY CHAIN ISOTYPES		
Mouse IgG2a IgG2b		
CH2 domain	*94*	*(17)*
CH3 domain	*61*	

Table 1 shows that proteins that behave as if they are products of allelic genes (allotypes) are as similar to or as different from each other in primary sequence as proteins that are clearly products of distinct but linked constant region genes (isotypes). Protein sequences of rabbit kappa light chain constant regions of different allotypes range from those about as dissimilar as the mouse λ1 and λ2 isotypes to those about as similar as mouse λ2 and λ3. The b4 and b5 constant regions are 66% and 62% homologous to b9 and λ1 and λ2 are 64% homologous (8-10). The b4 and $b4^{var}$ allotypes differ at 2 positions in the constant region and λ2 and λ3 differ at 5 of 95 constant region positions that have been sequenced (10).

Occasional experimental observations of expression of unexpected b allotypes in rabbits have led to proposals that there are several copies of the kappa chain constant region gene per haploid genome in rabbits and that allelic behavior of the b allotypes results from allelic regulation of expression of isotypic forms (18-21). It is indeed true that BALB/c mice express more λ1 than λ2 and λ3 isotype (22). There are also differences in the relative expression of human λ isotypes (3). The situation would approach that proposed to explain latent b allotype expression in rabbits, if different strains of mice or different individuals had genetically controlled allelic differences in the "pecking order" of isotype expression. A more definitive understanding of the genetic organization of the genes for rabbit kappa chain constant region allotypes will hopefully come from studies now in progress to address the questions by cloning rabbit kappa chain genes and using techniques of molecular biology that have indicated the presence of only one copy of the gene for kappa chain constant regions per haploid genome of mice and humans.

Table 1 shows that heavy chain allotypes and isotypes can also differ by similar percentages. Comparing the heavy chains of mouse IgG2a to IgG2b, the CH2 and CH3 domains of the two subclasses appear to have diverged in sequence to different degrees (94% and 61% homology). The greater homology of CH2 domains compared to CH3 is also observed in the comparison of the IgG2a heavy chains of BALB/c (1^a) and Black/Ka (1^b) allotypes (Dognin et al., this symposium [16]). Such different degrees of homology could reflect evolutionary pressures to maintain protein sequence in the CH2 domain to greater degree than in the CH3 domain. However, comparable degrees of divergence of third bases of codons in the nucleotide sequences of the two subclasses (23) would argue against selection pressure of this type being the sole explanation. Rather, recombinations between genes for two different subclasses may have occurred. Tsuzukida et al. (24) have shown that the A2m(1) allotype of the human IgA2 subclass has the exact protein sequence in the CH3 domain that the IgA1 subclass has whereas IgA2 of the A2m(2) allotype differs at four positions. They proposed that such a recombination generated the nA2m(2) isoallotype. That is, structural identity of the CH3 domains of the α1 and α2 chains of A2m(1) allotype results in the marker that is antithetic to A2m(2) being an isoallotype nA2m(2) shared by the IgA1 and IgA2 subclasses (25). Molecules which may have resulted from recombinations between genes for different subclasses have been observed in earlier

studies in humans (26, 27) and mice (28) as well as in the experimentally induced variants discussed by Kenter and Birshtein (this symposium [29]). The variety of allotypic determinant distributions observed by Herzenberg et al. (this symposium [30]) and others (31) in wild mice, as well as studies that demonstrated different mosaics of b allotypic determinants on kappa chains of genera of Lagomorphs related to rabbits (32, 33) have also been interpreted as reflections of recombination events leading to relatively rapid evolution of new constant region sequences. Kenter and Birshtein postulate that recombinations may be more frequent because of the observed prevalence of Chi sequences in the vicinity of Ig genes. Chi sequences are known to promote generalized recombination in their vicinity when present in bacteriophage lambda and E. coli (34, 35). It remains for future work to determine whether special sites that play a role in phage recombination play similar roles in eucaryotes. There is little doubt, however, that continued application of molecular biological approaches will provide additional insights into the evolutionary origins of the diversity of constant region genes for Igs.

REFERENCES

1. Ein, D. (1968). *Proc. Natl. Acad. Sci. USA* 60, 982.
2. Hess, M., Hilschmann, N., Rivat, L., Rivat, C., and Ropartz, C. (1971). *Nature* 234, 58.
3. Gibson, D., Levanon, M., and Smithies, O. (1971). *Biochemistry* 10, 3114.
4. Fett, J. W., and Deutsch, H. F. (1975). *Immunochemistry* 12, 643.
5. Hieter, P., Korsmeyer, S., Maizel, J., Max, E., Waldmann, T., and Leder, P. (1981). In Immunoglobulin Idiotypes and Their Expression. (Janeway, C. A., Sercarz, E., Wigzell, H., and Fox, F. C., eds.) Proceedings of the ICN-UCLA Symposium. Academic Press, New York.
6. Steinberg, A. G., Milstein, C. P., McLaughlin, C. L., and Solomon, A. (1974). *Immunogenetics* 1, 108.
7. Hieter, P. A., Max, E. E., Seidman, J. G., Maizel, J. V., and Leder, P. (1980). *Cell* 22, 197.
8. Appella, E. (1971). *Proc. Natl. Acad. Sci. USA* 68, 590.
9. Dugan, E. S., Bradshaw, R. A., Simms, E. S., and Eisen, H. N. (1972). *Biochemistry* 12, 5400.
10. Azuma, T., Steiner, L. A., and Eisen, H. N. (1981). *Proc. Natl. Acad. Sci. USA* 78, 569.

11. Chen, K. C. S., Kindt, T. J., and Krause, R. M. (1975). *J. Biol. Chem.* 250, 3289.
12. Farnsworth, V., Goodfleish, R. M., Rodkey, S., and Hood, L. (1976). *Proc. Natl. Acad. Sci. USA* 73, 1293.
13. Emorine, L., Dutka, S., Paroutaud, P., and Strosberg, A. D. (1979). *Molec. Immunol.* 16, 997.
14. Chersi, A., Alexander, C., and Mage, R. (1980). *Molec. Immunol.* 17, 1515.
15. Sogn, J., and Kindt, T. (1976). *J. Exp. Med.* 143, 1475.
16. Dognin, J. M., Lauwereys, M., and Strosberg, A. (1981). In Immunoglobulin Idiotypes and Their Expression. (Janeway,, C. A., Sercarz, E., Wigzell, H., and Fox, F. C., eds) Proceedings of the ICN-UCLA Symposium. Academic Press, New York.
17. Sikorav, J. L., Auffray, C., and Rougeon, F. (1980). *Nucleic Acids Res.* 8, 3143.
18. Bell, C., and Dray, S. (1971). *Science* 171, 199.
19. Strosberg, A. D. (1977). *Immunogenetics* 4, 449.
20. Yarmush, M. L., and Kindt, T. J. (1978). *J. Exp. Med.* 148, 522.
21. McCartney-Francis, N., and Mandy, W. J. (1979). *Ann. Immunol. Inst. Pasteur* 130c, 115.
22. Blaser, K., and Eisen, H. N. (1978). *Proc. Natl. Acad. Sci. USA* 75, 1495.
23. Tucker, P. W., Marcu, K. B., Slightom, J. L., and Blattner, F. R. (1979). *Science* 206, 1299.
24. Tsuzukida, Y., Wang, C. C., and Putnam, F. (1979). *Proc. Natl. Acad. Sci. USA* 76, 1104.
25. van Loghem, E., De Lange, G., and Koistinen, J. (1976). *Scand. J. Immunol.* 5, 161.
26. Kunkel, H. G., Natvig, J. B., and Joslin, F. G. (1969). *Proc. Natl. Acad. Sci. USA* 62, 144.
27. Natvig, J. B., and Kunkel, H. G. (1974). *J. Immunol.* 112, 1277.
28. Warner, N. L., Herzenberg, L. A., and Goldstein, G. (1966). *J. Exp. Med.* 123, 707.
29. Kenter, A., and Birshtein, B. K. (1981). In Immunoglobulin Idiotypes and Their Expression. (Janeway, C. A., Sercarz, E., Wigzell, H., and Fox, F. C., eds.) Proceedings of the ICN-UCLA Symposium. Academic Press, New York.
30. Herzenberg, L. A., Parsons, M., Huang, C. M., and Oi, V. T. (1981). In Immunoglobulin Idiotypes and Their Expression. (Janeway, C. A., Sercarz, E., Wigzell, H., and Fox, F. C. eds.) Proceedings of the ICN-UCLA Symposium. Academic Press, New York.
31. Lieberman, R., and Potter, M. (1969). *J. Exp. Med.* 130, 519.

32. Tosi, S. L., Mage, R. G., and Tosi, R. M. (1976). *J. Immunol.* 117, 679.
33. Mage, R. G., Young-Cooper, G. O., Rejnek, J., Ansari, A. A., Alexander, C. B., Appella, E., Carta-Sorcini, M., Landucci-Tosi, S., and Tosi, R. M. (1977). Cold Spring Harbor Symp. Quant. Biol. 41, 677.
34. Stahl, F. W. (1979). *Annu. Rev. Genet.* 13, 7.
35. Smith, G. R., Schultz, D. W., and Craseman, J. M. (1980). *Cell* 19, 785.

THE STRUCTURE AND GENETICS OF MOUSE IMMUNOGLOBULIN HEAVY CHAIN CONSTANT REGIONS DEFINED BY MONOCLONAL ANTI-ALLOTYPE ANTIBODIES

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In 1964 we postulated a chromosomal region for immunoglobulin heavy chains based on the finding that newly discovered allotypes of IgG_{2a} and IgA were closely linked (1). At that same Cold Spring Harbor meeting Henry Kunkel presented similar evidence of linkage for human heavy chain genes (2). Since that time allotypes have been found for 7 of the 8 known heavy chain genes in the mouse. The loci thus defined are now called Igh-1 through Igh-7 for, respectively, γ_{2a}, α, γ_{2b}, γ_1, δ, μ, and ε. Only γ_3 has yet to be shown to be polymorphic. Of course, the V_H genes as well as all the C_H genes are known to be clustered in one region on chromosome 12 of the mouse (3).

With the advent of hybridoma technology we have generated a series of monoclonal antibodies which recognize allotypic determinants (allotopes) on the gene products of the Igh-1, -3, and -4 loci (4,5, and Parsons et al., in preparation) (see ref. 6 for nomenclature). In this communication we discuss the use of these antibodies to examine the structure of these polymorphic proteins. Several key findings have emerged. The polymorphisms, as typified by the Igh-1a and Igh-1b proteins, are structurally complex, with allotopes scattered along the molecule. We have found that natural populations of mice provide a reservoir of genetic heterogeneity at the Igh loci as revealed by the relatively high levels of polymorphism in these populations as well as the existence of novel Igh phenotypes, allotypes, and haplotypes. We have also found situations where the structure of one Ig domain affects the presentation of allotypic determinants on another domain. Finally, our recent finding of membrane IgG_{2a} (7) can be extended to other classes of IgG.

There are extensive polymorphic differences throughout the Fc regions of the Igh-1a and Igh-1b alleles of IgG_{2a} immunoglobulins. This was determined by mapping the allotypic surface topography of these molecules with monoclonal anti-allotype antibodies (Fig. 1). Five distinct determinants were found on Igh-1a molecules and eight on Igh-1b molecules. The mapping of these determinants was done in two ways. Anti-

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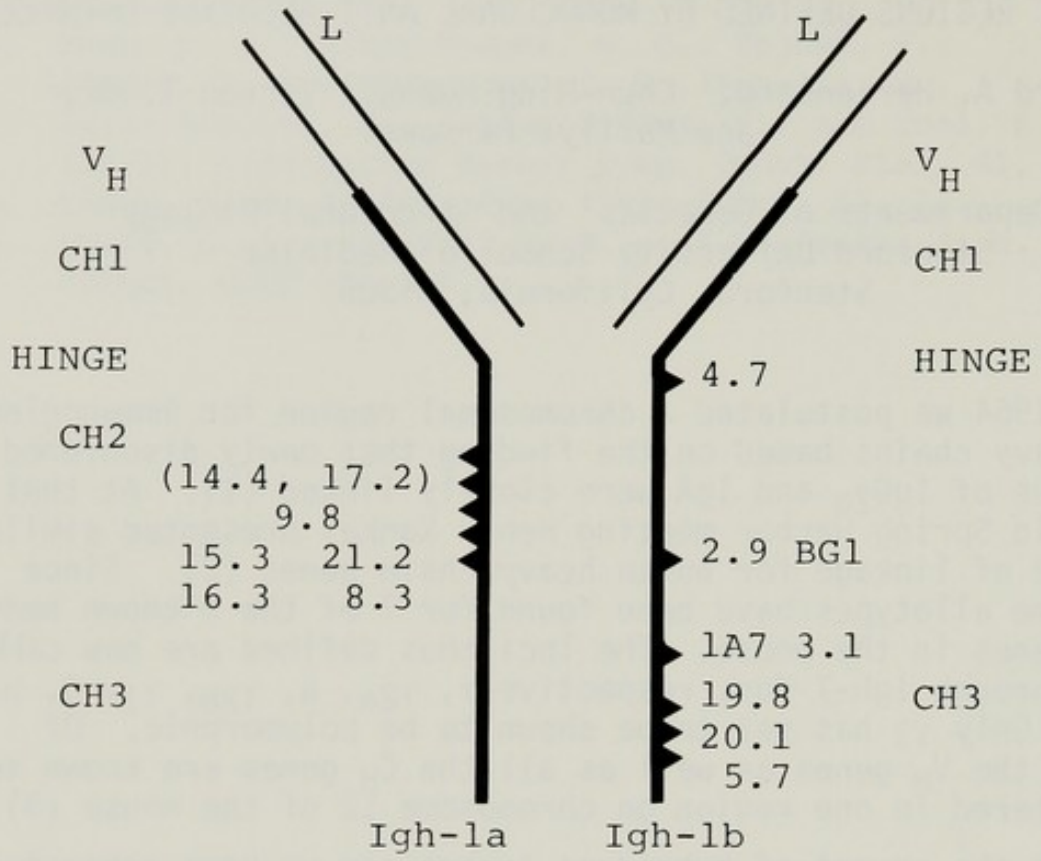

Figure 1. Location of allotype determinants on IgG_{2a}. As described in the text, Igh-1a and Igh-1b allotypic determinants were roughly localized to immunoglobulin domains by examining antibody activity with various proteolytic fragments of the antigen. Studies examining the ability of each antibody to hinder the binding of other antibodies to antigen also aided us in clustering the allotopes. Anti-Igh 1a antibodies 14.4, 17.2, and 9.8 may recognize the same determinant. Antibodies 2.9 and BG1, 3.1 and 1A7 react with distinct, but closely related determinants. While only one heavy chain of each immunoglobulin is depicted, we do not wish to imply that the determinants are expressed on single heavy chains.

* * *

body blocking assays (4) were used to determine whether individual monoclonal antibodies obscure determinants that are recognized by other monoclonal antibodies. In this case masking of determinants can be attributed to steric considerations or induced conformational changes. To roughly localize the regions of the IgG_{2a} molecules which display these allotypic determinants, we examined polypeptide fragments of the Fc regions for binding to the anti-allotype antibodies (4).

While conventional serology indicated a single allotype specificity unique to Igh-1b proteins (8), with monoclonal antibodies we have divided this into 8 distinct allotopes (allotypic determinants). Since almost every independently derived monoclonal antibody defined a new allotope, the number of allotopes must be very large. The extensive differences between the Igh-1a and Igh-1b alleles have been well substantiated by the recent nucleotide and amino acid sequence information (see articles of Bothwell et al., and Strosberg et al., this volume). In fact, these data demonstrate that more structural differences exist between the two Igh-1 allotypic molecules described than between Igh-1 (IgG_{2a}) and Igh-3 (IgG_{2b}) proteins.

The 20 monoclonal antibodies now available (including 2 kindly provided by M. Bosma, I.C.R., Philadelphia) divide the Igh-type strains into 5 pheno groups (Table 1): a,g,h,j; b; c; f; and d,e,n,o. After examining the allotopes of inbred strains, we decided to search in wild mice for further genetic variation at the Igh loci (9). Sera from 122 wild mice (Mus musculus) were kindly provided by Dr. J. Klein (Max Planck Inst., Tübingen, W. Germany) and Dr. E. K. Wakeland (U. of Florida, Gainesville, FL).

Most of the wild mice have Igh phenotypes similar to those of inbred strains or heterozygotes thereof. However, we have found new allotypes of the Igh-1 and Igh-4 loci. We have also found mice with unusual combinations of allotopes as well as new haplotypes which could be explained by recombination and/or gene duplication.

Table 1 shows reactivity patterns (Ig phenotypes) of some of these unusual wild mice. All of the mice from Poland that we have tested possess the Igh-a determinants of Igh-a or -d-like strains. In addition they have all eight Igh-1b allotopes, but not the Igh-4b allotope. In this population, the a-like and b-like determinants do not occur separately in individual mice, so it is unlikely that the animals are heterozygous. This would imply duplicated Igh-1 loci. Preliminary progeny testing from one of these Polish mice indicates that the Igh-1a and Igh-1b genes are segregating together, providing further evidence that the two a and b Igh alleles are on the same chromosome. The Polish mice are thus reminiscent of the Kyushu mice reported by Lieberman and Potter (10).

Interestingly, three Egyptian mice were found to possess only 3 of the 8 Igh-1b allotopes. These determinants have been localized to the CH3 domain of the Igh-1b molecule (Fig. 1). Although this variant Igh-1b molecule lacks the CH2 Igh-1b determinants, the molecular weight of its heavy

TABLE 1

REACTIVITY OF MONOCLONAL ANTI-ALLOTYPIC ANTIBODIES: Inbred Strains and Unusual Wild Mice

Igh Loci:	Igh-4		Igh-3		Igh-1																	
Determinants:	a	b	a	b	a								b									
	1 1 8	0	2 2	1 6	2 3	2 4	8 1 6	1 9 4	1 1 7	2 1 5			4	2 B G		3 1 A		1 2 9	5 0			
Igh Haplotypes																						
a,g,h,j	+	+	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	
c	+	+	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	
f	+	+	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	
d,e,n,o	+	+	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	
b	-	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	
Wild Mice																						
Egypt	+	+	-	+	-	-	+	+	-	-	-	*	+	-	-	-	-	-	+	+	+	
Poland	+	+	-	+	+	-	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	
Poland	+	+	-	+	+	-	+	+	-	-	-	*	+	+	+	+	+	+	+	+	+	
Taiwan	+	+	-	+	+	-	+	+	-	-	-	*	+	+	+	+	+	+	+	+	+	
Poland	+	-	-	+	+	-	+	+	-	-	-	*	+	+	+	+	+	+	+	+	+	

*Not tested

chain indicates that no substantial deletion has occurred. We examined this molecule for other allotypic determinants and found that it also bears a determinant common to Igh-1e and Igh-3e (16.3). It thus appears that this molecule resulted from an Igh-1e/Igh-1b or Igh-3e/Igh-1b recombination. These and other interesting Igh-haplotypes will be the subject of another communication (Huang et al., in preparation). With mutation creating new alleles as indicated by the loss of allotopes and recombination assorting allotopes to form new allotypes, the Igh gene complex shows an extensive polymorphism in the natural wild mice populations. It will be interesting to further explore the extent of polymorphism and try to determine what selective advantage, if any, it represents for the species.

Possible Domain Interactions. An interesting interaction between the Fab and Fc domains of individual molecules was discovered during our analysis of allotypic determinants. Individual monoclonal anti-Igh-4b (IgG_1 of allotype) antibodies react with different monoclonal Igh-4b proteins (presumably derived from the same Igh-4b gene) in quantitatively distinct patterns. A good example of this kind of reactivity pattern is the binding of anti-Igh-4b 22.9 to different Igh-4b proteins (Table 2). MOPC-245T, MOPC-300, and anti-DNP hybridoma 29-B5 react equally well with antibody 22.9. However, anti-dansyl hybridoma 44-26.2 reacts much more strongly. When the Fc fragment of this molecule was prepared, it reacted in a

* * *

TABLE 2

REACTIVITY OF ANTI-Igh-4b 22.9 WITH VARIOUS Igh-4b PROTEINS

Protein	50% Inhibition*
MOPC-245T	3.0
MOPC-300	2.5
29-B5	2.6
44-26.2	0.45
44-26.2 Fc	4.3
44-26.2 Fab	†

*pmoles required for 50% inhibition of 22.9 binding to antigen (44-26.2)

†no inhibition at highest concentration tested

fashion similar to the other Igh-4b proteins. Mixtures of 44-26.2 Fab and Fc resemble the Fc rather than the intact molecule, demonstrating that the structural linkage of these two moieties is necessary to influence the expression of the Fc allotypic determinant. These data indicate that the variable region can alter the presentation of distal determinants, located as far away as CH2.

Serological heterogeneity within Fc fragments of different Igh-1a proteins has also been observed. In this case, intact molecules of 29-B1, an Igh-1a anti-DNP hybridoma protein, and GPC-8, also an Igh-1a protein both react with anti-Igh-1a 15.3. However, the Fc fragment of GPC-8 reacts poorly with 15.3, while the Fc of 29-B1 reacts as well as the intact molecule. Two other Igh-1a Fc fragments were also examined: one behaves like GPC-8 and the other like 29-B1.

Two-dimensional gel analysis (11) of these Fc fragments reveal differences between the reactive and non-reactive Fc's (Fig. 2). No relative molecular weight differences are obvious from these analyses, but there are clear differences in the isoelectric points possibly due to deamidation differences. Any biological significance of the serological and charge heterogeneity is not known. Although glycosylation differences are possible, we do know that the determinant with which 15.3 reacts is pronase-sensitive and made in tunicamycin-containing cultures. Further work on the effect of these differences on a variety of immunoglobulin effector functions, such as complement or protein A binding and anisotropy decay studies, may provide more insight into this problem.

We have also reported that Igh-1a is expressed on the surface of hybridoma cells in a form which is serologically and biochemically distinct from that which is secreted (7). Membrane Igh-1a is approximately 10K daltons larger than secreted Igh-1a, and was shown to lack the determinant recognized by antibody 15.3. Although we know that this determinant is located in the CH2 domain (4), the loss of reactivity with antibody 15.3 does not necessarily mean that the primary sequence of membrane CH2 differs from secreted CH2. However, it does imply that the topography is different. Our preliminary evidence indicates that the Fc fragment of surface Igh-1a is both larger and more acidic than secreted Fc fragments made by the same cells.

We have looked for membrane IgG on hybridomas of other allotypes and isotypes. Fig. 3 shows the two-dimensional gel pattern of surface and secreted Igh-1b derived from an Igh-1b anti-eosin hybridoma. As in the case of Igh-1a, the membrane Igh-1b is both larger (10K daltons) and more acidic. Membrane

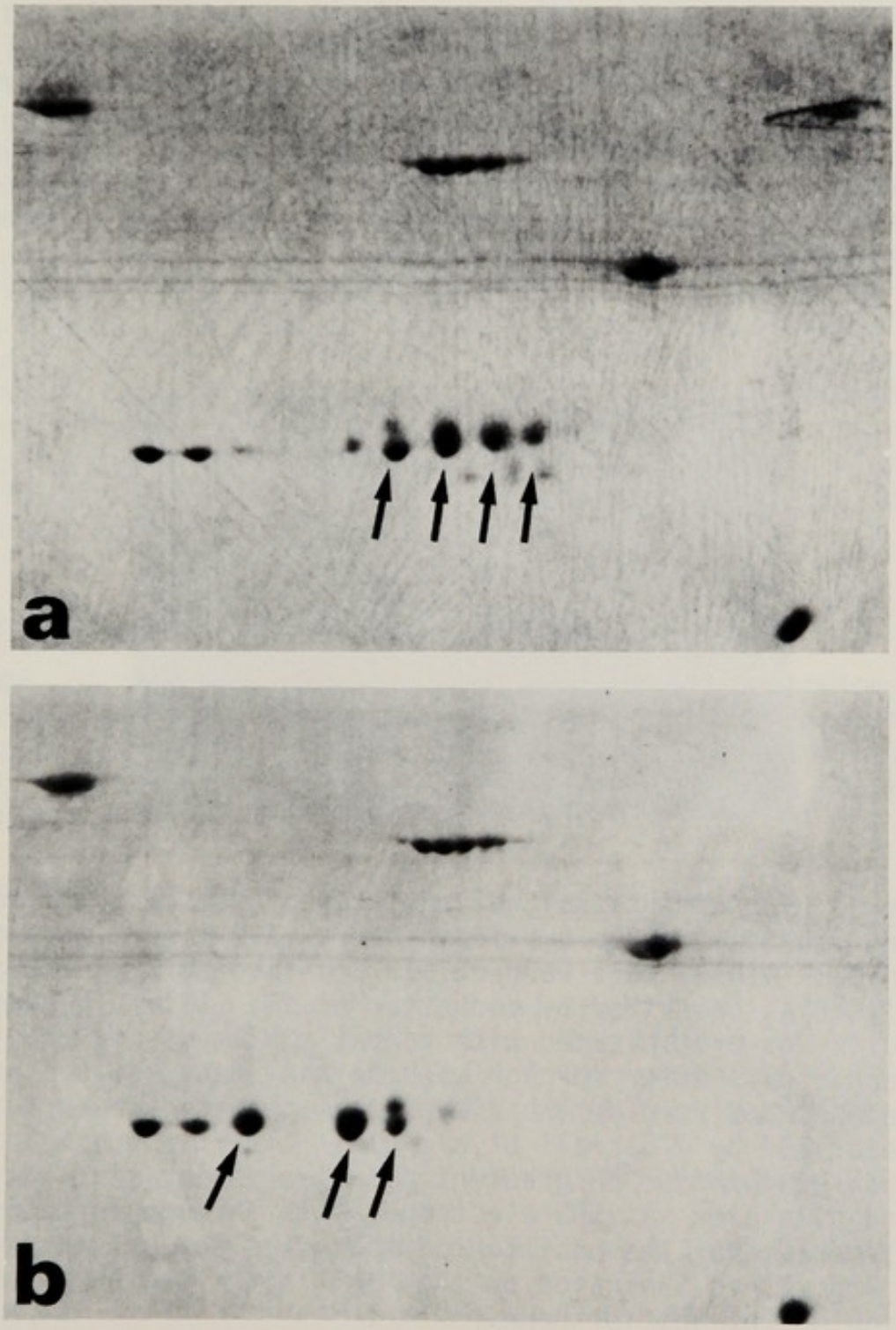

Figure 2. Two-dimensional gel analysis of Igh-1a Fc's. Purified Igh-1a proteins 29-B1 and GPC-8 were digested for 10 min at 37°C with papain as previously described (4). The Fc was separated from Fab and intact molecule by gel chromatography. Two-dimensional gel analysis under reducing conditions was as described by O'Farrell (11), with isoelectric focusing as the first (horizontal) dimension and SDS gel electrophoresis (12.5% acrylamide) as the second dimension.

The gels were stained with Coomassie Blue and Crocein scarlet to reveal protein bands. The arrows indicate the Fc molecules. Other stained bands are marker proteins (Pharmacia): phosphorylase B (94K Mr), bovine serum albumin (67K Mr), ovalbumin (45K Mr), carbonic anhydrase (30K Mr), and soybean trypsin inhibitor (20K Mr). The carbonic anhydrase migrates similarly to the Fc's in the second dimension, but has a different isoelectric point.
2-a: GPC-8 Fc 2-b: 29-B1 Fc

* * *

Figure 3. Two-dimensional gel analysis of membrane and secreted Igh-1b. Igh-1b hybridoma cells (149-1.5.5, an anti-eosin cell line), were washed, surface-labeled with ^{125}I, and lysed as described by Ledbetter et al. (12). The immunoglobulin was precipitated with rabbit anti-mouse Ig coupled to Sepharose. After through washing the bound antigen was eluted and analyzed by two-dimensional gel electrophoresis as described by O'Farrell et al. (13). The first dimension was non-equilibrium pH gradient gel electrophoresis; the second dimension was SDS electrophoresis through 10% polyacrylamide gel. The position of molecular weight standards (see Fig. 1) is indicated on the right. Only the heavy chain portion of the gel is shown.

* * *

Igh-1b possesses all 8 allotopes detected by the available anti-Igh-1b monoclonal antibodies. We have also found similar surface IgG on hybridoma cells producing IgG_1. It should be noted that different cell lines express different levels of membrane IgG and that culture conditions, particularly variations in serum, affect this expression.

The availability of well characterized monoclonal anti-allotypic antibodies will be useful in the task of explaining genetic and structural polymorphisms of murine immunoglobulins. We may expect new Ig C_H genes and arrangements of these genes in wild mouse populations. This information may increase our understanding of the evolution of Ig genes and the functional significance of allotypic and haplotypic variation. The differential regulation of isotype and allotype by T cells (14, 15) indicates the functional importance of allotopes and perhaps allotopic differences between membrane and secreted IgG's, IgE and possibly IgA. Finally, it is worth mentioning that allotypic determinants are equivalent to isotypic determinants in individual mouse strains. Monoclonal anti-allotopes are isotypic-specific probes in allotypically homozygous mice, and should thus have a valuable future in the study of the immune response and its regulation.

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REFERENCES

1. Herzenberg, L. A. (1964) Cold Spring Harbor Symp. Quant. Biol. 29, 455.
2. Kunkel, H. G., Allen, J. C., and Grey, H. M. (1964) Cold Spring Harbor Symp. Quant. Biol. 29, 443.
3. Meo, T., Johnson, J., Beechey, C. V., Andrews, S. J., Peters, J., and Searle, A. G. (1980) Proc. Natl. Acad. Sci. USA 77, 550.
4. Oi, V. T. and Herzenberg, L. A. (1979) Molecular Immunol. 16, 1005.
5. Parson, M. and Herzenberg, L. A. (1981) Immunogenetics 12, 207.
6. Green, M. C. (1979) Immunogenetics 8, 89.
7. Oi, V. T., Bryan, V., M., Herzenberg, L. A. and Herzenberg, (1980) J. Exp. Med. 151, 1260.
8. Herzenberg, L. A., and Herzenberg, L. A. (1978) In: Handbook of Experimental Immunology, 3rd Edition, (ed) Weir, D. M., Blackwell Scientific Pub., Oxford, 12.1.
9. Huang, C.-M., Parsons, M., Wakeland, E. K. and Herzenberg, L. A. (1981) J. Supramol. Structure and Cellular Biochem., Suppl. 5, 25.

10. Lieberman, R., and Potter, M. (1969) J. Exp. Med. 130, 519.
11. O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007.
12. Ledbetter, J. A., Seaman, W. E., Tsu, T. T., and Herzenberg, L. A. (1981) J. Exp. Med. (in press).
13. O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977) Cell 12, 1133.
14. Black, S. J., Tokuhisa, T., Herzenberg, L. A., and Herzenberg, L. A. (1980) Eur. J. Immunol. 10, 846.
15. Herzenberg, L. A., Black, S. J., Tokuhisa, T., and Herzenberg, L. A. (1980) J. Exp. Med. 151, 1071.

MULTIPLE AMINO ACID DIFFERENCES BETWEEN PRODUCTS OF ALLELIC CONSTANT REGION GENES : CLUES FOR EVOLUTION ?

by

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Products of allelic genes, that is of genes present at the same locus on homologous chromosomes, were in the past generally thought to differ only slightly in their amino acid sequence. This idea stemmed from examples such as the hemoglobins where single, or at the most, double amino acid substitutions would distinguish the variants from the normal protein. Allotypes, the genetic markers of immunoglobulins seemed at first to follow this pattern : the d allotype of the rabbit gamma heavy chain corresponds respectively to a methionine/threonine (d_{11}/d_{12}) substitution, the e marker to a threonine/alanine (e_{14}/e_{15}) substitution.

Recent work from our laboratory and from other groups has established that these "simple" allotypes are not necessarily preponderent. Thus the four serological forms of rabbit kappa light chains, b4, b5, b6 and b9 display between 20 and 35 % differences in the amino acid sequences of their constant regions (1-5). Other examples of "complex" allotypes have been described for the rabbit variable region a markers (6), and the rat kappa light chain constant region markers (7).

We have most recently determined the sequence of the Fc region of the murine $\gamma 2a$ heavy chains of the Ig-1^b allotype and have shown that it also differs from the previously analyzed Ig-1^a form by multiple substitutions (8). In this report we discuss these results together with those obtained in earlier work in the light of today's concepts of the evolution of immunoglobulin genes.

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a) Multiple differences between the γ2a Ig-1^a and Ig-1^b Fc sequences. We have determined the amino acid sequence of the Fc region of myeloma γ2a protein CBPC-101, obtained from CB20 mice which have the b haplotype of C57 Bl/6 mice. The primary structure was established by automated and manual Edman degradation of fragments and peptides obtained by enzymatic and chemical cleavages. The sequence strategy was as follows :

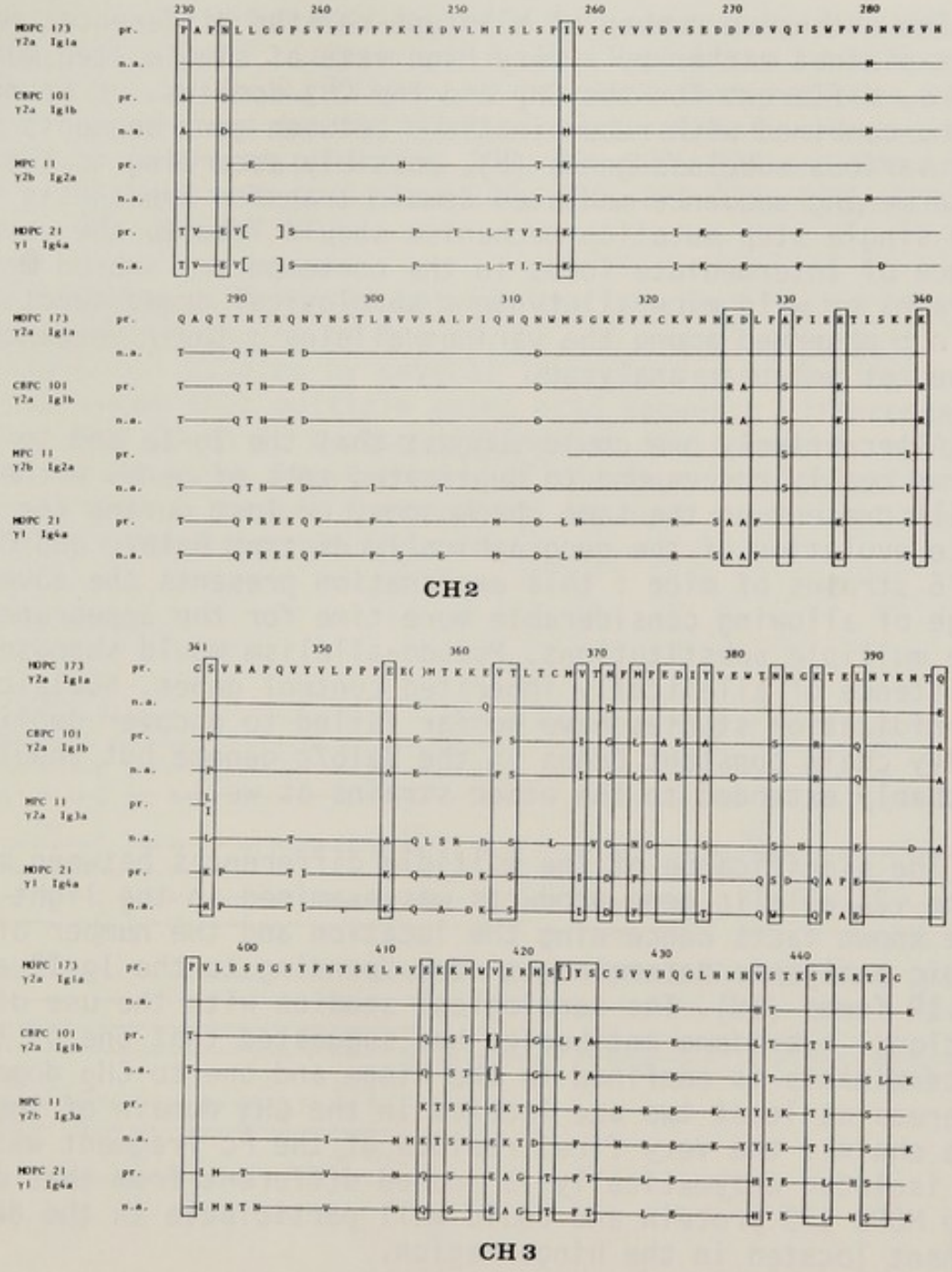
We first prepared the Fc fragment by papain digestion of the whole immunoglobulin. This fragment was then reduced and radioalkylated and further degraded by digestion with trypsin which cleaves the lysyl and arginyl peptide bonds or by chemical hydrolysis with CNBr in HCOOH/HFBA 1:1 which results in the cleavage of the methionyl and tryptophanyl peptide bonds.

Alternatively, we prepared the Fc part by subjecting the whole protein immediately to CNBr cleavage in 70 % formic acid.

Peptides were purified by gel filtration followed by high pressure liquid chromatography and sequenced either by the manual Edman degradation procedure using the DABIT reagent described by Chang (9) or by the automated procedure using the protein sequenator.

The comparison with the previously described (10,11) sequence of myeloma protein MOPC 173, a γ2a immunoglobulin of allotype Ig-1^a, obtained from Balb/c mice revealed eight amino acid substitutions in the CH_2 domain, i.e. 7 % differences (fig. 1a) and twenty-eight substitutions in the CH_3 domain, i.e. 27 % differences (fig. 1b). Many of the observed differences occur at positions at which murine γ heavy chains of other classes also differ from the γ2a chains.

The comparison was based on the sequences obtained from the analyses of the proteins as well as those predicted from the study of the nucleic acids. The amino acid sequence of the γ2a Ig-1^b protein which we analyzed (8) differed only at two positions from that predicted by Schreier and his collaborators from the nucleotide sequence of a γ2a gene obtained from hybridoma S43 cells (12). Whereas these two differences may result either from somatic mutations or the different origin of the corresponding gene, the excellent agreement in the rest of the Fc region establishes beyond doubt that the γ2a Ig-1^a and Ig-1^b germ-line genes really differ at multiple positions.



Figures 1a and 1b : Comparison of all the available sequences of the murine γ heavy chain CH2 and CH3 domains obtained by the analysis of both the proteins (pr.) and the nucleic acids (n.a.). Boxed residues enclose substitutions between the γ2a Ig-1^a and Ig-1^b forms as well as the corresponding residues in the chains of the other classes. References are : MOPC173 Pr. (10), n.a. (11), CBPC-101 pr. (8), γ2a Ig-1^b n.a. (12), MPC 11 pr. (14), γ2b, n.a. (15), MOPC21 pr. (16), γ1 n.a. (17).

The extensive number of allotype-related differences may be explained either by a very high rate of single step mutations, different for the CH_2 and the CH_3 domains, or by mutations combined with recombinations between gene segments from the various subclass genes (8), possibly according to the intervening sequence-mediated domain transfer hypothesis (13). The single step mutation mechanism should lead to the emergence of intermediate forms in the contemporary inbred mouse strains or wild mice allotypic : serological cross-reactivity is not observed among the various alleles ; their sequences have not yet been analyzed.

Alternatively one could suggest that the Ig-1a and Ig-1b forms really correspond to duplicated sets of genes either still present on the same chromosome, or lost during the separate evolution of the geographically distant Balb/c and C57 Bl/6 strains of mice : this explanation presents the advantage of allowing considerable more time for the appearance of the multiple substitutions. Pseudo-allelism would suppose the existence of allelically inherited control genes. Nucleic acid hybridization studies have so far failed to uncover duplicated heavy chain constant genes in the Balb/c genome but should be probably extended to the other strains as well.

The significance of the multiple differences between murine $\gamma 2a$ allelic gene products was examined in the light of the known facts concerning the location and the number of allotypic antigenic determinants corresponding to the $Ig\text{-}1^a$ and $Ig\text{-}1^b$ forms (18). The serological studies with the use of monoclonal hybridoma antibodies had suggested that one of the determinants is confined to the hinge and one to CH_2 domain whereas at least two are located in the CH_3 domain of the $\gamma 2a$ chain : the very first residue of the Fc fragment which we isolated enzymatically is indeed different from that of the MOPC 173 protein and could well participate in the determinant located in the hinge region.

Several of the other substitutions also occur in portions of the chain predicted not only to be exposed to water molecules but more importantly, to be accessible to much large molecules such as for instance anti-isotype or anti-allotype antibodies. These predictions were based on the studies of the tridimensional models of the human Fc region derived from the X-ray diffraction analyses by Deisenhofer et al. (19) and further discussed by Burton et al. (20) and on the studies of the guinea pig F'c fragment analyzed by Phizackerley et al. (21).

From a study which will be published separately in collaboration with Drs E. Lebrun and R. Van Rapenbusch, and from discussions with Dr R. Poljak, we conclude that residues likely to be involved in the allotypic determinants are located in the CH_2 domain at positions 258, 326-327, 334 and 340 and in the CH_3 domain at positions 342, 355, 375-376, 378, 413, 415-416, 437 and 441-442.

b) Multiple differences between the constant region of rabbit kappa light chains of the b4, b5, b6 and b9 allotypic forms. Previous studies by several laboratories including our own have shown that multiple amino acid sequence differences ranging from 20 to 35 % also distinguish the constant regions of the rabbit kappa light chains of the four serologically distinguished allotypic forms (1-5). We have summarized the available information in figure 2.

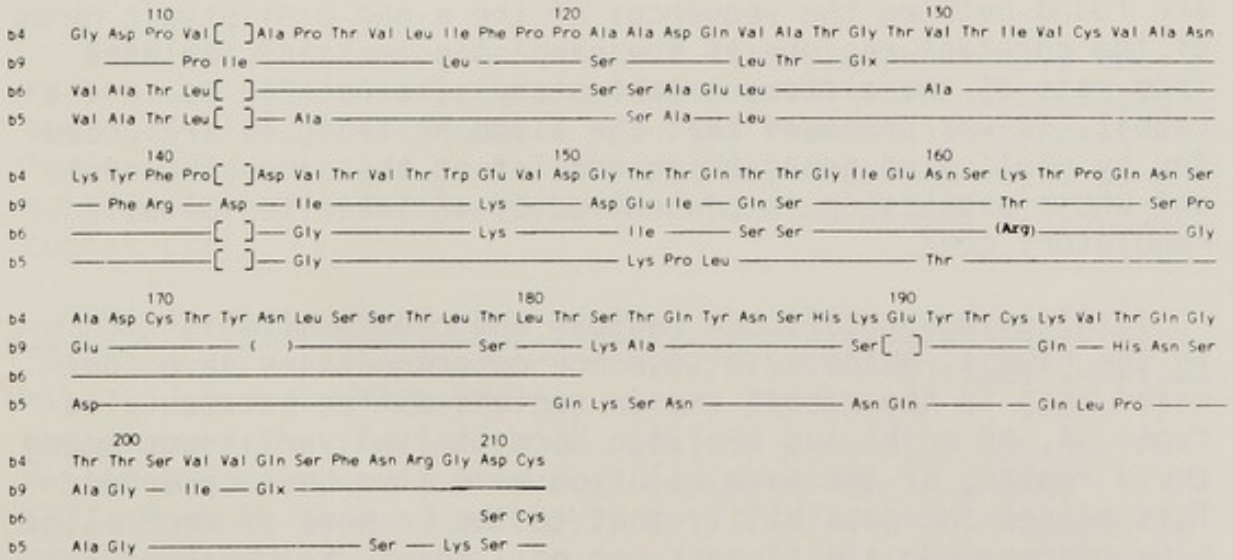


Figure 2 : Comparison of the amino acid sequences of the rabbit kappa light chain constant regions of the four allotypic forms. Data are from the following references : b4 (2), b9 (2,3), b6 (4), b5 (4,5).

The extensive variations among the rabbit Cκ sequences is difficult to account for by single-step mutations occuring since the emergence of the rabbit species from the lagomorphs since hares which diverged from rabbits at speciation-time only weakly react with specific anti-rabbit allotype antibodies. Alternative explanations have included the prolonged coexistence of several Cκ genes on the lagomorph chromosome, thus allowing the independent accumulation of multiple stepwise mutations or the emergence of variations through more complex mechanisms such as recombinations. Whether the various Cκ genes still coexist in contemporary rabbits, with an apparent allelism regulated hereditarily, or whether redistribution resulted in the expression of different alleles in different populations of animals is still actively under discussion (22-25). The occasional expression of latent allotypes in various rabbit colonies does however favor the coexistence of usually silent genes.

c) Multiple substitutions between the rat kappa light chain constant regions of allotypes a and b. About 15 % differences are found between the sequences of the a and b allotypic forms of the constant regions of the kappa light chains isolated from rats of the different laboratory strains (7). As in the rabbit, it was proposed that the a and b structure are coded for by duplicated genes which coexist on the same chromosome and whose expression is controlled by an inherited allelic regulatory gene.

d) Allotype-specific Jκ regions and pseudo-allelic λ chains in the rabbit. Amino acid sequence determinations have indicated that in the rabbit the Jκ regions differ between allotypes b4, b6 or b5 and b9 (26). More limited variations among the J regions of the same allotype have also been observed. This raises the possibility that to the Cκ gene of each allotype corresponds a different set of J genes, in contrast to what was described for the murine heavy chain genes for which only a single set of J regions serve to "join" V and C region genes (27). A situation similar to that in the rabbit was however described for the lambda chains in man (28).

Several genes code for lambda chain constant regions in the human genome, as had been predicted from the serological detection of the isotypic Oz and Kern genetic markers. Structural studies have confirmed the existence of amino acid differences between the lambda chain constant regions and have shown in addition that lambda-associated variable regions have also structural properties which distinguish them from kappa variable regions. More recently it was found that Jλ

regions, corresponding to residues 97 to 110 of the chains are coded for by distinct nucleotide sequences which are apparently specific for and located close to the various lambda constant regions (28).

The similarity between the rabbit system and the human lambda chain structure again raises the possibility that the rabbit constant regions are not products of allelic genes but may actually coexist on the genome. It is worth recalling here that the rabbit λ chain allotypes c7 and c21 were found to behave like alleles in some breeding studies, but as isotypes (pseudo-alleles) in others (29).

The data reviewed here, obtained either for the mouse, the rat or the rabbit light or heavy chain J or C regions all indicate that allelic differences may far exceed the single or double amino acid substitutions that had been identified for either protein variants such as the various hemoglobins, or for so called "simple allotypes" such as the human Km or the rabbit d or e genetic markers.

When we discussed some of these findings previously we raised the possibility that multiple amino acid substitutions among allelic genes products resulted from an independent evolution of duplicated genes. Present-day allelism would be the consequence of selective loss or silencing of one of the gene copies (22).

The development of gene cloning and sequencing techniques together with that of powerful hybridization analyses now permits to actually identify the nature and number of genes in the genome. The first results obtained with mice has not revealed the existence of several copies of the γ2a gene (13, 17), of which in our hypothesis only the Ig-1^a form would be expressed in Balb/c mice and only the Ig-1^b form would be utilized in B157/6 animals. The data thus suggest that if multiple differences arose in duplicated genes, loss of the alternate forms explains the present day allelism.

Alternatively the differences could be explained by simple divergence of the genes in separate animal lines. Such an explanation is particularly applicable to the mice which had different geographical origins. It is much harder to apply to rabbits in which a selective advantage for each of the allelic forms would necessarily have led to recombinations to yield permanent heterozygotes which would have incorporated on a single chromosome the advantage provided by each allele.

In addition, the loss of the alternate forms is hard to reconcile with the now well described expression of "latent allotypes". Indeed a large number of laboratories have established that in well controlled conditions, allotypes are found in animals which by breeding are not expected to possess these markers (22-25, 30-31).

It is quite apparent that the study of the actual structure of the genome of animals expressing the "wrong" allotypes will finally constitute the ultimate way to resolve the apparent paradox raised by the existence of multiple differences between products of allelic genes and the occasional appearance of the latent allotypes.

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References

(1) Zeeuws, R. and Strosberg, A.D. (1975) *Arch. Int. Physiol. Biochem.* 83, 205.
(2) Chen, K.C.S., Kindt, T.J. and Krause, R.M. (1975) *J. Biol. Chem.* 250, 3289.
(3) Farnsworth, V., Goodfliesh, R., Rodkey, S. and Hood, L. (1976) *Proc. Natl. Acad. Sci. USA* 73, 1293.
(4) Emorine, L., Dutka, S., Paroutaud, P. and Strosberg, A.D. (1979) *Molecul. Immunol.* 16, 997.
(5) Chersi, A., Alexander, C.B. and Mage, R. (1980) *Molecul. Immunol.* 17, 1515.
(6) Jaton, J.C., Braun, D.G., Strosberg, A.D., Haber, E. and Morris, J.E. (1973) *J. Immunol.* 111, 1838.
(7) Gutman, G.A., Loh, E. and Hood, L. (1975) *Proc. Natl. Acad. Sci. USA* 72, 5046.
(8) Dognin, M.J., Lauwereys, M. and Strosberg, A.D. (1981) *Proc. Natl. Acad. Sci.* In press.
(9) Chang, J.Y. (1979) *Biochem. Biophys. Acta* 578, 175.
(10) Fougereau, M., Bourgois, A., de Préval, C., Rocca-Serra, J. and Schiff, C. (1976) *Ann. Immunol. (Paris)* 127C, 607.
(11) Sikarov, J.L., Auffray, Ch. and Rougeon, F. (1980) *Nucleic Acid Res.* 8, 3143.
(12) Schreier, P., Bothwell, A., Mueller-Hill, B., and Baltimore, D. (1981) *Proc. Natl. Acad. Sci. USA.* In press.

(13) Yamawaki-Katacka, Y., Miyata, T. and Honjo, T. (1981) *Nucl. Ac. Res.* In press.
(14) Francus, T. and Birshtein, B.K. (1978) *Biochemistry* 17, 4324.
(15) Tucker, P.W., Marcu, K.B., Newell, N., Richards, J. and Blattner, F.R. (1979) *Science* 206, 1303.
(16) Adetugbo, K. (1978) *J. Biol. Chem.* 253, 6068.
(17) Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N. and Mano, Y. (1979) *Cell* 18, 559.
(18) Oi, V.T. and Herzenberg, L.A. (1979) *Molecul. Immunol.* 16, 1005.
(19) Deisenhofer, J., Jones, T.A., Huber, R., Sjödahl, J. and Sjöquist, J. (1978) *Hoppe-Seyler's Z. physiol. Chem.* 359, 975.
(20) Burton, D.R., Boyd, J., Brampton, A.D., Easterbrook-Smith, S.B., Emanuel, E.J., Novotny, J., Rademacher, T.W., van Schravendijk, M.R., Sternberg, M.J.E. Sternberg and Dwek, R.A. (1980) *Nature* 288, 338.
(21) Phizackerley, R.P., Wishner, B.C., Bryant, S.H., Amzel, L.M., Lopez de Castro, J.A. and Poljak, R.J. (1979) *Molec. Immunol.* 16, 841.
(22) Strosberg, A.D. (1977) *Immunogenetics* 4, 499.
(23) Strosberg, A.D., Emorine, L. and Zeeuws, R. (1979) *Ann. Immunol. (Paris)* 130C, 157.
(24) Mc. Cartney-Francis, N. and Mandy, W.H. (1979) *Ann. Immunol. (Inst. Pasteur)* 130C, 115.
(25) Yarmush, M.L., Sogn, J.A. and Kindt, T.J. (1979) *Ann. Immunol. (Inst. Pasteur)* 130C, 143.
(26) Emorine, L. and Strosberg, A.D. (1980) *Immunol. Lett.* 2, 107.
(27) Seidman, J.G., Max, E.E. and Leder, P. (1979) *Nature* 280, 370.
(28) Miller, J.F. and Storb, U.B. (1981) *J. Supramolecul. Struct. and Cell. Biochem.* suppl. 5 : Abst. 27, p. 13.
(29) Gilman-Sachs, A., Mage, R.G., Young, G.O., Alexander, C. and Dray, S. (1969) *J. Immunol. 103*, 1159.
(30) Bosma, M. and Bosma, G.C. (1974) *J. Exp. Med.* 139, 512.
(31) Kolb, C. and Weiler, E. (1979) *Ann. Immunol. (Inst. Pasteur)* 130C, 133.

REARRANGEMENT AND EXPRESSION OF A PRE-B CELL LIGHT CHAIN GENE IN PRE-B X MYELOMA HYBRIDS

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ABSTRACT - An Abelson virus-transformed mouse cell line (18-81) synthesizes μ heavy (H) chain in the absence of light (L) chain. When 18-81 is fused to variant myeloma cells which do not express L chain, 50% of the somatic hybrids express κ L chain. Southern blots of DNA from 18-81 cells show that the Cκ genes are on a 13kb Bam HI fragment which comigrates with the 13kb embryonic Bam HI fragment in splenic DNA. The 13kb Bam HI fragment is not detected in pre-B cell x variant myeloma (L^-) hybrids which express κ L chain. Instead a new Bam HI fragment is detected which is not found in either 18-81 pre-B cells or the myeloma cells. The de novo rearranged Cκ gene is on different-sized Bam HI fragments in three independent hybrids. This data supports the hypothesis that a pre-B cell, which is committed to synthesis of a particular H chain, acts as stem cell for expression of different L chains. When 18-81 is fused to myeloma cells which express κ L chain, the pre-B cell κ L chain gene is not rearranged or expressed. Thus, rearrangement and expression of a pre-B cell L chain gene may be affected by the presence of a properly rearranged L chain gene in a pre-B cell x myeloma hybrid.

INTRODUCTION

Murine pre-B cells synthesize μ H chain in the absence of

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L chain synthesis (1-3). Recent evidence from fetal liver hybridomas indicates that pre-B cell L chain genes are not rearranged (4, 5). Since pre-B cells are thought to be precursors of immature B lymphocytes which express surface immunoglobulin (Ig)(6-9), rearrangement and expression of L chain genes must be a primary event in pre-B development. Lymphoid cell lines with pre-B cell characteristics have been produced by in vitro transformation of murine bone marrow with Abelson murine leukemia virus (10, 11). In an attempt to induce L chain expression, we have used one of these cloned cell lines, 18-81, to make hybrids with myeloma cells. The rationale for this approach is the observation that hybridization of B-lymphoma cells with myeloma cells usually produces a hybrid with codominant Ig expression and the phenotype of the more differentiated myeloma parent (12-14). In our experiments the fusion of the pre-B cell line to myeloma cells resulted in hybrids expressing κ L chains encoded by the 18-81 Cκ genes. Expression of λ light chain is not detected in these hybrids. These studies with pre-B cell x myeloma hybrids provide a model system for studying the step in differentiation involving L chain gene rearrangement and expression.

RESULTS AND DISCUSSION

A. Functional Light Chain Gene Rearrangement Occurs At A High Frequency In Pre-B Cell X Variant Myeloma (L^-) Hybrids

When 18-81 (μ^+, L^-) is fused to variant myeloma cells which do not express a normal L chain (L^-) approximately 50% of the somatic hybrids express a κ L chain protein which is not produced by either of the parents (Fig. 1)(15). To determine whether this L chain is coded by the pre-B cell or the myeloma genome, the κ L chain genes were analyzed by Southern blots. Southern blots of DNA from 18-81 pre-B cells show that the Cκ genes are on a 13kb Bam HI fragment (Fig. 2) which is indistinguishable from the embryonic Cκ Bam HI fragment generated from spleen DNA (data not shown). Therefore, like fetal liver hybridomas, 18-81 contains unrearranged κ L chain genes.

The κ L chain genes in the L^- myeloma variants were also analyzed by Southern blots. NT_2 (an H^-, L^- variant of MPC11) has one Cκ gene (3.2kb Bam HI fragment) which is aberrantly rearranged and expressed as L chain constant region fragment (F_{CL}). NT_2 has lost the normal κ L chain gene (7.7kb Bam HI fragment) which is expressed by the parental L^+ MPC11 myeloma cells (16, 17). The other L^- myeloma variant, CBOHC (γ_{2a}^+, L^-), has two unexpressed (and presumably aberrantly rearrang-

ed) Cκ genes represented by Bam HI fragments of 5.7kb and 4.7kb (Fig. 2), while CBO (the γ_{2a}^+, κ^+ parent of CBOHC) has three Cκ genes represented by Bam HI fragments of 5.7kb, 5.1kb and 4.7kb (Fig. 2). Since CBOHC does not express L chain, the 5.1kb Bam HI fragment probably contains the functionally rearranged κ L chain gene expressed by CBO.

The NT_2 x 18-81.B4 and CBOHC x 18-81.T5 hybrids which express L chain were analyzed by Southern blots. Most hybrids contain the same Bam HI Cκ fragments as the myeloma parent, but the 13kb Bam HI embryonic Cκ fragment was not detected in any of the hybrids examined (Table 1, Figs. 2, 3). However, in each of these hybrids the 13kb fragment is replaced by a new Bam HI fragment which is not in the myeloma parent. This new Bam HI fragment probably contains the functionally rearranged Cκ gene expressed as a de novo L chain. The Cκ gene is considered to be functionally rearranged because in all hybrids studied the de novo L chain is assembled to H chain and

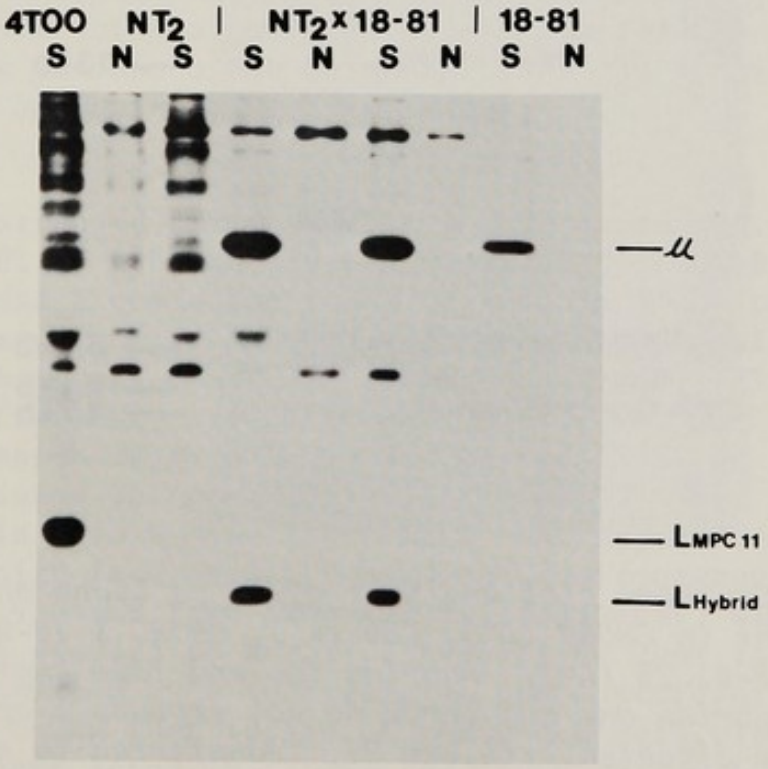


FIGURE 1. SDS-polyacrylamide gel electrophoretic analysis of Ig synthesized by 4T00.A (H^-, κ^+), NT_2 X 18-81.B4 hybrids and 18-81.B4(μ^+, L^-). Cells were labeled, lysed in 1% NP40 and immunoprecipitated with rabbit antibodies to murine μ, γ_2, κ and λ chains and goat antiserum to rabbit Ig(18). The precipitates were dissolved in SDS, reduced, electrophoresed on SDS-tris-glycine-polyacrylamide gels (19) and analyzed by photofluorography. N = normal rabbit serum; S = rabbit anti-MPC11 (γ_2, κ) + MOPC 104 (μ, λ).

can be secreted from the cell. These results also suggest that 18-81 has only one Cκ gene because if more than one Cκ gene were present, probably one Cκ gene would remain unrearranged (13kb Bam HI fragment) while the other gene was rearranged.

In 50% of 18-81 x L^- myeloma hybrids L chain is not expressed (Table 1). There are four possible reasons why these hybrids do not express L chain: 1) the embryonic Cκ gene from 18-81 is on a chromosome which is segregated from the hybrid; 2) the Cκ gene from 18-81 is not rearranged; 3) the Cκ gene from 18-81 is rearranged aberrantly; (4) the Cκ gene from

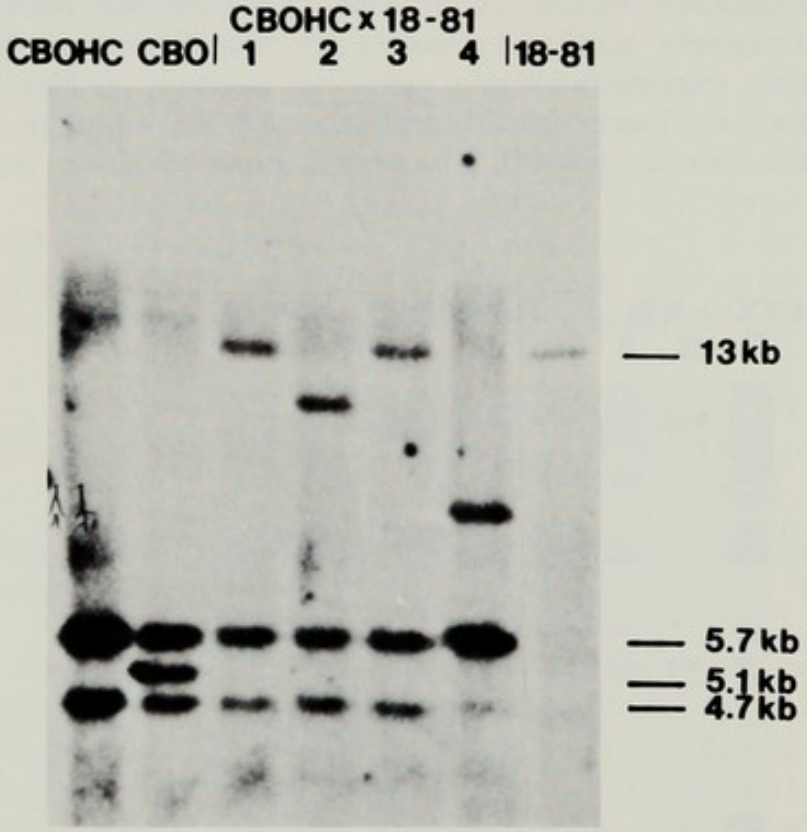


FIGURE 2. Southern blot analysis of kappa light chain gene rearrangement in CBOHC (L^-), CBO (κ^+), CBOHC X 18-81.T5 hybrids and 18-81.T5 (L^-). Hybrids two and four express κ L chain and hybrids one and three do not express L chain. Cellular DNA was digested with Bam HI, transferred to nitrocellulose (20) and hybridized (21) with a ^{32}P-nick translated Bam HI-Xba I fragment derived from an embryonic Cκ genomic clone as probe (22). The unrearraned Cκ gene is on a 13kb Bam HI fragment. CBO contains three rearranged Cκ genes (5.7kb, 5.1kb and 4.7kb), and CBOHC contains two of these Bam HI fragments (5.7kb and 4.7kb). The 5.1kb Bam HI fragment presumably contains the functionally rearranged Cκ gene and the 5.7kb and 4.7kb Bam HI fragment presumably contain aberrantly rearranged Cκ genes.

TABLE 1 - Rearrangement of the 18-81 Kappa Light Chain Gene in 18-81 (μ^+, L^-) X Variant Myeloma (L^-) Hybrids

Myeloma Parent	Number Hybrids	L Chain Expression	18-81 Cκ gene Rearranged	18-81 Cκ gene Not Rearranged	18-81 Cκ gene Lost
CBOHC	21	+	14	-	-
CBOHC	20	-	-	8	-
NT_2	2	+	2	-	-
NT_2	3	-	-	1	2

Synthesis of light chain protein was analyzed by SDS-polyacrylamide gel electrophoresis of immunoprecipitates isolated from (35S) Met radiolabeled postnuclear supernatants. Kappa light chain gene rearrangement was analyzed by Southern blots using a Bam HI-Xba I fragment derived from an embryonic Cκ genomic clone as probe. Not all hybrids have been tested by Southern blot analysis.

18-81 is rearranged correctly but is not expressed. In eight CBOHC x 18-81.T5 hybrids and one NT_2 x 18-81.T5 hybrid which do not express L chain the 18-81 Cκ gene is found on a 13kb Bam HI fragment. In two NT_2 x 18-81.B4 hybrids which do not express L chain the 13kb Bam HI fragment from 18-81 is lost and no new fragments are detected. Therefore, the lack of L chain expression appears to be due to the 18-81 Cκ gene either remaining unrearranged or being lost.

Thus, in this system functional rearrangement appears to occur at a high frequency. Functional rearrangement is also a more likely event than aberrant rearrangement, since in every hybrid studied so far if the 18-81 Cκ gene is rearranged it is expressed as a functional L chain protein. However, we have not shown that fusion of the pre-B cell with a L^- myeloma variant actually causes the pre-B cell κ L chain gene to rearrange. An alternative possibility is that 18-81 cells can spontaneously rearrange κ L chain genes in culture and that these cells are selected when 18-81 is fused to L^- myeloma variants.

B. Pre-B Cells Serve As Stem Cells For Light Chain Expression

Pre-B cells (like 18-81) which are committed to expres-

sion of a particular H chain V region have been postulated to serve as stem cells for generation of immature B lymphocytes which express different L chain V regions with the same H chain V region (2, 23). Analysis of the Cκ gene from three independent hybrids (Fig. 2, Fig. 3) by Southern blots show that the 18-81 Cκ gene is present on three different Bam HI fragments. It appears, therefore, that the 18-81 Cκ gene can rearrange with at least three different Vκ genes. On the other hand, we have found that two independent CBOHC x 18-81.T5 hybrids have the 18-81 Cκ gene rearranged to the same size Bam HI restriction fragment (data not shown). This result suggests that the rearrangement of a particular Vκ gene to the 18-81 Cκ gene is nonrandom, so that some Vκ genes may

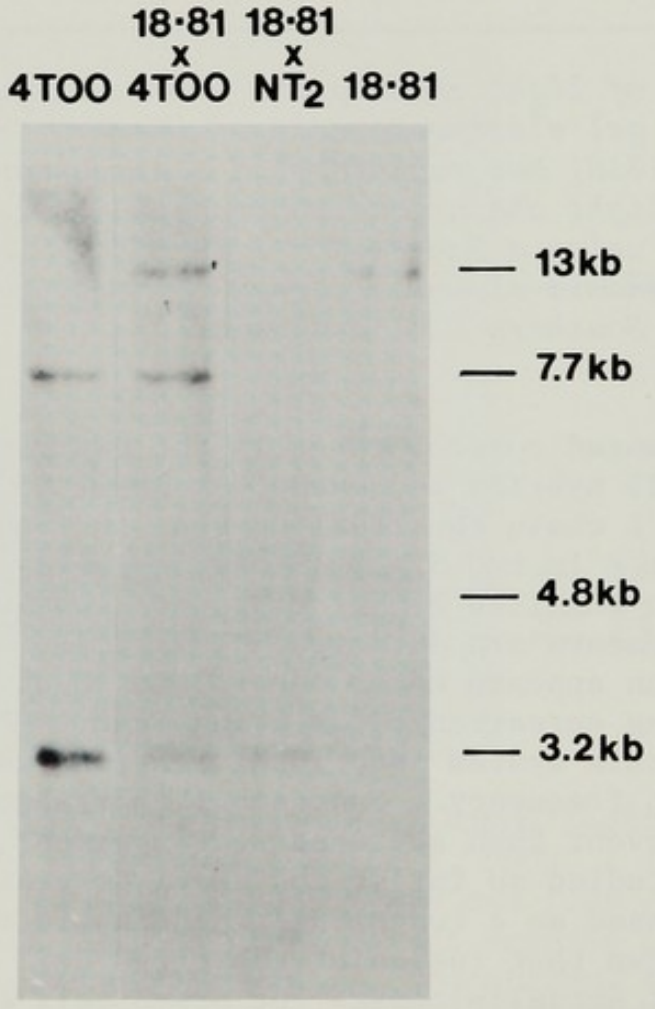


FIGURE 3. Southern blot analysis of kappa light chain rearrangement in 4T00.A (κ^+), 4T00.A X 18-81.B4 hybrids, NT_2 X 18-81.B4 hybrid and 18-81.B4 (L^-). The same conditions were used as described in Figure 2 legend. The 3.2kb Bam HI fragment contains an aberrantly rearranged Cκ gene which is found in the MPC11 myeloma and its variants and is expressed as a light chain constant region fragment.

be rearranged at a higher frequency than other Vκ genes. If further studies confirm that rearrangement of Vκ genes is nonrandom, it would suggest that the structure and organization of Vκ genes or some factor in the parental cell (e.g. H chain) favors rearrangement of certain Vκ genes.

C. The Pre-B Light Chain Gene Is Not Rearranged In Hybrids With Myeloma (L^+) Cells

In the experiments described above, when 18-81 is fused with an L^- myeloma variant approximately 50% of the hybrids rearrange and express the 18-81 Cκ gene as functional L chain. In contrast to this result, when 18-81 is fused with L^+ myelomas (the parents of the L^- myelomas) the 18-81 κ L chain is not rearranged or expressed in any of the hybrids which have been analyzed. For example, of 19 18-81.B4 x 4T00.A (an H^-, κ^+ MPC11 variant) hybrids none express a de novo L chain (which should be distinguishable from the MPC11 L chain because MPC11 κ L chain is larger than most κ L chains (Fig. 1)). Southern blots of DNA from 15 of these hybrids show that the 18-81 Cκ gene remains on the 13kb Bam HI fragment (Fig. 3, Table 2). Six 18-81.T5 x CBO (the γ_{2a}^+, κ^+ parent of CBOHC) hybrids also have the 18-81 Cκ gene on the 13kb Bam HI fragment (Table 2).

TABLE 2 - The 18-81 Kappa Light Chain Gene is Not Rearranged in 18-81 (μ^+, L^-) X Myeloma (L^+) Hybrids

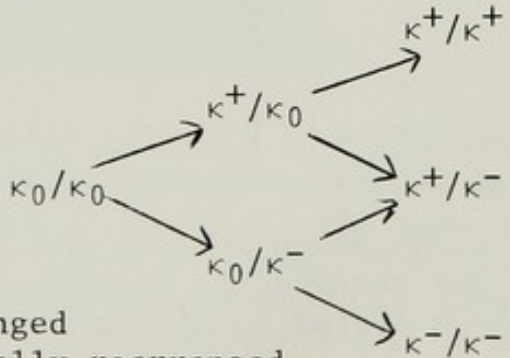
Myeloma Parent	Number Hybrids	L Chain Expression	18-81 Cκ gene Rearranged	18-81 Cκ gene Not Rearranged	18-81 Cκ gene Lost
CBO	0	+	-	-	-
CBO	24	-	-	6	-
4T00.A	0	+	-	-	-
4T00.A	19	-	-	15	-

Synthesis of light chain protein and rearrangement of κ light chain genes was analyzed as described in Table 1. Not all hybrids have been tested by Southern blot analysis.

C. Implications For Allelic Exclusion

B lymphoid cells synthesize and secrete an immunoglobulin which is encoded by only one L gene and only one H gene (24). Thus, L chain is subject to both isotypic (e.g. κ or λ) and allelic exclusion, so that only one functional L chain is expressed. When 18-81 is fused with a myeloma cell which contains a functionally rearranged κ L chain gene (4T00.A, CBO), the 18-81 Cκ gene remains unrearranged. However, when 18-81 is fused with a myeloma cell which contains only aberrantly rearranged κ L chain genes and does not express L chain (NT_2, CBOHC), the 18-81 Cκ gene is functionally rearranged in 50% of the hybrids. Thus, rearrangement and expression of a pre-B L chain appears to be prevented by the presence of a functionally rearranged L chain gene (or its product) in a pre-B cell x myeloma hybrid. It is possible that if the κ L chain gene can spontaneously rearrange in 18-81 cells during culture these cells are not selected when 18-81 is fused to L^+ myeloma cells. Therefore, the presence of a functionally rearranged myeloma gene is not preventing rearrangement of the 18-81 κ L chain gene; rather it does not give a selective advantage to hybrids derived from 18-81 cells that have rearranged the κ L chain gene.

Both normal murine lymphocytes and murine plasmacytomas have been examined to determine the basis of allelic exclusion for κ L chains. In a normal B-cell population about half of the Cκ genes are found to be unrearranged (25). This result indicates that rearrangement occurs on only one allele for the majority of normal B cells. Approximately half of the plasmacytomas studied have one rearranged and one unrearranged Cκ allele (26). The other plasmacytomas studied have more than one rearranged Cκ allele, but only one Cκ allele is rearranged so that it expresses a functional L chain (16, 17, 26-30). Plasmacytomas that have more than one functionally rearranged Cκ allele are extremely rare (31). A scheme has been proposed by Perry and co-workers to account for the data on Cκ gene rearrangements (26).



Where κ_0 = unrearranged
κ^+ = functionally rearranged
κ^- = aberrantly rearranged

Aberrant rearrangements ($\kappa_0 \rightarrow \kappa^-$) may occur either before or after functional rearrangements ($\kappa_0 \rightarrow \kappa^+$). If functional rearrangements occur at a much higher frequency than aberrant rearrangements, as is suggested by our data (Table 1), the high proportion of plasmacytomas containing aberrantly rearranged Cκ genes may result from aberrant rearrangements occuring after functional rearrangements.

There are two basic models which could account for allelic exclusion, i.e. the absence of two functional Cκ alleles in a single cell. One model proposes that rearrangement is a stochastic process so that each Cκ allele rearranges independently and functional rearrangements ($\kappa_0 \rightarrow \kappa^+$) occur at a low frequency. The second model proposes that the functional rearrangement of one Cκ allele actively prevents functional rearrangement at a second Cκ allele. Our data suggest that: 1) functional rearrangement can occur at a high frequency (50% of hybrids); 2) the probability of a functional rearrangement ($\kappa_0 \rightarrow \kappa^+$) is much higher than aberrant rearrangement ($\kappa_0 \rightarrow \kappa^-$); and 3) a second functional rearrangement may not occur in the presence of a functionally rearranged allele.

ACKNOWLEDGMENTS

We thank Mr. James Remenick for technical assistance and Drs. N. Rosenberg and D. Baltimore for the 18-81 cell line. This work was supported by NIH Research Grant AI12525. SCR is supported by NIH Training Grant 2T32-GM07082. WMK is a recipient of a Research Career Development Award A100293.

REFERENCES

1. Raff, M.D., Megson, M., Owen, J.J.T. and Cooper, M.D. (1976). *Nature 259*, 224.
2. Burrows, P., LeJeune, M. and Kearney, J.F. (1979). *Nature 280*, 838.
3. Levitt, D. and Cooper, M.D. (1980). *Cell 19*, 617.
4. Maki, R., Kearney, J., Paige, C. and Tonegawa, S. (1980). *Science 209*, 1366.
5. Perry, R.P., Kelley, D.E., Coleclough, C. and Kearney, J.F. (1981). *Proc. Natl. Acad. Sci. U.S.A. 78*, 247.
6. Osmond, D.G. and Nossai, G.J.V. (1974). *Cell Immun. 13*, 132.
7. Ryser, J-E. and Vassalli, P.J. (1974). *Immun. 113*, 719.

8. Owen, J.J.T., Wright, D.E., Habu, S., Raff, M.C., and Cooper, M.D. (1977). *J. Immun. 118*, 2067.
9. Burrows, P.D., Kearney, J.F., Lawton, A.R. and Cooper, M. D. (1978). *J. Immun. 120*, 1526.
10. Siden, E.J., Baltimore, D., Clark, D. and Rosenberg, N.E. (1979). *Cell 16*, 389.
11. Baltimore, D., Rosenberg, N. and Witte, O.N. (1979). *Immun. Rev. 48*, 3.
12. Eshar, Z., Blatt, C., Bergman, Y. and Haimovich, J. (1979). *J. Immun. 122*, 2430.
13. Laskov, R., Kim, K.J. and Asofsky, R. (1979). *Proc. Natl. Acad. Sci. U.S.A. 76*, 915.
14. Raschke, W.C., Mather, E.L. and Koshland, M.E. (1979). *Proc. Natl. Acad. Sci. U.S.A. 76*, 3469.
15. Riley, S.C., Brock, E.J. and Kuehl, W.M. (1981). *Nature 289*, 804.
16. Choi, E., Kuehl, M. and Wall, R. (1980). *Nature 286*, 776.
17. Seidman, J.G. and Leder, P. (1980). *Nature 286*, 779.
18. Kuehl, W.M. and Scharff, M.D. (1974). *J. Mol. Biol. 89*, 409.
19. Laemmli, U.K. (1970). *Nature 227*, 680.
20. Southern, E.M. (1975). *J. Mol. Biol. 98*, 503.
21. Jeffreys, A.J. and Flavell, R.A. (1977). *Cell 12*, 1097.
22. Seidman, J.G. and Leder, P. (1978). *Nature 276*, 790.
23. Cooper, M.D., Burrows, P.D. and Lawton, A.R. (1980). In *"Biological Basis of Immunodeficiency"* (Gelfand, E.W. and Dosch, H.-M., eds) p. 57, Raven Press, New York.
24. Pernis, B., Chiappino, G., Kelus, A.S. and Gell, P.G.H. (1965). *J. Exp. Med. 122*, 853.
25. Joho, R. and Weissman, I.L. (1980). *Nature 284*, 179.
26. Perry, R.P., Kelley, D.E., Coleclough, C., Seidman, J.G. Leder, P., Tonegawa, S., Matthyssens, G. and Weigert, M. (1980). *Proc. Natl. Acad. Sci. U.S.A. 77*, 1937.
27. Schnell, H., Steinmetz, M. and Zachau, H.G. (1980). *Nature 286*, 170.
28. Altenburger, W., Steinmetz, M. and Zachau, H.G. (1980). *Nature 287*, 603.
29. Max, E.E., Seidman, J.G., Miller, H. and Leder, P. (1980). *Cell 21*, 793.
30. Walfield, A.M., Storb, U., Selsing, E. and Zentgraf, H. (1980). *Nucl. Acids. Res. 8*, 4689.
31. Morse III, H.C., Goode, Jr., J.H. and Rudikoff, S.J. (1977). *J. Immunol. 119*, 361.

ISOLATION AND CHARACTERIZATION OF CLASS SWITCH VARIANTS OF MYELOMA AND HYBRIDOMA CELLS

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ABSTRACT. In order to follow immunoglobulin (Ig) heavy chain class switching in a single plasma cell clone we isolated spontaneous class switch variants from mouse myeloma and hybridoma cells. An IgG2a producing variant was isolated from MPC11 (expressing IgG2b). X63 cells switched from $\gamma 1$ to $\gamma 2b$ to $\gamma 2a$ and $\gamma 2a$ plus $\gamma 2b$ expression, from $\gamma 2b$ to $\gamma 1$ and from $\gamma 2a$ to $\gamma 1$ plus $\gamma 2a$ expression. From the hybrid cell line B1-8.64 (IgM) we isolated a variant producing IgD and from the hybrid cell line S24/63/63 (IgG3) a variant producing IgG1.

The class switch variants were selected by fluorescence-activated cell sorting with fluorescein-labelled class-specific antisera and isolated by cloning. Representative clones were chosen for further serological characterization, karyotyping, determination of heavy chain size and primary structure and analysis of the genomic DNA by Southern blotting.

Three major points emerge from the analysis:

(1) The variant heavy chains are composed of the wild type VDJ-region joined to the complete C-region of the acquired class.

(2) Spontaneous class switching is accompanied by DNA rearrangements. In the hybrid cell lines B1-8 and S24 the genes encoding the previously expressed C_H regions are found deleted upon class switching.

(3) As a rule, "forward" class switches follow the order of C_H genes on chromosome 12. However, in the case of X63 we also find double producers and revertants at rather high frequencies. The involvement of several chromosomes in the production of the latter variants is discussed.

The isolation of a class switch variant that secretes IgD (B1-8.$\delta 1$) enabled us to determine the C-terminal amino acid sequence of the secreted δ chain of the mouse. It corresponds to the CδDC exon.

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INTRODUCTION

Differentiation of immunoglobulin expression in B cells includes the "class switch" phenomenon. In many immune responses, in particular T-dependent ones, class switching occurs in the majority of the B cell clones. The B cells initially express IgM and IgD and switch to the expression of IgG or IgA. Because C_H-gene rearrangements and deletions have been found in myelomas, hybrid cell lines and B cells, it has been proposed that class switching is caused by C_H gene deletion, possibly involving sister chromatid exchange (1-8). The cells investigated in those experiments represented one differentiation stage of a given B-cell clone, i.e. cells before or after the class switch. Our approach is to isolate myeloma and hybridoma cells that have undergone a spontaneous class switch in vitro. We are thus able to analyse the same cells before and after class switching.

MATERIALS AND METHODS

Isolation of variants. For selection of class switch variants populations of cloned cell lines were stained in direct or indirect immunofluorescence with antisera or antibodies specific for one or several Ig classes not expressed by the wild type cells. Stained cells were separated by fluorescence-activated cell sorting, grown up and sorted again. When positive cells had been enriched to several percent the sorted cells were cloned. Clones of variant cells were identified by determining the class of Ig secreted into the culture medium. The technology of cell culture, antiserum production, cell sorting, antibody purification by DEAE-cellulose or affinity chromatography, karyotyping, IEF and SDS-PAGE has been described in detail as well as fluorescence labelling and microscopy (9-11). The X63 cell line was a gift of Dr. C. Milstein, the MPC11 cell line was donated by Dr. S. Tonegawa and the hybridomas B1-8 and S24 by Drs. T. Imanishi-Kari and M. Reth.

Serology. Source and purification of specific antibodies, radioactive immunosorbents and precipitation assays and enzyme linked immunosorbent assays have been previously described (9, 10). We thank Dr. L.A. Herzenberg for gifts of monoclonal antibodies.

Recombinant DNA plasmids. The plasmid pBRγ2b-7 was constructed with cDNA synthesized on a template from X63.2b-7 poly(A)$^+$RNA. This plasmid contains an almost complete copy (missing the first 31 base pairs) of the coding sequence for the BALB/c γ2b heavy chain. The details of the preparation and the structure of the plasmid will be published elsewhere (12).

For DNA sequence determination fragments subcloned in M13mp2 were sequenced by the chain termination procedure (13-15).

For Southern blot analysis DNA of cell lines and liver cells was isolated as described by Steffan et al. (16) with minor modifications. DNA samples of 10 µg were digested with various restriction endonucleases ((5 u/µg) like Eco RI, Bam H1, Hind III (Boehringer Mannneim, Mannheim, F.R.G.), Kpn I, Bgl I, Bgl II (BRL, Bethesda, MD, U.S.A.) and separated according to size on an 0.8% agarose gel (100 mM, 40-50 V, 12 hrs) As specific probes we used cDNA corresponding to the 3' ends of the Cµ, Cγ3, Cγ1 (gift of A.L.M. Bothwell (17)) and Cγ2b heavy chain genes cloned in pBR322 and a 1.2 kb Sac I - Eco RI fragment corresponding to a sequence starting about 100 base pairs from the 3' end of the J_H4 gene (gift of R. Maki (18)). All probes were labelled by the nick translation procedure as described by Rigby et al. (19). Southern blotting was done as described by Wahl et al. (20).

Protein sequencing. Cyanogen bromide cleavage of complete immunoglobulin molecules or isolated heavy chains was performed in 70% formic acid with a five fold excess (w/w) of cyanogen bromide. The resulting fragments were subjected to gel filtration on Sephadex G50 and G100 in formic acid/urea (Pharmacia Fine Chemicals, Uppsala, Sweden). Tryptic peptides were isolated from cyanogen bromide fragments containing blocked amino termini. Automated Edman degradations of the cyanogen bromide fragments or their tryptic peptides were performed in the presence of the non-protein carrier polybren (21) using a 0.2 M Quadrol program in the sequencer (22). Phenylthiohydantoin derivates were identitied by thin layer chromatography and by HPLC (12, 21-23).

RESULTS

The cell lines used in this study and the variants isolated from them appear in Fig. 1. A detailed description of the isolated of most of these cells has been published (9-11).

MPC11.We screened MPC11 cell populations (γ2b, κ) by double immunofluorecence microscopy for variant cells expressing γ2a in the cytoplasm and found cells staining for γ2a but not for γ2b at a frequency of approximately 10^{-4}. Such cells were isolated by 5 rounds of selection with an antiserum specific for γ1 and γ2a chains and subsequent cloning. According to IEF analysis all 5 variant clones secreted Ig with the same γ2a chain. Clone 1B6 was further characterized.

X63. In X63.5.3.1 (γ1, κ) cells IgG2a producing variants arise with a frequency of 2.3×10^{-7} per cell per generation. Such cells were isolated using a cocktail of antisera against µ, γ3, γ2b and γ2a and α chain determinants in 9 rounds of selec-

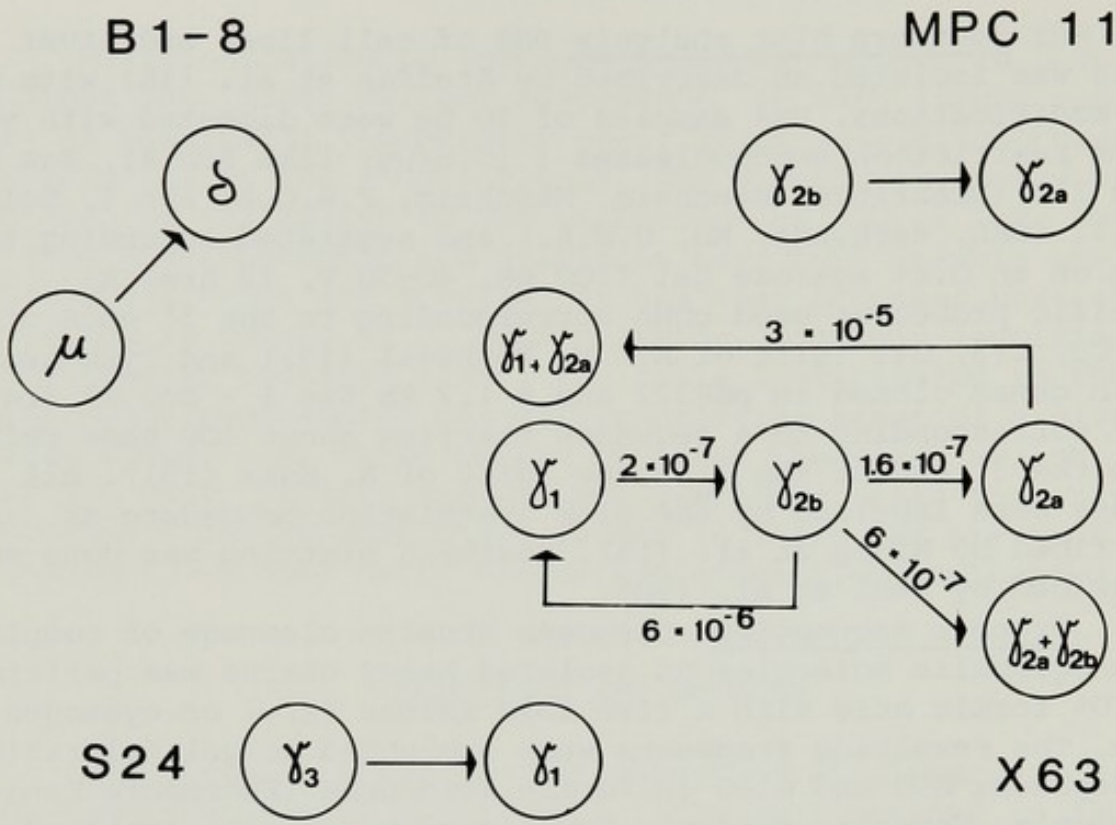

Fig. 1. Pedigree of class switch variants.
Cells producing Ig of the class enclosed by circles have been isolated. Arrows indicate descendancy and numbers the frequency of class switching per cell per generation.

tion. Again all variant clones secreted Ig of identical IEF patterns. In the variant clone X63.2b-7.8.4 (γ2b, κ) we detected IgG2a producing cells in a frequency of about 5.6×10^{-6} per cell per generation. The latter cells were isolated from X63.2b-7.8.4 using a mixture of antisera to μ, γ3, γ1, γ2a and α. After 3 rounds of sorting 25 IgG1-producing clones could be isolated and X63.R1-18 was chosen as a representative clone. In order to isolate also γ2a producers from X63.2b-7.8.4 we subjected these cells after one sorting with the antiserum cocktail to further selection with affinity purified anti-γ2a antibodies. The resulting cell population was cloned. 11% of the clones produced γ2a, 50% γ2b and 39% γ2a and γ2b heavy chains. X63.2a-25 was representative for 13 γ2a producing clones and X63.2a-93 for 44 double producers. From 3 times subcloned X63.2a-25 cells we isolated variants expressing γ2a and γ1

TABLE 1

COMPARISON OF CLASS SWITCH VARIANTS OF B1-8 AND S24
WITH B1-8 and S24 WILD TYPE CELLS

Characteristics	B1-8.64	B1-8.δ1	S24/63/63	S24-1/47
Affinity for NP[1)]	2×10^{-6} M	1.6×10^{-6} M	6×10^{-7} M	1.2×10^{-6} M
Affinity for NIP[1)]	1×10^{-7} M	1.2×10^{-7} M	2×10^{-7} M	2×10^{-7} M
Absorbance shift[2)]	yes	yes	yes	yes
App. molecular weight of heavy chain	75 000	61 000 44 000 (ungly.)[3)]	51 000	50 000
Surface Ig	-	+	+	+
Secreted Ig	+	+	+	+

1) NP: (4-hydroxy-3-nitrophenyl)acetyl, NIP: (4-hydroxy-5-iodo-3-nitrophenyl)acetyl
2) The absorbance maximum of NIP-cap changes upon binding to B1-8 and S24 (24).
3) Tunicamycin treatment

heavy chains by selection with anti-γ1. A representative clone out of 23 was X63.2aR1-16.

S24. The hybrid cell line S24 was originally obtained by fusing a X63.Ag8 cell with a C57BL/6 spleen cell producing an IgG3 antibody with λ1 light chains and specificity for the hapten 4-hydroxy-5-iodo-3-nitro-phenylacetyl (NIP) (24). We isolated a subclone of this hybridoma, S24/63/63, which had lost the expression of γ1 and κ chains of X63 origin. Two out of 4×10^5 S24/63/63 cells screened for class of cytoplasmic Ig expressed γ1 but not γ3 determinants. Such variants were isolated in five rounds of cell sorting. They produced a NIP-specific IgG1 antibody. S24-1/47 was a representative clone.

B1-8. The hybrid cell line B1-8 was obtained by fusing a X63.Ag8 cell to a C57BL/6 spleen cell producing a NIP-specific IgM with λ1 light chains (24). Subclone B1-8.64 had lost the expression of X63 γ1 and κ chains but still produced the IgM of C57BL/6 origin. B1-8.64 cells cannot be stained for IgM on the surface but secrete large amounts of specific antibody. In three rounds of sorting with a monoclonal antibody (Ls136, (25)) we enriched cells expressing λ and δ determinants on the surface and in the cytoplasm. The cells were then sorted twice with an $Ig5^b$ specific antibody (26) and cloned. Clone B1-8.δ1 was chosen for further characterization.

Characterization of variants. The immunoglobulins produced by the various class switch variants were investigated for size, isotype and allotype of the heavy chains and for idiotope and hapten binding properties. Most of the results of this comparison are published in detail elsewhere (9-11). The Ig secreted by X63.2a-93 could be separated into 3 fractions by chromatography on DEAE cellulose. Two of these fractions contained IgG2a and IgG2b respectively. The third (major) fraction consisted of hybrid molecules. We conclude that X63.2a-93 cells produce both γ2a and γ2b chains. Similarly, as shown by protein A affinity chromatography, X63.2aR1-16 cells secrete IgG1, IgG2a and hybrid molecules containing a γ1 and a γ2a heavy chain. The app. molecular weight of the γ1 and/or γ2a chains of X63, MPC11 and S24 origin is about 50 000 Dalton and that of X63 γ2b chains and S24 γ3 chains about 52 000 Dalton. The δ chain of B1-8.δ1 is secreted in a heavily glycosylated form and has an apparent molecular weight of 61 000 Dalton. The size of the unglycosylated δ-chain is about 44 000 Dalton.

The isotype of all variant heavy chains was determined serologically in radioactive binding inhibition assays. We never found a reaction with antisera against determinants of the previously expressed class. However, all variant heavy chains inhibited binding of antisera specific for the assigned class to standard Igs of that class completely.

The δ chains of the B1-8.δ1 and the γ1 chains of S24-1/47 carry the b allotype as determined by monoclonal anti-allotype

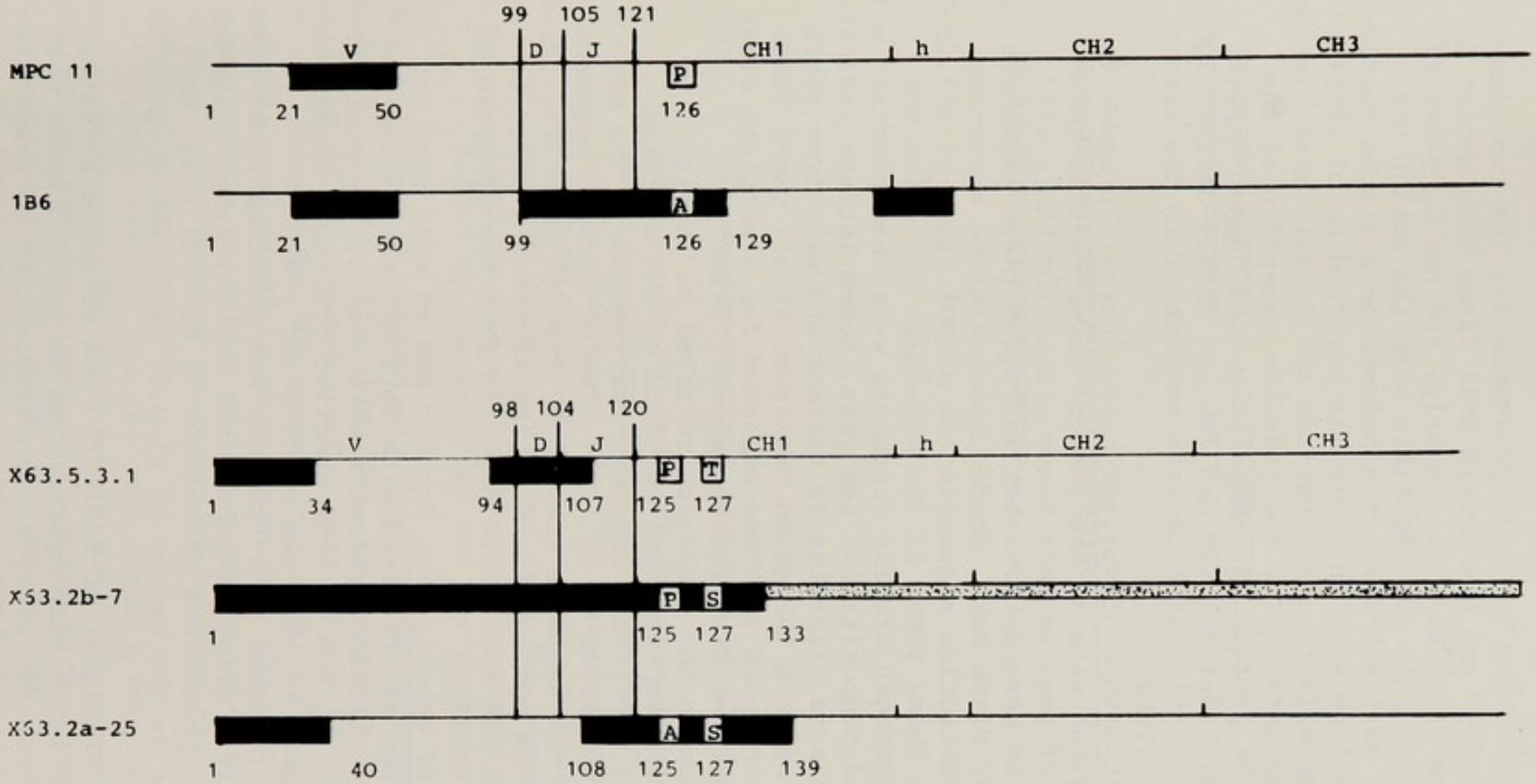

Fig. 2. Primary structure of heavy chains from MPC11 and X63 cell lines.
The heavy chains are given as horizontal lines and subdivided into V, D, J CH domains (CH1-3) and hinge (h) by vertical lines. Sequences determined by amino acid sequencing appear as black bars. Shaded bars represent sequences obtained from a cDNA clone. Numbers refer to sequence positions. Subclass-specific amino acids are given in the one letter code.

antibodies (11-6.3.1) and Ig(4a)10.9) (Table 1). The two class switches in the B1-8 and S24 hybrid cell lines thus appear to involve C_H genes of C57BL/6 origin only, and not C_H genes of the BALB/c parent. This is compatible with the notion that "forward" class switches reflect a process on a single chromosome No. 12. In line with this view is our finding of idiotypic identity between wild type and variant Igs in all cases (including the X63 variants), with the possible exception of the MPC11 derived 1B6 protein (9). Similarly, the hapten-binding properties of B1-8-derived IgM and IgD and of S24-derived IgG3 and IgG1 were indistinguishable from each other in the limits of our analysis (Table 1).

Primary structure of variant heavy chains. In order to pinpoint the switch site at the protein level partial amino acid sequences of some variant heavy chains and their wild type counterparts were determined. In Fig. 2 these sequences are indicated by black bars. Shaded bars refer to sequences determined by nucleotide sequencing of a cDNA clone of X63.2b-7. Lines represent those parts of the molecules for which the corresponding cyanogen bromide fragments were characterized only by end group and compositional analysis. Boxed letters indicate the first amino acid differing in variant and wild type chain sequences reading from the animo terminus. In all cases these replacements correspond to those expected for a complete class switch from published C-region sequences (27-29).

In addition to the sequences referred to in Fig. 2 we determined the carboxy-terminal sequence of the secreted chain of B1-8.δ1 (30). This sequence corresponds to the DC-exon described by Tucker et al. (31). The DC encoded sequence is preceeded by the sequence encoded by the Cδ3-exon (Fig. 3). The AC sequence is not expressed in the secreted δ chain in contrast to what one might have expected from the analysis of secreted μ chains (32).

Analysis of genomic DNA. Genomic DNA of wild type and variant cell lines was digested with various restriction enzymes and the restriction fragments were analysed by Southern blotting with V_H, J_H and C_H gene specific probes. A clear-cut result was obtained in the case of B1-8 and S24.

An Eco RI fragment of 14 kb size was detected in B1-8.64 with a Cμ- specific probe. Neither this nor any other fragment containing Cμ sequences was detectable in B1-8.δ1 (Fig. 4a). We conclude that in the genome of B1-8.δ1 cells the Cμ gene is deleted.

In an Eco RI digest of S24/63/63 DNA we found a restriction fragment of 14 kb size hybridizing to our Cγ3 probe that could not be accounted for by crosshybridization to fragments carrying germ line Cγ2b, Cγ3, Cγ2a or rearranged Cγ2b and Cγ2a genes of X63.Ag8 origin. This fragment which presumably contains

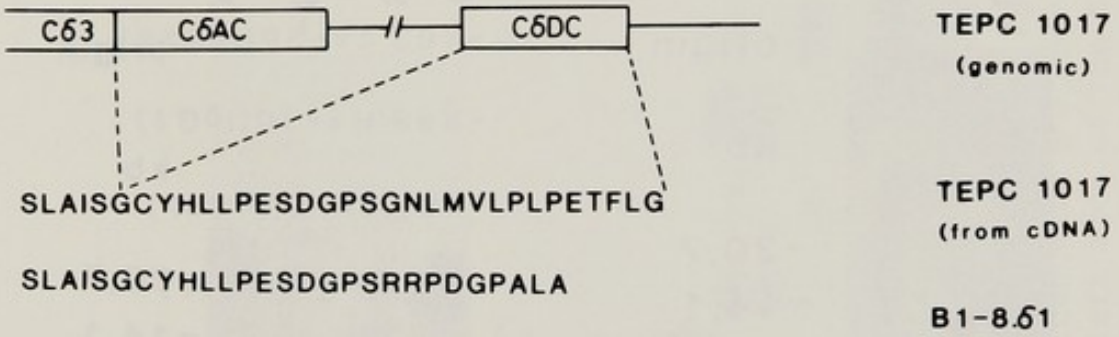

Fig. 3. The carboxy-terminal sequence of the secreted δ chain of B1-8.δ1.
Arrangement and designation of exons and amino acid sequence deduced from cDNA of TEPC1017 δ chain according to Tucker et al. (31) The nine carboxy-terminal residues of the B1-8.δ1 sequence differ from the cDNA deduced sequence but not from the sequence deduced from germline DNA (38). The cDNA and germline DNA sequence differ by one inserted and one exhanged nucleotide.

the Cγ3 gene has selectively disappeared from the genome of S24-1/47 (IgG1) cells (Fig. 4b). We interpret this to reflect deletion of the rearranged Cγ3 gene of S24/63/63 upon switching to Cγ1 expression. The possibility that the Cγ3 gene in S24-1/47 cells is rearranged in such a way that the corresponding restriction fragment coincides with one of the other bands on the blot is unlikely since the bands shared between the two cell lines have similar intensities.

In the case of the X63 cell lines a more complex situation was encountered. We found essentially the same patterns when DNA of the various lines was digested with a variety of restriction enzymes (Eco RI, Hind III, Bam HI, Bgl I, Kpn I) and analyzed by Southern blotting with Cγ1 and Cγ2b specific probes.

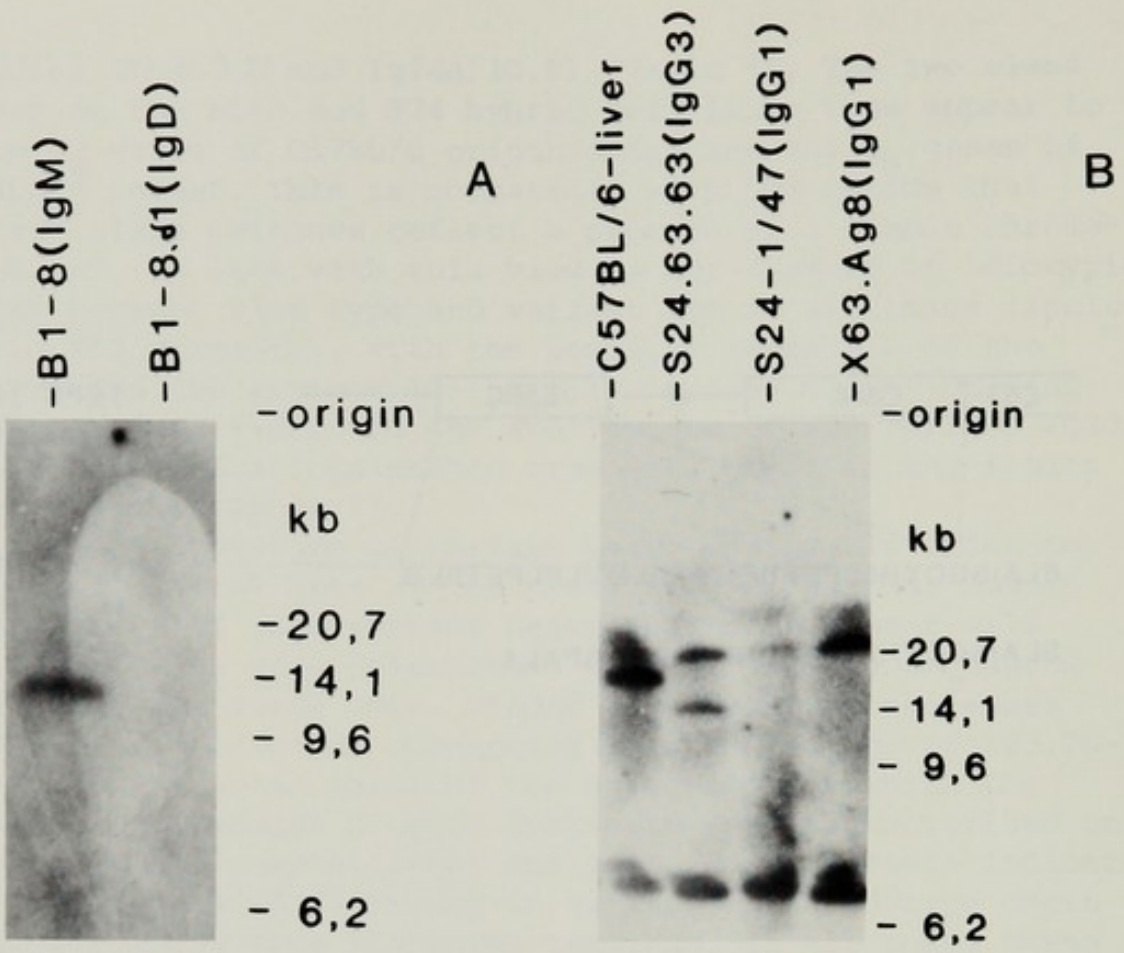

Fig. 4. 10 µg DNA from the cell lines indicated at the top was cleaved with Eco RI. Hybridization was done either with a Cµ specific probe (A) or with a cDNA probe containing the 3' end of Cγ3 (B) which crosshybridized with Cγ2a and Cγ2b. Nick translated Adenovirus 2 DNA cleaved with Eco RI or with Bam HI was used as size standard.

(A typical blot is shown in Fig. 5). However, differences were detected with a probe corresponding to a 1.2 kb sequence located 3' of J_H4 (18). In Fig. 6 we show the banding patterns obtained with this probe in the analysis of Kpn I digested DNA from several X63-derived lines. These include the wild type from which the class switch variants were derived (X63.5.3.1), several class switch variants (X63.2b-7 (IgG2b), X63.2a-25 (IgG2a), X63.R1-18 (IgG1 revertant), X63.2aR1-16 (γ2a + γ1)), X63.6.5.3 (a non-producer line (33) and X63.Ag8 (34). According to their presence in or absence from certain cell lines we number the restriction fragments from 1 to 4 (Fig. 6). Fragment 1 is found in all X63 cell lines, also in X63.6.5.3 (the non-producer) and X63.Ag8. Fragment 2 is found only in X63.Ag8, X63.5.3.1, X63.R1-18 and X63.2aR1-16, i.e. in cells producing IgG1. It is replaced in X63.2b-7 (IgG2b) and X63.2a-25 (IgG2a)

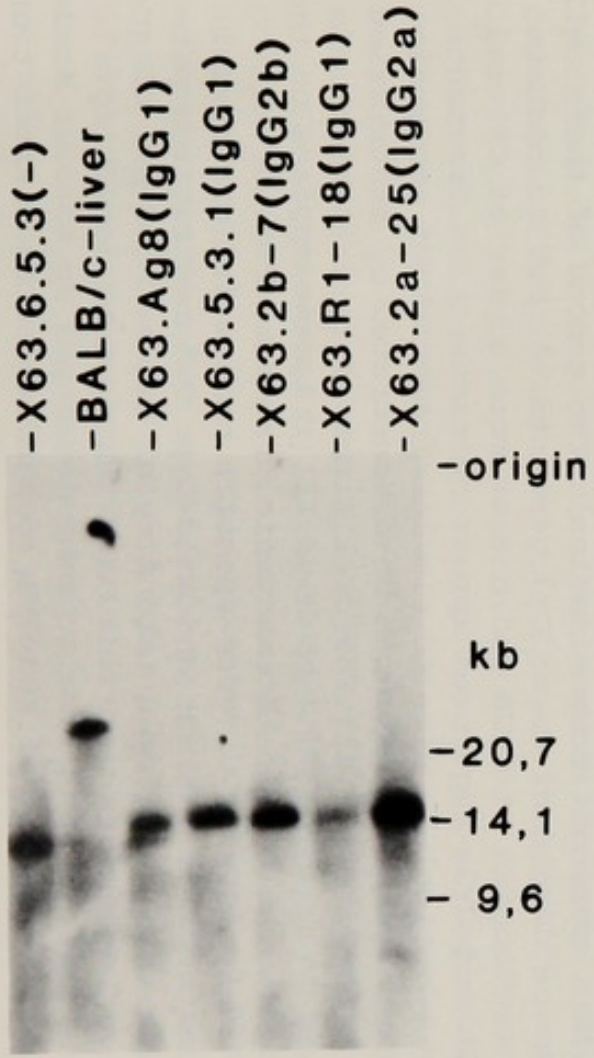

Fig. 5. Southern blot hybridization of Hind III digested DNA of X63 cell lines with a Cγ1 specific probe.

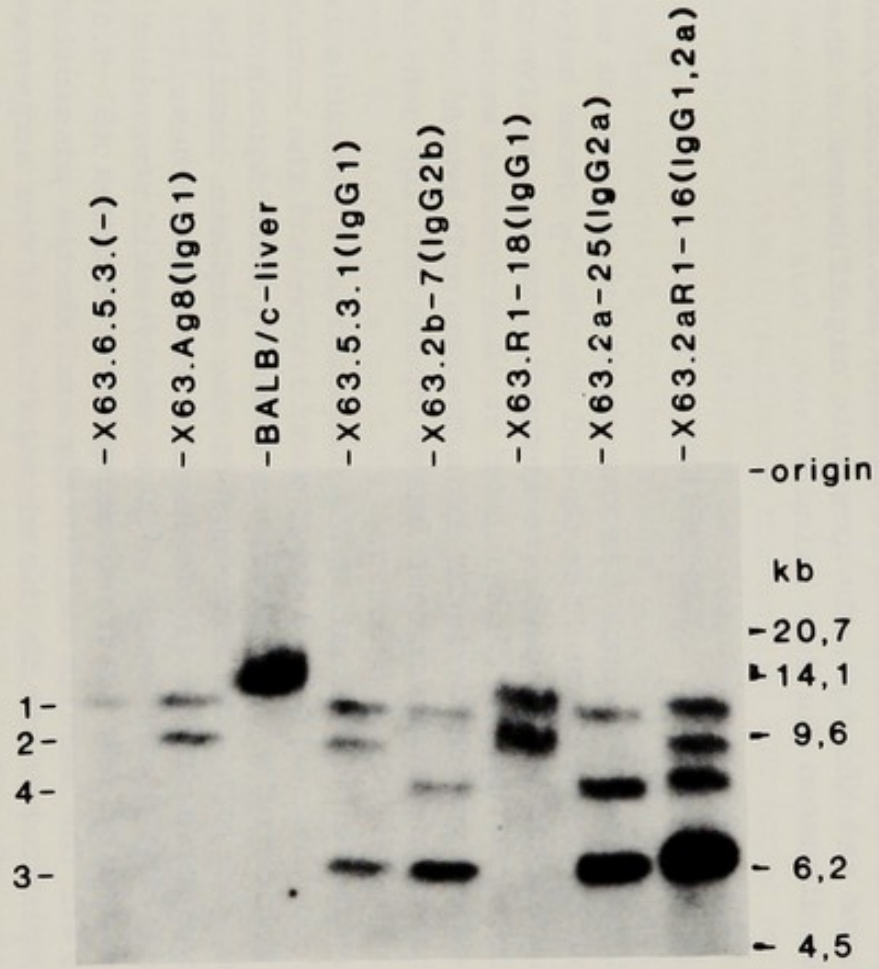

Fig. 6. Southern blot hybridization of Kpn I digested DNA from X63 cell lines with a J_H specific probe (18). Numbers on the left side of the blot refer to our numbering of bands (see text). Bands 1 and 2 of X63.R1-18 are slightly out of position on this particular blot.

by fragment 4 which is also found in X63.2aR1-16 producing γ1 and γ2a chains. Fragment 3 is found in all cell lines except X63.R1-18, the IgG1 producing revertant, X63.Ag8.6.5.3, the non-producer and X63.Ag8. The possible significance of these results is dicussed further below.

DISCUSSION

Work of several groups has now established that in cells expressing C_H genes other than Cμ and Cδ the C_H genes between J_H and the expressed C_H gene are removed from the active chromosome (1-8). To take a closer look at this process we have isolated class switch variants from myeloma and hybrid cell lines and analysed the variant heavy chains and Igh gene organisation.

The variant heavy chains are composed of a variable region identical to that expressed in the wild type and the entire constant region of a new class, as shown by serological and structural analysis. The exchange of the complete constant region is typical also for physiological class switches.

In all cell lines which we have analyzed class switching is accompanied by DNA rearrangements. In two cases (B1-8.δ1 and S24-1/47) the results definitely exclude the possibility that RNA splicing leads to the elimination of the sequences encoding the previously expressed C_H gene at the level of mRNA. These two cell lines have lost the corresponding C_H gene from the genome.

In the case of the switch variants derived from X63.5.3.1 the situation is a priori complicated since all X63 cell lines have multiple copies of chromosome 12 (A. Radbruch, unpublished data, T. Meo, personal communication). The genome of these cells appears to be devoid of Cμ or Cγ3 genes (3, 4) but to contain at least two copies of Cγ2b and one or more copies of Cγ1 and Cγ2a. Cγ2b and Cγ2a sequences appear on restriction fragments which are identical in size to similar fragments of embryonic DNA. A rearranged Cγ2b gene can be demonstrated by restriction analysis with Bgl I in all X63 cell lines (data not shown). In the case of Cγ1 a single 14 kb Hind III fragment appears on the blots, instead of the 21 kb embryonic fragment (Fig. 5).

The analysis with C_H gene-specific probes did not reveal differences between variant and wild-type DNA restriction patterns other than changes in intensity of bands. However, clear-cut DNA rearrangements were observed with a probe detecting a 1.2 kb fragment 3' of J_H. This probe developed up to four bands in restriction digests of X63 DNA, depending on the restriction enzyme. None of the restriction enzymes used cuts the 1.2 kb fragment itself, and therefore, since the Igh locus contains a single group of J_Hs (34, 35), different bands observed in the

blots should represent different Igh loci. Band 1 is present in all X63 lines including the non-producer line X63.Ag8.6.5.3. We think that the corresponding Igh locus is not involved in the class switches which our variants have undergone. Band 2 is present exclusively and invariably in cell lines expressing Cγ1 and thus most likely reflects the active Igh locus in these cells. Band 3 is only present in X63 cell lines producing γ2b or γ2a heavy chains. X63.2b-7 and X63.2a-25 (producing γ2b and γ2a respectively) do not have band 2. This may reflect a change in the active Igh locus connected to the class switch, most likely a deletion of the Cγ1-gene. The Cγ1-genes are, however, not completely removed from the genome of these cells since a 14 kb restriction fragment hybridizing with a γ1 specific probe is still found (Fig. 5). The Igh locus carrying the Cγ1-gene in X63.2b-7 and X63.2a-25 cells is perhaps the one represented by band 4. Since this band is not found in X63.R1-18 DNA we suppose that the corresponding Igh locus is involved in the production of the revertant phenotype.

The observed J_H rearrangement in the X63 cell lines X63.5.3.1 and X63.2b-7 could be caused by deletion of the Cγ1 gene from the active Igh locus. For reversion to γ1 production a different Igh locus must then donate a Cγ1 gene by transchromosomal recombination, placing it 3' to the J_H of the active chromosome in a position very similar to that of the active Cγ1 gene in the X63.5.3.1 parental cells. Gene cloning, R-loop analysis and sequencing of the appropriate DNA fragments is underway in order to analyse whether this model reflects the actual situation.

It is not clear whether class switching in vivo involves rapid sequential expression of several C_H-genes or proceeds directly to the finally expressed C_H gene (37). In myeloma and hybridoma cells spontaneous class switch variants other than revertants always expressed the C_H-gene located 3' next to the C_H-gene previously expressed in wild-type cells. We may observe in these cells a slow motion picture of physiological class switching, due to the absence of class switch promoting agents. However, it is also possible that such hypothetical promoting agents would lead to removal of several C_H-genes at a time.

The Ig produced by class switch variants provides a unique possibility to analyse the influence of different C-regions on antigen binding and on other effector functions of the antibody molecule. We found no difference in fine specificity and affinity for hapten between IgM and IgD of B1-8 and IgG3 and IgG1 of S24. Also, in general, the idiotype of variant Igs is the same as that of the corresponding wild type Ig. We presently use class switch variants in order to analyse the regulatory role of antibody constant regions in antibody-mediated feedback inhibition and idiotypic control.

Finally, the isolation of class switch variants may help to analyse differentiation processes that are connected with class switching. For example, B1-8.64 IgM is only secreted while B1-8.δ1 IgD is both secreted and inserted into the membrane. Thus, the machinary for differential splicing (32) of Igh transcripts is present in B1-8.64 cells but apparently cannot produce mRNA for membrane-bound μ chains. We suggest that Cμ membrane exons have been modified or deleted in B1-8.64 and that this process may be a prerequisite for the exclusive expression of secreted IgM (and possibly also IgD) in plasma cells.

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REFERENCES

1. Honjo, T., and Kataoka, T. (1978). Proc. Nat. Acad. Sci. US 75, 2140.
2. Early, P.W., David, M.M., Kaback, D.B., Davidson, W., and Hood, L. (1979). Proc. Nat. Acad. Sci. US 76, 857.
3. Rabbitts, T.A., Forster, A., Dunnick, W., and Bentley, D.L. (1980). Nature 283, 351.
4. Cory, S., Jackson, K., and Adams, J.M. (1980). Nature 285, 450.
5. Hurwitz, J.L., Coleclough, C., and Cebra, J.J. (1980). Cell 22, 349.
6. Nottenburg, C., and Weissmann, J.L. (1981). Proc. Nat. Acad. Sci. US 78, 484.
7. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
8. Davis, M.M., Kim, S.K., and Hood, K. (1980). Science 209, 1360.
9. Liesegang, B., Radbruch, A., and Rajewsky, K. (1978). Proc. Nat. Acad. Sci.US 75, 3901.
10. Radbruch, A., Liesegang, B., and Rajwsky, K. (1980). Proc. Nat. Acad. Sci. US 77, 2909.
11. Neuberger, M.S., and Rajewsky, K. (1981). Proc. Nat. Acad. Sci. US, in press.

12. Schreier, P.H., Bothwell, A.L.M., Geske, T., Dildrop, R., and Beyreuther, K., manuscript in preparation.
13. Sanger, F., Nicklen, S., and Coulson, A.R. (1977). Proc. Nat. Acad. Sci. US 74, 5463.
14. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980). J. Mol. Biol. 143, 161.
15. Schreier, P.H., and Cortese, R. (1979). J. Mol. Biol.129, 169.
16. Steffen, D., Bird, S., Rowe, W.P., and Weinberg, R.A. (1979). Proc. Nat. Acad. Sci. US 76, 4554.
17. Bothwell, A.L.M., Alt, F., Enea, V., and Baltimore, D. (1981). Cell, in press
18. Maki, R., Traunecker, A., Sakano, H., Roeder, W., and Tonegawa, S. (1980). Proc. Nat. Acad. Sci. US 77, 2183.
19. Rigby, P.J.W., Dieckmann, M., Rhodes, C., and Berg, P. (1977). J. Mol. Biol. 113, 237.
20. Wahl, G.M., Stern, M., and Stark, G.R. (1979). Proc. Nat. Acad. Sci. US 76, 3683.
21. Tarr, D.E., Beecher, J.F., Bell, M., and McKean, D.J. (1978). Anal. Biochem. 84, 622
22. Beyreuther, K., Raufuss, H., Schreicher, D., and Hengstenberg, W. (1977). Eur. J. Biochem. 75, 275.
23. Johnson, N.D., Hunkapillar, M.W., and Hood, L. (1979). Anal. Biochem. 100, 335.
24. Reth, M., Hämmerling, G.J., and Rajewsky, K. (1978). Eur. J. Immunol. 8, 393.
25. Reth, M., Imanishi-Kari, T., and Rajewsky, K. (1979). Eur. J. Immunol. 9, 1004.
26. Oi, V.T., Jones, P.P., Goding, J.W., Herzenberg, L.A., and Herzenberg, L.A. (1978). Curr. Top. Microbiol. Immunol. 81, 115.
27. Fougereau, M., Bourgois, A., de Preval, C., Rocca-Serra, J., and Schiff, C. (1976). Ann. Immunol. (Inst. Pasteur) 127C, 41.
28. Adetugbo, K., Milstein, C., and Secher, D.S. (1977). Nature 265, 299.
29. Tucker, P.W., Marcu, K.B., Newell, N., Richards, J., and Blattner, F.R. (1979). Science 206, 1303.
30. Dildrop, R., and Beyreuther, K. (1981)., submitted.
31. Tucker, P.W., Liu, C., Mushinski, J.F., and Blattner, F.R. (1980). Science 209, 1348.
32. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, T., and Hood, L. (1980). Cell 20, 313.
33. Kearney, J.F., Radbruch, A., Liesegang, B., and Rajewsky, K. (1979). J. Immunol. 123, 1548.
34. Köhler, G., and Milstein, C. (1975). Nature 256, 495.
35. Shimizu, A., Takahashi, N., Yamawaki-Kataoka, Y., Nishida, Y., Kataoka, T., and Honjo, T. (1981). Nature 289, 150.

36. Davis, M.M., Calame, K., Early, P.W., Livant, D.L., Joho, R., Weissmann, I.L., and Hood, L. (1980). Nature 283, 733.
37. Gearhart, P.J., Hurwitz, J.L., and Cebra J.J. (1980). Proc. Nat. Acad. Sci. US 77, 5424.
38. Tucker, P.W., Cheng, H.L., Mushinski, J.F., Fitzmaurice, L., Liu, C.P., and Blattner, F.R. (1981). In The Lymphocytes in the Immune Response, D. Mosier, N. Klinman, E. Sher and E. Vitetta (eds.) Elsevier-North-Holland, in press.

HUMAN ANTIBODY GENES: HEAVY CHAIN CONSTANT REGION GENE SWITCHING BY RNA SPLICING AND DNA REARRANGEMENT

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ABSTRACT

We have studied the structural arrangement for the human heavy chain Cμ and Cδ genes in isolated clones prepared from foetal liver DNA. The Cμ gene is made up of distinct domains for Cμ1, Cμ2, Cμ3 and Cμ4 + tp separated by short intervening sequences. About two kilobases (kb) downstream from Cμ4, additional segments occur which encode the membrane form of μ. The derived protein sequence of the first of the membrane segments is identical to an analogous segment of mouse, suggesting that production of membrane ($C\mu_m$) in human, like mouse, occurs by differential RNA splicing of a common nuclear RNA precursor. A further 2 kb downstream from $C\mu_m$ in the human DNA we detected the 5'-terminus of the Cδ1 domain, showing that the Cμ and Cδ gene segments are closely linked in human DNA.

Analysis of the intervening sequence to the 5'-side of the Cμ1 domain revealed a tandemly repeated sequence very homologous to the previously identified 'switch segment' found near mouse CH genes. By molecular hybridisation experiments we were able to detect a related but divergent sequence near to a human Cγ gene, while no related sequence was found near to Cδ. These results suggest that while μ/γ switching in human DNA involves recombination between partially homologous tandem repeats, μ/δ expression is most likely to occur through differential RNA splicing.

INTRODUCTION

Apart from V gene diversification in the immune response, a different kind of alteration occurs in the expression of antibody genes; a single VH gene is initially expressed with Cμ (1), which for a time is co-expressed with Cδ (2,3) and later the same VH sequence is switched to Cγ or Cα (1,4,5). The class switch then allows an antibody-producing cell to maintain the specificity with regard to antigen binding whilst developing different constant region functions during lymphocyte maturation (e.g. complement fixation or secretion through

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the mucosal membranes). A further developmental change that operates in lymphocytes is the alteration from surface immunoglobulin to the secreted form (e.g. in the case of IgM). Studies on these processes in mice have shown that switching from μ to γ or α involves some type of recombination event between an area of the large intervening sequence of the gene and an area on the 5' flanking side of the γ or α genes (6-8). Nucleotide sequences from these areas have failed to reveal definitive sequences that might be regarded as singals for this recombination but stretches of tandemly imperfectly repeated sequences were found near μ and γ genes (9,7). It was therefore suggested that these sequences might mediate homologous recombination (9) by allowing chromosomal alignment of the DNA segments that are to undergo the class switch. In this way sequential deletion of CH genes (10-13) would be a consequence of this recombination. The process by which the μ and δ genes are co-expressed does not readily fit into this scheme and it is not known at present how this is achieved. Recently, however, the mouse δ gene has been located just downstream of the μ gene (14) and it seems likely that co-expression results from RNA splicing of a common precursor rather than by DNA rearrangement. This possibility is given more weight by the recent finding that mouse membrane μ (μ_m) and secreted μ (μ_s) result from differential RNA splicing of one nuclear RNA transcript (15,16).

Our studies of human CH genes have shown that only about 5 kb of DNA separate the Cμ and Cδ genes which are adjacent in the genome. In addition segments coding for μ_m lie about 2 kb downstread from Cμ. The sequence of the μ_m coding segments indicates an identical protein sequence for this region in both mouse and human. Sequence and hybridisation analysis of a segment of DNA about 3 kb upstream (i.e. within the intervening sequence) of the human Cμ gene reveals the presence of analogous trandem repeat sequences (one basic structure being $GGG\overset{C}{\underset{T}{}}T\ (GAGCT)_n$) to those found near mouse CH genes. A related but more divergent sequence was also detected near a human Cγ gene. No related sequence was found near the δ gene. These results indicate that the tandemly repeated sequences are indeed functionally important for switch recombination since they are reasonably conserved between species and that μ/δ expression is not facilitated by such mechanisms but more likely by RNA processing.

The Isolation of Human Antibody Genes

Early studies of cross-hybridisation of cloned cDNA probes containing mouse immunoglobulin genes showed that mouse VH, VK (17) and mouse CH gene probes (unpublished) detect the

homologous genes in human nuclear DNA. We have, therefore, exploited this facility to derive isolated clones of human immunoglobulin genes from a clone library of human foetal liver DNA (a gift from T. Maniatis). This human DNA library was constructed in λ phage Charon 4A (18) using partially digested foetal liver DNA to which EcoRI linkers had been added; the library of phage, therefore, represents individual clones of essentially the entire human genome. The relevant human gene was identified within this library by *in situ* hybridisation (19) with a mouse cDNA clone: each clone thus obtained consists of a coding segment flanked on either side by regions of DNA.

The Structure of the Human μ CH Gene

We have studied two λ phage clones containing human DNA in which the coding segments for the Cμ occur (λC75 and λC76). The presence of the Cμ gene in both clones was assessed by hybridisation to a mouse cDNA plasmid containing Cμ sequences (20) and by nucleotide sequencing (21). One of the clones (λC75) was found to hybridise additional fragments with cDNA from DAUDI cell mRNA: these cells make membrane IgM and the additional hybridisation indicated the presence of membrane μ_m-coding segments in the λC75. This conclusion was also confirmed by sequence data. Table 1 summarises the domain structure of the human Cμ gene. As in the case of mouse, the $C\mu_S$ human gene is divided into four segments by short regions encoding Cμ1, Cμ2, Cμ3 and Cμ4 + tp domains separated by short intervening sequences (IVS).

Table 1

Domain structure of the human $C\mu_S$ gene

Genetic domain	Size of IVS	First codon	Last codon
Cμ1		Gly(114)	Pro(216)
	130		
Cμ2		Val(217)	Pro(328)
	242		
Cμ3		Asp(329)	Lys(433)
	150		
Cμ4 + tp		Gly(434)	Tyr(565)

The sizes of the IVS are deduced from restriction mapping and nucleotide sequencing. The first codon of each domain is created by RNA splicing. The numbers in brackets refer to the amino acid number according to the sequence G-A-L (22).

Restriction enzyme analysis of λC75 and λC76 enabled us to locate the μ_m coding segments in human DNA about 1.9 kb downstream from the end of $C\mu_s$; these distances approximate very closely to the analogous distances in mouse (16). The partial map of this region is shown in Figure 1. Nucleotide sequence data of the first μ_m coding segment demonstrated, in addition, a remarkable conservation between mouse and man. Figure 2 compares the first coding segment of the human μ_m with the analogous region of the mouse and the intra-Cμ4 splicing position in these species. Both regions are identical when translated in protein sequence, indicating

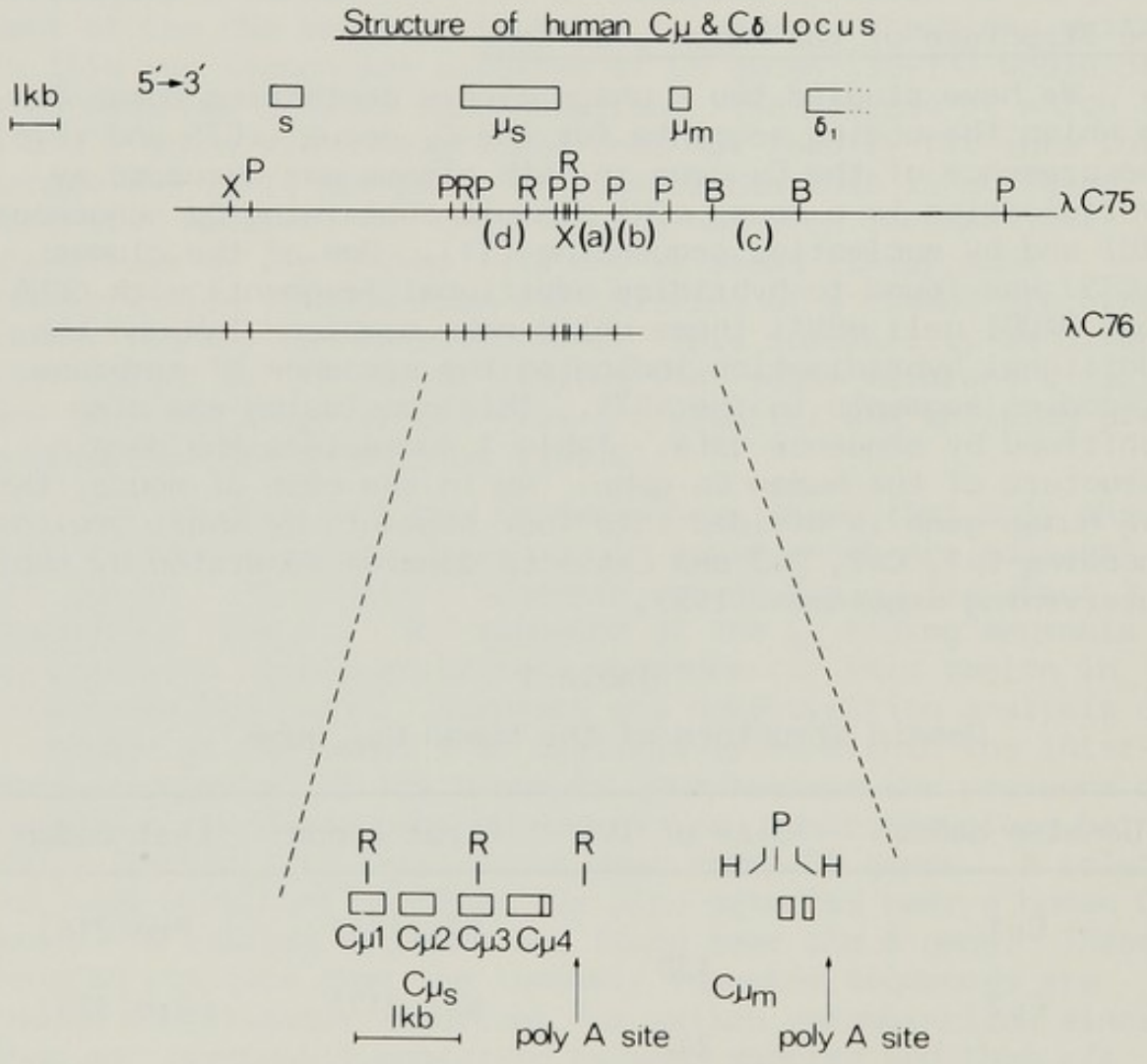

FIGURE 1. Partial map of the human Cμ and Cδ locus determined from cloned DNA. The orientation of the Cμ coding region is that the 5' end is shown on the left hand side of the drawing. Restriction sites are: R = EcoRI, P = PstI, X = XbaI, B = BamHI or BstI, and H = HpaII. S refers to sequences related to the H-chain switch.

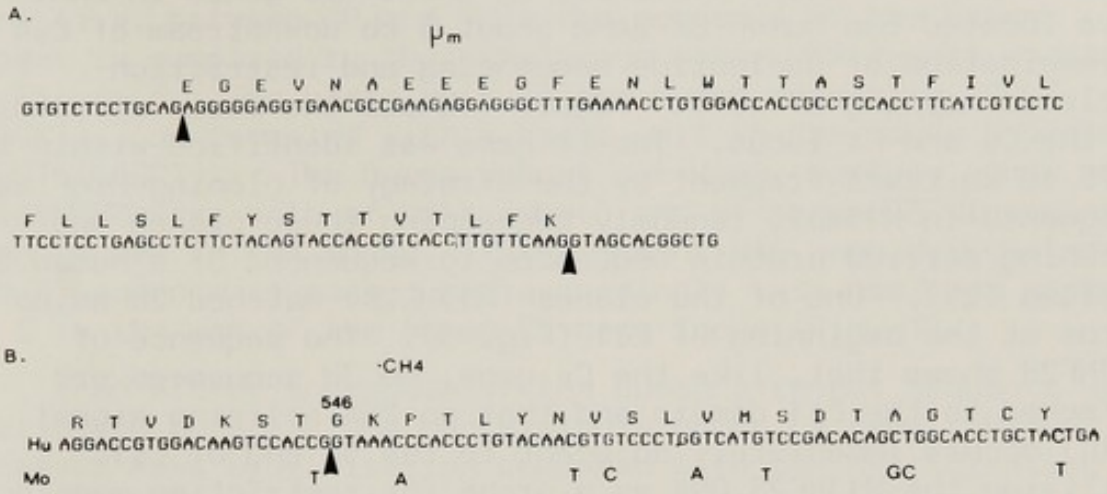

FIGURE 2. Comparison of nucleotide sequences of human and mouse μ_m coding segments. The nucleotide sequences of (a) the first coding segment of μ_m and (b) the intra-Cμ4 splicing position, for μ_m, are compared between human and mouse (16). The Cμ_m coding segment was identified within a random population of HpaII-digested DNA clones in M13mp7 (23) by sequencing as described (21) and comparison with the sequence of mouse μ_m.

strong evolutionary pressures being exerted to maintain the very hydrophobic nature of this region: the suggested role for this segment of polypeptide as an anchor for the μ_m (16) presumably accounts for this conservation. These experiments suggest therefore that the human μ_m production follows the same pathway as described in mouse (16) viz. two μ_m specific coding segments exist downstream of the μ_s poly(A) addition site. Production of μ_s or μ_m occurs by differential RNA splicing, μ_s being generated simply by the removal of inter-domain intervening sequences, while μ_m formation results from splicing a site within Cμ4 (GGT at codon 546) to the end of the first μ_m-specific segment and removal of the IVS between the first and second μ_m segments.

The Location of Cδ about 5 kb Downstream from Cμ in Human DNA

Previous experiments on mouse CH genes indicated that deletion of these genes occurs as a consequence of the class switch: μ/δ co-expression presents a possible violation of this scheme. An explanation of this latter phenomenon could be that the μ and δ genes are co-transcribed in order to allow differential RNA splicing of the V-segment to either of the C genes. Large precursor molecules containing μ and γ CH segments apparently do not exist (24) so a putative μ/δ precursor

would imply the close proximity of these two genes in DNA. We have located the human Cδ gene about 5 kb downstream of Cμ4 by a combination of nucleotide sequencing and restriction nuclease mapping of λC75. Figure 1 shows the basic structure of the Cμ and Cδ locus. The Cδ gene was identified within the λC75 10 kb EcoRI fragment by the strategy of cloning HinF sub-fragments in M13mp7, randomly sequencing these clones and matching derived protein sequences to sequences of a human IgD myeloma (25). One of the clones (M13HF28) matched 28 amino acids at the beginning of Cδ1 (Fig. 3). The sequence of M13HF28 shows that, like the Cμ gene, no JH sequences are attached to the Cδ1 domain and that an RNA splicing signal (CAG) occurs immediately adjacent to the 5' end of Cδ1. Utilising the M13HF28 DNA as a probe for restriction mapping [applying the Southern hybridisation procedure (26)], the Cδ1 domain was located approximately 5 kb from the end of Cμ4 (Fig. 1). The μ_m coding segments are therefore situated approximately equidistant from Cμ4 and Cδ1 domains.

Sequences Important for H Chain Class Switch

The H chain class switch has been demonstrated in mice to occur at a number of sites within the IVS between JH and Cμ and the 5' flanking regions of the various CH genes. Tandemly repeated sequences have been observed in these areas in mouse Cμ, Cγ1 and Cα genes (8,9) and a role in the switch for such sequences by homologous recombination has been suggested (9). Using a mouse DNA fragment containing a 20-nucleotide repeat sequence (mouse S probe), were able to identify and sequence

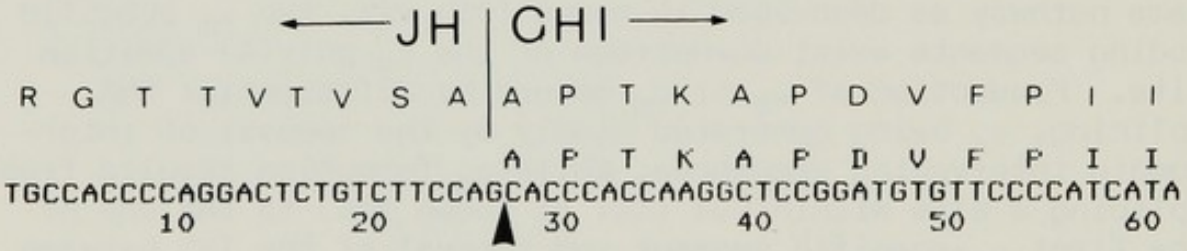

FIGURE 3. Partial sequence of M13HF28 allowing the identification of the human Cδ gene in the λC75 genomic clone. M13HF28 was isolated from a random selection of HinF clones in M13mp7, the nucleotide sequence derived and protein sequence derived in all six phases for comparison with human Cδ sequences. The amino acids are given as the one-letter code and the RNA splicing position at the 5' end of Cδ1 is shown. The top line refers to IgD protein sequence and the second line to the amino acid sequence derived from the nucleotide sequence of M13HF28.

an area of DNA lying about 3 kb upstream from the human Cμ gene (i.e. between JH and Cμ): the sequence of the human repeat is compared to the homologous mouse DNA repeat present in Figure 4. The basic unit of the human repeat is GGG$\frac{C}{T}$T $(GAGCT)_n$, as compared with a basic unit in the mouse S probe GGGGT $(GAGCT)_n$. The human repeat set does, however, show more heterogeneity, both of unit length and of sequence divergence, than occurs in the mouse. This segment has been designated the S sequence in mouse, and accordingly this has been marked as S in the map of the human Cμ gene locus (Fig. 1).

When either the mouse or the human μ repeat sequence was hybridised to a λ clone containing a human Cγ gene (λHγ4-6) we observed weak hybridisation compared with the hybridisation signal obtained with the region upstream of the human Cμ gene (compare Fig. 5, panel A and panel B lanes 1-3, which show the hybridisation of human or mouse S probes to the fragments upstream of Cμ in λC75 or λC76, with panel C lane 4 which shows the hybridisation of a mouse S probe to λHγ4-6). This result implies that the tandem repeat sequence near the Cμ genes is more conserved between species than those near Cγ genes. The fact that evolutionary conservation of these sequences has occurred between species and that these sequences occur in areas where the class switch occurs implies that they indeed play a functional role in the switch, particularly as in general intervening sequences drift much more rapidly than functional sequences (27). The functional role for such

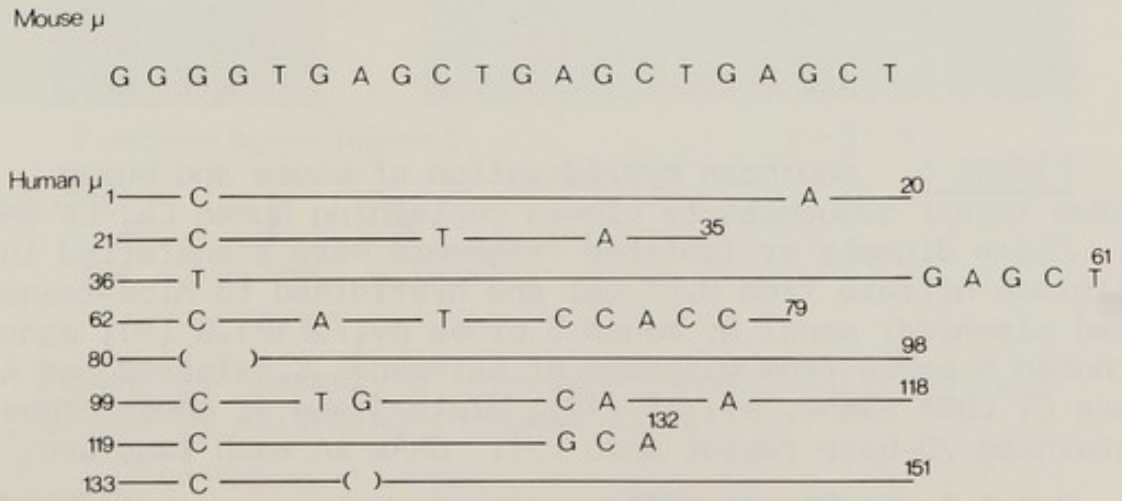

FIGURE 4. Comparison of the nucleotide sequence of tandemly repeated units near human and mouse Cμ genes. The tabulated comparison is between a repeat to the 5' side of human and mouse Cμ (7). The continuous line implies sequence identity and brackets represent positions of base deletions in the human sequence relative to those of mouse.

repeated sequences is likely to be mediation of homologous recombination by means of staggered and irregular alignment of these imperfectly homologous units allowing recombination across the IVS regions resulting in a CH switch.

Figure 5 also shows that neither the human (panel B, lane 4), nor the mouse S probe (panel C, lane 3) was capable of

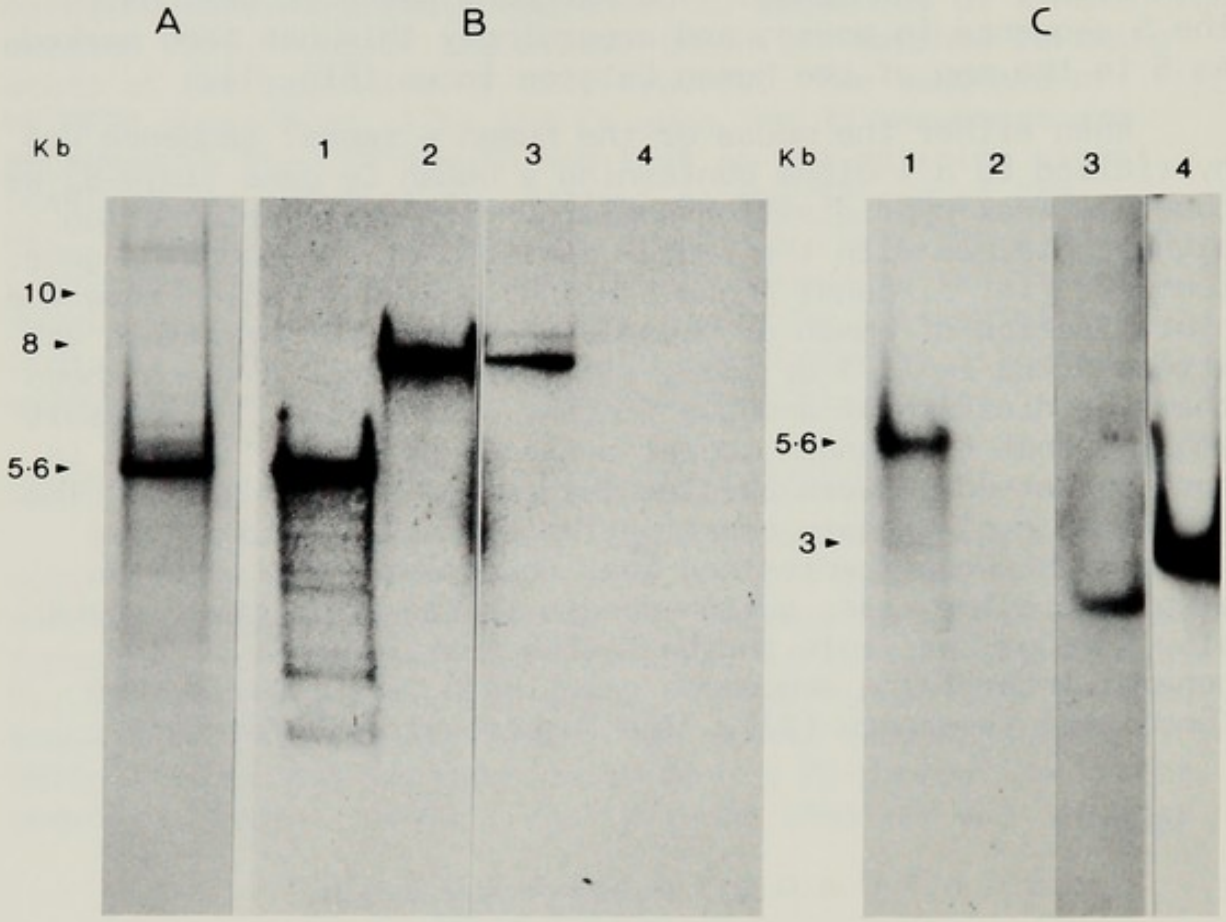

FIGURE 5. Southern hybridisation of mouse and human tandem repeat sequences to clones containing human Cμ, Cδ and Cγ. Clone digests or isolated fragments were transferred to cellulose nitrate from 0.8% gel and hybridised to nick-translated plasmids: panel A, mouse S probe $p\gamma_1$/2F1P1.8 (9); panel B, human S probe from upstream of Cμ; panel C, slots 2 and 4, mouse Cγ cDNA clone, $p\gamma_1$/A5 (18), slots 1 and 3, mouse probe containing 20-base repeat unit (9). DNAs in each lane are:

Panel A: EcoRI cut λC75
Panel B: lane 1, EcoRI cut λC75
2, EcoRI cut λC76
3, 8 kb EcoRI fragment from λC76
4, 10 kb EcoRI fragment (containing Cδ) from λC75
Panel C: lane 1/2, EcoRI cut λC75
3/4, PstI cut λHγ4-6

detecting a related sequence in regions of DNA between μ and δ genes. Furthermore, a JH probe that detected the human JH segments upstream from the Cμ gene failed to identify any JH sequences between Cμ and Cδ (T.H.Rabbitts, unpublished). Thus it seems unlikely that μ/δ switching occurs by either switch recombination or by sequential or simultaneous integration of a VH segment to Cμ and Cδ, but rather by RNA splicing involving alternative poly(A) addition sites.

Two dissimilar forms of class switching therefore appear to operate in human lymphocytes (Fig. 6). The first type, which involves alteration from membrane-bound to secreted μ, and which also appears to involve δ expression, can result from the formation of a nascent or complete RNA precursor molecule, including μ_m, μ_s and δ (δ_m and δ_s if the analogy with μ can be drawn): differential RNA termination or splicing can then generate μ or δ sequences with the identical VH sequence. This type of switching need not involve further DNA deletion (other than that presumed to occur when the VH integration events take place). The second type seems likely to involve the tandemly repeated type of sequence in homologous recombination to switch from, for example, Cμ to Cγ production with resultant DNA deletion in an analogous fashion to mouse DNA.

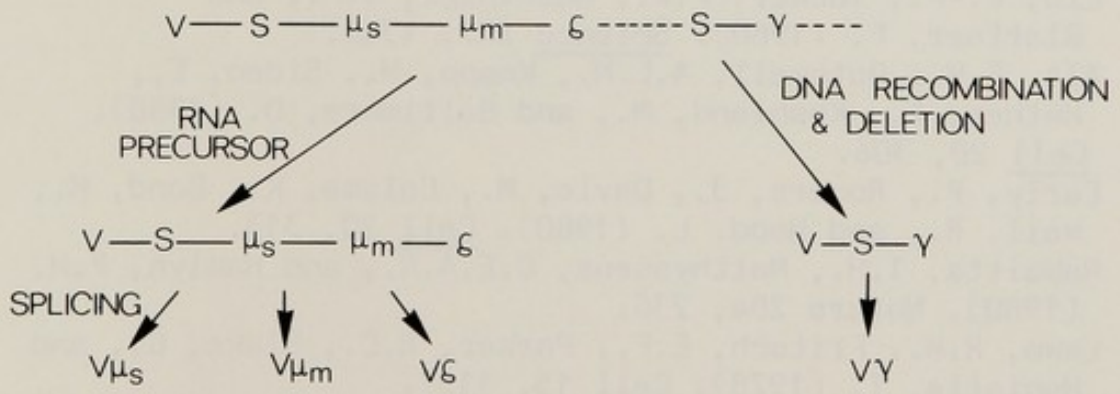

FIGURE 6. Summary of proposed events mediating $C\mu_s$, $C\mu_m$, Cδ and Cγ switching in human cells.

References

1. Cooper, M.D., Kearney, J.F., Lydyard, P.M., Grossi, C.E., and Lawton, A.R. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 139.
2. Pernis, B., Forni, L., and Luzzati, A.L. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 175.

3. Herzenberg, L.A., Herzenberg, L.A., Black, S.J., Loken, M.R., Okumura, K., van der Loo, W., Osborne, B.A., Hewgill, D., Goding, J.W., Gutman, G., and Warner, N.L. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 33.
4. Wang, A.C., Wilson, S.K., Hopper, J.E., Fudenberg, H.H., and Nisonoff, A. (1970). Proc. Nat. Acad. Sci. USA 66, 337.
5. Sledge, C., Fair, D.S., Black, B., Krueger, R.G., and Hood, L. (1976). Proc. Nat. Acad. Sci. USA 73, 923.
6. Davis, M.M., Calame, K., Early, P.W., Livant, D.L., Joho, R., Weissman, I.L., and Hood, L. (1980). Nature 283, 733.
7. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
8. Kataoka, T., Kawakami, T., Takahashi, N., and Honjo, T. (1980). Proc. Nat. Acad. Sci. USA 77, 919.
9. Dunnick, W., Rabbitts, T.H., and Milstein, C. (1980). Nature 286, 669.
10. Honjo, T., and Kataoka, T. (1978). Proc. Nat. Acad. Sci. USA 75, 2140.
11. Rabbitts, T.H., Forster, A., Dunnick, W., and Bentley,D.L. Nature 283, 351.
12. Coleclough, C., Cooper, D., and Perry, R.P. (1980). Proc. Nat. Acad. Sci. USA 77, 1422.
13. Cory, S., and Adams, J.M. (1980). Cell 19, 37.
14. Liu, C.-P., Tucker, P.W., Mushinski, J.F., and Blattner, F. (1980). Science 209, 1348.
15. Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980). Cell 20, 306.
16. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). Cell 20, 313.
17. Rabbitts, T.H., Matthyssens, G.E.A.R., and Hamlyn, P.H. (1980). Nature 284, 238.
18. Lawn, R.M.. Fritsch, E.F., Parker, R.C., Blake, G., and Maniatis, T. (1978). Cell 15, 1157.
19. Benton, W.D., and Davis, R.W. (1977). Science 196, 180.
20. Matthyssens, G.E.A.R., and Rabbitts, T.H. (1980). Nucleic Acids Res. 8, 703.
21. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980). J. Mol. Biol. 143, 161.
22. Watanabe, S., Barnikol, H.U., Horn, J., Bertram, J., and Hilschmann, N. (1973). Z. Physiol. Chem. 354, 1505.
23. Messing, J., Crea, R., and Seeburg, P.H. (1981). Nucleic Acids. Res., in press.
24. Marcu, K.B., Schibler, U., and Perry, R.P. (1979). Science 204, 1087.

25. Milstein, C.P., and Deverson, E.V. (1980). Immunology 40, 657.
26. Southern, E.M. (1975). J. Mol. Biol. 98, 503.
27. van den Berg, J., van Ooyen, A., Mantei, N., Schambock, A., Grosveld, G., Falvell, R.A., and Weissmann, C. (1978). Nature 276, 37.
28. Dunnick, W., Rabbitts, T.H., and Milstein, C. (1980). Nucleic Acids Res. 8, 1475.

Structure of HLA Antigens and Cloning of HLA Antigen Genes: a Brief Summary

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The major barrier to transplantation of tissues in various animals is what are called the class 1 histocompatibility antigens. These proteins are expressed on the surfaces of almost all cells of higher animals and are characterized by an unusual degree of polymorphism. Their major role is, of course, not in allograft rejection, but probably in the rejection of modified syngeneic cells, particularly in the elimination of virus-infected cells. In man, the genes encoding these antigens, the HLA-A, -B, and -C antigens, are located on chromosome 6, and in the mouse, genes for the corresponding H-2K, -D, and -L antigens are located on chromosome 17. Both of these sets of genes are encoded within a large region, perhaps 3,000 kilobase pairs in size, called the major histocompatibility complex. This region specifies a number of other proteins expressed on the surfaces of cells or involved in various immune functions.

The structure of these molecules is of great interest because only through this knowledge can the manner in which these molecules function be understood. During the past five years the complete primary structure of a human histocompatibility antigen and of a mouse histocompatibility antigen have been elucidated. Some information is also available about the secondary, tertiary, and quaternary structure. The antigen is composed of two chains, a heavy chain, a glycoprotein of about 44,000 daltons which contains two intrachain disulfide bridges; and a light chain, a peptide of about 12,000 daltons (β_2-microglobulin) which has one intrachain disulfide bridge. Each of these chains has a high content of β-pleated sheet structure. The chain interaction has many interesting features.

The primary amino acid sequence and other gross structural features have permitted the dissection of the

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heavy chain of these molecules into five regions. There are three extracellular regions of the heavy chain, α_1, α_2, and α_3, each approximately the size of an immunoglobulin domain. The N-terminal region, α_1, contains the only N-linked carbohydrate of the HLA molecule. In addition to this carbohydrate, a second one is attached in the α_2 segment of the H-2 heavy chain. α_2 and α_3 each have an intrachain disulfide bridge of about the same size as an immunoglobulin disulfide bridge. α_3 has very strong sequence homology to immunoglobulin C-region domains. In fact, statistical data indicate that the homology of α_3 to these domains is as high as the homology between different C-region domains. There is little doubt that α_3 and immunoglobulin domains have descended from the same ancestral gene. α_1 and α_2 have some sequence homology to each other and, therefore, probably arose by gene duplication, as in the case of immunoglobulin domains. However, neither α_1 nor α_2 has any sequence homology to immunoglobulin C or V region domains. α_3 is remarkably constant in structure when different H-2 or HLA antigens are compared, but α_1 and α_2 each contain regions of amino acid diversity (residues 65 to 80 in α_1 and residues 105 to 115 and 174 to 178 in α_2) which presumably contribute to the alloantigenic sites. Thus, α_1 and α_2 appear to be the regions of the molecule upon which evolutionary pressure has been placed and we assume that they have, therefore, diverged considerably from the ancestral genes from which they evolved, possibly the same ancestral gene which was involved in the evolution of α_3 and immunoglobulin domains. In addition to these three extracellular regions, the histocompatibility antigens contain a fourth region, a sequence of 25 hydrophobic amino acid residues which are located within the cell membrane and a fifth region of 31 hydrophilic amino acid residues which are located inside the membrane in the cytoplasm of the cell. One of the serine residues in the latter is phosphorylated and this fact may point to some function of this region. This division of the molecule into five regions is likely to be reflected in the organization of the genes encoding these antigens.

Several years ago a number of considerations focused attention on the use of gene cloning techniques to study structure of these antigens as well as the organization of the genes encoding them. In the first place, the DNA sequencing methods which had been developed made DNA sequencing a far more rapid means for obtaining primary structural information than protein sequencing, i.e., the DNA sequence can be decoded into the primary amino acid

sequence of the protein. Moreover, unlike the immunoglobulins, the histocompatibility antigens can be obtained only with difficulty, in small amounts, and at large expense. Therefore, it seemed likely that comparison of the primary sequences of a minimum of 20 molecules from analysis of the protein would be nearly prohibitive, although it might be obtained with much more facility from cloned DNA sequences. Such comparative information is necessary to localize firmly the sites and range of amino acid diversity which contribute to the high degree of polymorphism of these molecules and which must play an important role in their functions. In addition, a number of mutants or variants with altered function have been detected both in man and in mouse, and again localization of regions of alteration through DNA sequencing seemed an important way to determine the structures of these variant molecules. However, in addition to the need for knowledge about the primary amino acid sequences, there were other special reasons to pursue DNA cloning. The major histocompatibility complex itself is a region of large size which encodes a variety of genes involved in immune functions. A fairly large number of human diseases have been linked to one or another gene within this region. Thus, a study of the genetic organization of this region is likely to contribute greatly to understanding and analysis of these diseases, as the study of globulin gene clusters has contributed to understanding of the hemoglobinopathies. Moreover, the relationship of the genetic map of the MHC to a physical map needs to be elucidated. The organization of a single HLA-A, -B, or -C or H-2K, -D, or -L antigen within this region was also of considerable interest, particularly with respect to the question of whether or not, by analogy with other systems (e.g., immunoglobulins), the introns and exons of a single gene might correspond to the structural regions which had been defined.

The first approach to gene cloning was to establish a cell free system in which the heavy and light chains of these antigens were synthesized. The heavy chain was synthesized as a non-glycosylated precursor with a leader sequence of about 25 amino acid residues and could be processed by dog pancreatic microsomes to remove the leader sequence and add a glycan. The light chain precursor was processed simply by removal of its leader sequence. These studies provided the background and the reagents for cloning a cDNA sequence corresponding to an HLA antigen gene. First, polyadenylated mRNA was obtained on an oligo-dT cellulose column, and then partially purified by size fractionation

on an agarose gel. The fractions containing the HLA heavy chain poly-A mRNA were identified by cell free translation. They were used as templates for the synthesis of double stranded cDNA which was then dC tailed, and inserted into the Pst-cut and dG-tailed pBR322 vector. Recombinant DNA molecules were used to transform E. coli cells which were selected for tetracycline resistance. A clone containing a cDNA insert corresponding to an HLA antigen was identified by positive selection of HLA mRNA. Finally, to prove that the cDNA clone was an HLA antigen clone, the insert was excised and sequenced. The decoded amino acid sequence corresponded exactly to the last 46 residues of the HLA-B7 antigen, thus confirming that we had in fact isolated a cloned cDNA sequence corresponding to an HLA antigen.

Since publication of these data, the cloned cDNA probe has been used for a variety of purposes:

1. Southern blots have established that pHLA-1 hybridizes to a single major band after four different restriction enzyme digests of several different human DNAs. In addition to this major band, some 10 to 12 additional bands hybridize more weakly to the probe. These data suggested that the major band corresponded to an HLA-B antigen gene and that the minor bands corresponded to other genes or pseudogenes in the major histocompatibility complex which had sequence homology to the HLA-B antigen gene.

2. Further evidence in support of the concept that the probe might be relatively specific for the HLA-B antigen gene was provided in experiments employing DNA from radiation-induced mutations at the HLA-B locus (obtained from P. Kavathas and R. DeMars) in Southern blots. In a mutant in which expression of the HLA-B antigen of one haplotype had been lost, the intensity of the band was reduced to about one half relative to its parent. In a second mutant in which the expression of HLA-B locus antigen of both haplotypes had been lost, only a weakly hybridizing band remained at this position.

3. Northern blots established that only a single mRNA of about 1,640 base pairs hybridized strongly with pHLA-1. It is possible that a second minor mRNA species of about 1,620 base pairs hybridized more weakly to the probe.

4. Experiments carried out with human-mouse hybrid cell DNA have established that hybrids which retain chromosome 6 contain the major DNA fragment hybridizing with pHLA-1 and

most, if not all, of the minor bands found in the human parental DNA. No hybridization was seen in cell hybrids containing chromosome 15 in the absence of chromosome 6 or with mouse parental DNA under the hybridization conditions employed. (Experiments carried out by Dario Grossberger in collaboration with J. Barbosa, M. Kamarck, and F. Ruddle.)

5. Two different human genomic libraries constructed in phage lambda have been screened with pHLA-1. Four genomic clones were isolated from each library (by David Arnot from the second library). One set of four clones from an individual who has been HLA typed has been subjected to further study. Two of these clones hybridized strongly to pHLA-1 (suggesting that they may contain sequences corresponding to an HLA-B antigen) while the other two hybridize to pHLA-1 only weakly. The two strongly hybridizing clones are undergoing more extensive analysis. One of them (clone 1-3) contains only a single restriction fragment which hybridizes to the pHLA-1 although it contains several fragments which hybridize to pHLA-2 (our terminology for the clone isolated by Sood et al., see this volume). For example, Pst-cut DNA of clone 1-3 contains four restriction fragments of 1.5-2.5 kb, each of which hybridizes strongly to pHLA-2 and only one of these hybridizes to pHLA-1. DNA sequence analysis of these and other clones will tell us a great deal about the organization of an HLA antigen gene. Initial sequence analysis of clones 1-3 and 1-14B, the two strongly hybridizing clones, indicate that they share a region of about 400 base pairs of DNA sequence and differ in adjacent regions. It is possible that they represent two HLA-B locus alleles of the heterozygous individual from whom they were obtained.

No references have been provided, but a complete list and further details can be found in a recent review (1) as well as in two shorter reviews (2,3) and several recent papers (4,5).

References

1. Strominger, J.L., Engelhard, V.H., Fuks, A., Guild, B. C., Hyafil, F., Kaufman, J.F., Korman, A.J., Kostyk, T.G., Krangel, M.S., Lancet, D., Lopez de Castro, J.A., Mann, D.L., Orr, H.T., Parham, P.R., Parker, K.C., Ploegh, H.L., Pober, J.S., Robb, R.J., and Shackelford, D.A. (1981) In: The Role of the Major Histocompatibility Complex in Immunobiology M.E. Dorf, ed. (Garland Press, New York), pp. 115-172.

2. Strominger, J.L. (1980) In: Immunology 80. Progress in Immunology IV, M. Fougereau and J. Dausset, eds. (Academic Press, New York), pp. 541-554.

3. Ploegh, H.L., Orr, H.T., and Strominger, J.L. (1981) Cell, in press.

4. Ploegh, H.L., Orr, H.T., and Strominger, J.L. (1981) J. Immunol. 126, 270-275.

5. Ploegh, H.L., Orr, H.T., and Strominger, J.L. (1980) Proc. Natl. Acad. Sci. USA 77, 6081-6085.

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TRANSCRIPTION OF δ CHAIN GENES IN MOUSE MYELOMA CELLS AND NORMAL SPLEEN

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ABSTRACT Hybridization studies of the four δ chain RNAs found in IgD-secreting plasmacytomas and the two found in normal spleens indicate that different δ gene segments code for alternate 3' termini in the RNAs. These RNAs probably represent mRNAs for the different forms of heavy chain found in secreted and membrane-bound IgD.

INTRODUCTION

IgD, although present in very small quantities in serum, is found on the surface of most B lymphocytes and, hence, is thought to play a major role in the control of B cell function. The recent identification of two murine plasmacytomas, TEPC 1017 and TEPC 1033, which synthesize and secrete IgD (1), has provided a model system for the study of IgD expression.

A cDNA clone, pδ54J, containing a major portion of the mouse δ chain constant region derived from mouse myeloma TEPC 1017 mRNA has been constructed, characterized (2, 3), and used to identify genomic sequences in a mouse liver DNA library (4). The δ gene has been shown to be comprised of exons which encode two constant region domains, an exon encoding the hinge separating the two domains, and at least two exons which may encode alternate 3' termini for δ chain protein.

The cDNA clone, pδ54J, has been used to identify the δ RNAs present in plasmacytomas by hybridization of RNAs separated by electrophoresis on methyl mercury hydroxide agarose

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gels; one major and three minor RNA species have been observed in TEPC 1017 RNA (2). The demonstration of these multiple RNA species prompted an investigation of their relationship to one another as well as a comparison with RNAs synthesized by TEPC 1033 and by BALB/c spleen cells. The cDNA clone and subcloned fragments of the δ gene have been used to probe the structure of these RNAs and to examine the processing pathways that result in their production. Some of these hybridization studies and the initial characterization of the RNAs synthesized by these cells are presented in this communication.

MATERIALS AND METHODS

RNA Preparation

Cytoplasmic RNA was prepared from TEPC 1017 and TEPC 1033 cells as described (2). Total cellular RNA was prepared by homogenizing pulverized, frozen tumors or BALB/c spleens in 4 M guanidinium thiocyanate and precipitating with ethanol (5).

Subcloning of Genomic DNA Fragments

Fragments of genomic DNA indicated in Figure 1 were subcloned between the Xba I and Hind III sites of plasmid pACYC184 (6) (AC probe) or into the Bam Hl site of plasmid pBR322 (7) (DC, VDC, and VVDC probes). Prior to nick-translation (8), the subcloned genomic DNA fragments were prepared (9) from restriction enzyme-digested plasmid DNA which had been fractionated on acrylamide gels.

Methyl Mercury Hydroxide Agarose Gel Electrophoresis

As described previously (2), 5 μg RNA samples were treated with methyl mercury hydroxide, subjected to electrophoresis on 1% agarose gels containing 5 mM methyl mercury hydroxide, transferred to diazophenylthioether paper, and hybridized with nick-translated DNA probes as indicated in the legend to Figure 2.

RESULTS

TEPC 1017 poly(A)$^+$ RNA contains four RNA species which hybridize with pδ54J DNA (2). The major RNA species contains approximately 1750 nucleotides (NT); the three larger species contain approximately 2100, 2900, and 3200 NT. Because TEPC 1017 secretes large amounts of δ protein and has little surface δ, it seemed likely that the 1750 NT RNA species represented mRNA encoding secreted δ protein and that one or more of the less abundant RNA species might represent mRNA encoding membrane-bound δ protein. To test this hypothesis and to search for the putative membrane terminus, the δ gene was subfractionated, and genomic fragments were subcloned in plasmids. The origin of the subcloned fragments is indicated schematically in Figure 1. AC indicates adjacently coded; DC, distantly coded; VDC, very distantly coded; and VVDC, very, very distantly coded.

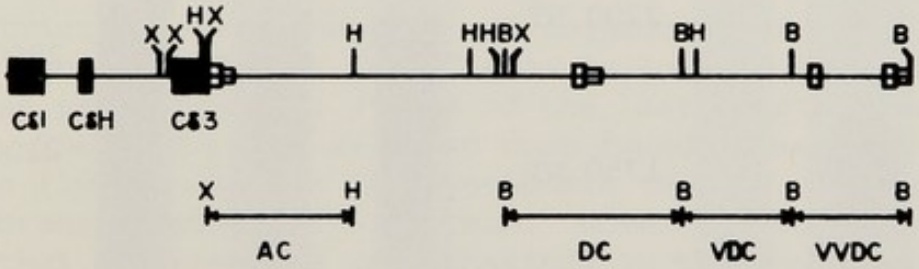

Figure 1. Restriction map showing the BALB/c δ gene (4) and the genomic fragments subcloned from it. B = Bam H1, H = Hind III, and X = Xba I.

The results of hybridization of ^{32}P-labelled DC and VVDC DNA to poly(A)$^+$ RNA prepared from TEPC 1017, TEPC 1033, and BALB/c spleen are shown in Figure 2. The DC probe hybridized to both a 1750 NT and a 3200 NT RNA species in TEPC 1017 and TEPC 1033; only a faint, blurred hybridization pattern to higher molecular weight RNA species was seen in the spleen RNA. The VVDC probe hybridized to a 2100 and to a 2900 NT RNA species in the two tumors. In spleen RNA, a 2900 NT RNA species clearly hybridized with VVDC, and a more faintly hybridizing 3200 NT RNA species also was observed.

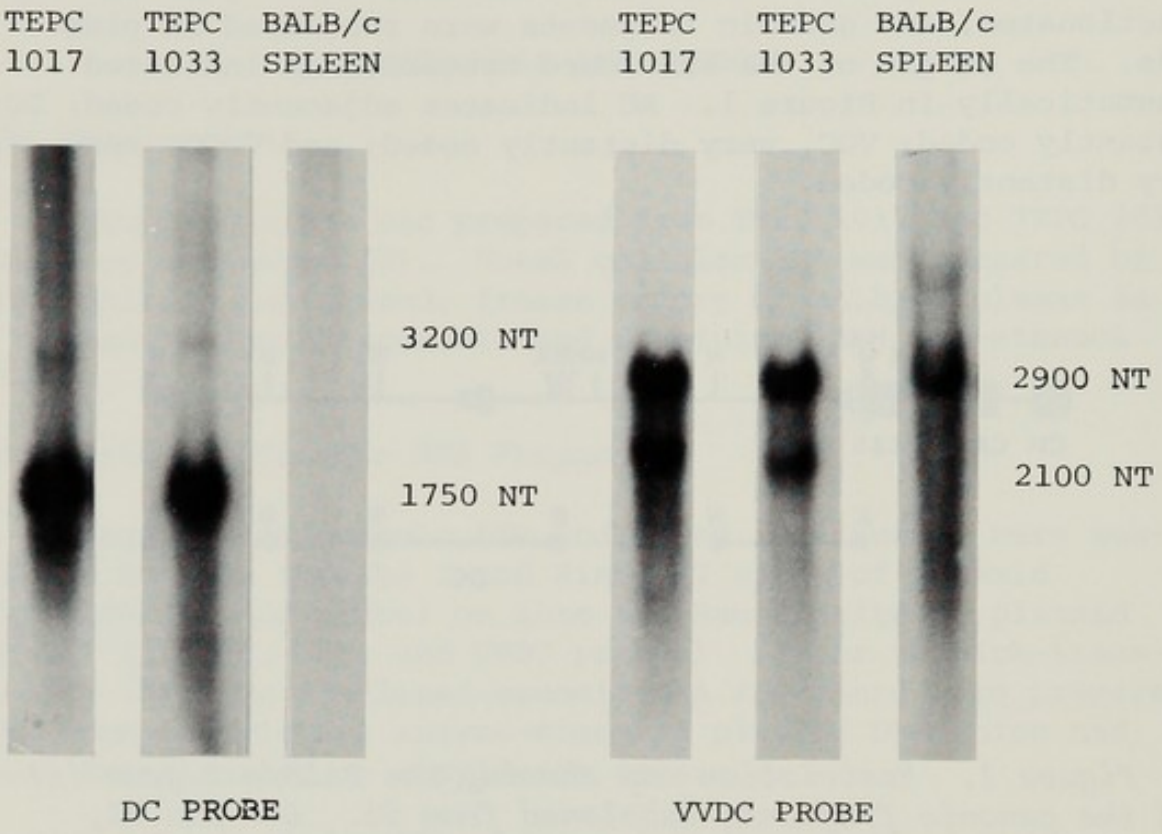

Figure 2. Methyl mercury hydroxide agarose gel electrophoresis of poly(A)$^+$ RNA prepared from TEPC 1017, TEPC 1033, and BALB/c spleen followed by transfer to diazophenylthioether paper and hybridization with ^{32}P-labelled DC probe (panel DC) and VVDC probe (panel VVDC). Approximate sizes of RNA species (in nucleotides) are indicated to the right of each panel.

Hybridization data for the AC, DC, VDC, and VVDC probes is summarized in Table 1.

TABLE 1

	AC	DC	VDC	VVDC
TEPC 1017	*3200 NT*	*3200 NT*	----	*2900 NT*
	2900 NT	*1750 NT*	----	*2100 NT*
TEPC 1033	*3200 NT*	*3200 NT*	----	*2900 NT*
	2900 NT	*1750 NT*	----	*2100 NT*
BALB/c Spleen	----	----	----	*3200 NT*
	----	----	----	*2900 NT*

DISCUSSION

Restriction enzyme mapping and sequencing of the δ gene (3, 4) demonstrated that the DC exon is located approximately 4600 base pairs from the 3' end of the last constant region domain. The μ membrane exon had been found in an analogous position (10), prompting the hypothesis that DC might encode the membrane terminus of δ protein. However, the determination that DC sequences are contained in the most abundant δ RNA species in both TEPC 1017 and TEPC 1033 suggested instead that the DC sequence encodes the carboxyl terminus of secreted δ protein. The lack of demonstrable DC sequences in spleen RNA and the determination that the nucleotide sequence (3) of the DC exon encoded an amino acid sequence of limited hydrophobicity further suggested that DC was not membrane related.

A renewed search for the exon encoding the postulated δ membrane terminus resulted in the hybridization data presented above. Initially it was postulated that the AC genomic fragment, found between the C_δ gene segments and DC, might contain a membrane sequence and that the organization of the two termini might be reversed as compared with that for the μ gene. However, this apparently is incorrect. The AC probe has been shown to hybridize only to the 2900 NT and the 3200 NT RNA species in the tumors, and little if any hybridization is seen with spleen RNA.

When genomic fragments on the 3' side of the DC fragment were used as hybridization probes, the VDC probe did not hybridize at all, but VVDC sequences were found in RNA species which did not contain DC sequences.

Both the 2100 NT and the 2900 NT tumor δ RNAs hybridized with the VVDC probe. In spleen RNA, the VVDC probe hybridized with the 2900 NT RNA species but no 2100 NT RNA species was observed. The strong hybridization of the VVDC probe to this spleen poly(A)$^+$ δ RNA is consistent with the hypothesis that VVDC exons encode a membrane terminus. Preliminary nucleotide sequence data is also consistent with this possibility.

The 2900 NT δ RNA species may represent the mature mRNA encoding normal spleen membrane δ. Since this RNA species appears identical in spleen and tumors, the tumors represent very useful models for the study of the regulation of membrane δ biosynthesis as well as secreted δ biosynthesis.

The hybridization data underline a potentially significant difference between the tumors and normal spleen. The 3200 NT δ RNA species observed in spleen RNA is different from that seen in the tumors. It contains VVDC sequences but not DC sequences; the reverse is true of the 3200 NT δ RNA species derived from the tumors. This difference in sequence content may indicate a tumor-specific difference or may reflect the relative infrequency of normal spleen cells differentiated to produce secreted IgD.

The processing pathways for generating mature mRNAs for secreted and membrane-bound δ chain are being studied. It is possible that combinations of different exons may result in the production of more than one type of membrane-bound δ chain. It is also possible that neither the 3200 NT nor the 2900 NT RNA species represent mature mRNAs. Rather they may be precursors of the 1750 NT and 2100 NT RNA species in the myelomas. Studies are in progress to determine the biological significance of these different RNAs in the tumors and in normal spleens.

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REFERENCES

1. Finkelman, F. D., Kessler, S. W., Mushinski , J. F., and Potter, M. (1981). *J. Immunol.* 126, 680.

2. Mushinski, J. F., Blattner, F. R., Owens, J. D., Finkelman, F. D., Kessler, S. W., Fitzmaurice, L., Potter, M., and Tucker, P. W. (1980). *Proc. Natl. Acad. Sci.* 77, 7405.
3. Tucker, P. W., Liu, C. P., Mushinski, J. F., and Blattner, F. R. (1980). *Science* 209, 1353.
4. Liu, C. P., Tucker, P. W., Mushinski, J. F., and Blattner, F. R., (1980). *Science* 209, 1348.
5. Chirgwin, J. M., Przybyla, A. E., MacDonald R. J., and Rutter, W. J. (1979). *Biochemistry* 18, 5294.
6. Chang, A. C. Y., and Cohen, S. N. (1978). *J. Bacteriol.* 134, 1141.
7. Bolivar, F., Rodriquez, R. L., Green, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. (1977). *Gene* 2, 95.
8. Rigby, P. J. W., Dieckmann, M., Rhodes, C., and Berg, P. (1977). *J. Mol. Biol.* 113, 237.
9. Maxam, A., and Gilbert, W. (1980). *Methods Enzymol.* 65, 499.
10. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). *Cell* 20, 313.

THE ROLE OF GENERALIZED RECOMBINATION IN IMMUNOGLOBULIN GENE EXPRESSION[1]

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We have isolated a number of variants of the MPC 11 mouse myeloma cell line which synthesize altered immunoglobulin heavy chains. In contrast to the parent γ2b heavy chain, several variant chains have serological characteristics of the γ2a subclass, indicative of the expression of previously unexpressed genetic information. Although some "γ2a" variant chains may have the complete γ2a constant region, others are actually γ2b-γ2a hybrid chains. For one such variant, the junction between γ2b and γ2a sequences has been shown to lie within the C_H2 domain. This junction comprises 24 amino acids which are identical both at the protein and nucleic acid level between γ2b and γ2a subclasses and therefore provides a large region of sequence identity which can serve as a target for recombination events. Efforts to determine whether recombination has taken place at the gene level are in progress. We have noted that there exists within the γ2b gene a DNA sequence, Chi, which in E. coli, facilitates generalized recombination. This same sequence occurs in V_HIII of mouse and human heavy chains, in the 3' mouse κ constant region, and was generated during the heavy chain class switch which gave rise to MC 101. Since the frequency of Chi is higher in immunoglobulin genes than in bulk DNA, we, therefore, consider: 1) whether the Chi sequence that works in E. coli plays a similar role in eukaryotic cells 2) whether Chi could play a particular role in immunoglobulins and 3) how homologous recombination may be related to unique features of immunoglobulin gene expression, in particular, the heavy chain class switch.

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INTRODUCTION

The mechanisms by which a eukaryotic cell governs the synthesis, assembly, and secretion of a molecule such as an immunoglobulin are clearly complex and intriguing. One way to gain insight into these mechanisms is to induce mutations and characterize the resulting variant cell.

We have utilized the MPC11 mouse myeloma cell line as a model system. From it, we have isolated several variants which synthesize altered immunoglobulin heavy chains. Characterization of these variant chains has given us insight into the process of mutation in eukaryotic cells, the mechanism of the heavy chain class switch, and the role of generalized recombination in immunoglobulin gene expression.

RESULTS AND DISCUSSION

Isolation of Variants

Primary variants, for the most part, have been isolated after treatment of the parent cell line with mutagens, such as Melphalan, a phenylalanine mustard used to treat human myeloma, and ICR-191, an acridine mustard. Following exposure to the drug, cells were cloned in soft agar and overlayed with antisera against the MPC 11 γ_{2b} heavy chain or Fc region. The secreted immunoglobulin from parental "stained" clones is detected in the form of a precipitate which looks, microscopically, like pepper dots over the clone. By definition, "unstained" clones are variants and include clones which have lost heavy chain synthesis completely as well as those making altered heavy chains. We have focused our studies entirely on the latter group. Altered heavy chains include those which are shorter than the parent (1) and others which have sero-

logical and structural determinants characteristic of the γ2a subclass (2,3). From certain primary variants, which may have a built-in instability, we have isolated spontaneously arising secondary variants (4). The variant families are depicted in Figure 1.

Primary Structural Studies

Primary structural studies of γ2a (MOPC 173) (5) and γ2b (MPC 11) (6,7) heavy chains and nucleic acid sequence determination of the corresponding germ line genes (8,9,10,11) have led to a complete description of these immunoglobulins. These studies from many laboratories as well as our own have laid the foundation for examining several variant chains.

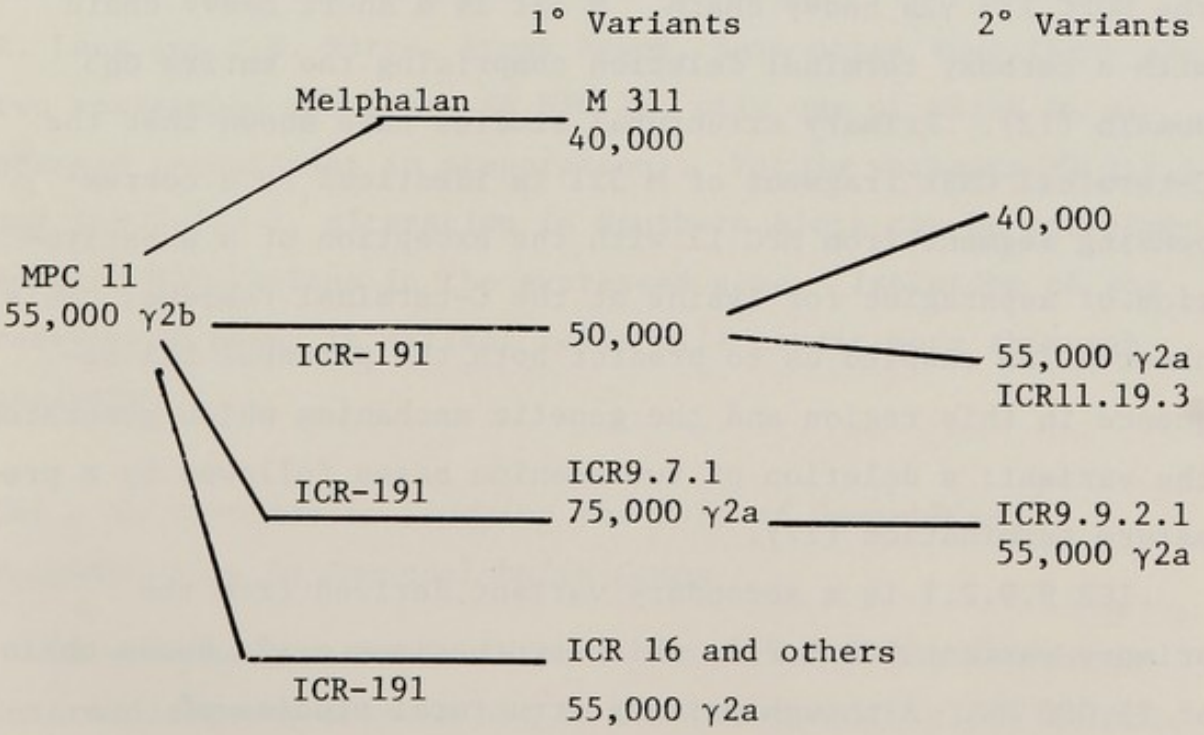

FIGURE 1. MPC 11 and variants derived from it: size and serological characteristics of heavy chain.

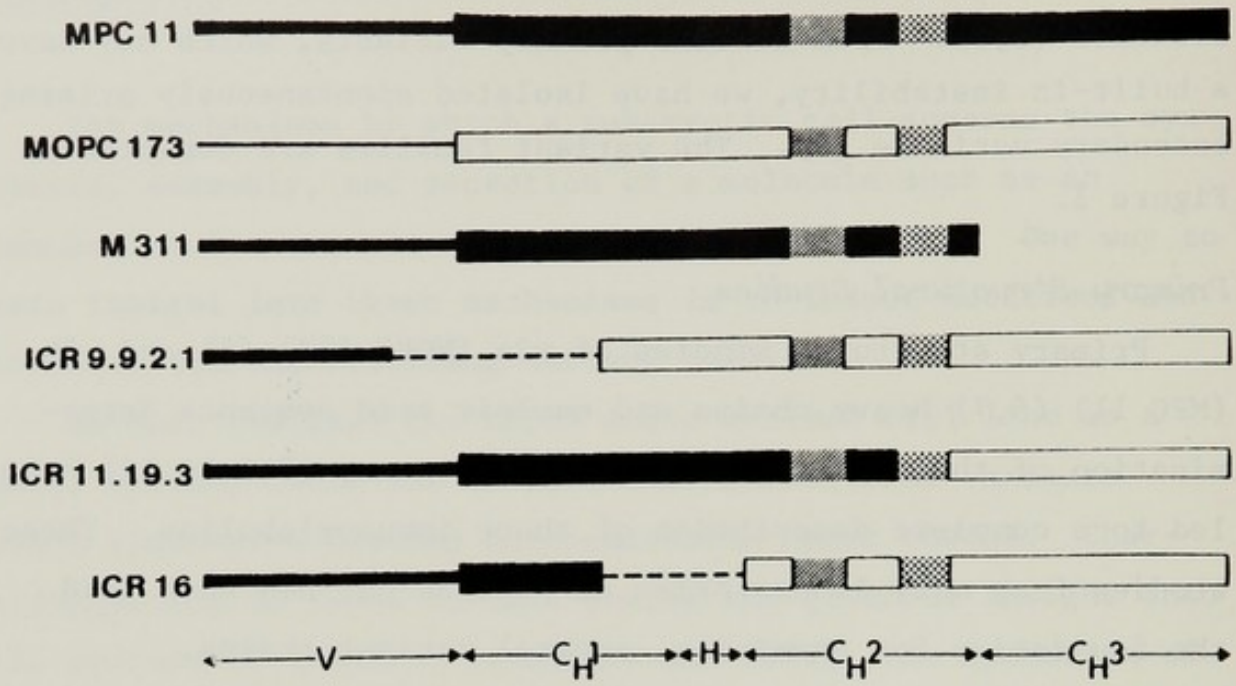

FIGURE 2. Schematic of the heavy chain of MPC 11 and several variants as compared with MOPC 173.

Figure 2 is a schematic of the structural studies of four variant chains, compared to the parental γ2b MPC 11 chain and the MOPC 173 γ2a heavy chain. M 311 is a short heavy chain with a carboxy terminal deletion comprising the entire C_H3 domain (12). Primary structural studies have shown that the C-terminal CNBr fragment of M 311 is identical to a corresponding segment from MPC 11 with the exception of a substitution of asparagine for lysine at the C-terminal residue. This observation enabled us to predict both the parental DNA sequence in this region and the genetic mechanism which generated the variant: a deletion of two adenine bases followed by a premature termination (12).

ICR 9.9.2.1 is a secondary variant derived from the primary variant ICR 9.7.1, which synthesizes a γ2a heavy chain of 75,000 MW. Although primary structural studies of ICR 9.9.2.1 are incomplete, it very likely represents a complete γ2a heavy chain of normal size (6).

Both ICR 16 and ICR 11.19.3 make γ2b-γ2a hybrid heavy chains. In ICR 16, the junction between the two sequences lies within a CNBr fragment that contains the hinge region and portions of both C_H1 and C_H2 domains. ICR 11.19.3 is one of a group of four variant molecules with γ2b-γ2a hybrid heavy chains which are distinct from ICR 16 and essentially identical to each other in terms of charge and serological markers (3). In ICR 11.19.3, the junction has been localized within the C_H2 domain (7). This junction comprises 24 amino acids and 72 nucleotides which are identical in γ2b and γ2a sequences, thus providing a potential site for recombination events.

DNA Rearrangements in Variant Cells

Efforts to determine whether recombination has taken place at the gene level are in progress. Examination of genomic DNA by Southern blots using probes for γ2b and γ2a genes has indicated that rearrangements are occurring. Very recently R. Lang and K.B. Marcu, Stony Brook, have noted that there are two rearranged γ2b genes in MPC 11, only one of which is expressed (manuscript in preparation). For the variants ICR 9.7.1 and ICR 9.9.2.1, alteration in Southern blots can be correlated with alterations in the expressed gene. Isolation of the rearranged genes in variant cells will permit more thorough analysis.

Chi - An Approach to Studying the Role of Generalized Recombination in Immunoglobulin Genes

A working hypothesis is that our variants may represent intermediates or culs de sac in the heavy chain class switch, or utilize mechanisms integral to that process. As noted previously, variants making γ2b-γ2a hybrid chains have arisen relatively frequently, and most of them have their γ2b-γ2a

junction in a region of sequence identity for the γ2b and γ2a subclasses. This fact suggests that generalized recombination may play a major role in Ig gene expression, particularly in the heavy chain class switch, and has led us to investigate this possibility.

Recombination has been studied in detail using lambda phage, and we were attracted by this well-characterized system as a model for studying the apparently more complex mechanism in eukaryotic cells. Dr. Julius Marmur introduced us to the observation of Dr. Frank Stahl and colleagues on Chi, a genetic element which, in lambda phage, promotes generalized recombination over a distance of five to ten kilobases (13,14). Although absent in wild type lambda, Chi can arise at several sites by a single base change: Dr. Gerald Smith has found the nucleotide sequence of Chi to be $\begin{matrix}5'\text{GCTGGTGG}5'\\3'\text{CGACCACC}5'\end{matrix}$ (15,16). A DNA sequence notably homologous to Chi, GCTGGATGG, occurs at the junction between γ2b and γ2a sequences in ICR 11.19.3: this observation initiated our studies on Chi. Although in lambda phage this nine-mer lacks Chi activity (16), a perfect Chi octamer exists on the nontranscribed strand in the C_H3 domain of the γ2b constant region at nucleotides 1373-1380. A lambda vector which contains the γ2b germ line gene, constructed by Dr. Kenneth Marcu, Stony Brook (17), has Chi activity, which indicates that Chi can function even when embedded in the context of immunoglobulin DNA.

A search of DNA sequences in the literature showed Chi sequences to be present in the 3' flanking region of the mouse C_k gene (18), and in the V_{HIII} subgroups of mouse myelomas S107 (19) and M603 (19) and the human hybridoma SP1/HL (20) at a position corresponding to amino acid residues N-3 to 6 of FR1. In addition, a Chi sequence is present in the switch DNA of the γ1 producing myeloma MC101 (21). The frequency of Chi

in the immunoglobulin genes is calculated to be once in 3 kb. When a library of total mouse genomic DNA was screened for Chi activity, the frequency was found to be once in 17 kb. Thus, Chi seems to be more frequent in immunoglobulin genes than in bulk DNA.

It is important to note that, currently, the only assay for Chi activity involves the infection of E. coli with lambda in a prokaryotic system, and thus one is stymied from concluding that Chi sequences in eukaryotic DNA similarly facilitate generalized recombination. However, an examination of the location of Chi sequences in immunoglobulin genes may permit speculation about their role. For example, it is of interest that proteins of the human V_{HIII} subgroup constitute one-half of serum immunoglobulins (22). Perhaps Chi serves to preferentially expand the number of genes of this subgroup or to promote its expression.

As noted, it may be that the γ2b-γ2a hybrid chains are the molecular consequences of an aberrant attempt at the heavy chain class switch, and it is quite possible that the Chi sequence in the γ2b constant region gene has promoted their generation. Although of the heavy chain constant region genes only the γ2b gene has been shown to contain Chi, the observation that Chi was formed in the rearrangement of switch (S) DNA, leading to the expression of the γ1 heavy chain in the MC101 myeloma, suggests a more general role. Figure 3 shows how Chi was generated in MC101 and illustrates that the elements of Chi are present in switch DNA 5' to all the immunoglobulin genes. It is therefore possible to generate a Chi sequence in almost every class switch although of the four switch regions sequenced in rearranged genes, a Chi sequence has been formed in only one, MC101. Since Chi stimulates homologous recombination, it may be that, if it is formed, it can facilitate subsequent heavy chain class switches.

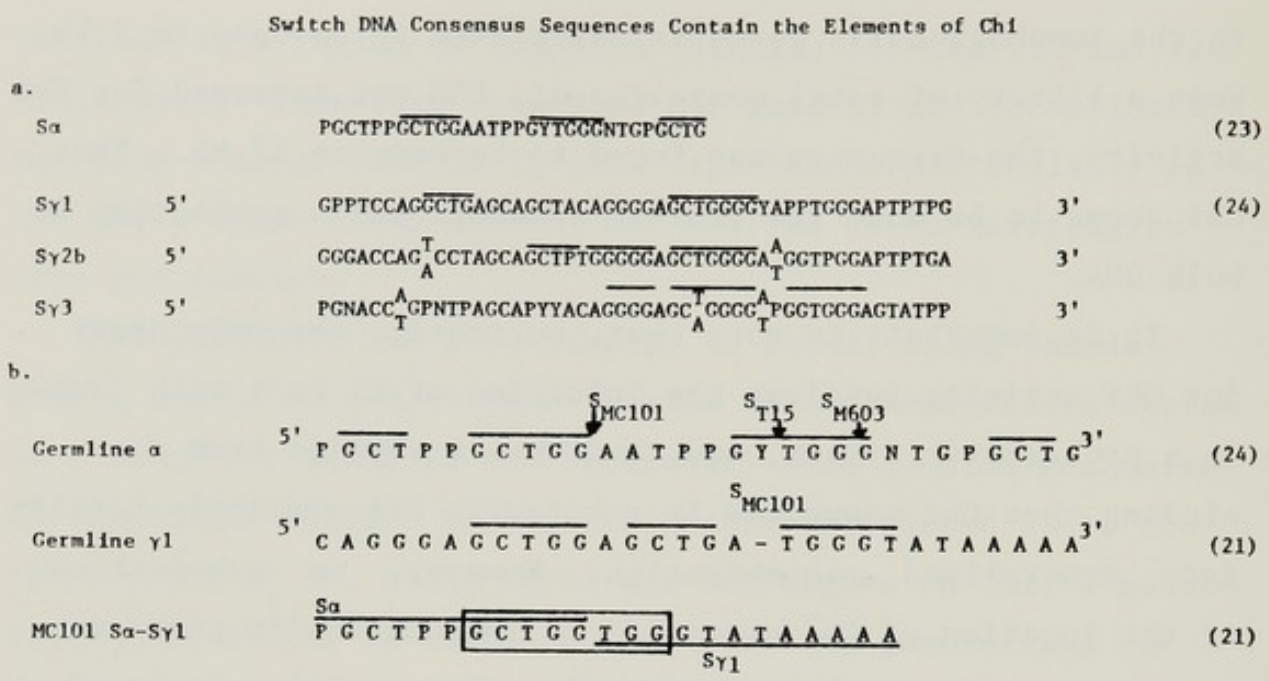

FIGURE 3. a) Sμ and Sα are notably homologous to each other and contain blocks of repetitive sequences which frequently repeat the first seven out of eight Chi nucleotides. Comparison of the γ family S DNA to Sμ and Sα shows that the short sequences AGCT, TGGG and AGCTGGG occur at the recombination sites and are shared among all S DNA types. Therefore, Chi can potentially be formed in any heavy chain class switch just as it was in MC 101. b) A Chi sequence was formed at the juxtaposition of Sα and Sγ1 in the myeloma MC101 which could have been facilitated by Chi.

Figure 4 illustrates how an intrachromosomal recombination intermediate may be stabilized by homologies shared by the constant region genes and by the switch DNAs. Although Chi might increase the frequency of such an intermediate, it is not necessary for its formation. Resolution of this intermediate could lead to the formation of hybrid chains or to a complete heavy chain class switch.

REFERENCES

1. Birshtein, B.K., Preud'homme, J.-L. and Scharff, M.D. (1974). *Proc. Natl. Acad. Sci. USA 71*, 3478.
2. Preud'homme, J.-L., Birshtein, B.K. and Scharff, M.D. (1975). *Proc. Natl. Acad. Sci. USA 72*, 1427.
3. Francus, T., Dharmgrongartama, B., Campbell, R., Scharff, M.D. and Birshtein, B.K. (1978). *J. Exptl. Med. 147*, 1535.

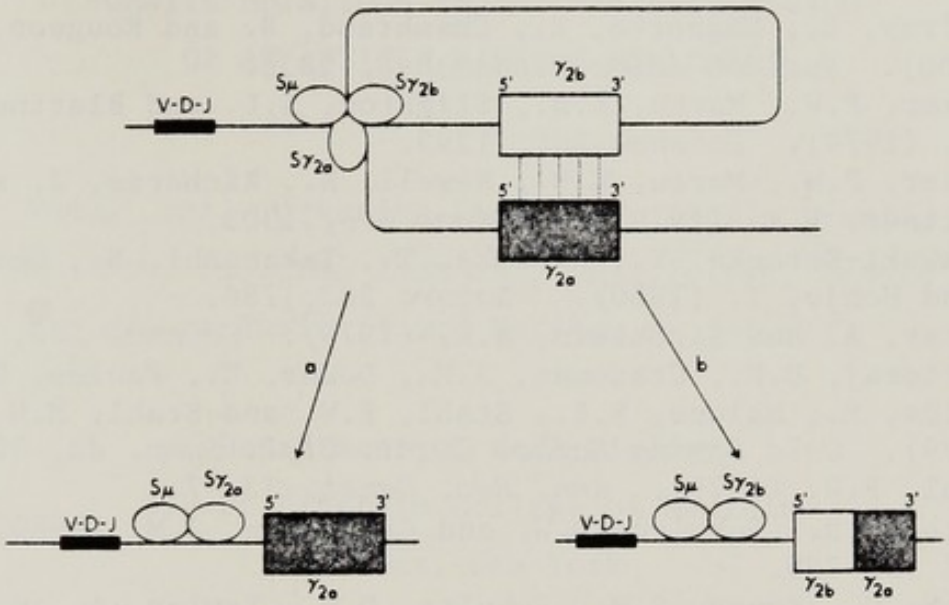

FIGURE 4. Proposed intermediate in heavy chain class switch. The example is based on the MPC 11 variant ICR11.19.3 and shows that Sμ, Sγ2b and Sγ2a switch region DNA are in close proximity. The whole structure is presumed to be stabilized by homologies in the various switching regions and particularly by homologies amongst the constant region genes. Chi may promote but is not necessary for the generation of this intermediate.

a. Hypothetical switching enzymes are induced and recognize, bind to and cause recombination in switch DNA resulting in a new variable region-constant region combination.

b. Putative recombination enzymes are activated which recognize and act on DNA homologies to promote generalized recombination between the constant regions resulting in a hybrid constant region.

4. Koskimies, S. and Birshtein, B.K. (1976). *Nature (London) 246*, 480.
5. Fougereau, M., Bourgois, A., de Preval, C., Rocca-Sera, J., and Schiff, C. (1976). *Ann. Immunol. (Inst. Past.) 127C*, 607.
6. Francus, T. and Birshtein, B.K. (1978). *Biochemistry 17*, 4331.
7. Birshtein, B.K., Campbell, R. and Greenberg, M.L. (1980). *Biochemistry 19*, 1730.

8. Auffray, C., Nageotte, R., Chambraud, B. and Rougeon, F. (1980). *Nucleic Acids Research 8*, 1231.
9. Tucker, P.W., Marcu, K.B., Slightom, J.L. and Blattner, F.R. (1979). *Science 206*, 1299.
10. Tucker, P.W., Marcu, K.B., Newell, N., Richards, J. and Blattner, F.R. (1979). *Science 206*, 1303.
11. Yamawaki-Kataoka, Y., Kataoka, T., Takahashi, N., Obata, M and Honjo, T. (1980). *Nature 283*, 786.
12. Kenter, A. and Birshtein, B.K. (1979). *Science 206*, 1307.
13. Chattoraj, D.K., Craseman, J.M., Dower, N., Faulds, D., Faulds, P., Malone, R.E., Stahl, F.W. and Stahl, M.M. (1979). *Cold Spring Harbor Quant. Biol. Symp. 43*, 1063.
14. Stahl, F.W. (1979). *Ann. Rev. Genet. 13*, 7.
15. Smith, G.R., Schultz, D.W. and Grasemann, J.M. (1980). *Cell 19*, 785.
16. Smith, G., Kunes, S.M., Schultz, D.W., Taylor, A. and Triman, K.L. (1981). *Cell* (in press).
17. Marcu, K. et al. (1980). *Cold Spring Harbor Quant. Biol. Symp.* (in press).
18. Hamlyn, P.H., Brownlee, G.G., Chen, C., Gait, M.J. and Milstein, C. (1978). *Cell 15*, 1067.
19. Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. (1980). *Cell 19*, 981.
20. Rabbitts, T.H., Mattyssens, G. and Hamlyn, P.H. (1980). *Nature 284*, 238.
21. Kataoka, T., Kawakanii, T., Takahashi, N. and Honjo, T. (1980). *Proc. Natl. Acad. Sci. (USA) 77*, 919.
22. Forre, O., Natvig, J.B. and Kunkel, H.G. (1976). *J. Exptl. Med. 144*, 897.
23. Davis, M.M., Kim, S.K. and Hood, L.E. (1980). *Science 209*, 1360.
24. Kataoka, T., Mugata, T. and Honjo, T. (1981). *Cell 23*, 357.

SOMATIC MUTATIONS IN THE VARIABLE REGION OF AN ANTIGEN BINDING MYELOMA[1]

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Mutations which result in changes in immunoglobulin production and structure arise frequently in cultured mouse myeloma cells and hybridomas. The S107 cell line, which synthesizes an IgA k antibody that binds phosphocholine and bears the T-15 idiotype, was examined for mutants with changes in their ability to bind antigen. Such mutants arise spontaneously and frequently. These changes in antigen binding are associated with single amino acid substitutions in the heavy chain variable region. The mutation rate for one of these events is $\sim 10^{-4}$*/cell/generation.*

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INTRODUCTION

The role of somatic mutation in the generation of antibody diversity has been vigorously debated for years and still remains unresolved. The discovery that immunoglobulin genes are composed of separate pieces of DNA temporarily focused attention on the role of gene rearrangement in generating amino acid sequence diversity in the third hypervariable region of both the light and heavy chain. The results summarized elsewhere in this monograph leave little doubt that a very large number of antibody molecules bearing different sequences in their third hypervariable region arise through the recombination of germ line variable region genes with four J (joining) segments in mouse kappa (κ) light chains and with a limited number of D (diversity) and J gene segments in mouse heavy chains. Even though these rearrangements at the DNA level could in principle generate a sufficient number of antibody molecules, they do not explain the amino acid diversity which exists in the first and second hypervariable regions. Furthermore, it is still not clear whether the sequence diversity in the third hypervariable region plays a crucial role in determining specificity in the recognition of antigen by all or even most antibody molecules.

The original work on mouse lambda (λ) light chains (1,2) suggested that some mechanism which produced what appeared to be point mutations was also important in producing the sequence diversity found in antibody molecules. For λ_1 light chains this has been confirmed at the DNA level by the observation that the mouse germ line contains only one V and one J region capable of coding for λ_1 light chains while λ_1 genes and λ_1 proteins with sequence changes are found in plasma cells (1,2,3). A number of workers have determined either the amino acid sequences or the nucleotide sequences of germ line

V region genes and compared them to the rearranged progeny of these genes as they are expressed in myelomas or hybridomas. The importance of this sort of pedigree analysis has recently been discussed in detail by Mel Cohn and his colleagues (4) and specific examples are presented elsewhere in this volume. These studies reveal that just as with mouse λ chains, κ light chain (5) and heavy chain (6) germ line genes undergo base substitutions in their first and second hypervariable regions and probably in their framework residues as well. Such pedigree analysis should ultimately provide information on the mechanism of these changes and on their relative importance in antigen recognition.

An alternative and complementary approach to in vivo pedigree analysis is to take an antibody forming cell growing in tissue culture and to identify and analyze progeny of this cell which are expressing antibody molecules that have undergone changes in their amino acid sequence. While this somatic cell genetic approach is even more artificial than the analysis of antibodies produced by hybridomas and myelomas, it does provide information which is not otherwise available. First it is possible to determine the exact rate at which individual amino acid substitutions are arising. Second, it is possible, at least in principle, to recover all of the progeny of the putative mutational events including those which would be missed in the analysis of hybridomas or normal antibodies because they no longer bind antigen. An example of this will be provided below. Third, it is possible to study the mechanisms responsible for the changes by manipulating the cells in vitro.

RESULTS AND DISCUSSION

For many years we and others have been carrying out such in vitro pedigree analyses using a number of different cultured mouse myeloma and hybridoma cell lines. These studies have recently been reviewed (7) and will not be discussed in detail here. However, Table 1 summarizes the phenotypes of

Table 1

PHENOTYPES OF MOUSE MYELOMA AND HYBRIDOMA VARIANTS

	MYELOMA	HYBRIDOMA
A. *Changes in gene expression*		
1. *Loss variants* (H^-L^+, H^+L^-, H^-L^-)	+	+
2. *Quantitative variants (decreased H and/or L synthesis)*	+	+
B. *Changes in immunoglobulin structure*		
1. *Deletions*		
C-terminal	+	+
internal	+	+
2. *Recombinants (class switch)*	+	+
3. *Long Chains*	+	
4. *Amino and substitutions*	+	
5. *Carbohydrate*	+	

the variants which have been identified. The points that are important to emphasize here are that: 1) variants with many of these phenotypes arise in myeloma cells at spontaneous frequencies of 10^{-2}-10^{-3}. In the few cases where accurate rates have been determined they are between 10^{-3} and 10^{-4}/cell/generation (8); 2) many of the phenotypes that have been identified in myeloma cell lines also arise in hybridomas (9,10, 11) although the frequency of class or subclass switches (12) and of deletions (9) are in some cases reported to be lower. We believe that hybridomas with point mutations and carbohydrate differences will soon be identified and our own experience is that the frequency of deletion and some class switch variants in hybridomas is as high as that found in myelomas (11); 3) this extreme genetic instability seems to be restricted to the immunoglobulin genes since mutations in enzymes such as hyproxanthine phorphoribosyl transferase, thymidine kinase, and Na-K ATPase arise at frequencies lower than 10^{-6}-10^{-7} (8); and 4) most of the somatic structural mutants identified so far are in the constant rather than the variable region (7).

We have therefore looked directly for variable region mutants arising in the S107 cell line. This cell line produces an IgA, κ antibody which binds the hapten phosphocholine (PC) and bears the T-15 idiotype. It thus closely resembles or is identical to most of the antibodies normally made by BALB/c mice in response to immunization with PC attached to protein carriers such as KLH (13). It is important to note here that we have recently found that the S107 cell line synthesizes two κ light chains in approximately equal amounts. We have cloned and sequenced the variable region genes that code for both of these light chains and studied their intracellular metabolism (14,15). The light chain relevant to these studies, which we call S107A, codes for the light chain which associates with

the S107 heavy chain to form the PC-binding antibody and is secreted by the S107.3.4 parental clone and all of the variants to be described below (14). The second chain, called S107B, shares only half of its variable region amino acids with S107A, contains a 6 base deletion at the V-J recombination site with the consequent deletion of two amino acids in J, does not assemble to the S107 heavy chain, and is not secreted by either S107.3.4 or any of the variants described below (15).

When the S107.3.4 cells are cloned in soft agar and overlayed with PC-KLH, between 0.1 and 1.0% of the clones are not surrounded by an antigen-antibody precipitate and are therefore presumptive antigen binding variants (16). Subsequent experiments revealed that 60-70% of these variants were still secreting similar amounts of IgA as the parent and that these IgA molecules were assembled into polymers to the same degree as the parent (16). Two of these 1° antigen-binding mutants, called U_4 and U_1, have been characterized in more detail. The mutant cells were recovered from the agar, recloned, and injected into mice. The variant antibody was purified from ascites fluid and compared to purified parental S107.3.4 protein. As can be seen in Table 2, U_4 does not agglutinate PC-sheep red blood cells (SRBC) and cannot compete with parental protein for binding to PC-KLH. There is no detectable binding of free hapten as measured by equilibrium dialysis. The fact that U_4 reacts less well with binding site specific rabbit antibody confirms that there has been a change in the configuration of the antigen binding site. This change in conformation does not, however, significantly affect the serology of the rest of the variable region since a number of monoclonal anti-T15 antibodies react equally well with parent and U_4. Both the arginine- and lysine-containing tryptic peptides of the light chains of the parent and the variant were indistinguishable, but there were a few differences between the arginine

Table 2

CHARACTERIZATION OF S107 VARIANTS

GENERATION	CELL LINE	HA TITER[a]	RIA PC-KLH (relative binding[b])	Ka ($10^5 M^{-1}$)	TRYPTIC PEPTIDE DIFFERENCES H	L
P	S107.3.4	8192	100%	2.3		
1^o	U_4	0	0.01	0	+	-
1^o	U_1	128	9.0	2.5	+	-
2^o	S_3S_1	512	26.	2.1	+	-

[a] 250 µg of purified protein examined in two fold dilutions.

[b] % relative binding $\left(\frac{\text{ng parent required for 50\% inhibition}}{\text{ng variant required for 50\% inhibition}} \times 100\right)$

containing tryptic peptides of heavy chains of the variant and parent. Chain recombination experiments confirmed that the heavy chain was responsible for the loss of antigen binding. Sequence analysis of the entire variable region of the U_4 heavy chain revealed a single amino acid substitution in the first hypervariable region at residue 35 (17). The first hypervariable region of the heavy chain must play an important role in PC-binding since all PC binding heavy chains have the sequence Phe-Tyr-Met-Glu at residues 32-35 and this sequence only appears in PC binding proteins (18,19).

This non-antigen binding variant nicely illustrates a point made earlier. It has arisen from a gene coding for a PC binding antibody but, since the mutant no longer binds PC, it would not be recognized and characterized if one were looking at PC binding hybridomas. Thus, in the pedigree analysis of the T-15 heavy chain germ line variable region gene in vivo, this is a potential progeny with a change in the first hypervariable region that would be lost to analysis. It is possible that this amino acid substitution has produced an antibody that will react with another antigen.

Some of the characteristics of a second 1° variant, called U_1, are also presented in Table 2. It was also originally identified because it did not give a visible precipitate when overlayed with antigen in the plate assay. However, the purified protein from this variant does react with PC-SRBC and PC-KLH, though much less well than the parental S107.3.4 protein. Its heavy chain contains a few tryptic peptides which differ from the parental heavy chain (16). Amino acid sequence analysis of the whole heavy chain variable region reveals a single amino acid substitution at the fifth residue of the J segment (20). This variant is particularly interesting because it reacts much less well than the parent with PC attached to carriers such as SRBC or KLH, but its affinity for the hapten

alone is identical to that of the parent (Table 2). This surprising finding suggests that the hapten binding site itself is unchanged but that the interaction of portions of the antibody with carrier is altered in such a way that hapten can no longer be bound when attached to carrier. However, the important conclusion is that variants with single amino acid substitutions in the variable region are arising at a high spontaneous frequency in the S107 cell line.

We are attempting to address two other questions about the somatic mutations which occur in the S107 immunoglobulin genes: 1) are there hot spots or some sort of preprogrammed (21) sets of mutations; and 2) what is the rate of individual mutational events? It would be much easier to study these questions if we could develop a positive rather than a negative assay for individual mutational events. This is in fact possible because the U_1 low antigen binding variant generates 2° variants with increased antigen binding. These were identified by cloning U_1 in soft agar and overlaying the subclones with PC-KLH. Most of the clones were not surrounded by an antigen-antibody precipitate but between 0.1 and 2.0% the clones did precipitate the PC-KLH (16). As can be seen in Table 2, one of these, S_3S_1, binds antigen better than U_1 but less well than S107.3.4. Four independent 2° variants of U_1 have been identified and all four have the same increase in antigen binding, are indistinguishable from each other serologically, and differ from U_1 by the same 2 or 3 heavy chain tryptic peptides (22). This suggests that a similar mutational event occurs repeatedly and at a high frequency. However, since we have only screened for variants with increased binding, we do not know whether other sorts of mutations are occurring at a similar high frequency.

We have determined the exact rate at which 2° variants with increased binding arise by carrying out a fluctuation analysis (23). This became possible when we were able to

define conditions under which U_1 cells did not form a plaque with PC-SRBC while the 2° variants did plaque using the Cunningham modification of the Jerne plaque technique (24). Many independent subclones of U_1 were examined for plaque forming cells. The mutation rate was calculated to be 1.4×10^{-4}/cell/generation (24). It is important to reiterate that this assay only identified that small subset of mutants which had increased their binding compared to U_1 above the minimum threshold required for plaquing.

The studies described above indicate that it is possible to use tissue culture cells to carry out a pedigree analysis of the gene that codes for the S107 PC binding antibody. Variants with changes in antigen binding and single amino acid substitutions in their heavy chain variable region arise spontaneously at a high frequency. Even though very few variants have been analyzed, it is clear that amino acid substitutions will not be limited to the hypervariable region. In studies not described here we, and others, have also identified constant region mutants in a variety of myeloma proteins including S107 (7).

Since this is a malignant cell growing continuously in culture, it is difficult to be certain whether the events we are observing are related to the normal generation of antibody diversity. It will be important to establish the relative frequency of point mutations in the hypervariable regions, framework residues, and constant regions of this and other cell lines. It will also be interesting tc compare the results of pedigree analysis carried out in culture to the results being obtained by Gearhart and Hood (6) and their colleagues with PC-binding hybridomas. However, at this time it seems likely that the antigen binding diversity we are observing in culture will provide significant insight in the

role of somatic mutation in the normal generation of antibody diversity.

REFERENCES

1. Weigert, M.G., Cesari, I.M., Youkovitch, S. and Cohn, M. (1970). *Nature 228*, 780.
2. Cesari, I.M. and Weigert, M. (1973). *Proc. Natl. Acad. Sci. (USA) 70*, 2112.
3. Bernard, O., Hozumi, N. and Tonegawa, S. (1978). *Cell 15*, 1133.
4. Cohn, M., Langman, R., and Geckeler, W. (1980). *Prog. Imm. 4*, 153.
5. Weigert, M., Gatmartan, L., Loh, E., Schilling, J. and Hood, L. (1978). *Nature 276*, 785.
6. Gearhart, P.J., Johnson, M.P., Douglas, R. and Hood, L. (1980). In press.
7. Morrison, S.L. and Scharff, M.D. (1981). *Critical Reviews in Imm.* In press.
8. Baumal, R., Birshtein, B., Coffino, P. and Scharff, M.D. (1973). *Nature 245*, 320.
9. Kohler, G. and Schulman, M. (1980). *Eur. J. of Immunol. 10*, 467.
10. Yelton, D.E., Cook, W.D. and Scharff, M.D. (1980). *Transpl. Proc. 23*, 439.
11. Yelton, D.E. and Scharff, M.D. In preparation.
12. Rajewski, K. This volume.
13. Kohler, H. (1975). *Transpl. Rev. 27*, 24.
14. Kwan, S.-P., Rudikoff, S., Seidman, J.G., Leder, P. and Scharff, M.D. (1981). *J. Exp. Med.* In press.
15. Kwan, S.-P., Max, E., Leder, P. and Scharff, M.D. Submitted.

16. Cook, W.D. and Scharff, M.D. (1977).*Proc. Natl. Acad. Sci. (USA) 74*, 5687.
17. Rudikoff, S., Giusti, A., Cook, W.D., and Scharff, M.D. In preparation.
18. Kabat, E.A., Wu, T.T., Bilofsky, H. (1976).*Proc. Natl. Acad. Sci. (USA) 73*, 617.
19. Rudikoff, S. and Potter, M. (1978).*Proc. Natl. Acad. Sci. (USA) 73*, 2109.
20. Cook, W.D., Rudikoff, S., Giusti, A. and Scharff, M.D. In preparation.
21. Klinman, N.R., Press, J.L., Segal, N.J. and Gearhart, P.J. (1976). *In: The Generation of Antibody Diversity (ed. A.J. Cunningham) Academic Press*, p. 127.
22. Cook, W.D. (1979). Ph.D. Thesis.
23. Luria, S.E. and Delbruck, M. (1943).*Genetics 28*, 491.
24. Zack, D., Cook, W.D. and Scharff, M.D. In preparation.

SOMATIC MUTATION INCREASES DIVERSITY IN IgG AND IgA BUT NOT IgM ANTIBODIES

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ABSTRACT The N-terminal amino acid sequences of the V_H and V_L regions from 16 hybridoma proteins which bind phosphorylcholine as well as the complete sequence analyses of 9 of these V_H regions are presented. There are more V_H regions participating in the response to phosphorylcholine than are encoded by germline V_H gene segments. Moreover, the V regions from IgG and IgA antibodies are considerably more variable than those from their IgM counterparts. These observations raise the possibility that a somatic mutation mechanism for V region diversification produces greater diversity in IgG and IgA than in IgM antibodies.

INTRODUCTION

Recent advances in DNA technology have greatly increased our understanding of the mechanisms responsible for antibody diversification. The antibody molecule contains a pair of heavy and light chains, which are each composed of a variable (V) and constant region. The V region is further divided into gene segments. The V region of the heavy chain (V_H) is formed from three gene segments: the V_H segment, encoding amino acids 1-95; the diversity (D) segment, encoding a variable number of amino acids in the third hypervariable region; and the joining (J_H) segment, encoding approximately amino acids 100-113 (1). The V region of the light chain (V_L) is formed from two gene segments: the V_L segment, encoding approximately amino acids 1-95; and the J_L segment, encoding amino acids 96-108 (2).

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Antibody diversity may be generated by the following mechanisms. (i) Heavy and light chains may pair combinatorially, allowing a given heavy chain to pair with many light chains and vice versa (3). (ii) Multiple germline genes exist for the V_H, D, J_H, V_L, and J_L segments (4). (iii) A V_L gene segment may associate combinatorially with several J_L gene segments (5), and a V_H gene segment may associate with several J_H gene segments (6). In addition, combinatorial joining of D gene segments with V_H and J_H gene segments may amplify diversity. (iv) Junctional diversity may be created at the joining sites of V_H, D, and J_H gene segments, and at the joining site of V_L and J_L gene segments. Gene segments may be joined at different points in their nucleotide sequences, thus generating hybrid codons as well as codon insertions or deletions (1,7). (v) Somatic mutation may increase diversity in germline gene segments (8).

In order to determine the extent that each of these mechanisms contributes to the generation of antibody diversity, we have produced hybridoma antibodies to the phosphorylcholine antigen in BALB/c mice, and then determined the amino acid sequence of the V_H and V_L regions (9). The results indicate that somatic mutation plays a major role in V region diversification. The most striking observation is that the IgG and IgA antibodies exhibit far more diversity than their IgM counterparts.

DIVERSITY IS GENERATED IN ANTI-PHOSPHORYLCHOLINE ANTIBODIES BY FIVE DIFFERENT MECHANISMS

1. Combinatorial pairing of heavy and light chains. HPCM2 and HPCM3 employ the same heavy chain in conjunction with two very different light chains (Fig. 1 & 2). This combinatorial association of two different light chains with the same heavy chain changes the nature of the antigen-binding site since the affinities of HPCM2 and HPCM3 antibodies for phosphorylcholine differ by 10-fold (9). The assumption has long been held that most heavy and light chains could associate combinatorially to generate diversity (3). Although it has not been formally demonstrated that every light chain can pair with every heavy

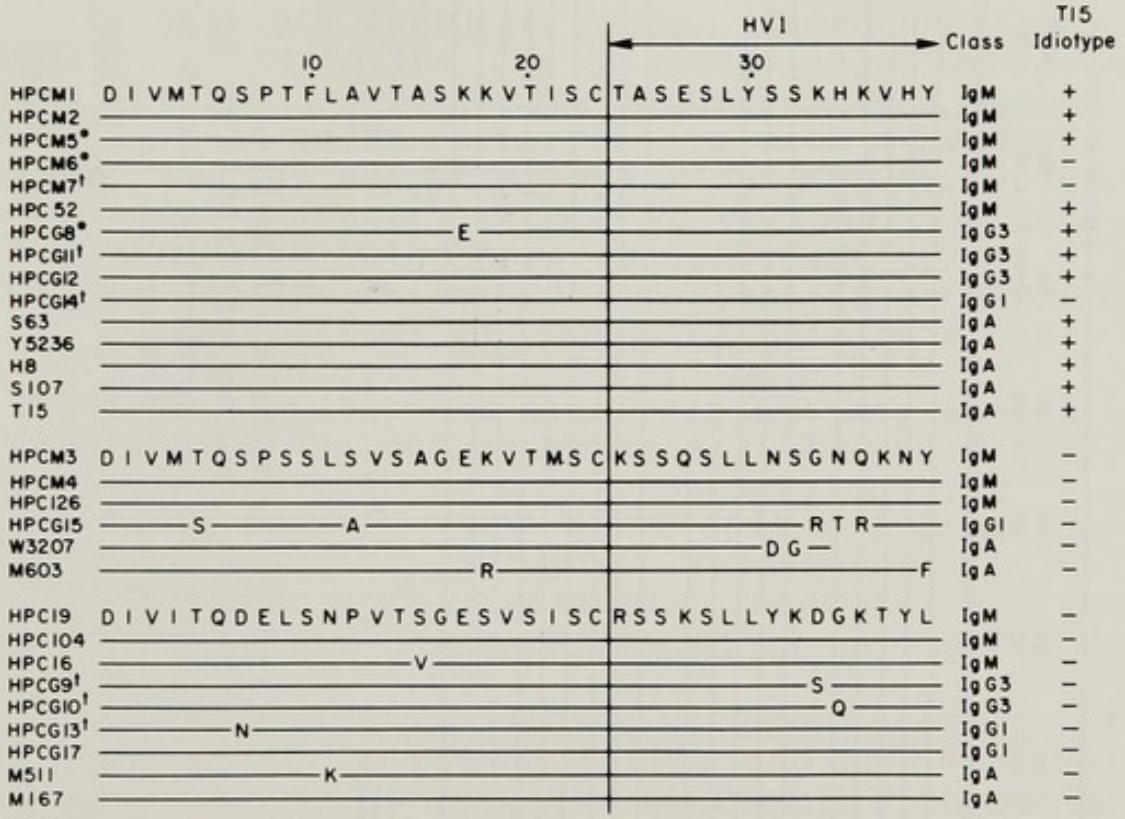

Fig. 1. V_L regions of anti-phosphorylcholine antibodies. Hybridomas were made from BALB/c mice and sequenced as described (9). Amino acids are noted by the one-letter code, and hypervariable (HV) regions are designated according to Kabat et al. (10). All * proteins come from one mouse, while the + proteins come from a second mouse. Other proteins each come from separate individuals. The V_L sequences are categorized according to the T15, M603, and M167 groups with the most typical protein of each group used as the prototype (11). A solid line indicates identity with the prototype sequence. The sequence of HPC16 is written assuming asx residues are identical to prototype asparagine or aspartic residues. The sequences HPC52, HPC126, HPC19, HPC104 and HPC16 are from ref. 12; the sequences of S63, Y5236, H8, S107, T15, W3207, M603, M511, and M167 are from ref. 13.

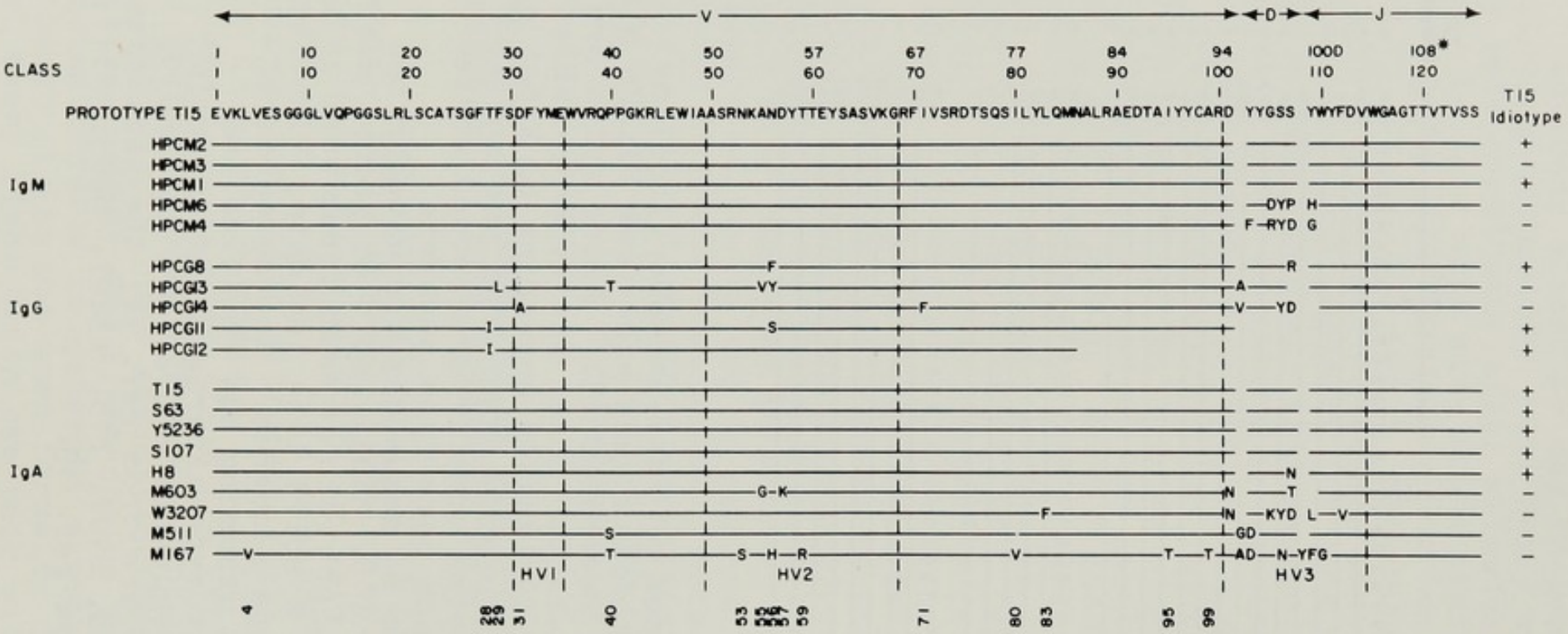

Fig. 2. Protein sequences of the V_H, D, and J_H segments of anti-phosphorylcholine antibodies (9). The numbering system of Kabat *et al.* (10) is shown on the first line (*). The consecutive numbering shown on the second line is for M167. All other V_H regions are shorter as indicated by the deletions at the V_H-D and D-J_H boundaries. Positions of amino acid substitutions are labeled at the bottom of the figure except in HV3. The asx reported at position 101 in M167 (10) has been changed to asparagine based on the DNA sequence (1).

chain, these data suggest that combinatorial association does increase diversity for some antibody responses.

2. Multiple germline genes. There are four germline genes in the T15 V_H group (14). One gene codes for the T15 prototype sequence. The second germline gene codes for a protein with a sequence similar to, but not identical with, HPCG15, which is the most different of the V_H regions (9) (data not shown). The third gene is different by 10% from the T15 and HPCG15-like nucleotide sequences; a hybridoma antibody representing this gene has not been found yet. The fourth gene is a pseudogene. Multiple D gene segments may encode the various D regions shown in Fig. 2, while only one J_H gene is used by anti-phosphorylcholine antibodies. There appears to be only one germline gene for the M167 V_L group (I. Weissman and U. Storb, personal communication). There is no information about the germline genes in the T15 V_L and M603 V_L groups.

3. Combinatorial joining of gene segments. As shown in Fig. 2, HPCM2, HPCM6, and HPCM4 share the same V_H segment but have different D segments. Although the numbers and sequences of the germline D genes are not known, the D segments in Fig. 2 exhibit sufficient diversity to suggest that there may be several genes. Thus, combinatorial joining of a V_H segment with different D segments appears to be a way of amplifying diversity.

4. Junctional diversity. The insertions and deletions found at either end of the D segment on the heavy chain (Fig. 2) as well as codon substitutions (1,9) probably arise from the site-specific recombination mechanism that joins the V_H and D as well as the D and J_H gene segments. This diversity probably occurs during the joining of gene segments at different sites within codons.

5. Somatic mutation in V_H and V_L regions. A comparison of the sequences of the germline gene segments in the T15 V_H, J_H, and M167 V_L groups to the protein sequences indicates that somatic mutation occurs to generate diversity. In the T15 V_H family, there are four different germline gene sequences. All of the 9 variant proteins shown in Fig. 2 appear to be derived from one T15 germline gene (14). The extent of variation ranges from one amino acid substitution to as many as seven

substitutions in the M167 V_H region. In the J_H region (15), there is one germline gene used by all anti-phosphorylcholine antibodies, and there are two apparent somatic variants of this gene (W3207 and M. Scharff, this volume). In the M167 V_L group, it has been reported that there is one germline gene (I. Weissman and U. Storb, personal communication), and six different protein sequences have already been identified (Fig. 1).

THE NATURE OF SOMATIC MUTATION IN V SEGMENTS

Somatic mutation may be generated by two processes. (i) Normal somatic mutation may act on all DNA in somatic cells. The increased diversity seen in antibody V regions relative to other proteins could be the result of strong selection operating on immunoglobulin-producing cells. (ii) A hypermutational mechanism may act specifically on antibody V regions. An examination of the data in Fig. 2 shows several interesting points concerning the nature of variability in V_H segments. As noted before, all of the different protein sequences are coded by somatic variant genes derived from one germline gene.

1. Somatic mutation occurs outside as well as within hypervariable regions. Of the 22 V_H segment substitutions (not including those at the V-D junction), ten are inside and 12 are outside the hypervariable regions. Therefore, the mechanism for generating somatic diversity is not limited to residues in the hypervariable regions.

2. Certain positions are more variable than others. Two substitutions occur at position 28, three at position 40, four at position 56, and nine at positions 53-59 in the second hypervariable region. In some of these codons, somatic mutation gives rise to identical amino acid substitutions, e.g., isoleucine at position 28 in HPCG11 and HPCG12. In other codons, somatic mutation generates different substitutions, e.g., phenylalanine, tyrosine, serine, and histidine at position 56 in HPCG8, HPCG13, HPCG11, and M167. The three distinct occurrences at position 40 of a proline to serine or threonine substitution correlate in an interesting manner with heavy-light chain pairing. The proline to serine or

threonine interchange at 40 occurs only in those heavy chains which pair with M167-like light chains. In addition, this potentially non-conservative amino acid interchange occurs in a loop which interacts strongly with the light chain (16). Thus, selection on substitutions in particular positions may determine which somatic mutants are ultimately expanded.

3. Variants from the germline sequence are found in V_L as well as in V_H gene segments. Therefore, the mechanism for somatic mutation must be activated in a trans fashion on different chromosomes.

IgG AND IgA ANTIBODIES ARE MORE DIVERSE THAN THEIR IgM COUNTERPARTS

When does somatic mutation occur? A comparison of the sequences of the heavy and light chains from IgM, IgG, and IgA antibodies suggests that mutation is correlated with the heavy chain class switch from IgM to other isotypes. In IgM antibodies, none of the 11 V_H segments is different from the prototype sequence (Fig. 2 and ref. 9), and only one of 12 V_L segments is different (Fig. 1). In contrast, in IgG antibodies, six of nine V_H segments (9) and five of nine V_L segments are different from the prototype sequence. In IgA antibodies, four of nine V_H segments and three of nine V_L segments are different. Thus, one of 12 IgM antibodies varies from the V group prototype sequence in either the heavy or light chain, whereas eight of nine IgG antibodies and five of nine IgA antibodies vary.

Cellular and recombinant DNA studies have suggested that B cells initially produce IgM and then switch to produce IgG or IgA after antigen stimulation (17). The increased diversity in IgG and IgA antibodies compared to IgM antibodies may occur by one of two general mechanisms. (i) The nonprototype V regions may arise by somatic diversification in antibodies of all classes at a low frequency. If so, B cells that express variant antibodies must be amplified by selection relative to B cells expressing germline antibodies after the class switch from IgM to IgG or IgA production. (ii) The nonprototype V regions may occur only in IgG or IgA antibodies. A somatic

mutation mechanism may be activated in conjunction with the molecular events mediating the switching of heavy chain classes, or at some time after the switch. Alternatively, somatic variants may accumulate in IgG or IgA-producing cells because they have undergone more clonal expansion than IgM-producing cells (18).

Two types of selection may operate on infrequent germline or somatic variants to produce the observed differences between IgM and IgG or IgA. (i) Antigen-driven selection. B-cell clones expressing variant antibodies may be expanded because they have a higher affinity for antigen than B cells expressing prototype antibodies. As IgG and IgA-producing cell populations may have undergone more antigen-driven selection than IgM populations, IgG and IgA antibodies should have a higher affinity for antigen than IgM antibodies. On the surface, the data presented in Fig. 3 would

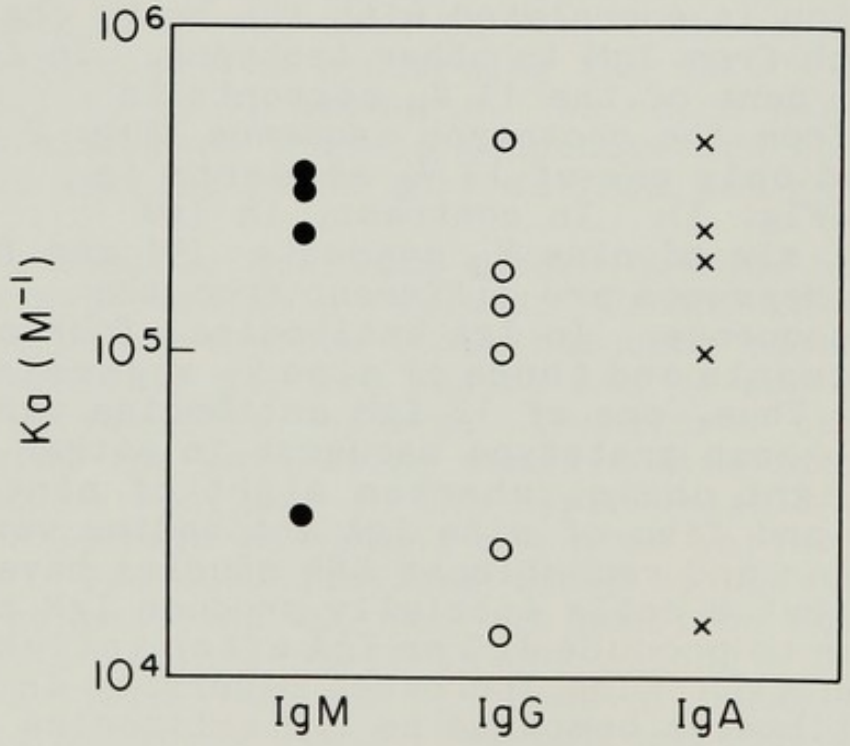

Fig. 3. IgM, IgG, and IgA antibodies have similar affinities for phosphorylcholine. The affinity of hybridoma and myeloma antibodies for phosphorylcholine was measured by equilibrium dialysis as described in ref. 9. The mean values of 2.36×10^5 for IgM, 1.48×10^5 for IgG, and 1.97×10^5 for IgA antibodies are not significantly different from one another ($p > .15$, Mann-Whitney U test).

appear to argue against this possibility. The range of affinity constants seen in the IgM, IgG and IgA antibodies is not significantly different. However, these data do not exclude the possibility that affinity constants of antibody in solution for free hapten could be significantly different from affinity constants of antibody in cell membranes for the hapten-carrier conjugate. Chang and Rittenberg (19) have observed that IgG and IgA anti-phosphorylcholine antibodies have a broader range of avidity for phosphorylcholine than IgM antibodies. Selection for variants in nonbinding site regions may occur whenever framework residues modify the positions of binding-site residues. (ii) Idiotype selection. Selection of infrequent variants may occur by idiotype-specific regulation. For example, if idiotype-specific suppression controls the level of predominant clones expressing the prototype sequences, then B cells with altered sequences may be selectively expanded.

CONCLUSION

The immune system seems capable of generating an enormous amount of variability by the somatic mutation of a limited number of germline genes. An animal may first respond to antigen with IgM antibodies using a finite number of germline V genes. After antigen stimulation, increased diversity may be introduced by mutation during the prolonged clonal expansion of IgG or IgA-producing cells, or perhaps during the class switch itself. The purpose of somatic mutation may be to introduce random substitutions throughout V regions in order to increase the repertoire of antibodies available to bind to antigen. Only a small minority of the variants may have a higher affinity for antigen.

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REFERENCES

1. Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). Cell 19, 981.
2. Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. (1978). Cell 15, 1.
3. Gally, J.A., and Edelman, G. (1972). Ann. Rev. Genet. 6, 1.
4. Seidman, J., Leder, A., Nau, M., Norman, B., and Leder, P. (1978). Science 202, 11.
5. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978). Nature 276, 785.
6. Schilling, J., Clevinger, B., Davie, J.M., and Hood, L. (1980). Nature 283, 35.
7. Max, E., Seidman, J. and Leder, P. (1979). Proc. Natl. Acad. Sci. USA 76, 3450.
8. Weigert, M., and Riblet, R. (1976). Cold Spring Harb. Symp. Quant. Biol. 41, 837.
9. Gearhart, P.J., Johnson, N.D., Douglas, R.
10. Kabat, E.A., Wu, T.T., and Bilofsky, H. (1979). Sequences of Immunoglobulin Chains (U.S. Dept. HEW).
11. Potter, M. (1977). Adv. Immunol. 25, 141.
12. Kocher, H.P., Berek, C., Schreier, M.H., Cosenza, H., and Jaton, J-C. (1980). Eur. J. Immunol. 10, 264.
13. Hood, L., Loh, E., Hubert, J., Barstad, P., Eaton, B., Early, P., Fuhrman, J., Johnson, N., Kronenberg, M., and Schilling, J. (1976). Cold Spring Harb. Symp. Quant. Biol. 41, 817.
14. Crews, S. Griffin, J., Huang, H., Calame, K., and Hood, L. Submitted.
15. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
16. Segal, D.M., Padlan, E.A., Cohen, G., Rudikoff, S., Potter, M., and Davies, D. (1974). Proc. Natl. Acad. Sci. USA 71, 4298.
17. Davis, M., Calame, K., Early, P., Livant, D., Joho, R., Weissman, I., and Hood, L. (1980). Nature 283, 733.
18. Rodwell, J.D., and Karush, F. (1980). Molec. Immunol. 17, 1553.
19. Chang, S.P., and Rittenberg, M.B. (1981). J. Immunol. 126, 975.

Antigen Binding Variants of the Dextran Binding Mouse Plasmacytoma J558[1]

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ABSTRACT

Variants producing an immunoglobulin altered in its reactivity with polymeric dextran have been isolated from the mouse myeloma, J558 (IgA, λ; anti-α1→3 dextran). No difference can be demonstrated in the primary structure of the mutant immunoglobulin (Ig) but a difference can be demonstrated in the composition of the carbohydrate on the mutant heavy (H) chain. This difference in glycosylation is shown to result from altered availability of the enzymes involved in glycosylation.

INTRODUCTION

Variants producing structurally altered immunoglobulin occur in cultured myeloma cells (1-7) at a high frequency, with loss of production of H chain occurring at approximately 10^{-3}/cell/generation and loss of production of light (L) chain occurring at a frequency of approximately 0.4×10^{-3}/cell/generation (8, 9).

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Most mutants of Ig producing cells fail in the synthesis of H chains, L chains, or both (8-10) or produce H chains with alterations in the constant region (2, 4, 6-7). Recently, the first description of a variable region mutant of a mouse myeloma cell line was reported (11). Mutants of the S107 myeloma protein (anti-phosphorylcholine [PC] altered in ability to interact with antigen were isolated, found to have an altered primary structure (see Scharff et al., this volume). It is also possible to isolate mutants of the J558 myeloma with altered ability to interact with antigen. We characterized these mutants and found them to differ from the wild type not in primary structure, but instead in carbohydrate content. This altered glycosylation was the consequence of altered availability of enzymes involved in glycosylation.

EXPERIMENTAL PROCEDURES

The J558 tumor (12) was obtained from the Salk Institute, San Diego, California and adapted to continuous growth in tissue culture in our laboratory. The drug marked derivative of 45.6, 4TOOl, resistant to 2.5 mM ouabain and 5 μg/ml thioguanine was obtained from M. D. Scharff, Albert Einstein College of Medicine, Bronx, New York.

Somatic cell hybrids between myeloma cell lines were made by the method described by Margulies et al. (13) and modified by Sharon et al. (14). Cells were maintained and radiolabelled as previously described except that labelling with ^{3}H- or ^{14}C-glucosamine (Schwartz-Mann Radiochemicals, Orangeburg, New York) was in complete medium (7).

Myeloma cells were cloned in soft agarose by the method of Coffino and Scharff (8, 15) using primary rat embryo fibroblasts as a feeder layer. Growing clones were overlaid with 1 ml of agarose medium containing 200 μg of dextran B1355 S and L (16). Immunoprecipitates formed around clones synthesizing and secreting immunoglobulin that specifically reacted with the dextran.

Polyacrylamide gels without SDS were prepared as modified by Takeo and Nakamura (17). Gels were fixed and stained as described by Maizel (18). ^{14}C-glucosamine

labelled protein were digested with pronase and neuraminidase and analyzed on tris-borate gels as described by Weitzman (19). The position of ^{14}C-labelled peaks was determined by autoradiography. Peptide map analysis was as previously described (20).

RESULTS

To identify mutants with altered ability to interact with dextran, myeloma cells cloned in soft agarose were overlaid with dextran B1355. Clones over which no precipitate formed were recovered and characterized. The majority (approximately 96 percent) were synthesizing only L chain and so were not further studied. Cytoplasmic lysates from those synthesizing both H and L chain were characterized by a radioimmunoassay (21). On the average, three times as much mutant Ig as wild type was required to bind 50 percent of the ^{14}C-dextran used in the assay. The size of the combining site of one mutant Ig (L187) was determined by assaying the relative ability of oligosaccharides of differing structure to inhibit precipitation (22). The combining sites of both mutant and wild type were found to accommodate between 4 and 5 sugar groups. The binding studies suggest that the antigen binding mutants have decreased reactivity with polymeric dextran while maintaining approximately the same site size and reactivity with small oligosaccharides as the wild type.

The Ig synthesized by the mutant (L187) was examined to identify the structural changes responsible for alterations in its ability to bind antigen. L187 produced H and L chains that were indistinguishable on SDS polyacrylamide gels from those of J558. However, when secreted Igs labelled with ^{14}C-amino acids from L187 and J558 cells were examined on polyacrylamide gels that did not contain SDS (17), a difference in the migration pattern of the Ig bands was observed (Fig. 1, lanes A and C). To investigate the contribution of carbohydrate to the differences in migration, carbohydrate minus (CHO-) Ig was prepared (23). In the absence of carbohyrate (Fig. 1, lanes B and D), J558 and L187 Ig migrate similarly, indicating that the difference in migration observed in lanes A and C was due to differences in the carbohydrate content of the two.

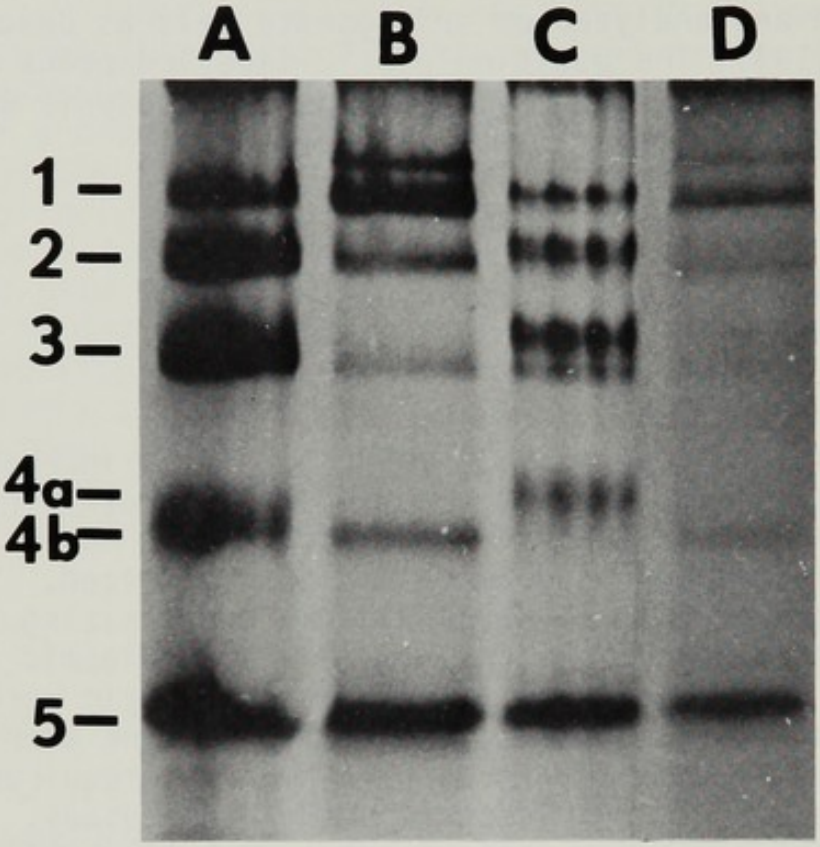

Fig. 1. Analysis of Ig secreted by L187 and J558 on polyacrylamide gels. Lane A, L187; B, L187 CHO⁻; C, J558, D, J558 CHO⁻. Bands 1 through 5 were recovered from these gels and their composition determined by analysis on SDS-polyacrylamide gels before and after disruption of disulfide bonds. Bands 1, 2, and 3 contained polymeric IgA, $(H_2L_2)_n$, with $n_1 > n_2 > n_3$. Band 4 contains $(H_2L_2)_1$. Band 5, which migrated identically in mutant and wild type cells, contained free L chain.

Analysis of Fab and Fc fragments demonstrated that this difference was located in the Fab region of the molecule.

Analysis of Glycopeptides of L187 and J558 Ig

The carbohydrate on J558 and L187 Igs was labelled by incubating the cells in media containing ^{14}C-glucosamine and labelled H chains were purified from cell secretions.

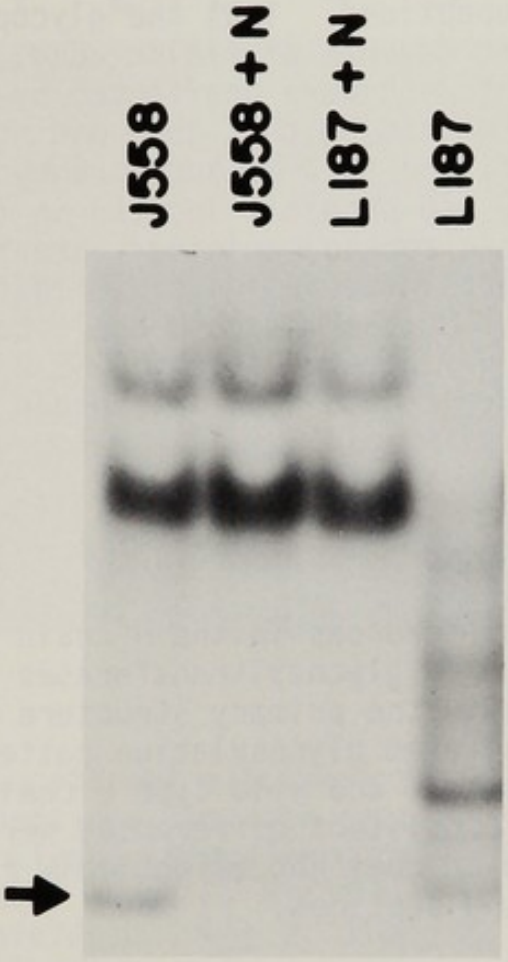

Fig. 2. Analysis of the glycopeptides of L187 and J558 on tris-borate polyacrylamide gels. Glycopeptides treated with neuraminidase are indicated by +N.

The L chains of both L187 and J558 do not contain carbohydrate. H chains were digested exhaustively with pronase and the glycopeptides examined on 10 percent tris-borate polyacrylamide gels (19). On these gels larger carbohydrates and carbohydrates containing sialic acid migrate more rapidly. As shown in Fig. 2, glycopeptides derived from L187 migrate more rapidly than those from J558. This difference in migration was also observed when glycopeptides from the Fab were compared (data not shown), suggesting a difference in glycosylation of the Fab fragments of L187 and J558. One fast moving glycopeptide was common to both L187 and J558 (marked by arrow) but absent from the Fab, indicating that it was most probably

located on the Fc. After digestion with neuraminidase, L187 glycopeptides migrated at the same rate as the two major wild type glycopeptides. Thus the glycopeptides of L187 contained varying amounts of sialic acid. The two major glycopeptides of J558 were unaffected by digestion with neuraminidase, indicating that they did not contain sialic acid. However, the fast moving carbohydrate present on both L187 and J558 and probably located in the Fc region changed its migration after neuraminidase treatment indicating that it contained sialic acid. Thus it appears that the glycopeptides of the mutant H chain contain more sialic acid than the glycopeptides from J558 H chain. However, the wild type H chain does not appear to be entirely devoid of sialic acid.

Peptide Map Analysis

The carbohyrate differences in the H chain of L187 could result from altered glycosyltransferases within the cell or from a change in the primary structure of the H chain leading to an altered glycosylation pattern. When the peptide maps of mutant and wild type L chain, Fd, and Fc were compared, no consistent differences were seen. However, the method used does not effectively resolve carbohydrate containing peptides.

Examination of the Available Complement of Glycosyltransferases in Mutant and Wild Type Cells

To determine if the enzyme content of L187 led to the differences in glycosylation of the H chain, two expriments were performed: Firstly, mutant and wild-type cells were infected with vesicular stomatitis virus (VSV) and the carbohydrates on the viral glycoprotein obtained from these cells compared. A difference in the enzyme complement of cells might be reflected in the carbohydrate of the virus since the virus relies on host cell enzymes for glycosylation (24, 25).

L187, J558, and BSC40 (permissive monkey fibroblast) cells incubated in media containing ^{14}C-glucosamine, were infected with VSV, the viral proteins immunoprecipitated with guinea pig anti-VSV and examined on tris-borate polyacrylamide gels before and after neuraminidase

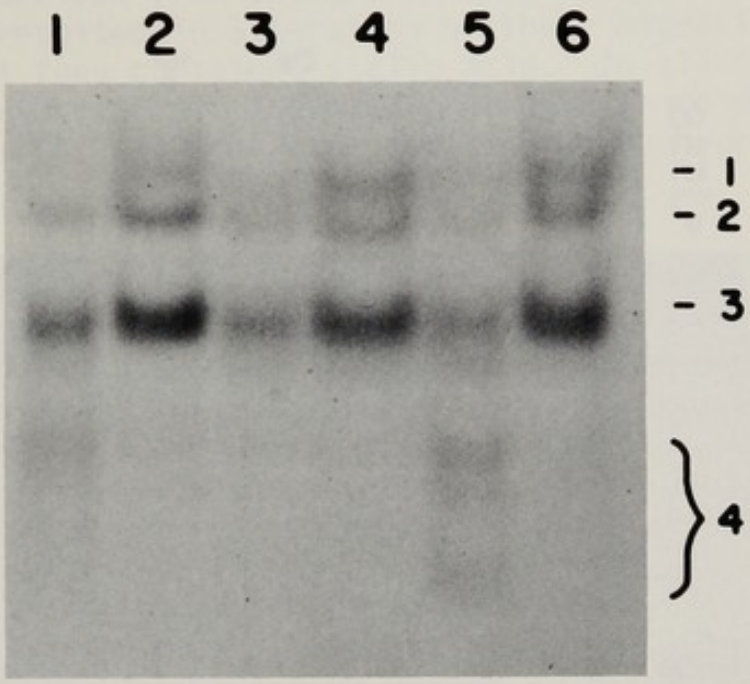

Fig. 3. Tris-borate gel electrophoresis pattern of glycopeptides isolated from glycoprotein G of VSV. Glycopeptides were prepared as described in the text. Lane 1, VSV grown in BSC40; 2, BSC40 + neuraminidase (+N); 3, J558; 4, J558 + N; 5, La87; 6, L187 + N.

treatment (Fig. 3). Glycoprotein G from virus grown in BSC40 (lanes 1 and 2) and L187 cells (lanes 5 and 6) contained sialic acid which was cleaved by neuraminidase, while glycoprotein G from VSV grown in J558 did not (lanes 3 and 4). These results indicated that there was a difference between the enzymes available for use in glycosylation in L187 and J558 cells.

To further examine the enzyme content of the myeloma cells, somatic cell hybrids were made between J558 and 4TOO1 (IgG_{2b}, κ) and L187 and 4TOO1. In the hybrid between L187 and 4TOO1, and in the hybrid between J558 and 4TOO1, both the IgA and IgG derived glycopeptides contained sialic acid. These data indicated that the IgA of wild type J558 could be sialated in the proper environment. J558 and L187 Ig isolated from the somatic cell hybrids bound antigen equivalently. Since Igs synthesized in the

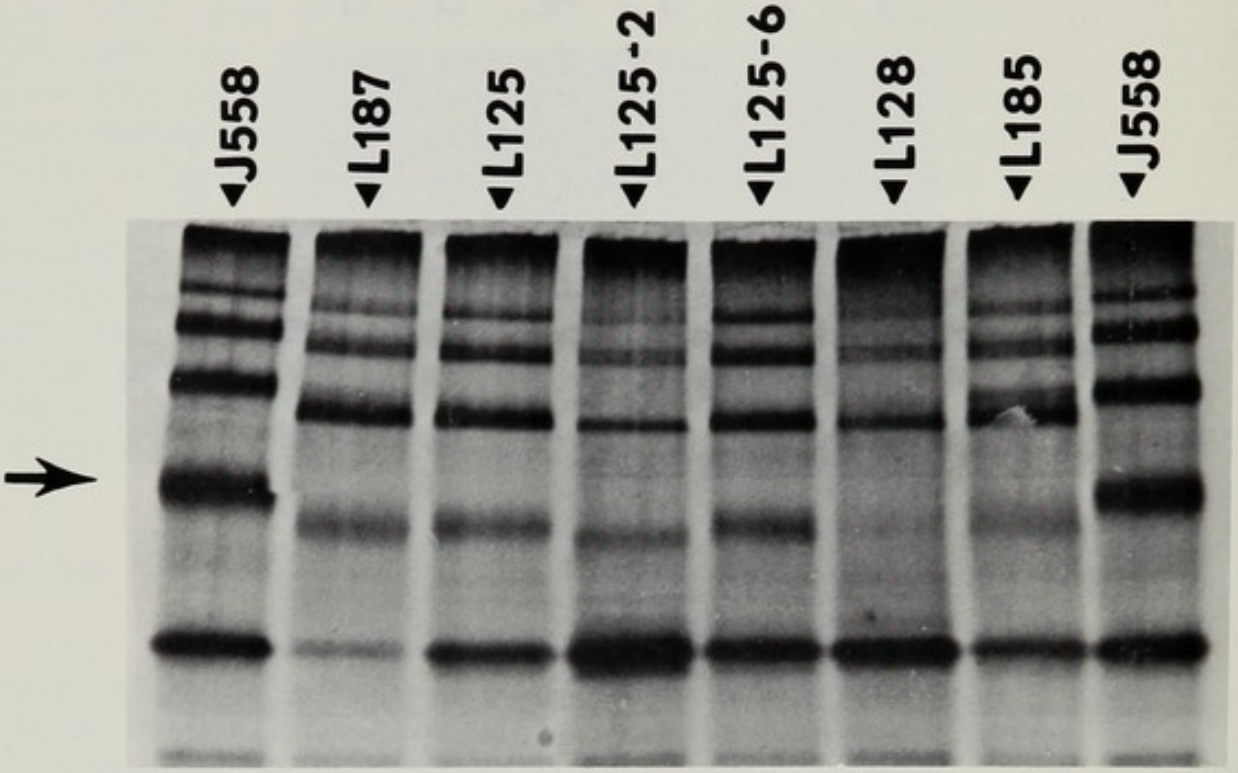

Fig. 4. Analysis on polyacrylamide gels of Ig secreted by J558 and mutants isolated from J558 altered in their reactivity with dextran.

somatic cell hybrids contained sialic acid, it appears that the undersialation of J558 results from a deficiency in an enzyme involved in glycosylation and not from an inhibitor of glycosylation.

Isolation of Additional Antigen Binding Mutants

Additional mutants were isolated from J558 that showed decreased reactivity with antigen in a ^{14}C-dextran binding radioimmunoassay. The secreted Ig from these mutants migrated similarly to the Ig from L187 when analyzed on tris-glycine gels without SDS (Fig. 4). When these mutants were labelled with ^{14}C-glucosamine and the glycopeptides analyzed on tris-borate gels, a heterogeneous group of glycopeptides like those of L187 was observed (data not shown); glycopeptides derived from other types of mutants (quantitative and H/L ratio mutants) migrated like the glycopeptides derived from J558. Treatment of the

glycopeptides derived from antigen binding mutants with neuraminidase resulted in alteration of their migration, indicating that they contained sialic acid. In all cases which we examined, mutants isolated from J558 which did not bind antigen as well as wild type contained carbohydrate moieties with additional sialic acid.

DISCUSSION

When an antigen binding mutant of J558 was characterized no difference in the amino acid sequence of the mutant protein could be demonstrated by peptide mapping. However, the mutant Ig was found to contain more sialic acid than the wild type Ig. J558 has been shown to contain carbohydrate at residue 55 of the hypervariable region. X-ray diffraction studies of the antibody combining site of the anti-PC protein M603 have placed amino acid number 55 of the H chain at the lip of the antibody combining site (26). The J558 combining site has not been examined by X-ray diffraction, but it is reasonable to assume that amino acid 55 may be located in an analogous position in the J558 combining site. A difference in the structure of the carbohydrate located at this position could thus affect the antibody combining site.

The size of the antibody combining site of L187 does not appear to be significantly different from that of J558 and both J558 and L187 react equivalently with small oligosaccharides. However, the Ig of L187 did not react as well with polymeric dextran as did the Ig produced by J558. The alteration in binding observed in the mutant is not strictly an alteration in specificity for reactivity with haptenic groups appears unchanged. Instead it is an alteration in reactivity with the haptenic group once it is attached to a large molecule. This could result from steric problems brought about by the presence of the large carbohydrate. A clear difference between the complement of enzymes available in mutant and wild type cells could be shown by comparing the glycosylation of the same protein (VSV glycoprotein G) in different cellular environments (J558, L187, or BSC40 cells). Glycoprotein G synthesized in L187 and BSC40 cells, contained sialic acid while glycoprotein G synthesized in J558 cells did not contain

sialic acid. Additional evidence for the availability of different glycosyltransferases in different myeloma cell lines was obtained using somatic cell hybrids.

Igs from several independently arising mutants of J558 were also shown to be altered in their ability to react with dextran and, like L187, to have altered glycopeptides, suggesting that the lesion(s) resulting in altered glycosylation was not a rare event. It remains to be shown if alterations in glycosylation is important in vivo in generating altered antibody-antigen reactivity. However, it should be noted, that most biologically important antigens exist in vivo not as free hapten, but associated with larger carrier proteins or cell surfaces.

REFERENCES

1. Cotton, R.G.H., Secher, D.S., and Milstein, C. (1973). Eur. J. Immunol. 3, 135.
2. Secher, D.S., Cotton, R.G.H., and Milstein, C. (1973). FEBS Letters 37, 311.
3. Scharff, M.D., Birshtein, B., Dharmgrongartama, B., Frank, L., Kelly, T., Kuehl, W.M., Margulies, D., Morrison, S.L., Preud'homme, J.-L., and Weitzman, S. (1975). In: Molecular Approaches to Immunology, Academic Press, New York, pp. 109.
4. Preud'homme, J.-L., Birshtein, B.K., and Scharff, M.D. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 1427.
5. Francus, T., Dharmgrongartama, B., Campbell, R., Scharff,M.D., and Birshtein, B.E. (1978). J. Exp. Med. 147, 1535.
6. Morrison, S.L. (1978). Eur. J. Immunol. 8, 194.
7. Morrison, S.L. (1979). J. Immunol. 123, 793.
8. Coffino, P., and Scharff, M.D. (1971). Proc. Natl. Acad. Sci. U.S.A. 68, 219.
9. Baumal, R., Birshtein, B.K., Coffino, P., and Scharff, M.D. (1973). Science 182, 164.
10. Morrison, S.L., and Scharff, M.D. (1975). J. Immunol. 114, 655.
11. Cook, W.D., and Scharff, M.D. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 5687.
12. Lundblad, A., Steller, R., Kabat, E.A., Hirst, J.W. Weigert, M.G., and Cohn, M. (1972). Immunochemistry 9, 535.

13. Margulies, D.H., Kuehl, W.M., and Scharff, M.D. (1976). Cell 8, 405.
14. Sharon, J., Morrison, S.L., and Kabat, E.A. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 1420.
15. Coffino, P., Baumal, R., Laskov, R., and Scharff, M.D. (1971). J. Cell Phys. 79, 429.
16. Jeanes, A., Haynes, W.C., Wilham, C.A., Rankin, J.C., Melvin, E.H., Austen, M.J., Cluskey, J.E., Fisher, B.E., Tsuchiya, H.M., and Rist, C.E. (1954) J. Am. Chem. Soc. 76, 5041.
17. Takeo, K., and Nakamura, S. (1972). Arch. Biochem. Biophys. 153, 1.
18. Maizel, J.V. (1969). In: Fundamental Techniques in Virology (Habel, K., and Salzman, N., eds.) Academic Press, New York, pp. 334.
19. Weitzman, S., Scott, V., and Keegstra, K. (1979). Anal. Biochem. 97, 438.
20. Wims, L.A., and Morrison, S.L. (1981) Mut. Res. in press.
21. Matsuuchi, L., and Morrison, S.L. (1978). J. Immunol. 121, 962.
22. Kabat, E.A. 1961. Kabat and Mayer's Experimental Immunochemistry. 2nd Ed., Charles C. Thomas, Publisher, Springfield, Illinois.
23. Monk, R.J., Morrison, S.L., and Milcarek, C. (1981). Biochem. in press.
24. Moyer, S.A., and Summers, D.F. (1974). Cell 1, 63.
25. Etichison, J.R., and Holland, J.J. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 4011.
26. Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M., and Davies, D.R. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 4298.

THE STRUCTURE OF THE HUMAN MAJOR HISTOCOMPATIBILITY LOCUS*

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ABSTRACT

We have been studying the genomic organization of sequences complementary to a cDNA clone of HLA-B mRNA isolated from a human cell line. The positive cDNA clone was initially identified using an extended 11 base synthetic oligonucleotide probe.

Southern blotting of human DNA isolated from a variety of sources shows multiple bands complementary to the HLA probe. Approximately 16 bands can be identified using any of a number of different restriction enzymes. Specific differences between individuals can be detected and these may have possible clinical significance. Studies with a human/hamster hybrid cell line suggest nearly all the observed bands reside on chromosome 6 which contains the MHC locus.

Genomic cloning of DNA from a human library shows

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that some of the more intense bands detected by blotting are composed of more than one type of fragment, suggesting some degree of long-range duplication of DNA sequences in the genome. Most of the fragments contain a complete gene which spans about 3 K.B. of chromosomal DNA and includes a number of intervening sequences.

It will now be possible to compare the HLA with the immunoglobulin gene complexes and determine possible evolutionary relationships between them.

INTRODUCTION

The major human histocompatibility locus (the HLA complex) encodes many antigenic determinants, some of which are involved in cell-cell recognition and some in the regulation of the immune response (for review see Ref. 1). Five major groups of MHC encoded antigens have been described, HLA-A,-B,-C,-D and -DR. The best characterized of these are the HLA-A and HLA-B loci which code for two series of structurally similar but highly polymorphic proteins.These two loci are the human equivalent of the murine H-2D and H-2K series.

The study of the HLA complex at the molecular level would help in our understanding of some of the processes involved in these areas of immunogenetics. To begin this analysis a cDNA clone of an mRNA for an HLA-B antigen was constructed (2) and used to investigate the genomic organization of the HLA locus.

MATERIALS AND METHODS

Sources of RNA and cell lines are as described in Ref. 2. All other techniques were performed by standard published procedures. Activated APT paper was used as the support medium for blotting experiments (B. Seed, personal communication).

RESULTS

The construction of the HLA cDNA clone has been described in detail elsewhere (2). Briefly, a synthetic oligonucleotide primer 11 bases long corresponding to a

C-terminal portion of the amino-acid sequence of an HLA protein (3) which would have the least amount of codon degeneracy was synthesized using the triester method (4). The primer was hybridized to mRNA from a human lymphoblastoid cell line and extended by reverse transcriptase in the presence of dideoxythymidine triphosphate and an excess of the other three ordinary deoxynucleoside triphosphates. In this way we isolated an extended 30 base oligonucleotide whose sequence corresponded to the preliminary amino-acid sequence of the HLA-B7 protein.

The 30-mer was used to screen cDNA libraries and positive HLA-like clones identified. One such clone corresponding to a B antigen (pDP 001) was found to have an insert approximately 1300 b.p. in length spanning the distance from the polyadenylic acid residues at the 3' end of the message to amino-acid 39 from the N terminal end of the protein. This clone was used in the studies described below.

A. Southern Blotting

Samples of human DNA were cleaved by different restriction enzymes and used in Southern blotting studies. A representative blot using pDP 001 as probe is shown in Fig. 1.

A number of observations can be made about the hybridization pattern which was obtained under low stringency washing conditions. Each enzyme gave rise to several bands (up to 16) which hybridized to the probe, showing that there are multiple genomic copies of sequences complementary to at least a part of the cDNA clone.

The bands are of different intensities, possibly reflecting either different lengths, or different extents of homology between the probe and the genomic sequences, or reflecting the number of copies of the sequence present at any given position. Preliminary studies on cloned genomic fragments show that the final possibility certainly makes a contribution to the differences. Two genomic EcoRI fragments of the same length were cloned and found to differ in internal sequence.

The more intense bands may correspond to HLA genes such as HLA-A or HLA-B and the fainter bands to related genes, for example, HLA-C or HLA-D/DR genes, or they could represent pseudogenes.

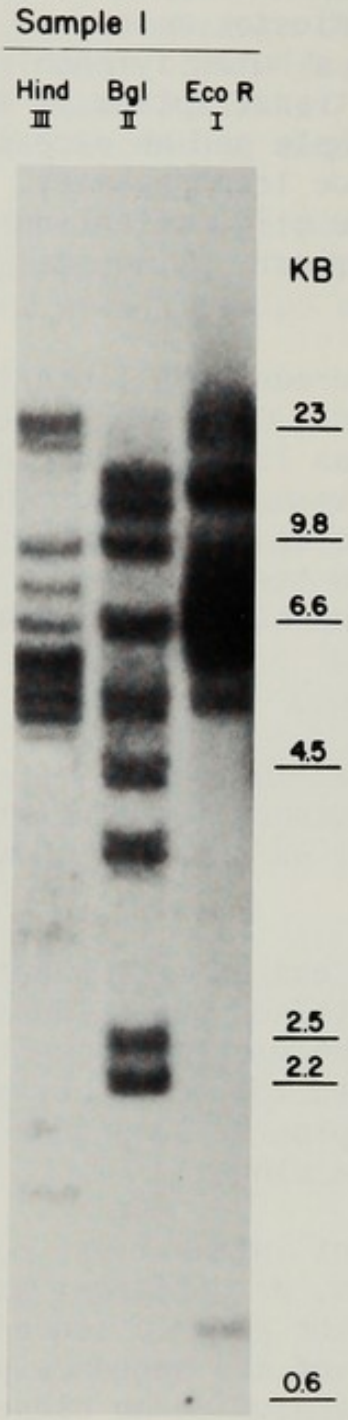

Fig. 1. Southern blot showing bands which hybridize to the HLA cDNA clone.

Owing to sequence polymorphisms and the possible presence of intervening sequences, it is not possible to estimate either the number of copies of any HLA gene or

to demonstrate the actual presence of intervening sequences. However, the large number of bands obtained suggests that the cDNA probe is detecting more than the very minimum number of possible genes which would be detected by this probe, which would be the HLA-A and HLA-B genes.

A preliminary blot using the 3' portion of the cDNA probe corresponding to the untranslated region of the mRNA showed a very similar pattern to that obtained with the whole probe. This suggests that each band contains at least a 3'sequence and may contain a complete gene. Data from a sequence analysis of genomic clones supports this conclusion and demonstrates strong conservation of the 3' untranslated sequences between different clones.

B. Polymorphism Studies

The HLA antigens are highly polymorphic (5), and therefore DNAs from different individuals were tested for possible variations in the banding pattern. Fig. 2 illustrates some of the differences obtained between individuals using three restriction enzymes.

Southern blotting is not a quantitative technique and hence heterozygous loci might not be detected by intensity differences but only by the presence or absence of a given band. Differences in band intensities may also be caused by variability in extents of homology between polymorphic HLA sequences. Nevertheless, a number of striking differences between intense bands can be observed, especially with EcoRI and BamHl enzymes (Fig. 2). It is not known whether the polymorphism resides in the transcribed or in the flanking sequences or in any intervening sequences, nor can any correlation be made between any polymorphism and any particular HLA-type. Family studies would be needed to answer this question.

Furthermore, it may be possible to use restriction enzyme polymorphism to study possible relationships between HLA type and diseases (6) such as alkylosing spondylitis. It may also be possible to "fingerprint" individuals by using several different enzymes to search for polymorphisms. A preliminary survey of some polymorphisms has shown that the prominent band "A" in the EcoRI (cf. Fig. 2) digest was found in 2 out of 10 individuals examined and band "B" in only one of the 10. Frequencies in this range would be useful in such studies.

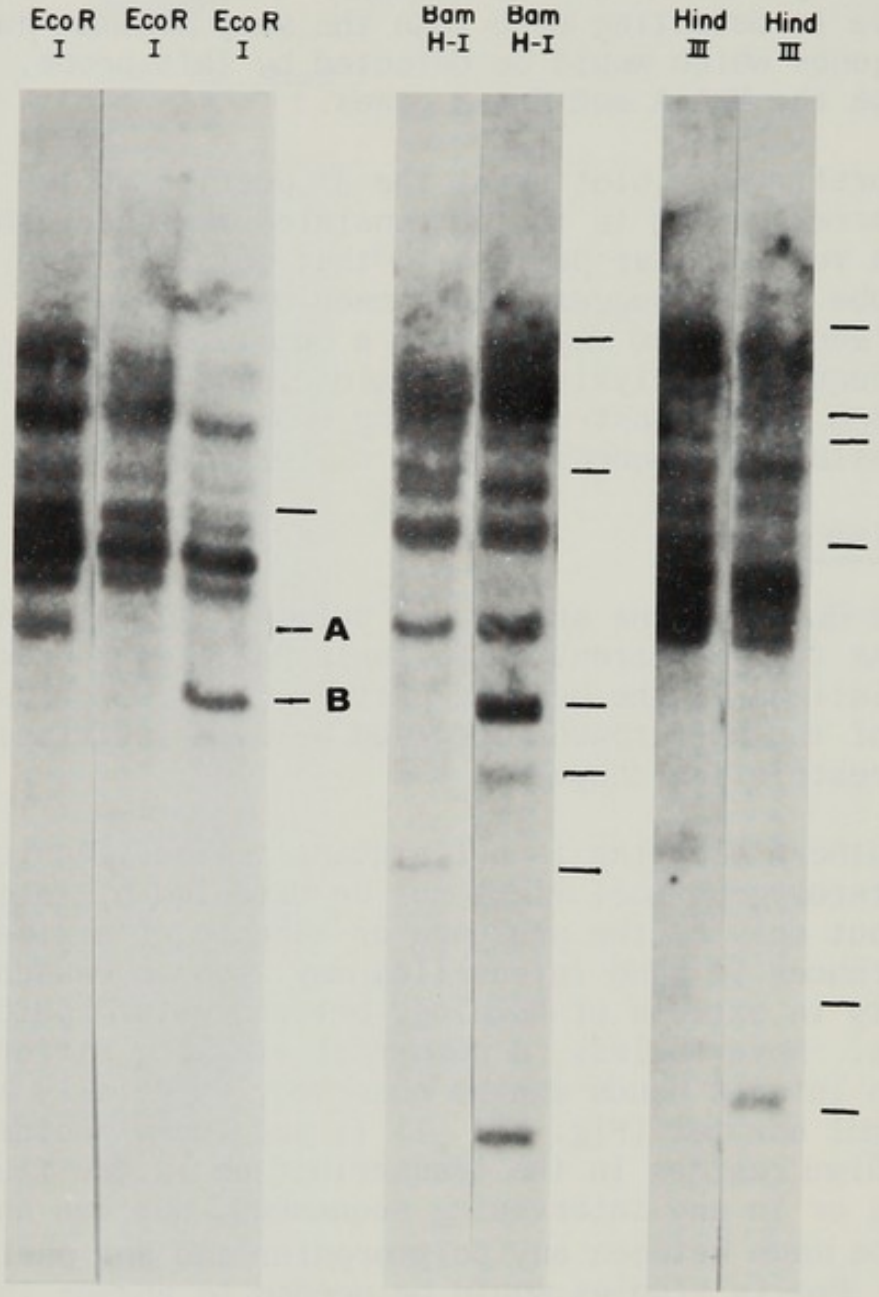

Fig. 2. Southern blot demonstrating HLA polymorphisms. Black bars at side indicate polymorphic bands. For explanation of bands A and B see text.

C. Chromosomal Localization

The number of bands observed by Southern blotting is greater than the minimum number (3-6) necessary to account for the HLA genes. Not all of these bands may encode HLA genes; some may code for other genes, and it was not certain *a priori* that all were linked to the MHC lows.

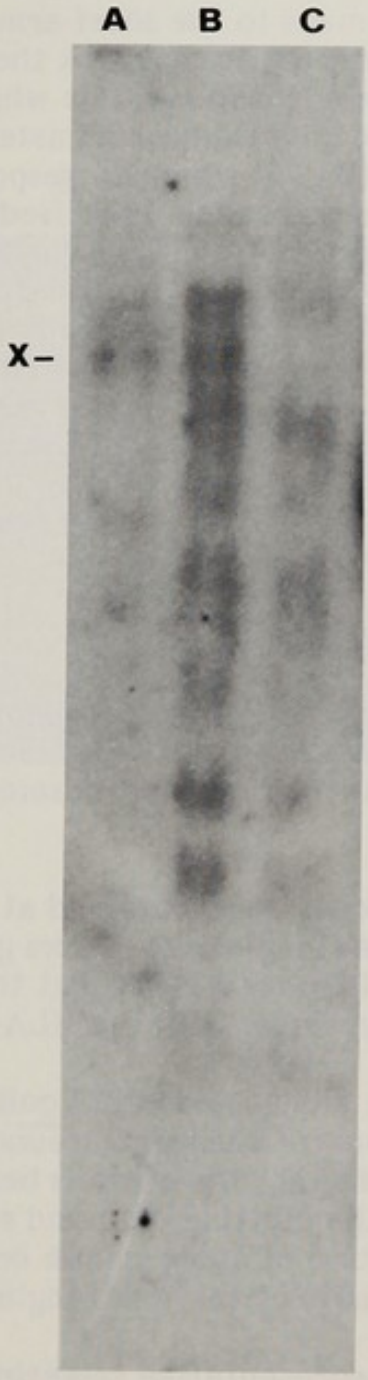

Figure 3. Southern blot of human/hamster cell line.
A. Human parental DNA cut with Bam Hl.
B. Hybrid DNA cut with Bam Hl.
C. Hamster parental DNA cut with Bam Hl.
X indicates position of major human specific band.

The MHC locus maps to the short arm of chromosome 6 (7). Therefore, as a preliminary experiment the cDNA clone was hybridized to the DNA from a hybrid cell line which contains only human chromosomes 3 and 6 on a Chinese hamster background. Many bands were detected (Fig. 3) which corresponded to both human and hamster genes. The darkest human-specific band (X) in the parental cell line was clearly present in the hybrid, indicating this band is probably linked to the HLA locus. Many of the fainter bands observed in the human parental sample cannot be unambiguously identified in the hybrid DNA because they co-migrated with hamster-specific bands. A more detailed analysis using several restriction enzymes is under way to clarify this point. Although the major human band can be assigned to chromosome 6, it is possible that some of the fainter bands may not be HLA linked and may reside on other chromosomes.

D. Genomic Cloning.

A library of human DNA from an EcoRI partial digest was cloned in the Charon vector 4A and screened for HLA-like genes. Many positive clones were identified and ten selected for further examination.

HLA positive clones were obtained at approximately 10-fold higher frequency than single copy B-like globin clones. This agrees with the blotting data and suggests that there are many genomic sequences which cross-hybridize with the HLA-B cDNA clone.

The sizes of the cloned genomic EcoRI fragments which hybridize to the cDNA clone are clustered around two major classes. One group is between 11 and 13 kb; the other is between 5 and 6 kb. This is an agreement with the blotting data and suggests that the clones are truly representative of the genomic organization of the genes. The significance,if any, of this grouping is unknown.

Fig. 4 shows a representative selection of 6 genomic clones cleaved with Xba and EcoRI and Xba. Two of the clones,A and B, have a small EcoRI generated fragment and hence may be overlapping. Clones B, D and E each reveal four small fragments generated by EcoRI and Xba double digests and may also be overlapping, although clone E does not share an EcoRI fragment with clones B and D and may be an allelic variant. Alternatively, there may be sequence repetition between different clones.

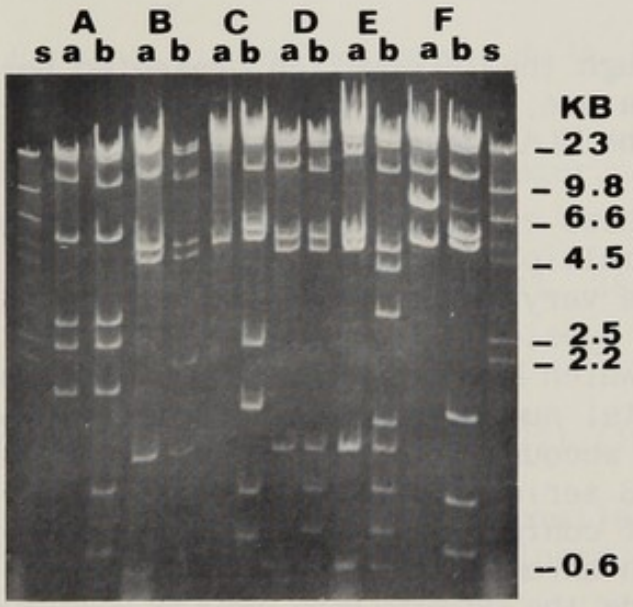

Fig. 4. Representative selection of HLA positive genomic clones.

a. cleaved by Xba.
b. cleaved by Xba plus EcoRI.

s. size marker.

Clones A and D have more than one copy of the 3' untranslated regions and may therefore contain more than one complete gene.

Nucleotide sequence data generated from the 6.5 kb EcoRI fragment of clone D has revealed the presence of at least three intervening sequences which separate the "domains" of the proteins (8) and would also be consistent with additional intervening sequences immediately preceding

and following the segments of DNA encoding the mature protein. Intervening sequences also separate the different domains of immunoglobulins and a number of other proteins (9), in agreement with Gilbert's model (10).

CONCLUSION

Although the information presented here is of a preliminary nature, it does provide some insights into the nature of the HLA locus.

Sequences complementary to the HLA-B cDNA clone are present in multiple copies in the genome. Different individuals have very similar patterns of DNA restriction fragments which hybridize to the HLA probe, but there is some variation which may prove to be a useful polymorphic system. The total number of such sequences is probably insufficient to account for all possible allelic variants of the HLA-A, -B series. Thus, for example, any given individual would not contain all possible HLA-B genes of which only one on each chromosome would be expressed. However, we cannot exclude the possibility that a subset of all HLA-B variants may be present in the genome, nor can we exclude a cassette model of gene activation.

Alternative explanations for the apparent multiplicity of sequences are pseudogenes or functional but only related genes. The limited data suggests that each band represents a complete gene spanning about 3 kb of genomic DNA and including intervening sequences which separate the different domains of the protein molecule. In this respect the HLA genes resemble "orthodox" genes such as the globin genes and neither contain very large intervening sequences such as the murine dihydrofolate reductase (11) or HGPRT (12) genes, nor very numerous intervening sequences, as in collagen (13), nor do they appear to be in separate widely dispersed sections which may need to undergo somatic rearrangement prior to expression (14).

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REFERENCES

1. Amos, B. and Kostyu, D. The genetics of histocompatibility. In "Advances in Human Genetics," Vol. 10 (H. Harris and K. Hirschhorn, eds.). Plenum Press, New York and London (1980).

2. Sood, A., Pereira, D. and Weissman, S. M. Proc. Natl. Acad. Sci. USA 78, 616 (1981).

3. Robb, R. J., Terhorst, C. and Strominger, J. L. J. Biol. Chem. 253, 5319 (1978).

4. Miyoshi, K. and Itakura, K. Tet. Lett. 38, 3635 (1979).

5. Klein, J. Science 203, 516 (1979).

6. Dick, H. M. Brit. Med. Bulletin 34, 271 (1978).

7. Van Someren, H., Westerveld, A., Hagemaijer, A., Mees, J. R., Meera Khan, P., and Zaalberg, O. B. Proc. Natl. Acad. Sci. USA 71, 962 (1974).

8. Ploegh, H. L., Orr, H. T. and Strominger, J. L. In press.

9. Artymiuk, P. J., Blake, C. C-T. and Sippel, A. E. Nature 290, 287 (1981).

10. Gilbert, W. In "Eukaryotic Gene Regulation." In ICN-UCLA Symposium on Molecular and Cellular Biology," Vol. 14.1 (Axel, R., Maniatis, T. and Fox, C. F., eds). Academic Press, New York (1979).

11. Nunberg, J. H., Kaufman, R. J., Chang, A. C. Y., Cohen, S. N. and R. T. Schimke. Cell 19, 355 (1980).

12. Liskay, M. Personal communication.

13. Yamada, Y., Avvedimento, V. E., Mudryj, M., Ohkubo, H., Vogeli, G., Irani, M., Pastan, I. and de Crombrugghe, B. Cell 22, 887 (1980).

14. Brack, C. and Tonegawa, S. Proc. Natl. Acad. Sci. 74, 5652 (1977).

THE BETA-2 MICROGLOBULIN GENE: EVOLUTIONARY RELATIONSHIPS

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Beta-2 microglobulin is encoded by a single gene in the mouse. This gene consists of at least four coding blocks separated by intervening sequences. However, the major portion of the protein (amino acids 3-95) is encoded as a single unit analogous to the discrete coding blocks for immunoglobulin constant and variable region domains and H-2 domains. Nucleotide sequence comparisons provide further evidence for a common evolutionary origin of the genes for beta-2 microglobulin, immunoglobulins and H-2.

INTRODUCTION

Beta-2 microglobulin is a small non-glycosylated polypeptide (11,800 daltons) present in serum and on the surface of almost all mammalian cells. It is not an integral membrane protein, but is found in close, non-covalent association with several membrane glycosylated polypeptides. In the mouse the proteins known to contain a beta-2 microglobulin subunit include the classical transplantation antigens (H-2 K, D, and L) and certain cellular differentiation antigens (TL, Qa-1, and Qa-2). These polypeptides are all encoded on chromosome 17 within the major histocompatibility complex (MHC) and most, if not all, are polymorphic. In contrast, beta-2 microglobulin is not linked to the MHC, and its polymorphism is extremely limited. Recently, an electrophoretically distinct variant of beta-2 microglobulin has been identified in C57Bl mice (1-3). This polymorphism, which is the result of a single amino acid substitution at residue 85 (Ala vs. Asp) (4), has enabled several groups to map the gene encoding beta-2 microglobulin to chromosome 2, closely linked to the loci for Ly-4 (a lymphocyte differentiation antigen) and H-3 (a minor histocompatibility antigen) (5,6). The association of

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beta-2 microglobulin with several distinct, although related cell surface molecules raises the question of how many genes encode molecules which have been identified as beta-2 microglobulin. Are the beta-2 microglobulin subunits of all of these proteins identical? Are they encoded at the same or different loci?

The structure of beta-2 microglobulin (99 amino acids with a disulfide loop of 56 residues) is reminiscent of immunoglobulin constant and variable region domains (7-8). Direct amino acid sequence comparison indicates as much as 28% homology between beta-2 microglobulin and immunoglobulin constant region domains (8,9). Similar protein structural features and amino acid sequence homologies to both beta-2 microglobulin and immunoglobulin domains have been described for the third domain (that closest to the plasma membrane) of HL-A (human) and H-2 (mouse) histocompatibility antigens (10-13). These protein studies have led to the hypothesis that beta-2 microglobulin, MHC antigens, and immunoglobulin domains all evolved from a common ancestral gene and are therefore all members of a genetic "super-family" (13-15).

We were interested in cloning the beta-2 microglobulin gene to determine how many genes encode this ubiquitous molecule and to see whether the postulated evolutionary relationship of beta-2 microglobulin , histocompatibility antigens, and immunoglobulins could be verified by comparisons of genetic organization and nucleotide sequence.

RESULTS AND DISCUSSION

Beta-2 microglobulin is produced by virtually all cells, but it represents less than 0.1% of the total cellular protein (16,17). These scant quantities of protein, and presumably mRNA, suggested that it would be difficult to obtain a molecular (cDNA) probe to study the beta-2 microglobulin gene. We therefore developed a positive selection technique by which large numbers of cDNA clones could be screened simultaneously to identify those containing sequences corresponding to beta-2 microglobulin (18). Once such cDNA clones were obtained, we could use them as hybridization probes in Southern blotting (19) experiments to determine the number of beta-2 microglobulin genes. Using a variety of different restriction endonucleases to digest genomic DNA from several strains of mice, we have been able to show that is a single gene for beta-2 microglobulin per haploid genome (data not shown). We have been unable to detect any closely related (>50% nucleotide sequence homology) genes or pseudogenes. It

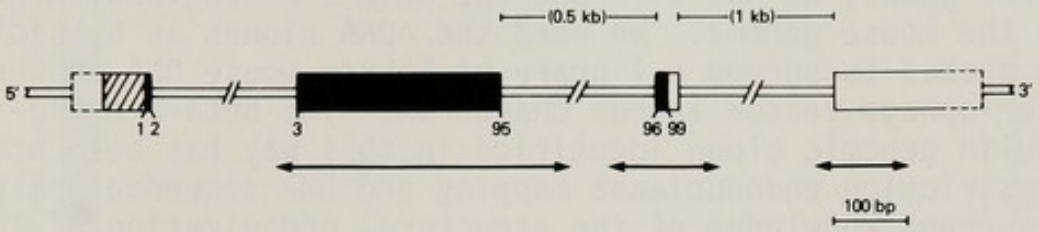

Figure 1. The structural organization of the beta-2 microglobulin gene. The parts of the gene encoding the secreted protein are indicated by boxes with black shading, and the numbers below refer to the encoded amino acid residues. The intervening sequences are represented by the bars connecting the coding blocks. The hatched area represents the sequence encoding the signal peptide. Open boxes indicate the 5' and 3' untranslated regions. The dotted lines indicate that the lengths of these untranslated regions have not yet been determined. The precise location of the coding block at the 5' end is not known, but it lies between 0.8 and 2 kilobases (kb) 5' of the main coding block. Although this 5' coding region is shown as a single unit, it is possible that there are more intervening sequences separating it into discrete coding blocks. The arrows below indicate the regions of the gene which have been sequenced.

is likely that the limited polymorphism of beta-2 microglobulin reflects the existence of only one gene for this protein. In contrast, the markedly polymorphic H-2 antigens have been shown to be part of a large multi-gene family (20-22). The gene duplications that have resulted in many germline H-2-related genes have provided the substrate for both DNA recombination and mutation. These processes could generate new variants which could then be either silently carried or expressed, and either functional or not. These new variants could provide even more substrate for the same diversifying processes, increasing the polymorphism without necessarily affecting the viability of the organism. In contrast, the single gene for beta-2 microglobulin provides a smaller target for mutation and less chance for recombination. In addition, beta-2 microglobulin must interact with several distinct (although related) proteins. It is therefore likely that many kinds of alterations in this gene will decrease viability and consequently be lost from the gene pool. Selection will therefore further limit the generation of polymorphic variants.

In order to investigate the evolutionary relationships of the single beta-2 microglobulin gene to both immunoglobulin and H-2 genes, we had to clone the beta-2 microglobulin gene from the mouse genome. We used the cDNA clones as hybridization probes to screen a library of BALB/c mouse DNA in the bacteriophage vector lambda Charon 4A. The beta-2 microglobulin genomic clone identified in this way has been studied by restriction endonuclease mapping and DNA sequence analysis. Our current knowledge of the structural organization of the beta-2 microglobulin gene as determined by these studies is indicated in Figure 1. The gene consists of at least four coding blocks separated by at least three intervening sequences. The junctions between the identified coding blocks and intervening sequences all contain the appropriate RNA splicing acceptor and donor sequences in accordance with the established consensus sequences for such junctions (23,24). Almost all of the structural protein, i.e., amino acids 3-95, is encoded in a single unit flanked by intervening sequences. This main coding block, which represents a domain-sized unit including the disulfide loop, is clearly analogous to the coding blocks for immunoglobulin variable and constant region domains (25-31). Recent evidence indicates that the domains of H-2 molecules, as recognized at the protein level, are also encoded in blocks separated by intervening sequences (22).

For comparisons of actual nucleotide sequences we have made use of a computer "best-fit" matrix routine (20) designed to compare distantly related sequences and emphasize regions of less than perfect homology. Figure 2 shows such a comparison of the nucleotide sequences of beta-2 microglobulin and the third constant region domain (CH3) of the mouse immunoglobulin heavy chain gamma 1. Each base of the nucleotide sequence along the Y axis is compared with every base along the X axis and a score given for each homology. Each time this is done the 5' and 3' flanking sequences are also compared and an additional score (in an inverse relationship to distance from the test bases) is added for matches in these regions. A dot is then placed at the position of highest score. The regions of greatest homology are indicated by the dotted diaggonal lines in the figure. The two most significant regions (longest lines), labelled 1 and 2, correspond in both genes to the sequences surrounding the codons for each of the two Cys residues involved in the disulfide loop. The homology is 57% over a region of 69 base pairs (bp) for the first Cys and 51 bp for the second. The amino acid sequence homology for both of the corresponding regions is approximately 30%. The actual nucleotide sequences of the homology region around the first Cys is shown in Figure 3. We have also compared the

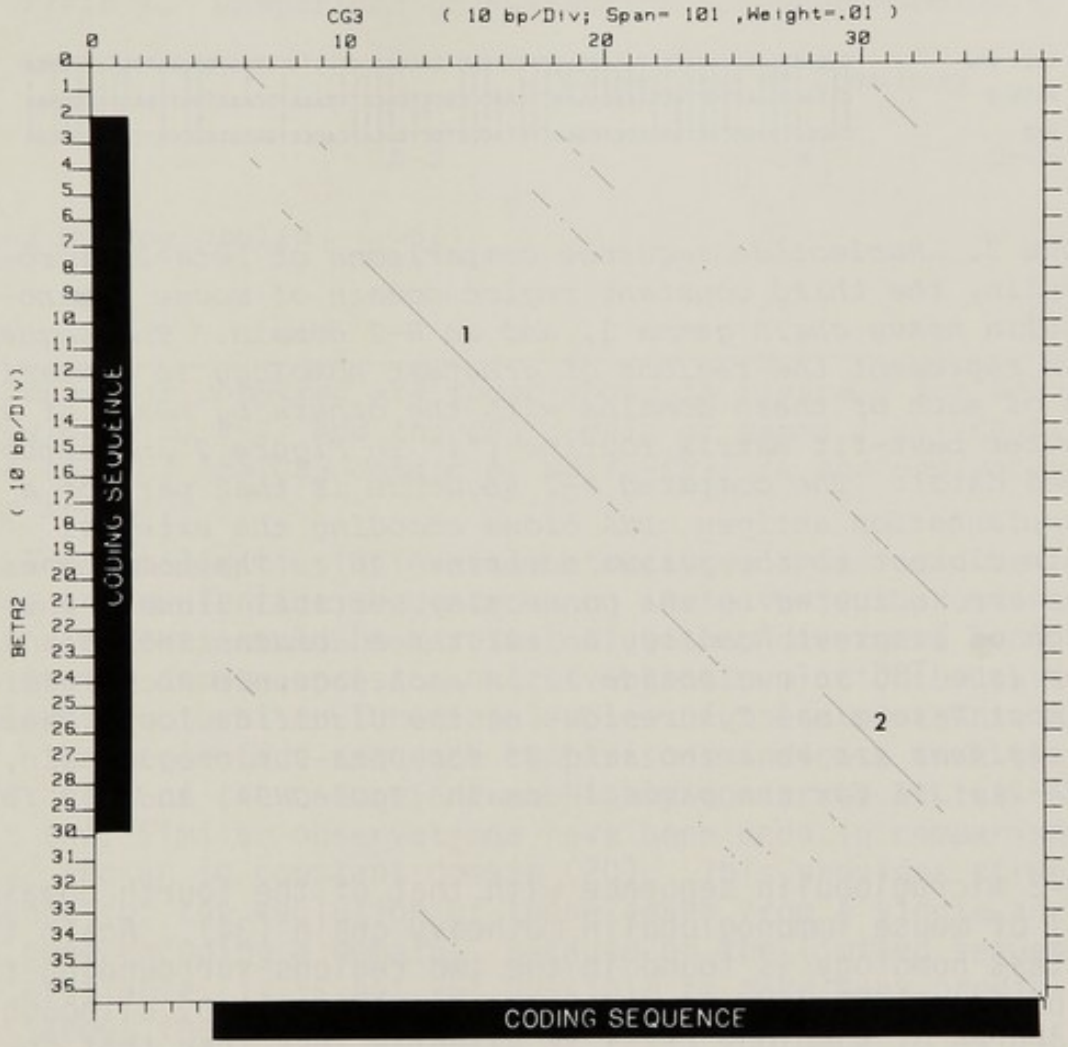

Figure 2. Homology between the nucleotide sequences of beta-2 microglobulin and the third constant region domain of mouse immunoglobulin heavy chain gamma 1. The nucleotide sequences of the main coding block (amino acids 3-95) of beta-2 microglobulin (BETA2) (32) and the third constant region domain of gamma 1 (CG3) (33) were compared using the best-fit computer matrix routine (20). The regions of homology are indicated by the dotted lines with a -45° slope. The two most significant regions of homology are labelled "1" and "2", and correlate with the sequences surrounding the Cys residues involved in the disulfide loops of both proteins. The numbering of the nucleotide sequences begins in the intervening sequences preceding the domains. The coding sequences are indicated by the shaded boxes in the figure and represent nucleotides 20-298 for beta-2 microglobulin and 47-367 for the gamma 1 third domain. The homologous Cys residues are encoded beginning at nucleotides 95 and 260 for beta-2 microglobulin and 125 and 299 for the gamma 1 domain. The divisions along the axes represent units of 10 bp.

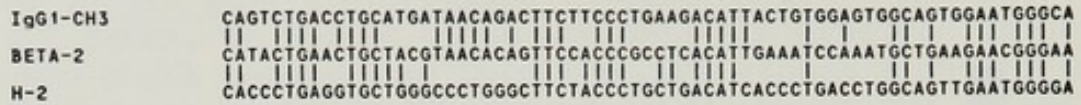

Figure 3. Nucleotide sequence comparisons of beta-2 microglobulin, the third constant region domain of mouse immunoglobulin heavy chain gamma 1, and an H-2 domain. The sequences shown represent the regions of greatest homology in comparisons of each of these domains with the others by means of a computer best-fit matrix routine ("1" in Figure 2 and unpublished data). The compared H-2 sequence is that part of a transplantation antigen cDNA clone encoding the external domain closest to the plasma membrane (20). The homologous bases are indicated by the connecting vertical lines. The region of greatest homology in all three domains includes the codon (the TGC *at nucleotide 11 in each sequence above) for the most N-terminal Cys residue of the disulfide loop. These Cys residues are at amino acid 25 for beta-2 microglobulin (BETA-2), 396 for the gamma 1 domain (IgG1-CH3), and 203 for H-2.*

beta-2 microglobulin sequence with that of the fourth domain (CH4) of mouse immunoglobulin mu heavy chain (34). Again the greatest homology is found in the two regions surrounding the codons for the Cys residues of the disulfide loop, although the degree of homology (54%) is slightly less than that observed for CH3 of gamma 1 (data not shown). The computer routine does not reveal any areas of significant homology between the beta-2 microglobulin sequence and either kappa (MOPC 41) (35) or lambda 1 (36) variable region sequences. Direct comparison of the sequences around the Cys codons shows only short stretches of up to 50% homology. Finally, we have compared the beta-2 microglobulin sequence to that part of the sequence of an H-2 family cDNA clone (20) encoding the third domain (the region that is most homologous to beta-2 microglobulin and immunoglobulins at the protein level). The regions of greatest nucleotide homology were the same as those observed in the comparisons with immunoglobulin constant domains, i.e., surrounding the codons for the two Cys residues. The nucleotide sequences were 51% and 60% homologous for the first and second such regions. Figure 3 shows the sequence comparison for the region around the first Cys codon. Of note, the direct comparison between H-2 and either CH3 of gamma 1 or CH4 of mu reveals even greater homology (65% and 62%, respectively) than that between beta-2 microglobulin and any of these domains (Table I).

Table I. Comparison of Nucleotide Sequence Homologies

	Percentage of Homology		
	H-2	γ_1-CH_3	μ-CH_4
Beta-2 Microglobulin	*51*	*57*	*54*
H-2		*65*	*62*

The sequences compared are those shown in Figure 3 for beta-2 microglobulin, H-2, and the CH_3 domain of gamma 1 (γ_1-CH_3), while that of the CH_4 domain of mu (μ-CH_4) is the equivalent 69 bp region.

The similarities in overall structure, amino acid sequence and nucleotide sequence among these various proteins and their genes could be a result of either divergent evolution from a common ancestor or convergent evolution because of similar structural or functional roles of these proteins. However, the nucleotide sequence comparisons indicate that the third bases of the codons are as highly conserved as the first two. Similar observations have been made in comparisons of H-2 and an Ig constant domain (20). This provides strong evidence for the evolution of these genes from a single ancestral gene encoding a domain. Because of the limited sequence data available, it is not yet possible to make many predictions about the order in which the genes encoding these various proteins diverged from each other. However, it does seem likely that immunoglobulin variable region genes either diverged earlier or evolved much more rapidly than the other members of the family, since they appear to have retained the least homology. Similarly, the gene for beta-2 microglobulin may have diverged earlier than the split between H-2 and heavy chain constant regions, since the latter two appear to be more homologous. More H-2 sequences will have to be compared as they become available in order to make more than tentative conclusions. Regardless of the order in which these genes diverged from one another, the homologies previously described at the level of protein structure and amino acid sequence and now for the genetic structure and nucleotide sequence indicate that they have all evolved from a common gene encoding a primitive domain.

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REFERENCES

1. Michaelson, J., Rothenberg, E., and Boyse, E.A. (1980). *Immunogenetics 11,* 93.

2. Goding, J.W. and Walker, I.D. (1980). *Proc. Natl. Acad. Sci. U.S.A. 77,* 7395.

3. Robinson, P.J., Graf, L., and Sege, K. (1981). *Proc. Natl. Acad. Sci. U.S.A. 78,* 1167.

4. Gates III, F.T., Coligan, J.E., and Kindt, T.J. (1981). *Proc. Natl. Acad. Sci. U.S.A. 78,* 554.

5. Goding, J.W. (1981). *J. Immunol. 126,* 1643.

6. Michaelson, J. *Immunogenetics,* in press.

7. Smithies, O., and Poulik, M.D. (1972). *Science 175,* 187.

8. Peterson, P.A., Cunningham, B.A., Berggard, I., and Edelman, G.M. (1972). *Proc. Natl. Acad. Sci. U.S.A. 69,* 1697.

9. Appella, E., Tanigaki, N., Natori, T., and Pressman, D. (1976). *Biochem. Biophys. Res. Commun. 70,* 425.

10. Gally, J.A., and Edelman, G.M. (1972). *Ann. Rev. Genet. 6,* 1.

11. Strominger, J.L., Cresswell, P., Grey, H., Humphreys, R.E. Mann, D., McCune, J., Parham, P., Robb, R., Sanderson, A.L., Springer, T.A., Terhorst, C., and Turner, M.J. (1974). *Transplant. Rev. 21,* 126.

12. Peterson, P.A., Rask, L., Sege, K., Klareskog, L., Anundi, H., and Ostberg, L. (1975). *Proc. Natl. Acad. Sci. U.S.A. 72,* 1612.

13. Orr, H.T., Lancet, D., Robb, R.J., Lopez de Castro, J.A. and Strominger, J.L. (1979). *Nature 282,* 266.

14. Burnet, F.M. (1970). *Nature 226,* 123.

15. Barker, W.C., and Dayhoff, M.O. (1976) in *Atlas of Protein Sequence and Structure,* Vol. 5, Suppl. 2, pp. 165-190.

16. Dubois, P., Fellous, M., Gachelin, G., Jacob, F., Kemler, R., Pressman, D., and Tanigaki, N. (1976). *Transplantation 22,* 467.

17. Appella, E. and Ramanathan, L., unpublished results.

18. Parnes, J.R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E., and Seidman, J.G. (1981). *Proc. Natl. Acad. Sci. U.S.A. 78,* 2253.

19. Southern, E.M. (1975). *J. Mol. Biol. 98,* 503.

20. Steinmetz, M., Frelinger, J.G., Fisher, D., Hunkapiller, T., Pereira, D., Weissman, S.M., Uehara, H., Nathenson, S., and Hood, L. (1981). *Cell 24,* 125.

21. Kvist, S., Bregegere, F., Rask, L., Cami, B., Garoff, H., Daniel, F., Wiman, K., Larhammar, D., Abastado, J.P., Gachelin, G., Peterson, P.A., Dobberstein, B., and Kourilsky, P. *Proc. Natl. Acad. Sci. U.S.A.,* in press.

22. Evans, G., Margulies, D., and Seidman, J.G., unpublished results.

23. Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., and Chambon, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 4853.

24. Seif, I., Khoury, G., and Dhar, R. (1979) *Nucl. Acids Res. 6,* 3387.

25. Brack, C., and Tonegawa, S. (1977). *Proc. Natl. Acad. Sci. U.S.A. 74,* 5652.

26. Seidman, J.G., and Leder, P. (1978). *Nature 276,* 790.

27. Sakano, H., Rogers, J.H., Hüppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., and Tonegawa, S. (1979). *Nature 277,* 627.

28. Early, P.W., Davis, M.D., Kaback, D., Davidson, N., and Hood, L. (1979). *Proc. Natl. Acad. Sci. U.S.A. 76,* 857.

29. Kataoka, T., Yamawaki-Kataoka, Y., Yamagishi, H., and Honjo, T. (1979). *Proc. Natl. Acad. Sci. U.S.A. 76,* 4240.

30. Tucker, P.W., Marcu, K.B., Newell, N., Richards, J., and Blattner, F. (1979). *Science 206,* 1303.

31. Calame, K., Rogers, J., Early, P., Davis, M., Livant, D., Wall, R., and Hood, L. (1980). *Nature 284,* 452.

32. Parnes, J.R., and Seidman, J.G., unpublished results.

33. Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N., and Mano, Y. (1979). *Cell 18,* 559.

34. Kawakami, T., Takahashi, N., and Honjo, T. (1980). *Nucl. Acids. Res. 8,* 3933.

35. Seidman, J.G., Max, E.E., and Leder, P. (1979). *Nature 280,* 370.

36. Bernard, O., Hozumi, N., and Tonegawa, S. (1978). *Cell 15,* 1133.

WORKSHOP SUMMARY 1-2
IMMUNOGLOBULIN IDIOTYPES AND THEIR EXPRESSION
VARIABILITY OF IMMUNOGLOBULIN GENE PRODUCTS

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THE PUZZLE OF THE T-CELL RECEPTOR (J.M.A.)

Despite a decade of research, the genetic basis for the antigen specificity of T cells remains a mystery. In particular, it is not established whether any conventional immunoglobulin gene contributes to the T cell receptor. Indirect lines of evidence do implicate V_H genes; the most persuasive are the recent identification of some monoclonal antibodies capable of recognizing idiotypes shared by B and T cells, and the existence in some systems of related heteroclitic responses in the two lymphoid populations, a finding which might not be expected for receptors that were similar but not identical. On the other hand, since some idiotypic determinants are now known to represent only a couple of amino acid residues, the possibility of convergent evolution within separate V region families for B and T cells has to be entertained.

It is generally expected that T cell receptors will require multiple V segments and one or more constant (C_T) genes. By analogy with B cells, this argues for DNA rearrangement, probably involving deletions, and some type of joining (J_T) element. The relation of the C_T to the C_H locus can be expressed in four models, three of which equate V_T with V_H.

Model 1 $V_H\ J_H\ C_H\ C_T$ $(J_T = J_H)$

Model 2 $V_H\ J_H\ C_H\ J_T\ C_T$

Model 3 $V_H\ J_T\ C_T\ J_H\ C_H$

Model 4 $V_H\ J_H\ C_H\ //\ V_T\ J_T\ C_T$

Model 1 now appears unlikely, since several functional killer and helper T lines studied by the laboratories of Hood and Tonegawa maintain the J_H region in germline context, or involve $D_H\ J_H$ fusion without V_H. Similarly the absence of

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JH (or C_H) deletion in those lines tends to argue against model 2. Whether suppressor T cells are likely to have the same genetic basis as killers or helpers is however debatable. No evidence bearing on models 3 and 4 is available.

A potential new approach to the T cell receptor was opened by the report by F. Owen and associates of an allotypic determinant displayed on suppressor T cells which is encoded 2 map units to the right of the C_H locus. The properties of this determinant suggest that it corresponds either to a C_T element or to a surface component closely associated with the receptor. If Tsu^d does correspond to a C_T gene, the mapping results would be compatible with either models 2 or 4.

ANTIBODY DIVERSITY I (O.M.)

Many radically different mechanisms can now be identified as contributing to the generation of antibody diversity. In addition to identifying these mechanisms it is important to quantitate the relative contribution to each. Some of the mechanisms generate grossly different Ig molecules (germ-line V genes) whereas others (somatic mutations) generate variants that deviate only slightly from the unmutated sequence (fine variation). The contributions of the mechanisms have been crudely estimated in Table 1. For example the

Table 1.

Germ-line	*Gross variation*	*Finer variation*
V_H *and* V_L	+++	++
J_H *and* J_L	+	+
D_H		++
Allelic differences in all		
Gene rearrangements		
V_L J_L	−	+
V_H D_H		
(D_H D_H)		
V D_H J_H	−	+
H-L combinations	+++	+++
Somatic mutations	+	+++

table suggests that the free combination of H and L chains generates a large number of grossly different Ig-molecules and a large number of slightly different ones. Blank spaces indicate an unwillingness to guess the magnitude.

ANTIBODY DIVERSITY II (A.R.W.)

The nature and generation of antibody diversity continues to be a fascinating problem central to the understanding of immune responsiveness and the self regulation of immune responses. It is important to remember that the generation of antibody diversity shapes not only the repertoire of antibody specificities but also the repertoire of idiotype antigenicity.

Antibody diversity has been studied in a variety of systems and using many methods of analysis. A distinct change in the nature of both systems and analytical approaches underlie the new and deeper understanding of antibody diversity that emerged during the course of this meeting.

In the past, myeloma proteins have provided an insight into antibody diversity at the amino acid level, although the possible limitations of such information were widely acknowledged. The emphasis in the study of myeloma protein sequences has shifted to the comparison of collections of myeloma proteins that specifically bind particular antigens. Until a few years ago the availability of individual homogeneous antibody molecules was limited. The advent of hybridoma technology now provides an unlimited supply of individual monoclonal antibodies specific for any and all antigens. The exploitation of this new resource is accelerating with the application of recombinant DNA technology.

The present view of antibody diversity is based upon: 1) a knowledge of amino acid sequences for collections of myeloma proteins and monoclonal antibodies sharing a common antibody specificity, 2) DNA sequences corresponding to these sets of proteins, 3) identification, counting and DNA sequencing of sets of homologous germ-line gene segments coding for the V, D & J portions of the V-region. A selection of the novel and updated information presented at this meeting is shown in Table 2. This information provided the background for the discussions of antibody diversity at the summary workshop.

The information presented in Table 2 provided definitive evidence for somatic mutations increasing the diversity antibodies derived from the germ-line gene elements. The distribution of somatic point mutations through framework and hypervariable portions of the V-region and the occurrence

Table 2.

SYSTEM/SET:	V_{λ_1} (Tonegawa, Weigert)		V_K21 (Tonegawa, Weigert)		V_K167 (Hood)		PC (Hood, Davie)		Dex (Hood, Davie)	NP (Bothwell)	ARS (Capra, Gefter)
Germ-line segments	DNA	Protein	DNA	Protein	DNA	Protein	DNA	Protein	Protein	DNA	Protein
V	1		8		1		5(4)			7	
D	–		–					>10	~11		
J	1		4		2		1		4		
Mutations FR	0	0		12			11		50%	5	10
HVR	2	12		48			11		50%	5	10
Silent	0						2			3	
Codon changing	2						2			7	
Ratio: Prototype / variant	2/1		1/3		1/2	1/5	1/9		1/6	1/2	1/10

of silent mutations in addition to codon changing mutations appear to be more consistent with a random mutational model assuming a high rate of point mutation and do not require the construction of a model for directed mutagenic events.

The stage of lymphocyte differentiation during which the somatic mutational mechanisms operate was a matter for much conjecture. At present the evidence is consistent with the idea that mutations accumulate throughout the expansion of B cell clones. Gearhart & Hood reported that in the anti-phosphorylcholine system the V_H regions of IgG and IgA antibodies are usually variants of the germ-line T15 V_H sequence. By contrast IgM anti-phosphorylcholine antibodies expressed only the T15 V_H sequence with no evidence for accumulated somatic mutations. The light chains of these antibodies correspond to only three prototype sequences in the IgM set but numerous mutants of these three prototype sequences are found in the IgG and IgA sets of anti-phosphorylcholine antibodies. The appearance of somatic mutants of V-H subsequent to isotype switching raised the possibility that there is a switch-related mutational mechanism. However, the concomitant appearance of mutations in associated V_L regions implies that the hypothetical switch related mechanism can act *trans* as well as *cis*. At present it is simpler to accept an elapsed time model in which mutations accumulate randomly throughout the proliferative history of a clone. The finding of one mutant V_H in an IgM anti-dextran antibody is consistent with the elapsed time model. The demonstration (by Scharff) that somatic mutations affecting antigen binding occur during the culture *in vitro* of S107 plasmacytoma cells appears to support a random somatic mutation model. The findings with S107 were discussed in comparison with the previous lack of V-region mutations in myeloma proteins when sought for either in random screening of clones *in vitro* or as revealed by the sequences of products and genes derived from myeloma cells at intervals of many generations. It is possible that S107 may be a special plasmacytoma line that has retained the mutational mechanisms.

The possibility that hybridomas would reveal more somatic mutation than myelomas was also considered. The monoclonal antibody produced by each hybridoma represents a single expressed V-gene prototype or variant that is assumed to be functional during an antigen driven proliferation of B lymphocytes. However, it cannot be assumed that each V-gene variant rescued in a hybridoma would have had any selective advantage in an immune response to the antigen eliciting the B cell proliferation. The serum antibody response to an antigen is probably not the sum of all variants found in hybridomas. Frequently arising variants probably tell us

more about the mechanism of somatic mutation than about the best way of making an antibody of a given specificity. The serum antibody response on the other hand should reflect antigen directed clonal selection superimposed on the somatic mutational mechanism. The sequence differences observed between the serum antibody and individual monoclonal antibodies specific for ARS may be interpretable in these terms (Capra, Gefter). Continued analysis of hybridoma products collected in a single response should provide an openended set, analysis of which may be very revealing of the mechanisms generating somatic diversity and of their potential.

The antibody repertoire should involve considerable overlap between individually analyzed subsets of the repertoire. Polyfunctionality of individual monoclonal antibodies was mentioned at this meeting only transiently. Further analysis of hybridoma products should clarify the extent to which this property contributes to the repertoire. Sharing of idiotypes between antibodies of different specificities (usually observed as the sharing of idiotype between a specific antibody and non-antibody immunoglobulin) may reflect changes in specificity effected by different V_L-V_H associations or changes in specificity determined by somatic mutations outside idiotopes. The use of germ-line V genes in response to a single hapten appears to be limited. Although an appropriate DNA probe detects several germ-line V genes that might be able to contribute to the anti-phosphorylcholine or anti NP responses (Table 2) the data collected so far suggest that only a single V-gene from each of these sets is being expressed either in its germ-line sequence or as a series of mutants. In the phosphorylcholine related V-gene set it should be noted that there are two identical T15 V-gene sequences represented in the germ-line. In the response to NP the analysis of three expressed V_H genes analyzed at the DNA level was reported at this meeting by Bothwell. Two of these sequences correspond to antibodies chosen as being the most idiotypically distinct amongst a large family of anti-NP hybridoma products. Even so, all three expressed V_H genes appear to be derived either directly or by a small number of somatic mutations from a single germ-line V_H gene in the NP set. Flanking sequence analysis of germ-line and expressed V-genes can provide more conclusive evidence for the origin of particular somatic V-gene variants. Such sequence analysis has already been used by Hood to confirm some of the anti-phosphorylcholine V-gene assignments.

The extent of functional somatic variation of V-genes relative to the noise level in the mechanism of generating diversity evoked some discussion. The concensus seemed to be that most variation could be selected for function in the

immune system. A random hypermutation mechanism does have high potential noise level but many functions can be subserved by V region sequence variation. Thus V_H mutants can be selective for: 1) changes in antibody specificity - gross or fine changes in specificity may be effected mainly by hypervariable region amino acid sequences but the effect of mutations in framework regions cannot be ignored; 2) V_L - V_H combinatorial association component of antibody diversity probably depends largely on framework region requences and hence will be affected by mutations in framework regions; 3) mutations affecting the amino acid sequence in any part of the V region may affect the idiotypes expressed on an antibody and thus may have important effects on the shaping of the antibody repertoire by idiotype - anti-idiotype interactions.

ALLELIC EXCLUSION (M.W.)

Expression of a functional antibody gene depends on precise deletions that join variable gene segments (V_H, V_κ, and V_λ) to joining gene segments (J_κ, J_λ, J_H, and D_H). The key to allelic exclusion lies in the configuration of the silent allele. Surveys of plasmacytomas show these to be in either of two states, germ-line (κ^o, λ^o, or H^o) or aberrantly rearranged (κ^-, λ^-, or H^-). A minimal model for allelic exclusion is that expression of a locus such as the κ-chain locus depends on the rate of the rearrangement event and the relative frequencies of rearrangement to either κ^+ or κ^-. This probabilistic model can be extended to isotypic exclusion, i.e. κ versus λ, and perhaps to other phenotypes such as the precendence of H-chain expression in pre-B lymphocytes. The focus of this symposium was to test directly and indirectly the validity of this model. New results were presented on the nature and rate of formation of aberrant rearrangements, on other levels of gene control, such as transcriptional control, on the state of silent alleles in normal lymphocyte populations, and on systems that test the predictions of the probabilistic model.

ABERRANT REARARRANGEMENTS

The variety of forms of aberrant rearrangements continues to grow, each case representing a different defect in the complex series of events leading to a functional antibody. The joining process can itself be defective, leading to reading frames that result in premature chain termination. This type of aberrant rearrangement has been described at the κ-chain locus (Max, Seidman, and Leder) and an analogous

example at the H-chain locus was described here by Nottenberg and Weissman.

Pseudo genes, examples of which have been found for the joining signals, joining gene segments and V gene segments, are often the reason for aberrant rearrangement. Sequences have been identified at the κ-locus that provide sites for illegitimate recombination of V gene segments to regions lacking joining gene segments (Seidman and Leder, Kennedy and Tonegawa). The consequence of one of these rearrangements is a truncated light chain consisting only of signal region and constant region, due to the absence of J region RNA processing signals (Perry and coworkers, Kuehl and coworkers). This may be a common form of rearrangement in lymphocytes (Alt and coworkers). Illegitimate recombination mediated by sequences within the V_K locus can also occur leading to joins of non-V region sequences to J sequences, as reported by Cory, and other forms of deletion produce "amputated genes", as described by Mach. A pseudo J_K sequence has been found in the complete analysis of the J_K region that could function as a site for aberrant rearrangement (Seidman, Sakano), and V regions may contain a high proportion of pseudo V genes. One out of three of related set of V_K genes appears to be nonfunctional (Bentley and Rabbitts) and two out of six of a related set of V_H genes are crippled (Bothwell et al., this symposium). These pseudo V's contain missense, deletions and insertion mutations, but in principle could be recombined to J gene segments to create aberrant rearrangements.

A most interesting form of aberrant rearrangement at the H-chain locus has been discovered by Kurosawa, Sakano and Tonegawa (Nature, in press). This type occurs at a high frequency in both plasmacytomas and T cell lines and is due to the three-segment nature of the V_H locus, V_H, D_H, and J_H. Thus, a complete V_H gene structure requires two joining events and these appear to occur independently, as D_H-J_H structures have been found at the silent H-chain locus (these in turn were used to identify at least part of the germ-line D_H gene locus). D_H-J_H rearrangement seems to be frequent, perhaps due to the D_H to J_H distance or other factors that could control the rate of rearrangement, such as the number of joining signals. V_H to D_H rearrangement would be predicted from Tonegawa's findings and V_H-D_H and D_H-J_H rearrangements on the same chromosome may lead to a permanently excluded locus.

Allelic exclusion appears not to be controlled at the transcriptional level. Although aberrant rearrangements are usually not transcribed (Perry and coworkers), this is due to the type of rearrangement rather than the transcriptional competence of the silent chromosome. For cases of κ chain

exclusion of the κ^o/κ^+ type, the κ^o allele is transcribed, however, not processed to mRNA. [This property is indirectly shown by the susceptibility of the κ^o locus to endonuclease (Storb, this symposium).] Thus, both loci are transcriptionally competent and κ^o transcription is initiated by germ-line promoter-like sequences near the J_κ locus that are perhaps supplied by the nearest germ-line V_κ gene (Van Ness and coworkers, this symposium). Transcription at the silent κ locus is usually shut down as a result of rearrangement, suggesting that aberrant alleles are often due to the translocation of segments lacking appropriate transcriptional signals.

ALLELIC EXCLUSION IN LYMPHOCYTE POPULATIONS

In view of the variety of opportunities for aberrant rearrangements due to the nature of V-region loci (pseudo genes) and the process of joining, it would seem reasonable that they play a major role in ensuring allelic exclusion. Furthermore, it would be expected that a system selected for to provide diversity through mutation, deletion and multiple genes should in addition incur deleterious cases, for example frameshift mutations. Nevertheless, the possibility that aberrant rearrangements result from a tumor-specific effect has been raised, as most aberrant rearrangements have been characterized in plasmacytomas or other cell lines. Indeed, from studies on the degree of rearrangement at κ loci in normal B lymphocytes it has been concluded that joining occurs on just one chromosome, and therefore aberrant rearrangements do not occur in normal lymphocytes (Joho and Weissman). This conclusion not only raises the question of how plasmacytomas generate different κ^+/κ^- genotypes but also obviates probabilistic explanations for allelic exclusion (see below). These dilemmas posed by the conclusions of Joho and Weissman have stimulated further examination of the state of antibody genes in normal lymphocytes. As reported at this symposium by Coleclough and coworkers, average values from a series of determinations of the state of κ alleles in κ-expressing, normal B lymphocytes indicate a high level of rearrangement at the silent κ allele. Thus, the frequency of what is likely to be aberrant rearrangement at the silent κ locus is not significantly different in normal B lymphocytes and plasmacytomas. Analysis of the H-chain locus in B lymphocytes also reveals aberrant rearrangements at the H-chain locus. In an extension of earlier findings (Hurwitz, Coleclough and Cebra) only low levels of the germline, H^o, are detected in keeping with the low frequency of H^o alleles found in plasmacytoma surveys (Coleclough et al., this symposium).

MODELS

Certain recent data on rearrangements at κ and H loci in mouse lymphocytes and plasmacytomas support the probabilistic model described above. At the κ locus both κ^{o} and κ^{-} configurations contribute to exclusion; at the H-chain locus both H^{o} and H^{-} types are found but the proportion of H^{+}/H^{-} to H^{+}/H^{o} genotypes is higher than κ^{+}/κ^{-} to κ^{+}/κ^{o}. Thus, the rate of rearrangement and frequency of aberrant rearrangement are higher at the H-chain locus, an effect that may be attributed to the nature and/or organization of the D_H locus. On the other hand, earlier conclusions claim lack of aberrant rearrangement at the κ locus (Joho and Weissman). Similarly, experimental data were presented here that are interpreted to mean that rearrangements occur at both H-chain loci per lymphocyte (Nottenberg and Weissman). Such all-or-none effects would require different sorts of models. In addition, results on the induction of κ chain expression by fusion of a pre-B cell line with myeloma cells suggest exclusion may be determined by an active process (Riley, Brock and Kuehl, this symposium). Depending on whether the myeloma expresses a κ chain, pre-B cell-myeloma hybrids either do not undergo κ rearrangement (if the myeloma expresses κ) or only undergo productive (κ^{+}) rearrangements (if the myeloma does not express κ). These results are believed to mean that expression suppresses subsequent rearrangement and that the frequency of κ^{+} rearrangements is far higher than κ^{-}.

Whether a probabilitistic model for allelic exclusion applies to isotypic exclusion may depend on the species examined. Coleclough et al. (this symposium) have analyzed the nature of both κ and λ genes in mouse plasmacytomas and hybridomas expressing either κ or λ chain. From the frequency of aberrant rearrangements at κ and λ loci it can be argued that isotypic exclusion depends on the relative rates of rearrangements at these loci; in mouse the rate of κ rearrangement far exceeds the rate of the λ locus. On the other hand, similar surveys in human lymphocyte lines described by Hieter and Leder (this symposium) show that lines expressing κ show no rearrangement at the λ locus, whereas λ-expressing lines have deleted both κ constant region genes. Again, these results would argue for special mechanisms that are either selectively induced in different lymphocytes or a sequential process for isotypic determination.

T CELL CLONES USED TO DISSECT THE MURINE I REGION

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Recent data from our laboratory have shown that it is possible to clone murine T cells reactive either with: (A) alloantigens or (B) soluble antigens, the recognition of which is restricted by the murine I region (1-5). These studies relied in great part upon our observation that there exist hybrid or combinatorial products of the murine I region (4,5). Such combinatorial products of one subregion of the I region (I-A) are schematically illustrated in Fig. 1. Data obtained from our laboratory suggests that such hybrid I region molecules are utilized as restriction determinants for the recognition of soluble antigens, are recognized as alloantigens and react with monoclonal antisera directed at Ia antigens (4,5).

The data presented in this report make use of T cell clones reactive both with alloantigens and a variety of soluble antigens: GAT, TGAL, sperm whale myoglobin and KLH. These studies made use of previous technology generated in our laboratory for the isolation and propagation of clones of murine T cells from long-term cultures of alloreactive T lymphocytes (1). These preliminary studies on alloreactive murine T cell clones provided important contributions to the concepts of the molecular composition of Ia molecules. These

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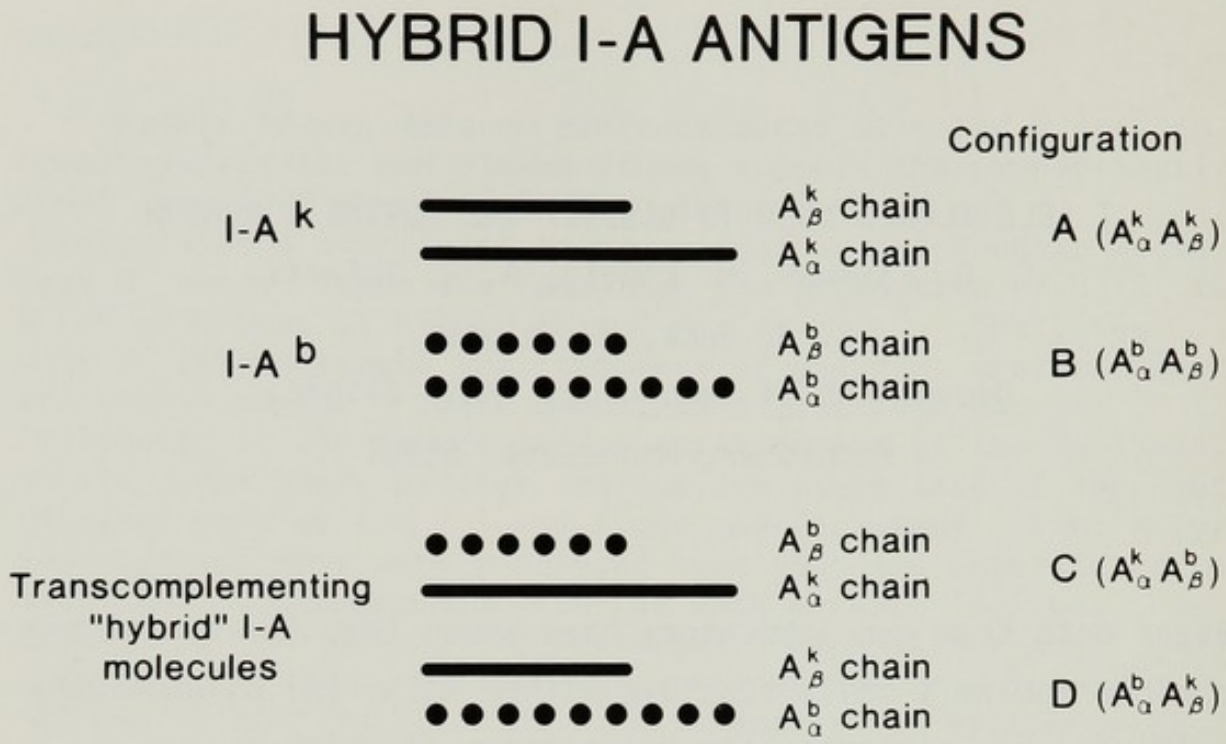

studies were the first to suggest that there were MLR stimulating determinants on cells from F_1 mice [(B6A)F_1] encoded within the I-A subregion which were not present on either strain A or B6 stimulator cells (6). The trans-complementing hybrid products detected by such alloreactive murine T cell clones have been demonstrated to be formed by free combinatorial association of alpha and beta chains encoded within the I-A subregion of heterozygote mice (5). This is diagramatically illustrated in Fig. 1. Thus, on the surface of cells from F_1 mice, one would expect at a minimum four separate I-A subregion products, both conventional cis-complementing products of each parental strain (Configuration A and B in our model), as well as two trans-complementing products which use the alpha and beta chain of each parent in free combinatorial association (Configuration C and D). This interpretation has been substantiated by the biochemical studies of Silver et al. (7). These studies showed that precipitation with monoclonal antibodies directed at a

specificity on the beta chain of the I-A^k molecule (A^k_β) in F_1 mice (who were I-A heterozygote for the $A^k_\alpha A^b_\beta$) precipitated two alpha chains in association with a single A^k beta chain. Subsequent investigation made use of the observation that the T cell proliferative response of mice to certain synthetic antigens was controlled by the I region of the MHC (8). This genetic control seemed to exist at the level of the antigen presenting cell and was thought to reflect I region restriction of antigen recognition. We thus attempted to show that such trans-complementing hybrid I region products could function in a T cell proliferative response. In these studies we immunized (B6A)F_1 mice with the random polymers GAT, TGAL and the native protein molecules KLH and sperm whale myoglobin and derived T cell clones from the immune lymph node cells. The T cell clones initially obtained in soft agar and subsequently recloned by limiting dilution in the presence of antigen, syngeneic filler cells and IL-2 allowed the following interpretations: (1) that there exist hybrid I-A antigens on antigen presenting cells formed by free combinatorial association of alpha and beta chains from heterozygote mice that function effectively to restrict antigen recognition by immune T cell clones. This interpretation makes use of data obtained using cells from the B6.C-H-2^{bm12} (bm12) mutant mouse which exhibits a defect in the normal A^b_β chain expression (5). Because of the difference demonstrable between A^{bm12}_β and A^b_β chains in heterozygote combination with A^k_α chains we could identify both combinatorial products within I-A (Configuration C and D, Fig. 1) (2,5); (2) both trans-complementing products exist and recognition of each hybrid I-A antigen is equally represented among clones obtained from long-term cultures of (B6A)F_1 lymph node cells immune to TGAL, KLH and sperm whale

myoglobin; (3) hybrid I-A products formed by free combinatorial association of alpha and beta chains on antigen presenting cells of (high responder x low responder)F_1 mice showed that low responsiveness to TGAL was not genotypically conferred by either the alpha or beta chain of low responder mice (i.e., hybrid I-A products bearing either low responder alpha with high responder beta chains or vice-versa were effective in antigen presentation to T cell clones) (3); (4) certain T cell clones derived from (B6A)F_1 mice that were immunized with TGAL could respond to TGAL in association with strain A (low responder phenotype) antigen presenting cells. This suggests that nonresponsiveness is not the inability of antigen presenting cells of strain A to present antigen but must reside in the inability of strain A T cells to recognize the association between I-A^k and TGAL (Kimoto and Fathman, manuscript in preparation); (5) restriction sites on antigen presenting cells and sites which stimulate alloreactive T cell clones can be blocked by monoclonal anti-Ia antibodies; (6) in addition to such heterozygote hybrid antigen restricting determinants there exist homozygous antigen restricting determinants that are present on mice homozygous for the I-A^b product that are not present on cells of mice having I-A^b as one haplotype in heterozygote combination with any other haplotype at I-A. That is, there is a homozygous antigen restricting determinant which is analogous and probably identical to the homozygous alloantigen stimulatirg determinant described by us several years ago (3,9); (7) murine T cell clones reactive with sperm whale myoglobin have allowed us to show that there are epitopes recognized by T cells in cyanogen bromide cleavage fragments which seem to be identical to the epitopes present in the native myoblobin molecule; (8) several different restriction sites encoded

within the I region ($A^b_\alpha A^b_\beta$ and $A^b_e E^k_\beta$) are capable of presenting a single cleavage fragment of sperm whale myoglobin; (9) a single I region restriction site may present several different antigens and different epitopes of a complex antigen (sperm whale myoglobin); (10) perhaps the most intriguing observation to be made concerning the data outlined above is that heterozygote mice must have a method of recognizing "self Ia antigens" among all possible combinations of Ia antigens that exist within the species. That is, the recognition of new hybrid products from such free combinatorial association of alpha and beta chains cannot be handed down in the germ line from either parent. Since the heterozygote mouse must recognize its own I region for restricted recognition of antigens, it must have the capability of recognizing any I region product that might be formed in the entire species.

The data summarized above have allowed us to suggest that Ir genes (defined as the ability of immune T cells to recognize antigen), Ia antigens and MLR stimulating determinants encoded within the I region are all associated with the same product. More importantly, the data described above would suggest that certain Ir gene phenomena (i.e., the inability of strain A to respond to TGAL), seemingly reside in the lack of T cell recognition and not in the inability of antigen (TGAL) to associate with I region (I-A^k) products. Thus, these data suggest that there is no antigen specific association between I-A products and soluble antigens; this association (restriction) exists only at the level of the T cell receptor.

REFERENCES

1. Fathman,CG and Hengartner,H. Clones of alloreactive T cells. Nature 272:617, 1978.
2. Kimoto,M. and Fathman,CG. Antigen-reactive T cell clones I. Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. J. Exp. Med. 152:759. 1980.
3. Kimoto,M. and Fathman,CG. Antigen-reactive T cell clones II. Unique homozygous and (high responder x low responder) F_1 hybrid antigen presenting determinants detected using (T,G)-A--L reactive T cell clones. J. Exp. Med. 153:375, 1981.
4. Fathman,CG and Kimoto,M. Studies utilizing murine T cell clones: Ir genes, Ia antigens and MLR stimulating determinants. Immunol. Rev. 54:57, 1981.
5. Fathman,CG, Kimoto,M, Melvold,R, and David,C. Reconstitution of Ir genes, Ia antigens and MLR determinants by gene complementation. Proc. Natl. Acad. Sci. (in press).
6. Fathman,CG and Nabholz,M. In vitro secondary mixed lymphocyte reaction (MLR) II. Interaction MLR determinants expressed by F_1 cells. Eur. J. Immunol. 7:370, 1977.
7. Silver,J, Swain, SL, and Hubert,JJ. Small subunit of I-A subregion antigens determines the allospecificity recognized by a monoclonal antibody. Nature 286:272. 1980.
8. Schwartz,RH and Paul,WE. T lymphocyte-enriched murine peritoneal exudate cells. II. Genetic control of antigen induced T lymphocyte proliferation. J. Exp. Med. 143:529, 1976.
9. Hengartner,H. and Fathman,CG. Clones of alloreactive T cells. I. A unique homozygous MLR stimulating determinant present on B6 stimulators. Immunogen. 10:175, 1980.

T CELL HYBRIDOMAS

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ABSTRACT. Our laboratory has prepared a series of inducible T cell hybridomas. They are of use not only as monoclonal sources of T cell factors such as interleukin-2, but also in experiments on the nature of the T cell receptor for self-H-2 plus antigen. From the experiments described in this paper we conclude that at least two factors, and possibly three, are required for B cell IgM responses to SRBC, IL-2, IL-X and perhaps IL-1. Another series of experiments has shown that T cells with specificity for antigen recognized in the context of self-H-2 do not recognize the two entities independently. A third set of observations has suggested an intriguing, if almost totally unfounded, idea to explain the origin of alloreactivity in our hybridomas, and self-H-2 plus antigen reactivity in normal T cells.

INTRODUCTION

One of the problems of studies on T cell specificity, function and heterogeneity in the past has been the lack of monoclonal lines of T cells producing particular factors, or with well defined specificity, analogous to the plasmacytomas which have been so useful in studies on B cell specificity. Although reagents which define subsets of T cells have been in use for some years now (1) it is clear that these cannot easily be used to define T cell populations with only one function (if, indeed, such exist) and certainly the isolation of T cells with a single specificity for antigen has not been possible with these reagents. Conventional techniques in which antigen specific T cells are isolated on antigen or anti-idiotype-coated matrices have also been of limited use, especially as many T cells,

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restricted to recognize H-2-associated antigen, probably can only be bound to free antigen with great difficulty, if at all.

Recently three advances have allowed the acquisition of useful monoclonal T cell lines (2-3). These advances have been the use of interleukin-2 (IL-2) as a growth stimulant for T cells *in vitro* (6), the appropriate testing of well established T cell lines for function (7-9) and the development of techniques for preparing T cell hybridomas (10,11). It is the purpose of this manuscript to discuss briefly our recent experiments using the last of these techniques to analyze T cell function and specificity.

MATERIALS AND METHODS

Mice. Mice were obtained from the Jackson Laboratories or were bred in our own vivarium using breeding triplets given to us very kindly by various investigators throughout the country (listed in 12).

Cell Preparation and Culture. B cells were prepared by pretreating mice with anti-thymocyte serum, followed by treatment of spleen cells *in vitro* with a cocktail containing heterologous anti-T cell, and monoclonal anti-Thy 1 (kindly given to us by Dr. I. Trowbridge), anti-Qa 4 and anti-Qa5 (kindly given to us by Dr. U. Hammerling) and a Qa-like antibody, MK 2.2. Sometimes macrophages (MØ) were also removed from these cells by passage over G-10 Sephadex columns (13).

Preparation of Antigen-specific T Cell Hybridomas. T cells from antigen-primed lymph nodes were enriched *in vitro* for response to antigen as described by Schrier *et al.*, (14). They were then fused by standard techniques (10,11), to one of three HAT-sensitive lines developed in this laboratory, FS6-14.13.AG2 (11,12), A040/AG (12) or A040.10.AG1 (12). All three parent tumor cell lines secreted IL-2 in response to concanavalin A (Con A) stimulation. In addition, A040.10.AG1 secreted IL-2 in response to $I\text{-}A^{k}$-antigen presenting cells plus ovalbumin (OVA) or to $H\text{-}2^{b}$-antigen presenting cells.

Hybrids were selected in HAT, tested for IL-2 production in the presence of the appropriate antigen plus antigen presenting cells and cloned at limiting dilution.

Assay for IL-2. Supernatants were assayed for IL-2 concentration by titrating their ability to support, for 24 hours, the growth of HT-2 cells, scored microscopically (3,11). HT-2, a cell line given to us kindly by Dr. James

Watson, grows in IL-2 but dies within 24 hours if the lymphokine is withdrawn. In our assays all negative supernatants contained no detectable IL-2 at any concentration tested including 80%. Positive supernatants contained detectable IL-2 at concentrations down to between 20 and 0.6%, with almost all supernatants positive at 10%.

Preparation of Supernatants. IL-2 containing supernatants were prepared from FS6-14.13 by incubating the cells at an initial concentration of 5×10^5/ml with 5 ug/ml Con A for 24 hours at 37°C. IL-X containing supernatants were prepared in similar fashion from DT1.15 except that the adherent cells from B10.D2 peritoneal washings were also present at approximately 10^5/ml.

IL-2 containing supernatants from P388D1 were prepared by growing the line up to 1/3 confluence, replacing the medium with new medium containing 2.5×10^{-5} M indomethacin, and continuing the incubation for a further 2 days before harvesting. Con A SN were prepared from normal spleen cells by standard techniques (11).

Where necessary IL-X was prepared free of IL-2 and IL-1 by absorbing IL-2 for 24 hours at 37°C with T cell blasts, followed by G-200 Sephadex gel filtration to separate IL-X (MW ~ 40 KD) from IL-1 (MW15 KD).

α-methyl mannoside was added to all supernatants prepared with Con A (11).

RESULTS

Use of T Cell Hybridomas as Sources of Lymphokines.

Recently it was realized that most T cell lines must be induced before they can be used as sources of T cell factors (7-9,11). Our own experiments showed that the hybridoma, FS6-14.13, only secreted IL-2 when stimulated with Con A (11). Although it is not clear how many other factors this hybridoma secretes upon stimulation, for example it also makes basophil growth factor (personal communication, Dr. Sredni), it certainly does not make every factor normally found in the supernatants of Con A-stimulated spleen cells. This is exemplified by the results shown in Tables 1 and 2. Con A induced supernatants from normal mouse spleens stimulated anti-sheep red blood cell (SRBC) responses in B cells, indicating that all factors required for IgM anti-SRBC response were probably in such supernatants. By contrast, a Con A-induced supernatant from FS6-14.13 did not stimulate anti-SRBC plaque forming cells (PFC) in B cells, providing T cells were stringently removed from these

populations. IL-2 could be removed from Con A SN by absorption with T cell blasts. When this was done, it was apparent that a factor (IL-X?) remained which could synergize with IL-2 in stimulating the responses of B cells and MØ to SRBC (Table 1). It is noteworthy that this factor was ineffective in the absence of IL-2.

Table 1

B Cells Responding to SRBC Need at Least Two Helper Factors

Helper Factors Added	Anti-SRBC PFC/12 Microcultures[a]
None	0
Normal Con A SN[c]	270 ± 49[b]
FS6-14.13 Con A SN[c]	14 ± 5
IL-2 depleted[cd] normal Con A SN	5 ± 5
FS6-14.13 Con A + IL-2 depleted normal Con A SN[cd]	277 ± 64

[a] 1.5×10^5 B cells and MØ/culture well.

[b] Results shown are the average ± SE of four similar experiments.

[c] Factors used at optimal concentrations (20-60%, depending on batch).

[d] IL-2-depleted Con A SN from normal spleen cells prepared by absorption at 37° with various T cell blast populations.

Recently we have isolated a cloned hybridoma, DT1.15, which caused the production of IL-X in the presence of Con A and MØ, as shown in Table 2. Also shown in Table 2 is the fact that a macrophage product, IL-1, failed to syngerize with IL-2 in anti-SRBC responses, although, if B cells were thoroughly depleted of MØ, it would increase a response stimulated by IL-2 plus IL-X. This fact strongly suggests

that IL-X is not IL-1, a suggestion which is strengthened by the fact that other experiments, not shown here, have demonstrated that IL-X has a molecular weight of about 40 KD.

Table 2

Presence of IL-X in supernatants of a Hybridoma Incubated with MØ and Con A

Helper Factors Added	Anti-SRBC PFC/12 Microcultures[a]
-	0
IL-1[b]	0
IL-2[c]	0
IL-X[d]	0
IL-1 + IL-2	19
IL-1 + IL-X	49
IL-2 + IL-X	124
IL-1 + IL-2 + IL-X	513

[a] 1.5×10^5 G-10 Sephadex purified B cells/well.

[b] 20% SN of P388D1 incubated with 2.5×10^{-5} indomethacin.

[c] 30% SN of FS6-14.13 incubated with Con A.

[d] 30% SN of hybridoma DT1-15 incubated with Con A and MØ, purified by absorption with T cell blasts (HT-2 cells) at 37°C followed by G-200 Sephadex chromatography.

Altogether these experiments suggest a role for at least two factors in B cell anti-SRBC IgM responses, IL-2 and IL-X. A third factor, IL-1, may also be implicated.

Use of T Cell Hybridomas in Studies on the T Cell Receptor(s).

The nature of the T cell receptor(s) for H-2-associated antigen is still in doubt. Steps forward in studies of this entity would be the establishment of cloned cell lines with defined specificity, and secondly the discovery of a reliable and easy antigen binding assay. Although cloned T cell lines (2-6) and the efforts of Elliott and Lonai and their coworkers (15,16) have filled both these needs we felt that the existence of antigen specific T cell hybridomas would also be of some benefit in these investigations.

We reasoned that it has been hard to make such hybrids in the past because antigen binding has been so difficult to detect. We therefore prepared hybridomas of this type using inducible IL-2 production as an assay for acquisition of antigen-specific receptors by our hybrids.

Con A-inducible, IL-2 secreting, HAT-sensitive hybridomas were fused to antigen-specific T cell blasts, and products tested for their ability to secrete IL-2 in response to antigen in the presence of the appropriate antigen presenting cells. Table 3 contains a list of some of the cloned hybrids we have made so far by this method. All hybridomas were exquisitely specific not only for antigen, but also for H-2 haplotype and subregion, where this has been mapped. They obeyed all the rules for antigen-specific H-2 associated recognition established for normal T cells and T cell lines.

Table 3

Antigen-specific H-2 Restricted T Cell Hybridomas

Tumor Cell	T Cell Blast	Name	Specificites
FS6-14.13.AG2	B10.A + OVA	A040.10	OVA/I-A^k -/H-2^b
FS6-14.13.AG2	BALB/c + cyto c	DC 1.18.3	cyto c/I-A^d
FS6-14.13.AG2	B10 + Sendai	BS12.11	Sendai/H-2^b
A040/AG	B10.D2 + KLH	AODK1.16	KLH/I-B^d-I-C^d
A040/AG	B10.D2 + KLH	AODK10.4	KLH/I-A^d
A040.10.AG1	B10.D2 + HGG	AODH7.1	HGG/H-2^d

Having made these hybrids we set out to prepare cell lines with specificity for two different antigens associated with two different H-2 haplotypes, by fusing one of our antigen-specific, H-2 restricted T cell hybridomas to normal T cell blasts with different specificities. This was attempted in a number of fusions. In all of these a number of cell lines were prepared which had inherited specificities for antigen plus H-2 from both parents. In no case was a cell line obtained with specificity for antigen inherited from one parent, and for H-2 inherited from the other. These results strongly suggested that antigen and H-2 recognition did not occur independently by means of two independent receptors on the T cell surface.

A stringent test of this conclusion, however, required that one of these cell lines be cloned, in order to prove unequivocally that the specificities inherited from both parents were being expressed, without mixing, in the same cells. Although cloning these by now triple hybrids and simultaneously retaining both parental specificities proved difficult, it was achieved for one line, AOFK11, a line that was the product of a fusion between A040.10.AG1 and B10.M (H-2^f) T cells specific for KLH. The antigen recognition properties of this line and some of its cloned descendants are shown in Table 4. More details of this lines are given in a recent publication (12).

Table 4

Properties of AOFK11 and its Relatives

Line	IL-2 Production in Response to:			
	$H\text{-}2^a$ + OVA	$H\text{-}2^f$ + KLH	$H\text{-}2^a$ + KLH	$H\text{-}2^f$ + OVA
A040.10.AG1	+	-	-	-
AOFK11	+	+	-	-
AOFK11.11[a]	+	+	-	-
AOFK11.11.1[b]	+	+	-	-

[a]Cloned at 0.5 cells/well, 30% plating efficiency.

[b]Cloned at 0.125 cells/well, 30% plating efficiency.

The properties of AOFK11.11.1 demonstrate that antigen and H-2 recognition are not independent events on the T cell surface.

Alloreactivity Displayed by Antigen Specific, H-2 Restricted T Cell Hybridomas.

Several investigations have reported frequent occurence of anti-allo H-2 reactivity in cloned T cell lines specific for antigen plus self H-2 (5). Such observations lend weight to the idea that anti-allo H-2 recognition is the consequence of crossreaction with self-H-2 plus antigen. We have looked for anti-allo H-2 recognition amongst our hybridomas and so far have found no examples of recognition of $H\text{-}2^a$, $H\text{-}2^d$, or $H\text{-}2^s$ amongst between 8 and 80 lines tested for response to each haplotype. In contrast, we have observed two independent occurences of anti-$H\text{-}2^b$ reactivity, and three other related occurences of the same specificity. The fusions leading to these lines are shown in Table 5.

Table 5

Production of Anti-H-2^b Reactive Hybridomas

Tumor Cell	Normal T Cell Blast	Name	H-2^b Specificity
FS6-14.13.AG2	B10.A + OVA	A040.10	K^b or I-A^b
FS6-14.13.AG2	B10.D2 + (TG)-A--L	2DT9	not mapped
A040/AG	B10.D2 + KLH	AODK18.1	K^b or I-A^b
A040/AG	B10.D2 + KLH	AODK20.1	K^b or I-A^b
A040/AG	B10.D2 + KLH	AODK	not mapped

As shown in Table 5, in two independent fusions to FS6-14.13.AG2 hybrids were obtained which responded to H-2^b in the absence of additional antigen. One of these fusions involved B10.A T cell blasts specific for H-2^a plus ovalbumin (OVA), and resulted in a cloned line, A040.10 which responds to H-2^b (K^b or I-A^b) or I-A^k plus OVA. The other involved B10.D2 T cell blasts specific for H-2^d plus (TG)-A--L. One resulting line, 2DT9, responded to H-2^b only.

Interestingly enough three other H-2^b reactive lines occured in a fusion of T cell blasts specific for KLH plus H-2^d to an uncloned, azaguanine resistant relative of A040.10, A040/AG. Although this uncloned line started its career with the characteristics of A040.10, namely the ability to respond to I-A^k plus OVA, or H-2^b, it had lost these properties by the time it was used in this fusion. Curiously of the 13 hybrids picked in this fusion, three responded to H-2^b alone and not I-A^k plus OVA or H-2^d plus KLH. This is the only occasion on which we have been able to split the H-2^b reactivity of A040 from its reactivity for I-A^k plus OVA, if, indeed, that is what has happened.

Although we realize that these are inadequate data on which to base a theory, we cannot resist speculating that the high frequency of anti-H-2^b in our hybridomas is something to do with the fact that they are all derived from the line, FS6-14.13, which was itself the product of a

fusion between BW5147 ($\underline{H\text{-}2}^k$) and BDF_1 ($\underline{H\text{-}2}^b \times \underline{H\text{-}2}^b$) T cells. We therefore hypothesize that FS6-14.13 inherited a specificity for $\underline{H\text{-}2}^b$ plus an unknown antigen from its normal T cell parent, and that one component(s) of this receptor is responsible for anti-$\underline{H\text{-}2}^b$ specificity which is not completely expressed in FS6-14.13 because of the nature of the other component(s) of the receptor. New receptor components, introduced by fusion of FS6-14.13 to antigen specific T cell blasts, complement the receptor on FS6-14.13 and sometimes by chance allow full expression of anti-$\underline{H\text{-}2}^b$ reactivity.

For example, we would suggest that in the fusion which created A040.10 the incoming T cell blasts brought a fully fledged receptor for $\underline{I\text{-}A}^k$ plus OVA, one chain of which complimented the (partial?) receptor on FS6-14.13.AG2 to create full anti-$\underline{H\text{-}2}^b$ reactivity. During the creation of A040/AG some of the blast-derived chains were lost, resulting in loss of both reactivities. Anti-$\underline{H\text{-}2}^b$ but not anti-$\underline{I\text{-}A}^k$ plus OVA reactivity was restored in some cases by the introduction of yet more receptor material from blasts specific for $\underline{H\text{-}2}^d$ plus KLH.

This complex receptor model suggests that what we were doing when we complimented the receptor allowing anti-$\underline{H\text{-}2}^b$ reactivity during fusion, may have been the reverse of what happened in the thymus, where full anti-self reactivity may have been changed to anti-self plus antigen reactivity (17) by completely replacing one of two (or more) polypeptide chains which make up the T cell receptor responsible for associative recognition.

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REFERENCES

1. Cantor, H., and Boyse, E.A. (1975) J. Exp. Med. 141, 1390.
2. Fathman, C.G., and Hengartner, H., Nabholz, M., Lernhardt, W., Schrier, M.H., and Haas, W. (1979) Europ. J. Immunol. 9, 592.
3. Watson, J. (1979) J. Exp. Med. 150, 1510.
4. von Boehmer, H., Hengartner, H., Nabholz, M., Lernhardt, W., Schreier, M.H., and Haas, W. (1979) Europ. J. Immunol. 9, 592.
5. Sredni, B., Tse, H., and Schwartz, R. (1980) Nature 283, 581.
6. (1980) Immunol. Rev. 51.
7. Shimizu, S., Konaka, Y., and Smith, R.T. (1980) J. Exp. Med. 152, 1436.
8. Farrar, J.J., Fuller-Farrar, J., Simon, P.L., Hilfiker, M.L., Stadler, B.M., and Farrar, W.L. (1980) J. Immunol. 125, 2555.
9. Gillis, S., Scheid, M., and Watson, J. (1980) J. Immunol. 125, 2570.
10. Kontianen, S., Simpson, E., Bohrer, E., Beverely, P., Herzenberg, L., Fitzpatrick, W., Vogt, P., Torano, A., McKenzie, I., and Feldmann, M. (1978) Nature 274, 477.
11. Harwell, L., Skidmore, B., Marrack, P., and Kappler, J.W. (1980) J. Exp. Med. 152, 893.
12. Kappler, J.W., Skidmore, B., White, J., and Marrack, P. (1981) J. Exp. Med. in press.
13. Ly, I., and Mishell, R.I. (1974) J. Immunol. Methods 5, 239.
14. Schrier, R.P., Skidmore, B.J., Kurnick, J.T., Goldstine, S.N., and Chiller, J.M. (1979) J. Immunol. 123, 2525.
15. Elliott, B.W., Takaco, B., and Nagy, Z. (1979) Europ. J. Immunol. 9, 646.
16. Lonai, P., and Steinman, L. (1977) Proc. Natl. Acad. Sci. USA 74, 5662.
17. Jerne, N.K. (1971) Europ. J. Immunol. 1, 1.

ANTIGEN BINDING T CELL HYBRIDS[1]

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INTRODUCTION

Hybridization of an antigen-specific T cell with a continually replicating T lymphoma permits the establishment of antigen-specific continual cell lines. One can use these lines to obtain answers to some questions concerning T cells. These include: What is the molecular nature of the T cell receptor for antigen? What, in addition to antigen, does the receptor recognize? What is the relationship of the receptor to antigen binding factors? Do T cells recognize antigen in a different context than do suppressor factors? The latter is suggested by the observation that though T cells preferentially recognize antigen in the context of self (1) or after processing by macrophages (2), antigen-suppressor factors have been described which are completely removed by antigen affinity methods (3).

Two major theories concerning T cell receptors have developed. According to one, there are two separate receptors on T cells, one which recognizes self H-2, the other a nominal antigen. In the other proposal, one unit recognizes both antigen and self. Neither theory in its simplest form accounts satisfactorily for the observation that some antigen-specific T cell clones can be stimulated by nominal antigen plus self H-2 as well as by allogeneic cells. This suggests that a given T cell may recognize more than 1 antigen (4).

A number of investigators have described T cell hybrids which produce antigen binding factors (3,5,6). We have produced such antigen-specific hybrids which produce suppressor factors in order to study the interrelationship of T cell receptors and antigen-specific factors. This communication will focus predominately on parameters which regulate recognition of cellular moietys and soluble antigen by such antigen-specific T cell hybrids.

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METHODS

TABLE 1. ANALYSIS OF T CELL HYBRIDS

PARAMETER	*CHROMOSOME*	*METHOD*	*REFERENCE*
Hypoxanthine guanine phosphoribosyltransferase	*X*	*Growth in HAT medium*	*11*
Isozymes			
Glucose phosphate isomerase	*7*	*Starch gel electrophoresis*	*11*
Isocitrate dehydrogenase	*1*	*Starch gel electrophoresis*	*9*
Antigens			
H-2	*17*	*Quantitative absorption of cytotoxicity; Direct cytotoxicity*	*7,11*
Thy-1	*9*	*Quantitative absorption of cytotoxicity; Immunofluorescence*	*7,11*
"Self-recognition"		*Rosetting mouse RBC Binding to macrophage monolayers*	*7*
Antigen recognition		*Rosetting antigen-coupled RBC. Binding to antigen pulsed monolayers*	*8,9*
Lymphokines			
Suppressor Factors		*Inhibition of PFC to ABA-Brucella abortus*	*10*
		Inhibition of PFC to SRBC	*11*
		Inhibition of IgE α OVA	*12*
Biosynthetically labelled proteins		*Absorption of lysates to antigen; SDS-PAGE*	*13*
RNA		*Northern blot analysis with cloned immunoglobulin gene probes*	*14,15*

Preparation of T and B Cell Hybrids: T cell hybrids made between primary T cells and BW5147, an AKR HGPRT⁻ T lymphoma, have been described previously. These hybrids include Hyb 29P whose primary parent was derived from T cells educated in vitro to SRBC (7); Hyb 49A whose primary parent was a T cell from mice suppressed for the IgE response to ovalbumin (OVA)

(8); and cells of the Hyb 51 series whose primary parents were obtained from a population of azobenzenearsonate (ABA) specific T suppressor cells enriched on antigen coated plates (9,10).

Preincubation of Hybrids on Macrophages: Peritoneal exudates were prepared in mice by injection of 3ml thioglycollate broth. Three to six days later, the cells were washed and absorbed to tissue culture plates for 1 to 2 hours at 37° in RPMI with 5% fetal calf serum. The adherent cells (mainly macrophages) were pulsed with 50μg ABA-BSA or PBS and incubated at 37° for an additional hour. The monolayers were then washed and 2 x 10^5 T or B cell hybrids added. The plates were spun at 500 rpm for 4 minutes and then placed at 4° or 37°. Under these conditions, T cells bind optimally at 37°, B cells at 4°. At the end of 1 hour, non-adherent cells were removed by gentle aspiration, washed once, counted and then tested for rosetting capacity in the usual fashion.

RESULTS

Confirmation of the Hybrid Nature of T Cell Lines: The coexpression of constitutive and differentiated functions of both parents, including isozymes, Thy-1 and H-2, has been described previously (7,8,9,10,11). Another marker used to analyze the hybrid nature of the cells, which may also have implications concerning T cell function, is transcription of a new species of polyadenylated RNA in the hybrids. Some lymphomas synthesize polyadenylated RNA which hybridizes with a DNA probe to Cμ (16). BW5147 does not express this RNA as detected by Northern blot analysis, but 4 of 9 T cell hybrids made with BW5147, including Hyb 51 H7D, do contain Cμ RNA. Since the lymphoma parent does not synthesize this RNA, it is likely that the new RNA in the hybrid is contributed by the primary parent (14,15).

Recognition of Red Cell Antigens: We have previously reported that, though BW5147 does not bind red cells, many T cell hybrids do bind sheep and mouse but not human erythrocytes (7). This phenomenon is quantitated by counting the number of T cells which form rosettes with RBC. A rosette is scored as such if it binds at least 3 red cells. In most cases the number bound is considerably higher, up to approximately 20 erythrocytes. This phenomenon is regulated by culture conditions; both culture phase, and cell cycle (7). Cells in late logarthmic phase of culture express the activity most prominently. These cells rosette in G2 and continue to bind red cells through M.

The rosetting phenomenon is influenced by factors coded by both H-2-linked and non-H-2-linked genes. Of the T cell

hybrids so far examined, those which rosette bind C57BL/6 erythrocytes well. This is true whether or not the primary parent is C57BL/6. When one normalizes rosetting to C57BL/6 RBC, one can compare rosetting activity with red cells of various strains (Table 2). Normalized frequency of Hyb 29P rosette-forming cells with AKR RBC (H-2^k) is 100%, but only 22% with B10Br (also H-2^k). Likewise 29P does not form rosettes with BALB/c RBC (H-2^d) yet 56% of input 29P form rosettes with B10·D2 RBC (also H-2^d).

Antigen Recognition: The interaction of antigen with T cell hybrids can be visualized when that antigen is presented on an erythrocyte. Hyb 49A, a hybrid between BW5147 and BDF1 T cells, binds OVA-coupled BDF1 and OVA-sheep RBC to a greater extent than it binds sham treated RBC, and this binding to antigen-coupled RBC is inhibited by preincubation with soluble antigen (8). The same phenomenon is observed with hybrids of the Hyb 51 series which bind ABA-RBC (9). This rosetting is under the same culture phase and cell cycle regulation as the binding to RBC which are not coupled with antigen.

Antigen-Specific Binding is Only Seen When Facilitated by Red Cell Binding: The ABA-specific B cell hybrid AK 2.2 binds ABA-coupled mouse, sheep and human RBC, and this binding is completely inhibitable by preincubation of the hybrid with ABA-BSA. T cell hybrid antigen-specific binding occurs only when the antigen is coupled to either mouse or sheep, but not human RBC (Table 3). This experiment suggests that visualization of antigen-specific binding is only possible when the antigen is presented on a cell with which the T cell can interact.

TABLE 2. T CELL HYBRID BINDING TO MURINE ERYTHROCYTES IS INFLUENCED BY FACTORS CODED BY H-2 AND OTHER NON-H-2 LOCI

	% Rosette Forming Cells[1]								
H-2	b	b	k	k	a	d	r	s	d
RBC	B6	B10	AKR	B10Br	B10A	B10D2	B10RIII	B10S	BALB/C
BW[2]	0	0	0	0	0	0	0	0	0
29[3]	100	50	100	22	50	56	110	25	0

[1] *These results represent at least 4 experiments. The % rosetting with C57BL/6 erythrocytes in 11 experiments was 32.2 ± 3.9.*

[2] *BW = BW5147 AKR H-2^k*

[3] *29 = Hyb 29P C57BL/6 x AKR H-2^b x H-2^k*

TABLE 3. ROSETTING OF ABA SPECIFIC T AND B CELL HYBRIDS WITH ERYTHROCYTES OF VARIOUS SPECIES

		% ROSETTES			
	ROSETTING CONDITIONS				
	INHIBITOR	—	—	ABA-HSA	OVA
	ERYTHROCYTE	RBC	ABA-RBC	ABA-RBC	ABA-RBC
HYBRID	SOURCE OF RBC				
Hyb 51 A5 (T)	MOUSE	25	27	12	21
	SHEEP	17	34	18	31
	HUMAN	0	0	0	0
Hyb AK 2.2 (B)	MOUSE	2	39	0	34
	SHEEP	0	53	0	56
	HUMAN	0	43	0	38

Experiments were carried out to determine whether blocking of non-specific binding would inhibit antigen-specific binding. This was accomplished by preincubating ABA-specific hybrids for 1 hour on antigen-pulsed or unpulsed macrophages and then testing them for their ability to rosette with antigen-coupled red cells.

B cell hybrids were inhibited in their ability to form antigen-specific rosettes when the hybrids were absorbed to antigen-pulsed but not normal macrophages (Table 4). Presumably this is due to physical depletion of the B cells, as approximately 60% of the cells bound to the antigen-pulsed macrophages. The other possibility is that the macrophages have released antigen which physically blocks the B cell surface immunogloculin and prevents specific rosetting.

The T cell hybrid rosetting was completely inhibited after absorption to macrophages whether the monolayers were pulsed with antigen or not. The blockade of rosetting was complete and included both antigen-specific and non-specific rosetting. The mechanism was probably not depletion of rosette-positive cells since it occurred whether the T cells were incubated on the macrophages at 37° or 4°. In the experiment described in Table 4, only 2% of the T cells absorbed to macrophages at 4°, and 17% absorbed at 37°, but rosetting was completely abolished after absorption at either temperature. This is consistent with the interpretation that inhibition of rosetting is due to an alteration of the T cell "self" receptor in a similar fashion as inhibition occurs after preincubation with soluble antigen. It is also consistent with the interpretation that the antigen-specific and non-specific activities are dependent upon each other and could even be mediated by the same structure.

TABLE 4. PREINCUBATION WITH SPECIFIC ANTIGEN INHIBITS B AND T RFC
PREINCUBATION WITH MACROPHAGES INHIBITS T RFC[1]

B CELL HYBRID	*AK 2.2*	*α ABA*
Preincubation	*% ABA-RBC RFC*	*% Inhibition*
PBS	*52%*	——
ABA BSA	*0*	*100%*
PBS macrophages	*64%*	*0*
ABA BSA macrophages	*0*	*100%*

T CELL HYBRID	*51H7*	*α ABA*
Preincubation	*% ABA-RBC RFC*	*% Inhibition*
PBS	*24%*	——
ABA BSA	*6%*	*75%*
PBS macrophages	*0*	*100%*
ABA BSA macrophages	*0*	*100%*

[1] *Hybrids were preincubated with inhibitors (PBS, soluble antigen or mouse macrophages) for 1 hour at 4°, and then tested for their ability to form rosettes with ABA-coupled RBC.*

DISCUSSION

The experiments reported here and in previous publications have demonstrated that T cell hybrids which produce antigen-specific suppressor factors are also capable of binding that antigen at the cell surface. This binding can only be seen when the antigen is presented on a cell membrane since binding of labelled soluble antigen has not been demonstrated reproducibly by autoradiography. Presumably the T cell interacts with soluble antigen, since preincubation with soluble antigen inhibits later rosetting. We interpret our results to indicate that the surface on the red cell acts to facilitate antigen-specific binding, and thereby stabilizes the receptor-antigen complex. The experiments presented seek to determine the nature of the facilitating structure present on certain red cells. Though expression of the structure is under H-2 control, it is probably not an Ia determinant. Restriction of rosetting has been described in two other studies. These markers are "R" which maps between G and D (17), and an element described by Sia and Parrish which maps to the right of D in the L locus (18). The struc- we describe is not necessarily limited to red cells, since

the data presented here suggest it is present on macrophages as well. It will be interesting to determine whether the glycophorin-like structure, W 3/13, described on rat T, but not B cells (19) bears any functional or antigenic relationship to the molecule detected by "non-specific" rosetting. These may be members of a class of cellular interaction structures.

Our experiments do not yet allow us to distinguish between "self" and antigen-specific binding. Both occur in late logarithmic phase of growth, both are removed by absorption to macrophages. Moreover, incubation of cell lysates with SRBC and antigen-SRBC results in enrichment of the same polypeptides (62,000 46,000, 42,000 and 33,000 molecular weight as determined by SDS gel electrophoresis) (13). Future experiments will concentrate on characterization of and comparison of these molecules with antigen-specific suppressor factors. Parameters investigated will be size, genetic regulation and reactivity with antisera which recognize candidates for T cell constant region determinants (20,21).

REFERENCES

1. Zinkernagel, R.M. and Doherty, P.C. (1974). *Nature(Lond), 248*, 701.
2. Paul, W. and Benacerraf, B. (1977). *Science 195*, 1293.
3. Taniguchi, M., Saito, T., and Tada, T. (1979). *Nature (Lond), 278*, 555.
4. Sredni, B. and Schwartz, R.H. (1980). *Nature 287*,855.
5. Pacifico, A. and Capra, J.D. (1980). *J. Exp. Med. 152*, 1289.
6. Kapp, J.A., Arraneo, B.A. and Clevinger, B.L. (1980). *J. Exp. Med. 152*, 235.
7. Ruddle, N.H., Beezley, B.B., and Eardley, D.D. (1980). *Cell. Immunol. 55*, 42.
8. Whitaker, R.B. and Ruddle, N.H. (1980). *Cell. Immunol. 55*, 56.
9. Ruddle, N.H., Beezley, B., Lewis, G.K. and Goodman, J.W. (1980). *Molec. Immunol. 17*, 925.
10. Goodman, J.W., Lewis, G.K., Primi, D., Hornbeck, P. and Ruddle, N.H. (1980). *Molec. Immunol. 17*, 933.
11. Ruddle, N.H. (1978). *Current Topics Microbiol. Immunol. 81*, 203.
12. Whitaker, R.B. (1980). Thesis, Yale University.
13. Ruddle, N.H., Whitaker, R.B., and Zuniga, M.C. Lymphokine Reports, in press.
14. Zúñiga, M.C., D'Eustachio, P., and Ruddle, N.H. (1981). *J. Supramolec. Struct. suppl. 5*, 21.

15. Zuniga, M.C., D'Eustachio, P., and Ruddle, N.H., manuscript in preparation.
16. Kemp, D.J., Harris, A.W., Cory, S., and Adams, J.M. (1980). *Proc. Natl. Acad. Sci. 77*, 2876.
17. Primi, D., Lewis, G.K., Triglia, R. and Goodman, J.W. (1979). *J. Exp. Med. 149*, 1349.
18. Sia, D.Y. and Parrish, C.R. (1980). *J. Exp. Med. 151*, 553.
19. Brown, R.A., Barclay, A.N., Sunderland, C.A. and Williams, A.F. *Nature 289*, 456.
20. Cone, R., Rosenstein, R., Murray, J., Iverson, G.M., Ptak, W., and Gershon, R. *PNAS* in press, (1981).
21. Owen, F.L., Finnegan, A., Gates, E.R., and Gottlieb, P.D. (1979). *Europ. J. Immunol. 9*, 948.

T-CELL CLONES: GENERATION AND ANALYSIS OF ANTIGEN-SPECIFIC MOLECULES

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Abbreviations used in this paper: BM, bone marrow; C, centrigrade; CCM, complete conditioned medium; CFU-C, colony forming units-culture; CFU-S, colony forming units-spleen; CE, cloning efficiency; Ci, curie; CM, conditioned medium; Con A, concanavalin A; ^{51}Chromium-51; DME, Dulbecco's modified Eagles media; DMSO, dimethyl sulfoxide; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FL, fetal liver; HCL, hydrochloric acid; ^{3}H-T, ^{3}H-methylthymidine; Ig, immunoglobulin; IL-II, interleukin-II; 2ME; 2-mercaptoethanol; MEM, minimum essential media, mM, millimolar; MW, molecular weight; N_2, nitrogen; NK cells, natural killer cells; PAS, periodic acid-Schiff; pp IL-II, partially purified interleukin II; SRBC, sheep red blood cells; R, rads; TdT, terminal deoxynucleotidyl transferase; μ, micron; ug, microgram.

INTRODUCTION

Mammalian lymphocytes are divisible into two major classes. One class requires the presence of an intact thymus gland for normal maturation ('T cells') while a second class, ('B œlls'), develops elsewhere. Fully differentiated T cells and B cells both carry, at their membrane surface, receptor molecules capable of binding to different chemical determinants, and are responsible for initiating specific immunologic responses. Recently, neoplastic clones of B-cells ('myeloma cells') have been used to analyze the genes that govern immunoglobulin synthesis, with particular emphasis on genetic mechanisms that might account for immunoglobulin diversity(1-3).

Thymus-dependent lymphocytes have several features that make them a more attractive model for analysis of genetic mechanism that govern cellular differentiation. First, glycoproteins expressed at the surface of cells within the thymus-dependent lineage have been identified using both conventional and monoclonal antibodies (4). Many of the genes coding for these glycoproteins have been precisely mapped, and are ex-

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pressed exclusively on cells undergoing thymus-dependent differentiation. Assessment of the immunologic function of several mature T-cell populations, each carrying unique molecular labels at their membrane surface, has demonstrated that each is specialized to carry out a particular immunologic activity (4).

These glycoproteins have also been used to partially delineate several immature cell types that represent early stages of thymus-dependent differentiation. This analysis has indicated that stem cells migrate from yolk sac and liver in the embryo (5) and from bone marrow in the adult (6) into the thymus where they differentiate to thymocytes (7); one result of intrathymic differentiation is the export of different 'T cell sets' that are specialized to mediate one or another immunologic activity (4).

Although this approach has successfully delineated cells at different stages of thymus-dependent maturation, analysis of each of these populations has been difficult. First, many cell types are difficult to isolate and expand to significant numbers in long term in vitro cultures. Second, mature T lymphocyte sets that carry distinctive cell surface glycoprotein 'markers' often do not represent homogenous populations, and thus are liable to confound biochemical analysis.

We have developed a general method for producing cloned populations of cells bearing surface glycoproteins found at different stages of thymus dependent differentiation (8). The procedure does not require hybridization to tumor cells. We reasoned that expression of receptors for a T-cell specific growth factor might be part of the genetic program of all cells within the thymus dependent lineage, including precursor populations; therefore, this growth factor might selectively stimulate proliferation of these cells from a heterogeneous cell population. In addition, particular types of irradiated cells monolayers are necessary to initiate efficient clonal growth of cells at different stages of thymus-dependent differentiation. This approach has resulted in the production, in vitro, of cloned populations that are stable with respect to expression of unique patterns of cell surface glycoproteins, histochemical reactions, and immunologic function and can be expanded in culture to numbers $>10^8$. These cell populations represent a unique source of material for biochemical analysis of the cellular events required to generate specialized cellular progeny from precursor populations and, in some cases, the specialized differentiated functions of these cells.

RESULTS

I. GENERATION OF CLONED POPULATIONS THAT EXPRESS DIFFERENTIATED FUNCTION (TABLE 1, 2)

A. Distribution compared with heterogeneous sets (Table 1).

Table 1

Expression of Different Ly Glycoproteins on Heterogeneous Spleen T-Cells and Colonies Derived from this Population

	Surface Phenotype (% positive)			
Cell Populations	$Thy1^+$ $Ly1^+2^-$	$Thy1^+$ $Ly1^+2^+$	$Thy1^+$ $Ly1^-Ly2^+$	$Thy1^+$ $Ly1^-2^-$
Ig^- Spleen cells	25	40-50	5-10	10-20
Colonies derived from Ig^- spleen cells	20	46	13	20

The proportion of cells within heterogeneous spleen T-cell population expressing different Ly glycoproteins was determined by immunofluorescein on the FACS-I. Fifteen colonies derived from this starting population were similarly tested for expression of different Ly surface glycoproteins.

C. Cloned cells expressing inhibitory activity.

B. Cloned cells expressing inducer activity.

All $Thy1^+Ly1^+2^-$ cloned populations tested (total of 5) augmented the secretion of immunoglobulin by B cell 10-100 fold. Cloned cells expressing the $Ly1^+2^+$ or $Ly1^-2^+$ surface phenotype did not. (Table 2)

All $Thy1^+Ly1^-2^+$ cloned populations suppressed Ig secretion. A portion (4/7) of $Ly1^+2^+$ clones, the surface phenotype associated with precursors of suppressive cells also inhibited secretion of Ig. By contrast, none of the five $Ly1^+2^-$ clones suppressed the response. (Table 3,4)

D. Cloned cells expressing natural cytolytic activity (Table 5).

Spleen cells from non-immune mice (or blood lymphocytes from humans) can lyse or damage a variety of target cells, notably malignant cells, in vitro, and have been termed natural killer (NK) cells (25,26,29,30). This activity is independent of antibody and complement and does not reflect con-

Table 2

Cloned Populations Expressing the $Ly1^+2^-$ Phenotype: Immunologic Function

No. of Cloned Cells/Culture ($x10^4$)		No. of Immunoglobulin-Secreting Cells in Culture after Incubation with: a) B cells	b) Ly1 cells + B cells
Expt. 1	0	320 ± 29	2520 ± 197
	1	3040 ± 413	4240 ± 357
	5	4680 ± 423	4280 ± 482
Expt. 2	0	500 ± 72	7400 ± 592
	2	2600 ± 210	8300 ± 913
	5	5600 ± 632	7232 ± 774

Isolated B cells or mixtures of Ly1 and B cells were incubated x 4 days in vitro with increasing numbers of cloned $Ly1^+2^-$ cells. The number of Ig-secreting B cells was then enumerated. This activity of Ly1-D9.1 is representative of the activities of four other $Ly1^+2^-$ clones.

Table 3

Cloned Populations Expressing the $Ly1^-2^+$ Phenotype: Immunologic Function

No. of Cloned cells/Culture ($x10^4$)		No. of Immunoglobulin Secreting Cells after Incubation of: a) Ly1 + B cells	b) B cells
Expt. 1	0	7233 ± 770	2100 ± 452
	2	7100 ± 640	n.d.
	5	1600 ± 173	1600 ± 321
Expt. 2	0	2601 ± 302	500 ± 221
	2	2408 ± 133	n.d.
	5	799 ± 98	800 ± 133

Immunologic function of Cl.$Ly1^-2^+$/6: Immunoglobulin secretion was detected using the hemolytic plaque assay. Ly1 cells + B cells or B cells alone were incubated x four days in vitro in the presence or absence of the indicated numbers of cloned cells. This activity is representative of the activity of three additional $Ly1^-2^+$ clones.

Table 4

Cloned Populations Expressing the $Ly1^+2^+$ Phenotype
Immunologic Function

No. of Cloned Cells/Culture ($x10^4$)		No. of Immunoglobulin Secreting Cells After Incubation of: a) Ly1 + B cells	b) B cells
A. Clone $Ly1^+2^+$-/12			
Expt. 1	0	7233 ± 940	1600 ± 192
	1	12600 ± 1285	1100 ± 198
Expt. 2	0	1122 ± 181	500 ± 65
	1	100 ± 150	700 ± 56
B. Clone $Ly1^+2^+$-/11			
Expt. 1	0	1476 ± 221	390 ± 35
	10	260 ± 13	104 ± 13
Expt. 2	0	5700 ± 456	3000 ± 302'
	0.2	2600 ± 364	n.d.
	10	1600 ± 96	550 ± 61

Immunologic function of 2 representative $Ly1^+2^+$ clones (a total of 7 were tested) are shown: A. Cl.$Ly1^+2^+$-12 and B. Cl.$Ly1^+2^+$-11. Immunoglobulin secreting B cells were enumerated after 4 days in vitro incubation in the presence of Ly1 cells (as a source of inducer activity) or in their absence. Two patterns of activities were observed. Three of five $Ly1^+2^+$ clones had activity A and two/five activity B.

ventional phagocytic activity. Cells responsible for this reaction are said to resemble small lymphocytes morphologically, and are found mainly in spleens of young animals. Numerous studies have indicated that this cell set represents an immature cell within the thymus lineage, although the possibility that at least a portion of NK cells represents monocyte/macrophage precursors has not been ruled out. The surface phenotype of cells carrying NK activity in heterogeneous spleen cell populations is $TL^-, Ly1^-2^-Ig^-Ly5^+Qat5^+NK1^+$ and a substantial fraction may also express Thy1. Cells mediating NK activity were enriched from heterogeneous spleen cell suspensions before initiation of the cell growth in cultures irradiated spleen cell monolayers. Cloning efficiency of cells that a) expressed the NK glycoprotein pattern and b) expressed lytic ('NK') function, was 27%. Lytic activity of cloned NK cells was 100 fold more potent that unselected spleen cell populations. Cl.NK/11 was also rested for its ability to lyse several cellular targets. Lysis ($\geq$ 50%) was seen at effector to target rations of less than 1 (Table 5). Conventional tar-

Table 5

Cytolytic Activity against YAC-1 and RL-12 Lymphoma Target Cells

	% SPECIFIC LYSIS				
	YAC-1 lymphoma target				R12 lymphoma target
	Effector: target ratio				Effector: target ratio
Source of cells tested:	50:1	12.5:1	6.25:1	.5:1	6.25:1
Spleen	29	10	<1	<1	0
Ig^-Ly5^+ fraction of spleen	nd	28	14	<1	0
Clone NK-2 ($Thy1^+Ly1^-2^-5^+$)	nd	39	40	28	0
Clone FL-1 ($Thy1^-Ly1^-2^-5^+$)	nd	0	0	0	0
Clone BM-I-1 ($Thy1^-2^-5^+$)	nd	0	0	0	0

Heterogeneous cell populations from spleen and the Ig^-Ly5^+ fraction of spleen cells were prepared as previously described (19). Cloned population NK-2 was derived by positive selection from spleen cells; FL-1 clone was derived from fetal liver; BM-I-1 clone was derived from a coloney from bone marrow cells. All populations were tested for cytolytic activity against the NK sensitive YAC-1 lymphoma and the NK resistant RL-12 lymphoma.

gets YAC-1 and MBL2 lymphomas were susceptible. In addition, P815 mastocytoma and EL-4 lymphoma cells, targets of 'activated' NK cells, were also lysed. Syngeneic LPS-activated B lymphocytes were very susceptible to NK lysis (>50% Lysis at E/T=1); Con A activated T lymphocytes, thymocytes and bone marrow cells were relatively resistant. No activity was observed against an NK resistant radiation-induced lymphoma (R12) or the K562 erythroleukemia, a human NK target cell. The properties of these cells are examined in more detail elsewhere (10).

II. GENERATION OF CLONED POPULATIONS AT EARLY STAGES OF LYMPHOCYTE DIFFERENTIATION

A. Cloned populations from fetal liver cells.

Cell suspensions obtained from the liver of day 13 mouse fetuses were distributed at limiting dilution into microwells containing 0.1ml CM and monolayers of irradiated cells from either fetal liver, bone marrow or spleen.

The mean cloning efficiency of cultures containing these three types of cell monolayers was .16%, .63% and .42% respectively; preincubation of fetal liver cells before cloning on irradiated bone marrow cells resulted in a CE of 33%. Examination of 10 randomly selected clones from three experiments showed that all expressed the glycoprotein pattern of cells within the thymic lineage ($Ig^-Lyb2^-/TL^-Th1^-Ly1^-2^-/Ly5^+NK1^-$). No other surface pattern was observed.

B. Cloned populations from bone marrow cells.

Two types of colonies were detected after initiation of growth on irradiated BM monolayers. "Type I" cells accounted for approximately 60% of colonies and expressed surface glycoproteins identical to the pattern of clones from F.L. -vida supra. Type II cells expressed the surface glycoproteins characteristic of immature thymocytes ($Ig^-Lyb2^-/Thy1^+TL^+Ly1^+2^+/Ly5^+$). These cells did not contain detectable levels of the enzyme terminal deoxynucleotidyl transferase (TdT) by chemical assay, although slight positivity was detected by immunofluorescence. Growth on irradiated thymocyte or spleen monolayers increased the proportion of Type II (thymocyte) colonies to 80-90%. In all cases, cloned populations derived from Type I or II colonies maintained the cell surface glycoprotein pattern and TdT activity of the parent colony.

C. Cloned populations of thymocytes from heterogeneous thymocytes suspensions.

Attempts to initiate efficient cell growth on thymocytes using different irradiated cell monolayers failed (C.E.+.03%). However, preincubation of thymocytes in vitro before initiation of clonal growth was initiated on irradiated spleen cell monolayers. All cloned populations tested expressed the glycoprotein markers characteristic of immature thymocytes. Whether a portion of these cloned lines contains TdT is currently being tested.

III. DEFINITION OF ANTIGEN-SPECIFIC SUPPRESSOR MOLECULES PRODUCED BY CLONED $Ly2^+$ T-CELLS (11,12)

Activation of antibody forming (B) lymphocytes to most antigens requires induction by thymus-derived (T) lymphocytes. In addition, analysis of T-cells has shown that this class of lymphocytes is capable of exerting specific suppressive effects, and that these two functions are mediated by different sets of T lymphocytes that each express a different and characteristic pattern of cell-surface glycoprotein (4). In addition, unlike antigen-specific T-helper cells, antigen-specific suppressor T cells bind to antigen-coated columns. Recently, supernatants of cultures containing T-cells have been shown to mimic the action of T suppressor cells. However, so far, analysis of supernatant material has not allowed insight into the structural basis of antigen-specific suppression.

We have produced several clones of $Ly2^+$ T-suppressor cells from donors immunized with sheep red blood cells (SRBC); each binds specifically to sheep glycophorin, a major erythrocyte membrane protein. All of these clones secrete a polypeptide that binds specifically to SRBC and this binding is inhibited by glycophorin purified from sheep erythrocytes but not other erythrocytes. Analysis by column chromatography shows the polypeptide runs slightly slower than bovine serum albumin (68,000 daltons). After elution with 0.2M sodium phosphate pH 7.2, from DEAE-cellulose and preparative flat bed isoelectrofocusing (IEF), an internally labeled polypeptide responsible for antigen binding and specific suppression has been purified to virtual homogeneity. The purified protein has an apparent MW of 70,000 daltons under reducing as well as non-reducing conditions, and shows two very close bands in analytical IEF (pI=5.0). It is likely that one of the two represents a postranscriptional modification (Figure 1).

Ninety-five per cent of this internally labelled material binds to sheep erythrocytes but not to horse or burro erythrocytes. Binding to SRBC is completely inhibited by glycophorin from sheep but not horse or human erythrocytes, nor neuraminidase-treated sheep erythrocyte glycophorin, strongly suggest-

ing that the factor recognize glycophorin determinants in the surface of the erythrocyte membrane and that the sugar moiety plays a very important role in this recognition (Table 6). In addition, the polypeptide is retained by sheep glycophorin but not human glycophorin-coated sepharose columns. This purified antigen binding material is retained by lentil-lectin-sepharose columns, but not by columns coupled with antisera directed against the I-region or I-J subregions of the major histocompatibility locus. Sepharose columns coupled with antisera raised against immunoglobulins from mice, also failed to retain the factor. Taken together, the above data indicate that the purified antigen binding material is glycoprotein in nature and does not bear either conventional Ig or I region determinants.

Table 6

Immunological Characteristic of Purified Suppressor Polypeptide

	Immunoadsorbent	*^{35}S methionine labeled SF bound (% cpm added)*
A.	*lentil-lectin-sepharose*	*100*
	R anti MIg sepharose	*0*
	anti I-J^b sepharose	*0*
B.	*SRBC*	*100*
	HRBC	*5*
	BRBC	*3*
C.	*SRBC + Sheep glycophorin*	*0*
	SRBC + Horse glycophorin	*95*

1000 cpm of purified suppressor polypeptide were incubated with: A) 1 ml sepharose beads/ml B) 10^7 RBC/ml C) 10^7 RBC/ml in presence of 10 ug/ml of indicated glycoproteins.

Incubation of Lyl cells from SRBC-immune donors with the purified 70K glycopeptide (final concentration 100 pcg/ml) x 18 hrs. results in 90% reduction in the ability of these helper cells to induce B-cells to secrete anti-SRBC immunoglobulins. Identical incubation of Lyl cells from donors immune to burro erythrocytes (BRBC) has no effect on anti-BRBC specific helper function (Table 7). Antigen is required for the 70K glycopeptide to suppress T_H activity: preincubation of Ly cells with the 70K molecule in the absence of antigen (or in the presence of the 'wrong' antigen) has no effect on subsequent SRBC specific T helper activity (Table 7). Because preincubation of B cwels under identical conditions does not inhibit B-cell secretion of anti-RBC antibodies, the main target of the 70K glycopeptide appears to be antigen-specific inducer T-cells.

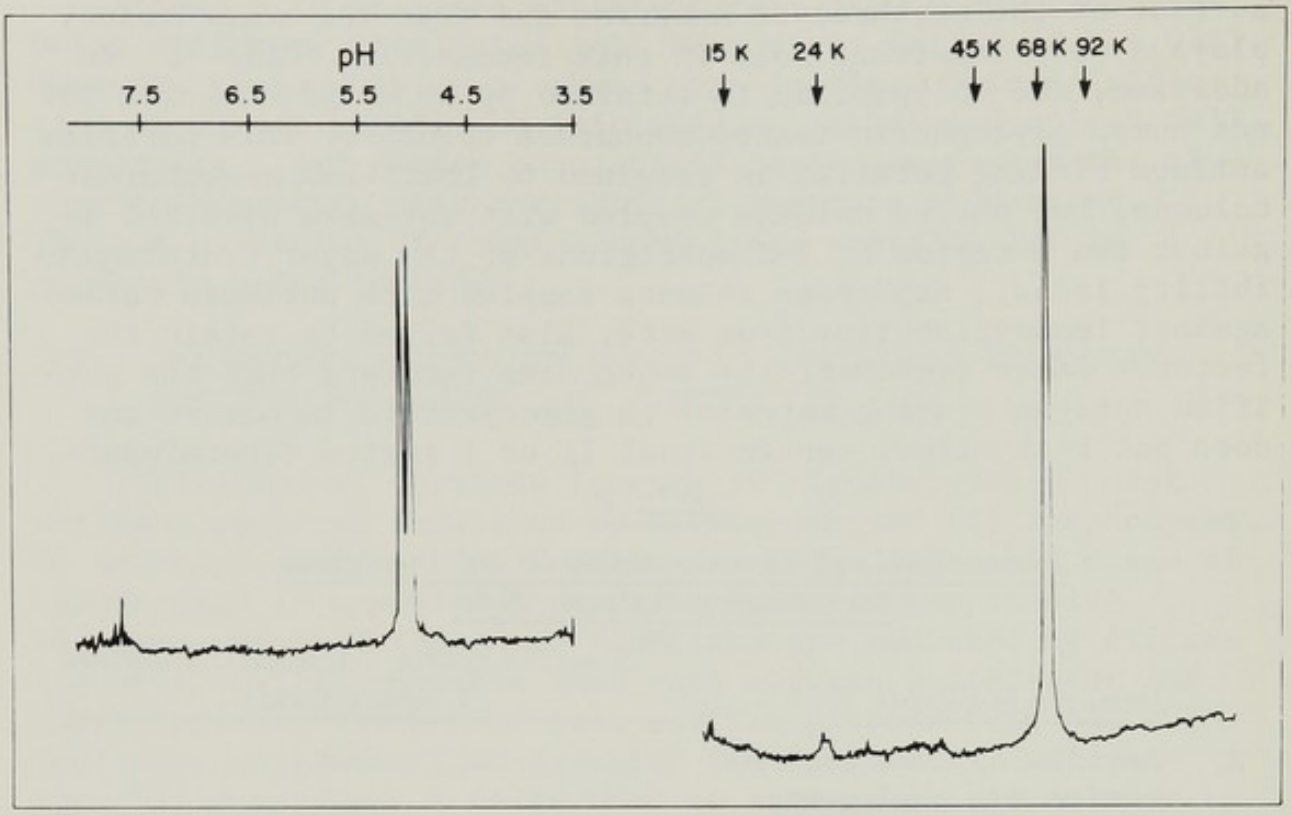

Figure 1
Densitometric scans of fluorograms of purified ^{35}S-methionine labelled suppressor peptide. Right, SDS-polyacrylamide gel electrophoresis. Left. isolectrofocusing on polyacrylamide gels, pH gradient 3-8.

These data show that a cloned T-cell population synthesizes (and probably secretes) a glycopeptide of 70,000 daltons and pI of 5.0 that binds specifically to sheep erythrocyte glycoprotein and that this single polypeptide preempts the T-helper response to the whole erythrocyte. The structural basis of T cell recognition has been the object of many studies. Two main approaches have been followed: analysis of extracts from suppressor cells or analysis of supernatants from heterogeneous populations of T-cells. These have not provided insights into the biochemical structure of T-cell molecules that directly suppress immune function due to the lack of homogeneity of the preparations. More recently, T-cell hybrids have been analyzed. However, in general T-cell hybrids rapidly lose chromosomes and express as mainly the phenotype of the tumor partner: it is extremely difficult to demonstrate biological significance of hybrid T-cell products. Nonetheless, the above approaches have indicated a major type of suppressor factor. It can be obtained from suppressor cell extracts, or supernatants, and T-cell hybridomas. The factor has a mean size of 45-50K, bears I-J

Table 7

Effect of the Suppressor Polypeptide on Inducer T Cells and B Cells: Specific Inhibition of Inducer T Cell Activation in the Presence of Antigen

	First Incubation (24 hr)	Anti-SRBC Ig Secretion (% Suppression)	
A) Ly1 Target	$Ly1_{is}$ + SRBC	675 ± 33	(--)
	$Ly1_{is}$ + SRBC + SF-70K	54 ± 8	(93)
	$Ly1_{is}$	650 ± 51	(--)
	$Ly1_{is}$ + SF-70K	459 ± 40	(29)
	$Ly1_{ib}$ + BRBC	850 ± 85*	(--)
	$Ly1_{ib}$ + BRBC	1000 ± 100*	(0)
B) B Cell Target	B	350 ± 18	(--)
	B + SF-70K	300 ± 37	(7)
	B + SRBC	720 ± 56	(--)
	B + SRBC + SF-70K	630 ± 48	(12)

Cells were incubated for 24 hr in the first culture in the presence or absence of purified 70,000 dalton suppressor molecule, washed and transferred to a second culture containing the indicated cells. After a further 72 hr incubation, anti-SRBC or anti-BRBC specific (*) Ig secretion by B cell enumerated. $Ly1^+$ cells (10^5) immune to SRBC ($Ly1_{is}$) or to BRBC (Ly_{ib}) non-immune B-cells (10^6) and homologous RBC (10^6) were included in culture.

regions products, and suppresses by recruiting more suppressor cells from precursor $Ly1^+2^+$ populations.

The suppressive molecule described here directly suppresses T_H activity. The use of cloned T-cell populations has allowed us to purify this molecule to virtual homogeneity and will provide a more clear insight into the biochemical basis of the structure of antigen specific T-cell products. The data summarized in Table 8 are instructive in this regard. The molecule is very sensitive to proteolytic as well as spontaneous degradation; it gives rise to two main peptides of apparent MW 45,000 and 24,000 daltons according to SDS polyacrylamide gel electrophoresis. The 45K moiety (Peptide A) cannot bind antigen but retains suppressive activity which is no longer specific for SRBC. By contrast, the 24K peptide (Peptide B) retains SRBC specific binding activity, but lacks

Table 8

Presence of Two Different Domains in the Suppressor Factor Molecules

	MW	PI	Antigen binding	Suppression		Reaction	
				Antigen-specific	Non-specfic	αTSF	αFv
Purified TSF	70K	5.0	+	++	-	+	+
Peptide A	45K	5.6	-	-	+	+	-
Peptide B	24K	?	+	-	-	-	+

suppressor function.

Two types of molecules that may play important roles in the generation and expression of Ts activity and in antigen recognition. The first includes material that may have two chains and I region products; these molecules are important in T-T interactions that generate suppressor T-cells from $Ly2^+$ precursors. The second directly mediates suppression and is composed of a single 70K chain, lacks I-region determinants, and is likely to represent a secreted form of the T-cell receptor.

The molecule described here belongs to this latter group. Picogram amounts of this glycoprotein can specifically suppress the Th response to a foreign erythrocyte. A molecular explanation of this activity comes from the finding that the 70K molecule does not suppress Ly Th cells in the absence of the correct erythrocyte. Antigen is not required to stimulate Th cells and perhaps render them more sensitive to suppression because Th cells from BRBC or HRBC-immune donors are not suppressed after restimulation in vitro with the 70K protein and the erythrocyte used for immunization. Recent studies have indicated that Lyl cells can bind to radiolabelled antigen that has been "processed" to macrophages (14). Since the 70K molecule specifically binds to SRBC, antigen can form a 'bridge' between the Ts molecule and antigen-specific Th cells. Possibly, antigen bound to Lyl cells may focus Ts molecules, resulting in specific suppression of anti-SRBC Th activity.

Several mechanisms might account for the ability of a single glycoprotein to preempt the entire primary antibody response to a foreign erythrocyte. The explanation that we favor is based on the structural properties of the 70K molecule. After interaction with the antigen, the 70K protein breaks down into two peptides, having molecular weights of 45K and 24K. The former peptide displays non-specific suppression and does not bind to antigen; the latter does not possess detectable Ts activity but binds specifically to SRBC. These findings suggest that occupation of the antigen-binding portion of the Ts molecule may result in activation of the 45K portion and suppression of Lyl cells, regardless of their specficity for antigen.

Although suppression may result from a direct interaction between the Ts molecule and Lyl cells, a second possible mechanism comes from studies of antigen-recognition by T helper cells. Lyl^+ Th cells are thought to recognize antigen in association with Ia molecules on the surface of antigen-presenting cells. Though positively selected Lyl cells incubated with the 70K protein are >98% Lyl^+2^- according to immunofluorescence, contamination by macrophages cannot be excluded. Possibly, Lyl cells may recognize complexes of the 70K molecule and antigen on the surface of macrophages.

Suppression of Lyl cells may thus reflect either direct inhibition by the 70K-antigen complex or non-specific inhibitory molecules produced by macrophages after interaction with the 70K molecule-antigen complex. Experiments aimed at pinning down the role of antigen-presenting cells in suppression and further analysis of the biologic functions of different regions of the suppressive molecule should resolve these questions.

REFERENCES

1. Sakano, H., Huppi, K., Heinrich, G., and Tonegawa, S. (1970). *Nature 280*, 288.
2. Schilling, J., Clevinger, B, Davie, J. and Hood, L. (1980). *Nature 283*, 35.
3. Seidman, J., and Leder, P. (1978). *Nature 276*, 790.
3a. Bernard, O., Hozumi, N., and Tonegawa, C. (1978). *Cell 15*, 1133.
4. Cantor, H., and Boyse, E.A. (1977). *Imm. Rev. 33*, 105.
5. Owen, J.J.T., and Ritter, M.A. (1969). *J. Exp. Med. 129*, 431.
6. Ford, C.E., Micklem, H.S., Evans, E.P., Grax, J.G., and Ogden, D.A. (1966). *Ann. NY Acad. Sci. 229*, 283.
7. Owen, J.J.T., and Raff, M.C. (1970). *J. Exp. Med. 132*, 1216.
8. Nabel, G., Fresno, M., Chessman, A., and Cantor, H. (1981). *Cell 23*, 19.
9. Nabel, G., Greenberger, J.S., Sakakeeny, M.A., and Cantor, H. (1981). *Proc. Natl. Acad. Sci. USA 78*, 1157.
10. Nabel, G., Bucalo, L.R., Allard, J., Wigzell, H., and Cantor, H. (1981). *J. Exp. Med.* in the press.
11. Fresno, M., Nabel, G., Mc-Vay Boudreau, L. Furthmayer, H., and Cantor, H. (1981). *J. Exp. Med.* in the press.
12. Fresno, M., Mc-Vay Boudreau, L., Nabel, G., and Cantor, H. (1981). *J. Exp. Med.* in the press.
13. Pacifico, A., and Capra, J.D. (1980). *J. Exp. Med. 152*, 1289.

IMMUNOGENETICS OF MONOCLONAL SUPPRESSOR T CELL PRODUCTS[1]

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The synthetic polymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) and L-glutamic acid^{50}-L-tyrosine^{50} (GT) stimulate specific suppressor T cells in certain strains of mice. Extracts from these T cells contain factors (TsF) that inhibit GAT- or GT-specific antibody responses by normal spleen cells or proliferative responses by primed T cells. We have produced T cell hybridoma lines that constitutively synthesize GAT-TsF or GT-TsF which functionally and serologically resemble the factors extracted from suppressor T cells. Data presented demonstrate that these monoclonal products bind to GAT and can discriminate between the related polymers, GA and GT. Furthermore this antigen binding specificity correlates with the expression of the idiotypic determinants GA-1 and CGAT, respectively.

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INTRODUCTION

Immune responses to the synthetic polymers of GAT and GT are controlled genes linked to the major histocompatibility gene complex (1). GAT fails to stimulate development of antibody responses or to prime T cells for proliferative responses in nonresponder mice bearing the H-$2^{p,q,s}$ haplotypes. However, GAT complexed to methylated bovine serum albumin (GAT-MBSA) stimulates GAT-specific antibody responses and primes for GAT-specific T cell proliferative responses in nonresponder mice. GT does not stimulate antibody production or prime for proliferative responses in any inbred mice (2) but, like GAT-MBSA, GT-MBSA can elicit antibody responses and prime for proliferative responses. GAT stimulates GAT-specific suppressor T cells in nonresponder H-$2^{p,q,s}$ mice that can inhibit development of GAT-specific antibody and proliferative responses normally stimulated by GAT-MBSA (3). GT-specific suppressor T cells can be elicited by GT in some (H-$2^{d,k,s}$), but not other (H-$2^{a,b,q}$) strains of mice (4).

Extracts from suppressor T cells contain antigen-specific T cell-derived suppressor factors (GAT-TsF or GT-TsF) that inhibit development of primary antibody responses or proliferative responses (3). The active mediator is a protein that binds to insolublilized antigen, bears determinants encoded by the I-J subregion of the MHC but does not bear detectable determinants encoded by the IgCH genes (5).

The immunoglobulin genetics of antibodies specific for GAT have been analyzed by producing guinea pig anti-idiotypic antibodies to serum antibodies from D1.LP mice. This reagent detects a common determinant, called CGAT, in GAT-specific antibodies from all strains of mice, regardless of whether they are responder or nonresponder strains (6). Antibodies

bearing the CGAT idiotype bind to GAT and GT but not GA, whereas GA-binding antibodies also bind to GAT but not GT and do not bear the CGAT determinant (7). GA-specific antibodies bear a common idiotypic determinant, GA-1, that is expressed by all but one of the inbred strains tested (8).

Antigen-binding TsF molecules share cross-reactive determinants with anti-GAT antibodies since GAT-TsF from either SJL or DBA/1 mice could be absorbed by the guinea pig anti-CGAT antibodies (9). Since extracts containing GAT-TsF probably contain a heterogeneous mixture of suppressor factors that differ in fine specificity, we have compared the antigen-binding characteristics and expression of cross-reactive idiotypic determinants of monoclonal GAT-TsF produced by a DBA/1 T cell hybrid, and GT-TsF produced by a BALB/c T cell hybridoma.

MATERIALS AND METHODS

Production of T Cell Hybridomas

Seven days after injection of GT or GAT mice were sacrificed and splenic T lymphocytes (enriched by passage over nylon wool columns) were fused to the HAT-sensitive AKR thymoma, BW5147, and clones producing TsF were selected as previously described (5).

Antisera and Immunoadsorbents

Alloantisera specific for the I^q, $I\text{-}J^s$ and $I\text{-}J^k$ subregions were prepared by Dr. Donald C. Shreffler, Washington University School of Medicine (St. Louis, MO). Guinea pig antibodies specific for the CGAT and GA-1 idiotypic determinants were prepared as previously described (6,8). It should be noted that the serum containing anti-CGAT antibodies also contains a

small but detectable amount of anti-GA-1 idiotypic antibody (8). Anti-GAT-TsF was produced by hyperimmunization of rabbits with affinity-purified, concentrated monoclonal GAT-TsF from the culture supernate of the DBA/1 hybrid, C4#4. Antibodies were coupled to CnBr-Sepharose 4B (Sigma Chemical Co.) and the synthetic polymers GAT, GA, and GT were coupled to amino-hexyl Sepharose 4B (Sigma Chemical Co.) as previously described (5).

Cell Cultures and Assay of TsF

Primary PFC responses were assayed in cultures containing normal spleen cells stimulated with GAT-MBSA, GT-MBSA or SRBC (10). Proliferative responses by lymph node T cells from mice primed with GT-MBSA or GAT-MBSA were measured by incorporation of ^{3}H-thymidine (^{3}H-TdR).(3). Various dilutions of hybridoma supernatant fluids were added to PFC or proliferative cultures at initiation. The data have been expressed as the inverse of the final dilution of TsF that causes 50% specific suppression of the control response (S_{50}U/ml).

RESULTS AND DISCUSSION

Suppression of GAT-Specific PFC and Proliferative Responses

The fusion of BW5147 and GAT-primed DBA/1 T cells resulted in growth in several primary cultures from which three clones with suppressive activity were derived (5). Supernatant fluids from clone C4#4 inhibited the GAT-specific PFC response to GAT-MBSA and the GAT-specific proliferative response to GAT in cultures of DBA/1 lymphoid cells (Table I). PFC and proliferative responses were reduced by 50% at dilutions of 1:20,000 and 1:10,000, respectively. These supernates provide a highly enriched source of suppressor factor compared to extracts

Table I. Culture Supernate from Clone C4#4 Inhibits GAT-Specific Responses

	S_{50} U/ml	
	GAT-Specific IgG PFC Response*	GAT-Specific Proliferative Response‡
Untreated Supernate	20,000	10,000
Effluent from GAT-Seph	<5,000	<1,000
Eluate from GAT-Seph	100,000	75,000

**The GAT-specific PFC response of these cultures in the absence of supernate was 320 PFC/culture.*

‡The GAT-specific proliferative response of cultures in the absence of supernate was 27,000 cpm.

prepared by sonication of splenic T cells (yielding ~1000 S_{50}U/mouse). The GAT-TsF produced by clone C4#4 is retained by a GAT-sepharose immunoadsorbent and activity can be eluted with 2M KCL. The five to ten-fold increase in suppressor activity in the eluate is due in part to concentration during purification. GAT-TsF from C4#4 supernates is qualitatively identical to T cell extracts in its ability to suppress both the GAT-specific PFC and proliferative responses and its binding affinity for GAT. In addition, these T cell hybrids produce enough suppressor factor to meet the requirements for biochemical and structural analyses (12).

To identify MHC encoded determinants on monoclonal GAT-TsF, C4#4 supernate was affinity-purified, concentrated and filtered through insolubilized alloantisera or normal mouse Ig and the effluents tested for suppressive activity in PFC and proliferative responses. The affinity-purified, unadsorbed factor and the factor adsorbed with normal mouse Ig inhibited the

GAT-specific PFC and proliferative responses without inhibiting responses to SRBC or MBSA (Table II). The immunoadsorbent containing anti-I^q antisera retained most of the activity, whereas the anti-I-J^s and anti-I-J^k adsorbents did not. The anti-I-J^s and anti-I-J^k sera have been shown independently to bind TsF produced by mice bearing the appropriate haplotype (not shown). These results demonstrate that the monoclonal GAT-TsF is qualitatively identical to GAT-TsF in lymphoid cell extracts in that the biologically active molecule has both an antigen-binding site(s) and

Table II. Monoclonal GAT-TsF Bears MHC Encoded Determinants

	S_{50}U/ml			
	PFC Response*		Proliferative Response‡	
Purified GAT-TsF Absorbed With	GAT	SRBC	GAT	MBSA
-	600,000	≤ 1,000#	640,000	≤ 10,000#
N Ig-Seph	640,000	≤ 1,000	630,000	≤ 10,000
Anti-I^q Seph	4,300	≤ 1,000	10,000	≤ 10,000
Anti-I-J^s Seph	580,000	≤ 1,000	400,000	≤ 10,000
Anti-I-J^k Seph	800,000	≤ 1,000	610,000	≤ 10,000

**PFC responses to GAT-MBSA by normal DBA/1 spleen cells in the absence of factor was 505 GAT-specific IgG PFC/culture and 1250 IgG PFC to SRBC.*

‡Proliferation to GAT by lymph node cells from DBA/1 mice in the absence of factor was 20,130 cpm over a background of 4975 cpm.

#The lowest dilutions tested (1/1,000 or 1/10,000) gave no detectable suppression.

determinants encoded by the I region of the MHC. The I-J subregion of the H-2^q haplotype has not been defined by recombination, but we assume that C4#4 bears determinants encoded by the I-J^q subregion because all of the GAT-specific and GT-specific suppressor factors that can be tested bear determinants encoded by the I-J subregion of the appropriate haplotype. It is interesting, although not surprising, that the biologically active hybridoma product does not bear I-J determinants encoded by the AKR thymoma BW5147.

Comparison of Fine Specificity and Expression of Idiotype by Two Monoclonal TsF

We have produced several hybridoma T cell clones from GAT- or GT-primed suppressor cells and preliminary experiments indicated that these TsF displayed the ability to distinguish between GA and GT. This observation has led us to compare the antigenic fine specificity and expression of the CGAT and GA-1 idiotypic determinants. The first monoclonal factor examined was C4#4, the product described above. This factor binds to GAT-Sepharose and GA-Sepharose, but not GT-Sepharose, and it is bound by both guinea pig anti-CGAT and anti-GA-1 antibodies (Table III). Since the guinea pig anti-CGAT contains antibodies to both CGAT and GA-1 idiotypic determinants, and since C4#4 is bound by anti-GA-1 sera, it is most likely that the binding of C4#4 to the anti-CGAT immunoadsorbent is via anti-GA-1 antibodies. As expected rabbit anti-C4#4 binds C4#4.

A second clone derived by fusion of GT-primed BALB/c T cells with BW5147 produces a suppressor factor that binds equally well to GT and GAT but not GA (Table III). In addition, this factor is not recognized by an anti-GA-1 idiotype but is bound by the guinea pig anti-CGAT, suggesting that this factor is CGAT positive. Rabbit anti-C4#4 did not

Table III. Monoclonal GAT-TsF and Monoclonal GT-TsF Exhibit Different Antigen Binding Specificities and Idiotypic Determinants

	S_{50}U/ml			
	DBA/1 GAT-TsF‡		BALB/c GT-TsF*	
Immunoadsorbent	PFC	Proliferation	PFC	Proliferation
None	600,000	580,000	27,000	32,000
GAT-Sepharose	<1,000	<10,000	<5,000	<5,000
GA-Sepharose	1,000	<10,000	45,000	30,000
GT-Sepharose	250,000	650,000	<5,000	<5,000
Anti-GA-1 id	<10,000	<10,000	22,000	35,000
Anti-CGAT id	<10,000	<10,000	8,800	<5,000
NGP Ig	800,000	600,000	22,000	43,000
Anti-GAT-TsF C4#4	<1,000	<10,000	24,000	37,000

‡*Affinity-purified concentrated supernate from DBA/1 T cell hybridoma (C4#4)*

**Unpurified supernate from a BALB/c T cell hybridoma(B1#5)*

bind the BALB/c GT-TsF suggesting that the rabbit antibody is not a class-specific reagent that combines with all suppressor factors. Other studies suggest that it is not uniquely reactive for C4#4 or H-2^q factors since it does bind GAT-TsF extracted from GAT-primed B10.S mice (not shown). In fact, it appears that there is positive correlation between binding of factors by anti-GA-1 and anti-C4#4. However, preliminary results suggest that these two antibodies recognize distinct determinants since anti-C4#4 antibodies do not bind anti-GAT antibodies whether or not they express the GA-1 idiotype.

There are two important conclusions that can be drawn from these data. First, that monoclonal TsF exhibit remarkable fine specificity illustrated by the fact that both of these

monoclonal factors bind to GAT and one reacts with GA not GT, whereas the other reacts with GT not GA. Monoclonal anti-GAT antibodies produced by B cell hybridomas also display the ability to discriminate between GA and GT determinants (7). Lymphoid cell extracts from GAT-primed nonresponder mice have been shown to be more cross-reactive than the monoclonal TsF since they bind to GAT, GA and GT (11) which suggests that suppressor factors produced in vivo represent a collection of heterogeneous molecules of precise antigenic specificity.

The second conclusion is that the correlation between GT-binding activity and expression of the CGAT idiotypic determinant and GA-binding activity and the GA-1 idiotype originally defined for GAT-specific antibodies is also reflected by the monoclonal supressor factors. Admittedly, this study is restricted to products of only two clones, but the validity of this conclusion continues to be supported by data from the survey of monoclonal suppressor factors currently in progress.

Assuming that the correlation between antigen-binding specificity and expression of idiotypic determinants is verified in other monoclonal suppressor T cell products and that the anti-idiotypic antibodies that bind GAT-specific antibody are the same as those that bind GAT-TsF, what is the significance of shared determinants between B and T cell products? Are there two variable region repertoires - one linked to IgCH genes and one linked to the MHC genes that share structurally related determinants because they bind to the same epitope? Or, is their one variable region repertoire utilized by both T cells and B cells? We anticipate that answers to these questions will be derived by comparing the amino acid sequences of monoclonal T and B cell products that bear cross-reactive idiotypic determinants and ultimately by studying regulation of gene expression in these cell lines.

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REFERENCES

1. Benacerraf, B. and Germain, R.N. (1977) *Immunol. Rev. 38:* 70.
2. Debre, P.A., Kapp, J.A., Dorf, M.E. and Benacerraf, B. (1975) *J. Exp. Med. 142:*1447.
3. Araneo, B.A. and Kapp, J.A. (1980) *J. Immunol. 125:*118.
4. Debre, P.A., Waltenbaugh, C., Dorf, M.E., Benacerraf, B. (1976) *J. Exp. Med. 144:*272
5. Kapp, J.A., Araneo, B.A. and Clevinger, B.L. (1980) *J. Exp. Med. 152:*235.
6. Ju, S-T., Benacerraf, B. and Dorf, M.E. (1978) *Proc. Natl. Acad. Sci. 75:*6192.
7. Pierres, M., Ju, S-T., Waltenbaugh, C., Dorf, M.E., Benacerraf, B. and Germain,R.N. (1979) *Proc. Natl. Acad. Sci. 76:*2425.
8. Ju, S-T., Pierres, M., Germain, R.N., Benacerraf, B. and Dorf, M.E. (1979) *J. Immunol. 123:*2505.
9. Germain, R.N., Ju, S-T., Kipps, T., Benacerraf, B. and Dorf, M.E. (1979) *J. Exp. Med. 149:*613.
10. Kapp, J.A., Pierce, C.W. and Benacerraf, B. (1973) *J. Exp. Med. 138:*1107.
11. Theze, J., Kapp, J.A., and Benacerraf, B. (1977) *J. Exp. Med. 145:*839.
12. Krupen, K., Araneo, B.A., Kapp, J.A., Stein, S., Weider, K. and Webb, D. (1981) *J.Supramol.Struct.Suppl. 5:*118.

ACTIVATION OF AN ACCEPTOR T CELL HYBRIDOMA BY A V_H^+ I-J^+ MONOCLONAL SUPPRESSOR FACTOR*

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Functional and structural properties of an antigen-specific suppressor T cell factor(TsF) and its mode of action was examined by using T cell hybridomas. The hybridoma-derived TsF specific for keyhole limpet hemocyanin(KLH) consists of two distinct polypeptide chains either of which is encoded by genes in the Ig heavy chain linkage group or those in the I-J subregion of the H-2 complex. These two gene products of the secreted form of the TsF are linked in covalent association with disulfide bonds, while those of the extracted TsF are noncovalently linked. The secreted TsF cleaves into the two polypeptide chains by the reduction with dithiothreitol. The cleaved chains are easy to reassociate to compose the active form of the TsF.

The KLH-TsF(TsF_1) per se could stimulate another I-J^+ T cell hybridoma bearing acceptor for TsF to produce new TsF(TsF_2). The TsF_2 has KLH-specific suppressor activity and the H-2 restriction specificity like TsF_1. The TsF_2 bears I-J determinants but does not carry antigen-binding capacity. Since the activity of the TsF_2 was absorbed with the TsF_1-producer, it is possible that TsF_2 has anti-idiotypic receptor for the antigen-binding structure of TsF_1. Therefore, TsF_1 seems to act as signals to stimulate and activate the acceptor hybridoma in the resting state, in which both idiotype-anti-idiotype and I-J-I-J interactions must be involved.

INTRODUCTION

It has been known that suppressor T cells play a crucial role in the regulation of immune response. Suppressor T cells have been shown to express certain Ly phenotypes and the products encoded by genes in the I-J subregion of the H-2 complex(1). Furthermore, the factors derived from antigen-

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specific suppressor T cells have also been found to bear antigen-binding capacity and I-J encoded products(2-6). Despite the above mentioned information, the mechanism of immune suppression induced by the suppressor T cells(Ts) or their factors(TsF) is not completely understood. However, it has been demonstrated that a series of interactions among different populations of regulatory T cells is involved in the immunoregulatory suppressor pathway. Our previous studies(7) have shown that the I-J$^+$ TsF specific for keyhole limpet hemocyanin(KLH) derived from Ts with I-J and Ly23 phenotypes acts on I-J$^+$, Ly1$^+$2$^+$3$^+$ acceptor T cells to induce new Ts acting as effector cell type. Sy et al.(8) and Germain et al.(9) have also demonstrated that idiotype bearing I-J$^+$ TsF induces second order suppressor T cells(Ts$_2$). Thus, it has been postulated that an antigen-specific TsF acts as the signal which mediates the cell to cell communication in the suppressor pathway.

In an attempt to elucidate the biological role of the antigen-specific TsF and the mechanisms of its action, we have established I-J positive T cell hybridomas with different functional activities: one the TsF producer, another the acceptor for TsF. Experiments in this communication characterized a monoclonal KLH-specific TsF and the mechanisms of activation of an acceptor T cell hybridoma by KLH-TsF. It is demonstrated that the KLH-binding and the I-J encoded polypeptide chains compose the KLH-TsF which activates and stimulates the acceptor T cell hybridoma(34S-281) in the resting state to produce new TsF(TsF$_2$), and that the TsF$_2$ bears the I-J determinants together with the anti-idiotypic structure for the TsF$_1$.

MATERIALS AND METHODS

T Cell Hybridomas Expressing I-J Subregion Gene Products.

I-J^b positive T cell hybridomas were established by the fusion of AKR-derived thymoma cell line BW5147 and suppressor T cells from keyhole limpet hemocyanin(KLH)-primed C57BL/6 mice enriched with KLH-coated Petri dishes. The hybrid cells were stained with anti-I-J^b(B10.A(5R) anti-B10.A(3R)) followed by reacting with fluorescein-conjugated rabbit anti-mouse immunoglobulins. They were selected by a fluorescence activated cell sorter(FACS) and cloned by a single cell manipulation. There are several I-J^b positive T cell hybrid clones with different functional activities. The clone 34S-18 has KLH-specific suppressor activity. The clone 34S-281 does not have any functional activity per se, but does have the capacity

to absorb the KLH-TsF derived from 34S-18. Therefore, the cell line 34S-281 was found to be an acceptor-bearing T cell hybridoma.

Preparation and Characterization of the Hybridoma-Derived TsF.

The cell-free extracts were obtained by ultracentrifugation of the frozen and thawed materials of the hybridoma cells, and were used as the cellular form of the TsF. As the secreted TsF, the culture supernatant of the hybridoma cells or the ascitic fluid from the hybridoma-bearing mice was used. For analysis of immunochemical properties of the hybridoma-derived factor, the factor was absorbed with and eluted from the immunoadsorbent columns of antigens and antibodies. The effluent or the acid-eluate from the columns was tested for its activity in the *in vitro* secondary anti-DNP PFC response.

Activation of The Acceptor Bearing Hybridoma Cell Line (34S-281) with KLH-Specific TsF.

In order to characterize the interaction between KLH-TsF and acceptor T cell hybridoma, $1x10^7$ acceptor bearing hybridoma cells were incubated with 200µl of the secreted TsF on ice for 1hr in the absence of the antigen KLH. The cells were washed and cultured in a fresh medium. The cell-free extract or the culture supernatant from the activated acceptor hybridoma was collected and tested for its activity in the *in vitro* secondary anti-DNP PFC response.

RESULTS AND DISCUSSIONS

Structural Properties of the KLH-Specific Suppressor Factor (KLH-TsF) Derived from a T Cell Hybridoma (34S-18).

Since KLH-specific suppressor activity was obtained in the cell-free extract or in the ascites from the hybridoma-bearing mice, the functional and structural properties of the KLH-TsF derived from the clone 34S-18 was characterized by using immunoadsorbent columns composed of antigens and antibodies. The results clearly showed that KLH-TsF consists of two characteristic determinants, i.e., the antigen-binding moiety and the product of genes in the I-J subregion of the H-2 complex, since the activity of the extract or the ascites was completely absorbed with the KLH and the anti-I-J^b columns but not with anti-immunoglobulins nor the anti-I-J^k columns. Therefore, the TsF was found not to be an immunoglobulin despite it's antigen-binding capacity. However, recent reports from various laboratories indicate that the T cell factor carries determinants analogous to the V region of the immunoglobulin heavy chain.

Germain et al.(9) have shown that the suppressor T cell factor specific for synthetic copolymer, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}(GAT), possessed a common idiotypic structure shared by the anti-GAT antibodies. Eshhar et al.(10) have also demonstrated that (T,G)--A-L specific helper factor derived from T cell hybridoma carried the determinants detected by the rabbit antibodies against the framework structure of the variable region of the immunoglobulin heavy chain.

The KLH-TsF derived from the hybridoma was also found to carry V_H determinants, since the suppressor activity of the KLH-TsF was absorbed with and eluted from the columns composed of antibodies against V_H determinants of the immunoglobulin, as shown in Fig.1. Thus the antigen-binding structure of the functional T cell factor seemed to have an identical or comparable structure to the antigen-binding site of antibodies. It is, therefore, conceivable that two gene products are involved in the structural entity of the KLH-TsF: those encoded for by genes in the Ig heavy chain linkage group and the other the products of genes in the I-J subregion of the H-2 complex. These two distinct determinants detected on the KLH-TsF was found to be on different polypeptide chains. As shown in Table 1, the mixture of the effluents of the extracted TsF with the KLH and the anti-I-J^b columns, each of which had no detectable activity, could reconstitute the strong suppressor activity. The effluent from the KLH column does not contain KLH binding moiety while I-J products are present. Similarly, after

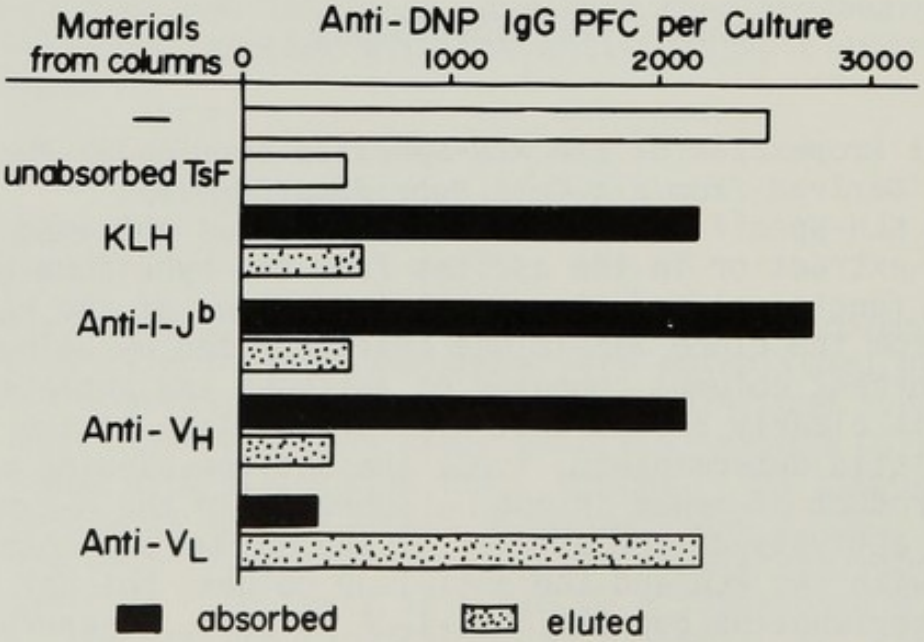

FIGURE 1. Absorption of a monoclonal KLH-TsF with immunoadsorbent columns of antigen and antibodies. Note that the activity was absorbed with and eluted from KLH, anti-I-J^b and anti-V_H but not anti-V_L.

TABLE 1. TWO GENE PRODUCTS OF KLH-TsF WITH NONCOVALENT ASSOCIATION IN THE EXTRACT BUT WITH COVALENT LINKAGE IN ASCITES.

Materials added	Anti-DNP IgG	
	Ascites	Extract
Not added	1774	1211
unabsorbed	313	146
KLH absorbed	2236	1255
Anti-I-J^b absorbed	2043	1342
1/2 KLH absorbed and 1/2 Anti-I-J^b absorbed	2118	100

TABLE 2. REDUCTION AND ALKYLATION OF THE SECRETED TsF.

Purified suppressor molecules treated with		Anti-DNP IgG PFC/culture
——		2394
untreated		798
Reduction and alkylation	5mM DTT 10mM Iodoacetamide	2100
Reduction	5mM DTT	751
Alkylation	10mM Iodoacetamide	651

absorption with anti-I-J^b column, I-J products are removed, leaving KLH-binding moiety in the effluent. Hence, the combination of the two effluents resulted in the mixture of the two components, which constituted the suppressor activity. These results indicates that the extract contains two distinct polypeptide chains in non-covalent association. On the other hand, the secreted TsF has different features from the extracted TsF, possessing the two determinants on the same molecule, since

the mixture of the effluents of the secreted TsF from the KLH and the anti-I-J^b columns could not show any suppressive activity, suggesting that the two polypeptide chains are linked in covalent association.

The secreted TsF was investigated to corroborate the presence of the interchain S-S bonds and whether it cleaves into the two polypeptide chains. The secreted TsF purified with the KLH column was reduced with 5mM dithiothreitol(DTT) and alkylated with 10mM iodoacetamide. In some experiments, the purified TsF was treated only with 5mM DTT or 10mM iodoacetamide under the same conditions as above. The materials thus obtained were dialysed with buffer to remove chemical reagents, and tested for their activity. The results shown in Table 2 clearly demonstrated that the activity of the purified KLH-TsF was abrogated by the reduction and alkylation. In contrast, the treatment with DTT or iodoacetamide by itself did not affect the TsF activity. The data strongly suggest that the interchain S-S bonds are present between the antigen-binding and the I-J encoded polypeptide chains of the secreted TsF, and that the dissociated two chains successfully reconstitute the TsF activity.

As the experiments did not show the fact that the suppressor activity obtained by the reduced TsF was due to the recombination of the antigen-binding and the I-J encoded polypeptide chains, the following experiments were designed to test this possibility. The reduced TsF was at first fractionated

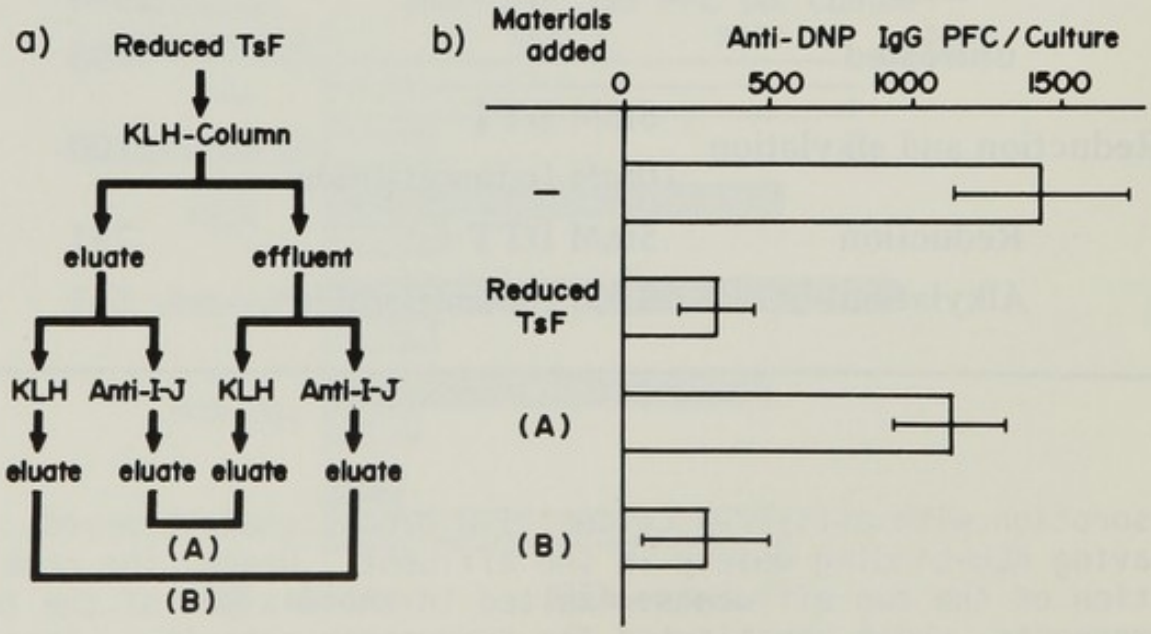

FIGURE 2. Successful reconstitution of suppressive activity with the mixture of antigen-binding and I-J encoded chains of the reduced TsF after fractionation with the KLH column.

by the KLH column equilibrated with Tris-HCl buffer containing 0.5mM DTT to separate the antigen-binding polypeptide chains in the acid eluate from the I-J products in the effluent. The effluent and the acid eluate from the KLH column were further applied to the KLH and anti-I-J^b columns. The materials bound to the columns were eluted and mixed to make two groups (A and B) as indicated in Fig.2a. The mixture A or B was tested for its activity in the *in vitro* secondary response. As shown in Fig.2b, group B, which theoretically consists of the combination of the antigen-binding and I-J encoded chains, reconstituted strong suppressive activity, while no significant activity was observed by group A(control). From these results, it is clear that the antigen-binding and the I-J encoded polypeptide chains of the secreted TsF are cleaved by the reduction of the interchain S-S bonds and are easy to reassociate to compose the active TsF molecules, and that the association of the two chains is essential for the expression of the TsF function.

A T Cell Hybridoma with Acceptor for the KLH-TsF.

Our previous studies(7) have shown that the suppression of antibody response involves the interactions of several subsets of suppressor T cells bearing I-J subregion gene products on their cell surface. In particular, KLH-TsF derived from $Ly2^+3^+$, $I\text{-}J^+$ Ts acts on the acceptor T cells expressing I-J determinants and $Ly1^+2^+3^+$ phenotypes that stimulate another population of I-J positive Ts to develop into an actual effector cell type. Similar observations were demonstrated in the GAT or Azobenzenarsonate(ABA) specific suppressor systems reported by Germain et al.(9), and Sy et al.(8).

In order to characterize the mode of action of the antigen-specific TsF and the biological role of the acceptor T cells, a T cell hybridoma having acceptor for the KLH-TsF was used. Among I-J^b positive T cell hybridomas which has been established by the fusion of BW5147 and enriched C57BL/6 Ts, there are several clones with different functional activities. The particular clone 34S-28l expresses I-J determinants on the cell surface but does not have any functional activity per se. However, this hybridoma was found to possess the acceptor for the KLH-TsF(TsF_1) derived from the clone 34S-18 as described in the previous section, since the 34S-18 derived TsF was completely absorbed with the clone 34S-28l but not with other I-J positive T cell hybridomas 34S-18 or 34S-704. The acceptor cell line was found to be killed by anti-Ly1.2 or anti-Ly2.2 and rabbit complement. However, since parental cells(BW5147) express Ly1.2 phenotypes which are identical to those of C57BL/6 suppressor T cells, it could not be determined whether the Ly1.2 antigens on the hybridoma derive from C57BL/6 or the

parental cell line(BW5147).

Production and Characterization of New Suppressor Factor(TsF_2) Produced by the Acceptor T Cell Hybridoma(34S-281) after Stimulation with KLH-TsF(TsF_1).

The acceptor T cell hybridoma(34S-281) was incubated on ice with KLH-TsF(TsF_1) for 1hr in the absence of the antigen KLH. The cells were extensively washed and cultured in a fresh medium. For the detection of the activity of the activated acceptor hybrids, the culture supernatant or the cell-free extract from the cells was collected at various time intervals after the stimulation, and tested for its activity in the *in vitro* secondary response. As shown in Fig.3, the acceptor hybridoma started to produce new suppressor factor, referred as TsF_2, at 8hr after the stimulation with KLH-TsF, while no suppressor activity was obtained by the supernatant nor the cell-free extracts from the unstimulated 34S-281. The production of TsF_2 continued up to 17 days or more after the stimulation. These results suggest that the cloned hybrid cells with acceptor site start to produce another TsF after they were activated by KLH-TsF. This implies that the KLH-TsF(TsF_1) per se has the capacity to stimulate the acceptor T cells in the resting state.

The TsF_2 produced by the activated acceptor hybridoma was found to be KLH-specific and to have the restriction specificity, since the TsF_2 suppressed the antibody response against DNP-KLH but not DNP-EA. Furthermore, the TsF_2 suppressed the

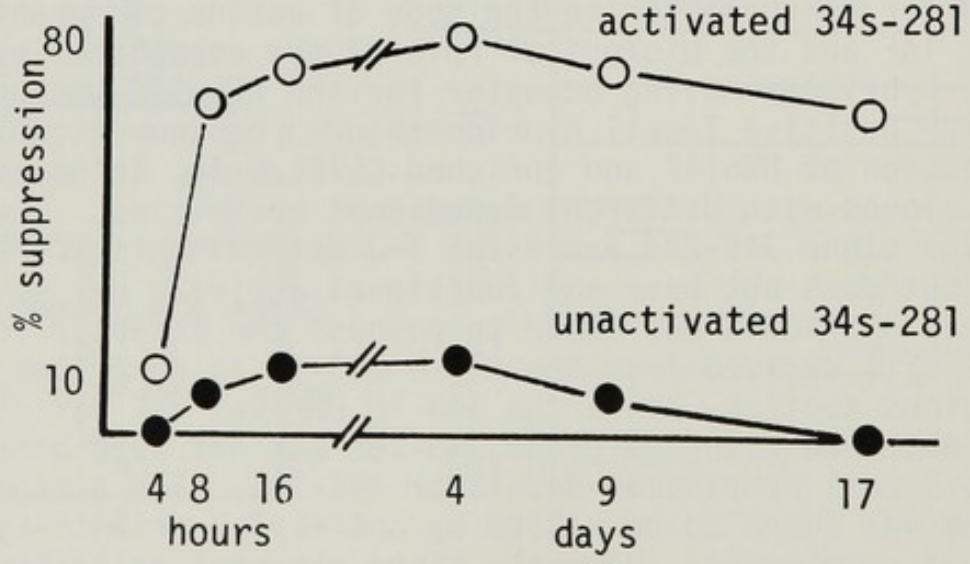

FIGURE 3. Continuous production of TsF_2 from the acceptor T cell hybridoma(34S-281) after stimulation with the KLH-TsF(TsF_1)

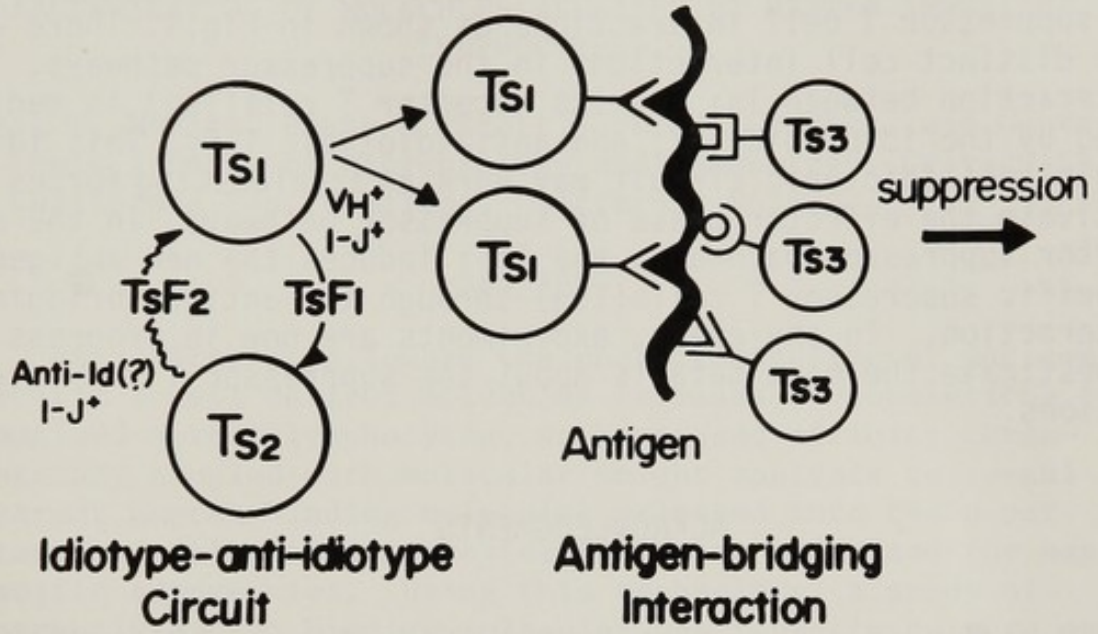

FIGURE 4. Possible model of suppressor T cell interaction

response of the syngeneic or the semisyngeneic but not the allogeneic spleen cells. From these results, no functional difference between TsF_1 and TsF_2 was observed. However, the immunochemical analyses of TsF_2 by using the immunoadsorbent columns of antibodies and antigens have shown that the TsF_2 possesses the I-J determinants but does not have the KLH-binding capacity. The questions which arise at this point are how the monoclonal TsF_2 exerts the KLH-specific suppressor activity despite its inability to bind to the KLH-column and how the TsF_2 suppresses the whole anti-DNP responses in the presence of DNP-KLH. So far no definitive evidence has been obtained to provide a satisfactory answer. However, in our recent experiments, the activity of the TsF_2 was absorbed with the TsF_1 producer cell line (34S-18). It is, therefore, most likely that the TsF_2 possesses the anti-idiotypic receptor for the antigen-binding structure on the TsF_1. The anti-idiotypic TsF_2 may be able to stimulate and proliferate the TsF_1 producer cells having the proper idiotypic receptor, in which idiotypic-anti-idiotypic circuit should be involved.

Another important observation concerning the mechamism of suppression by the factor is that the monoclonal TsF_1 could induce the hapten specific suppressor T cells as reported by Herzenberg et al.(12). Thus, the monoclonal TsF_1 could induce another subset of suppressor T cells(Ts_3) which recognize the different epitopes(including DNP or other KLH determinants) on the DNP-coupled KLH molecules through the antigen-bridging interaction. The Ts_3 should act as the effector suppressor T cells.

From these results, we would like to postulate the possible model of suppressor T cell interactions as shown in Fig.4. There are two distinct cell interactions in the suppressor pathways. The interaction between Ts_1 and its acceptor T cell(Ts_2) is mediated by the idiotypic TsF_1 and anti-idiotypic TsF_2. This idiotypic-anti-idiotypic circuit may work as accelerating forces to activate the effector phase of suppressor pathways. In the effector suppressor pathways, the TsF_1 induces the new antigen-specific suppressor T cells(Ts_3) through the antigen-bridging interaction. In any event, experiments are now in progress to investigate the more details about the suppressor T cell interactions.

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REFERENCES

1. Tada, T. and K.Okumura(1979). Advances in Immunology. Vol.28. P.1.
2. Tada, T., M.Taniguchi and C.S.David(1976). J.Exp.Med. 144:713.
3. Theze, J., C.Waltenbaugh, R.N.Germain and B.Benacerraf (1977). Eur.J.Immunol. 7:705.
4. Araneo, B.A. and J.A.Kapp(1980). J.Immunol. 125:118.
5. Taniguchi, M., T.Saito and T.Tada(1979). Nature. 278:555.
6. Taniguchi, M., I.Takei and T.Tada(1980). Nature. 283:227.
7. Taniguchi, M. and T.Tokuhisa(1980). J.Exp.Med. 151:517.
8. Sy, M-S, M.H.Dietz, R.N.Germain, B.Benacerraf and M.I. Greene(1980). J.Exp.Med. 151:1183.
9. Germain, R.N., S.Ju, T.J.Kipps, B.Benacerraf and M.E.Dorf (1979). J.Exp.Med. 149:613.
10. Eshhar, Z., R.N.Apte, I.Lowy, Y.Ben-Nerah, D.Givol and E.Mozes(1980). Nature. 286:270.
11. Taniguchi, M., T.Tada and T.Tokuhisa(1976). J.Exp.Med. 144:20.
12. Herzenberg, L.A., T.Tokuhisa and L.A.Herzenberg(1981). in this volume.

CHARACTERIZATION OF MOLECULES RELEASED BY HAPTEN IMMUNIZED LYMPHOCYTES[1]

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ABSTRACT

As a first step toward the characterization of antigen-specific T cell derived molecules released by populations of immunized normal lymphocytes, we have used affinity chromatography coupled with molecular weight analysis to reveal apparent hapten binding molecules released into the supernatants of cultured lymphocytes from mice immunized for hapten specific suppression. Using this technology, a group of apparently hapten binding molecules was found in culture supernatants of lymphoid cells from animals suppressed for either the azobenzenearsonate (ABA) or the dinitrophenyl (DNP) haptens. The dominant species had apparent molecular weights of 92kd, 68-72kd, 42kd, and 14kd after reduction and alkylation and SDS-PAGE. The culture supernatants were invariably contaminated with hapten specific immunoglobulin (Ig) if spleen cells or mesenteric lymph node cells were used as sources of cells. In contrast, no Ig was found in culture supernatants of superficial lymph node cells. The relative Ig content of the cell culture supernatants were reflected by their relative abilities to neutralize hapten conjugated bacteriophage, where spleen cell supernatants were more active than supernatants from a mixture of spleen cells plus lymph node cells. Supernatants from superficial lymph nodes cultured alone were ineffective at phage neutralization despite the presence of readily demonstrable hapten binding molecules. Since phage neutralization requires a bivalent interaction (1), it is postulated that the T cell derived hapten binding molecules are functionally monovalent in solution. While hapten binding molecules are readily revealed using the above methods, their precise biological functions remain to be worked out.

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INTRODUCTION

The structural nature of T cell factors and of antigen receptors still remains obscure after rather intensive study by numerous investigators. Recent reviews summarizing results in this field underscore the large gaps in our knowledge (2-5). As a first step toward elucidating the biochemical nature of antigen-specific T cell products, we have examined the electrophoretic profiles (in SDS) of affinity-purified hapten specific molecules released by cells from various lymphoid organs of mice suppressed for DTH (6-8). Antigen-specific suppressor factors have been demonstrated in suppressed animals by biological and ligand binding criteria (6-8). By utilizing affinity chromatography and the extremely sensitive protein detection techniques of fluorography, silver staining, and haptenated phage inhibition (HPI), it has been possible to detect molecules specifically released into culture supernatants in response to a secondary antigenic stimulus. These molecules have apparent antigen binding activity and are enriched when purified T cells derived from superficial lymph nodes are used to generate culture supernatants.

MATERIALS AND METHODS

DNP and TNP specific suppressors were generated as described by Moorhead (6) for DNP, and by Zembala and Asherson (7) for TNP. Briefly, single (day 0) or multiple (days 0 and 3) intravenous injections of dinitrobenzene sulfonic acid (DNBS, 15mg/mouse) or of picrylsulfonic acid (TNBS, 5mg/mouse) were followed on day 5 (single injection) or on day 6 (double injection) by skin painting with .5% (w/v) dinitrofluorobenzene (DNFB) or with 7% (w/v) picryl chloride. ABA specific suppressors were induced according to Bach et. al.(9). These animals were boosted by the subcutaneous application of 10mM ABA diazonium salt six days after priming for suppression. Sixteen hours after skin painting or boosting, spleen and lymph node suspensions were prepared and cultured for 48hr without additional antigen. The culture medium was serum free Click's EHAA supplemented with 10mM HEPES and 50ug/ml Gentamycin. Cultures were incubated in an atmosphere of 7%O_2, 10%CO_2, and 83%N_2. For biosynthetic labelling, the appropriate amino acid was omitted. After 48hr of culture, the supernatants were harvested by sequential centrifugations at 400g and 10,000g. Protease inhibitors were added to 1mM (TPCK,TLCK, PMSF) or to .5mM (pepstatin A). Seventy-five microliters of affinity matrix was added per ml of supernatant and the mixture was rotated overnight at 4C. The

affinity matrix was collected in a cotton plugged 1ml pipette tip and washed with 100 column volumes of PBS. One column volume of 50mM DNP-glycine, 1mM TNP-SAC (6-aminocaproic acid), or 10mM ABA-phenylacetic acid in PBS was added. After 2hr at 23C, the column was eluted by centrifugation. The samples were reduced and alkylated (10) immediately and analysed by SDS-PAGE using 5-20% gradient gels (11). Proteins were detected by silver staining (12) or by fluorography (13). HPI was performed by the decision technique (14) with DNP-SAC substituted MS-2 phage. HPI titers were read as the reciprocal of the dilution which inactivated 50% of the phage as compared to an uninhibited control.

RESULTS

Molecules isolated by affinity chromatography from cell culture supernatants: Molecules were isolated by affinity chromatography on DNP-BSA-Sepharose 4B from radiolabelled cell culture supernatants derived from DNP suppressed CBA mice. SDS slab gel analysis of reduced and alkylated hapten eluates is shown in fig. 1. Track A1 shows the absence of DNP specific material in lymph node culture supernatants derived from mice which had not been skin painted with DNFB 16hr earlier. By contrast, skin painting DNBS tolerized mice with DNFB 16hr prior to culture results in the appearance of several bands which bind specifically to the DNP column (track A2, fig.1). These molecules failed to specifically bind to BSA-Sepharose 4B columns. The dominant bands in track A2 are 92kd, 68-72kd, 42kd, and 14kd. The differences between DNBS-tolerized/DNFB-painted mice and mice tolerized with DNBS but not painted with DNFB (tracks A1 and A2, fig.1) are not due to poor labelling or to low amounts of starting material since both groups of mice had approximately the same amount of TCA-insoluble radioactivity in the supernatants and in the material applied to the affinity columns. The relatively large number of bands seen in track A2 calls into suspicion the specificity of each of the proteins which are eluted from the DNP column.

Initially, two approaches were taken to investigate the problem of specificity. First, T cells were enriched by panning on anti-Ig coated plates (15) to the point that the preparations contained less than 1% detectable B cells. This procedure resulted in an enrichment of the dominant 92kd, 72kd, 42kd, and 14kd bands. Curiously, there seemed to be a shift in the relative dominance of the bands in the 68-72kd range after panning. In unfractionated lymph node cell cultures the dominant band in this region ran at 68kd; in the panned preparation the dominant band ran at 72kd. The reason

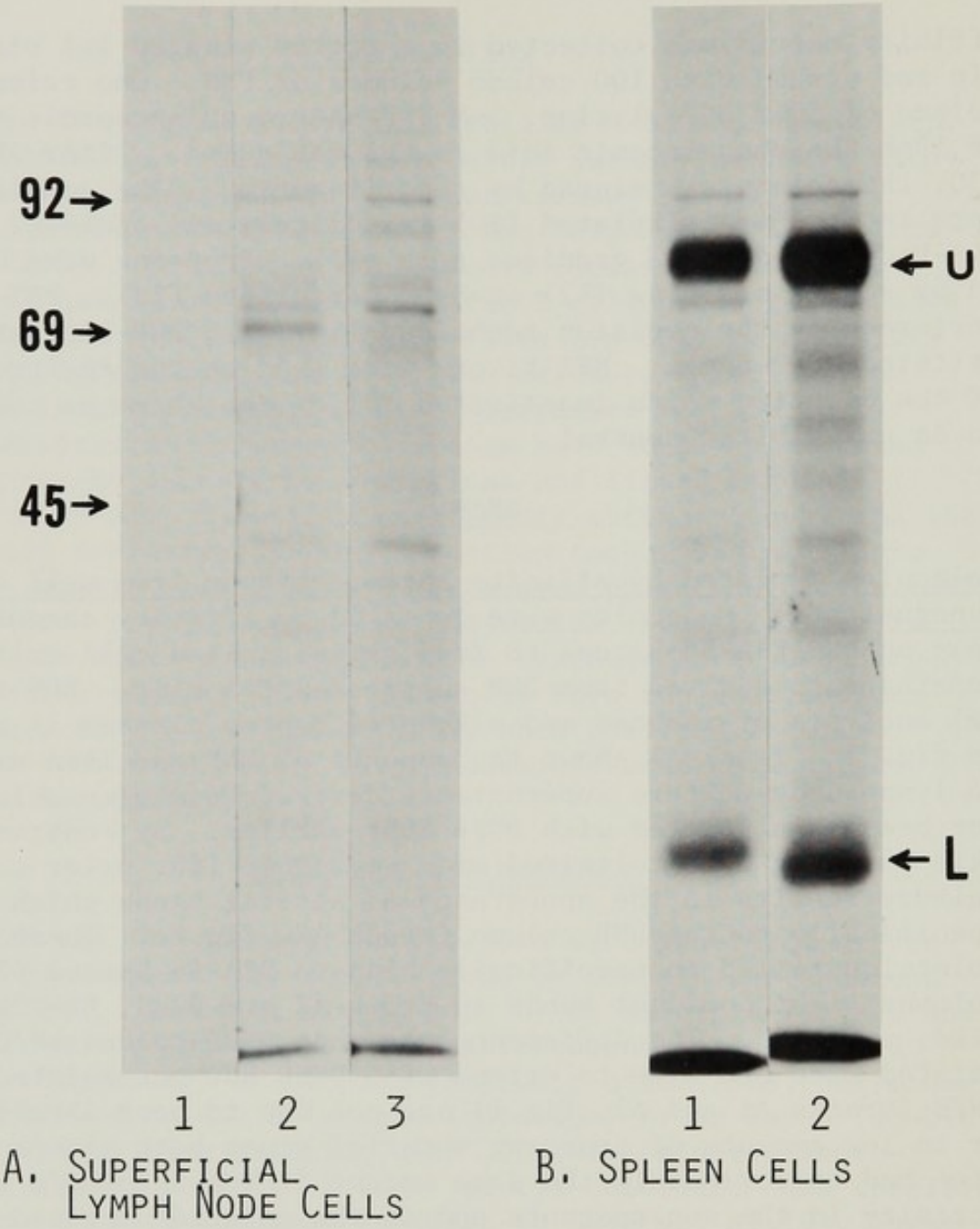

FIGURE 1. ^{35}S-methionine labelled culture supernatants from superficial lymph node cells (A) or from spleen cells (B) from mice tolerized with DNBS. The mice were either not skin painted (1) or skin painted (2&3) with DNFB prior to culture. Track A3 shows the DNP binding molecules from the supernatant of T cells enriched by panning on anti-Ig plates. All samples were reduced and alkylated after elution from a DNP-BSA-Sepharose 4B column by DNP-glycine. The fluorogram was exposed for 10 days.

for this shift is unclear. However, preliminary experiments suggest that these two molecules may be related since they have similar leucine/methionine ratios. Nevertheless, pulse chase experiments and structural studies are required for clarification of this point.

In the second approach, the question of specificity has been addressed by changing the type of affinity matrix as well as the hapten system. In an extensive series of experiments, similar banding patterns were seen for the TNP and ABA haptens when the appropriate suppression protocol was used. Also, changing the linkage between the hapten and the matrix from a protein linked hapten to an aminoethylcarbamyl-ethyl linker(for TNP and DNP) or to a tyraminyl linker for ABA had no effect on the protein pattern seen after fluorography. These results clearly point to the specificity of the affinity step for the hapten in question. However, it is still possible that not all of the observed bands are truly specific since some might be accounted for by molecules which have an affinity for other molecules in the supernatant which bind to the hapten. Hapten elution would bring all of these molecules off of the matrix, resulting in the apparent hapten specificity of all species. This question is being pursued by experiments using hapten blotting after electrophoretic separation.

Another point raised by the data in fig.1 is the effect of skin painting with DNFB on the pattern of DNP binding molecules from culture supernatants of superficial lymph node cells as compared to those from supernatants of spleen cells. Skin painting is essential for the efficient production of suppressor factor in culture (6,7). When spleen cells from DNBS tolerized (but not DNFB painted) mice were cultured, several of the dominant bands were found in addition to DNP specific IgM (track B1, fig.1). Supernatants from superficial lymph nodes coming from the same animals had no detectable DNP binding material (track A1, fig.1). Skin painting with DNFB caused some increase of the bands from spleen supernatants while causing the appearance of DNP binding molecules in supernatants from lymph node cells. Since all but the 69kd band are found in the spleen supernatants of unpainted mice, it is possible that the other bands play no role in the suppression of DTH. Another possibility is that the relevant molecules are made at a reduced rate in the spleens of DNBS tolerized animals and that the role of skin painting is to increase the rate of synthesis of these molecules in this organ while directing the traffic of the factor secreting cells to the superficial nodes which drain the sites of stimulation. It is interesting that the B cells secreting DNP-specific IgM are not similarly directed to the

superficial nodes in skin painted mice (fig.1, tracks A2 and B2).

Presence of hapten specific immunoglobulin in suppressor supernatants: An unexpected finding in the present investigation was the presence of hapten-specific Ig in the supernatants of spleen cells or in the supernatants of spleen cells admixed with lymph node cells. Hapten specific IgM has been found in supernatants from spleen cells tolerized with either ABA-modified spleen cells (A/J mice) or DNBS (CBA and Balb/c mice). Preliminary experiments in the TNP system suggest that less IgM is made after tolerization with TNBS than with either DNBS or ABA-modified spleen cells. DNP specific IgM is apparent in panel B of fig. 1. Its presence is also revealed by experiments using haptenated phage neutralization as the assay. When supernatants from a mixture of spleen cells and lymph node cells coming from tolerized and painted mice are passed over an polyvalent anti-mouse Ig column, the HPI titer was reduced by over 80% (Exp.1, table I). The presence of relatively large amounts of hapten specific IgM in at least two hapten-specific suppressor systems signals the need for caution in the generation of antisera against putative T cell factors, and in the design of experiments aimed toward the purification of these factors.

Are T cell factors functionally monovalent? Once it had been determined that suppressor supernatants derived from superficial lymph nodes did not contain detectable classical Ig, it was of interest to characterize these supernatants with haptenated phage neutralization. Surprisingly, minimal HPI activity was found in superficial lymph node supernatants when compared to supernatants derived from suppressed spleen cells (Exp. 2, table I). These same supernatants contained a minimum of 1-5ng/ml of any single DNP binding band as judged by the standardized silver staining of SDS-PAGE electropherograms. Since the HPI technique can detect MOPC-315 at the level of 12.5pg/ml (50% inhibition titer), it was expected that superficial lymph node supernatants should effectively neutralize the phage. In contrast, spleen cell supernatants had an HPI titer of 31.6 (Exp. 2, table I). Several explanations for the low levels of HPI in the superficial lymph node supernatants can be advanced. The presence of inhibitiory substances is ruled out by mixing experiments where mixtures of spleen cells and superficial lymph node cells gave the predicted titer of HPI (Exp 1, table I). Another explanation is that the T cell derived molecules are functionally monovalent in solution. Earlier studies using antibody and Fab fragments to neutralize phage suggested that monovalent ligands could neutralize. Indeed, it was for this

TABLE I

LEVELS OF DNP-PHAGE NEUTRALIZING ACTIVITY IN LYMPHOID CELL SUPERNATANTS

	Source[a]	HPI Titer/ml[b]	Treatment	%HPI Titer
Exp.1	Serum	350	-	-
	Spleen-Lymph Node	15.9	-	100
	" "	1.9	Absorbtion with anti-Ig	11.9
Exp.2	Spleen	31.6	-	100
	Superficial Lymph Node	.2	-	.6

a. CBA/J mice were immunized with DNBS-DNFB for suppression of DTH. Serum was collected on day 6 after painting with DNFB 16hr earlier.
b. HPI titer = reciprocal of the dilution giving 50% neutralization of control phage
c. Supernatants were passed over a rabbit anti-Ig column. The effluent was concentrated to the original volume.

reason that we originally chose the HPI assay as a method to study hapten binding by T cell derived molecules. However, recent studies by Cramer and Krawinkel (1) and ourselves suggest that Fab fragments are unable to neutralize phage. The earlier data reporting phage neutralizaion by Fab fragments can be explained by undigested bivalent antibody in the Fab preparations. This explanation is supported by our kinetic evidence on the reactivation of neutralized phage by free hapten. Fab fragments of MOPC-315 had the same reactivation rate constant as intact antibody, although there was approximately a 1000 fold reduction in HPI titer. These data suggest that the reduction in titer was not due to an increased dissociation rate, as proposed earlier (17), but

rather to the loss of multivalent antibody. The residual titer is easily explained by undigested MOPC-315.

The low levels of HPI seen in the supernatants coming from superficial lymph node cells are compatible with the hapten binding molecules being functionally monovalent. This is especially puzzling since none of the hapten binding molecules enter non-reducing SDS gels, suggesting that the native forms are larger than 250-300kd. This evidence, though admittedly indirect, suggests that hapten specific suppressor molecules are functionally monovalent in solution. If this is true, then a possible role for MHC restriction in factor activity could be to provide for a multivalent interaction necessary to stably focus a factor-antigen complex onto a cell surface.

DISCUSSION

In this report, we have presented data which suggest the presence of apparent hapten binding molecules in cell supernatants from animals immunized for hapten specific suppression. When considered in the light of possible chromatographic artifacts and the uncertainty about which molecules are responsible for biological activity, these data should be viewed cautiously. The points raised herein will be clarified only when we have characterized biologically active factors from either cloned T cell lines or from T cell hybridomas.

Despite these reservations, we have sufficient evidence to believe that some of the molecules are hapten specific and related to suppression. In summary: a) molecules of the same apparent molecular weight have been found in three haptenic systems (eg. ABA, DNP, and TNP); b) hapten columns specifically absorb at least 10 times the amount of radioactivity from supernatants as that absorbed by control columns, this radioactivity is specifically eluted by free hapten; c) there is an absolute requirement for skin painting to obtain hapten-binding molecules in superficial lymph node supernatants; d) partial T cell purification concomitantly enriches the hapten-binding molecules; and e) the hapten binding molecules can be distinguished from conventional Ig by both molecular weight and by serological criteria.

It is useful to compare the molecular weights found by other investigators for T cell factors with those reported here. Recently, Adorini *et. al.* (18) reported the synthesis of a 85-92kd molecule by a suppressor T cell lymphoma specific for hen egg white lysozyme. This molecule binds specifically to lysozyme columns and it is also bound by an anti-V_H immunosorbent. On the other hand it has been recently report-

ed that T cells have a glycophorin like 95kd molecule on their surface which contains sialic acid (19). It will be important in the future to compare these two molecules with our 92kd band by partial peptide mapping. Cone et. al. (20) isolated a TNP-specific suppressor factor using techniques similar to those described in this report. This factor degrades into two fragments of 45kd and 25kd. A similar molecule has been described by Cantor and Gershon (21) which is specific for sheep erythrocyte glycophorin. Taken together, these findings are remarkably similar to those reported in the present communication since a band of 68-72kd is the dominant species in supernatants derived from superficial lymph node cells. It is possible that the 42kd band and the trace 25kd band that we see are identical to those found by other investigators. It is perhaps not surprising that we find several bands on our electropherograms of affinity purified supernatants since we are dealing with a mixed population of cells; however, it is hoped that this analysis will be useful in identifying the biologically relevant molecules released by functional T cell lines and T cell hybridomas.

The role of skin painting with reactive hapten 16hr prior to harvesting cells for culture should be interpreted with regard to the effect of splenectomy on the ability of a mouse to make DNP-specific suppressor cells. Sy et.al.(16) showed that adult splenectomy abrogated the ability to generate suppressor cells in response to skin painting and that the spleen must be present for at least 3 days after tolerization with DNBS in order to develop suppressors in the peripheral lymph nodes. It should also be noted that antigen restimulation is important in the development of effector suppressor cells (Ts_2, see ref.(22)) and that soluble suppressor factors must be complexed with antigen in order to be active (23). Our data, when coupled with the findings cited above, prompt the following interpretation. Once an animal is tolerized with DNBS, pre-Ts_2 cells are generated in the spleen. These cells are the immediate precursors to the suppressor effector cell (Ts_2). Skin painting with DNFB stimulates pre-Ts_2 to become Ts_2 which then migrate to the peripheral lymph nodes. These cells may be more active with regard to the synthesis of DNP specific molecules than unstimulated cells. Skin painting also provides DNP which may complex with anti-DNP specific factors which are responsible for effector suppression. Since DNP is administered as the highly reactive DNFB, it is possible that the DNP must first react with a self component (eg.MHC) before it can form a functional complex with the anti-DNP specific suppressor factor. Clearly, the molecules in the

culture supernatant responsible for biological activity must be identified before this mechanism can be verified.

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REFERENCES

1. Cramer, M., and Krawinkel, U. (1980). In Regulatory T Lymphocytes (B.Pernis and H.Vogel, eds.), pp.39-53, Academic Press, Inc., New York.
2. Germain, R.N., and Benacerraf, B. (1980). Springer Semin. Immunopathol. 3, 93.
3. Taussig, M.J. (1980). Immunology 41, 759.
4. Altman, A., and Katz, D.H. (1980). J. Immunol. Methods 38,9.
5. Tada, T., and Okumura, K. (1979). Adv. Immunol. 28,1.
6. Moorhead, J.W. (1977). J. Immunol. 119,315.
7. Zembala, M., and Asherson, G.L. (1974). Eur. J. Immunol. 4, 799.
8. Zembala, M., Asherson, G.L., Mayhew, B., and Krejci, J. (1975). Nature 253, 72.
9. Bach, B.A., Sherman, L., Benacerraf, B., and Greene, M.I. (1978). J. Immunol. 121, 1460.
10. Lane, L.C. (1978). Anal. Biochem. 86,655.
11. Laemmli, U.K. (1970). Nature 227, 680.
12. Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980). Anal. Biochem. 105, 361.
13. Chamberlain, J.P. (1979). Anal. Biochem. 98,132.
14. Witte, O.N., and Slobin, L.I. (1972). J. Immunol. 108, 927.
15. Wysocki, L.J., and Sato, V.L. (1978). PNAS. 75, 2844.
16. Sy, M.S., Miller, S.D., Kowach, H.B., and Claman, H.N. (1977). J. Immunol. 119, 2095.
17. Hornick, C.L., and Karush, F. (1972). Immunochem. 9,325.
18. Adorini, L., Doria, G., and Ricciardi-Castagnoli, P. (1981). Lymphokine Reports 5, in press.
19. Brown, W.R.A., Barclay, A.N., Sunderland, C.A., and Williams, A.F. (1981). Nature 289, 456.
20. Cone, R.E., Murray, J.H., Rosenstein, R.W., Ptak, W., Iverson, G.M., and Gershon, R.K. (1981). J.Supramol. Struct. 13, Supplement 5, 6.
21. Cantor, H., and Gershon, R.K. (1980). In Regulatory T Lymphocytes (B. Pernis and H. Vogel, eds.), pp. 15-37, Academic Press, Inc., New York.

22. Germain, R.N., and Benacerraf, B. (1981). Scand. J. Immunol. 13, 1.
23. Waltenbaugh, C., and Lei, H.Y. (1981). J. Supramol. Struct. 13, Supplement 5, 46.

EVIDENCE FOR A T CELL CONSTANT REGION GENE FAMILY: CHARACTERIZATION OF CELL SURFACE ANTIGENS BY IMMUNOPRECIPITATION WITH ALLOANTISERA AND MONOCLONAL ANTIBODIES[1]

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INTRODUCTION

The relationship between antigen receptors on T and B cells has long been debated (1,2,3,4). Immunoglobulin on the surface of B cells clearly acts as an antigen recognition structure (5). T and B cell receptors closely resemble one another in that at least part of the polypeptide chain coding for antigenic specificity of each cell type carries idiotypic determinants (6). Although the V_H genes expressed on T and B cells overlap (7,8), the fine specificity of the repertoire may not be identical (9,10). It is well documented that the constant region markers of known immunoglobulin classes are not part of the T cell receptor (11,12). One must hypothesize that T and B cells share a common cellular origin in order to share the genes coding for V_H determinants, but that diversification occurs very early in the lineage of T-B differentiation so that each cell line (T or B) can then continue to modify and expand its V_H germ line gene repertoire along separate pathways. We have presented evidence from biological experiments that there are constant region genes on T cell receptors which are antigenically unique to the T cell and possible analogs for the B cell isotypes. This evidence was obtained by making antiserum in BALB/c animals which reacted with C.AL-20 T cells. Briefly, our antiserum reacts with mature Lyt2+ cells in Igh-1^d and Igh-1^e mice (13) and blocks the T cell receptor for the arsonate cross-reactive idiotype (14,15). This antiserum acts as a polyclonal trigger for suppressor T cells for many T dependent antigens (16,17), which are Igh-V restricted in their cell recognition require-

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ments (18). The gene(s) which codes for this determinant has been mapped two units downstream from Igh-1 on Chromosome 12 (19). We have suggested there is a complex of constant region genes in this region of the chromosome, tentatively designated IgT, which may code for a variety of functionally nonoverlapping T cell receptors on helpers, suppressors or cytotoxic T cells.

Evidence is presented here that two serologically separable polypeptides are coded for by genes mapping to this region. We have identified a 68,000 D antigen which corresponds to the specificity previously designated Tsu^d and a second antigen, $Tind^d$, of 62,000, 45,000 and 17,000 D which reacts with a monoclonal antibody.

METHODS

Antisera: The production and specificity of anti-Tsu^d has been previously described (13,16,19). Monoclonal anti-$Tind^d$ (clone $9IIIA_2$) was produced in our laboratory.[1] Briefly, a sensitive cell surface radioimmunoassay was used to detect secretion of anti-T cell antibodies reacting with either BALB/c or C.AL-20 Con A blasts. The positive clone, secreting $\gamma_1 k$ immunoglobulin, was subcloned by limiting dilution into microtiter plates with thymocyte feeder layers. Anti-isotype reagents were provided through the generosity of Drs. H. H. Wortis of Tufts U. and D. Parker, U. of Mass. and have been described (20).

Mice: C.AL-20 mice were developed by Dr. M. Potter at the NIH in Bethesda, MD and propagated in our breeding facilities at Tufts since 1978. CAL.B mice, $H\text{-}2^b$ congenic mice on a C.AL-20 background, were obtained as breeding pairs from M. Cohn at the Salk Institute, San Diego, CA (21). C.B.AL-1 mice were derived from a cross between two BALB/c allotype congenic strains, BAB/14 x C.AL-9 by R. Riblet, Institute for Cancer Research, Philadelphia, PA (22) and were defined to have a double recombination event, one between V_HDex and Igh-1 and a second between Tsu^d and Pre (see Figure 2 inset and map in Figure 3) (19). BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA).

Immunoprecipitation: Staphalococcal A immunosorbent (Staph A) was purchased from the Enzyme Center, Tufts New England Medical Center, Boston, MA. Sheep anti-mouse IgG, was incubated with Staph A and the washed complex was used to preabsorb all cell extracts. Cell extracts were made by in-

[1] *Spurll, G.M. and Owen, F.L. Evidence from monoclonal antibodies for a T cell constant region gene family, manuscript in preparation.*

ducing 48 hr. T cell blasts with Concanavalin A, labeling the cells with ^{35}S-methionine in methionine free RRMI, and extracting washed cells with a detergent cocktail plus 5mM PMSF. The extracts were ultracentrifuged at 35,000 g for 2 hours and preabsorbed 24 hours with Staph A-anti-IgG$_1$. Cell extracts were incubated with 5λ of anti-Tsu^d alloantiserum or 100λ of 9IIIA$_2$ tissue culture supernatant for 12 hours at 4°C. The Staph A-anti-IgG$_1$ was added for 2 hours at 4°C and the Staph A-anti-IgG$_1$-^{35}S-antigen complex was pelleted and washed 4 X in lysis buffer containing PMSF. The labeled complex was eluted in sample buffer and electrophoresed on DATD cross-linked (12%) polyacrylamide tube gels according to the method of Laemmli (23) as modified by Emerson and Cone (24). Tube gels were sliced and counted in Econofluor with 5% Protosol (New England Nuclear, Boston, MA). Molecular weight standards were a ^{14}C-labeled cocktail of phosphorylase B (92,500 D), bovine serum albumin (69,000 D), ovalbumin (46,000 D), carbonic anhydrase (30,000 D) and cytochrome C (12,000 D).

RESULTS

Immunoprecipitation of Tsu^d with alloantisera is strain specific

Alloantisera, shown in biological studies to have activity against the product of the Tsu^d gene, precipitated a single 68,000 MW polypeptide from ^{35}S-methionine labeled extracts of Con A induced T cell blasts. The precipitation of a single chain was strain specific: C.AL-20 T cell blasts incorporate label into an identifiable product, while BALB/c T cells do not (Figure 1). The extracts were treated with proteolytic inhibitors (PMSF) at the time of cell lysis and the precipitates were treated with 2-ME when eluted from Staph A. Each panel in Figure 1 represents the average of duplicate tube acrylamide gels and is corrected only for the counter background cpm. Although this pattern of precipitation was seen with one pool of antiserum, various other pools precipitated additional components with the 68,000 MW determinant (Figure 2). Characteristically a second pool of serum made at a later time precipitated four major polypeptides of 68,000 MW, 62,000 MW, 45,000 MW and 17,000 MW. All of these were strain specific in C.AL-20 and absent in BALB/c, and their relative ratio in cpm fluctuated widely in repeated labelings of cell preparations. The C.AL.B mouse, which has the H-2 type characteristic of the BAL.B (H-2^b) mouse, also carries these 4 determinants, suggesting either the H-2 complex does not encode these determinants, although determinants shared by H-2 components may be part of the nonalloanti-

genic framework, or additional noncovalently bonded components may bear H-2 encoded antigens.

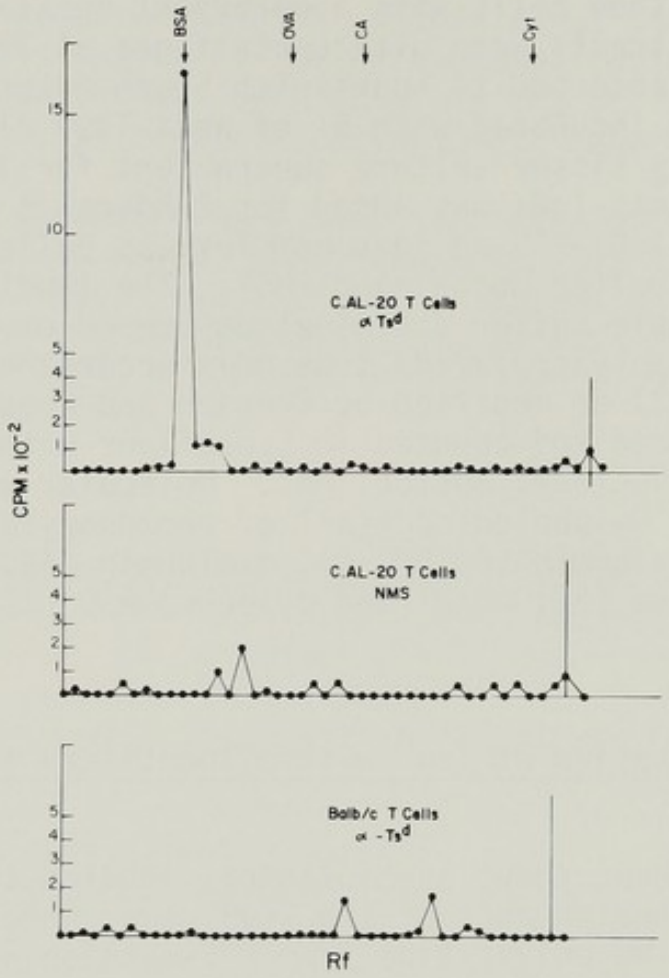

FIGURE 1. Immunoprecipitation of Tsu^d with alloantiserum.

A monoclonal (γ_1k) antibody was selected from mice immunized with a protocol known to produce anti-Tsu^d. This antibody (9IIIA$_2$) precipitated 3 of the 4 major ^{35}S-methionine labeled components detected with the second preparation of anti-Tsu^d. The 62,000 MW, 45,000 MW and 17,000 MW components are precipitated in C.AL-20 and C.AL-B but not BALB/c animals. The two anti-Tsu^d sera which precipitate only 68,000 D polypeptides or 68,000 D and 62,000, 45,000 and 17,000 suggest that there are a minimum of two specificities in some of our anti-Tsu^d serum. The 9IIIA$_2$ monoclonal confirms this finding. and suggests the 62,000, 45,000 and 17,000 pieces are antigenically related to one another. The specificity detected by 9IIIA$_2$ has been designated anti-Tind^d based upon the biology of the cell which expresses this marker (data not shown).

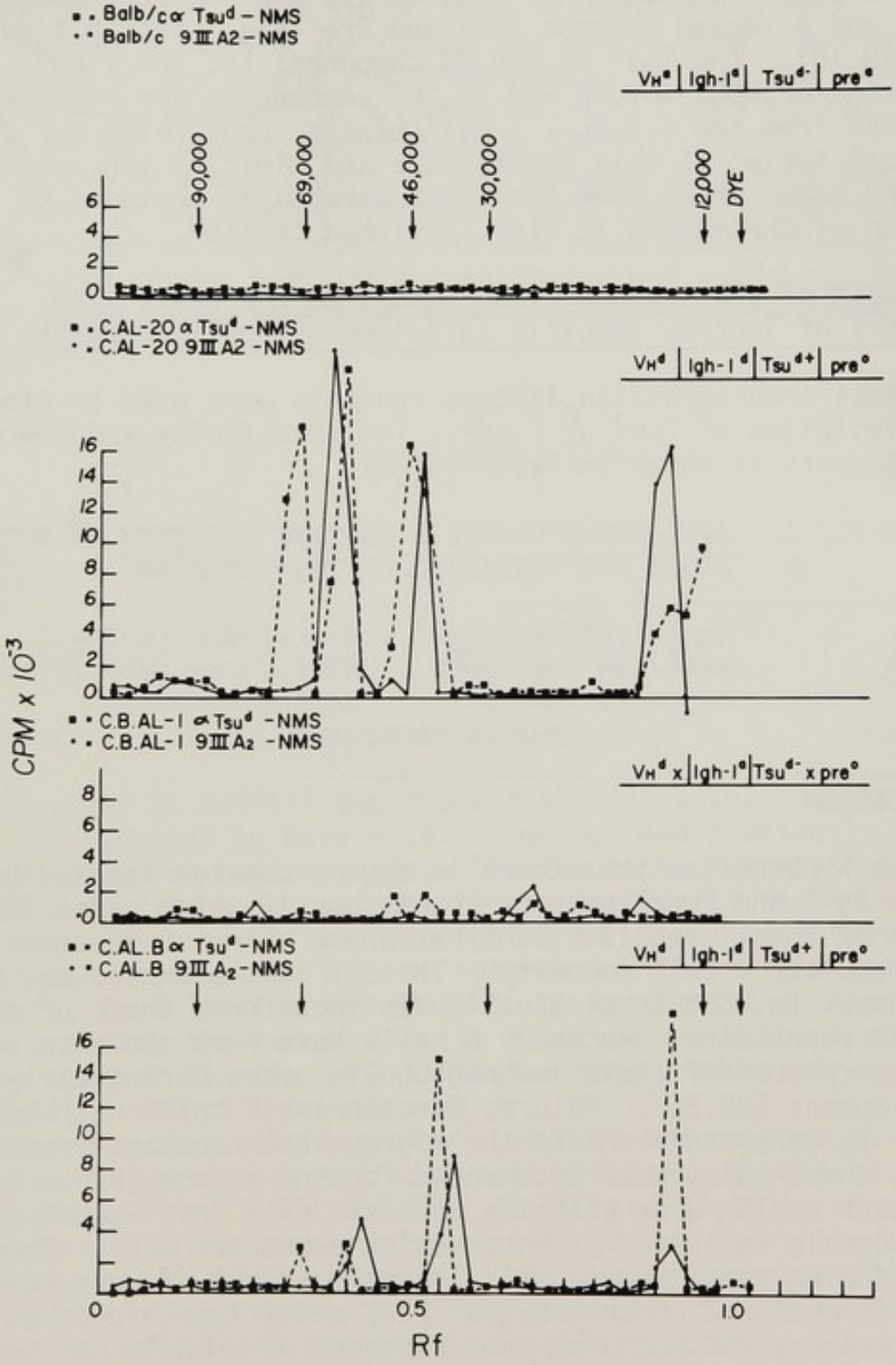

FIGURE 2. Strain specific precipitation of Tind^d with monoclonal antibody and alloantisera.

Tsu^d and Tind^d are both encoded by linked genes in the proposed IgT region of Chromosome 12

A recombinant mouse (C.B.AL-1) was defined to have two

recombination events on Chromosome 12; one between V_HDex and Ig-1 and a second between Tsu^d and Pre (see inset in figure 2) (19,18). The Tsu^d 68,000 MW component and the $Tind^d$ ($9IIIA_2$) 62,000, 45,000 and 17,000 components are not precipitated from the C.B.AL-1 T cell blasts, confirming our biological typing of this strain (19) and limiting the position of the genes which code for these structural proteins to a region of Chromosome 12 closely linked to Tsu^d.

Products of the Tsu^d and $Tind^d$ genes do not react with a panel of isotype specific anti-immunoglobulin reagents

Anti-immunoglobulin isotype reagents were used to block precipitation of Tsu^d or $Tind^d$. The specificity and source of reagents is shown in Table 1 (20).

TABLE 1. ANTI-IMMUNOGLOBULIN ISOTYPE REAGENTS DO NOT BLOCK PRECIPITATION OF Tsu^d OR $Tind^d$

Anti-IgD (MOPC1017)	*Anti-IgG2a (HOPC-1)*
Anti-IgM (MOPC104E)	*Anti-IgG2b (MPC-11)*
Anti-IgG3 (J606)	*Anti-k (MPC-11)*
Anti-λ (MOPC104E)	

DISCUSSION

We hypothesize that there is a gene complex located between Ig-1 and Prealbumin on Chromosome 12 which codes for a group of T cell specific isotypes whose effector functions may regulate T cell immunity. These T cell products may be analogous to, but serologically distinct from, those of the B cell repertoire. Recently B cells have been shown to rearrange pieces of their immunoglobulin genes during early development (25,26). Mature, functioning B cells rearrange their V_H genes adjacent to the appropriate constant region gene, a mechanism well documented for the α gene (27). Each constant region gene actually consists of a "mini-gene" for each domain separated by intervening sequences (28). Recently the finding that thymus cells (largely a pool of very early undifferentiated prothymocytes) (29) and T lymphoid lines (30) express RNA encoding some segments of μ chain has been used as an argument that T cells utilize IgM as antigen receptors. Alternative interpretations could be that a) the μ gene is nonproductively rearranged in these cells (12); b) the cells used for these studies represent intermediate steps in the differentiation pathway of T and B cells; or c) translocation of V_H genes in T cell precursors takes place in an analogous way as do the B cell gene segments, but that the

V_H gene is translocated twice; once adjacent to μ, where it incorporates a segment (or domain and one or more intervening sequences) of the μ gene and translocates it a second time along with V_H adjacent to a characteristic T cell constant region marker further downstream for Igh-1.

Since only a small part of the constant region of the μ gene would be enclosed within the T cell constant region gene, the conformation and subsequently the resultant antigenic specificity of the translated protein product would not be recognizable as μ while hybridization studies might suggest it is μ.

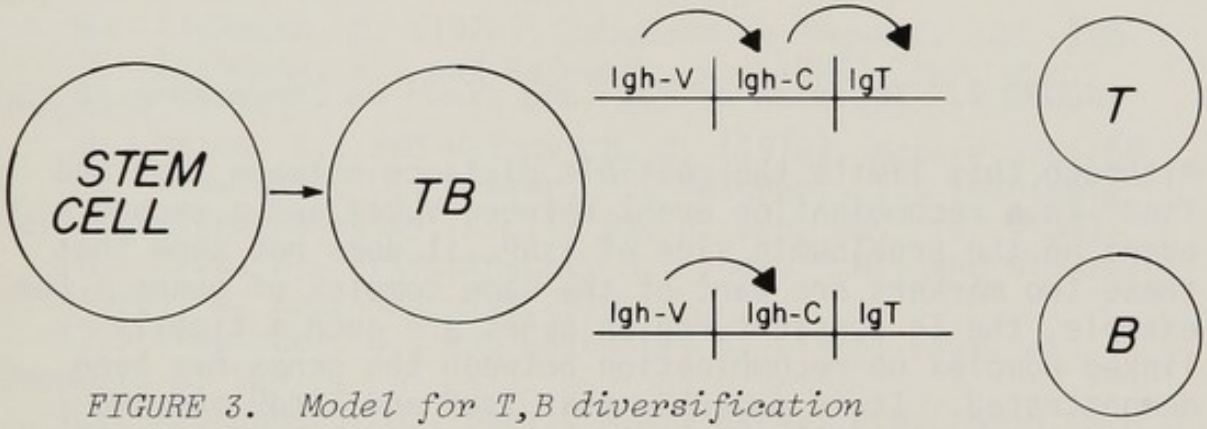

FIGURE 3. Model for T,B diversification

In order to fulfill the model outlined above, T cells would be required to have a set of unique and previously undefined constant region genes. The immunoprecipitation studies presented here demonstrate two antigenically distinct molecules which are likely candidates for T cell constant region gene products. They do not react with anti-isotype reagents, Tsu^d is clearly separable by recombination from the genes encoding the immunoglobulin allotypes, and the precipitation studies clearly show these two gene products are antigenically distinct from one another.

The molecular weight of Tsu^d (68,000) corresponds to that reported for antigen specific T cell receptors by others (31, 32), and that for $Tind^d$ (62,000, 45,000 and 17,000) corresponds to the marker reported by Pacificio and Capra for a secreted molecule (33). However, we have no direct evidence that either of our molecules binds to antigen. The finding that anti-Tsu^d blocks binding to T cell rosettes to the arsonate cross-reactive idiotype (14) is indirect and could indicate that this molecule is stearically related to the T cell antigen receptor at the cell surface in analogy with the finding that anti-Lyt2 blocks cytotoxic T cells (34). Experiments are in progress to further clarify this point.

The experiments which map both Tsu^d and $Tind^d$ to the same region of Chromosome 12 are based on the use of recombinant

congenic mice (C.B.AL-1) (see map in figure 4).

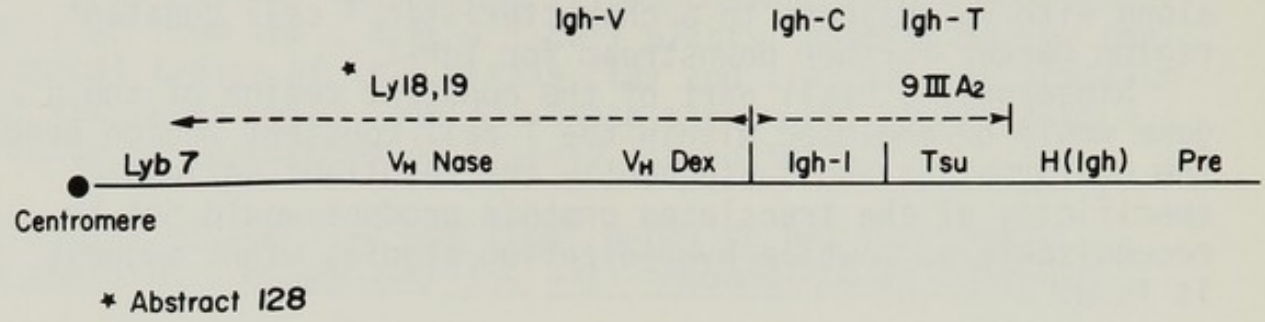

FIGURE 4. Position of Tsu^d and Tind^d on Chromosome 12

Although this limits the possible distance between Tsu^d and Tind^d to a recombination event between V_HDex and a second event on the prealbumin side of Tsu^d, it does not show that these two markers are part of the same complex of genes. For example, the Ig constant region genes are such a tightly linked complex no recombination between the genes has been demonstrated. It is *possible* that Tsu^d and Tind^d are two members of such a T cell complex, but estimates of recombination frequency have not been attempted. There are other serologically detectable markers linked to Ig-1. The position of a tentatively designated Ly18 and Ly19 have been indicated (35,36).[2] These cell cycle dependent antigens have not been shown to be related to immunoglobulins or to T cell receptors. It is still possible that Tind^d may be an unrelated antigen which simply has a close genetic proximity to Tsu^d on Chromosome 12.

The possibility that Tsu^d and Tind^d may be membrane and secreted forms of the same molecule was considered. This seems improbable since some preparations of antisera recognize anti-Tsu^d but not Tind^d while 9IIIA$_2$ recognizes Tind^d but not Tsu^d. It is probable that the 62,000, 45,000 and 17,000 MW components of Tind^d are antigenically related. If Tind^d is a T cell receptor, degradation of the native molecule of 62,000 to two smaller and possibly nonfunctional molecules could be an adaptive advantage.

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[2] *Finnegan, A. and Owen, F.L. Manuscript in preparation.*

REFERENCES

1. Marchalonis, J.J., Atwell, J.L., and Cone, R.E. (1972). *Nature New Biol. 235*, 240.
2. Vitetta, E.S., and Uhr, J.W. (1973). *Fed. Proc. (Symp.) 32*, 35.
3. Merrill, J.E., and Ashman, R.F. (1979). *J. Immunol. 123*, 434.
4. Putnam, D., Clagett, J., and Storb, U. (1980). *J. Immunol. 124*, 902.
5. Roelants, G., Forni, L., and Pernis, B. (1973). *J. Exp. Med. 137*, 1060.
6. Eichmann, K. (1978). *Advances in Immunol. 126*, 195.
7. Eichmann, K., and Rajewsky, K. (1975). *Eur. J. Immunol. 5*, 661.
8. Mozes, E., and Haimovich, J. (1979). *Nature 278*, 56.
9. Krawinkel, U., Cramer, M., Melchers, I., Imanishi-Kari, T., and Rajewsky, K. (1978). *J. Exp. Med. 147*, 1341.
10. Binz, H., Frischknecht, H., Shen, F.W., and Wigzell, H. (1979). *J. Exp. Med. 149*, 910.
11. Krawinkel, U., and Rajewsky, K. (1976). *Eur. J. Immunol. 6*, 529.
12. Kronenberg, M., Davis, M.M., Early, P.W., Hood, L.E., and Watson, J.D. (1981). *J. Exp. Med. 152*, 1745.
13. Owen, F.L., Finnegan, A., Gates, E.R., and Gottlieb, P.D. (1979). *Eur. J. Immunol. 9*, 948.
14. Owen, F.L., Ju, S.-T., and Nisonoff, A. (1978). *P.N.A.S. 74*, 2084.
15. Kuettner, M.G., Wang, A.L., and Nisonoff, A. (1972). *J. Exp. Med. 135*, 579.
16. Owen, F.L. (1980). *J. Immunol. 124*, 1411.
17. Owen, F.L. (1980). *J. Supra. Mol. Struct. 14*, 175.
18. Owen, F.L., Riblet, R., and Gottlieb, P.D. Manuscript Submitted.
19. Owen, F.L., Riblet, R., and Taylor, B. (1981). *J. Exp. Med. 153*, 0000.
20. Spurll, G.M., and Owen, F.L. (1980). *J. Immunol. Methods 35*, 335.
21. Geckler, W., Blomberg, B., DePreval, C., and Cohn, M. (1976). *Cold Spring Harbor Symposium on Quantitative Biology 51*, 748.
22. Wiegert, M., and Riblet, R. (1978) *Springer Seminars in Immunopathology 1*, 133.
23. Laemmli, U.K. (1970). *Nature (Lond.) 227*, 680.
24. Emerson, S.G., and Cone, R.E. (1979). *J. Immunol. 105*, 1116.
25. Tonegawa, S., Maxam, A.M., Tizard, R., Bernard, O., and Gilbert, W. (1978). *Proc. Natl. Acad. Sci. USA, 75*, 1485.

26. Gilbert, W. (1978). *Nature 277*, 501.
27. Early, P.W., Davis, M.M., Kaback, D.B., Davidson, N., and Hood, L. (1979). *Proc. Natl. Acad. Sci. USA, 76*, 857.
28. Cory, S., and Adams, J.M. (1980). *Cell 19*, 37.
29. Storb, U., Hager, D., Putnam, L., Buck, L., Farin, F., and Clagett, J. (1976). *P.N.A.S. 73*, 2467.
30. Kemp, D.J., Harris, A.W., Cory, S., and Adams, J.M. (1980). *P.N.A.S. 77*, 2876.
31. Rubin, B., Hertel-Wulff, B., and Kimura, A. (1979). *J. Exp. Med. 150*, 307.
32. Binz, H., Frischknecht, H., Mercolli, C., Dunst, S., and Wigzell, H. (1979). *J. Exp. Med. 150*, 1084.
33. Pacificio, A., and Capra, D.A. (1980). *J. Exp. Med. 152*, 1289.
34. Shinohara, N., and Sachs, D.H. (1979). *J. Exp. Med. 150*, 432.
35. Finnegan, A., and Owen, F.L. (1980). *Fed. Proc. 39*, 4877 (Abstract).
36. Finnegan, A., and Owen, F.L. (1981). *J. Supr. Mol. Struct., Supplement 4*, 129 (Abstract).

T CELL V_H VERSUS B CELL V_H [1]

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Using isolated, hapten-specific T cell receptor material we showed in earlier studies that the variable region of immunoglobulin heavy chains is shared between B- and T-cell receptors as analysed for three genetic V_H-markers. We here report on an apparently "allotypic" reaction of T cell receptor material with an antiserum against immunoglobulin V_H as well as on the analysis of such receptors with monoclonal anti-idiotopic reagents. We thus have accumulated serological and/or genetical evidences for the presence of presumably all segments - hypervariable and framework - of the B cell V_H-region in receptors derived from a functionally as yet undefined T cell population. Idiotypic analysis of primary and hyperimmune receptor materials demonstrated, however, that as suggested earlier the rules of V_H expression are quite different in B and T lymphocytes.

INTRODUCTION

The first evidence that the variable regions of immunoglobulin (Ig) heavy chains (V_H) are present on functionally defined T cell receptors was obtained in 1975 (1). Since then numerous reports confirmed and extended this original finding (2-4). Despite enormous efforts, however, no proteinchemical or comparable structural information became available to proof or disprove this point. Therefore we still have to define the T cell V_H in serological terms.

Since a serological crossreaction between a V_H-region expressed on serum immunoglobulin and a T cell product is by itself not a solid evidence for the identity or even similari-

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TABLE 1

RABBIT ANTI-V_H ANTIBODIES DO NOT REACT WITH AKR-DERIVED T-CELL RECEPTOR MATERIAL

	% absorption by[a]		
	Rabbit anti-V_H	Rabbit anti-V_L	Rabbit anti-NP[b]
A. (C57BL/6 x CBA)F_2, Ig-1^{bb} T cell receptor fraction[b]	78	10	86
B. SJL anti-NP T cell receptor fraction	74	5	78
C. BALB/c anti-NP T cell receptor fraction	76	24	n.d.[c]
D. AKR anti-NP T cell receptor fraction	4	19	n.d.
E. C57BL/6 anti-NP antibodies	6	56	72
F. AKR anti-NP antiserum	0	n.d.	n.d.
G. MOPC 315 protein[d]	64	69	0

a) Absorption is determined by HPI titers before and after incubation with insolubilized antisera or antibodies; values are corrected for unspecific absorption on normal rabbit serum immunosorbent (0-8% unspecific absorption).

b) Total receptor material is first absorbed on polyspecific anti-Ig immunosorbent to remove the B cell receptors. The supernatant of the absorption represents the T cell receptor fraction (7), since - as verified in every single experiment - further absorption of this material on fresh anti-Ig does not result in detectable binding. This supernatant is then treated as stated in the table.

c) n.d. = not determined in this set of experiments

d) MOPC 315 was titrated with DNP-cap T4 bacteriophages.

ty of the two molecules in question such serological analyses have to be carried out with a couple of independent reagents and - most importantly - the V_H-markers analysed must be followed by genetic analysis on both B cell V_H and T cell V_H, i.e., an IgH-allotype linkage must be established.

Our approach to the T cell receptor analysis is the isolation and subsequent serological characterization of hapten-specific T cell receptor material (5-7). Up to now we have three genetic V_H-markers on such isolated materials: 1) Receptor material isolated from sensitized rabbit T cells carries a allotypes (8). 2) 4-Hydroxy-3-nitro-phenylacetyl (NP)-specific T cell receptors obtained from C57BL/6 spleens carry a fine specificity marker (6) as well as 3) an idiotypic marker, called NP^b, defined by highly specific but heterogenous guinea pig and rabbit anti-idiotypic reagents (6, 9).

The idiotopic analysis of the NP^b idiotype was recently made possible by the development of a set of mouse (10, 11) or rat monoclonal anti-idio typic antibodies each reacting with one of the probably many idiotopes comprising the NP^b idiotype. We thus have the possibility to study the question of the extent of overlap between B cell V_H determinants and T cell V_H determinants with such highly specific serological tools which, with all likelihood, detect V_H-idiotopes (12). In addition we now studied a rabbit anti-mouse-V_H antiserum which we used in our previous work (13) with AKR-derived T cell material and seem to get additional genetic evidence for the fact that B cell V_H and T cell V_H are certainly very similar if not identical.

As suggested by the work with primary T cell receptor material and hyperimmune serum antibodies (9) the idiotypic analysis of receptor material obtained from hyperimmune animals was performed to verify our previous conclusion that the regulation of V_H expression is more conservative in T cells then in B cells and their products.

MATERIALS AND METHODS

Receptor preparation and analysis. The isolation of B- and T-cell receptor material on hapten-coupled nylon discs, the immunoabsorption techniques as well as the titrations of hapten-specific materials with hapten-coupled T4 bacteriophages (HPI) have been described previously (5-9, 13).

Immunizations. Primary immunization (1^o): C57BL/6 mice were immunized with 100 µg NP-coupled chicken gammaglobulin (CG)

TABLE 2

IDIOTOPE EXPRESSION IN ISOLATED NP-SPECIFIC T CELL RECEPTOR MATERIAL

	% absorption by [a]			
	Ac106[b] (idiotope 2)	Ac146[b] (idiotope 1)	R207[c]	Rabbit anti-NP[b]
A. C57BL/6 anti-NP T cell receptor fraction[d]	27	9	0	75
B. C57BL/6 anti-NP T cell receptor fraction	32	16	0	66
C. SJL anti-NP T cell receptor fraction	63	12	0	72
D. C57BL/6 anti-NP antibodies	38	9	10	83
E. SJL anti-NP antibodies	0	0	0	0
F. B1-8 protein	99	99	48	n.d.[e]

a) See Table 1, footnote a)
b) Monoclonal CBA anti-B1-8 idiotopic antibodies, ascites fluids
c) Monoclonal rat anti-B1-8 idiotopic antibody , culture supernatant
d) See Table 1, footnote b)
e) Not determined in this set of experiments

as described (14) and receptor material was isolated 4-8 weeks later. Secondary immunization (2^o): Mice were boosted with 100 µg NP-CG in an identical way 8-10 weeks after priming and receptor material was isolated 3 weeks later. Tertiary immunization (3^o): Animals were boosted with 50 µg ^{NP}-CG - omitting the bortedella pertussis - 6 weeks after 2^o injection. Receptor material was isolated 3 weeks later.

Antisera and antibodies. Purified rabbit anti-mouse V_H antibodies (15) and rabbit anti-mouse light chain variable region (V_L) antiserum (16) were gifts of Drs. Ben-Neriah and D. Givol and are those used in our previous work (13). The absorbed rabbit anti-NP^b idiotypic antiserum was a gift of Dr. T. Imanishi-Kari. The preparation and characterization of mouse and rat monoclonal anti-idiotopic antibodies Ac106, Ac146 (10) and R207 (Imanishi-Kari, T., and Grützmann,R. manuscript in preparation) are described in detail elsewhere.

RESULTS

Analysis of AKR-derived T cell receptor material with rabbit anti-V_H antiserum. In agreement with our previous studies (13) the rabbit antibodies induced against the V_H region of the mouse myeloma protein MOPC 315 (15) can be shown to react with a major proportion of isolated, hapten-specific T cell receptor material of C57BL/6, BALB/c and SJL origin (Tab. 1). In a recent publication, Ben-Neriah et al. have demonstrated that their anti-V_H reagents detect determinants on isolated H-chains and on functionally defined T-helper cell receptors which characterize "allotypic", IgH-linked differences between the mouse strain AKR and the other strains tested (17). We therefore included this particular strain in our analyses (Tab. 1). The clearcut negative result of our absorption experiments which are in concordance with Ben-Neriah's data add further weight on the conclusions drawn from such serological analyses because we now have the above-mentioned genetic argument available. A similar argument can not be made for the rabbit anti-V_L antiserum since no allotypic differences are found in this case (compare ref. 13).

Idiotopic analysis of NP specific receptor material. The use of monoclonal anti-idiotopic antibodies (10-12) in the analysis of isolated, NP-specific T cell receptor material opens three important new aspects: 1) The specificity of the antibodies is much less of a problem compared to conventional heterogenous anti-idiotypic reagents, 2) a set of different

TABLE 3

C57BL/6 DERIVED, NP-SPECIFIC T CELL RECEPTOR MATERIAL EXPRESSES NP^b IDIOTYPE EVEN AFTER REPEATED IMMUNIZATIONS

	% absorption by rabbit anti-NP^b a)		
	1^o b)	2^o	3^o
T cell receptor fraction c)	74	73	59
B cell receptor fraction d)	21	16	18
Serum antibodies	76	19	22

a) See Table 1, footnote a)
b) 1^o, 2^o, 3^o: Primary, secondary and tertiary materials. For details of immunization compare Materials and Methods section
c) See Table 1, footnote b)
d) % absorption of the B cell receptor fraction is calculated from the % absorption of total receptor material (i.e., B + T) on rabbit anti-NP^b minus % absorption of the T cell receptor fraction on the same immunosorbent as described in ref. 9

monoclonal anti-idiotopes can be used to screen for the overlap of the B cell V_H repertoire and the T cell V_H repertoire, and 3.) once the idiotopes as defined by the individual monoclonal antibodies are localized structurally on the B cell V_H, information about the detailed structure of T cell V_H may become available (see Discussion).

Using our absorption technology, we observe binding of 38%, 9%, and 10% of C57BL/6 anti-NP serum antibodies to the immobilized antibodies Ac106, Ac146, and R207, respectively (Tab. 2,D). A potent immunosorbent was difficult to prepare with the culture supernatants of cell line R207 (Tab. 2,F).

In C57BL/6-derived T cell receptor materials the percentage of Ac106 and Ac146 positive molecules was very similar to that in serum antibodies (Tab. 2). In SJL which serves as a negative control in NP^b expression at the antibody level (13, 18) the expression of Ac106 idiotope is even more pronounced at the T cell receptor level as compared to C57BL/6. Idiotope R207, however, is not present on our T cell derived molecules (Tab. 2). It may thus be concluded on the basis of these findings that a major proportion of B cell V_H determinants are indeed expressed on T cell V_H as well but that there certainly exist V_H determinants which are not shared by T cell V_H. It is important, however, to stress the uncertainties introduced by a possible influence of the V_L-region on the expression of the idiotopes studied.

NP^b expression on hyperimmune T cell receptors. The comparison of NP^b-idiotype frequencies in T- and B-cell receptor materials from primed C57BL/6 mice and serum antibodies of boosted animals led to the conclusions that under these conditions the B cell receptors are already NP^b negative - as reflected by the 2^o serum antibodies - while the T cell receptor material still expressed the major NP^b idiotype predominantly (9).

The data in Table3 confirm and considerably extend our previous results. Even after three injections of antigen resulting in a low but detectable NP^b expression in both B cell receptors and serum antibodies (compare ref. 19) 59% of isolated T cell receptor material is bound to our anti-NP^b immunosorbent (Tab. 3). Apart from the lack of R207 expression it is this obvious difference in V_H regulation which clearly distinguishes T cell V_H from B cell V_H.

It may be mentioned in passing that hyperimmune mice represent another case where the T cell compartment is expressing NP^b-idiotype in the absence of comparable amounts of circulating NP^b-positive B cell products, a situation similar to the chimeric mouse experiments which proved the T cell origin of the material under study (20).

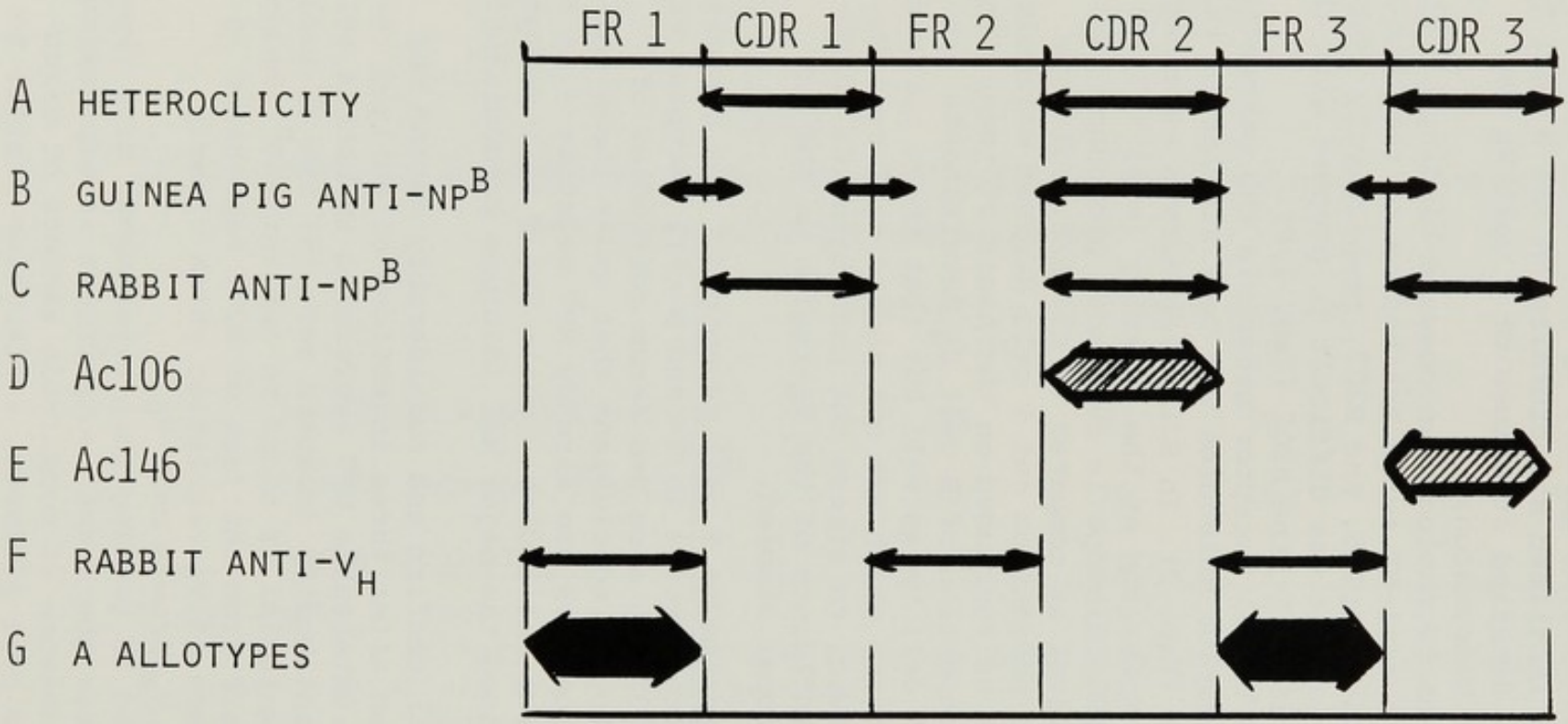

Figure 1. Assignment of T cell V_H markers to the various segments of a V_H region. Markers A - E are expressed on NP-specific T cell receptor material of C57BL/6 and SJL origin, markers F and G are expressed in mouse or rabbit T cell receptors regardless of hapten-specificity. FR : framework segment, CDR : complementarity determining region. The reaction of B and D with idiotype can not be inhibited by free hapten, the reaction of C and E can be inhibited by free hapten. The three types of bars are explained in the text.

DISCUSSION

The simple concept that T-cell and B-cell receptors carry the very same V_H region is challenged by several findings: a) On the Ig level the V_H region is associated with a V_L region to form an antigen binding site. V_L region determinants are, however, not found on the majority of T cell receptors. b) Helper and killer T lymphocytes are functionally restricted to recognize antigen only in the context of self-MHC structures while B cells do not show this restriction. Could one principle receptor structure - namely the V_H region(s) - be responsible for both types of recognition? And c) DNA rearrangements in the Ig-coding region of the mouse chromosome 12 which undergoes very characteristic translocation events in Ig-expressing B cells are not readily found in T cell lines (compare, e.g., ref. 21 and Tonegawa, S., this volume).

In the face of these problems we summarize our serological evidence concerning the T cell V_H in Figure 1. A hypothetical V_H region is divided into three framework (FR) segments and three complementarity determining regions (CDR) the third of which may be at least partially coded for by the D region, as documented for, e.g., the NP^b-V_H region (22). Four of the seven V_H markers found on T cell V_H are only vaguely correlated to these individual segments, e.g., the determinants detected by the rabbit anti-NP^b antibodies whose binding is readily inhibited by free hapten are probably localized in the CDR segments but not in the FR while no further correlation to a particular CDR can be postulated. Such a situation is indicated by slim lines in Fig. 1. The a allotype determinants on rabbit V_H, however, are most likely located in FR1 (amino acids 4-16) and FR3 (amino acids 67-71, 84-85) (23). This is indicated by strong solid bars in Fig. 1.

A recent sequence comparison of two NP^b-positive hybridoma antibodies (22 and Botwell, A., this volume) allowed a tentative localization of the idiotopes defined by monoclonal anti-idiotopic antibodies on the NP^b-V_H region (12). These locations are marked with shaded bars in Fig. 1 to emphasize the present uncertainties of these assignments.

The overall picture of the serological relatedness of T cell V_H and B cell V_H emerging from Fig. 1 is striking: There are serological reactions with <u>all</u> segments of the V_H region expressed in the isolated, hapten-specific T cell receptors. Since expression of idiotope R2O7 may require the presence of Cλ (24) together with the NP^b-V_H the lack of absorption of T cell receptor material (Table 2) to this reagent can not be used as an argument for gross structural differences in T-cell V_H as

compared to B cell V_H.

All our data therefore suggest to continue the search for B-cell derived DNA-V_H probes and the corresponding IgH-allotype- and therefore V_H-matched T cells, T cell lines, or T cell hybridomas which have to be shown to actually express the respective V_H gene product. To our knowledge these critical experiments have not been completed so far.

As demonstrated by the data in Table 3 the rules of V_H expression in T and B cells are so different that it is not difficult to envisage situations where an original (primary) close relationship of B cell V_H and T cell V_H becomes more and more obscured in the course of an ongoing immune response. In other words, the lack of a common idiotype in B- and T-cell V_H does by no means exclude the possibility that both V_H regions are derived from the same original gene pool.

The level(s) at which these clearly different expression patterns are regulated are unknown. An attractive idea would be to envisage differences at the level of gene organization and/or reorganization which would take place in V_H genes of B cells but only to a limited extent in T cells. An alternative explanation would be that T cell populations have a much higher threshold as far as antigen stimulation is concerned in order to express V_H genes other than germline V_H. An ultimate analysis of V_H genes expressed in at least certain subsets of T cells may help to answer these questions.

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REFERENCES

1 Eichmann, K., and Rajewsky, K. (1975). Eur. J. Immunol. 5, 661.
2 Rajewsky, K., and Eichmann, K. (1977). Contemp. Top. Immunobiol. 7, 69.
3 Binz, H., and Wigzell, H. (1977). Contemp. Top. Immunobiol. 7, 113.
4 Lindahl, K.F., and Rajewsky, K. (1979). Int. Rev. Biochem. 22, 97.

5 Krawinkel, U., and Rajewsky, K. (1976). Eur. J. Immunol. 6, 529.
6 Krawinkel, U., Cramer, M., Imanishi-Kari, T., Jack, R.S., Rajewsky, K., and Mäkelä, O. (1977). Eur. J. Immunol. 7, 566.
7 Cramer, M.,and Krawinkel,U. (1980).In Regulatory T Lymphocytes, Pernis, B., and Vogel, H.J. (eds.), Academic Press, New York, p. 39.
8 Krawinkel, U., Cramer, M., Mage, R.G., Kelus, A.S., and Rajewsky, K. (1977). J. Exp. Med. 146, 192.
9 Krawinkel, U., Cramer, M., Melchers, I., Imanishi-Kari, T., and Rajewsky, K. (1978). J. Exp. Med. 147, 134.
10 Reth, M., Imanishi-Kari, T., and Rajewsky, K. (1979). Eur. J. Immunol. 9, 1004.
11 Kelsoe, G.H., Reth, M., and Rajewsky, K. (1980). Immunol. Rev. 52, 75.
12 Reth, M., Bothwell, A., and Rajewsky, K. (1981). This volume.
13 Cramer, M., Krawinkel, U., Melchers, I., Imanishi-Kari, T., Ben-Neriah, Y., Givol, D., and Rajewsky, K. (1979). Eur. J. Immunol. 9, 332.
14 Jack, R.S., Imanishi-Kari, T., and Rajewsky, K. (1977). Eur. J. Immunol. 7, 559.
15 Ben-Neriah, Y., Wuilmart, C., Lonai, P., and Givol, D. (1978). Eur. J. Immunol. 8, 797.
16 Ben-Neriah, Y., Lonai, P., Gavish, M., and Givol, D. (1978). Eur. J. Immunol. 8, 792.
17 Ben-Neriah, Y., Givol, D., Lonai, P., Simon, M.M., and Eichmann, K. (1980).Nature, 285, 257.
18 Imanishi-Kari, T., Rajnavölgyi, E., Takemori, T., Jack, R.S., and Rajewsky, K. (1979).Eur. J. Immunol. 9, 324.
19 Mäkelä, O., and Karjalainen, K. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 735.
20 Krawinkel, U., Cramer, M., Kindred, B., and Rajewsky, K. (1979). Eur. J. Immunol. 9, 815.
21 Kronenberg, M., Davies, M.M., Early, P.W., Hood, L.E., and Watson, J.D. (1980). J. Exp. Med. 152, 1745.
22 Bothwell, A., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., and Baltimore, D. (1981). Cell, in press.
23 Kindt, T.J. (1975). Advanc. Immunol. 21, 35.
24 Reth, M. (1981). Doctoral thesis. University of Cologne.

RECEPTORS AND REGULATION OF MAJOR HISTOCOMPATIBILITY-RESTRICTED CYTOTOXIC T LYMPHOCYTES

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ABSTRACT

We discuss the experimental evidence on the nature of the antigen receptor of H-2 restricted cytotoxic T lymphocytes (CTL) and a system for the detection of such receptors on AKR anti-AKR-TNP CTL by anti-idiotypic antisera (aId) generated by immunization of syngeneic AKR mice with T cell blasts enriched for anti-TNP CTL. In addition, a limiting dilution system is described in which the regulation of the TNP-specific CTL response upon immunization is analysed. The results demonstrate that with antigen priming a shift from suppressable to non-suppressable CTL at high frequency can be observed. It is pointed out that the regulatory events underlying this response could eventually be studied within the concept of the immune network by using aId against such CTL.

INTRODUCTION

Antisera raised against idiotypes (Id) on immunoglobulin (Ig) molecules have been used by a number of investigators to demonstrate the presence of similar determinants on receptors and soluble factors of T cells with the same antigen specificity. The finding that antisera against framework determinants of the variable region of the Ig heavy chain (anti-V_H antisera) also reacted with antigen specific T cell factors and receptors (1) strengthened the assumption that the T cell uses the entire variable region of the Ig heavy chain to build part of its antigen receptor. The serological analysis could be supported by data indicating that the expression of Id and V_H determinants on T cells was genetically controlled by genes linked to the Ig allotype locus. This seems to constitute the common notion agreed upon by most specialists

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working in that field. Since a biochemical purification of the T cell receptor has still been achieved, the controversy, whether other parts of the Ig molecule like a light chain and the Ig constant part are found on T cell receptors, might persist further. In view of the strong serological and genetic evidence for the entire V_H region claimed to be expressed on factors and receptors of T helper and suppressor cells and hybridomas with identical function it is surprising that a definite purification of the molecules has not sufficiently progressed. This might be attributed, however, to the possibility that T cells produce receptors in quantity orders of magnitude lower than B cells (for review see 1,2). Recent evidence from experiments using the tools of molecular biology adds further complications to the prevailing concept of the T cell receptor. These experiments demonstrated that neither a T helper cell or a CTL clone, growing in medium substituted with T cell growth factors (TCGF), nor a T cell tumor Wehi-22 rearranged the Ig genes in a way characteristic for mature B cells with Ig expression (3). Furthermore, the absence of mRNA for the constant region of the u-chain excluded transcription from these nonrearranged genes in germ-line configuration. These findings taken together with the serological and genetic analysis narrow the concept of the T cell receptor to the following model. The T cell uses only part of the Ig variable region genes which accomodates reactivity of the T cell receptor with anti-Id and anti-V_H antisera. Putative joining region (J) genes of the T cell, however, are different from those of the B cell and link up to unique T cell constant region genes. This would imply that Id unique to T cells might exist and, hence, have a considerable impact on network regulation of the immune system.

Another problem arises with the available experimental evidence on T cell receptors of major histocompatibility complex (MHC)-restricted CTL. As pointed out above, CTL clones were found negative for Ig gene rearrangement. In addition, it has been unsuccessful so far to demonstrate the B cell Ig defined major cross-reactive idiotypes (MCRI) ARS and NP^b on A/J and C57Bl/b (B6) CTL specific for the haptens p-azophenylarsonate and (4-hydroxy-3-nitro-phenyl) acetyl (NP), respectively (4,5). Similarly, NP-primed B6 CTL were not heteroclitic, i.e., they did not show better lysis of syngeneic target cells coupled with the crossreactive hapten (4-hydroxy-5-iodo-3-nitrophenyl) acetyl (NIP) than of target cells coupled with the immunizing hapten NP (6). Heteroclicity, however, is a fine specificity marker for NP-induced primary B6 IgM antibodies. Furthermore, anti-V_H antisera did not conclusively react with CTL clones from long-term cultures (H.v.Boehmer, personal communication).

In retrospect, the results from negative experiments cited above provide sufficient justification for the attempt to use T cells as immunogens to raise anti-Id antisera against Id on their antigen-specific membrane-bound receptors directly. This approach has been tried with alloreactive and MHC-restricted T cells (7,8,9,10). This communication, however, will be restricted to a discussion of Id on receptors of MHC-restricted T cells and the methodological aspects of their detection. In addition, we shall describe a limiting dilution system in which the functional aspects of the regulation of the 2,4,6-trinitrophenyl (TNP)-specific CTL response upon immunization are studied. We hope from this system to supply a basis for a future detailed analysis of a MHC-restricted CTL response within the frame of the immunological network.

METHODS

To raise anti-Id antisera T cells were enriched for AKR anti-AKR-TNP CTL derived from 5 day in vitro cultures without antigen restimulation of draining lymph node cells from AKR mice 5 days previously painted with 2,4,6-trinitrophenylchloride (picrylchloride = PC). These cells were injected (i.p.) into syngeneic virgin AKR mice. The first injection was in complete and further injections at intervals of at least two weeks were in incomplete Freund's adiuvant. Each mouse received at least 5×10^6 cells at each immunization. The mice were bled two to four weeks after 5 successive immunizations. The antisera, abbreviated as AKRa(AKRaAKR-TNP), were tested for reactivity with syngeneic and allogeneic CTL raised against the stimulating or control antigens as well as against alloreactive CTL by immunofluorescence and complement dependent lysis. In the latter case, subsequent depression of CTL function was determined. These methods have been described in detail (9).

To study the details of the regulation of the TNP-specific CTL response upon immunization with PC the following system was used. At different times of sensitization of the abdominal skin of AKR mice with PC and subsequent in vitro culture of the draining lymph node cells samples of cell populations containing antigen primed cells were further expanded by stimulation with Concanavalin A for two days and subsequent limiting dilution in the presence of filler cells and TCGF (11). At the end of the limiting dilution period individual wells were tested for a TNP-specific cytotoxic response.

RESULTS AND DISCUSSION

Receptors of TNP-specific CTL. We have designed a new approach to raise antisera, referred to as AKRa(AKRaAKR-TNP), against Id on receptors of TNP-specific, H-2-restricted AKR CTL by using cell fractions enriched for T cell blasts containing such CTL as immunogens in syngeneic mice. This experimental situation is complex and the antisera need to be controlled for a number of 'contaminating' antibodies. Since we used enriched fractions of antigen-activated blast cells as immunogens it had to be excluded that the antisera were directed against: 1) the antigen TNP, which might have persisted on the surface of the immunizing cells from antigen activation. TNP might have been inserted into the cell membrane or be bound by antigen-specific receptors, 2) neoantigenic determinants (NAD) formed by association of persisting TNP with cell surface molecules of the T cell blasts, and 3) blast cell antigens expressed on most T cell blasts.

Our experiments did not provide any evidence for these reactivities. We were unable to demonstrate any TNP on the cell surface of AKR anti-AKR-TNP CTL as well as any reactivity of AKRa(AKR-AKR-TNP) with TNP-coupled sheep red blood cells. Furthermore, the antisera did not lyse B6 anti-B6-TNP CTL stimulated in a way identical to AKR anti-AKR CTL. Detection of putative NAD formed by association of TNP with cell surface molecules of AKR anti-AKR CTL was made unlikely since AKRa (AKRaAKRTNP) were unreactive with AKR_{TNP} anti-B6 CTL derived from draining lymph nodes of mice sensitized with PC. Contaminating antibodies against TNP-NAD chemically modified by antigen processing cells and bound to AKR anti-AKR-TNP CTL receptors were excluded by the following experiment. Trypsin treatment had efficiently removed CTL receptors and, consequently, any possible processed TNP-NAD. AKRa(AKRaAKR-TNP), however, still lysed AKR anti-AKR CTL after resynthesis of CTL receptors after recovery in overnight culture from trypsin treatment. Since after absorption on irrelevant AKR blasts AKRa(AKRaAKR-TNP) did not react with TNP, blast cell antigens on other activated T cells of AKR type, alloreactive AKR CTL and FITC-specific, H-2 restricted AKR CTL used for specificity controls, but lysed a substantial fraction of AKR anti-AKR-TNP CTL, we concluded that the antisera were directed against Id on AKR anti-AKR-TNP CTL receptors (Table 1).

TABLE 1

Detection of specificity associated determinants (idiotypes) with AKRa(AKRaAKR-TNP) antisera

CTL	restriction	reactivity with AKRa(AKRaAKR-TNP)
AKR anti-AKR-TNP	H-$2K^k$	+
AKR anti-B6		-
AKR_{TNP} anti-B6		-
AKR anti-AKR-FITC	H-$2K^k$	-
B6 anti-B6-TNP	H-2^b (poor)	-

These data have a number of implications which we shall briefly mention and which have been discussed in detail elsewhere (2,9). The fact that AKRa(AKRaAKR-TNP) reacted with a sizable proportion of AKR anti-AKR-TNP CTL could be interpreted as the activity of many aId antibodies against a variety of Id. Alternatively, a restricted number of antibodies could detect a MCRI on most AKR anti-AKR-TNP CTL which might consist of only a limited number of different clonotypes. Although, AKRa(AKRaAKR-TNP) seem to differentiate between Id of two H-2 restricted CTL of the same strain, AKR anti-TNP and anti-FITC CTL, respectively, they do not provide an answer to the validity of the one or two receptor model suggested for H2 restricted recognition (12). If we assume that putative anti-self receptors on AKR anti-AKR-TNP and AKR anti-AKR-FITC CTL are identical and not immunogenic in our immunization protocol for aId we are still left with the possibility that the antisera detect receptors either for TNP (in case of the two receptor model) or for TNP-self (in case of the one receptor model). This could effectively be studied with TNP-specific AKR CTL from chimaeric mice. In addition, further experiments on the genetic control of Id expression on AKR anti-AKR-TNP CTL might have some bearing on the same question. They have a basis in the finding that Id determined by AKRa(AKRaAKR-TNP) show strain specificity with AKR anti-AKR-TNP and B6 anti-B6 TNP typing Id positive and negative, respectively. Any results in this area could shape our ideas of whether T and B cell Id are completely overlapping or whether Id unique to T cells exist. The latter assumption would be supported by our inability to find any reactivity of AKRa (AKRaAKR-TNP) with AKR anti-TNP antibodies. The definition of unique T cell Id would have definite consequences for the regulation of the immune system as a whole within the frame of the network concept.

Since the original publication of the above data for Id on TNP-specific CTL (9) very similar experiments have been carried out and showed that Id on Newcastle Disease Virus (NDV)-specific CTL could be detected by essentially the same approach (10). In both systems vigorous repeated i.p. immunizations of syngeneic mice with high cell numbers of T cell blast fractions enriched for specifically antigen activated CTL in complete and incomplete Freund's adiuvant lead to the generation of a limited number of antisera specific for Id on receptors of TNP- or NDV-specific, H-2 restricted CTL. These immunization schedules with subsequent numerous test bleedings put a considerable strain on the health of the immunized animals. Nevertheless, we have recently been able to select some active antisera from individual bleedings from single mice repeatedly immunized with 10^7 T cell blasts containing AKR anti-AKR-TNP CTL. We found antisera with marked activity against CTL and hardly any effect on other TNP-activated T cells. When the activity of single bleedings was compared with the one of pools of these single bleedings we found additive effects indicating that perhaps two Id determinants are expressed. Few antisera, however, reacted with a large fraction of TNP-activated T cells without any effect on anti-TNP CTL. This indicated that, similar to Id on alloreactive T cells (13), Id on proliferative and cytotoxic TNP-specific H-2 restricted T cells might differ.

We believe that this system will provide us with a further understanding of the genetic control of T cell receptor expression on H-2 restricted CTL and a better evaluation of the one and two receptor model of MHC-restricted recognition. A biochemical analysis of T cell receptors from these cells, however, will greatly rely on the possibility to generate a sufficient supply of monoclonal aId antibodies with a corresponding CTL clone from long term cultures in medium substituted with TCGF.

Regulation of MHC-restricted, TNP-specific CTL. To study the regulation of a cytotoxic response on the Id level and follow idiotypically defined clones upon immunization it would be best to have an experimental situation in which a substantial proportion of the CTL carries a dominant MCRI, or a restricted number of Id. That AKRa(AKRaAKR-TNP) aId consistently reacts with a sizable proportion of AKR anti-AKR-TNP CTL could be interpreted in this sense. To prepare the ground for such a future study we set up a system in which the details of the regulation of AKR anti-AKR-TNP CTL upon antigen priming can be analysed. At different times of sensitization of the abdominal skin of AKR mice with PC and subsequent in vitro culture of the draining lymph node cells samples of cell populations containing antigen-primed cells

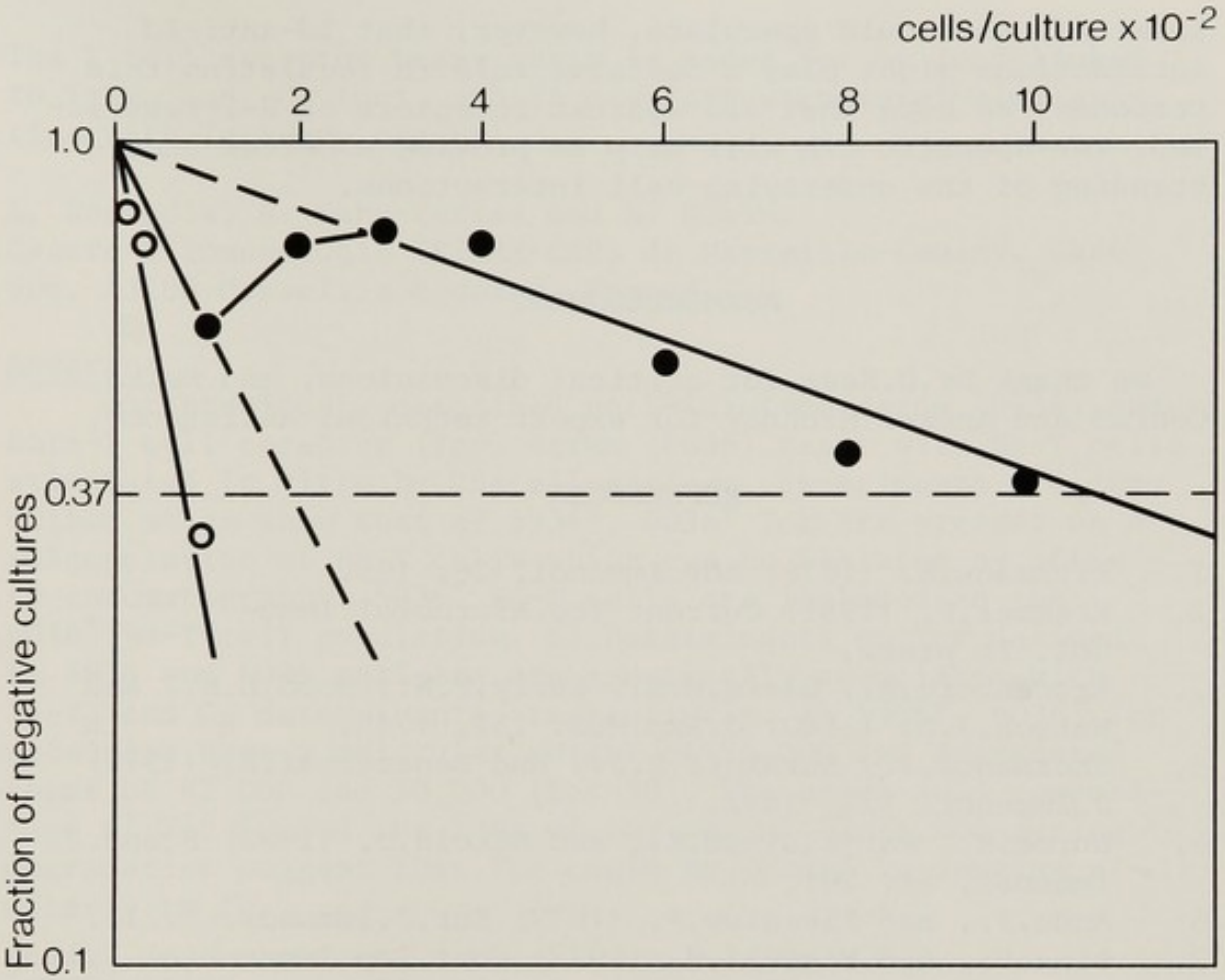

FIGURE 1. Frequency of AKR anti-TNP CTL of normal AKR lymph node cells, ●—●, and of lymph node cells from AKR mice 5 d previously sensitized with PC on the abdominal skin, O—O.

were further expanded by stimulation with Concanavalin A for two days and subsequent limiting dilution in the presence of filler cells and TCGF (11,14). At the end of the limiting dilution period cells from individual culture wells were tested for a TNP-specific cytotoxic response.

These experiments are depicted in Fig.1 and showed that in normal cells or in cell populations tested early after in vivo PC-sensitization, two distinct fractions of TNP-specific CTL could be observed: CTL_{ss} sensitive, and CTL_{rs} resistent to suppression. CTL_{ss} occurred at a higher frequency than CTL_{rs}. With additional time (day 5) after sensitization only one frequent CTL population, resistent to suppression or unsuppressed because of the elimination of suppressor cells, was evident. These data could be interpreted in the sense that antigen priming had turned a suppressable virgin TNP-specific precursor CTL population into primed, non-suppressable effector cells. The nature of the suppressor cell and the mechanism of its action in this system remain to be de-

termined. One could speculate, however, that Id-anti-Id interactions might play a decisive role in regulating this response. We hope that aId against receptors of H-2 restricted, TNP-specific CTL will help to provide a better understanding of the underlying cell interactions.

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REFERENCES

1. Eichmann,K. (1978) Adv.Immunol. 26, 195.
2. Krammer,P. (1981) Current Top.Microbiol.Immunol. In press.
3. Kronenberg,M., Davis,M.M., Early,P.W., Hood,L.E., and Watson,J.D. (1980) J.Exp.Med. 152, 1745.
4. Sherman,J.I., Burakoff,S.J., and Benacerraf,B. (1978) J.Immunol. 121. 1432.
5. Hurme,M., Karjalainen,K., and Mäkelä,O. (1980) Scand.J. Immunol. 11, 241.
6. Ando,J., and Kisielow,P. (1979) Eur.J.Immunol. 9,211.
7. Binz,H., and Wigzell,H. (1977) Cont.Top.Immunobiol.7,113.
8. Krammer,P.H. (1978) J.Exp.Med. 147, 25.
9. Krammer,P.H., Rehberger,R., and Eichmann,K. (1980) J.Exp. Med. 151, 1166.
10. Kees, U. J.Exp.Med. In press.
11. Eichmann,K., Falk,I., Melchers,I., and Simon,M.M. (1980) J.Exp.Med. 152, 477.
12. Zinkernagel,R.M., Callahan,G.N., Althage,A., Cooper,J., Klein,P.A., and Klein,J. (1978) J.Exp.Med. 147, 882.
13. Binz,H., Frischknecht,H., Shen,F.W., and Wigzell,H. (1979) J.Exp.Med. 149, 910.
14. Goronzy,J., Schäfer,U., Eichmann,K., and Simon,M.M. (1981) J.Exp.Med. In press.

The T cell receptor heavy chain is coded for by loci linked to Ig-V_H and -C_H loci, and it displays structural similarities with Ig heavy chains.

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Abstract.

Our previously described anti-idiotype serum (5936) and anti-T cell receptor (Tcr) serum (6036) react with B6-T cells stimulated in vitro by CBA alloantigen. Experiments are described which show that a) 5936^+, 6036^+ Tcr are present on a subpopulation of B6-T cells which can be isolated by affinity chromatography. 5936^+ B6-T cells are included in the 6036^+ B6-T cell population. b) Determinants on Tcr defined by 5936 and 6036 antisera are genetically associated with Ig-V_H and C_H determinants, respectively. c) 5936^+, 6036^+ Tcr molecules have a molecular weight of 75,000 and degradated forms of 62,000 and 50,000 (Tcr-50). The amino acid composition of Tcr-50 and the loss of regions of 12-13,000 during degradation suggest that Tcr could be folded in domains similarly to Ig.

Introduction

The molecular structure of the antigen-binding T-cell receptor (Tcr) is a subject of controversy (1). Most experiments indicate that the Tcr contains at least one heavy chain with a molecular weight (MW) of 50-75,000, carrying an antigen-combining site and Ig-related idiotypes (Id) (1-4). It has however, very recently been shown that Ig-J and -C genes are not expressed in various T cell clones, and suggested that Ig-V_H genes could be rearranged to join Tcr J and C genes (5). Rearrangement adjacent to C_μ but not adjacent to C_k genes was indeed observed (6). Thus, though the genes responsible for the expression of Tcr heavy chains have been shown to be linked to Ig allotype genes (1-4), an eventual rearrangement of the Tcr V and C_T genes does not seem to implicate Ig-J and-C_H genes.

We have described two types of antisera which react against B6-Tcr : a) antiserum 5936 recognizing Ig Id in B6 anti-CBA antisera and in B6 anti-CBA MLC T cell populations and b) antiserum 6036 recognizing allotypic/isotypic determinants on 5936^+ B6-Tcr but not 5936^+ B6-Ig (7). A priori, these results suggest a) that 5936^+ B6-T cells are included in the 6036^+ B6-T cell population, b) that the anti-Id reactivity of 5936 antiserum against Tcr should segregate with

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B6-V_H markers and the 6036 antiserum reactivity against Tcr should segregate with B6-Ig-C_H markers, and c) that, if Tcr and Ig genes have arisen by duplication of a common ancestral gene, some degree of similarity between the Tcr and Ig heavy chains is expected. The present experiments substantiate these expectations.

Results.

All methods have been described elsewhere (cf 1). Three types of experiments were performed in order to test the above three predictions :

1. 5936-Id$^+$ B6-T cells are included in antiserum 6036-reactive B6-T cell populations.

It is established that 5936-Id$^+$ B6 T cells after incubation with antiserum 5936 can be fractionated on rabbit Ig (RIg)-anti-RIg columns (1). Likewise, antiserum 6036 coated B6-T cells can be fractionated on RIg anti-RIg columns. In the present experiments, 6036$^+$ B6 anti-CBA T cells were isolated, cultured for 10 days and restimulated with CBA alloantigen for 4 days. These cells were passed through RIg

Table 1

Specific fractionation of antisera 5936/6036$^+$ B6 T cells stimulated with CBA alloantigen

6036$^+$ B6 $\bar{a}$ CBA cells[a]	RIg $\bar{a}$-RIg fractionation[b]	Recovery	Reactivity with antisera 5936	6036
+ (-)	-	100	8,293	13,914
+ (-)	+	92	8,317	15,718
+ (6036)	+ (P)	1	493	897
+ (6036)	+ (E)	78	12,471	30,903
+ (5936)	+ (P)	21	507	5,852
+ (5936)	+ (E)	49	19,839	27,418

6036$^+$ B6 anti-CBA T cells were pretreated with (a) fractionated (b) and assayed as mentioned in the text. The reactivity of the cells against antisera 5936 and 6036 was measured using the protein A assay. cpm for cells plus normal rabbit serum < 300, for cells plus anti-thymocyte antiserum ≈ 52,000.

anti-RIg columns. One half of the cells were incubated with antiserum 6036, the other half of the cells were incubated with antiserum 5936. The two populations of cells were subsequently fractionated on two separate RIg anti-RIg columns. The data in Table 1 show that practically all of the 6036^+ B6-T cells upon re-incubation with antiserum 6036 are retained on the RIg anti-RIg column. The majority of the cells can be recovered and react with both 5936 and 6036 antiserum. Of the 6036^+ B6-T cells which were pre-incubated with antiserum 5936, 21% was recovered in the passed cell population, i.e. they were 5936^-, 6036^+ and 49% was recovered by elution, i.e. they were 5936^+, 6036^+. These results have been verified by the protein A assay. Control experiments were the following : a) 6036^+ B6 anti-CBA cells were incubated with normal rabbit serum. Here, 91% of the cells were found in the RIg anti-RIg column passed fraction. b) 6036^+ B6 anti-$C_3H.Q$ T cells were incubated with antisera 5936 and 6036 and subsequently fractionated on RIg anti-RIg columns. 93% and 2% of these cells were found in the passed populations respectively, whereas 5% and 81% of these cells were eluted, respectively. Thus, these results demonstrate that 5936^+ B6 anti-CBA T cells are contained in the 6036^+ cell population and is a distinct subpopulation. In addition, it was demonstrated that more than 80% of these cells were Lyt 1^+, 2.3^- as demonstrated earlier for non-fractionated 5936^+ B6 anti-CBA T cells (1).

2. Tcr and Ig heavy chain loci are linked.

In order to determine whether loci linked to Ig-V_H and -C_H loci are involved in the production of Tcr, we have made F1 hybrids between B6 mice (5936^+) and CBA (5936^-). These F1 hybrids were mated with B6 mice and H-2^b homozygous and Ig-1^b/Ig-1^j allotype heterozygous mice were selected. These mice were subsequently backcrossed to B6. After 5, 9, 14 and 20 backcross generations, the heterozygous mice were mated and Ig-1^j homozygous mice selected. From such animals, inbred strains of mice were produced viz B6.5a, B6.9a, B6.14a and B6.20a. In the present study, only B6.5a and B6.9a mice were used. These mice were analyzed for the V_H markers NP (8), S14 (9) and ABA-HOP (10) and always controlled for the Ig-1^j allotype (courtesy to Drs. U. Krawinkel, M. Cramer and O. Mäkelä). B6 mice express the NP marker but not the S14 and ABA-HOP markers, while CBA mice express the S14 and ABA-HOP markers but not the NP marker as expected (Table 2). B6.9a mice are phenotypically identical to CBA mice, whereas B6.5a mice behave like B6 mice as regards the NP and S14

Table 2

Antisera 5936 and 6036 defined determinants on B6 T cell receptors are genetically associated with Ig-V_H and C_H determinants.

MLC blast cells	Resp. cell phenotype				% cells killed by RC and antisera		
	C_H	V_H					
	Ig-1	ABA-HOP	S14	NP	5936	6036	ATG
B6 $\bar{a}$-CBA	b	-	-	+	29	35	98
B6 $\bar{a}$-C_3H.Q	b	-	-	+	2	31	98
C_3H.SW $\bar{a}$-CBA	j	ND	ND	-	1	-1	97
B6.5a $\bar{a}$-CBA	j	+	-	+	27	2	96
B6.5a $\bar{a}$-C_3H.Q	j	+	-	+	-3	-1	97
B6.9a $\bar{a}$-CBA	j	+	+	-	2	-2	94
B6.9a $\bar{a}$-C_3H.Q	j	+	+	-	1	3	95
CBA $\bar{a}$-B6	j	+	+	-	0	-2	98

Normal rabbit serum and RC killed 10-16% of the cells. These values have been subtracted. See also text for further details. ND = not done.

markers and like CBA mice as regards the ABA-HOP marker. B6, C_3H.SW, B6.5a, B6.9a and CBA-T cells were stimulated in MLC. On day 4 MLC blasts were tested for reactivity against antisera 5936 and 6036 (control ATG = anti-thymocyte antiserum) in the presence of rabbit complement (RC). The results in Table 2 show that antiserum 5936 reacted only with B6 and B6.5a anti-CBA T cells, whereas antiserum 6036 reacted only with B6 anti-CBA or anti-C_3H.Q T cells. Thus, the reactivity of antiserum 6036 with the MLC blasts segregate with the Ig-1^b allotype of the T cells, while the reactivity of antiserum 5936 with the MLC blasts segregate with the NP-V_H marker of B6 mice.

3. Biochemical analysis of the B6 Tcr heavy chain.

Supernatants of B6 anti-CBA T cell MLC were passed over 6036 antiserum-Sepharose columns. The 6036^+ molecules were

eluted from the columns, labelled with ^{125}I and immediately analyzed on SDS-PAGE (Figure 1a). A unique peak is obtained corresponding to a molecular weight (MW) of 75,000 daltons (d). When however, the same material (repeatedly frozen and thawed for various experiments) is reanalyzed, degrated forms appeared with MW of 62,000 d (Figure 1b and 1c) and 50,000 d (Figure 1c and 1d). The three different forms were called Tcr-75 (MW 75,000), Tcr-62 (MW 62,000) and Tcr-50 (MW 50,000). The molecular weight of these Tcr remained unchanged after chemical reduction (not shown).

The 6036 antiserum was also used to precipitate Tcr material from lysates of B6 anti-CBA T cells labelled either externally by the lactoperoxydase procedure or internally by incorporation of tritiated amino acids. The analysis of the precipitates on SDS-PAGE (Figure 2) shows that membrane Tcr (mTcr) molecules are similar in molecular weight to Tcr-75.

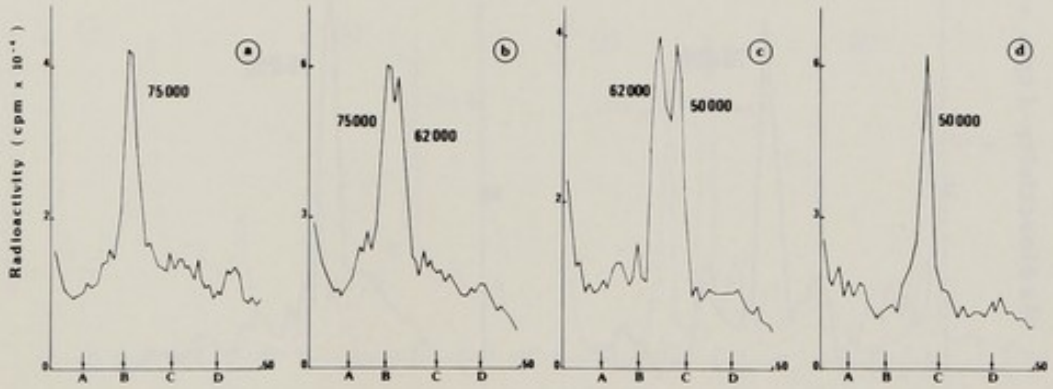

Figure 1. Fresh (a) and repeatedly frozen and thawed (b, c, d) ^{125}I-Tcr molecules in 6% gels. Internal ^{131}I markers are : A (IgG), B (Transferrin), C (Phosphocreatine kinase) and D (Ig light chain).

In addition, the molecular weight of mTcr is not modified by reduction (not shown). Thus, mTcr and Tcr-75 present structural similarities in addition to common antigenic determinants (anti-Tcr-75 recognize mTcr). These results suggest that the T cells of the present system synthesize single chain molecules of molecular weight 75,000 daltons, which are found on the membrane and can be released in the culture supernatants. The native Tcr released in the supernatant (Tcr-75) is spontaneously degrated to lower molecular weight molecules (Tcr-62 and Tcr-50).

A pool of 2,750 ml supernatant from B10 anti-B10.A(4R) MLC was used to isolate a preparation of 6036^+ Tcr material (Figure 3a). This material was passed over an anti-fetal calf serum-Sepharose column and subsequent analysis in SDS-PAGE of this preparation (Figure 3b) indicates that contaminants still represent more than 50% of the molecules and a fractionation on a Con A-Sepharose column was undertaken. Figure 3c demonstrates that the Tcr-50 molecules passed through the column indicating that these molecules have no or few carbohydrate moities (almost no 50,000 molecules were

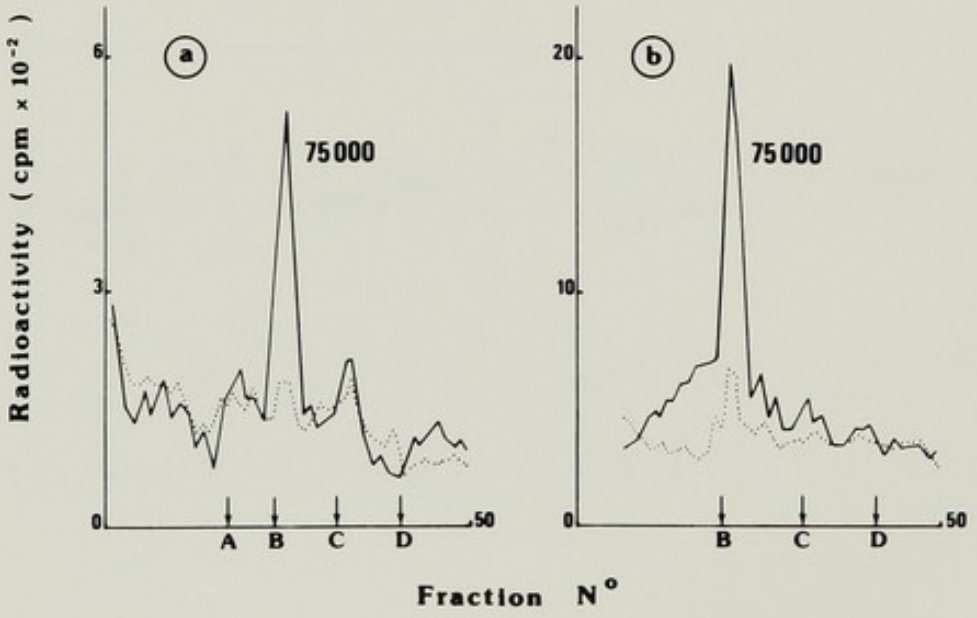

Figure 2. Lysate of B6 anti-CBA T blasts ^{125}I-surface labelled (a) or ^{3}H-Leu biosynthetically labelled (7) were precipitated with the 6036 anti-Tcr antiserum (full line) or an anti-HSA antiserum (broken line). 4.5% gels. Markers as in Figure 1.

eluted from the Con A-Sepharose column - not shown). The Con A-Sepharose column passed material was cleared of most of the contaminants but new contaminants appeared from the Con A column. This preparation was thus passed over a last Sepharose column (anti-RIg) to remove the last contaminants. The material which passed through that column was analyzed on SDS-PAGE and was shown to contain Tcr-50 with less than 10% detectable contamination. The Tcr-50 was then pure enough to undertake amino acid analysis and NH_2-terminal amino acid sequence (9 nanomoles of purified Tcr-50 were obtained from the 2,750 ml of supernatant).

An aliquot (0.3 nanomoles) of the purified Tcr-50 preparation was used to perform amino acid analysis while the rest was submitted to direct amino acid sequence in a Beckman 890 C sequencer (11). Unfortunately, no residue could be identified among ten steps of degradation carried out, thus suggesting that the NH_2-terminus of Tcr-50 was blocked.

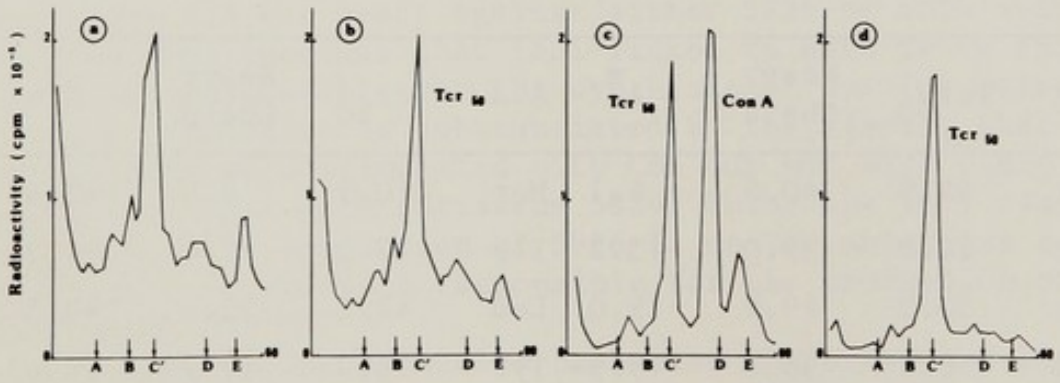

Figure 3. Tcr-50 eluted from a 6036-Sepharose column (a) was passed through anti-FCS (b), Con A (c) and anti-RIg (d) Sepharose columns. 5% gels. Markers were as in Figure 1 except C' (IgG heavy chain) and E (cytochrome C).

The amino acid composition of Tcr-50 was determined and compared (Table 3) to that of the MOPC 173 heavy chain (11). This comparison reveals numerous differences between Tcr-50 and this heavy chain (and other types of Ig heavy chains, not shown) : Tcr-50 possess a higher number of glutamyl, alanyl and histidyl residues and a lower number of seryl, threonyl and prolyl residues. Variations were also observed for the hydrophobic residues but this could not be of structural importance as the increase of the leucyl and phenylalanyl residues is balanced by the decrease of the valyl and tyrosyl residues, respectively. The very large difference observed for glutamyl residues (+ 20) is correlated to the isoelectric point of 5 observed for Tcr-50 (1).

Other residues are equally represented in Tcr-50 and MOPC 173 heavy chain, in particular the cysteyl residues. This result suggests that both molecules have a similar number of disulphide bridges and is in agreement with a possible structure in domains for the two molecules. This hypothesis is supported by the degradation of the Tcr-75 in Tcr-62 and Tcr-50 by loss of regions possessing the size of a domain (12,-13,000 daltons).

Table 3

Comparison of the amino acid (A.A) composition of Tcr-50 and the IgG2a MOPC 173 heavy chain.

A.A	Tcr_{50}	Heavy Chain	Δ^{*}	A.A	Tcr_{50}	Heavy Chain	Δ^{*}
Asx	44.9	40.8	+ 4.1	Met	10.9	8.9	+2.4
Thr	24.0	35.0	-13.2	Ile	13.2	17.4	-4.2
Ser	30.0	49.9	-19.0	Leu	42.2	32.7	+9.5
Glx	56.8	36.9	+19.9	Tyr	13.3	17.7	-4.4
Pro	22.2	35.7	-13.5	Phe	18.7	12.8	+5.9
Gly	28.7	24.6	+ 4.1	Try	ND	11.2	-
Ala	35.8	18.4	+17.4	Lys	32.8	32.1	+0.7
Cys	10.7	10.9	- 1.2	His	14.3	8.9	+5.4
Val	29.9	41.6	-11.9	Arg	16.2	12.8	+3.4

* : For each residue, number of Tcr-50 minus number of H chain.

Discussion.

Antiserum 5936 defines Id produced by B6 mice upon immunization with CBA but not with $C_3H.Q$ alloantigen. However, these Id are not carried by anti-CBA antibodies (1). The specificity of 5936-Id is thus not known, but they are associated with Ig-1b allotypes of the B6 mouse. Antiserum 5936 does also react with T cells present in B6 anti-CBA MLC but absent in B6 anti-$C_3H.Q$ MLC. The T cell-produced molecules that react with antiserum 5936 have previously been designated Tcr molecules for reasons discussed in ref. 1. The present results have demonstrated that 5936-Id^+ B6-T cells constitute a distinct subpopulation of antiserum 6036$^+$ B6-T cells most of which carry the Lyt 1^+ 2.3^- membrane marker. 5936-Id^+ B6-Tcr molecules are synthesized by T cells (Figure 2) and loci closely linked to the loci for the NP-V_H marker of B6-Ig are involved in the synthesis of 5936-Id on our B6-Tcr molecules (Table 2).

Antiserum 6036 was produced in a rabbit immunized with 5936-Id^+ B6-Tcr (7). This antiserum detected allotypic and isotypic determinants on 5936-Id^+ Tcr as well as on 5936-Id^- Tcr (Table 2). The allotypic determinants defined by antiserum 6036 appear to be genetically encoded by a locus linked to the C_H-allotype locus of B6 mice (1, 7). The data in Table 2 demonstrate that B6.9a T cells (possessing all B6 genes except the CBA Ig genes) stimulated by CBA or $C_3H.Q$ alloantigen did not react against either 5936 or 6036 antiserum. We thus conclude that loci linked to both B6-V_H and C_H loci are responsible for the synthesis of our Tcr molecules. This conclusion is substantiated by the finding that B6.5a T cells when stimulated with CBA but not with $C_3H.Q$ alloantigen react with antiserum 5936. Thus, the loci responsible for the production of 5936-Id on Tcr molecules seem to be linked to the loci responsible for the production of the B6-V_H marker NP.

Isolated B6-Tcr molecules have been characterized biochemically. They are single chain polypeptides which in their native form have a MW of 75,000 d. Tcr-75 were readily degraded into 62,000 and 50,000 d forms, and the Tcr-50 molecules were found to be non-glycoproteins with an isoelectric point of 5 or below. The NH_2-terminal amino acid on these Tcr-50 molecules were blocked, but the amino acid composition is in accordance with its low isoelectric point. As was discussed above, the Tcr-50 molecules have the same number of cysteyl residues and they could be built-up of domains of 12,000 MW as Ig heavy chain molecules. Thus, despite distinct differences in the amino acid composition between Tcr-50 and Ig heavy

chains they would seem to be members of the same family of molecules.

In conclusion, the presented data demonstrate that our B6-Tcr molecules are produced by a sub-population of B6-T cells and contain two types of determinants on the same polypeptide chain : Ig-V_H related determinants and non-Ig C_T determinants coded for by loci linked to C_H loci. These results and the biochemical data support the following hypotheses proposed by us (1) and others (5) : the loci coding for Tcr comprise Ig-V_H like loci and non-Ig C_T loci, are localized in the area of the Ig heavy chain loci and they could be of the same family of genes. The C_T and C_H genes could have emerged from a common ancestor by duplication and selected during evolution to different functions.

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References.

1. Rubin, B., Suzan, M., Kahn-Perles, B., Boyer, C., Schiff, C. and Bourgois, A. (1980) Bull. Inst. Pasteur, 78, 305.
2. Rajewsky, K. and Eichmann, K. (1977). Contemp. Topics Immunobiol. 7, 69.
3. Binz, H. and Wigzell, H. (1979). Contemp. Topics Immunobiol. 7, 113.
4. Pacifico, A. and Capra, J.D. (1980). J. Exp. Med. 152, 1289.
5. Kronenberg, M., Davis, M.M., Earley, P.W., Hood, L.E. and Watson, J.D. (1980). J. Exp. Med. 152, 1745.
6. Forster, A., Hobart, M., Hengartner, H. and Rabbits, T.H. (1980). Nature, 286, 897.
7. Rubin, B. and Bourgois, A. (1981) Scand. J. Immunol., 13 in press.
8. Imanishi, T. and Mäkelä, O. (1974) J. Exp. Med. 140, 1498.
9. Mäkelä, O., Julin, M. and Becker, M. (1976) J. Exp. Med. 143, 316.
10. Mäkelä, O., Pasanen, V.J., Sarvas, H. and Lehtonen, M. (1980) Scand. J. Immunol. 12, 155.
11. Bourgois, A. and Fougereau, M. (1970) Eur. J. Biochem. 12, 558.

IDIOTYPIC T RECEPTORS: RIGID ALLO-MHC BUT OPPORTUNE SELF-MHC REACTIVITY

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ABSTRACT. We have previously shown that molecules derived from normal Lewis T cells carrying the idiotypes of "Lewis-anti-DA" type display strong binding to the relevant DA MHC polypeptide chains without any detectable binding to third party, BN, MHC chains. However, weak but significant binding affinity to self heavy Ia chains, that is of Lewis type, was also found. Here we have extended this study to include T cell receptors isolated using the same anti-idiotypic immunosorbant but where the molecules are coming from the T lymphocytes of normal, congenic L.BN rats. Such molecules expressed the same strong binding to DA allo-MHC chains but did now fail to express any detectable reactivity towards Lewis derived MHC polypeptides. In contrast, significant but weak binding was noted to both heavy and light Ia chains of the "new" self MHC, that is BN type MHC. We conclude that T cells use the very same receptors for reactivity against allo-MHC as are used in self-MHC restricted responses. Whereas reactivity against allo-MHC reactivity would seem compulsory and with high avidity, reactivity of weak but select specificity for self-MHC determinants behaves in an "opportune" manner with several different pathways being possible starting from the same original and presumed germ line genes coding for allo-MHC reactivity.

INTRODUCTION

T cells are compared to B cells obsessed with reactivity towards both allo- and self-major histocompatibility antigens as evident by numerous studies (1,2,3,4,5). Cloned T cells can express both allo-MHC reactivity whilst at the same time being reactive against a soluble protein antigen in the context of self-MHC (6). The exact pathways through which T lymphocytes reach this striking, high level of MHC reactivity are debated but little doubt seem to exist that this in part is generated

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at the population levels during ontogeny (6-10). Here, thymic and peripheral lymphoid tissue experiences in the context of MHC surface antigens seem to play important parts in the selective processes.

We have here addressed a single issue in this complex area, namely the question whether at the molecular level T cell receptors for allo-MHC antigen also are able to display selective binding to self-MHC structures in some measurable manner as would be anticipated from the functional studies of T cell specificity. We have previously been able to demonstrate that T but not B cell -derived antigen-binding molecules (with similar specificity as measured as strong, specific allo-MHC reactivity) may have the ability to show significant, albeit weak self-MHC binding properties (11). Here we present data showing that T cells with receptors carrying a particular idiotypic marker and allo-MHC reactivity can maintain these two features whilst changing their weak "self"-MHC reactivity in accordance with the MHC structures of the T cell donor.

MATERIAL AND METHODS

The principal outline reads as follows: Internally labelled, single, MHC polypeptide chains defined as heavy Ag-B, heavy Ia and light Ia chains were purified using IgG alloantibody immunosorbants in combination with SDS-PAGE methodology (11). DA, BN and Lewis were used as three rat strains with distinct and different MHC types. In the present study we also included a fourth strain, L.BN, which differs with regard to MHC from Lewis otherwise being identical. IgG alloantibodies were raised and purified as described (11). Likewise, an anti-idiotypic serum with specificity Lewis-anti-(Lewis-anti-DA) was raised and used as immunosorbant coupled to CNBR-Sepharose for the purification of idiotype-positive, Ig-negative molecules (= T cell receptors) from large volumes of normal serum from Lewis or L.BN adult rats (for procedures see 12). Strong binding (+++) was considered to exist when a particular type of single MHC polypeptide chain was retained on an immunosorbant column in such a way as to require high ionic, low pH treatment to be recovered. Weak binding (+) was established when the MHC chains under scrutiny became retarded in their passage through the column beyond that of low molecular weight markers but eventually passed through the column without having to resort to a change in elution buffers.

RESULTS

A Summary of Previous Findings

We have previously carried out a study using T cell-derived material from Lewis normal rats comparing the reactivity of such

material to IgG alloantibodies from Lewis-anti-DA, Lewis-anti-BN and DA-anti-Lewis immune rats. Table 1 shows the findings in a summary form.

Table 1. Binding of single MHC polypeptide chains of various types using B or T cell derived antigen-binding immunosorbants

Type of immunosorbant	MHC chain type: DA			BN			Lewis		
	44K	33K	27K	44K	33K	27K	44K	33K	27K
IgG Lewis-anti-DA	+++	+++	+++	-	-	-	-	-	-
IgG Lewis-anti-BN	-	-	-	+++	+++	+++	-	-	-
IgG DA-anti-Lewis	-	-	-	-	-	-	+++	+++	+++
T Lewis-anti-DA	+++	+++	+++	-	-	-	-	+	-

+++=strong binding; +=weak binding; for details see 11. Note that 27K DA chain are retained in most experiments.

Our conclusion from these results was that idiotypic T cell molecules derived from normal Lewis T lymphocytes in a shed form do display strong allo-MHC reactivity encompassing all three types of MHC chains analyzed (DA) indicating that allo-antigenic determinants must still be expressed on such chains. This was also found true for the three different alloantisera used as measured at the single MHC polypeptide level of relevant Ag-B types. A unique difference between T and B cell derived antigen binding molecules was detected at the level of self-MHC Lewis polypeptide chains where significant but weak binding (expressed as retardation rather than retention) was observed for the heavy Ia chain group of molecules. This binding is not strong enough to be seen when studying the isolated receptor material for direct binding on cells of different MHC types as exemplified in table 2. We know from previous studies that the anti-idiotypic immunosorbant used to purify the "anti-DA" idiotype-positive molecules from normal or immune Lewis T lymphocytes contain anti-idiotypic antibodies reacting with both anti-Ag-B and anti-Ia specific receptors carrying distinct idiotypes (13). As the present T Lewis "anti-DA" material stems from normal rat Lewis serum we believe that the reason for anti-self reactivity being expressed in these studies only at the level of the heavy Ia chains may rather reflect matters of quantity than quality (anti-Ia idiotypic material being more prevalent than anti-Ag-B receptors for reasons of different frequencies of the two T cell populations (14). That this self-reactivity can extend to also include light Ia chains of self-MHC type will become apparent below. It should be remembered,

however, that despite the fact that anti-allo-Ag-B T receptors could be shown to display strong binding for allo-MHC Ag-B heavy chains of correct type we have so far failed to observe any measurable "self-MHC" reactivity in this T receptor group of molecules.

Table 2. T cell receptors isolated from normal Lewis serum by the use of anti-Lewis-anti-DA idiotypic immunosorbants display strong binding for DA cells

Binding material	Percent binding of input material*
Lewis spleen cells	20.6 %
DA spleen cells	81.7 %
BN spleen cells	12.8 %
Anti-Lewis-anti-DA sorbant	86.2 &

*^{125}I-idiotypic Lewis-anti-DA T derived molecules purified as described (11).

From table 2 we would also like to conclude that probably all T molecules with the Lewis-anti-DA idiotypes express antigen binding specificity of the expected type (percentage binding to idiotype-specific immunosorbant versus allogeneic cells of relevant type = DA is the same; we failed to demonstrate that the binding to self = Lewis was significantly higher than to third party = BN MHC when using cells as targets and carrying out several washes after the incubation). Such a strong association between idiotype and antigen-binding specificity is not the normal for most other idiotypic systems studied and may signify that there exist a close to obligatory association between idiotypes of this nature and relevant allo-MHC reactivity on the T cell derived molecules.

We have previously explored the inheritance pattern of the idiotypes detected on T lymphocytes using anti-Lewis-anti-DA idiotype specific antisera raised in LewisxDA F_1 hybrids or Lewis rats (15,16). A summary of the expression of these idiotypes in relation to MHC and heavy Ig chain genes is presented in table 3. As seen two factors are eminent. In order to at all be able to express the idiotypes the animals must carry the same heavy Ig genes (presumably V_H) as the Lewis rats. Secondly, introduction of the DA MHC genes into the host environment causes an absolute suppression of expression of the idiotypes of Lewis-anti-DA type. We have previously interpreted these results to mean that the actual cells with these idiotypes are permanently suppressed or eliminated. Alternative explanations such as absorptions by the overwhelming

amounts to antigen of idiotype-positive material are quite plausible.

We consider that these latter results tell us an important point with regard to flexibility of T cell receptors in relation to idiotypic determinants present on allo-MHC reactive molecules. It would thus not seem possible for the T cell molecules here being analyzed to retain the "Lewis-anti-DA" idiotypes if they could be selected away from strong DA allo-MHC reactivity. This would in essence mean that these idiotypes are antigen-binding site specific ones with regard to the relevant allo-MHC structures. This is important to bear in mind when considering the results of the next sets of experiments.

Table 3. Expression of "Lewis-anti-DA" T cell idiotypes as affected by heavy Ig genes and/or MHC genes*

	MHC composition			
Heavy Ig gene type	Lewis	DA	BN	LewisxDA F_1
DA	-	-	-	-
BN	-	-	-	-
Lewis	+	-	+	-

*+=presence of idiotypes: -=absence of idiotypes. For details see 15, 16.

The Lewis-anti-DA idiotypes are expressed in other strains of rats provided these carry heavy Ig genes of Lewis type and do not carry DA MHC genes. Accordingly we now isolated from large amounts of normal rat serum using the very same immunosorbant anti-Lewis-anti-DA column that was previously used (11) to select idiotype positive T cell derived molecules from adult Lewis and L.BN rats. L.BN is identical to Lewis except that it carries the MHC type of BN. Small immunosorbant columns were prepared of this T "receptor" material comparing the ability of such immunosorbants to retain MHC polypeptide chains of DA, BN or Lewis types. The question being asked was whether the idiotype identical molecules stemming from Lewis versus L.BN serum would still regard Lewis as self-MHC (see table 1, heavy Ia retention) of whether the presence of BN MHC in the L.BN rats would change this reactiivty to include BN rather than Lewis as "self".

The results presented in a summary form of six different batches of T cell derived, idiotypic immunosorbants are shown in table 4. The results are striking and clearcut. As before the Lewis derived "Lewis-anti-DA" molecules besides shows anti-DA string binding activity retarded the passage of significant proportion of self, Lewis heavy Ia chains without dis-

playing any measurable activity for any of the BN MHC polypeptide chains.In contrast,the "Lewis-anti-DA" idiotype positive molecules stemming from normal L.BN rats whilst maintaining their strong anti-DA MHC polypeptide chains now had changed their weak binding activity to BN (the new self-MHC) Ia chains in stead of Lewis.

Table 4. Opportune,weak self-MHC Ia binding activity of T cell derived molecules with the same idiotypic determinants and strong allo-MHC binding ability.

Immunosorbant[x]	MHC chain type[xx]								
	DA			BN			Lewis		
	44K	33K	27K	44K	33K	27K	44K	33K	27K
T,Lewis	62.4	62.4	46.4	31.9	29.1	24.0	27.2	58.2	27.4
T,L.BN	58.8	59.4	51.4	21.9	57.4	47.0	28.1	36.4	30.6

[x]CNBR-Sepharose columns to which T cell derived material with "Lewis-anti-DA" idiotypes from Lewis and L.BN rats respectively had been coupled.

[xx]Percent retention of applied material.Normal serum coupled Sepharose columns retain between 20-35% of applied material. ---= retention; _ _= retardation;both being statistically significant if underlined.Results denote mean of 6 experiments;

DISCUSSION

From the above data we would like to suggest the following scheme:Allo-MHC reactive T cell receptors with strong binding properties for the relevant antigens=true receptors in the biological context have site-related idiotypic determinants against we have raised anti-idiotypic reagents.Such idiotypes can not be changed without in parallel losing allo-MHC reactivity.This may not occur at all or alternatively there now is a loss of any selective force for such T cells as evidenced by their failure to be expressed in animals carrying the allo-MHC genes (=here DA)even if the animals carry Lewis Ig heavy chain genes.However,there exist in the variable regions of such antigen-binding,allo-MHC reactive T cell polypeptide chain the possibilities to select for minor variations during

ontogeny without losing allo-MHC reactivity but now also including select self-MHC reactivity.Data favouring such an interpretation have been presented in tables 1 and 4.We can not at present conclude that this occurs in all of the allo-MHC reactive T cells in a normal individual or even in the majority of the idiotype-positive T lymphocytes.Available data from functional studies of allo- versus self-MHC restricted responses we interpret to mean that the dominating part of strong allo-MHC reactive T lymphocytes indeed have been selected to include a measurable degree of self-MHC binding. Here,passage of T cell receptor material through allo-MHC versus third party and self-MHC immunosorbants would here be required measuring retention and retardation in a quantitative manner.

Nude mice have been reported to contain T lymphocytes with unusual reactivity with regard to MHC antigens,being maybe particularly low in self-MHC restricted T cells (17,18).Such findings in conjunction with the present receptor studies at the molecular level makes us feel that allo-MHC reactivity as studied here reflects germ line genes.Differentiation/selection of T cell specificity at the population level would thus move via "additions" rather than "subtractions" towards self-MHC reactivity.Our results do finally not tell whether T cells use a single antigen-binding receptor type for any kind of antigens.They certainly show,however,that the very same receptor that is being used for allo-MHC reactions can also be shown to be involved in select self-MHC reactions making it a strong candidate to also be directly involved in reactions against conventional,T-dependent antigens.

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REFERENCES

1.Wilson,D.B.,Blyth,I.L.,and Nowell,P.C.,(1968) J.Exp.Med.,128, 1157.
2.Ford,W.L.,Simmonds,S.I.,and Atkins,R.C.,(1975) J.Exp.Med,141, 681.
3.Nisbet,H.W.,Simonsen,M.,and Zaleski,M.(1969) J.Exp.Med.,129, 459.
4.Binz,H.,and Wigzell,H. (1975) J.Exp.Med.,142,1218.
5.Paul,W.E.,Shevach,E.,Thomas,D.W.,Pickeral,S.F. and Rosenthal, A.,(1976) Cold Spring Harbor Symp.Quant.Biol.,41,571.
6.Sredni,B.,and Schwartz,R.H.,(1980),Nature,287,855.

7.Fink,P.J.,and Bevan,M.,(1978),J.Exp.Med.,148,766.
8.Zinkernagel,R.M.,Callahan,G.N.,Althage,A.,Cooper,S.,KLein,P. A.,and Klein,J.,(1978),J.Exp.Med.,147,882.
9.Bevan,M.,(1977),Proc.Nat.Acad.Sci.U.S.A.,74,2094.
10.Finberg,R.,Burakoff,S.,Cantor,H.,and Benacerraf,B.,(1978), Proc.Nat.Acad.Sci.U.S.A.,75:5145.
11.Binz,H.,Frischknecht,H.,Mercolli,C.,Dunst,S.,and Wigzell,H., (1979) J.Exp.Med.,150:1084.
12.Binz,H.,and Wigzell,H. (1976) Scand.J.Immunol.,5:559.
13.Binz,H.,Frischknecht,H.,Shen,F. and Wigzell,H. (1979) J.Exp. Med.,149:910.
14.Ryser,J.E.,and MacDonald,H.R.,(1979) J.Immunol.,122:1691.
15.Binz,H.,Wigzell,H.,and Bazin,H.,(1976),Nature,264:778.
16.Binz,H.,Frischknecht,H.,Mercolli,C.,and Wigzell,H.,(1978), Scand.J.Immunol.,9:167.
17.Hünig,T.,and Bevan,M.J.,(1980),J.Exp.Med.,152:688.
18.Gillis,S.,and Watson,J.,(1981),J.Immunol.,126:4.

THE USE OF HETEROANTISERA TO T CELL ANTIGEN BINDING PROTEINS AS PROBES FOR T CELL RECEPTORS[1]

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INTRODUCTION

T and B lymphocytes respond to an antigenic stimulus in a highly specific manner which involves, initially, an interaction between the antigen and membrane-bound antigen recognition structures (1-3). As a consequence of antigenic stimulation, antigen-induced B cells differentiate into cells which secrete soluble products (immunoglobulins) which have combining sites identical to membrane-bound counterparts which serve as cell surface antigen recognition structures. However, B cell surface immunoglobulins can differ in their constant regions from secreted immunoglobulins. Some T cells also produce soluble molecules which can ligate the inducing antigen (4). However, unlike B cells, the nature of T cell-derived membrane and soluble antigen recognition structures

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has not been elucidated completely. It is evident that in the last ten years the T cell receptor for antigen is not the elusive entity (3) it once was. Nevertheless, it is fair to say that we can still say more about what T cell antigen recognition structures are *not*, than what they are.

Although no immunoglobulin constant region determinants have been unequivocally detected on T cell antigen recognition structures, epitopes associated with immunoglobulin heavy chain (5,6) and in some cases light chain (7) variable regions (V) have been demonstrated on both membrane-bound and soluble T cell derived molecules. Some soluble antigen-specific products derived from T cells (4) bear antigenic determinants encoded in the Major Histocompatibility Complex (MHC). In some cases, MHC-bearing components have been shown to be entities separate from polypeptides which bind antigen and in other instances a single polypeptide chain apparently bears both immunoglobulin V-region and MHC-associated antigens (8). The effector function of antigen-specific molecules released by T cells appears to be immunoregulation; either help or suppression. Detailed structural information, the genetic origin, and structure-function relationships of both membrane-associated and soluble antigen recognition structures derived from T cells remains to be determined. In many ways, we are, with T cell antigen recognition structures, where Immunology was with B-cell derived immunoglobulins fifty years ago.

The strategy employed to study B cell membrane antigen recognition structures was predicated on the hypothesis that B cell surface receptors for antigen are similar to their soluble counterparts. Since B-cell derived immunoglobulins, regardless of their ligating specificity for antigen, possess isotypic antigens, heterologous anti-isotype sera (particularly anti-light chain) were used to identify cell surface immunoglobulins as B lymphocyte receptors for antigens (1,2).

Attempts to demonstrate that T cell antigen recognition structures are related to immunoglobulins (using the same strategy applied to B cells) has had a long and controversial history (3). The difficulty with this approach is that T cell receptors for antigen are clearly not "garden-variety" immunoglobulins. The use of anti-V-region antisera (3,5,6) was clearly a breakthrough in this effort. However, this approach is limited severely by the amount of antigen specific material that might be isolated from T cells and could also restrict the spectrum of T cell receptors being analyzed. An alternative approach has been to purify T cell antigen recognition structures by their specific affinity for antigen (3,9-13). The advantage of this strategy is that the results are not biased towards a particular class of molecules (i.e., immunoglobulin related). However, like the anti-V-region approach, the amount of material isolated is severely limited. Moreover, in many cases, the molecules isolated are soluble products which may not be identical to membrane-bound receptors for antigen. However, drawing on the strategy used for B cells, if T cell antigen recognition structures shared antigenic determinants, "anti-isotype" sera might be used as a more general probe for both cell membrane and soluble T cell antigen receptors.

Our approach to the T cell receptor problem has been to isolate T cell products by their specific affinity for antigen and to produce heterologous antisera to these molecules which can be used as general probes for cell-membrane bound and soluble T cell receptors for antigen. The results of our studies indicate a striking structural and antigenic similarity between soluble T cell antigen recognition structures and their cell membrane analogues.

CHARACTERIZATION OF T CELL POLYPEPTIDES SPECIFIC FOR HAPTENS

To obtain antigen-specific, T cell derived products, we have utilized a modified protocol (14) developed originally by Zembala and Ashershon (15) to generate antigen-specific suppressor factors produced by T cells obtained from mice rendered tolerant to the haptens TNP, DNP (or Oxazalone). In this scheme, mice are injected I.V. with tri- or dinitrobenzene sulfonic acid and are painted subsequently with picrylchloride or DNFB respectively. Spleen and lymph node T cells obtained from these animals release, during a 48 hr culture period, an antigen-specific "factor" (TSF) which inhibits specifically the ability of hapten-sensitized T cells to transfer delayed hypersensitivity to naive recipients. The suppressive moiety can be *specifically* removed by absorption to sepharose beads coupled with TNP or DNP-conjugated bovine gammaglobulin, and can be eluted with the homologous hapten.

Hapten affinity purified TSF labeled biosynthetically with ^{3}H-leucine or ^{35}S-methionine, or by radioiodination is resolved by SDS-PAGE under reducing conditions into 68,000d polypeptide chains. Sizing by gel chromatography indicates that these molecules can occur in a dimeric or monomeric form. The characteristics of these molecules thus far delineated are shown in Table 1. By sensitive radioimmunoassy techniques neither immunoglobulin isotypic nor major histocompatibility determinants have been identified on these molecules. Preliminary evidence suggests that DNP-specific TSF resolved as a single peak by SDS-PAGE exhibits heterogeneity by isoelectric focussing and binds specifically to DNP with a binding constant of approximately 1 x 10^{5}k Cal/mole. As will be discussed further below, TSF shows marked sensitivity to proteolytic degradation with major cleavage products of 45,000

TABLE 1 CHARACTERISTICS OF TSF

	Property	Assays
1.	Antigen Specificity (14)	
	DNP	Bioassay, rebinding of radio-
	TNP	labeled molecules to hapten,
	Oxazalone	Equilibrium dialysis
2.	Molecular size (14)	
	68,000d	SDS-PAGE, gel chromatography
	140,000d	gel chromatography
3.	Sensitivity to Proteolysis (14)	
	Extremely sensitive, major breakdown products are 45,000 and 25,000d polypeptides	
4.	Blocked N-terminus (14)	N-terminal analysis
5.	Isoelectric Point	
	Multiple peaks	1-dimensional isoelectric focussing in polyacrylamide gels
	Broad band at 68,000d	2-D gel analysis
6.	Cytophilic for Macrophages (14)	Bioassay, binding of radiolabeled TSF to cells
7.	Immunoglobulin determinants (14)	
	Negative for kappa or lamda isotypes and IgM, IgA, IgG, IgD or IgE heavy chain isotypes	Radioimmune assay
8.	Alloantigenic determinants (14)	
	Negative for Ia, I-J Lyt1, Lyt2	Radioimmune assay. Absorption to antibody-conjugated Sepharose beads

and 25,000d. The hapten-specific molecules whose properties are outlined in Table 1 show marked similarities to antigen-specific products we have isolated from T cells sensitized *in vitro* to heterologous erythrocyte antigens (11).

USE OF HETEROLOGOUS ANTISERA TO T CELL ANTIGEN BINDING PROTEINS AS PROBES FOR THEIR MEMBRANE ANALOGUES

To characterize cell membrane analogues of the proteins described above, we have immunized rabbits with hapten-affinity purified, DNP-specific TSF. We reasoned that, by analogy with immunoglobulins, heteroantisera to TSF might contain antibodies which recognize determinants shared by T cell antigen recognition structures having diverse antigen binding specificity.

Rabbit anti TSF (RaTSF) has been generated which binds to soluble, radiolabeled TSF or to microtiter trays coated with TSF. Although the antisera do not contain antibody activities to immunoglobulin isotypic determinants or to mouse albumin, competition radioimmunoassay has demonstrated that mouse serum contains TSF+ material at approximately 200-500 ng/ml. Absorption of RaTSF by thymocytes and splenic T cells but not splenic B cells removes antibody binding activity for TSF indicating the presence of TSF determinants on the T cell surface. In agreement with the absorption studies, indirect immunofluorescence analysis of the lymphocyte-binding ability of RaTSF (15) indicates that the antiserum binds to 80% of thymocytes (with dull fluorescence), 60-70% of splenic T cells (bright fluorescence) but does not bind to B cells. Within the splenic T cell pool, all $Lyt2^+$ cells are stained brightly by this reagent while 50% of $Lyt1^+$, 2 cells are stained with dull fluorescence. These results suggest that either all $Lyt2^+$ cells and only some $Lyt1^+$ cells

bear cell surface molecules which share antigenic determinants with soluble TSF or that the site density and/or display of molecules recognized at the cell surface by anti-TSF differs for $Lyt1^+$ and $Lyt2^+$ cells.

The molecular identity of cell surface molecules bound by RaTSF has been ascertained by immunoprecipitation of radioiodinated proteins present in detergent lysates obtained from thymocytes, spleen or splenic T cells labeled by lactoperoxidase-catalyzed radioiodination. RaTSF specifically precipitates radiolabeled cell surface proteins obtained from thymocytes, spleen cells and splenic T cells, but not splenic B cells. Inclusion of 5 µg hapten-affinity purified (DNP-specific) TSF during immunoprecipitation of cell surface proteins blocks completely the precipitation of ^{125}I-labeled membrane proteins by RaTSF, providing further evidence for shared determinants between TSF and T cell surface proteins.

As shown in Fig. 1, the SDS-PAGE profile of reduced and alkylated T cell membrane proteins bound by RaTSF varies depending on how the proteins are precipitated. When the RaTSF membrane protein complexes are precipitated with sheep anti-rabbit immunoglobulin serum, the major molecular species detected have molecular weights of 45,000 and 25,000d. However, when the complexes are insolubilized with *S. aureus* Cowan Strain I (Protein A) or the membrane proteins are absorbed to RaTSF coupled to Sepharose 4B beads, the major molecular species isolated are 68,000d. Cross-clearing experiments using RaTSF and sheep anti-rabbit Ig serum in a first precipitation and RaTSF-Sepharose in a subsequent precipitation (or vice versa) indicates that the membrane proteins resolved by each precipitation method are the same species in different form. Support for this contention was obtained when it was observed that immunoprecipitation of 68,000d TSF with RaTSF

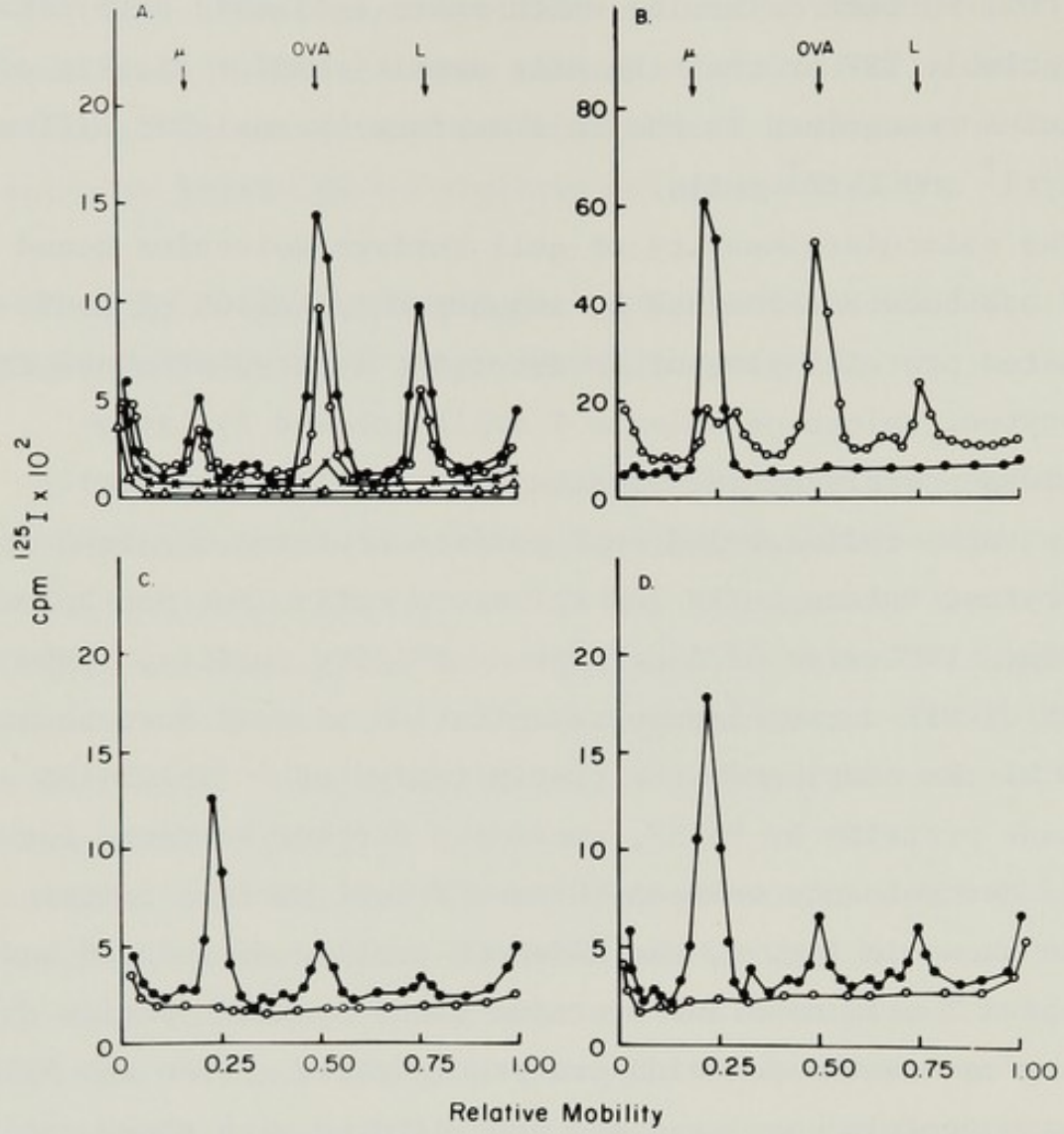

FIGURE 1. Immunoprecipitation of Lymphocyte Cell Membrane Proteins or TSF With RaTSF. ^{125}*I-labeled membrane proteins from T cells labeled by surface radioiodination (A,C,D) or* ^{125}*I-labeled TSF (B) were precipitated with: (A) sheep anti-rabbit Ig serum and RaTSF or NRS; (o-o)splenic T cells + RaTSF, (●-●)thymocytes + RaTSF, (x-x)splenic T cells + NRS, (Δ-Δ)thymocytes + NRS; (B) (●-●)* ^{125}*I-TSF, (o-o)* ^{125}*I-TSF precipitated with sheep anti-rabbit Ig serum and RaTSF; (C) (●-●)splenic T cell membrane proteins bound by RaTSF + S. aureus, (o-o) NRS + S. aureus; (D) (●-●)splenic T cell membrane proteins bound by RaTSF-Sepharose, (o-o)NRG-Sepharose.*

and sheep anti-rabbit Ig serum (panel B, Fig. 1) results in degradation of the molecule into 45,000 and 25,000d species.

Fractionation of ^{125}I-labeled splenic T cell or thymocyte membrane proteins by gel chromatography in detergent

followed by immunoprecipitation of column fractions with RaTSF Sepharose beads (Table 2) demonstrated that the RaTSF-bound proteins can occur in a dimeric form (150,000d) and a monomeric form. Resolution of the membrane proteins isolated from the column fractions by SDS-PAGE suggests that the dimeric forms may be covalently or non-covalently linked. However, inclusion of iodoacetamide in the detergents used to lyse the cells prevents occurrence of covalently linked dimers, suggesting that these structures may be formed by disulfide interchange during extraction and/or immunoprecipitation. The latter possibility is suggested further by the detection of covalently linked dimers obtained from column fractions corresponding to 70,000d species. It seems likely that such dimers were formed during immunoprecipitation.

TABLE 2 MOLECULAR SIZING OF CELL SURFACE PROTEINS BOUND BY RaTSF[a]

Column Fractions	*Polypeptides in SDS-PAGE (daltons)*	
Sephadex G-200	*Non-reduced*	*Reduced*
Void Volume	*150,000*	*68,000*
		30,000
IgG marker position	*140,000*	*68,000*
		45,000
		15,000
BSA marker position	*140,000*	*68,000*
	70,000	*35,000*
Sephacryl 200-S		
>BSA marker	*150,000*	*70,000*
	70,000	
BSA marker position	*150,000*	*70,000*
	70,000	

[a] *^{125}I-labeled, detergent soluble splenic T cell membrane proteins were fractionated by chromatography in Sephadex G-200 or Spehacryl 200-S equilibrated in 0.1% Triton X-100. Column fractions were mixed with RaTSF-Sepharose beads. ^{125}I-labeled membrane proteins bound by the beads were eluted with SDS and resolved by SDS-PAGE.*

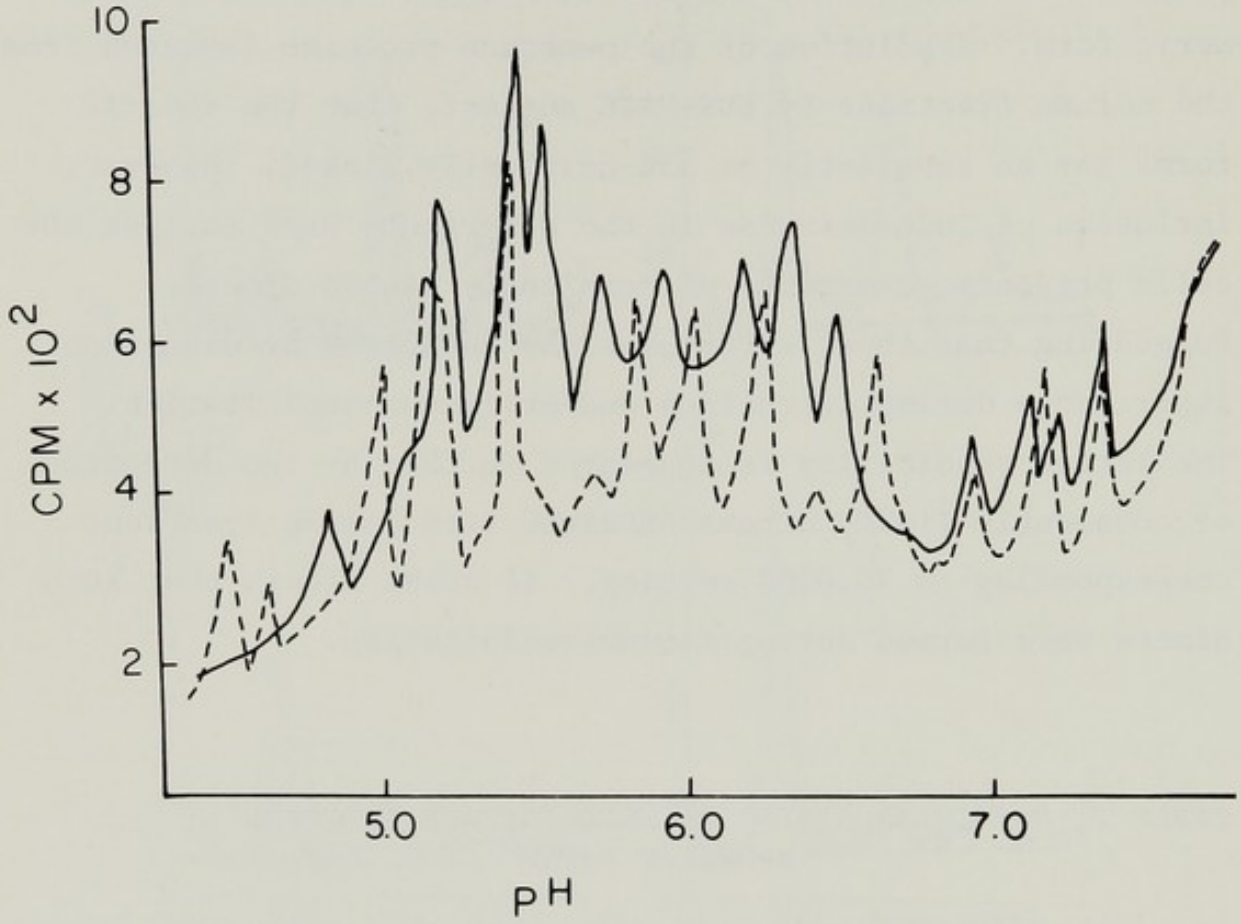

FIGURE 2. Isoelectric Focussing of Surface Radiolabeled T Cell Proteins Bound by RaTSF. Splenic T cells from BALB/c (—) and CB20 (--) mice were labeled with ^{125}I and ^{131}I respectively. The labeled cells were mixed, lysed and membrane proteins were bound to RaTSF-Sepharose. Bound proteins were eluted and resolved by isoelectric focussing in polyacrylamide gels.

Membrane proteins bound by RaTSF have also been subjected to isoelectric focussing (IEF) to ascertain charge heterogeneity and possible polymorphism. Fig. 2 shows the results of studies in which BALB/c and CB20 splenic T cells were labeled with ^{125}I and ^{131}I respectively. The labeled cells were mixed, lysed with Triton-X-100 and the lysates absorbed

to RaTSF-Sepharose. SDS-PAGE of the labeled proteins bound by RaTSF resolved 85% of the labeled polypeptides into a peak corresponding to 68,000d. However, the IEF pattern revealed marked charge heterogeneity. Moreover, some polymorphism in this pattern was observed since at least 50% of the resolved peaks of radioactivity for BALB/c and CB20 were not co-incident. Control experiments in which ^{125}I and ^{131}I labeled BALB/c cells were treated similarly resulted in no more than 10% of the peaks being non-coincident.

ANTISERA SPECIFIC FOR OTHER ANTIGEN SPECIFIC (T CELL) PRODUCTS BIND THE SAME CELL SURFACE PROTEINS

The observation that RaTSF possessed binding activity for many (but not all) T lymphocytes suggests that antigenic determinants carried by DNP-specific T cell-derived polypeptides were expressed by many T cell surface receptors.

TABLE 3 DIFFERENT ANTIGEN SPECIFIC (T CELL DERIVED) PRODUCTS SHARE ANTIGENIC DETERMINANTS

Antigen-Binding Protein	*Antiserum Binding*[a]		
	RaTSF	*Serum 2318*[b]	*Serum 801*[c]
Oxazalone-specific TSF	+	+	+
DNP-specific TSF	+	+	+
TNP-specific TSF	+	+	+
SRBC-specific (T cell clone) TSF[d]	+	*N.T.*	*N.T.*
Lewis anti DA, Ig^-, Id^+, 70Kd protein[e]	+	+	+

[a] *Binding determined by immunoprecipitation in solution or binding to coated plates.*
[b] *Sheep anti-mouse SRBC-specific, 70Kd TSF (Provided by Drs. C. Janeway and J. Mattingly).*
[c] *Rabbit anti-rat (Lewis anti-DA) allospecific, Ig^-, Id^+, 70Kd protein provided by Drs. H. Binz, H. Frischknecht and H. Wigzell.*
[d] *Material isolated and test done by Dr. M. Fresno, Harvard University.*
[e] *Provided by Drs. H. Binz, H. Frischknecht and H. Wigzell.*
+ = positive binding activity; N.T. = not tested
SRBC = sheep erythrocytes

Further support for a putative "anti-isotypic" nature of this antiserum was obtained when it was found that RaTSF would bind to soluble, T cell derived products with specificity for TNP, Oxazalone and sheep erythrocytes (Table 3). Of particular note is that RaTSF binds to a rat T cell-derived, allo-specific polypeptide isolated by absorption to anti-idiotype antibodies. Conversely, antisera against other antigen specific T cell products bind TSF. Thus, murine and rat T cell soluble antigen recognition structures share antigenic determinants recognized by rabbit or sheep antisera to different T cell derived antigen-specific molecules.

A similar situation obtains for murine T cell membrane proteins bound by these antisera. As shown in Table 4, radio-labeled T cell surface proteins isolated by absorption and elution from RaTSF-Sepharose are bound to rabbit antisera to rat T cell antigen-specific molecules and sheep antisera to SRBC-specific murine T cell polypeptides.

TABLE 4 T CELL MEMBRANE PROTEINS ISOLATED WITH RaTSF ARE BOUND BY ANTISERA TO OTHER ANTIGEN-SPECIFIC T CELL PRODUCTS

Antiserum	*CPM Bound RaTSF-Isolated Membrane Proteins*
Anti-TSF (SEPH)	2.6×10^4
RGG (SEPH)	0.4×10^4
2318 (SEPH)[a]	2.0×10^4
NSG (SEPH)[a,b]	0.5×10^4
801[b]	2.1×10^4
NRS[b]	0.5×10^4

^{125}I-labeled membrane proteins were extracted from splenic T cells by detergent lysis. Labeled membrane proteins were absorbed to RaTSF-Sepharose beads and eluted with 0.2m $NaCO_3$ buffer. After dialysis into 0.05% Triton X-100-Tris Cl buffer (pH 8.0), the labeled proteins were tested as above.

[a] *See Table 3 for characteristics.*

[b] *Assay by immunoprecipitation with sheep anti-rabbit Ig serum.*

NSG = normal sheep Ig

NRS = normal rabbit serum

Moreover, immunoprecipitation of radiolabeled cell membrane proteins with one reagent removes the majority of cell surface polypeptides bound by the others.

DISCUSSION AND CONCLUSIONS

The studies described in this brief review have attempted to clarify the molecular basis of T lymphocyte recognition of antigen by characterizing T cell products which bind antigen and generating antisera to these molecules which could be used as general probes for T cell membrane antigen recognition structures. The structural characteristics of soluble T cell polypeptides specific for the haptens TNP, DNP or oxazalone are similar to those described for allospecific murine (16) or rat T cell (6) products and for heterologous erythrocyte antigens (11). Antigen-specific T cell products larger (10) or smaller (15) than 68,000d have also been reported. Quite possibly variations in size may be due to the susceptibility of these molecules to aggregation and/or degradation. In addition, it is conceivable that several classes of T cell antigen recognition structures exist and (by analogy to immunoglobulin heavy chains) these classes may differ in size.

Several lines of evidence have been developed which suggest that T cell antigen recognition structures may share isotypic determinants. Rabbit antibodies raised against DNP-specific 68,000d polypeptides bind to antigen-specific (T cell derived) molecules of diverse specificities. The fact that such antibodies also bind rat T cell derived molecules indicates further a high degree of conservation of T cell antigen recognition molecules. Conversely, rabbit and sheep antibodies to other antigen-specific (T cell) products shows similar "broad-spectrum" reactivity. Moreover, RaTSF binds to a majority of splenic T lymphocytes and binding activity

for TSF can be removed by absorption with relatively few T cells. The preferential reactivity of RaTSF for $Lyt2^+$ cells could be due to differences in density, display and/or membrane dynamics of $Lyt2^+$ vs. $Lyt1^+$, 2^- receptors. On the other hand, some $Lyt1^+$, 2^- cells may have receptors of a different isotype from $Lyt2^+$ cells.

The structural properties of cell surface proteins bound by RaTSF bear striking similarities to soluble TSF and T cell and T cell-derived serum proteins which have been isolated with anti-idiotype antibodies (6,15,16). The predominant molecular species bound by RaTSF are 68,000d polypeptide chains which can occur in monomeric and dimeric form. (Whether one of these species is an artifact of preparation remains to be determined.) The occurrence of lower molecular weight species does, however, appear to be due to degradation which occurs during the isolation procedure. Moreover, the cell surface molecules bound by RaTSF exhibit charge heterogeneity by IEF. Comparisons of the IEF pattern of RaTSF-bound proteins obtained from mice which differ only in allotype indicates polymorphism in these proteins. This procedure should prove useful in ascertaining the genetic origin of these molecules.

Since soluble TSF completely blocks the binding of RaTSF to cell surface proteins, these proteins clearly share antigenic determinants with an antigen-specific T cell product. Moreover, sheep antisera to murine T cell, 70,000d SRBC-specific suppressor factors and rabbit antisera to 70,000d rat T cell-derived allospecific polypeptides bind to TSF and to cell membrane proteins isolated by RaTSF. These results provide further evidence for a commonality in antigenic determinants between different T cell antigen recognition structures and support further our contention that the membrane proteins bound by RaTSF represent T cell surface

receptors for antigen. Whether the soluble antigen recognition molecules are identical to the cell membrane structures or represent circulating analogues (like serum vs. surface immunoglobulins) remains to be determined.

REFERENCES

1. Warner, N.L. (1974). *Adv. Immunol. 19*:67.
2. Vitetta, E.S., Pure′, E., Isakson, P., Buck, L. and Uhr, J. (1980). *Immunol. Rev. 52*:211.
3. Cone, R.E. (1981). *Progr. Allergy*. In Press.
4. Tada, T. and Okumura, K. (1979). *Adv. Immunol. 26*:195.
5. Eichmann, K. (1978). *Adv. Immunol. 26*:195
6. Binz, H. and Wigzell, H. (1976). *Scand. J. Immunol. 5*:559
7. Lonai, P., Ben-Neriah, Y, Steinman, L. and Givol, D. (1978). *Eur. J. Immunol. 8*:827.
8. Taniguchi, M. (1980). *Transplant. Proc. XII*:423.
9. Cramer, M. and Krawinkel, U. (1980). In: Regulatory T Lymphocytes (B. Pernis and H. Vogel, Eds.) Acad. Press N.Y. pg.39.
10. Goodman, J.W., Lewis, G.K., Primi, D.,Hombeck, P. and Ruddle, N.H. (1980) *Mol. Immunol. 17*:933.
11. O'Connor, S., Eardley, D., Shen, F-W, Gershon, R.K. and Cone, R.E. (1980) *Molec. Immunol. 17*:913.
12. Callahan, H.T. and Maurer, P.H. (1980). *Molec. Immunol 17*:913.
13. Pacifico, A. and Capra, J.D. (1980). *J. Exp. Med. 152*: 1289.
14. Rosenstein, R.W., Cone, R.E., Murray, J.H., Ptak, W. and Gershon, R.K. (1981). *Proc. Nat. Acad. Sci. (USA)* In Press.
15. Zembala, M. and Asherson, G.L. (1974). *Eur. J. Immunol 4*:799.

16. Rubin, B., Bourgois, A., Kahn-Perles, B., Suzan, M. Boyer, C. and Schiff, C. (1981). *J. Supr. Mol. Struct. and Cell. Biochem. Suppl. 5* (abstr. 171).

17. Krupen, K., Areneo, B., Kapp, J., Stein, S., Wieder, K. and Webb, D. (1981). *J. Supr. Mol. Struct. and Cell. Biochem. Suppl. 5* (Abstr. 118).

USES OF ANTIFACTOR ANTISERA AND MONOCLONALS IN THE ANALYSIS OF T CELL PRODUCTS

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ABSTRACT

Serological analysis of T cell factors using rabbit or mouse antisera has yielded a two region model of antigen specific factors, with a constant and variable region, closely akin to the general model of immunoglobulin structure.

To avoid problems of specificity of these sera, and permit their more extensive use, attempts were made to convert them into monoclonals, which would provide a stable source of the same reagent for a variety of studies.

Rat x mouse hybridomas producing antifactor antibodies were generated and used for the biochemical analysis of factors and in developing new assays of T cell function, such as rosettes and reverse plaques.

INTRODUCTION

The fact that T cells produce meager amounts of their mediators, commonly termed 'factors', has greatly hampered detailed analysis of their structure and function. However, progress has been made chiefly by serological analysis, and it is now accepted (1) that factors have a variable region, which bears serological determinants in common with immunoglobulin-idiotypic (2) and framework V_H markers (2). The presence of a constant region is becoming accepted (3). Although its exact nature is still not known, circumstantial evidence points strongly to the constant region of factors being related to the Immunoglobulin gene cluster. Thus we and many others have found over the years that some anti Ig sera react with factors (e.g. 4,5). More recently we have found that some but not all anti human β_2M antisera, raised in different species, react with helper and suppressor factors produced in mice or monkeys (6), suggesting that T cell factors, belong to the same ancestral gene family as Ig. In the accompanying paper, we demonstrate that antifactor sera, raised in allogeneic mice, react with T cells in an IgC_H allotype restricted manner (7). Owen has obtained data that suppressor T cells bear IgC_H allotype-linked determinants (8). If, as seems likely, these are related to T cell receptors for antigen, this would add to the evidence that there is a gene cluster, related to the IgC_H gene, which controls specific T cell product(s) (receptor/factor) in constant regions.

The analysis of antigen-specific T cell products would be greatly enhanced if the antisera against factors were of undoubted and reproducible specificity. Hence we began to convert antifactor

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antisera to monoclonals, and report here our success in the generation of an anti helper factor and an antisuppressor factor monoclonal.

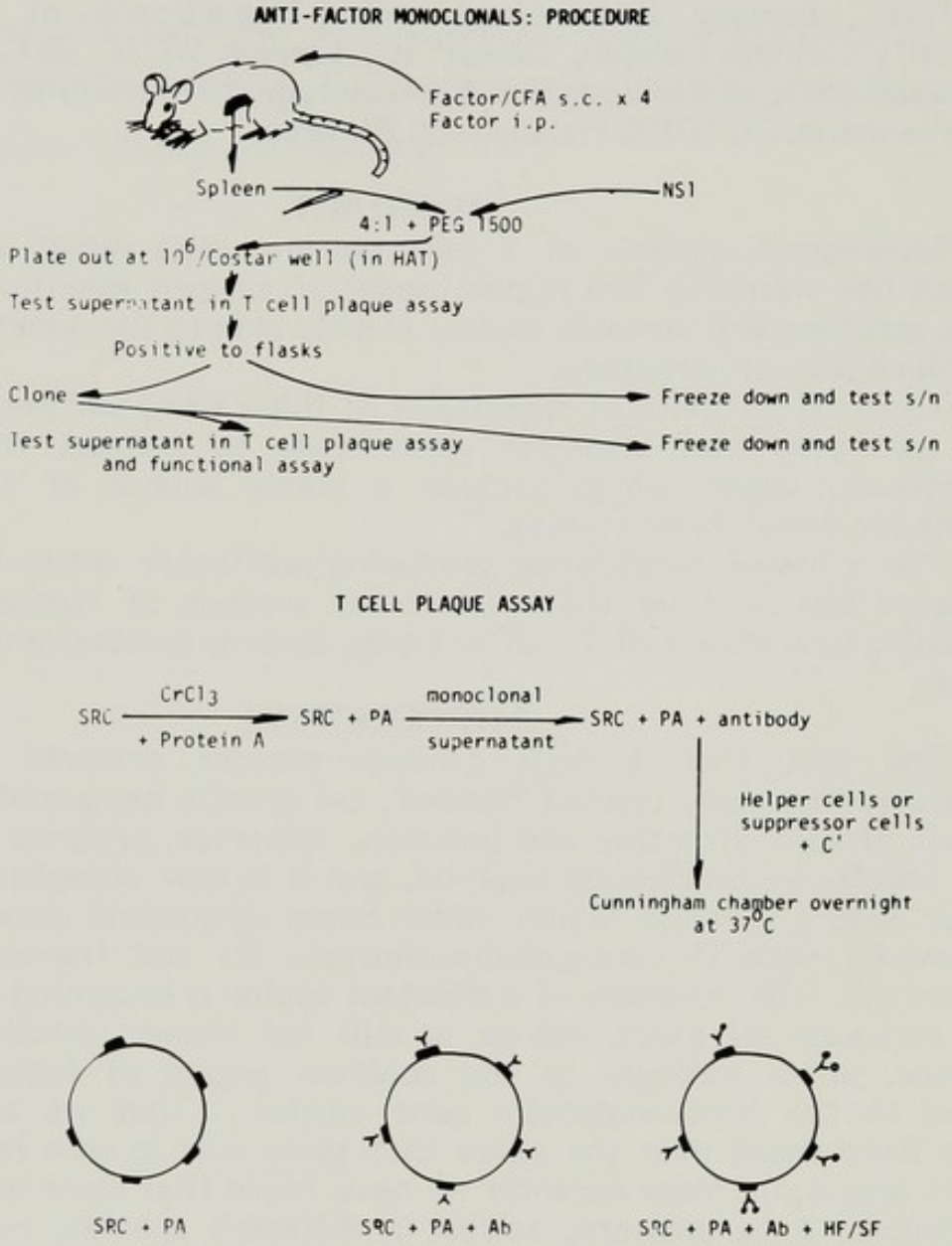

Fig. 1 Protocol for generating and screening anti-factor monoclonals.

METHODS

Culture techniques were described previously. Monoclonal rat antibodies against mouse helper factor (HF) or suppressor factor (SF) were prepared as summarized in Fig. 1. The screening procedure for detecting antifactor antisera and monoclonals was a reverse plaque procedure (9), summarized in Fig. 1.

Biochemical analysis of the products of a helper hybridoma, E1.6c12, was performed as described in detail elsewhere (10).

Briefly, El.6 cells were washed extensively in BSS containing 2ME, incubated for 2 hours with 0.25 mCi ^{35}S-methionine (Amersham) at 25 x 10^6/ml. Cells were then diluted in RPMI-1640 containing 10% foetal calf serum and 5 x 10^{-5} M 2ME and incubated for a further 18 hours. Labelled cells were washed thrice in BSS and digested in 0.25% NP-40 in Tris-buffered saline pH 7.0 for 20 minutes on ice. Insoluble material was removed by centrifugations. 20 µl of rabbit anti-HF was added to 300 µl for 18 hours at 4°C, and the complexes precipitated with 200 µl 10% Staph. aureus (Cowan-I strain). The precipitates were washed 5 times in NP-40 Tris saline and 100 µl SDS-urea added. This was boiled for 9 minutes; 50 µl of 3 X Laemmli buffer added, and polyacrylamide gel electrophoresis in the presence of 10% sodium dodecyl sulphate performed on individual tube gels. Gels were cut into 1 mm slices, samples eluted, and counted.

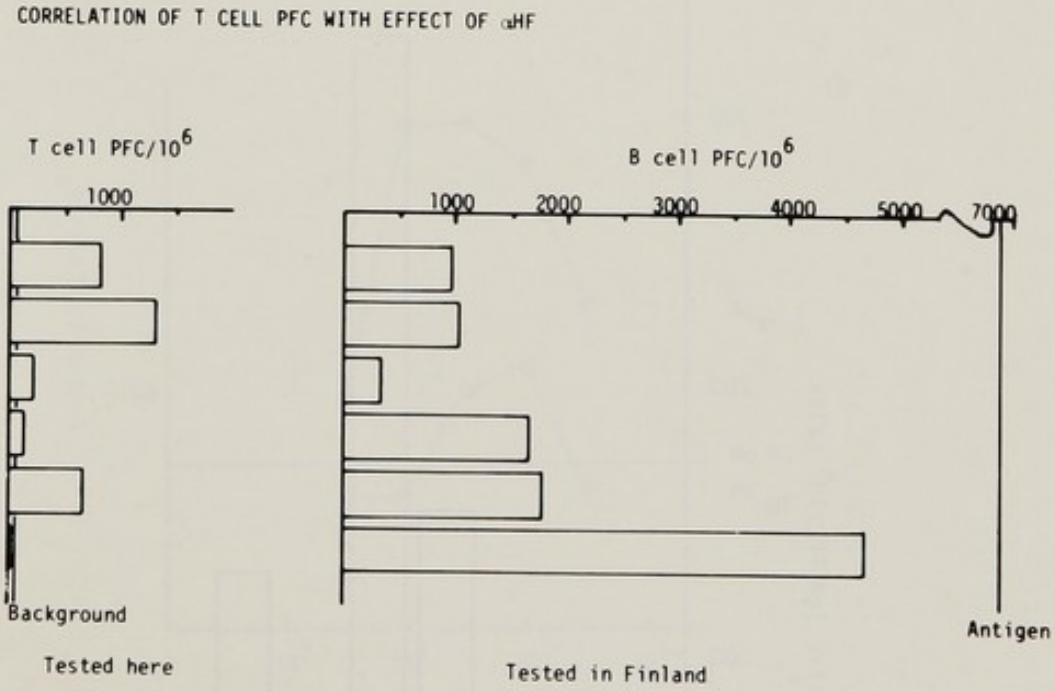

Fig. 2 Correlation of reverse T cell plaques with anti-helper factor effect. T cell plaques tested on cells from in vitro cultures, four days after initiation. B cell plaques on spleen cells of mice primed three times with TNP-KLH.

RESULTS

Detection of antifactor monoclonals

Having developed a reverse plaquing procedure for the detection of T cells releasing factors (Makidono et al., in preparation) using antifactor antibodies coupled to red cells, the same technique was used to screen for antifactor antibodies, using the supernatants

of rat x mouse fusions. From each fusion several wells were positive and these were cloned and retested.

Putative monoclonal antifactor supernatants were tested for their biological activities on antibody responses in vitro and in vivo. As shown in Fig. 2, there is a good correlation between the detection of T cell reverse'plaques' with an anti HF, and diminution of the IgG response to TNP KLH in vitro of primed mice and (not shown) secondary IgM response.

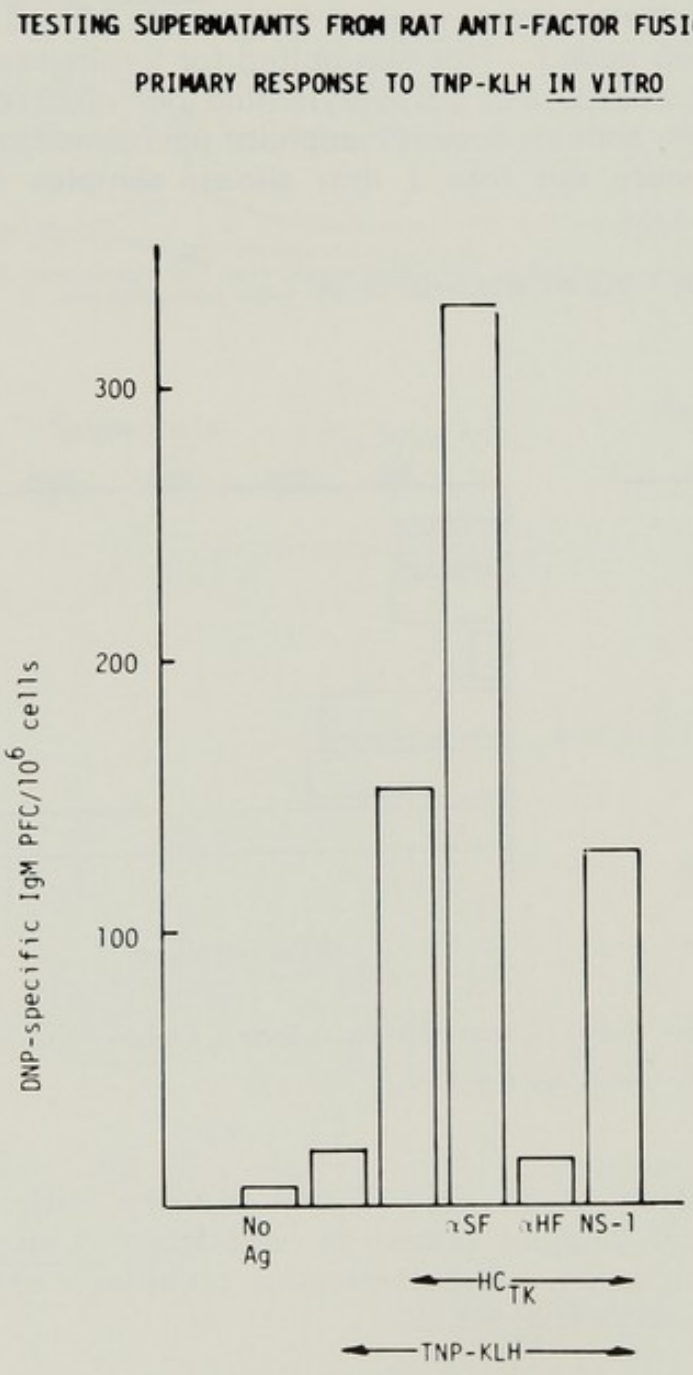

Fig. 3 In vitro effect of anti-factor monoclonals. PrimaryHC induced in vitro with KLH for four days, assayed on anti-theta treated unprimed spleen cells in vitro. TNP-KLH 0.1 μg/ml, anti-SF, anti-HF, NS-1 supernatants put into B cell culture at 1% (final v/v).

In other tests, the same supernatants were tested in in vitro primed helper cells and unprimed B cells, and the response was also inhibited (Fig. 3).

Monoclonal antifactor reagents may be a useful probe for the activity of these molecules in the antibody response in vivo. For this purpose we modified a protocol described by Sprent (11), injecting nylon wool purified T cells, together with sheep red cells (SRC), and antifactor into irradiated (850 rad, ^{60}Co source) mice. Five days later, their spleen cells were used as a source of helper cells for in vitro assays of the response to SRC. The results were quite clear-cut - there was virtually no help from T cells exposed to anti HF in vivo (Fig. 4).

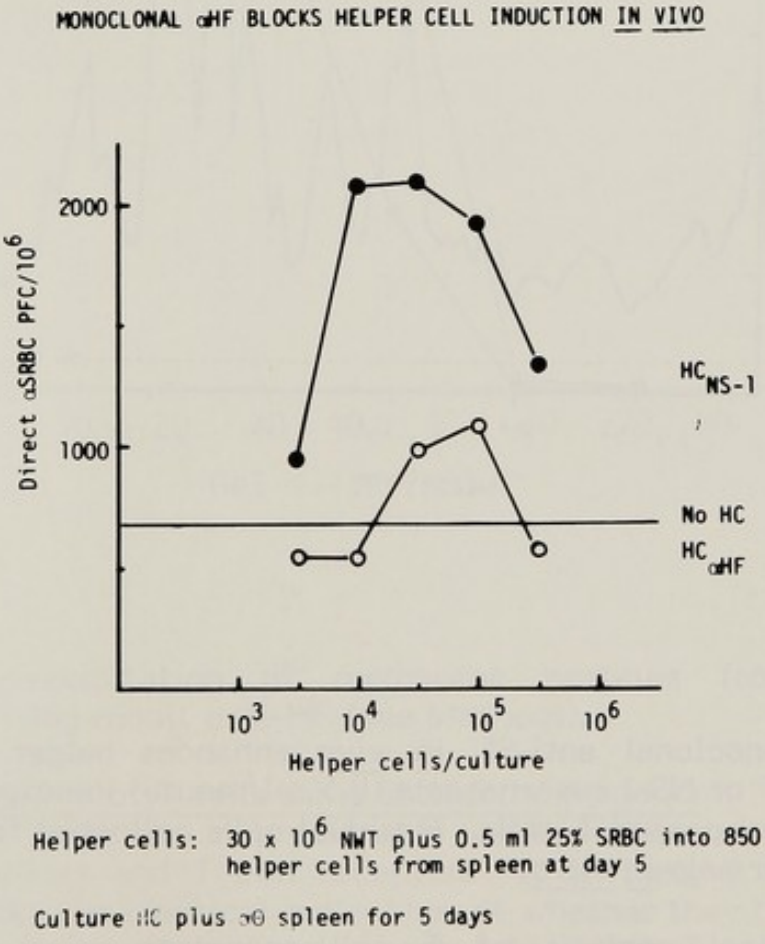

Fig. 4 Monoclonal anti-HF blocks helper cell induction in vivo. Anti-HF or NS-1 supernatants (0.5 ml/mouse) injected together with nylon wool passed T cells. Residual cells collected from spleen assayed in vitro.

In the same in vivo induction of help, anti SF augmented help, especially at low numbers of cells. The magnitude of the effect is not dramatic, but this is not a system which yields much suppression.

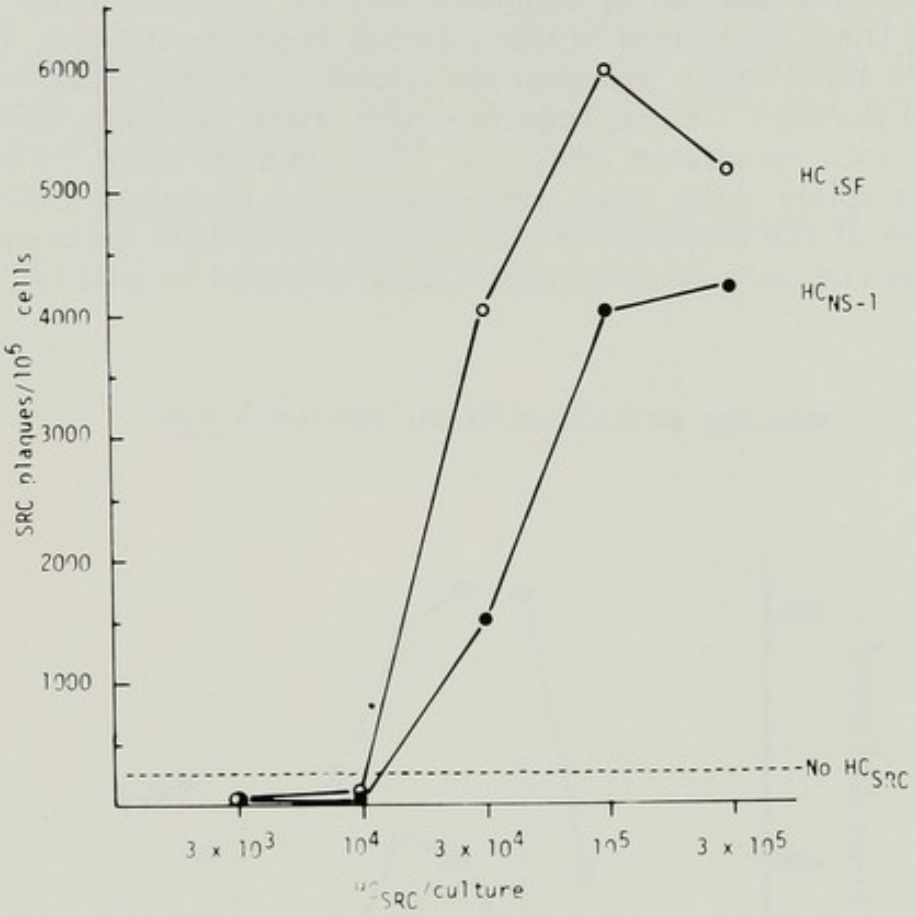

Fig. 5 Monoclonal anti-SF in vivo enhances helper effect in vitro. Anti-SF or NS-1 supernatants (0.5 ml/mouse) injected together with nylon wool passed T cells. Residual cells collected from spleen and assayed for help in vitro.

Biochemical analysis of T cell receptors using antifactor reagents

We have used antifactor reagents to examine T cells, both normal and T hybrids, for receptors and secreted factors. Depending on the exact cell used, different high molecular weight peaks were precipitated, ranging in molecular weight from 95,000 to 45,000. Results with a KLH-specific T cell hybridoma (E1.6c12) are shown in Fig. 6. The cells were labelled for two hours and chased for 18 hours, prior to digestion in).25% NP-40. Precipitation of two major peaks, of 68,000 and 60,000 daltons, with rabbit anti-HF is shown. From this cell line, analogous peak can also be precipitated using a monoclonal anti-HF (44.4).

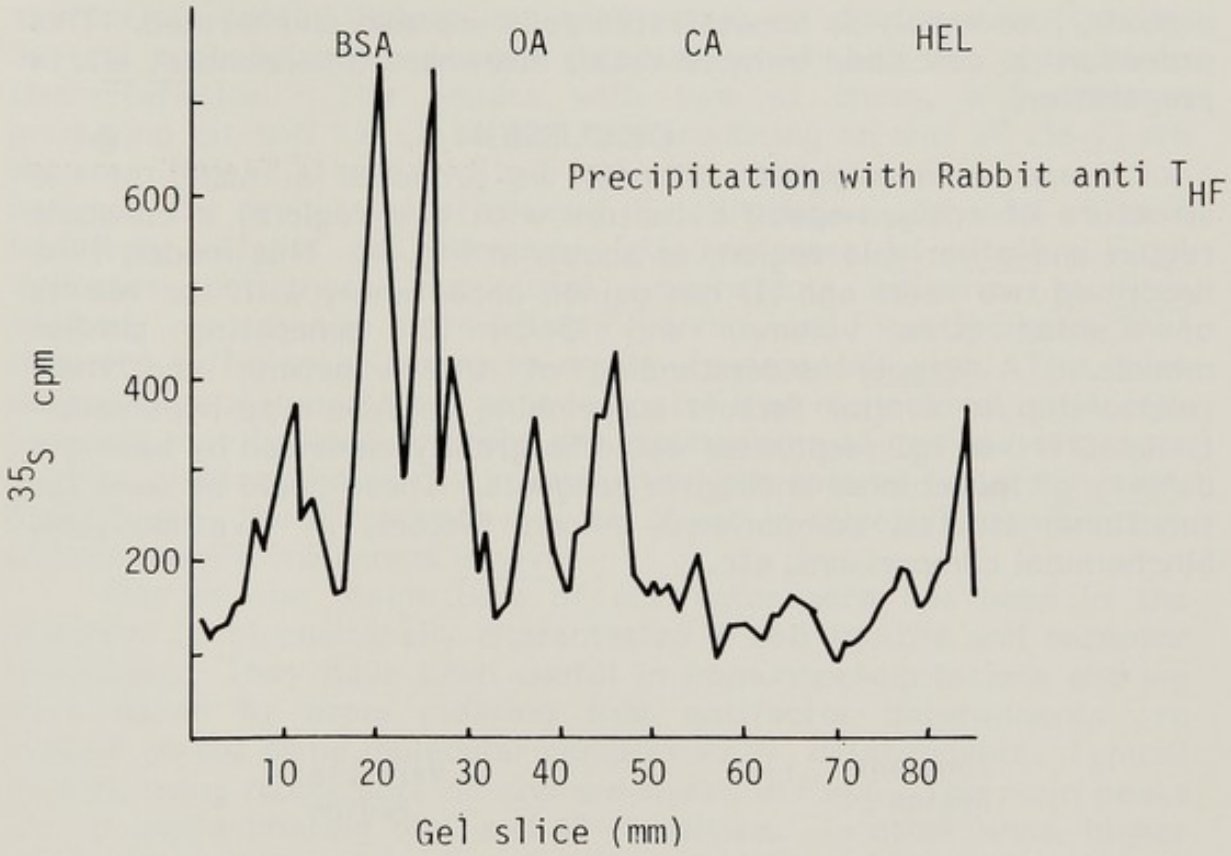

Fig. 6 Precipitation of membrane proteins from a helper hybridoma using rabbit anti-HF (see Methods).

New assays of T cells using antifactor reagents

While there is a predictable relationship between T cell released factors and T cell receptors for antigen, it is not known whether these are identical molecules, or whether they bear the same relationship as membrane and secreted Ig. Both T cell factors and receptors bear idiotypic V_H markers, and both react with antifactor antisera. It was thus of interest to determine whether antifactor antisera may be used to enumerate T cells rapidly shedding factor, or having factor determinants on their surface.

Cells shedding factor were detected by reverse plaques performed as shown in Fig.1. Despite much calibration and titration of conditions of coupling of Protein A, antifactor antibody, complement, red cell concentration and time of incubation, we find that the procedure still has problems of sporadic unexplained failure, chiefly due to lysis of all the red cells, and is time consuming and

cumbersome. However it does offer a new method of accurately enumerating activated T cells. This procedure is described in detail elsewhere (Makidono et al., in preparation).

Cells bearing factor determinants were enumerated by a rosette procedure. This detects many more cells than the reverse plaques, presumably as nonactivated cells are also enumerated. This procedure is described in more detail elsewhere (Makidono et al., in preparation).

DISCUSSION

Based on serological criteria, we proposed a model of the structure of antigen-specific factors with two regions, a constant region and a variable region, as shown in Fig. 7. This model, first described two years ago (1) has gained acceptance, with the results of Cantor (this volume) and Owen (8) generating similar models. A deeper understanding of these factors, and their relationship to similar factors suppressing delayed-type hypersensitivity (DTH) or IgE responses; would be greatly enhanced by having a battery of monoclonal antifactor reagents. These could be used for functional studies, comparisons among factors, in vivo analyses, biochemical comparisons, etc.

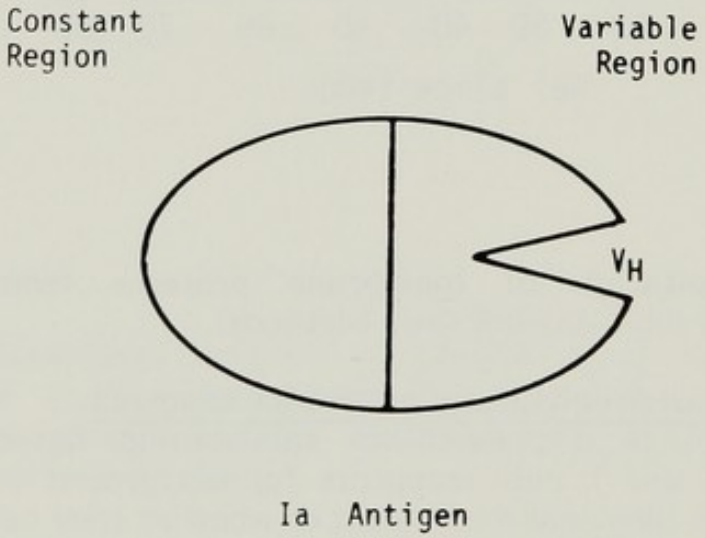

Fig. 7 Two region model of factor. The role of Ia antigens is not clear.

Monoclonal antibody reagents would not have problems associated with antisera, such as the presence of activity against the memrbane antigens (e.g. anti-H-2, anti-Ly), immunoglobulin, viral proteins or carbohydrates. Thus two years ago we embarked on the problem of converting the 'class' specific rabbit antifactor antisera, which discriminate between HF and SF, into monoclonals. As rabbit cells do not yield stable hybrids, we first ensured that rats immunized with factor, yielded factor class-specific antibodies.

The first major problem to overcome was the screening of hybridomas for antifactor activity - we chose two methods which though laborious would detect all such antibodies - effects of supernatants on in vitro immune responses, and the capacity to yield reverse plaques using Protein A coupled SRC and T cells releasing the appropriate factor (helper and suppressor), as discussed in Results. Several such hybridomas were detected which had the appropriate characteristics. The results with two of these, a hybridoma producing rat anti HF (1-13) and one producing an anti SF (36-1) are shown in Figs. 2, 3 and 4. Fig.2 indicates that there is a correlation between the production of reverse T cell plaques with anti HF and inhibitory effects on secondary IgG responses in vitro. Fig.3 indicates that these monoclonal supernatants influence in vitro antibody responses in a predictable manner, and Fig.4 indicates that they are sufficiently potent to modify responses in vivo. This is an observation of importance, as it indicates that monoclonal antifactor reagents may be used to probe the mechanisms of immune responses in vivo, and may be of use in evaluating the role of factors in vivo. Conceivably, these reagents may be of use in selective manipulation of immune responsiveness in vivo.

One of the major uses of antifactor sera has been in the attempts to biochemically characterize T cell factors and receptor molecules. They have been useful in immunoprecipitations and we have shown by cross clearing that antifactor determinants are present on the same molecular complex as V_H determinants. Typical results, using rabbit anti HF and are shown in Fig.6. The main peaks are of approximately 68K and 60 K daltons. In other lines, higher molecular weight peaks are found, indicative of heterogeneity of receptors, as found in B cells (Cecka et al., in preparation).

One other use of antifactor reagents (quite comparable to that of class specific anti Ig reagents for B cells) is in detection, enumeration and separation of functional classes of T cells releasing different classes of T cell factor. We have developed two procedures - reverse plaques for the enumeration of high rate releasing cells, and rosetting for the enumeration of cells not releasing factor, but bearing it, or crossreactive determinants on its surface. Both precedures have only been extensively calibrated using antisera and not as yet with monoclonals. Calibration of the parameters has proven to be highly important - for instance, parameters such as the source of red cells, concentration of protein A, concentration of antifactor reagent and the time of incubation to yield plaques or rosettes all make major differences in numbers detected. The former assay is much more time consuming and so has not yet lived up to its promise for the direct enumeration of functional T cells in various responses. It has been useful, however, for the detection of monoclonals.

REFERENCES

1. Feldmann, M., J.M. Cecka, H. Cosenza, C.S. David, P. Erb, R. James, S. Howie, S. Kontiainen, P. Maurer, I. McKenzie, C.R. Parish, A. Rees, I. Todd, A. Torano, L. Winger and J. Woody (1979) In "T and B Lymphocytes: Recongition and Functions", eds. F. Bach, B. Bonavida and E. Vitetta. New York: Academic Press, pp.343-360.

2.Eichmann, K. 1978 Adv. Immunol. **26:** 195.

3. Kontiainen, S. and M. Feldmann (1979) Thymus **1:** 59.

4. Feldmann, M. (1972) J. Exp. Med. **136:** 737.

5. Okumura, K. and T. Tada (1974) J. Immunol. **112:** 783.

6. Lamb, J.R., E.D. Zanders, A.R. Sanderson, P.J. Ward, M. Feldmann, S. Kontiainen, T. Lehner and J.N. Woody (1980) J. Immunol (submitted).

7. Cantor, H. (1981) This volume.

8. Owen, F. (1980) J. Immunol. **124:** 1411.

9. Gronowicz, E., A. Coutinho and F. Melchers (1976) Eur. J. Immunol. **6:** 588.

10. Cecka, J.M., S. Kontiainen and M. Feldmann (1981) Manuscript in preparation.

11. Sprent, J. (1978) J. Exp. Med. **147:** 1159.

12. Julius, M., E. Simpson and L.A. Herzenberg (1975) Eur. J. Immunol. **3:**645.

ANTISERA AGAINST ANTIGEN-SPECIFIC T-DERIVED FACTORS

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INTRODUCTION

In order to analyze the relationship of antigen receptor on B and T cells serological reagents raised against B cell receptor, surface antigens or antibody have been chiefly used (Table 1). Anti-IgFc does not regularly react with T cells or factors with the exception of chicken anti-μ. Some anti-μ sera have reacted but the reaction has not been precisely defined (1-4). In contrast, there is much evidence for shared or cross-reacting V_H determinants (using reagents prepared by Dr. Givol)

Table 1. Reactivity of antisera against B cells, T cells, or specific T derived factors.

Reagent raised against	*Reactivity with B cells*	*T cells*	*T factors*
secreted IgFc	+	−	−
V_H	+	+	+
V_L	+	−	−
Id	+	+	+
cell surface Ia I-A	+	+	+
I-J	−	+	+
T factors F constant	*NT*	+	+
F variable	*NT*	+	+
F allogroup	*NT*	*NT*	+
$\beta_2 M$	*NT*	*NT*	+

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and Id structures of B cells, T cells or T factors with many different antigens (5-18). V_L determinants (using $\alpha\lambda$ reagents by Dr. Givol) are usually not detected (5-8). The anti-factor preparations (raised against antigen specific suppressor, SF, or helper, HF, factors) seem to recognize determinants associated with factor isotypic (F constant) region, factor antigen binding site (F variable) and with factor allogroups. Determinants reacting with monoclonal anti-β_2M have likewise been shown on factors (19). These structures are discussed in this and related papers (see Feldmann et al., this volume).

MATERIALS AND METHODS

These have all been fully described elsewhere (20-25).

RESULTS

XENOGENEIC ANTI-FACTOR PREPARATIONS

Rabbit anti-SF raised against e.g. mouse SF_{KLH} (acid eluate of antigen columns) absorbs out the activity of all antigen specific SF whether of mouse, monkey or human origin (20,24, 25), but not of antigen specific HF. The anti-HF preparations raised against e.g. mouse HF_{KLH} (acid eluate from antigen columns) react in a similar fashion but only with HF (20,24,25). These antisera (ca.20 tested so far) thus recognize cross-reacting or shared determinants confined to a functionally distinct subclass of factors which is a situation analogous to the isotype or constant region of the immunoglobulins (26).

SYNGENEIC ANTI-FACTOR PREPARATIONS

Antisera raised against $CBASF_{KLH}$ or $CBASF_{GAT}$ only reacted with factors of the same specificity and of the same (or some unrelated, see below) strains (20, Table 2).

CBA anti-$CBASF_{KLH}$, when tested on $CBAHF_{KLH}$ also absorb out the activity of HF_{KLH} indicating shared or crossreacting determinants in the combining site of KLH-specific SF and HF (20).

When SF_{KLH} from other strains of mice were absorbed with CBA anti-$CBASF_{KLH}$ or CBA anti-$CBASF_{GAT}$ the latter antiserum regularly failed to absorb out the SF_{KLH} activity while the former did it in certain strains of mice. These strains do not all share Igh-C allotypes or H-2 with CBA (Table 3).

Table 2. Absorbtions of CBASF with syngeneic anti-factor sera

Stimulus		Suppression	Response	
Spl.cells	Antigen	SF	IgG AFC/10^6	%suppr.
TNP-KLH 1^o	-	-	0	-
"	TNP-KLH	-	1456 ± 180	-
"	"	$CBASF_{KLH}$	339 ± 106	77
"	"	abs $CBA\alpha CBASF_{KLH}$	1400 ± 133	4
"	"	elu "	NT	-
"	"	abs $CBA\alpha CBASF_{GAT}$	394 ± 111	73
"	"	elu "	NT	-
GAT 1^o	-	-	9 ± 4	-
"	GAT	-	116 ± 19	-
"	"	$CBASF_{GAT}$	60 ± 10	52
"	"	abs $CBA\alpha CBASF_{GAT}$	120 ± 7	0
"	"	elu "	51 ± 4	61
"	"	abs $CBA\alpha CBASF_{KLH}$	58 ± 2	54
"	"	elu "	111 ± 12	4

$3x10^6$ spleen cells from TNP-KLH or GAT primed CBA mice were cultured for 5 days in the presence or absence of SF absorbed and eluted from CBA anti-$CBASF_{GAT}$ or CBA anti-$CBASF_{KLH}$ together with 0.02 µg of TNP-KLH or 1.0 µg of GAT/well. Anti-DNP or anti-GAT antibody forming cells (AFC) were assayed on day 5 of the culture. First line in each experiment gives background (without added antigen), 2nd line response (without added SF). Only IgG AFC, expressed as AFC/10^6 in the starting cell population, are given.

Table 3. The ability of CBA anti-$CBASF_{KLH}$ to absorb out the activity of SF_{KLH} derived from different strains of mice

Strain	H-2	Igh-C allotype	SF activity
CBA	k	j	absorbed
C3H	k	j	not
BALB/c	d	a	absorbed
B10.BR	k	b	not
B10	b	b	not
SJL	s	b	absorbed
AKR	k	d	absorbed
A/J	a	e	not

Table 4. Reactivity of backcross SF_{KLH} with CBA anti-CBASF_{KLH}

No of mice tested	Igh-C allotype	H-2	SF_{KLH} activity
12	j/b (57%)	k/b	absorbed in 2/4
		b/b	" 2/8
9	b/b (43%)	k/b	" 5/7
		k/b	" 0/2

Table 5. Immunoglobulin allotype linkage of antifactor antisera

Serum	% fluorescent T cells		
	CBA.$Ig1^b$	B10.BR	CBA
CBAαB10.A(4R)SF_{KLH}	46	40	12
BALB/c αB10.A(4R)SF_{KLH}	32	27	11
Normal mouse serum	10	11	11

T cell from nylon wool passed spleen. Serum at 1/25 dilution. Two stage fluorescence with rhodamine goat αMIg. For Ref. see 30.

SF_{KLH} from about 20 backcross (CBAxB10)F_1xB10 mice have been tested so far. No obvious linkage to Igh-C allotype or H-2 was seen in this rather limited material (Table 4).

However, when alloantisera (raised in Igh-C and H-2 disparent strains) was used there seems to be an allotype linked recognizion (Table 5). This has only been done by fluorescence so far and the functional tests have not yet been performed. The combining site determinants recognized by syngeneic antifactor sera are thus distributed in a non-Igh-C allotype and non-H-2 linked fashion (Tables 3,4) while factor "allotypic" non-combining site determinants may be Igh-C allotype linked (Table 5). The distribution of these markers thus resembles the distribution of certain B cell idiotypes which are not closely allotype linked (27-29).

DISCUSSION

The antisera raised against specific factors (factors being only purified by absorbtion and elution from antigen columns) have proven useful tools in analysis of T derived factors, and revealed in addition of factor (F) constant region determinants also determinants of the factor (F) variable region. The nature of F constant determinants and their exact localization in the SF polypeptide chains is not solved, but they are likely to be part of the factor "heavy chain", and thus located in the same polypeptide chain as the combining site determinants (31).

The relationship of the "idiotypic" determinants of the factor combining site and the factor "allotype" determinants to Igh-C and MHC markers is interesting. The former show no obvious linkage to Igh-C (or MHC) while the latter appear to be Igh-C (but not MHC) linked. It is known that there are V_H genes 10 map units or more away from the IgC_H gene cluster. If the genes coding the factor combining site "V" markers are located even more distally from IgC_H, it is possible that with the small number of mice used, linkage would not be detected. However, if the constant region genes of the factors are more closely linked to IgC_H the linkage would be detected using the allotype congenic mice. It should be stressed, however, that the techniques used for detecting factor variable and constant region markers in backcross and congenic mice were quite different, and the backcross analysis has not been performed using the factor "allotype" antiserum. The results do not exclude the possibility that factor variable determinants are controlled by genes in the same chromosome as IgV_H and IgC_H. Our interpretation of the data is that T cell factor variable region genes are distant (10 units or more) from IgC_H genes, but the factor constant region genes much closer.

In addition to the functional analysis discussed above, these antisera have proved useful in the analysis of specific T hybridomas and their products, as fluorescent probes, or in rosetting and T cell plaque assays. These aspects are discussed in this volume by Feldmann et al. (32).

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REFERENCES

1. Marchalonis, J.J., Warr, G.W., Bucana, C., and Hoyer, L.C. (1979). *J.Immunogenet.6*, 289.
2. Feldmann, M., and Basten, A. (1972). *J.Exp.Med. 136*, 49.
3. Howie, S., Parish, C.R., David, C.S., McKenzie, I.F.C., Maurer, P.H., and Feldmann, M. (1979). *Eur.J.Immunol. 9*, 501.
4. Zanders, E.D., Lamb, J., Kontiainen, S., and Lehner, T. (1980). *Immunology 41*, 587.
5. Tada, T., and Hayakawa, K. (1980). *Progr.Immunol. 4*, 389.
6. Feldmann, M., Erb, P., Kontiainen, S., Todd, I., and Woody, J.N. (1979). *Ann.N.Y.Acad.Sci. 332*, 591.
7. Feldmann, M., Cecka, J.M., Cosenza, H., David, C.S., Erb, P., James, R., Howie, S., Kontiainen, S., Maurer, P., McKenzie, I., Parish, C., Rees, A., Todd, I., Torano, A., Winger, L., and Woody, J.N. (1979). In ICN-UCLA Symposia on Molecular and Cellular Biology, T and B lymphocytes: Recognition and Function, *vol. VII*, (B.Bonavida, F.Bach and E.Vitetta, eds.) pp.343-360. Academic Press. N.Y.
8. Krawinkel, U., Cramer, M., Imanishi-Kari, T., Jack, R.S., Rajewsky, K., and Mäkelä, O. (1977). *Eur.J.Immunol. 7*, 566.
9. Black,S.J., Hämmerling, G.J., Bereck, C., Rajewsky, K., and Eihmann, K. (1976). *J.Exp.Med. 143*, 846.
10. Weinberger, J.Z., Benacerraf, B., and Dorf, M.E. (1980). *J.Immunol. 151*, 1413.
11. Yoshikatsu, H., Nisonoff, A. (1980). *J.Immunol. 151*, 1231.
12. Ju, S.-T., Pierres, M., Germain, R., Benacerraf, B., and Dorf, M.E. (1981). *J.Exp.Med. 126*, 177.
13. Nisonoff, A., and Greene, M.I. (1980). *Progr.Immunol. 4*, 57.
14. Benacerraf, B. (1980). *Progr.Immunol. 4*, 419.
15. Julius, M.H., Cosenza, H.C., and Augustin, A.A. (1978). *Eur.J.Immunol. 8*, 484.
16. Mozes, E.(1978). In Ir genes and Ia antigens (H.O. McDevitt, ed.) p.475. Academic Press, N.Y.
17. Okumura, K., Tokuhisa, T., Takemori, T., and Tada, T. (1978). In Ir genes and Ia antigens (H.O. McDevitt, ed.) p.147. Academic Press, N.Y.
18. Binz, H., and Wigzell, H. (1975). *J.Exp.Med. 142*, 197.
19. Lamb, J.R., Zanders, E.D., Sanderson, A.R., Ward, P.J., Feldmann, M., Kontiainen, S., Lehner, T., and Woody, J.N. Submitted for publication.
20. Kontiainen, S., and Feldmann, M. (1979). *Thymus 1*, 59.
21. Feldmann, M., Cecka, J.M., James, R., Todd, I., and Kontiainen, S. (1980). In *Cont.Topics in Immunobiology*, (N.L. Warner, ed.), *11*, 275.

22. Kontiainen, S., and Feldmann, M. (1977). *Eur.J.Immunol. 7*, 310.
23. Kontiainen, S., and Feldmann, M. (1978). *J.Exp.Med. 147*, 110.
24. Kontiainen, S., Woody, J.N., Rees, A., and Feldmann, M. (1980). *Clin.Exp.Immunol.*, in press.
25. Lamb, J.R., Zanders, E.D., Kontiainen, S., and Lehner, T. (1980). *Infect.Immun. 30*, 766.
26. Milstein, C., and Pink, J.R.L. (1970). *Progr.Biophys. Mol.Biol. 21*, 209.
27. Karjalainen, K. (1980). *Eur.J.Immunol. 10*, 132.
28. Lieberman, R., Rudikoff, S., Humphrey, Jr., W., and Potter, W. (1981). *J.Immunol. 126*, 172.
29. Ju, S.-T., and Dorf, M.E. (1981). *J.Immunol. 126*, 183.
30. Culbert, E.I., Kontiainen, S., Cecka, M.J., James, R., Makidono, R., Simpson, E., and Feldmann, M. (1981). In *Hybridomas*, G.J. Hämmerling, U. Hämmerling and C.G. Kearney (eds.). Elsewier, North Holland, in press.
31. Cecka, M. et al. Manuscript in preparation.
32. Feldmann, M., James, R., Culbert, E., Cecka, M., Makidono, R., and Kontiainen, S. (1981), this volume.

MURINE AND PRIMATE T CELL-DERIVED PROTEINS RELATED TO IMMUNOGLOBULIN COMBINING SITE-REGION DETERMINANTS

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Certain xenoantisera specific for antigenic determinants lying either near or within the combining sites of serum immunoglobulins (Igs) of man and mouse were found to react with T cell products of M.W. 65,000-70,000. These T cell molecules were isolated using antisera reactive with Fd/L interaction determinants, V_H-fragments and idiotypes. Antigenic determinants of the T cell molecule cross-react with the Fd fragment of serum μ chain. The Fd-like fragment of the trypsin-treated T cell molecule, and smaller polypeptides produced by CNBr degradation, retain the cross-reactive antigenic determinant and were isolated by affinity chromatography. Our data indicate that the T cell heavy chain has an Fd-fragment related to those of certain serum Igs, but that the nature of its constant region (Fc) remains to be established.

INTRODUCTION

We have produced a battery of antisera specific for antigenic determinants lying either near or within the combining site of serum antibodies (1-4). Such antisera are not specific for serum isotypes, but tend to react with Fd/L interaction determinants. Although they usually react best with heavy chain-related determinants, both in the case of anti-idiotype (5) and "shotgun" reagents made against polyclonal Fab fragment pools in phylogenetically distant species (2,3), interaction between heavy chain and light chain is frequently required for optimal formation of the antigenic determinant. Such reagents are useful in the detection and isolation of immunoglobulin-related molecules of certain T cells. In addition to raising antisera against Fab-region determinants which crossreact with certain T cell components, it is possible to immunize with the isolated T cell product and to generate antibodies which react with corresponding portions of serum immunoglobulins (6). These reagents can be termed "anti-IgT" or "anti-Ig-related T cell factors". In the present report,

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we describe the types of products isolated using these antisera and outline steps in the characterization of the Fd-like fragments of these molecules.

Experimental: Fig. 1 illustrates the products isolated from various T cell preparations using the antisera reactive against Fd determinants (a), avian anti-$(Fab')_2$ (b), anti-ARS idiotype (c), anti-IgT (d,e,f) and anti-V_H (g). A, b and c are the products of murine T cells, where a is the ^{125}I-labeled Ig-crossreactive product isolated from the culture fluid in which the monoclonal T cell lymphoma WEHI 22 had been grown and b is the biosynthetically (^3H-Leu) labeled product of this cell isolated using chicken anti-$(Fab')_2$. In order to obtain the data given in c, peripheral T cells of A/J mice which had been immunized with the arsonate hapten coupled to Limulus hemocyanin (7) were stimulated with Concanavalin A and the biosynthetically labeled (^{75}Se-Met) product isolated from the culture fluid by precipitation using rabbit antibody against the ARS idiotype (7). The control is this experiment represents purified B cells from the same animal which were likewise treated with Concanavalin A. In D and E, culture fluid in which the marmoset amplifier T cell line 70-N2 had been grown was concentrated, passed through protein A-Sepharose to remove contaminating fetal calf IgG, radioiodinated and precipitated using rabbit antibody directed against the purified 70-N2 molecule (6). D shows the resolution of the precipitated material on a 5% acrylamide gel in SDS under non-reducing conditions and E is the reduced sample on 10% polyacrylamide. The data in F depict the radioiodinated molecules precipitated from culture fluid in which the monoclonal marmoset T cell line 1605L had been grown, by the antibody made against the IgT. The data in G represent the biosynthetically labeled (^3H-Leu) product of the T cell precipitated by chicken antibody directed against the V_H-fragments of a human Waldenstrom macroglobulin (1).

All the T cell samples have a predominant component of approximate mass 65-70,000 daltons. There is little evidence of lower molecular weight components in these patterns, although components of approximate mass 25,000 daltons are sometimes observed both in biosynthetically labeled and in radioiodinated preparations of the T Ig-related material. The intact, unreduced material has an approximate mass of about 140,000 and consists of a dimer of the 67,000 dalton heavy chain. Light chain is clearly present in the B cell Ig (F).

The antisera used here are not isotype specific, and it was important to determine whether there is an overall relationship between the T cell product and classical serum antibodies. This was tested in a number of ways, both serological and biochemical.

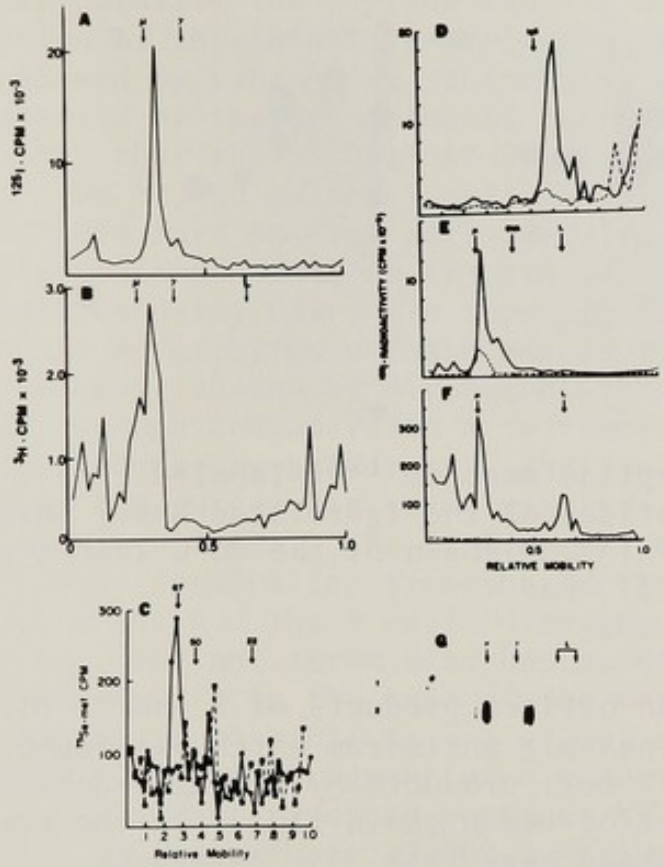

Fig. 1. Resolution on SDS-PAGE of murine and primate Ig-related molecules. A, radioiodinated Ig-related molecule of murine WEHI 22 T-lymphoma obtained from culture fluid isolated via Fd-region crossreaction (8); B, (^{3}H)-leu labeled product of WEHI 22 T-lymphoma isolated by affinity-chromatography on chicken anti (Fab) Sepharose (2); C, biosynthetically labeled (^{75}Se-Met) product of T cells of A/J mice bearing the ARS-idiotype (5,7). ●—●, purified T cell population stimulated with Con A; ■--■, purified Con A-stimulated B cell population. D, intact Ig-related product of marmoset in vitro grown line 70-N2 precipitated by rabbit anti-IgT (3,6). ——, specific ppt.; ----, normal rabbit IgG control ppt. E, reduced Ig-related product of 70-N2 line. Same symbols as D. F, ——, spec. ppt. IgM of marmoset B cell line 1605L; ----, cont. ppt. G, radioautograph (fluorograph) of (^{3}H)-Leu labeled products (2) of marmoset 70-N2 T cell line precipitated by anti-V_H (origin 2) or normal chicken globulin (origin 1).

In Fig. 2 radioiodinated T cell heavy chain prepared from the murine T cell lymphoma WEHI 7 was compared with radioiodinated μ chain of the (μλ) myeloma protein MOPC 104E and with viral glycoprotein gp 71 by two dimensional peptide mapping (8). gp 71 has a totally different profile of tyrosine containing peptides then does either the T cell product or the standard μ chain. There is an overall similarity between the T cell product and the μ chain but there is clearly a lack of identity. β2M and bovine serum albumin (not shown) also are clearly distinct from μ chain and T cell heavy chain.

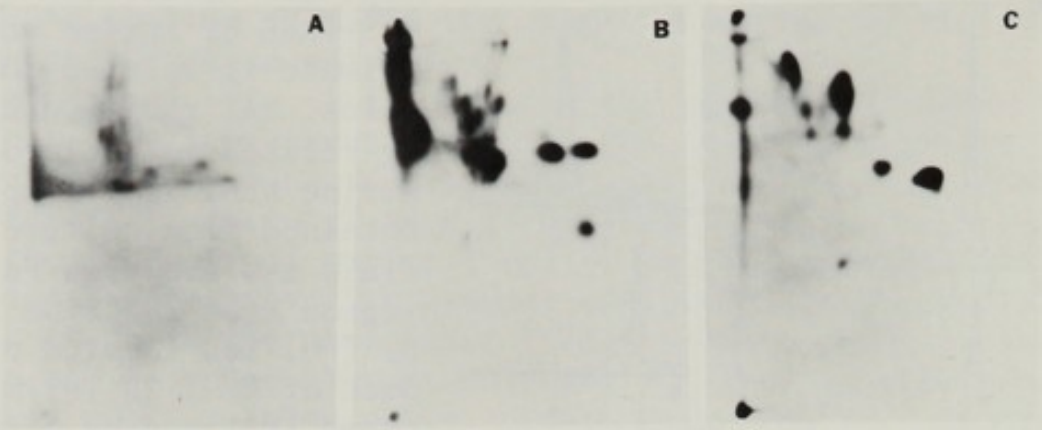

Fig. 2. Two dimensional peptide maps of ^{125}I-labeled tyrosine-containing tryptic peptides of the Ig-related heavy chain of murine T lymphoma WEHI 7 (A), μ chain of the MOPC 104E myeloma (B) and viral gp 71 (C). Ref. 8.

A serological comparison between products of B and T cells was carried out using a guinea pig antiserum which was found to react with both IgM and T cell products by a crossreaction localized to the Fd fragment of the μ chain (10). In the competition radioimmunoassay shown here (Fig. 3), detergent extracts were made of mouse spleen and thymus, and the UV-induced fibrosarcoma 112. Low concentrations of Triton X 100 (11) were used. The spleen (i.e. B cell) extract inhibits the precipitation of the IgM myeloma protein MOPC 104E with a slope identical to that of the standard IgM. The antiserum used here does not react detectably with λ chain so all the reactivity is against heavy chain determinants. The extract of the nonlymphoid tumor does not inhibit the precipitation at all. The T cell extract inhibits appreciably but with a significantly lower slope, thereby indicating that the T cell product is crossreactive with the μ chain. These serological

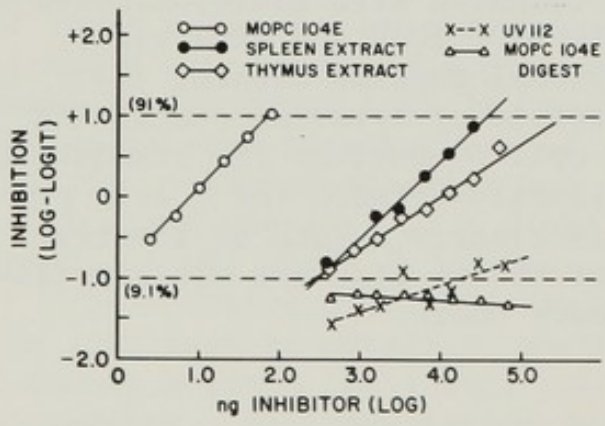

Fig. 3. Determination by competition radioimmunoassay of the capacities of B cell detergent extract (spleen), T cell extract (thymus), fibrosarcoma extract (UV112) and protease digest of denatured MOPC 104E (λμ), to inhibit the precipitation of ^{125}I-labeled MOPC 104E by guinea pig antiserum to polyclonal IgM molecules (4).

data parallel the peptide mapping data. In addition, denaturation of the intact IgM molecule or of the T cell extract followed by exhaustive proteolytic degradation destroys the capacity of these samples to inhibit the reaction, thus indicating that the reactivity is against peptide determinants.

The 67,000 dalton T cell Ig-related product can be degraded into smaller antigenic fragments by treatment either with proteases such as trypsin (4) or by cleavage at methionyl residues using CNBr. As shown in Fig. 4, fragments comparable to the Fd of serum μ chain can be isolated both from B and T cell heavy chains by digestion with trypsin, followed by passage through Concanavalin A to remove those peptides bearing high mannose carbohydrates, and finally by precipitation using the guinea pig serum reactive with the Fd fragment of the molecule (4). The Fd fragment of the τ chain differs slightly in mobility from the Fd fragment of the B cell surface μ chain. The B cell Fd fragment is of μ chain origin, because the antiserum used has no detectable reactivity with the δ chain. In the case of the marmoset T cell heavy chain, it has been possible to use a goat antiserum prepared against the Fab monomer fragment of a monoclonal human IgM to isolate Fd region CNBr-produced peptides of smaller molecular weight than the approximate 29,000 described above. Two peptides of approximate M.W. 10,000 and 6,000 can be isolated from CNBr-digests of solubilized marmoset T lymphoma cells by solid phase antibody specific for Fab monomer (Fig. 5).

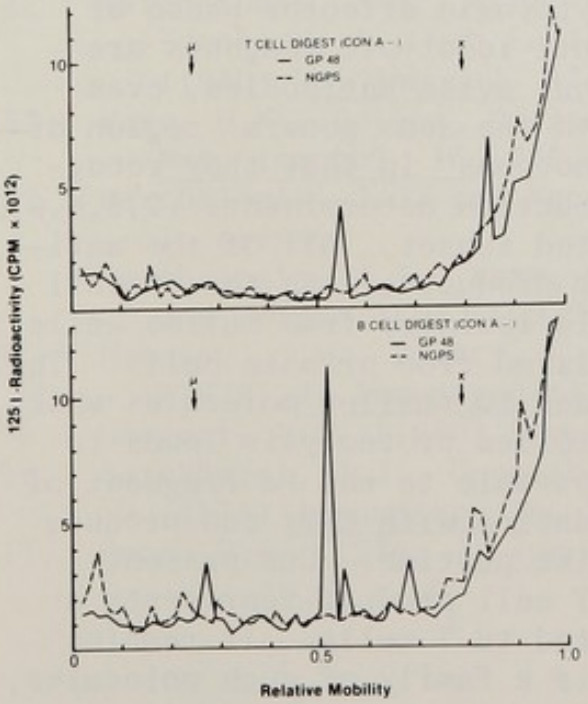

Fig. 4. Fd-like fragments of radioiodinated T cell surface Ig-like component (upper fig.) and B cell surface μ chain (lower fig.) resolved by SDS-PAGE. ——, precipitated by guinea pig anti-μ plus fixed S. aureus cells; ----, control precipitate of normal guinea pig serum plus fixed S. aureus cells (4)

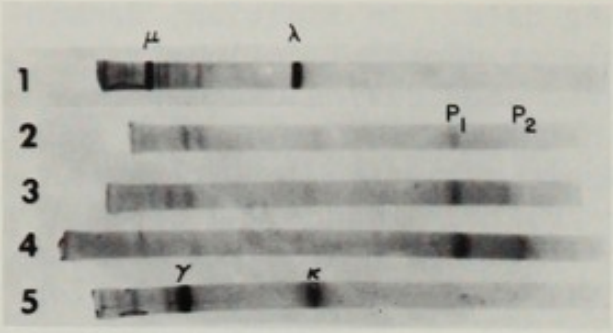

Fig. 5. Resolution by SDS-PAGE (14% gel) of reduced human IgM (λμ)-origin 1, reduced polyclonal human IgG- origin 5, and fragments produced by CNBr-digestion of marmoset 70-N2 cells which react with antisera directed against determinants located in the Fd fragment of human μ chains.

CONCLUSION

It has been possible to use antisera directed against antigenic determinants located in the vicinity of the combining site of serum antibodies to isolate products of certain T cells. Further, immunization of rabbits or goats with the Ig-like product of T cells isolated by the anti-combining site-related antibodies allowed the production of reagents which showed that the T cell products are clearly related to immunoglobulin variable regions, and also suggest the presence of constant regions which are not typical of any major serum or B cell isotype. Although we have not presented any of the functional data here, the various antisera described inhibit a number of T cell functions including binding of antigen (5), the initiation of the mixed lymphocyte reaction, (3) and, under certain conditions, the cytotoxic effector phase of specific T cell killing. The anti-idiotypic reagents are very restricted in specificity but avian antibodies, even though they apparently react with the same general region of the molecule (2,3,5), act as "shotguns" in that they recognize the majority of V_H/V_L interaction determinants (2,3,6,9), instead of an extremely restricted subset. All of the antisera isolate essentially similar products, with the overall properties of the T cell molecule isolated from murine cells being quite similar to that isolated from primate cells. The intact heavy chain can be degraded to smaller molecules which are antigenically active. Controlled proteolysis leads to the production of fragments comparable to the Fd fragment of serum immunoglobulins and degradation with CNBr can produce smaller fragments from the Fd-like peptide. Our present feeling is that the Ig-related T cell product represents a new Ig isotype which is restricted to T cells. It remains to be determined whether there is a family of such molecules,

with suppressor T cells possibly expressing different τ isotypes than do helper T cells, and the possibility must also be considered that the variable regions expressed by the T cell products, although antigenically related to those of serum antibodies, either represent a restricted subset of the universe of variable regions or are crossreacting molecules that diverged early in evolution from the set of variable regions expressed by B cells. Structural analyses designed to provide definitive information on these questions are in progress.

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REFERENCES

1. Marchalonis, J.J., Warr, G.W., Rodwell, J.D. and Karush, F. (1980) Proc. Natl. Acad. Sci. USA 77, 3625.
2. Warr, G.W., Marton, G., Szenberg, A. and Marchalonis, J.J. (1978) Immunochemistry 15, 615.
3. Marchalonis, J.J., Warr, G.W., Wang, A.C., Burns, W.H. and Burton, R.C. (1980) Mol. Immunol. 17, 877.
4. Marchalonis, J.J., Warr, G.W., Santucci, L.A., Szenberg, A., von Fellenberg, R. and Burckhardt, J.J. (1980) Mol. Immunol. 17, 985.
5. Warr, G.W., DeLuca, D. and Marchalonis, J.J. (1979) Mol. Immunol. 16, 735.
6. Marchalonis, J.J. and Wang, A.C. (1980) J. Immunogenetics in press.
7. Marchalonis, J.J., Warr, G.W., Smith, P., Begg, G.S. and Morgan, F.J. (1979) Biochemistry 18, 560.
8. Moseley, J.M., Beatty, E.A. and Marchalonis, J.J. (1979) J. Immunogenetics 6, 1.
9. Haegart, D.G. (1980) Immunology 41, 663.
10. Burckhardt, J.J., Guggesberg, E. and von Fellenberg, R. (1974) Immunology 26, 521.
11. Cone, R.E. (1976) J. Immunol. 116, 847.

SUMMARY WORKSHOP: THE T CELL RECEPTORS

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It should be clear from the foregoing chapters that the nature of the T cell receptors for antigen (including those for self antigens) has not been defined. Indeed the proceedings of this workshop were significantly less optimistic than those of the same workshop held at the 1977 ICN-UCLA conference in Park City. This atmosphere of doubt was attributable to three factors: 1. The paucity of significant new data from structural studies of T cell antigen binding proteins; 2. The bewildering variety of different subsets of T cells and of their secreted, antigen-specific products that were presented at this meeting; and 3. The negative evidence regarding activation-rearrangements in the Igh gene complex reported by several molecular biologists at this meeting.

In contrast to this negative impression, there were several major advances. The most significant are the three different techniques for the cloning of T cells: Cloning of functional T cell lines with growth factors; antigen-specific T cell hybridomas; and induction of antigen-specific T cell lymphomas with radiation leukemia virus. Secondly, many groups now have hetero- or allo-antibodies specific for what appear to be T cell allotypes or isotypes. Third, it seems likely that positive data will be forthcoming from several molecular biologists on the nature of gene rearrangements in cloned T cells using specific VH probes instead of the CH or JH probes thus far employed.

Against this background, the workshop on T cell receptors consisted of a lively discussion of several issues, which will be summarized here. The convenors of the workshop would like to thank all the participants.

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Do T Cells Use Igh-V Gene Products to Form Their Specific Receptors?

Four years ago, based on the combined use of antisera specific for VH-encoded idiotypic determinants and mapping of the genes controlling expression of these determinants in T cells to VH, it was widely agreed that T cells make use of VH genes in forming their receptors. At this workshop, this seemingly sensible conclusion was attacked by several participants on the following grounds.

First, it was noted that in several instances, antibodies specific for anti-hormone antibodies had reacted with the receptor for that hormone. Since the hormone receptor is not known to be encoded in VH, such findings weaken the serological evidence for VH encoded T cell receptors. Weissman and Pillemer presented a second example of the degeneracy of anti-idiotypic antibodies. They had prepared rat monoclonal antibodies to mouse T lymphoma cells. One of these reacted with the phosphorylcholine-binding myeloma TEPC 15 and not other myelomas. The determinant recognized is in VK, and such antibodies are not found in other anti-TEPC-15 idiotype preparations. This monoclonal antibody also reacted with all T lymphocytes. By immuno-precipitation, they demonstrated that this T cell surface structure was the Thy-1 antigen. Thus, reaction with an apparent anti-idiotypic antibody does not necessarily imply use of the same genetic material to form the receptor. However, the genetic linkage to VH observed in several studies (Eichmann, Cramer, Rubin, Sachs, and Hodes) would seem to favor such an interpretation. Not so, goes the argument, since it is now believed by many that cells having specificity for idiotype are selected for by serum idiotypic molecules (Ig) that are indeed encoded in VH. Such anti-idiotypic cells could in turn select those T cells bearing non-VH encoded receptors most closely resembling genuine VH encoded idiotypes via the idiotypic network, thus leading to the serological cross reactions and the genetic mapping data observed. This argument has appeal to many investigators who are uncomfortable with the negative data obtained thus far at the DNA level, as well as to several participants who felt that the absence of evidence for VL influences on the T cell receptor were incompatible with the behavior of immunoglobulin idiotypes, most of which require both VL and VH. However, where it has been tested, T cells from mice unable to make Ig bearing idiotypic determinants due to L chain polymorphisms or other genetic defects of idiotype expression unrelated to VH do still have T cells bearing idiotype. This would appear to argue against the premise that mapping of T cell idiotype to VH is secondary to expression of idiotype as Ig.

Do MHC-restricted T cells Bear VH-encoded Idiotypes on T Their Receptors for Antigen?

Given the uncertainty about the genetic origins of the idiotypic molecules found on T cells specific for particular antigens, it was difficult to approach the next issue, which was whether T cells recognizing antigen in an MHC-restricted fashion do so using receptors bearing Igh-V encoded idiotypes. However, if one accepts the genetic and serological evidence discussed previously at face value, then such a question can be approached. On close examination, most of the data concerning T cell idiotypic determinants relates either to alloreactive T cells and their receptors specific for non-self MHC encoded antigens or T cells not defined in terms of MHC restricted function or that are clearly not MHC restricted. This is important to resolve, since it is clear that if an MHC-restricted T cell recognized antigen using the same VH gene product as a B cell, then altered-self models of MHC restriction can not be supported, and a second recognition unit for MHC must be postulated. Unfortunately, this argument can not be resolved based on presently available evidence. Apart from the helper T cells characterized as idiotypic by Eichmann and his co-workers some years ago, the apparently similar phosphorylcholine specific helper T cells described by Augustin, Cosenza and Julius, and studies of staphylococcal nuclease specific, idiotypic helper T cells presented at this meeting by Sachs, Hodes and co-workers, no data exists. In none of these systems was MHC restriction proven, nor, indeed, was it demonstrated unequivocally that the idiotypic determinants were present on the T cell surface, although strong circumstantial evidence does exist for its presence. No one was aware of evidence for VH-encoded idiotypic determinants present on cytotoxic effector cells that were MHC restricted. Thus, there is at present no clear picture of the status of idiotypic determinants present on T cells that are MHC restricted. Hence, one can not argue strongly from such data for or against any particular model for the T cell receptors. It was agreed that the Lyt-2 molecule must sterically related to the binding receptor on cytolytic T cells, since anti-Lyt-2 can inhibit cytolysis. However, the failure of anti-Lyt-2 to react with T cell-derived antigen binding molecules suggested that Lyt-2 is not a component of the receptor on suppressor T cells involved in antigen binding.

Are T Cell Idiotypes Less Heterogeneous Than B Cell Idiotypes?

Several laboratories have presented evidence that T cell derived idiotypic materials are less heterogeneous than B cell derived idiotypic materials specific for a particular

antigen. Although this work has been primarily carried out using heterogeneous antibodies as anti-idiotypes, it is clear from the assembled data that T cells tend to express only the commonest or public idiotypic specificities, while B cells often express variant forms. While no one could present counter examples, this proposition was greeted with little enthusiasm by the workshop. However, the data in the literature are quite impressively consistent on this point, and the workshop convenors feel it is worthy of further consideration and experimental testing. Given the rapid redefinition of idiotypic determinants now possible using monoclonal idiotypes and anti-idiotypes, a clearer picture of T cell heterogeneity of idiotype expression should soon emerge.

T Cell Allotypes and T Cell Isotypes:

One of the most promising developments in the field of T cell receptors to emerge at this meeting was the description of several antisera and monoclonal antibodies showing specificity for T cell surface molecules that are candidates for the T cell receptor. The possibility that the T cell receptor might be a complex polypeptide encoded in both VH genes on chromosome 12 and H-2 genes on chromosome 17 was introduced and dismissed for lack of biological precedent. Therefore a model for antigen binding T cell receptor molecules must account for the observation of idiotypic similarity with products of the immunoglobulin heavy chain gene complex and reactivity with anti-Ia using a model which does not employ classical Ia antigen polypeptides as constant regions of receptors. No satisfactory agreement to the Ia question was reached although glycosylation was raised as a possible explanation. Here, use of monoclonal anti-Ia antibody should soon clarify these findings. One issue raised by antisera specific for T cell receptor molecules is whether T cells will express a variety of different receptor molecules, similar to immunoglobulin isotypes, and whether such T cell isotypic molecules will likewise be marked by allotypic determinants. Apart from the studies presented elsewhere in this book by Owen and co-workers, and studies of Givol and Eichmann using anti-VH antibody, there is at present very little information about the possibility of T cell receptor allotypes. However, the work of Cone *et al* does suggest polymorphism for the structural gene for T cell receptors. Whether Owen's sera truly define receptors, or are directed at differentiation antigens, remains to be determined, although there was enthusiasm for the concept that these defined receptor species. On the question of using antisera to define T cell isotypes, there was again some guarded enthusiasm, but no clear-cut examples.

The variation in the molecular weights reported for receptors on helper cells (75,000 from B. Rubin), suppressor cells 68,000 (Owen, Cantor) and inducers (62,000 Owen) suggest that functional T cells may utilize different receptors. The fact that antisera directed against these molecules do or do not react with various other T cell subsets or T cell derived immunoregulatory molecules is suggestive data that the molecules are different and restricted in their functional activity. One difficulty with these studies is clearly that the antisera had not been exchanged in a round robin so that molecular weight determinations in separate laboratory settings were being compared. In addition, negative data in bioassays are difficult to interpret when no positive control for the receptor in the same system is available. Nevertheless, in several studies, antisera or monoclonal antibodies appeared to react with some but not all T cell subsets, and with some but not all T cell derived immunoregulatory proteins. The generalization that T cells are functionally complex, that their functional complexity may be reflected in a chemical and genetic heterogeneity of their antigen-specific products, and that it may be useful to explore this complexity by preparation of clean reagents specific for these molecules does emerge from these studies. That Owen's antibodies map two such determinants to the 3' side of the Igh-C region is further enticement for immunologists to explore this approach. It also adds highly indirect evidence to the circumstantial case that T cell receptors form a variant family of conventional Ig molecules.

Are T Cell Factors Simply Soluble Forms of T Cell Receptors?

Since B cells use essentially the same molecule as an antigen-specific cell surface receptor and as a secreted effector molecule, differing only in the C terminus of the heavy chain, immunologists have tended to assume that T cells would behave in a similarly logical manner. Indeed the clonal selection theory would seem to demand that they do so. However, data emerging from studies of the molecular, antigenic and antigen binding characteristics of T cell factors have raised some questions in this regard. First, Ia antigens are often observed to be covalently associated with T cell antigen binding factors in contrast to the data reported for T cell receptors by Fresno and Cantor and by M. Cramer. Secondly, T cells appear to secrete very low quantities of antigen binding material. This was dramatically illustrated by the studies of Kapp, et al, showing that affinity purified suppressor factor comprised less than 0.1% of the secreted protein in a supernatant of a T cell hybrid, and that of this, only 0.1% was actually biologically active. That is, 99.9% of the putatively antigen-binding material was

not actually biologically active. The 0.1% that was active had to be separated on high pressure liquid chromatography, and this material had a molecular weight of 23,000d, bound antigen, bore idiotypic determinants, and was bound by anti-I-J antisera. This immediately raises the possibility that the molecules purified by antigen binding or by use of anti-idiotype antibody was mainly non-biologically active "carrier molecules". This, in turn, calls into question all the studies done using such methods for determination of molecular weight, etc., since the Kapp results suggest that all such values may be true only for the carrier material. Likewise, the same could be true for antisera raised against such molecules. Alternatively, T cell products obtained from monoclonal lines developed by fusion could bear more resemblance to the differentiation state of the malignant parent lines than to the in vivo functional product of a normal cell. BW5147, the most common tumor parent for such lines is derived from a spontaneously occuring thymoma. This immature cell may have no more resemblance genetically to a splenic T cell than an embryonic liver cell does to a secreting splenic B cell. In order to compare molecular weights of antigen binding materials it is critical to determine the biological properties of the cells which secrete them. We may someday have the opportunity to compare products of cells derived from one parental cell at different levels of differentiation.

These studies do raise several questions about the chemistry of the T cell receptor that remain to be answered. They point out the need for high resolution chemistry coupled to a sensitive biological assay in resolving questions in this field. The repeated reports of 68,000, 62,000, 45,000, and 23,000 molecular weights suggests that proteolysis of molecules to dispose of a "carrier" or "constant" region or as a premodification event for acquiring an Ia determinant may be a normal cellular function. The problems in isolating material of a consistent molecular weight may suggest that enzymatic post-translational modification is a crucial cell function. Those investigators not using proteolytic inhibitors (Kapp, Taniguchi, Cramer) may isolate fragmented molecules for valid biological reasons. Finally, they leave wide open the question of whether T cell factors are the soluble expression of the receptors found on the T cells that produced them. Taniguchi et al cited clear evidence for a difference in molecular characteristics of cell receptors and secreted products in a T cell system suggesting that the membrane bound and secreted products may differ in a manner similar to the membrane and secreted forms of IgM.

New Techniques for Obtaining Purified, Antigen-specific T Cells for Study:

T cell hybrids have proven very valuable in the study of T cell products, as have T cell clones for the study of function, both points illustrated many times in this book. However, it would be most valuable to be able to convert a T cell clone into a malignant T cell line, so that essentially unlimited amounts of receptor and if present of specific secreted product could be obtained without the attendant problems of hybrids (polyploidy, instability) or clones (difficulty in growth, freezing). A possible breakthrough discussed in this workshop involved the transformation of enriched T cell populations, and perhaps of T cell clones as well, with radiation leukemia virus. The transformation of cloned functional T cells is most attractive, since then both the functional, non-transformed, and the transformed versions of the same cell would be available for study, thus mitigating the disadvantages of each cell type. Such approaches should soon liberate studies of T cell receptors from the technological difficulties so far encountered, and coupled with hybridomas directed at T cell receptor idiotypes, allotypes and isotypes, congenic and recombinant inbred mouse strains, and cDNA clones specific for VH and CH genes, shed light on the mystery of the T cell receptor.

In summary, while the workshop was striking in its inability to generate agreement about even the simplest questions about T cell idiotypy, it is our feeling that this pessimism was really exaggerated by the feeling on the part of many of those present that facts obtained in the near future would revolutionize our understanding to such an extent that it was almost useless to speculate on the highly fragmentary information now available.

ACTIVATION OF THE IDIOTYPIC NETWORK: ENVIRONMENTAL AND REGULATORY INFLUENCES

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I. INTRODUCTION

It has been proposed that during ontogenic development of the immune system of an individual, the products of germ line genes, particularly those near or within the major histocompatibility complex (MHC) play a major role in the initial selection of antigen-sensitive lymphocytes (1). Subsequently, it was demonstrated that there is a commitment of T cells to recognize MHC encoded determinants existing on thymic epithelial cells (2,3), thus reinforcing the concept that the selective pressure for generating functional T cells must be exerted by "self" antigens. The theme of this paper is to expand upon this thinking by suggesting that selection of T cells need not be limited to those T cells with receptors for MHC encoded determinants but may include selection of T cells committed to recognizing other germ line encoded products, including self idiotypic determinants. This concept is based on the notion that the expression of certain idiotypes (V genes) has been important in evolutionary development and may influence the development of the T cell repertoire.

Recent studies have shown that the optimal production of certain idiotypes (Id) during antibody responses is dependent on the synergistic activity of two distinct helper T (Th) cell sets (4-8). One of the helper T cell sets interacts with other lymphoid cells (macrophages and/or B cells) by recognizing MHC encoded determinants,

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particularly Ia, on the target cell surface (9,11). The precision of this type of cell to cell interaction is determined by MHC, anti-MHC interactions. These studies have also described the presence of a Th cell set which acts directly on idiotypic B cells via an anti-idiotypic receptor (4). This latter type of cell-to-cell interaction suggests that the rules governing the interactions between Th, macrophages and B cells may be expanded to include Id, anti-Id interactions. This set of anti-idiotypic Th cells has thus far been shown only for those Ids that are known to be inherited and which may normally represent a substantial portion of circulating antibody - these Ids will be referred to as "germ line" or "regulatory" idiotypes. This paper will review evidence for two distinct antigen-specific Th cells involved in TEPC 15 Id-dominated antibody responses to the hapten phosphorylcholine and will suggest that the two sets of helper T cells are analogous in their specificities - both being specific for foreign antigen and for a self determinant. The analogy is based on the concomitant specificities of various T lymphocytes for antigen and autologous gene products and proposes that autologous gene products could include "regulatory" idiotypic determinants as well as MHC encoded determinants. For convenience, one can discriminate these cells by their putative anti-self specificities, calling the conventional Th cell ThMHC, and the idiotype-specific Th cell ThId (18).

II. HELPER T LYMPHOCYTES INVOLVED IN T-DEPENDENT ANTI-PHOSPHORYLCHOLINE RESPONSES.

A. Characteristics of the Two Helper T Cell Sets

The thymus-dependent antibody response to phosphorylcholine (PC) - containing antigens is dominated in BALB/c mice by antibody bearing a single idiotype characteristic of the BALB/c myeloma protein, TEPC 15 (T15). Helper T (Th) cell function required for an optimal T15 dominated anti-PC plaque-forming cell (PFC) response during an adoptive secondary response to PC-protein antigens has been shown to vary with the source of Th cells. While "conventional" Th cell (ThMHC) activity is required for induction of PC-specific B cells to produce antibody, the expression of antibody bearing predominantly the T15 idiotype depends on the presence of a second Th cell set (ThId) (6,7). Mice which fail to make naturally circulating antibody bearing the T15 idiotype have been shown to be deficient in the ThId cell set, and Th cells from such mice induce anti-PC responses which are not dominated by the T15 idiotype - suggesting a role for T15

bearing immunoglobulin (Ig) in the activation of the ThId cell set (12). A variety of systems have been used to evaluate ThId cell function in mice expressing low levels of circulating T15 Id (see Table I). These genetically and experimentally defined systems allowed an analysis of the contribution of the conventional (ThMHC) cell set to the anti-PC response in the absence of detectable ThId cell function. For example, Th cells from carrier-primed BALB mice suppressed from birth with anti-μ antibody will induce B cells from from PC-primed normal BALB donors to produce a substantial anti-PC response, but the proportion of T15 Id expressed is considerably less than that induced by Th cells from normal BALB mice (see line 2a) (7). These studies using Th cells from a variety of systems which lack ThId cell activity have demonstrated that the functional activity of the remaining Th cells is identical to conventional Th cells, requiring antigen and self MHC recognition as well as the presence of the appropriate hapten-carrier conjugate. In all senses this cell appears to be identical to the conventional Th cell originally described by Mitchison (13) and later shown to recognize antigen in the context of self I region encoded determinants on the macrophage and/or B cell surface (9-11). Similarly, the function of the ThId cell set was studied by restoring ThId cell activity to the ThId cell depleted T cell pool and evaluating the characteristics and activation requirements of this unique Th cell set.

It has been demonstrated that the reconstitution of ThId cell activity to the systems listed in Table I required the addition of an antigen-specific $Thy1^+$, $Ly1^+$ T cell from a T cell donor that expressed high levels of T15 Id (6,7,14). An example of such a restoration is summarized in Table II and restated specifically in Figure 1 (groups 1-4). Most importantly, as seen with the ThMHC cell set, ThId cell function is antigen specific, requiring antigen priming and re-exposure to the priming antigen in order to induce T15 bearing B cells to produce antibody. Other distinguishing features of the ThId cell set determined by similar depletion and restoration studies are listed in Table IIIa.

Clearly the ThId cell population appears to recognize T15 determinants as well as carrier protein since only T15 bearing B cells are activated in the presence of this cell set. The apparent recognition of auto-idiotype determinants as a self determinant on the B cell surface would predict that, unlike "conventional" Th cells (ThMHC) whose activity is restricted by recognition of MHC encoded determinants, ThId cell activity should not be controlled by genes mapping in the MHC. The results of one approach to evaluating the

TABLE I. SUMMARY OF SYSTEMS USED TO DELINATE TH CELLS REQUIRED FOR OPTIMAL T15 PROMOTION - Depletion of ThId Activity

System	Source of Th Cells	Mean of % T15 Expression	Number of Experiments	Range of % T15 Expression
1. GENETICALLY DEFINED				
a. CBA/N	F1 ♂	32	21	17-48
	F1 ♀	84		77-93
b. Igh^b haplotype	(C.B20 x B6)F1	33	5	21-45
	(BALB/c x B6)F1	85		82-91
2. EXPERIMENTALLY DEFINED				
a. Anti-μ treatment	Neonatally suppressed	36	11	29-44
	Normal	87		81-89
b. Blast repopulation	BALB/c nu/nu - blasts	27	3	19-33
	BALB/c nu/nu - small cells	89		83-93
3. CLONED T CELLS	BALB/c - OVA (8)	27	5	17-33
	BALB.B - OVA (1)	25	3	20-31

TABLE II. RECONSTITUTION OF ThId CELL ACTIVITY: ANTIGEN SPECIFICITY OF THE IDIOTYPE-RECOGNIZING HELPER T CELL

Group	Source of ThMHC Anti-μ treated mice primed to:	Source of ThId normal mice primed to:	Antigen	PC-PFC/ spleen*	%T15
1.	KLH	- (ThId deficient)	PC-KLH	++	30
2.	KLH	OVA	PC-KLH	++	33
3.	KLH	OVA (ThId restored)	PC-KLH + OVA	++++	81
4.	-	OVA	PC-KLH + OVA	±	98

*5 million B cells from PC-primed normal mice were transferred along with Lyl^+ T cells from anti-μ treated, keyhole limpet hemocyanin (KLH) - primed and normal, ovalbumin (OVA) - primed donors into irradiated syngeneic recipients. The number of anti-PC plaque-forming cells (PFC) was determined eight days after transfer.

ACTIVITY OF T_H CELL SUBSETS PRIMED WITH ANTIGENS CONTROLLED BY Ir GENES

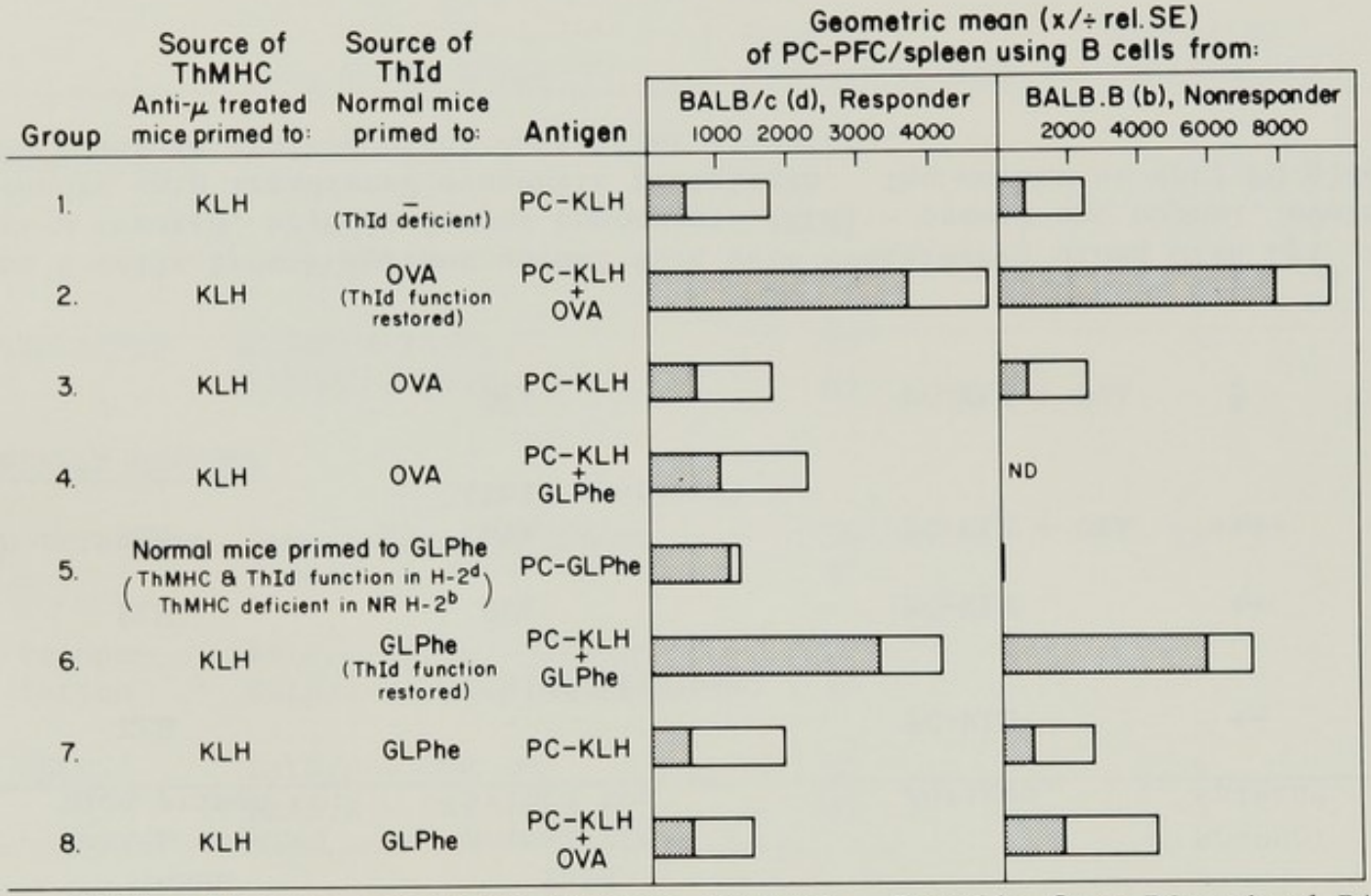

FIGURE 1. ThId cells are not under Ir gene control. B cells from PC-primed BALB/c or BALB.B donors were transferred along with syngeneic T cells from anti-μ treated KLH-primed and normal OVA or GLPhe-primed BALB/c or BALB.B donors into 500R irradiated syngeneic recipients. The number of PC-specific PFC was determined on day 8 after transfer. The shaded portion of each bar represents the proportion of the anti-PC PFC shown to be $T15^+$.

TABLE III. CHARACTERISTICS OF HELPER T CELLS INVOLVED IN THE T15 DOMINATED ANTI-PC RESPONSE

	ThMHC	ThId
A. Phenotype and Activation Requirements		
1. Ly1	+	+
2. Ly2	-	-
3. Thy1	+	+
4. Antigen specific	+	+
5. Requirement for hapten, carrier linkage	+	-
6. Presence in low T15 strains	+	-
7. Selective activation of $T15^+$ B cells	-	+
8. Activates B cells by itself	+	-
B. Anti-self specificity		
1. Antigen recognition under known Ir gene control	+	-
2. Antigen recognition is MHC-restricted	+	-
3. Recognition of T15 bearing B cells	-	?

role of MHC-linked genes in regulating T-dependent immune responses are shown in detail in Figure 1. Responder (BALB/c) and nonresponder (BALB.B) mice were primed with poly-(L-glutamic acid, L-lysine, L-phenylalanine) (GLPhe), the response to which is under the control of Ir genes (15,16). Th cells from responder and nonresponder mice primed to GLPhe were evaluated for their ability to collaborate effectively with syngeneic PC-primed B cells. It can be seen in Figure 1 (Group 5) that T cells from nonresponder mice primed with GLPhe could not provide "conventional" Th cell (ThMHC) activity as has been shown previously (17). By contrast, the same source of T cells could reconstitute

the ThId cell set which is missing from anti-μ treated mice (Group 6). Since both GLPhe primed responder and nonresponder mice could provide ThId cell activity to syngeneic B cells, it was concluded that the antigen-specific responses of the ThId cell set were not under the control of known Ir genes (18). Similarly, studies using F1 into parent chimeras have shown that the ThId cell set is apparently not MHC restricted (19) since ThId cells can collaborate effectively with B cells from both parental haplotypes. Based on these studies, it seems reasonable to propose that ThId-B cell collaboration does not involve recognition of MHC encoded products (Table IIIb). Furthermore the interaction between B cells and ThId cells may be analogous to the interaction between Ia restricted ThMHC and Ia bearing cells. Auto-idiotypic, in this case ThMHC, determinants on the cell surfaces would direct the interaction of the antigen-activated ThId cell set.

B. Comparison of the Two Helper T Cell Sets

While the experimental observations are consistent with the notion that ThId cells appear to be specific for autologous Id determinants and antigen, there are many interesting issues which remain unresolved: 1) Does the ThId cell set bear receptors which bind idiotypic or Ig associated determinants? 2) Does the precision of antigen recognition of ThMHC differ from ThId cells since antigen recognition by ThMHC cells is clearly influenced by their anti-self specificity? 3) Does one cell recognize both nominal antigen and idiotype or are the specificities characteristic of the ThId cell population due to interacting T cell subsets?

Since the antigen-specific ThId cell population defined here regulates exclusively the activation of T15 bearing PC-specific B cells, it can be postulated that ThId cells select B cells bearing the T15 idiotype by means of anti-idiotypic receptors. While this interpretation may explain the selective activation of T15 bearing B cells, it is possible that selected B cell subpopulations are triggered by signals from Th cells which involve the interaction of T cells and/or their products with unique B cell surface molecules. This interaction need not involve recognition of idiotypic determinants on the B cell. T15 dominance may be expressed if the B cell subpopulation triggered is mainly T15 bearing. However, evidence from other systems demonstrating that Ig recognizing Th cells can descriminate various isotypes (20), allotypes (21) or idiotypes (4-8) as well as studies that Id-specific Th cells can bind idiotype

directly (4) are compelling arguements in favor of the notion that ThId cells recognize auto-idiotypic determinants. Equally convincing is the evidence that the presence of ThId cells is linked to the levels of naturally occuring circulating idiotype suggesting that circulating Id may play a role in development (expansion and/or activation) of ThId cells (22). Moreover, it would seem unlikely that the triggering of a unique B cell subset by ThId cells or their products involving interaction molecules other than idiotype would be confined to only those strains expressing the T15 Id, especially since ThId cells appear to trigger exclusively T15 bearing B cells regardless of whether the source of B cells came from a strain expressing high or low levels of circulating idiotype. A final resolution of whether or not the ThId cell set recognizes B cell idiotype will depend on experiments demonstrating the fine specificity of ThId-B cell interaction.

Previous studies have shown that idiotype-recognizing Th cells can be generated by direct immunization with idiotype (23-26). It seems likely that these cells are distinctly different from the ThId cell set, possibly recognizing linear determinants rather than idiotypic or conformational determinants as they are represented on cell surfaces (27,28). Moreover, the activity of directly immunized idiotype-specific cells appears to be under H-2 gene control (26) and to be most efficient when the hapten and carrier (in this case, idiotype) are physically linked. These results indicate that Th cells immunized with idiotype resemble "conventional" H-2 restricted, carrier specific helper T cells (ThMHC), with little evidence to suggest that these cells are physiologically important in selective activation of idiotype bearing B cells. By contrast, the ThId cell set is important in selective activation of B cells bearing the T15 idiotype and is specific for an immunizing antigen unrelated to the T15 idiotype. Thus, the ThId cell set appears to recognize idiotypic determinants independent from its recognition of the immunizing antigen. Yet the concept that different sets of antigen-specific T lymphocytes may recognize autologous gene products not all of which are encoded in the MHC is feasible only if it can explain how, given the tremendous variability in idiotypes, the interaction between a ThId cell and an idiotype bearing B cell is possible. It can be observed during an anti-PC response that while there is selective activation of T15 bearing B cells, there appears to be no change in the activation of B cells bearing alternate idiotypes even when varying the source of the ThId cell set (Fig 1, line 1 v. line 2 and 6). These data suggest that certain idiotypes,

which occur in high frequency, are important in regulatory events. These idiotype bearing Igs may be promoted preferentially because they have protective properties against common environmental pathogens (29), thus limiting the number of idiotypes required to be recognized and selected by the ThId cell set.

Although the concept that distinct T cell sets may display similarities in their ability to recognize antigen and to interact with appropriate target cells by recognition of cell surface determinants, it seems unlikely that the ThMHC and ThId cell sets parallel one another in their fine specificity for antigen and the types of signals delivered to the B cell. Considering the role MHC-encoded determinants are thought to play in the specificity of antigen recognition by ThMHC cells, it might be predicted that the precision of recognition of T-dependent antigens by ThMHC and ThId cells would be different. The specificity of ThMHC cells for antigen seems to be very precise (30,31). This capacity to discriminate may be determined in part by the recognition of antigen in association with self MHC encoded gene products. By contrast, the recognition of antigen by ThId cells seems independent of the self specificity (here the T15Id). This difference in the form in which antigen is recognized by ThMHC as opposed to ThId cells suggests that the two sets of cells could recognize the same antigen using the same receptor with distinct specificity patterns as measured functionally. Moreover, preliminary data do suggest that ThMHC cells are less cross-reactive than ThId cells. While an independent evaluation of antigen activation of the ThId cell set has not been undertaken, the ThId cell set would appear not to require simultaneous recognition of Id and antigen to deliver its helper signal to a T15 bearing B cell. This concept is strongly supported by the observation that PC-specific B cell activation by antigen activated ThId cells does not require an association between the priming antigen and the hapten PC to promote the association of antigen with T15 bearing cells. Furthermore, differences between the two types of T cells is reflected in the types of signals each delivers to a B cell. This is particularly evident from the experiments described in Figure 1, where T cells from nonresponder mice lack GLPhe specific-ThMHC cells but possess functional, GLPhe-specific ThId cells; these nonresponder T cells are incapable of generating an anti-PC response in the presence of PC-GLPhe, but do provide ThId cell function when mixed with a source of ThMHC cells (Fig 1, lines 5 and 6). It appears, then, that there is a qualitative difference in the signals delivered by these two cell populations and the functional expression of the activity of the ThId cell set

may depend on the presence of the "conventional" ThMHC, H-2 restricted cell. While at present there is no evidence distinguishing the effects of each Th cell population on a PC-primed B cell, perhaps the presence of the ThMHC cell is required for the elicitation of growth factors which can act initially on either the B cell or the ThId cell set. Only then can the ThId cell deliver its signal to the B cell, possibly via the immunoglobulin receptor alone. While the nature of this signal (whether proliferative and/or differentiative) is at this time unknown, it may be able to allow for selective activation of Id bearing B cells initially during an immune reponse.

Finally, experiments establishing the notion that a single T cell bears two receptors which function independently will ultimately depend on cloning ThId cells and demonstrating their specificity for antigen and idiotype. At this time, the explanation that ThId cells bear two receptors is based on the following arguments: 1) The functional activity of the ThId cell set depends on antigen priming of the T cell donor in vivo and reexposure of the T cell population to the priming antigen to elicit a T15 dominated anti-PC response (6,7,18) (see Groups 1-4, Fig 1). The use of unprimed T cells or antigen-primed T cells which are confronted with inappropriate antigen or no antigen during the secondary response will not provide selective help to T15 bearing B cells. In addition, preliminary results indicate that the activity of the ThId cell set can be removed by antigen-coated petri dishes. 2) If two or more T cells account for the specificity requirements seen experimentally, perhaps one T cell being antigen-specific and one T cell being idiotype-specific, the antigen-specific cell would constitute another, as yet new and uncharacterized T cell population, distinct from the antigen-specific, MHC restricted, ThMHC cell set. This third cell would have to be lacking or deficient in low T15 strains and must not be H-2 restricted or under the control of known Ir genes. While this explanation for the results can not be ruled out at this time, it is the author's opinion that the concept of one cell bearing 2 receptors, one for Id and one for antigen, is the simplest explanation.

Taken together these experimental results strongly support the concept that there exist two functionally distinct Lyl^+ helper T cells necessary for a T15 dominated secondary T-dependent anti-PC response. The selective activation of T15 bearing B cells depends on the presence of the ThId cell set since depletion of ThId cells experimentally or by the use of T cell clones (see Table I) generates an anti-PC response which is predominantly non-T15 in nature (32). When considering why the immune system would choose to make an

idiotypically restricted response which clearly does not utilize the best product in terms of affinity for PC (6), it might be postulated that the ThId cell serves to activate early precursor PC-specific B cells most frequently represented during an immune response. The rapid activation of those T15 bearing B cells may have a protective role as has been demonstrated for anti-PC antibodies during pneumococcal infections (29). This suggests that ThId cell sets may only be represented for promotion of those antibody responses important in defense against common environmental antigens, there being no need to postulate that there is such a Th cell for all idiotypes expressed by B cells.

III. INFLUENCE OF ThId CELL SET ON CLONAL DOMINANCE

A. T-Independent and T-Dependent B Cell Responses

While it is clear that the ThId cell set has a potent effect on the idiotypic dominance of secondary T-dependent (TD) responses to PC, this mechanism appears not to be the explanation for the T15 dominance expressed by a separate population of B cells responding to T-independent (TI) antigens. With the use of antigen-specific, proliferating T cell clones (33) as a source of "conventional" helper T cells (ThMHC), it was possible to evaluate the activation of different subpopulations of PC-specific B cells in the relative absence of the ThId cell sets. To do this, ovalbumin (OVA)-specific T (T_{ova}) cells which recognize OVA in the context of self MHC encoded determinants (33) can be shown to induce a substantial anti-PC response *in vitro* in the presence of the linked hapten-carrier conjugate, PC-OVA (32). The presence of this cloned T cell, which appears to mimic ThMHC activity, generated TD anti-PC antibody responses which were predominantly non-T15 in nature (32). A summary of the data using various T_{ova} clones can be seen in Table IV. Using this *in vitro* system to evaluate the ability of a purified population of B cells in the presence of T_{ova} cells to respond to TI or TD forms of PC, it was possible to demonstrate a vast difference in T15 Id expression. While the anti-PC responses to TI or TD forms of PC could be shown to increase with increasing T_{ova} cell number (32), the response to PC coupled to *Brucella abortus* (BA), in contrast to the response to PC-OVA, was dominated by B cells producing T15 bearing antibody. This idiotypic dominance seemed not to be influenced by the absence of the ThId cell set. This suggests that the B cell subpopulations responding to TI forms of PC maintain T15 dominance by another mechanism.

TABLE IV. RESPONSE TO PC-OVA AND PC-BA IN VITRO IN THE PRESENCE OF CLONED, MHC RESTRICTED OVA-SPECIFIC, $Ly1^+$ T CELLS

	PC-primed B Cells Responding To:			
T Cell Clone	PC-OVA		PC-BA	
Added to B Cells				
T_{ova}	PFC	%T15	PFC	%T15
A3a	+	28	+	92
B2b	+	33	ND	ND
D4b	+	30	ND	ND
B6d	+	21	+	85
A3d	+	38	+	89
1C1	+	ND	+	89
C4d	+	15	+	93
B4d	+	33	+	88
A4d	+	42	+	86
B2d	+	36	+	91
A5d	+	31	+	90
C1a	-	-	ND	ND
0	-	-	+	93

$3x10^6$ B cells from PC-primed donors were mixed with $1x10^5$ T_{ova} cells along with 0.1 μg/ml PC-OVA or 10^{-4} dilution of stock PC-BA in 24 well (1ml) Costar plates.

From these data, it seems possible that PC bearing environmental antigens could resemble TI forms of PC and selectively stimulate T15 bearing B cells. Subsequent re-exposure to PC, triggering TI responding B cells, would yield a T15 dominated anti-PC response. This explanation might also account for high levels of naturally occuring circulating T15 Id seen in some strains, since PC is a component of gut and respiratory bacteria. Similarly, T cells have also been shown

to selectively express the "regulatory" or "germline" idiotypes in a variety of antigenic systems (24,34-36), and perhaps are selected and expanded by exposure to environmental antigens in much the same manner as TI responding B cells. One could postulate that the initiation of T15 dominance begins with certain subpopulations of lymphocytes responding to environmental pathogens (TI forms of PC) with the selection of T and B cells which are the most protective against these pathogens, in this case those bearing the T15 Id. Furthermore, the expansion of Id bearing T cells early in ontogeny may have effects on the differentiation of ThId cells which appear to recognize Id determinants and perhaps have been selected for by cell to cell interactions involving Id-bearing T cells and those Th cells bearing receptors for T cell Id. This suggests that there may be analogous differentiation of the ThId and ThMHC cells sets, leading to the selective expression of specificity repertoires for self determinants on these cells. Just as ThMHC cells are selected for possession of anti-self MHC specificity during their development in the thymus (2,3), so would ThId cells be selected for anti-self Id specificity by contact with self T cell idiotypic determinants. Since T cells appear to express "regulatory" or "germline" idiotypes, ThId cells would be focussed on providing help to B cells bearing the same or similar Ids. Once selected for, ThId cells would be expanded in the periphery by the circulating Id much in the same way ThMHC cells may be expanded by contact with peripheral MHC encoded antigens (37).

This hypothesis predicts that environmental antigens play a critical role in the initiation of clonal dominance as expressed by Id bearing T cells and TI responding B cells. Once initiated, the ThId cell set may be selected for by contact with the T cell "regulatory" Id and subsequently will selectively activate Id bearing B cells early during TD immune responses. While T cells and TI responding B cells may be restricted to the expression of "regulatory" or "germline" idiotypes, TD responding B cells may shift from a predominant idiotypic response to a more heterogeneous response due to suppression of the ThId cell set as the response proceeds.

REFERENCES

1. Jerne, N.K. Eur. J. Immunol. 1, 1 (1971).
2. Bevan, M.J. Nature 269, 417 (1978).
3. Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A. and Klein, J. J. Exp. Med. 147, 882 (1978).

4. Woodland, R. and Cantor, H. Eur. J. Immunol., 8, 600 (1978).
5. Hetzelberger, D. and Eichmann, K. Eur. J. Immunol. 8, 846 (1978).
6. Bottomly, K. and Mosier, D.E. J. Exp. Med. 150, 1399 (1979).
7. Bottomly, K., Janeway, C.A. Jr., Mathieson, B.J. and Mosier, D.E. Eur. J. Immunol. 10, 159 (1980).
8. Adorini, L., Harvey, M. and Sercarz, E.E. Eur. J. Immunol. 9, 906 (1979).
9. Katz, D.H., Graves, M., Dorf, M.E., DiMuzio, H., Benacerraf, B. J. Exp. Med. 141, 263 (1975).
10. Sprent, J. J. Exp. Med. 147, 1159 (1978).
11. Sprent, J. J. Exp. Med. 147, 1838 (1978).
12. Bottomly, K., Mathieson, B.J., Cosenza, H. and Mosier, D.E., in "B Lymphocytes in the Immune Response" (M. Cooper, D. Mosier, I. Scher and E. Vitetta, eds.), p. 323. Elsevier North Holland, New York (1979).
13. Mitchison, N.A. Eur. J. Immunol. 1, 18 (1971).
14. Bottomly, K. and Janeway, C.A. Jr., Eur. J. Immunol. In press.
15. Merryman, C., Maurer, P.H. and Bailey, D.W. J. Immunol. 108, 937 (1972).
16. Dorf, M.E., Stimpfling, J.H., Benacerraf, B. J. Exp. Med. 141, 1459 (1975).
17. Katz, D.H., Dorf, M.E. and Benacerraf, B. J. Exp. Med. 143, 906 (1976).
18. Bottomly, K. and Maurer, P.H. J. Exp. Med. 152,1571 (1980).
19. Bottomly, K. and Mosier, D.E. Manuscript submitted for publication.
20. Kishimoto, T. and Ishizaka, K. J. Immunol. 111, 720 (1973).
21. Herzenberg, L.A., Okumura, K., Cantor, H., Sato, V.L. Shen, F-W., Boyse, E.A. and Herzenberg, L.A. J. Exp. Med. 144, 330 (1976).
22. Bottomly, K. and Mosier, D.E. in "Strategies of Immune Regulation" (E. Sercarz and A. Cunningham, eds.) p. 487 Academic Press, New York (1980).
23. Janeway, C.A. Jr., Sakato, N. and Eisen, H.N. Proc. Natl. Acad. Sci. U.S.A. 72, 2357 (1975).
24. Cosenza, H., Julius, M.H. and Augustin, A.A. Immunol. Rev. 34, 3 (1977).
25. Eichmann, K., Falk, I. and Rajewsky, K. Eur. J. Immunol. 8, 853 (1978).
26. Hannestad, K. and Jorgensen, T. in "Immunoglobulin Idiotypes and Their Expression" (C.A. Janeway, E. Sercarz and H. Wigzell, eds.) Academic Press, New York,

in press.
27. Benacerraf, B. J. Immunol., 120, 1809 (1978).
28. Rosenthal, A.S. Immunol. Rev. 40, 16 (1978).
29. Briles, D.E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J. and Barletta, R. J. Exp. Med. 153, 694 (1981).
30. Hill, S.W. and Sercarz, E.E. Eur. J. Immunol. 5, 317 (1975).
31. Berzofsky, J.A., Pisetsky, D.S., Schwartz, R.H., Schechter, A.N. and Sachs, D.H. Adv. Exp. Med. Biol. 98, 241 (1978).
32. Bottomly, K. and Jones III, F. in "B Lymphocytes in the Immune Response: Functional Developmental and Interactive Properties" (N. Klinman, D. Mosier, I. Scher and E. Vitetta, eds.), p. 415. Elsevier North Holland, New York (1981).
33. Jones, B. and Janeway, C.A. Jr. Manuscript submitted for publication.
34. Black, S.J., Hämmerling, G.J., Berek, C., Rajewsky, K. and Eichmann, K. J. Exp. Med. 143, 846 (1976).
35. Krawinkel, U., Cramer, M., Imanishi-Kari, T., Jack, R.S., Rajewsky, K. and Mäkela, O. Eur. J. Immunol. 8, 565 (1977).
36. Mozes, E. and Haimovich, J. Nature 278, 56 (1979).
37. Zinkernagel, R.M. Immunol. Rev. 42, 224 (1978).

REGULATION OF ANTIBODY SPECIFICITY AND IDIOTYPY BY TWO INDEPENDENT T CELLS[1]

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When the B10.A mouse makes an antibody response to multideterminant hen eggwhite lysozyme (HEL) in complete Freund's adjuvant, the end product is not a random blend of antibodies directed against a variety of regions on the molecule, with varying idiotypy and isotypy. In fact, the predominant antibody response is primarily directed against a single region on the molecule, bears a predominant idiotype (IdX) and is more than 90% of the IgG1 isotype. We have no understanding of the isotypic regulation and will not discuss this further. However, we would like to propose that the highly restricted idiotypy and specificity of the response is determined by two different types of T cell: the MHC-restricted antigen-specific T helper cell, AgTh, and an idiotype-specific T helper cell, IdTh. It is interesting in this system that the AgTh belongs to an idiotypic universe distinct from IdX and thus AgTh interactions with other cells (e.g. B cells and Ts) involve mediation by antigen rather than idiotype.

DETERMINATION OF ANTI-HEL SPECIFICITY IN B10.A AND A/J MICE

a. Anti-HEL in B10.A and A/J Mice is Directed in Large Majority Against the N-C Region of HEL. We have utilized a peptide (termed "N-C"), derived by mild acid hydrolysis, containing (residues 1-17 disulfide bound to the C-terminal 120-129 (1), to show that the large proportion of the

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isoelectrically focused spectrum of anti-HEL antibodies binds to radiolabeled N-C. Furthermore, an N-C solid phase immunabsorbent removes most anti-HEL antibody whereas an absorbent made with the mixed disulfide of L2, the central portion of the HEL molecule (a.a. 13-105) and containing almost all the remainder of HEL, removes about 10% of the anti-lysozyme antibodies.

This amazing preference for the N-C region explained a mystifying result obtained a decade ago (2) in studying mouse anti-HEL and anti-TEL (turkey lysozyme). Whereas (see Table I below) mouse anti-TEL or rabbit anti-HEL sera failed to react with iodinated TEL, they reacted quite well with iodinated HEL. That I*TEL was still largely intact was indicated by its reactivity with antisera from rabbits immunized with TEL. TEL has a tyrosine at amino acid residue 3 instead of phenylalanine and we presume that interaction between mouse anti-HEL, reacting primarily at the N-C-terminus with I*-TEL was sterically hindered by preferential labelling of I* at position 3 in TEL.

Table 1. Iodination of Turkey Lysozyme Interferes with its Regional Reactivity.

	# animals	Direct-binding to: *I-HEL	*I-TEL	Avg ratio of reactivity with HEL/TEL [1]
Rabbit anti-HEL	3	+++	+	30
Rabbit anti-TEL	5	+++	++++	0.36
Mouse anti-TEL	5	+++	+	9

[1]Represents numerical ratio of antigen-binding values. Inhibition assays of these sera with unlabeled HEL + TEL gave ratios very close to 1.

It should be stressed that the conclusion that "mouse" anti-HEL is directed against N-C is nevertheless provisional. (a) We have only examined the specificity of anti-HEL in a very few mouse strains. (b) The affinity of the typical peptide-antibody interaction is two to three orders of magnitude lower than that for the native protein, making reactivity with peptides extremely hard to measure in the mouse. Isoelectric focusing is one of the few modalities which seems to allow the low affinity reactivity of peptides to be readily visualized. (c) We have not as yet been successful in any plaque forming cell assay exploiting peptide-coated red cells, or in the use of peptides as inhibitors of PFC. Although it is possible to compare the repertoires of different mouse strains by using a panel of closely or distantly related lysozymes, it is rare that an exact epitope can be specified by this technique. Despite these limitations, we are confident of the N-C specificity of the B10.A or A/J response to HEL.

b. The MHC Limits the Breadth of Response. We may ask why the response should be restricted in the mouse, whereas Atassi and his colleagues have shown that in the goat (3), there is evidence for an equal response to three major, widespread epitopes on the molecule. We feel that the answer to this query lies in the deficiency in the breadth of epitopes effectively presented by the major histocompatibility complex to T cells of a single mouse strain. A large amount of evidence has accumulated, inherent in the studies of Ir gene control of the immune response, which suggests that deficiencies of epitope presentation in any one strain of mouse are usually overcome in the F1 between two inbred strains. We suppose that there is a limitation of attachment sites on the peptide capable of combination with appropriate Ia molecules on the presenting cells. The result of this limitation is that only certain portions of the antigen are able to effectively address the T cell repertoire.

One of the most striking findings from our laboratory during the past several years has been the high degree of specificity restriction characteristic of a particular haplotype in its recognition of peptides within a molecule the size of HEL (129 amino acids). It must be remembered that from the viewpoint of foreignness, HEL with about 50 amino acid differences from mouse lysozyme would be expected to have many potential epitopes. However, when either HEL or the related ringnecked pheasant lysozyme (REL) is

presented to the B10.D2 or other H-2^d T cells, the only proliferative T cells that are activated are those with specificity for a circumscribed region within the third cyanogen bromide peptide of the lysozyme, (termed L3). In this instance, because of the fortunate alignment of amino acids within the panel of lysozymes, it was possible to designate two amino acids, asn-arg, (HEL) or lys-his, (REL) at residues 113 and 114 as crucial residues within the determinant in the L3 peptide. Results with L3 (a.a. 106-129) isolated from REL and HEL confirm the notion that this region of the lysozymes is uniquely recognized by the B10.D2 H-2^d mouse: L2 of HEL (a.a. 13-105) and REL (a.a. 16-105) do not raise a proliferative response in this strain. If this represents a fundamental inability of H-2^d strains to recognize such a large portion of this seemingly multideterminant antigen, it suggests that there must be a rare concomitance of events underlying immune recognition of proteins. In the case of the H-2^d mouse, the "regional specificity" is especially interesting since it was not expected that the unique reactivity site of two non-cross reactive antigens (HEL and REL for B10.D2 T cells) would be in the same place! To us this is suggestive of a strongly favored attachment site, identical on HEL and REL, and quite close to residues 113-114. It is possible that such attachment sites are not widespread and that only one or two might be found on a molecule the size of lysozyme.

That this isn't an isolated instance is evident from work of Krzych et al. (4) on the large protein antigen, beta-galactosidase (GZ). Examination of the several cyanogen bromide peptides with a molecular weight around that of L2(HEL) showed that one of these, CB-20(GZ) seemed to neither elicit a T helper, T proliferative nor a T suppressor response in the H-2^k mouse, whereas the other 11 peptides studied displayed one or two of these activities. A conceivable alternative with such "null" antigen fragments, is that suppressor cells could be raised by an epitope on the "null" fragment which have as their target another portion of the same seemingly non-immunogenic fragment. (This type of regulatory circuitry prevails for lysozyme as a whole, in which the non-responder $H2^b$ mouse does not respond at all to HEL because of a suppressor-inducing epitope at the N-terminus of the molecule (5)). Such targeted suppresson seems unlikely in the case of the H-2^d lack of response to L2. One approach to disclosing suppression would be to try to reveal smaller peptides

within L2-(HEL) or CB-20(GZ) which are able to induce a T cell proliferative response, suggesting some suppressive interaction which keeps the potential hidden.

An interesting example of possible confusion at the level of assessing the T repertoire comes from experiments in the B10.A strain. Here, immunization with HEL primarily addresses T cells with specificity for an epitope within L2, with little if any reactivity towards L3. However, B10.A mice are perfectly able to respond to L3 if primed originally with L3 in CFA. Thus, in the B10.A mouse primed with HEL a type of hierarchy is in effect with clear preference for one rather than other epitopes within the molecule. Whatever the reason for the phenomenon, it leads to an apparent singlemindedness in the response to the antigen.

c. Relationship Between AgTh Restriction and Limited B Cell Activation. Why should the antibodies made in the $H2^a$ mouse be directed against the N-C region of lysozyme? We postulate that at one stage in cell collaboration between the Th cell and the B cell, antigen-bridging occurs, and presume that the direction of the bridging is circumscribed by the limited recognition of lysozyme by the AgTh cells of this strain. The only lysozyme epitopes available for B cell reaction on a small protein of this size would be on the opposite side of the molecule. The general orientation of receptors to the antigen will be identical whether the T cell receptor moves to a macrophage, or if the antigen first reacts with T cell or B cell. According to this prescription, it should be an easy matter to test the specificity of antibody after immunization with L3 in the B10.D2, and such experiments are underway. Preliminary PFC results from experiments with the panel of lysozymes only indicates that the specificity in such cases is very different from usual, but more precise description of specificities is planned. One implicit idea in this formulation is that there are two independent steps in AgTh-B cell collaboration. The first step involves activation of the AgTh by an antigen fragment in association with Ia molecules of the MHC; the second is the interaction between the activated AgTh and the B cell occurring over an antigen bridge either of native antigen or "fractured antigen" (having a three dimensional site recognizable by B cells and a slightly unravelled end, capable of recognition

by the AgTh). If native antigen itself is seen by the AgTh, it has to be an epitope which can be recognized by the cell's receptor in the context of the molecule as a whole.

DETERMINATION OF THE IDIOTYPIC NATURE OF ANTI-HEL IN THE MOUSE

It is evident that we have assigned the "molecular steering" role in controlling the specificity of the anti-HEL response to the MHC-restricted AgTh. An equally challenging problem is understanding the source of the highly predominant idiotype, IdX-HEL. Initially, IdX-HEL predominance seemed to be inherent in the restricted N-C specificity of the response, but then it was discovered (6) in an examination of hybridomas with a distinct peptide specificity that both L2 and N-C binding monoclonal-antibodies could possess the IdX-HEL predominant idiotope. At this point, a historical description of the predominant idiotypy in this system would be informative.

a. Initial evidence that antibodies with differing specificity for HEL lysozyme could still share idiotypy. Before anti-HEL hybridomas were obtained in our laboratory, restricted immunogens for producing anti-Id were obtained by using rare cross reactive immunogens. For example anti-HUL serum from BALB/c mouse #43 was absorbed on HEL, and then eluted, capitalizing on the fact that HEL and HUL are extremely weakly cross-reactive (7). The resultant HEL-HUL cross-reactive antibody seemed "monoclonal" by isoelectric-focusing and served as the immunogen for guinea pigs and rabbits in raising anti-idiotypic antiserum. The anti-idiotypic serum obtained by this procedure was absorbed over normal mouse Ig columns as well as HEL columns. It was reactive with the immunogenic HEL-HUL cross-reactive spectrotype of BALB/c mouse #43, but in the IEF absorption assay, it appeared unable to react with any other anti-HUL antibodies in that serum. It also failed to react with anti-HUL antibodies in non-cross reactive mouse sera. Furthermore, it seemed to react with almost all the anti-HEL antibodies in assorted mouse strains, with BALB/c, C57BL/6, and six additional Igh allogroups.

We were aware that in the heterogeneous isoelectric spectra of anti-HEL antibodies, a variety of different fine specificities could be revealed by staining with a panel of diverse radioactive lysozymes (7). Yet each of the spectrotypes seemed to possess the idiotope (or set of idiotopes) characterizing anti-HEL antibodies. This was determined by absorption of the rabbit anti-idiotypic serum with one spectrotype at one extreme of the IEF spectrum and showing that all the anti-idiotypic activity was abrogated, followed by elution of the absorbed anti-idiotype and demonstration of its capability of reacting with a distinct spectrotype at the other pH extreme of the spectrum (7). This experiment was repeatable with other spectrotype pairs so we concluded that antibodies of differing pI as well as differing fine specificity could bear the same cross-reactive idiotype, which we termed IdX-HEL.

b. Evidence from hybridomas that antibodies with different peptide specificity for HEL also shared IdX-HEL.

As discussed above, most H-2^a anti-HEL antibodies possess specificity for the N-C region of HEL, but molecules of clearly varying fine specificity still bear IdX-HEL. However, to reveal whether IdX-HEL was wed to a particular determinant area on the molecule, it remained to isolate mouse monoclonal antibodies (MA) of different peptide specificity to show clearly that IdX-HEL was not indissociably linked to anti-N-C antibodies. MA of L2 specificity and MA of N-C specificity each could react with anti-IdX-HEL serum or could absorb out its activity. Among the six hybridomas with distinct regional specificity for lysozyme peptides, three possessed reactivity for N-C and three others for L2: each of these six were IdX-HEL+.

c. Secondary hybridomas are IdX+ whereas primary hybridomas are IdX-negative. A clarifying experiment involved the preparation of monoclonal lines by hybridization with lymphocytes undergoing a primary response, on day 9, ("primary" hybridomas) and "secondary" hybridomas, prepared with lymphocytes at day 3 or 5 of the secondary response (8,9). Almost all the secondary hybridomas were IdX-HEL+ while almost all the primary hybridomas were IdX-HEL-. It appears that a potent selective force must operate which promotes the dominance of IdX-HEL+ B cells, whatever their specificity for antigen.

d. The Existence of an IdX-HEL-Specific Th. Based on the precedent established in the azophenylarsonate (Ars) system, where Woodland and Cantor (10) demonstrated that KLH priming could induce a population of Ars-idiotype-recognizing Th cells, we asked whether HEL alone could raise populations of both IdX-HEL-specific T helper cells (IdXTh) as well as antigen-specific T helper cells (AgTh). In this set of experiments (11), the T cells were purified on anti-Ig plates and then mixed with normal B cells in culture with HEL coupled to SRBC as antigen. When the T cells were passed over an HEL-coated plate, the response was reduced four to five-fold. Evidence for the involvement of an IdXTh came from the fact that a similar reduction occurred if the T cells were passed over an IdX-HEL-coated plate, but not a normal Ig plate. Proof that two distinct cells were being removed from the T cell population was provided by a reconstitution experiment. If the non-adherent cells from each of the depleting procedures were mixed together, full responsiveness was restored.

These results suggest that after HEL immunization, not only do the MHC-restricted AgTh get activated, but also IdX-specific Th. We presume that it is this latter cohort of cells that provides the positive selective pressure leading to the predominance of IdX-HEL in the B cell population. The failure to deplete the idiotype-specific population on HEL-coated plates is an indication that the IdXTh does not bear receptors for HEL; the route leading to the induction of these IdXTh is now being studied in this laboratory.

e. Kinetics of Appearance of IdX-HEL in the Immune Response. Analysis of the proportion of IdX-HEL+ PFC was performed by using anti-idiotypic antiserum to inhibit IdX-HEL+ plaques. In the anti-HEL in vivo response, very little IgM antibody ever appears and the IgG1 isotype clearly exerts its hegemony. Even the earliest PFC are largely of the IgG1 isotype and are not inhibitable by anti-IdX-HEL prepared in rabbits or guinea pigs. In Fig. 1, it can also be noted that as the primary response wanes, the residual PFC are almost exclusively IdX-HEL+. Preliminary results show that PFC making the antibody of the highest affinity (top 5%) are also IdX-HEL+, suggesting that affinity selection and predominant idiotype selection are concurrent and linked. At the onset and peak of the secondary response, the overwhelming proportion of anti-HEL PFC are IdX-HEL+.

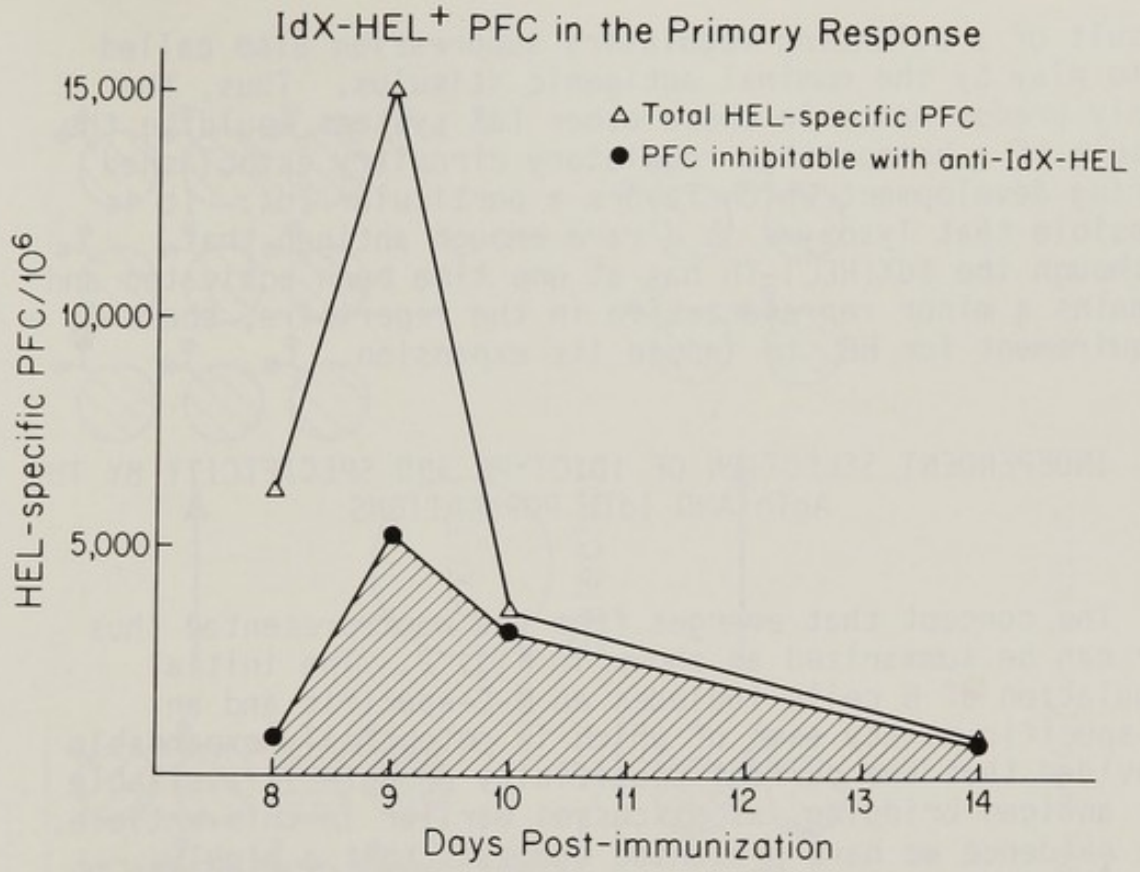

Figure 1. Shaded areas represent the IdX positive PFC. Rabbit anti-idiotype was used at a final dilution of 1-50.

This is different from the situation found in a variety of other IdX systems, e.g. the nitrophenyl (NP) or the azophenylarsonate (CRI) and the phosphorylcholine (T15) idiotypes. In these cases, the IdX appears very early in the primary response and then later in the secondary response, it is joined or replaced by other non-IdX species of antibodies, as it loses its predominance. The IdX-HEL situation is somewhat puzzling, since it exerts such a strong dominance after its positive selection, suggesting that the IdXTh already has been primed by environmental or autochthonous antigens. This would be a reasonable explanation for the early dominance of the T15, NP^b and CRI Ids; their subsequent downward modulation would be the

result of a balancing regulatory suppression also called into play by the nominal antigenic stimulus. Thus, the early predominance in these other IdX systems would be the result of a homeostatic regulatory circuitry established during development which favors a particular IdX. It is possible that lysozyme is a rare enough antigen that although the IdX(HEL)-Th has at one time been activated and remains a minor representative in the repertoire, there is a requirement for HEL to induce its expansion.

INDEPENDENT SELECTION OF IDIOTYPE AND SPECIFICITY BY THE AgTh AND IdTH POPULATIONS

The concept that emerges from the work presented thus far can be summarized as shown in Fig. 2. The initial population of B cells includes an N-C-specific and an L2-specific moiety each of which is potentially expandable provided that the correct specificity of AgTh is available for antigen bridging, as discussed earlier in this article. The evidence we have presented suggests that a highly limited area of the lysozyme molecule is utilized by the AgTh population to provide help in any particular strain. A "choice" by the MHC of which T cell to activate will indirectly determine the specificity of the B cell response. Therefore, since there is such a strong preference in the H-2^a strains for triggering a response by an epitope contained within the tryptic peptide a.a. 74-96, (M. Katz, unpublished) we assume that this explains the preference for expansion of B cells of N-C specificity.

In Figure 2, this is represented by a minimal expansion of L2-specific B cells by the AgTh of opposite (N-C) specificity. It is at this point that we postulate that AgTh-activated B cells displaying IdX will be positively selected by the IdX-recognizing Th. Therefore, after the IdXTh has reached its fully activated state, it will exert a potent selective force that leads to an overwhelming dominance of IdX^+ PFC. It is possible that an IdXTh is always required to stimulate B cell maturation to the point of antibody secretion. Thus, either L2-specific B cells or N-C specific B cells which display IdX-HEL will be driven to the final maturational stages of their development by the IdXTh.

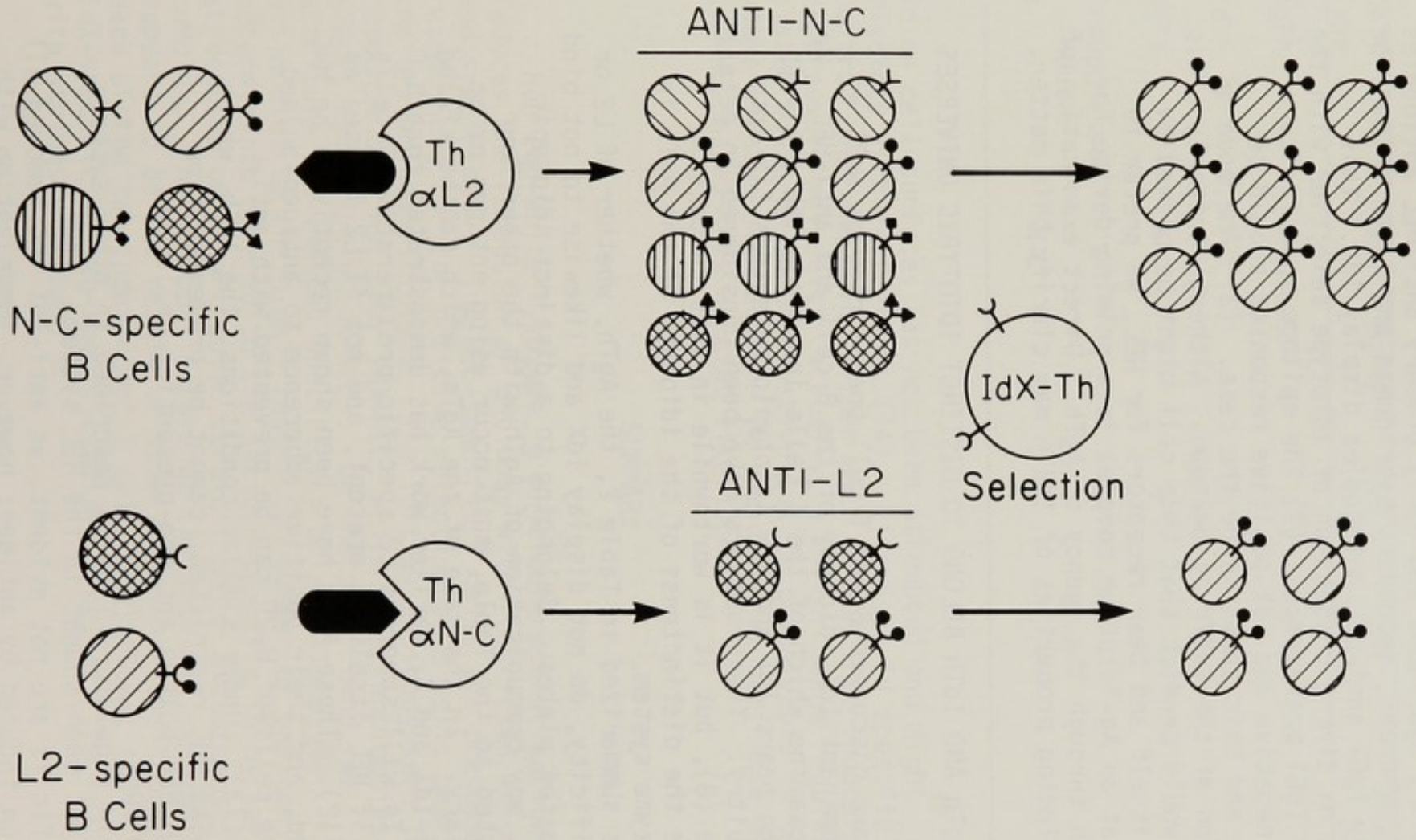

Figure 2. A scheme of AgTh and IdX-Th selection. See text for details.

Several additional points should be noted. (1) Both IgG1 primary hybridoma and early primary anti-HEL antibodies lack IdX, whereas, secondary hybridomas and later primary or secondary IgG1 anti-HEL antibodies display IdX. This suggests no direct connection of idiotype selection with the isotypic IgG1 preference. (2) The epitope specificity of the IdX-negative and IdX-positive responses should be similar, and this seems to be the case. (3) The mode of activation of the IdXTh is unknown. Although Bottomly (this volume) would predict that this cell might be primed by antigen itself and bear receptors for HEL, we prefer to think that an Ag-idiotype complex is a priming device for the IdXTh through the agency of AgTh. Direct examination of the Ag-binding properties of IdXTh may clarify this matter.

THE AgTh AND IdTh BELONG TO DISTINCT IDIOTYPIC UNIVERSES

We have discussed B and T cell specificity as well as the predominant IdX idiotypy of the B cell and anti-HEL. We can now examine which of the T cells involved with anti-HEL regulation bears IdX and how idiotypic receptors fit into the circuitry. These matters have been considered in detail elsewhere (8), but it is worthwhile in this context to emphasize the distinctness of the idiotypy of the AgTh in the lysozyme system.

As is summarized in Table 2, the AgTh, whether of L2 or N-C specificity, do not display IdX and likewise to not bind to IdX coated plates. Belonging to a distinct idiotypic universe, any communication of AgTh with the other four cells listed in the Table, must occur using antigen as an intermediary. In the case of the AgTs, which can be killed with anti-IdX and C, earlier work has demonstrated that N-C specific Ts will suppress L2 specific proliferative T cells, but only if HEL itself is present, and not if L2 is used as antigen (12). These AgTs have been shown recently to be MHC restricted, and their positive adherence to antigen-pulsed presenting cells of H-2^b can be prevented with anti-idiotype (13). Under similar conditions, the AgTh, which are likewise MHC restricted, cannot be prevented from binding to HEL-pulsed antigen-presenting cells by anti-idiotype.

Certain regulatory T cells described in other systems have not yet been sought in the HEL system. Thus, IdX-specific Ts are not evident, as anti-IdX + C removed all suppression induced by antigen; however, immunization with

Table 2.

Cell Type	Specificity	I-J	IdX on cell	Binds to IdX
AgTh	L2; N-C	-	-	-
IdTh	IdX	?	-	+
AgTs	N-C	±	++	-
T-suppressor inducer	?	++	-	?
B	N-C	-	++	-

Id on cell surfaces has not been attempted and might disclose such suppressors (14). Likewise, Id specific control over the AgTh, either with Id-specific inducers or suppressors may exist:.actually, a mirror-image regulatory system based on an alternative IdX is conceivable in certain mouse strains although the very widespread dominance of IdX-HEL makes this a faint likelihood.

SUMMARY

Hen egg-white lysozyme, a multideterminant antigen, induces the formation of antibody with restricted idiotypy and specificity. IdX-HEL-specific T cells are presumably responsible for the positive selection of B cells bearing the predominant idiotype. The initial antibody seen in the response does not bear IdX-HEL, while later antibodies are almost exclusively IdX-HEL+. On the other hand, we attribute the limited specificity of anti-lysozyme antibodies to the highly restricted number of antigenic determinants offered to the antigen-specific Th by Ia structures of a particular strain's major histocompatibility complex. The eventual nature of the anti-HEL response appears to be the result of steering mechanisms imposed by each of the two independent T cell populations, the IdX-specific and Ag-specific helper T cells. The help comes with strings attached!

REFERENCES

1. Harvey, M.A., L. Adorini, A. Miller, and E.E. Sercarz. Nature, 281:594-596. (1979).

2. Stratton, J.A., and A. Miller. Fed. Proc. 29:700 (1970).

3. Atassi, M.Z. Immunochemistry. 15:909-936. (1978).

4. Krzych, U., A. Fowler, A. Miller, and E.E. Sercarz. Proc., 4th International Congress of Immunology. Theme 17.4.09. (1980).

5. Adorini, L., M.A. Harvey, A. Miller, and E.E. Sercarz. J. Exp. Med. 150:293-306. (1979).

6. Metzger, D.W., A. Miller, and E.E. Sercarz. Nature. 287:541-542. (1980).

7. Benjamin, C., A. Miller, E.E. Sercarz, and M. Harvey. J. Immunol. 125:1017-1025.

8. Sercarz, E.E., and D.W. Metzger. Springer Seminars in Immunopathology. 3:145-170. (1980).

9. Metzger, D.W., A. Furman, A. Miller, and E.E. Sercarz. Submitted for publication. (1981).

10. Woodland, R., and H. Cantor. Eur. J. Immunol. 8:600. (1978).

11. Adorini, L., M.A. Harvey, and E.E. Sercarz. Europ. J. Immunol. 9:906-909. (1979).

12. Yowell, R.L., B.A. Araneo, A. Miller, and E.E. Sercarz. Nature 279:70-71. (1979).

13. Araneo, B.A., R.L. Yowell, D.W. Metzger, and E.E. Sercarz. Proc. Natl. Acad. Sci. USA 78:499-503. (1981).

14. Sy, M.-S., M.H. Dietz, R.N. Germain, B. Benacerraf, and M.I. Greene. J. Exp. Med. 151:1183-1195. (1980).

IDIOTYPE-SPECIFIC T CELL HELP IN THE ANTI-ARSONATE RESPONSE OF A/J MICE[1]

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The exposure of murine B cells to ligands which interact with surface Ig molecules potentiates the appropriate cell for activation (antibody synthesis and secretion) by T cell help, as demonstrated with anti-Ig and anti-idiotype reagents. T cell help can be provided by culture supernatants of T cells activated by Concanavalin A. Such supernatants contain both helper and suppressor factors, which are separable by gel filtration. Suppressor activity for the response to a particular antigen could be removed by and recovered from the antigen, whereas helper activity was unaffected by absorption with antigen. Moreover, the T cell-replacing helper fraction (TRF) induced antibody synthesis in cultures of spleen cells from normal or athymic mice in the absence of exogenous antigen. B cells from A/J mice cultured with TRF mounted anti-arsonate PFC responses which were >95% positive for a major idiotype (CRI), as opposed to the ~50% normally seen in antigen-induced responses. Passage of TRF through a CRI-Sepharose affinity column completely removed the CRI-inducing activity, which was recovered in a thiocyanate eluate of the column. The findings therefore indicate that TRF preparations contain helper factors which recognize and bind major idiotopes. It is not known whether the anti-idiotype and the differentiation signalling activities in TRF preparations are properties of the same molecule, but recovery of full activity from idiotype affinity columns suggests that such is the case.

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Idiotype-specific help may arise in the normal immune response when Ag causes expansion of particular B cell clones, presenting an antigenic stimulus for T cells recognizing the expanded set of surface idiotopes. B cells from Ag-primed mice, when cultured with normal T cells, induced id-specific help in the absence of Ag, indicating that T_H activation by B cell idiotopes may indeed occur. Kinetic studies of the anti-ABA response in vivo suggest that id-specific help may be operative in selecting B cells for isotype switching. Since TRF alone induced only IgM PFC formation, it appears that synergistic help (antigen-specific plus id-specific) may be required for early isotype switching by B cells.

INTRODUCTION

While it is generally accepted that the specificity of the antibody response is dependent on the binding of antigen by clonally distributed surface Ig molecules on B lymphocytes, there is as yet no consensus as to the role of Ig antigen receptors in B cell activation (reviewed in 1,2). We have approached this issue by monitoring the plaque-forming cell (PFC) response of murine B cells to ligands for surface Ig, employing anti-Ig or anti-idiotype reagents, in the presence and absence of T cells or T cell replacing factors (TRF), as well as the PFC response to "TRF" alone. It will be shown that anti-Ig reagents do, indeed, transmit a signal to B cells which potentiates their response to T cells or TRF, leading to the generation of PFC. Evidence will also be provided to show that TRF preparations obtained from Concanavalin A-stimulated T lymphocytes contain non-antibody helper factors with demonstrable anti-idiotypic specificity which, in the absence of additional T help, induce essentially idiotypically pure IgM PFC responses. A model will be developed to suggest how B cell surface Ig and anti-id T cell helper factors operate in the activation of B cells.

B Cell Activation by Anti-Ig and Anti-Idiotype Antibodies (3)

Spleen cells from BDF_1 mice cultured with varying quantities of affinity-purified polyvalent anti-mouse Ig antibodies or their $(Fab')_2$ fragments mounted polyclonal PFC responses, assayed as anti-fluorescein responses (3). The distinctive features of this response were as follows: (a) It was strictly T-dependent inasmuch as depletion of T cells with anti-Thy 1.2 serum completely abrogated the PFC response. (b) It was necessary to clean the cells of immune complexes by

trypsin-treatment or repeated washing. Cells cultured in the continuous presence of anti-Ig throughout the culture period did not yield PFC. (c) Addition of the "helper fraction" (see below) from a supernatant of spleen cells cultured with Concanavalin A for 48 hours completely restored the response of T-depleted cultures. (d) The use of three different anti-idiotypic sera in place of polyclonal anti-mouse Ig antibody selectively activated the specific idiotype in each instance. This was demonstrated for the anti-arsonate (ABA) idiotype (CRI) in A/J mice (Figure 1), the T-15 anti-phosphorylcholine idiotype in Balb/c mice, and the M-315 anti-dinitrophenyl idiotype in the Balb/c strain.

Thus, the interaction of B cell membrane Ig with appropriate ligands generates a signal to the cell which potentiates its response to T cell-derived differentiation signals.

Idiotype-Specific Help by Con A-Induced TRF

The T cell replacing helper activity (TRF) in culture supernatants from Con A-activated T cells could be resolved from suppressor activity, which was also present, on the basis of molecular size (4). Suppressor activity eluted from Sephadex G-100 columns at a molecular weight position of about 70 kd, whereas help appeared in a 15-30 kd fraction (Figure 2). The supernatants were generated by culturing unfractionated spleen cells, or cells from which B cells were largely eliminated by panning on anti-mouse Ig-coated dishes, for 48 hours in serum-free medium containing 1 μg/ml of Con A. Con A was subsequently removed by absorption with Sephadex G-50 and fluids were concentrated to 0.2 of the initial volume.

Suppressor activity for the response to sheep RBC could be removed by and recovered from the antigen, whereas helper activity was unaffected by absorption with antigen (5). Thus, suppressor molecules in Con A-induced supernatants have readily demonstrable antigen-binding activity, whereas helper factors do not. Moreover, Con A supernatant at high concentration induced polyclonal antibody synthesis in cultures of spleen cells from normal or athymic mice in the absence of exogenous antigen (Figure 3). The polyclonal effect required approximately 20X the concentration of supernatant needed for induction in the presence of anti-Ig reagents.

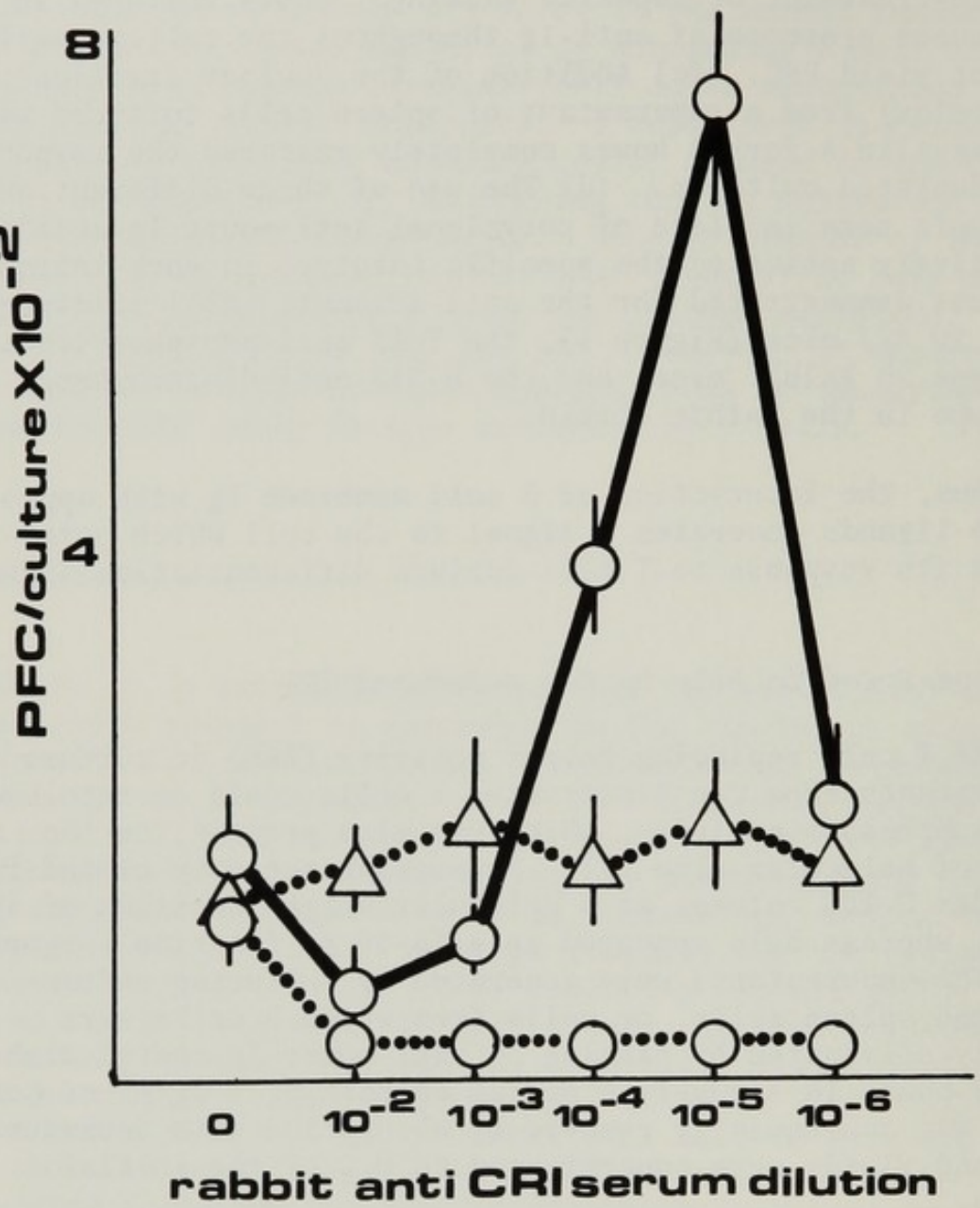

FIGURE 1. Spleen cells from A/J mice were cultured for 3 days with the indicated dilutions of rabbit anti-CRI serum. Thereafter, cells were harvested, trypsin-treated and recultured for 24 hr. PFC were detected against ABA-SRBC (O--O) and TNP-SRBC (△···△). The number of anti-ABA PFC was also determined in the presence of 10 µl of anti-CRI serum in the plaquing medium. (Reproduced from J. Immunol. (1980) 125: 1286 by copyright permission of The Williams and Wilkins Co.).

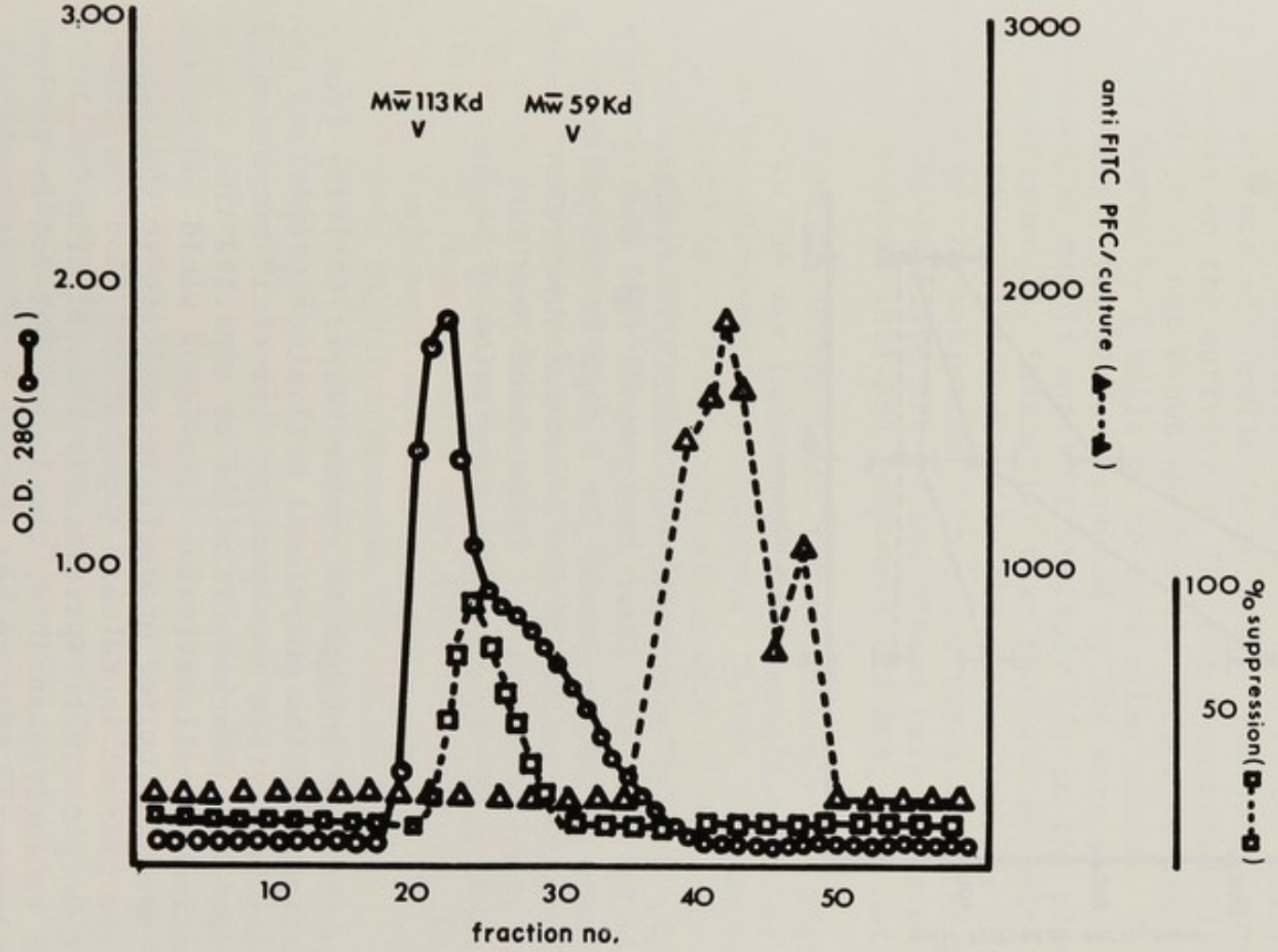

FIGURE 2. Sephadex G-100 chromatogram of Concanavalin A-induced culture supernatant of mouse spleen cells. Column fractions were assayed for helper activity in an in vitro anti-hapten response (△--△) and for suppressor activity in an in vitro antibody response to sheep RBC (□--□).
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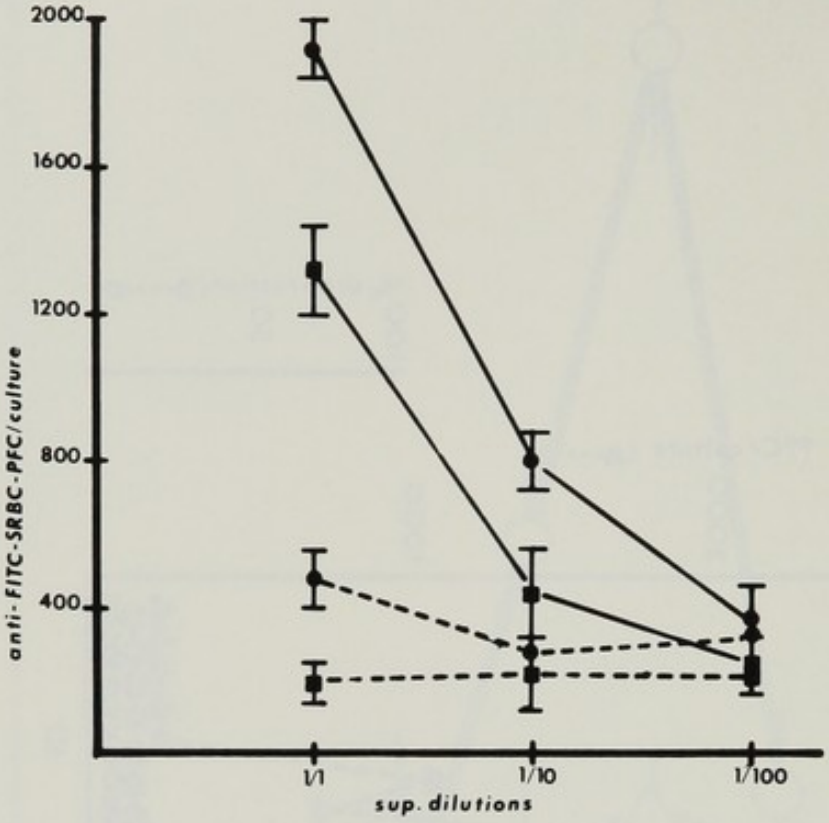

FIGURE 3. Anti-Fluocescein (FITC)-SRBC PFC response of spleen cells of nude mice (Balb background) (■) and of their normal littermates (●) cultured for 3 days in the presence of the indicated dilutions of Con A-induced supernatant (——) and control supernatant (---). (Reproduced from Eur. J. Immunol. (1979) 9:607 by copyright permission of Verlag Chemie GMBH).

The inability of antigen to remove helper activity from TRF preparations and the polyclonal activation properties of the materials point to a non-specific "hormonal" mechanism of B cell activation. However, it might be expected that a "broad spectrum" T cell activator like Con A would induce the release of specific helper products in addition to whatever else might be present in culture supernatants, and the observation that help could be specifically removed from Con A-induced TRF by antigen-antibody complexes, but not by antigen alone (6), suggested that helper factors in TRF preparations might recognize idiotypic determinants (idiotopes) on antibody molecules. We tested this hypothesis using the idiotypic system defined by the anti-ABA (azobenzenearsonate) response of A/J mice, designated CRI (7).

Spleen cells from A/J mice were treated either with monoclonal anti-Thy 1.2 serum and complement or with complement alone and cultured for 4 days with various concentrations of Con A-induced helper fraction (G-100 column) produced by syngeneic spleen cells (3). The two sets of cultures mounted significant anti-ABA PFC responses in the absence of antigen, assayed with ABA-SRBC conjugated target cells (3). The dose-response curves differed somewhat, less factor being required in the absence of T cells (Figure 4). The inhibitory effect of T cells on the activity of TRF has been noted elsewhere (3), but it is not known whether the effect is due to binding of the factor by T cells or to some other cause. Of particular interest, the plaques induced by TRF were almost totally inhibited by rabbit anti-CRI antibody (3) in the plaquing medium (Figure 1), indicating that the vast majority of anti-ABA secreting cells induced by the factor express idiotopes detected by the antiserum. This contrasts with the proportion of CRI-positive PFC we and others have observed in antigen-induced anti-ABA responses (20-80%, data not shown).

In order to determine if the exclusively CRI-positive response to TRF was induced by a CRI-specific factor, the following experiment was conducted. A CRI immunoadsorbent was prepared for affinity chromatography of molecules with anti-CRI specificity. Ascites fluid from A/J mice hyperimmunized with ABA-KLH was used to prepare anti-ABA antibody by affinity chromatography on ABA-BGG-Sepharose columns, elution being carried out with sodium thiocyanate. The anti-ABA antibody preparation, which was about 50% CRI-positive, was coupled to Sepharose for anti-CRI affinity chromatography. Spleen cells from A/J mice were treated with anti-Thy 1.2 serum and complement and cultured for 4 days with various concentrations of unfractionated TRF (G-100 helper fraction), with TRF which had been passed through a CRI column, or with the thiocyanate eluate from the column. As shown in Figure 5, absorption of TRF by CRI completely abrogated the idiotype-positive PFC response; a small residual CRI-negative PFC response was still detectable. Strikingly, the CRI inducing activity was completely recovered in the column eluate. Passage of TRF through a control column of normal mouse Ig had no effect on its CRI-inducing activity (data not shown). Therefore, the findings provide compelling evidence that the helper factor in Con A-induced TRF for the CRI-positive anti-ABA B cell response recognizes and binds the idiotope. This mediator is unlikely to be intact or fragmented Ig because it is found in a 15-30 kd molecular weight fraction and its activity is completely inconsistent with that of antibody in T cell-depleted cultures (3). Under these conditions, anti-Ig reagents did not induce PFC responses.

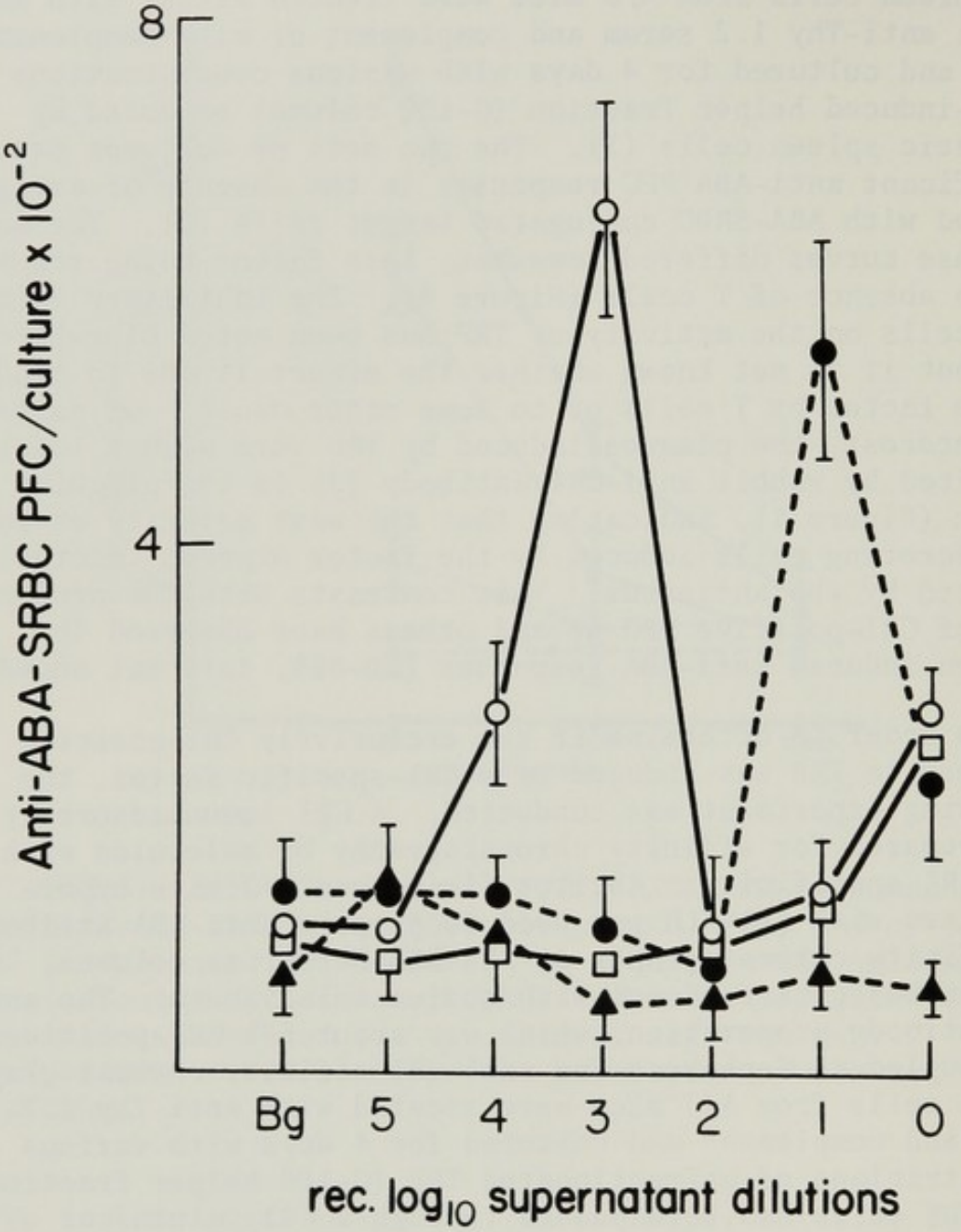

FIGURE 4. Spleen cells from A/J mice were treated either with complement alone (●--●) or with anti-Thy 1 serum and complement (O--O) and cultured in 0.5 ml volumes at a density of 10^7 cells/ml for 4 days in the presence of the indicated dilutions of Con A-induced helper factor. The number of anti-ABA-SRBC plaques was also determined in normal (▲--▲) and anti-Thy 1 treated (□--□) cultures with 15 µl of rabbit anti-CRI serum in the plaquing medium.

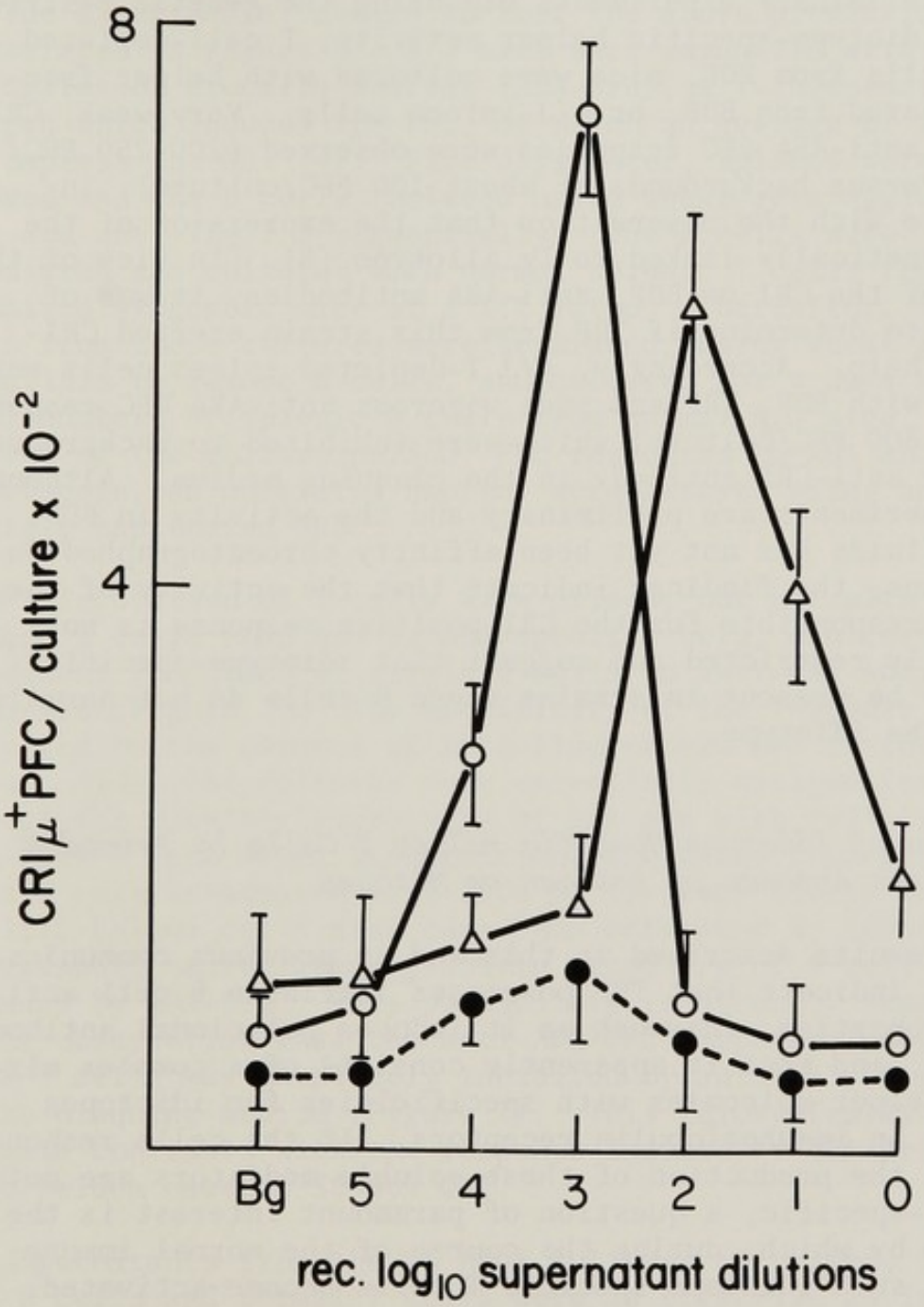

FIGURE 5. Spleen cells from A/J mice were treated with anti-Thy 1 serum and complement and cultured for 4 days in the presence of varying dilutions of crude helper factor (O--O), of helper factor passed through a CRI column (●--●) and with the eluate from this absorption (△--△). CRI+ PFC were determined by subtracting the number of PFC which formed in the presence of anti-CRI from the number which formed in its absence.

In preliminary experiments exploring the genetic restriction of idiotype-specific helper activity, T cell-depleted spleen cells from BDF_1 mice were cultured with helper fraction prepared from BDF_1 or A/J spleen cells. Very weak, CRI-negative anti-ABA PFC responses were observed (200-250 PFC/culture versus backgrounds of about 100 PFC/culture), in accordance with the observation that the expression of the CRI is genetically linked to Ig allotype (8). In view of the absence of the CRI on BDF_1 anti-ABA antibodies, it was of interest to determine if TRF from this strain exerted CRI-specific help. Accordingly, A/J T-depleted spleen cells were cultured with BDF_1 TRF and made vigorous anti-ABA PFC responses (600-800 PFC/culture) which were inhibited to background levels by anti-CRI antibody in the plaquing medium. Although these experiments are preliminary and the activity in BDF_1 culture fluids has not yet been affinity chromatographed on CRI columns, the findings indicate that the activity of the mediator responsible for the CRI-positive response is not genetically restricted and suggest that idiotype-specific T cells may be present in strains whose B cells do not normally express the idiotype.

Activation of Idiotype-Specific Helper T Cells by Primed B Cells in the Absence of Antigen or Mitogen

The results described in this and in previous communications (5) indicate that TRF possesses intrinsic B cell activating properties, inasmuch as it induces polyclonal antibody responses, and that it apparently consists of a complex mixture of helper molecules with specificities for idiotopes expressed on immunoglobulin receptors. If the cells responsible for the production of these soluble mediators are not "antigen"-specific, a question of paramount interest is the mechanism by which, during the course of the normal immune response, such idiotope-specific T cells become activated.

One might readily visualize that upon challenge with an exogenous antigen an expansion of specific B cell clones would take place, perhaps with the assistance of antigen-specific T cell help. This perturbation of the "steady state" could present an antigenic stimulus for T cells recognizing the expanded set of idiotopes, whose activation would release idiotope-specific factors which, in turn, would induce B cells to differentiate. Indeed, it has been shown that Ig or related surface molecules on activated B cells trigger Ly 1^+2^- T cell responses (9). A testable prediction based on this largely hypothetical sequence of events is that idiotype-specific help should be inducible by antigen-primed B cells in the absence of antigen.

The experimental design to test the above prediction is illustrated in Figure 6. A/J mice were immunized with ABA conjugates of Brucella abortus (ABA-Bru), a T-independent antigen which induces IgM PFC, of which an average of about 50% express the CRI (4). Five days later, spleens were removed and the B cells isolated using wheat germ agglutinin (10) and anti-Thy 1.2 sequentially. The B cells were irradiated and cultured with normal splenic T cells from immunized syngeneic mice at a 1:1 ratio. Thereafter, the T cells from these cultures were separated, using wheat germ agglutinin to remove B cells, and cultured for 4 days with equal numbers of splenic B cells from normal A/J mice. At the end of the culture period, PFC responses to ABA and to fluorescein, an unrelated hapten, were assayed using appropriately haptenated SRBC.

Preincubation of T cells with primed, but not with unprimed, B cells resulted in ABA-specific help, manifested by subsequent induction of virgin B cells to anti-ABA antibody synthesis (Figure 7). The specificity of the response was reflected by the absence of anti-fluorescein PFC in the cultures. Since the cultures were essentially antigen-free (except for possible carry-over of antigen with cells taken from mice 5 days after immunization) and cells from primed animals were irradiated with 2000 R, it seems likely that specific helper cells must have been activated by idiotypic determinants on the immunoglobulin receptors of B cell clones expanded by ABA-Bru. This possibility is strongly supported by the observation that the anti-ABA PFC response induced by these T cells was completely inhibited by anti-CRI antibody in the plaquing medium (Figure 7). This idiotypically pure response contrasts with the response induced by ABA-Bru, which seldom exceeds 50-60% CRI.

Supernatants from the 48 hour cultures of primed, irradiated B cells and normal T cells were as effective as intact T cells in inducing virgin B cells to make anti-ABA antibody, which, again, was almost completely CRI-positive (Figure 8). Anti-fluorescein PFC were not observed and supernatants of cultures containing unprimed B and T cells failed to exhibit activity.

Thus, the findings are remarkably concordant with T helper cell activation by B cell idiotopes and are, indeed, difficult to explain by any other mechanism.

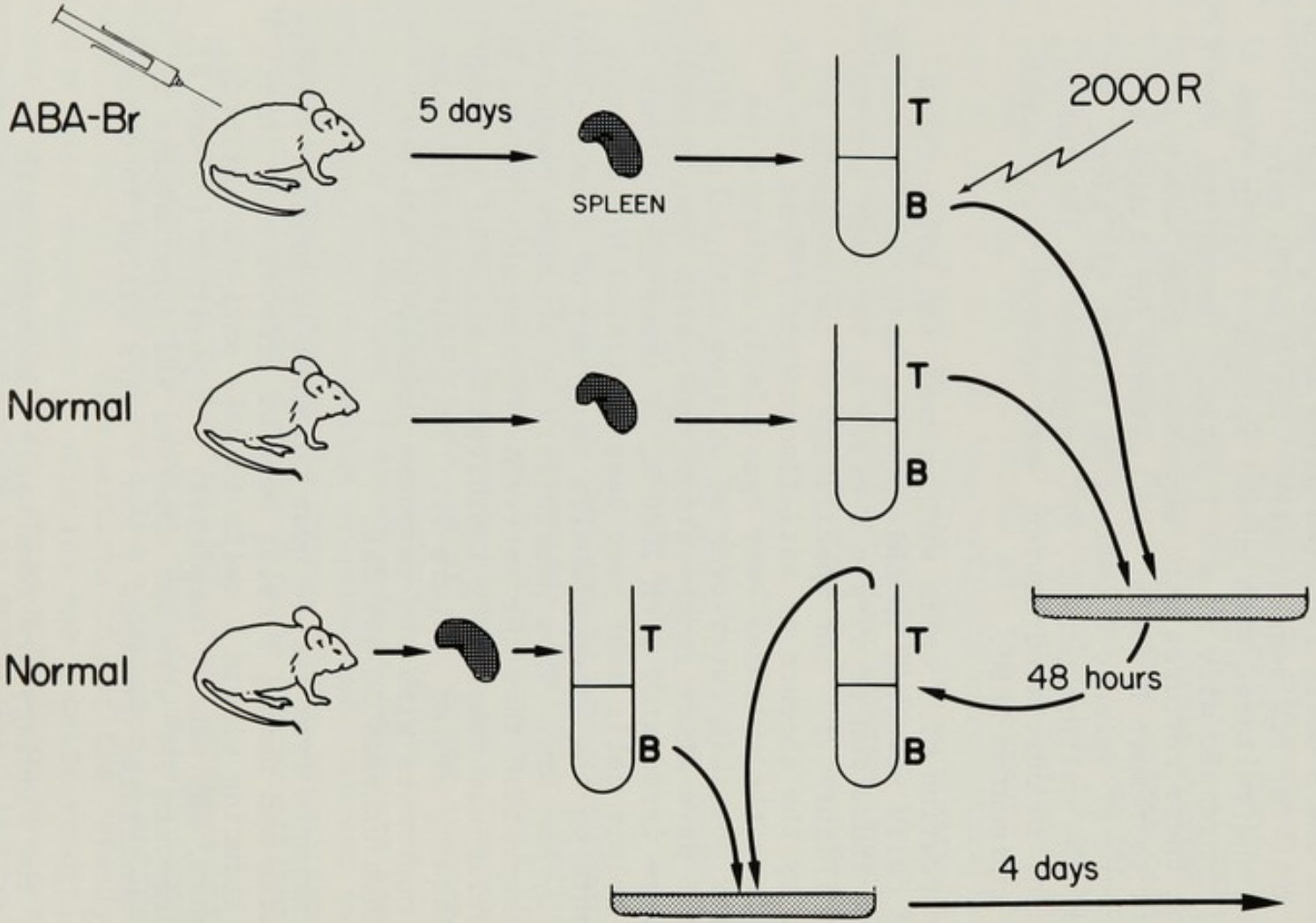

FIGURE 6. Schematic representation of the experimental protocol for assessing B cell induction of idiotype-specific T cell help.

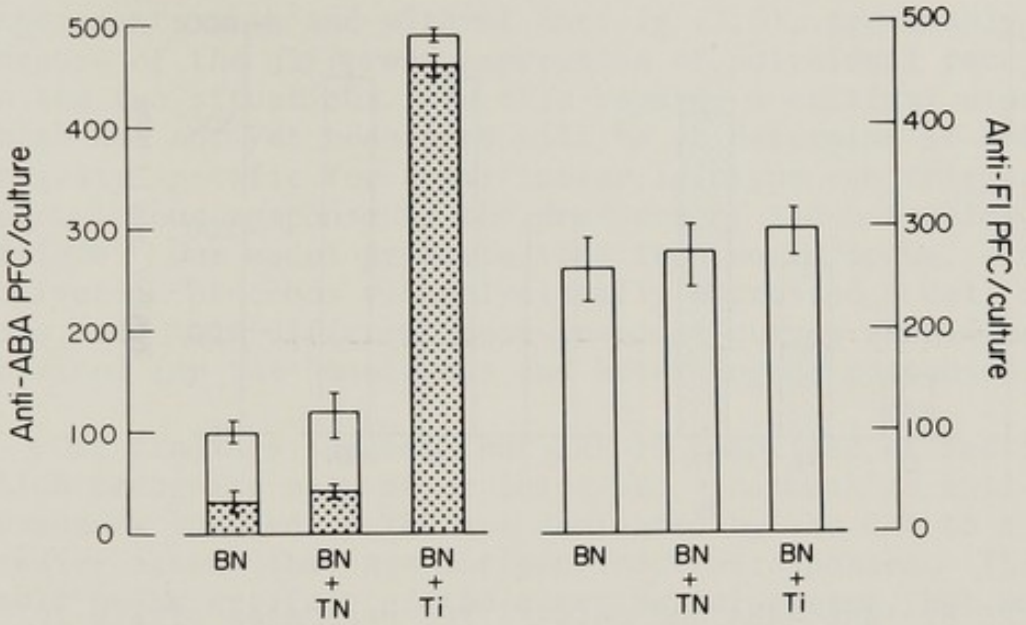

FIGURE 7. Anti-ABA and anti-FP PFC responses of 5 x 10^6 B lymphocytes from A/J mice cultured alone (BN), with 5 x 10^6 syngeneic T cells precultured with the same number of unprimed B cells (BN & TN) and with 5 x 10^6 syngeneic T lymphocytes precultured with equal numbers of B cells from A/J mice primed with ABA-Bru (BN & Ti). Stippled bars represent the number of plaques inhibitable by α-CRI antiserum in the plaquing medium.

Role of B Cell Surface Ig and of Idiotype Specific TRF in B Cell Activation

The interaction of B cell surface Ig with ligands apparently potentiates the cell for activation by T help. This has been demonstrated with specific anti-Ig reagents (3) and presumably also occurs with specific antigen. However, the surface Ig interactions are insufficient to induce antibody synthesis in the absence of T help (3), although there is evidence that they may trigger DNA synthesis (2). In our view, the findings are most readily explicable by postulating that surface Ig interactions induce or increase the expression of non-clonally distributed receptors for the helper factor, which provides the differentiation signal leading to Ig synthesis. The helper factor can activate B cells in the absence of antigen or other ligands for Ig receptors, as in the experiments described here, because it itself possesses anti-idiotype specificity and, hence, can interact with Ig receptors to induce the receptor for its activation moiety. Thus, the helper factor is postulated to recognize clonally

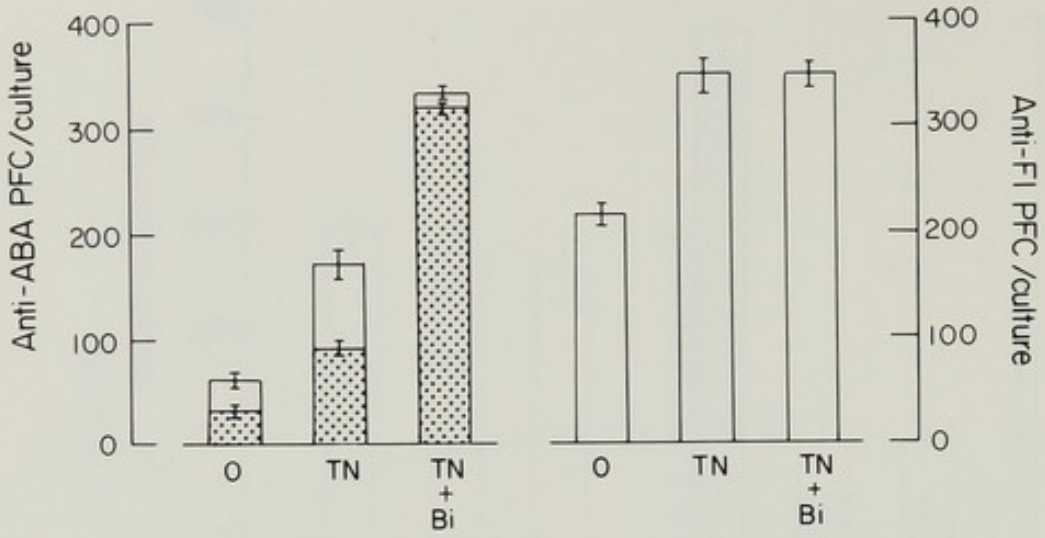

FIGURE 8. Anti-ABA and anti-Fl PFC responses of 5 x 10^6 B lymphocytes from A/J mice cultured for 4 days along (O) or with 10 l of supernatant fluid from 48 hour cultures of 5 x 10^6 normal T cells (TN) or 5 x 10^6 normal T cells plus 5 x 10^6 B cells from mice primed with ABA-Bru (TN & Bi). Stippled bars represent the numbers of PFC inhibitable by anti-CRI serum in the plaquing medium.

expressed idiotypes via an anti-idiotype binding site and non-clonally expressed receptors via a second binding site. It is this second interaction which triggers B cell differentiation.

There are at least two highly controversial aspects to this hypothesis. One is that we refer to a single "helper factor" when no such factor has been isolated and characterized. What we have demonstrated is a specific "activity" which binds to and is recoverable from an idiotype affinity matrix. Thus, we conclude that there are, among the lymphokines released by Con A-activated T cells, products with demonstrable inductive specificity. This is not to say that such products activate B cells by themselves, since macrophages (and residual T cells) are always present in the T-depleted cultures. However, the critical point in these experiments is the specificity of the responses. The second controversial aspect is the activation of B cells by TRF preparations in the absence of antigen or other external ligands for surface Ig. These results will clearly have to be confirmed and reconciled with the more general body of literature in the field before they are widely accepted. The direct activation of B cells by TRF required more than an order of magnitude higher concentration in parallel

experiments with and without anti-Ig (3,5), presumably because of the different expression of polyclonal receptors in the two situations. In this regard, a critical experiment which has not yet been done will be to determine if helper activity specific for a particular idiotype can trigger a heterologous response in the presence of the heterologous antigen. Our model predicts that this would occur, since activation proceeds via polyclonally expressed B cell receptors, and that different dose-response curves should be obtained for the homologous and heterologous responses.

Our findings suggest that TRF is comprised of factors which recognize only major idiotypes, inasmuch as anti-ABA responses induced by TRF are dominated by the CRI to a much greater extent than are antigen-induced responses. This could be an artifact of the experimental system, but more likely reflects different mechanisms of B cell activation. For one thing, only IgM responses are induced by TRF, even using B cells from antigen-primed mice. In other experiments using structurally defined synthetic bi- and tri-functional antigen molecules, it was found that at least two carrier epitopes were required for IgG anti-hapten responses in mice, whereas one sufficed for IgM responses (11). These findings are remarkably concordant with the concept of different helper T cells synergizing for optimal B cell responses (12). Indeed, the observation that an "allogeneic effect" replaced the need for an Ig-specific helper cell, but not for a carrier-specific cell (12), precisely dovetails with our conclusion that TRF provides idiotype-specific help, based on the assumption that the "allogeneic effect" is an alternative mechanism for generating TRF. Therefore, "minor" idiotypes may arise through the action of carrier-specific help exclusively, whereas "major" idiotypes assume dominance through the combined action of carrier-specific and id-specific help. Indeed, in a study of the idiotypic profile of the anti-ABA PFC response of A/J mice to ABA-KLH, the earliest IgG PFC were almost 100% CRI-positive, whereas the later response was only about 35% CRI-positive (13), suggesting that CRI-positive IgM producers are selectively switched by a mechanism which is likely to be idiotype-specific help. Thus, synergistic help may determine the kinetics of isotype switching. It remains to be seen if the idiotype-specific helper activity in Con A-induced TRF is the product of the "TH2" Ig-specific cell described by others (12).

REFERENCES

1. Melchers, F., Andersson, J., Lerukardt, W., and Schreier, M.H. (1980). *Immunol. Rev. 52*, 89.

2. Sieckmann, D.G. (1980). *Immunol. Rev. 52*, 181.

3. Primi, D., Lewis, G.K., and Goodman, J.W. (1980). *J. Immunol. 125*, 1286.

4. Goodman, J.W., Lewis, G.K., Primi, D., Hornbeck, P., and Ruddle, N.H. (1980). *Mol. Immunol. 17*, 933.

5. Primi, D., Lewis, G.K., and Goodman, J.W. (1979). *Eur. J. Immunol. 9*, 607.

6. Barnabe, R.R., Martinez-Alonso, C., and Coutinho, A. (1979). *Eur. J. Immunol. 9*, 546.

7. Nisonoff, A., Ju, S.-T., and Owen, F.L. (1977). *Immunol. Rev. 34*, 39.

8. Laskin, J.A., Gray, A., Nisonoff, A., Klinman, N.R., and Gottlieb, P.D. (1977). *Proc. Nat. Acad. Sci. USA 74*, 4600.

9. L'age-Stehr, J., Teichmann, H., Gershon, R.K., and Cantor, H. (1980). *Eur. J. Immunol. 10*, 21.

10. Bourgiugnon, L.Y.W., Rader, R.L., and McMahon, J.T. (1979). *J. Cell. Physiol. 99*, 95.

11. Chen, P., Nitecki, D.E., Lewis, G.K., and Goodman, J.W. (1980). *J. Exp. Med. 152*, 1670.

12. Janeway, C.A., Bert, D.L., and Mosier, D.E. (1980). *Eur. J. Immunol. 10*, 236.

13. Conger, J.D., Lewis, G.K., and Goodman, J.W. (1981). *J. Exp. Med.*, in press.

IMMUNOLOGICAL CIRCUITRY GOVERNED BY MHC AND V_H GENE PRODUCTS

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ABSTRACT Two separate experiments indicated the roles of anti-idiotypic B and T cells in the augmentation and suppression of the antibody response. 1) In the Ig^+ B cell population from normal as well as from nude mice existing is a novel cell type with Lyt-1 antigen (termed B' cell) that augments the antibody response mounted by IgV_H matched B cells under the T cell-limited condition. The anti-idiotypic nature of the B' cell was suggested. 2) Idiotype-positive suppressor T cells and their hybrid cell lines produce a factor (TsF) with an idiotype and I-J-coded determinants. Such TsF suppresses the antibody response of IgV_H- and/or MHC-matched responding cells. Evidence indicated that TsF activates the pre-existing anti-idiotypic T cells to become active suppressor effector cells. The deletion of anti-idiotypic T cells resulted in the failure of suppression induced by TsF.

INTRODUCTION

The immune cell interactions are mediated though recognition of polymorphic structures expressed on different subsets of lymphoid cells. Such mutual recognition imposes a restriction by which one cell type can find the right second cell type to be activated, and thus leading to a consecutive activation of a series of cells which finally neutralizes the initial stimulatory signal (circuit). One of the restricting elements has been determined to be the product of major histocompatibility complex (MHC). Our previous studies indicated that the suppression of antibody response by T cells is mediated by a soluble product (TsF) made by Lyt-2^+,3^+ T cells that carries determinants coded for by genes in the I-J subregion of murine MHC. TsF acts on Lyt-1^+,2^+,3^+ acceptor T cells that also carry I-J subregion products, and induces a new suppressor effector T cell of the Lyt-2^+,3^+ subclass. This amplification loop of the suppression is strictly restricted by the

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identity of genes in I-J subregion between TsF-producer and acceptor T cells. It is as yet undetermined whether this restriction is due to the presence of anti-I-J receptors or to the complementary (or like-like) interactions between I-J subregion gene products on different cells.

The second restrictive element having polymorphic structures is the idiotype as originally postulated by Jerne (1). This type of restriction is important not only in the cell to cell interactions to perform the specific immune response, but also relevant to consider the maintenance and increase of the specificity repertoire. Thus the immune system is viewed as a net composed of a variety of cells recognizing different MHC products and immunoglobulin variable(V) regions, and the circuitry is made among the constituent cell types. The dichotomy of MHC- and idiotype-restricted cell interactions has not been fully resolved.

In this presentation, we would present two pieces of experimental evidence that indicate that the interplay between MHC- and IgV region-restricted cell interactions plays important roles in the induction and suppression of the antibody response.

I. IgV_H restricted helper T cell is a B' cell

A previous report from our laboratory (2) demonstrated the existence of two different helper T cells that can help B cells independently or synergistically. They are termed as Th_1 and Th_2 based on the modes of their help and phenotypic expressions. Th_1 is a conventional helper T cell that helps B cells in the Mitchisonian type mode (cognate interaction). Th_2, on the other hand, can help B cells that are independently stimulated by a hapten which is not linked to the carrier molecule (polyclonal interaction). Th_1 has no detectable Ia determinants, while Th_2 carries Ia coded for by a gene in I-J subregion. A number of differences have been noticed as depicted in Table I, which also includes the properties of the third cell type (B' cell) which we describe below. Other studies also indicated the presence of two separate helper T cells (3-5), although the identity between them has not been critically examined. As the conventional helper T cell (Th_1) has been shown to be MHC-restricted under the physiologic condition, the question has been asked whether Th_2 is IgV-region restricted. Because of the polyclonality of the activation of B cells by Th_2, it is unlikely that Th_2 is the idiotype-specific helper T cell. In addition, it has been demonstrated that there is no Ig restriction in the Th_2 help in combinations of Ig congenic T and B cells.

Okumura *et al.* (manuscript in preparation) have looked

TABLE I

CHARACTERISTICS OF HELPER FAMILIES

Th_1	Th_2	B'
Nylon wool nonadherent	Nylon wool adherent	Nylon wool adherent
Ia^-	Ia^+(I-J)	Ia^-
Thy-1^+	Thy-1^+	Thy-1^-
Lyt-1^+	Lyt-1^+	Lyt-1^+
Carrier-specific	Carrier-specific	V_H-specific
Ig unrestricted	Ig unrestricted	Ig restricted
Ig^-	Ig^-	Ig^+

for allotype-specific helper T cells by utilizing allotype congenic splenic T and B cells, and have had serendipity to find a new cell type which helps B cells in an IgVH restricted fashion. As we discuss later, this cell type has several controversial properties that represent neither B nor T cell, and thus we tentatively term it as B' cell.

To detect the effect of B' cell, the following members of lymphocytes were cocultured: 1) B cell; dinitrophenylated-keyhole limpet hemocyanin (DNP-KLH)-primed spleen cells were treated with anti-Thy-1.2 and anti-Lyt-1.2 and complement(C). The treatment with anti-Lyt-1 was essential to detect the activity of B' cell (see below). 2) T cell; KLH-primed splenic T cells were obtained by plating in anti-mouse Ig-coated Petri dishes (Ig^- cells). Cells were separated into Th_1 and Th_2 by a nylon wool column passage (2). Either nylon wool nonadherent (Th_1) or adherent (Th_2) T cells can be used as the source of carrier-specific helper T cells. 3) B' cell; normal splenic B cell fraction was obtained by adherence to a nylon wool column followed by a treatment with anti-Thy-1.2 and C. In general 3×10^6 B cells and limited number ($0.5 \sim 1 \times 10^5$) of T cells were cocultured to mount a reasonable IgG secondary antibody response, where 1×10^6 B' cells from different Ig congenic mice were added to see their augmenting effect. Antibody responses of IgG_{2a} and IgG_1 classes were assessed by the radioimmunoassay with the 8-day culture supernatant using monoclonal anti-Igh-1 and anti-Igh-4 allotype reagents. The detailed methods and results will be published elsewhere.

Fig. 1 depicts a representative result of IgG_{2a} antibody

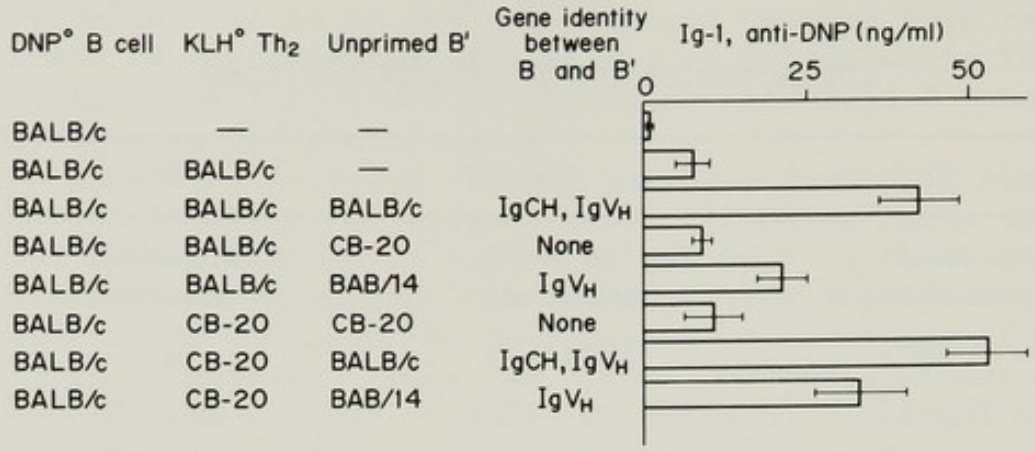

FIGURE 1. Ig gene restriction between B and B' cells

responses in various combinations of B, T and B' cells. The syngeneic combination of B and T cells could mount a low but significant anti-DNP antibody response. The addition of B' cell to the mixture of B and T cells augmented the response by several folds. Such an augmentation is achieved by B' cells only from strains sharing at least IgV_H genes with B cells (c. f., BALB/c B cell and BAB/14 B' cell), whereas those from completely Ig congenic strains were ineffective (c.f., BALB/c B cell and CB.20 B' cell). The minimal requirement for this augmentation was that B and B' cells had to derive from IgV_H identical strains regardless the source of Th.

The question arose as to whether the B' cells in this combination are conventional unprimed B cells. Okumura *et al.* studied the phenotype of B' cells that were incurred to this augmentation. As shown in Table I, B' cells were found to have interesting properties; they are Thy-1 negative but positive for Ig and Lyt-1 antigen as determined by both the cytotoxic and plating techniques. They are negative for Lyt-2, Ia and ThB antigens. The activity of B' cells was normal in nude mice of BALB/c background, suggesting that the B' cell belongs to the B cell lineage.

To learn the role of B' cells in the expression of idiotypes among the produced antibody, B cells were taken from C57BL/6 strain immunized with 4-hydroxy-3-nitro-phenyl acetyl (NP)-KLH. They were cocultured with KLH-primed T cells and syngeneic B' cells. As depicted in Fig. 2, normal B' cells augmented the anti-NP antibody response under the T cell-limited condition. When B' cells were plated in the NP^b idiotype-coated dish before addition to the coculture, the augmenting activity was greatly reduced indicating the anti-idiotypic nature of the B' cell. The plating the cells in CBA anti-NP-coated dishes as well as in normal C57BL/6 Ig-coated dishes

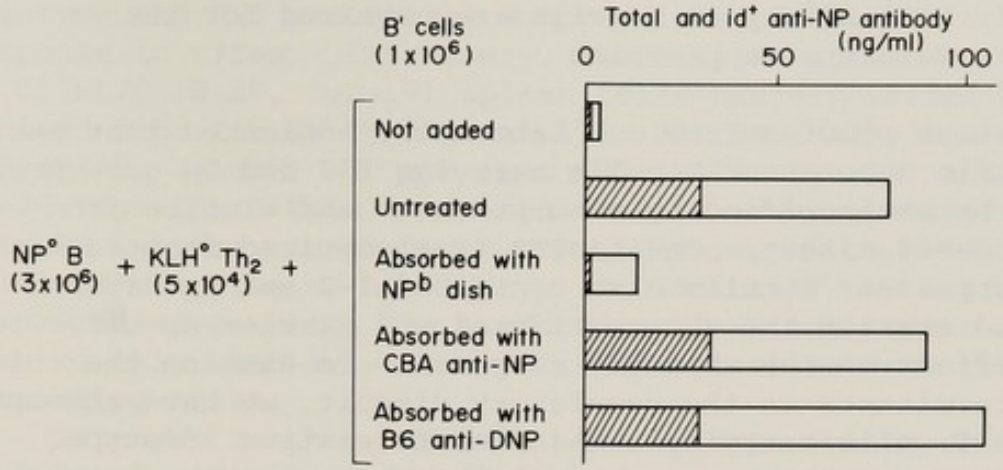

FIGURE 2. Regulation of id expression by B' cell

did not abolish the B' cell activity.

The above data indicate that IgV_H restricted 'apparent' helper activity is carried by a cell type obtainable by the conventional procedure to purify B cells. This cell type is, however, distinguishable from typical B cells by the presence of Lyt-1 antigen on its cell surface. The normal existence of such cells in nude mice suggests that B cell society has an endogenous network in which idiotype-bearing B cells have complementary anti-idiotypic B' cell sets (or *vice versa*) to maintain the B cell repertoire without participation of regulatory T cells. Thus, we think that B cells' inherent network is maintained even without T cells as originally postulated by Jerne. Helper T cells would help corresponding B cells by cognate and polyclonal interactions, but at the same time activate the endogenous B and B' cell interaction to manifest the full-blown antibody response with the major idiotype expression (Fig. 3).

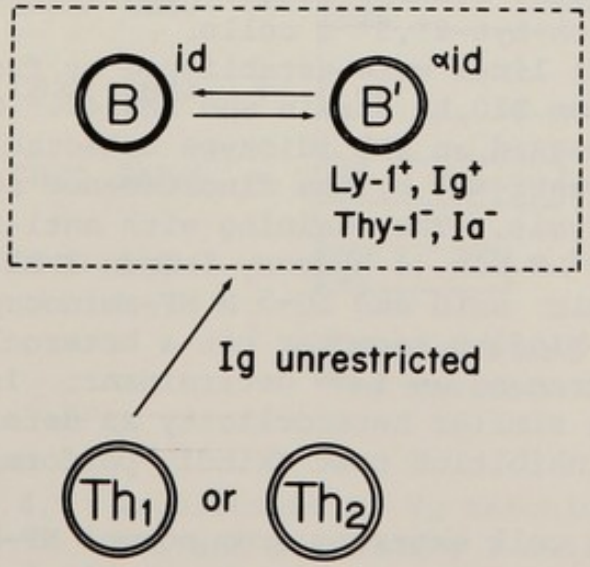

FIGURE 3. Minimal requirement for id expression

II. Anti-idiotypic T cells are required for the idiotype suppression

Previous studies from our laboratory indicated that antigen-specific suppressor T cells carrying I-J and V_H gene products can be semipurified by adsorption to and elution from antigen coated dishes. The factor (TsF) derived from such purified suppressor T cells also contained I-J and V_H determinants, and exerted the MHC-restricted and carrier-specific suppressor effect on the antibody response. To examine the role of V_H determinants in the regulatory circuit, we have attempted to obtain T cells carrying a major crossreactive idiotype.

C57BL/6 and B10.BR mice were immunized with NP coupled to gelatin and syngeneic mouse Ig. The T cell fraction of NP-primed spleen cells was incubated in Petri dishes coated with NP-bovine serum albumin, and NP-binding T cells were recovered by washing with cold medium (6). By fluorescence staining with various anti-idiotypic reagents, it was found that a portion of such NP-binding T cells carry major crossreactive idiotypes of NP^b. In general 5 to 10% of recovered T cells were stained with rabbit anti-NP^b, and the majority of them carry the idiotope detectable by a monoclonal anti-idiotype antibody (AC38) which was kindly provided by Drs. Michael Reth and Klaus Rajewsky. Similar percentages of cells were stained with anti-V_H provided by Dr. David Givol, while none of the monoclonal and conventional anti-allotype antibodies could react with them. The expression of NP^b idiotype on NP-binding T cells was found to be linked to Igh-1^b allele.

The NP-binding T cells thus obtained were suppressor T cells, since the cells as well as their extracts could suppress the secondary anti-NP but not anti-DNP antibody response *in vitro*, and were unable to help the NP-specific B cells in the presence or absence of other T cells. The NP^b idiotype was found exclusively on Lyt-2^+,3^+ T cells.

Hybridoma cell lines were established by fusion of NP-binding T cells from B10.BR strain and BW5147. One of the hybrid, 7C3-13, expressed an NP^b idiotype detectable by conventional guinea pig anti-NP^b by the fluorescence activated cell sorter (FACS) analysis. The staining with anti-idiotype was inhibitable by 10^{-7} M NIP (4-hydroxy-5-iodo-3-nitro-phenyl acetyl) aminocaproic acid and 10^{-5} M NP-aminocaproic acid, indicating the NP-binding receptor has a heteroclicity. The cell line also expressed an I-J^k determinant. Isolated receptor molecule had a similar heteroclicity as determined by the haptenated phage inhibition test (kindly performed by Dr. Mathias Cramer).

Utilizing the cell extracts from normal NP-binding T cells and 7C3-13 hybridoma cells, we have been able to show both

idiotype and MHC restricted suppressions of anti-NP antibody response *in vitro*. In summary, the anti-NP antibody response of C57BL/6 (H-2^b, Igh-1^b) spleen cells was suppressed by TsF derived from C3H.SW (H-2^b, Igh-1^j), B10.BR (H-2^k, Igh-1^b) and CWB (H-2^b, Igh-1^b) but not from C3H (H-2^k, Igh-1^j) and BALB/c (H-2^d, Igh-1^a). This indicates that in order to suppress the anti-NP antibody response of C57BL/6 mouse, either H-2 or Igh-allotype matching is necessary. In addition, the expression of NP^b idiotype among the produced anti-NP antibodies was suppressed only when Igh-allotype is identical between TsF and responding cells.

This was further substantiated by experiments using idiotype-bearing TsF from 7C3-13 hybridoma. In the experiment depicted in Fig. 4, we cocultured IgV_H congenic CB.20 B cells and BAB/14 T cells, and TsF from 7C3-13 was added to the culture. In the combination of CB.20 B and T cells was found a significant suppression of both total and idiotype positive anti-NP PFC, while the same TsF was unable to suppress the response mounted by CB.20 B cells and BAB/14 T cells. Since CB.20 B cells used in this experiment contain B' cells described in section I, the target of id^+ TsF is not the B' cell. On the other hand, the V_H gene identity is clearly required between T cells and TsF but not between B cells and TsF for the effective suppression, indicating that the V_H gene identical T cells mediate the TsF effect in the idiotype suppression of responding B cells.

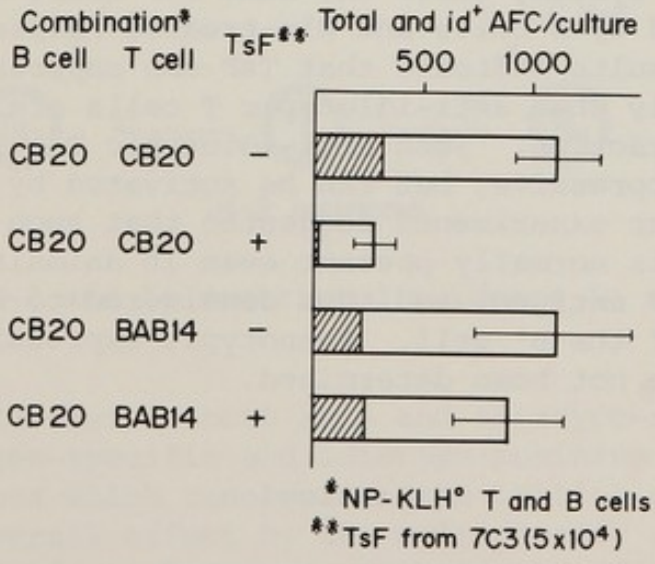

FIGURE 4. Requirement of V_H matching between TsF and responding T cell

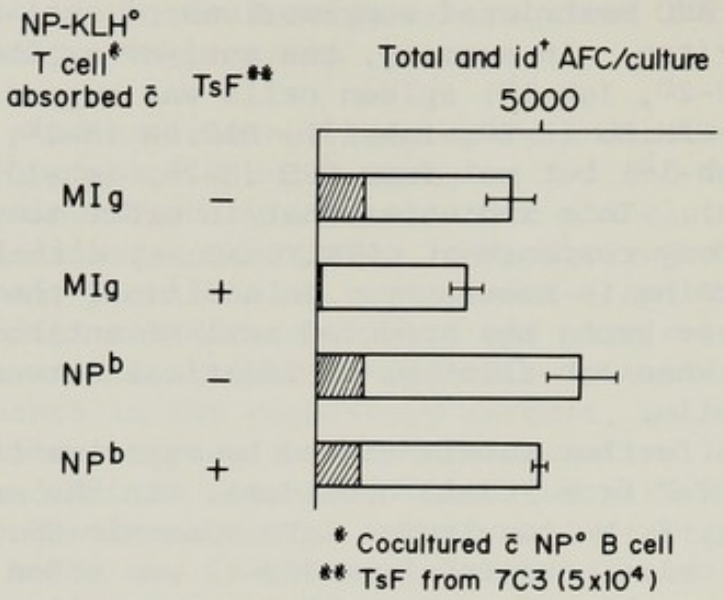

FIGURE 5. Requirement of anti-idiotypic T cells the suppression of NP^b idiotype

In order to prove the above postulate, we took NP-KLH-primed T cells as the helper cell source and incubated in Petri dishes coated with NP^b idiotype (primary anti-NP antibodies) or with normal C57BL/6 Ig (MIg). The nonadherent T cells were cocultured with NP-primed syngeneic (C57BL/6) B cells with or without TsF from 7C3-13 hybridoma. As shown in Fig. 5, after the removal of putative anti-idiotypic T cells by plating on NP^b dishes, TsF could no longer suppress the idiotype expression in the coculture system, while the same TsF could suppress the response mounted by B cells and MIg-treated helper T cells.

These results indicate that TsF can suppress the idiotype expression only when anti-idiotypic T cells are present in the helper cell fraction. Such anti-idiotypic T cells are not by themselves suppressive, but can be activated by idiotype-bearing TsF. Other experiments suggested that such an anti-idiotypic T cell is normally present even in animals primed with KLH (unrelated antigen) and thus considered to be a T cell counterpart of the B' cell. Phenotypic expressions of this cell type have not been determined.

Conclusion

Two lines of experiments presented in this paper suggest that both B and T cell societies are composed of cells expressing sets of V region determinants (idiotypes) and complementary V region determinants (anti-idiotypes) which are interacting in the absence of external antigens. B cells can maintain

their repertoire even without T cells as in nude mice by the presence of B' cells, while helper T cells can activate not only the specifically stimulated B cell clone, but also the whole network interactions maintained by the presence of B' cells (see section I). In parallel, idiotype-bearing suppressor T cells utilize the second set of normally present anti-idiotypic T cells to make the latter to become active suppressor T cells (idiotype specific Ts_2). This activation of idiotype specific suppressor T cells consequently interfares with the B cell network to suppress both overall and idiotype positive antibody responses.

Fig. 6 is a simplified model of the regulatory circuits governed by both MHC and VH. Our previous studies with KLH-specific TsF primarily envisaged to us the MHC-restricted pathway because of the lack of major crossreactive idiotypes, while with certain antigens such as NP we can envision the role of V_H gene products serving as the restrictive elements in the circuit. The latter results are consistent with the recent notion presented by Sy *et al.* (7) and Weinberger *et al.* (8).

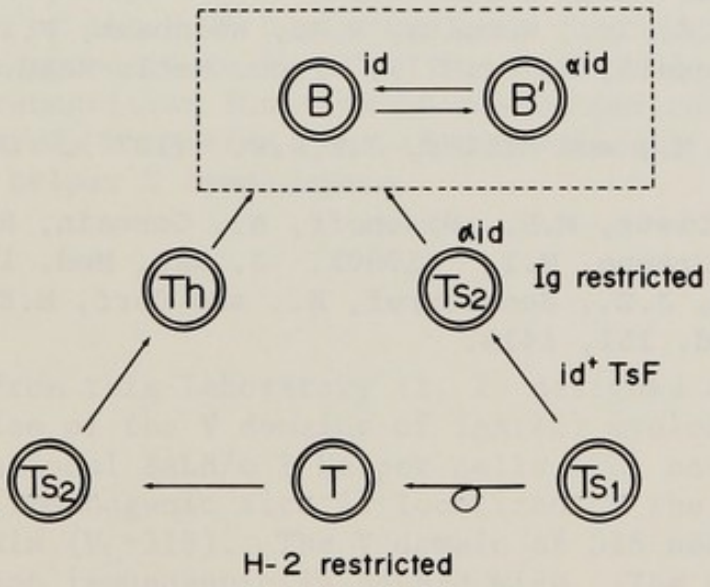

FIGURE 6. H-2 and Ig restricted pathways in suppression

It is noted that in both MHC- and idiotype-restricted pathways, antigen-specific and idiotype positive TsF utilizes second cell types which can broaden the initial narrow specificity to an overall effect by the multiplicity of I-J recognizing and anti-idiotypic sets. The understanding of the interplay of these two pathways may be of importance for the comprehension of the immunoregulatory system (supported by a grant from the Japanese Ministry of Education, Culture and Science).

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REFERENCES

1. Jerne, N.K. (1974). Ann. Immunol.(Inst. Pasteur) 125C, 373.
2. Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. (1978). J. Exp. Med. 147, 446.
3. Marrack, P., and Kappler, J.W. (1976). J. Immunol. 116, 1373.
4. Cantor, H., Hugenberger, J., McVay-Boudreau, L., Eardley, D.D., Kemp, J., Shen, F.W., and Gershon, R.K. (1978). J. Exp. Med. 148, 871.
5. Janeway, C.A.,Jr., Murgita, R.A., Weinbaum, F.I., Asofsky, R., and Wigzell, H. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 4582.
6. Taniguchi, M., and Miller, J.F.A.P. (1977). J. Exp. Med. 146, 1450.
7. Sy, M-S., Dietz, M.H., Nisonoff, A., Germain, R.N., Benacerraf, B., Greene, M.I. (1980). J. Exp. Med. 152, 1226.
8. Weinberger, J.Z., Benacerraf, B., and Dorf, M.E. (1980). J. Exp. Med. 151, 1413.

RECOGNITION OF VARIABLE (V) DOMAINS OF MYELOMA PROTEIN 315 BY B AND T LYMPHOCYTES[1]

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We compared the specificity of humoral and helper cell responses to the V_L domain of the isologous IgA(λ2) myeloma protein 315. Anti-native L-315 antibodies bound native V_L-315 but not the unfolded domain or the assembled domain of the complete myeloma protein. By contrast, helper cells did not distinguish between unfolded, native and assembled forms of V_L-315. Neither the antibodies nor the helper cells cross-reacted with the λ1 L-chain of the assembled myeloma protein J558. The helper cells of H-2^d haplotype mice responded to V_L but not to V_H-315; mice of the H-2^k haplotype were responders to V_H and non-responders to V_L-315. The data indicate that the antigen recognition function of the helper cells of the present study resembles that of conventional carrier-specific helper T lymphocytes.

INTRODUCTION

Studies from this laboratory (1, 2) designed to analyze the recognition of the V domains of IgA(λ2) myeloma protein 315 by conventional BALB/c T helper cells (Th) have revealed that a major immunogenic site is localized on the V domain of the L-315 chain (V_L-315). The V domain of 315 heavy chain (V_H-315) is not immunogenic in BALB/c mice. The major histocompatibility complex (MHC) genes play a major role for the recognition of the two V domains of M315 (3). Thus, mice of the H-2^d haplotype respond to V_L-315 but not to V_H; conversely, H-2^k haplotype mice are responders to V_H but not to V_L. The present experiments were designed to compare the specificity of humoral and helper cell responses to V_L-315, and to obtain further evidence for the control of immune response to V-domains of M315 by H-2-linked genes. The results are discussed in terms of the possibility that conventional helper cells against V domains may act directly on B cell receptors.

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MATERIALS AND METHODS

Mice. All mice were obtained commercially. BALB/c from Gml. Bomholtgård, Denmark, BALB.K from Olac 1976, England, C3H and C3H·H-2^o from The Jackson Laboratory, Maine.

Antigens. The following procedures have been described: purification of myeloma protein 315 (4), V_H and V_L domains of M315 (5), coupling of 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP)-azide to proteins, and iodination of NIP-caproic acid (6); separation of H and L chains (7), aggregation of L-315 by cross-linking with glutaraldehyde (8). Myeloma protein J558 (IgAλ1) was purified by a modification of the method described by Hiramoto et al. (9).

Unfolded V_L-315 was produced by reduction of the intrachain S-S bond in 5M guanidine HCl with 0.005 M dithiothreitol followed by alkylation with 0.01M iodoacetamide, pH 8.3.

Antisera. BALB/c antiserum against glutaraldehyde aggregated free L-315 was obtained as described (1).

Immunization. NIP-priming of B cells with NIP_7-BSA or NIP_{342}-KLH and priming of T cells with carrier (L-315, V_L-315) was done with a single intraperitoneal injection of immunogen in Freund's complete adjuvant 8 weeks before cell transfer as described (1, 2).

Adoptive cell transfer. Spleen cells from hapten - and carrier primed donors were transferred i.v. to 500 rad irradiated syngeneic recipients as described (1, 2). The recipients were boosted the day after transfer with 100-200 µg of NIP-coupled complete myeloma protein in saline.

Assay of antibody response. Anti-NIP antibody activity was measured 10-11 days after boost by a modified Farr assay as described (1).

RESULTS

Recognition of V_L-315 domain by B cells. To study the specificity of recognition of the V_L-315 domain by B lymphocytes, BALB/c mice were immunized with free L-315 and the specificity of the resulting antibodies was examined in a double antibody radioimmunoassay. Various unlabeled Ig chains were tested for ability to inhibit the reaction between ^{125}I-labeled V_L-315 or L-315 and the corresponding pooled antiserum. Free native V_L-315 was the only ligand that blocked the reaction (Fig 1).

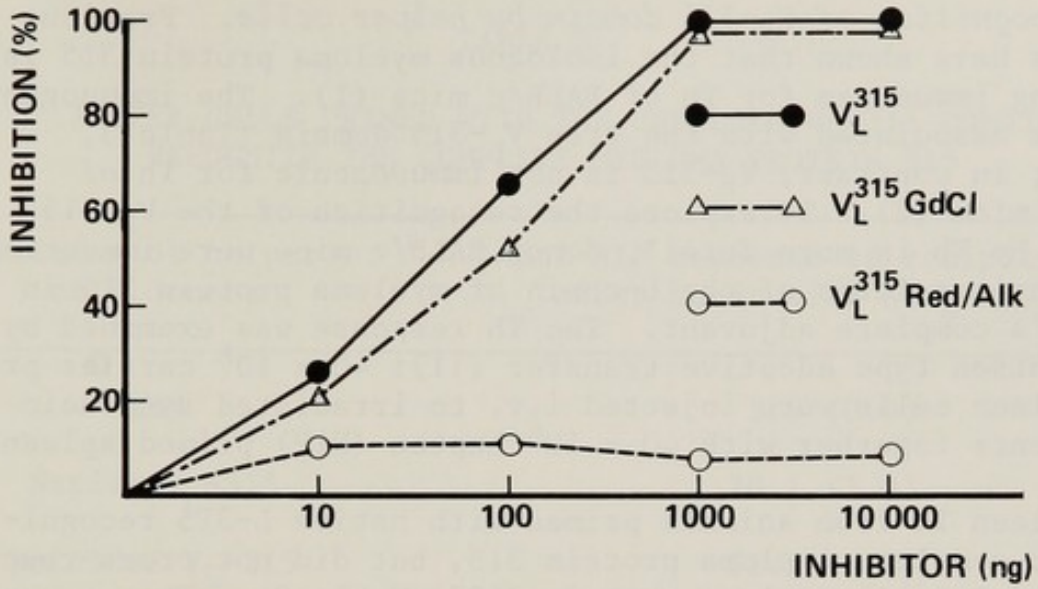

FIGURE 1. Native but not unfolded (Red/Alk) V_L-315 inhibits reaction between BALB/c anti-L-315 antiserum and radioactive V_L-315.

No cross-reacting inhibition was observed with native free L-J558 (Table 1), the V domain of which is 88% homologous with V_L-315 (10), suggesting that the anti-L-315 antibodies were specific for a private antigenic site (idiotype) of the antigen. Furthermore, unfolded (completely reduced and alkylated) V_L-315 (Fig 1) and L-315 associated with H-315 in the complete M315 myeloma protein (Table 1) did not inhibit at all. It appears therefore that this idiotype was displayed by free native L-315 chain, but that it was absent on the unfolded chain and in the complete myeloma protein 315.

TABLE 1

SPECIFICITY OF ANTIBODIES AGAINST THE ISOLOGOUS LIGHT CHAIN OF MYELOMA PROTEIN 315

Inhibitor	Amount (ng)	^{125}I-labeled L-315 bound§ (%)
None	-	38.8
L-315	1000	4.5
Complete M315	1250	34.8
L-J558	1000	47.8
Complete J558	1000	53.0

§*The assay was done with 30 ng radioactive antigen.*

Recognition of V_L-315 domain by helper cells. Previous studies have shown that the isologous myeloma protein 315 is a strong immunogen for Th of BALB/c mice (1). The immunogenicity is associated with the free V_L-315 domain (Table 3, Ref 2); in contrast, V_H-315 is not immunogenic for Th of BALB/c mice (2). To explore the recognition of the V_L-315 domain by Th in more detail, donor BALB/c mice were immunized with various forms of the L-chain of myeloma protein 315 in Freund's complete adjuvant. The Th response was examined by a Mitchison type adoptive transfer (11): 40×10^6 carrier primed spleen cells were injected i.v. to irradiated syngeneic recipients together with 40×10^6 hapten (NIP) primed spleen cells.

Spleen Th from animals primed with native L-315 recognized the complete myeloma protein 315, but did not cross-react with the IgA(λ1) myeloma protein J558 (Table 2). This suggested that the Th, like the B cells, recognized a private antigenic determinant (idiotype) of V_L-315.

Spleen Th from mice primed with the unfolded (completely reduced and alkylated) V_L-315 domain cross-reacted with the native M315 myeloma protein (Table 3). This could mean that Th, unlike B cells, recognized an antigenic site determined by the primary structure of the antigen, and secondly, that the native assembled myeloma protein and the denatured V_L-315 domain shared this determinant.

TABLE 2

HELPER CELLS PRIMED WITH NATIVE L-315 DO NOT CROSS-REACT WITH IgA(λ1) MYELOMA PROTEIN J558

Priming of helper cell donors	Boost of recipients	Anti-NIP-response of recipients
MEM[+]	$NIP_4$315	0.6 (0.6)
MEM	NIP_{10}J558	1.5 (0.8)
L-315	$NIP_4$315	89.1 (3.5)
L-315	NIP_{10}J558	5.4 (1.9)

[+]*Minimum essential medium in FCA. Numbers in parenthesis are standard errors.*

TABLE 3

HELPER CELLS PRIMED WITH THE UNFOLDED V_L-315 DOMAIN RECOGNIZE THE COMPLETE MYELOMA PROTEIN 315

Priming of helper cell donors	Anti-NIP response of recipients after boost with NIP_4-M315
MEM	3.5 (0.9)
Native V_L-315	70.1 (3.9)
Unfolded V_L-315	62.2 (1.8)

H-2 linked genes control Th responses to V domains of myeloma protein 315. To further confirm that MHC-genes regulate the Th responses to the isolated V domains of myeloma protein 315 (3), H-2 congenic strains were primed with either V_L-315 or V_H-315 domain and the Th responses examined in a Mitchison type adoptive transfer as described above. The results are shown in Table 4. Mice of the $H\text{-}2^d$ haplotype (BALB/c) were responders to V_L-315 and non-responders to V_H-315; by contrast, mice of the $H\text{-}2^k$ haplotype (C3H) were non-responders to V_L-315 and responders to V_H-315. The H-2 congenic strain BALB.K has the BALB/c genome except for genes of the MHC complex which are derived from C3H (12); it exhibited a response pattern like $H\text{-}2^k$ (C3H) mice. The congenic strain C3H-H-2^o, which has the H-2 haplotype of B10·OH on C3H background (Ref. 12; se Table 4), responded like $H\text{-}2^d$ (BALB/c) mice. These data provide strong evidence that genes within the MHC control the Th responses to these antigens.

DISCUSSION

The present experiments indicate that helper cells and B cells immunized with the isologous V_L-315 domain differ in terms of their recognition of this antigen. The antibodies elicited by the L-315 chain reacted with the isolated native, but not with the unfolded, V domain. The antigenic site was only expressed on the free L-315 chain or its V_L domain, since the associated (V_L + V_H) domains of the complete 7S myeloma protein failed to react with the antibodies. The anti-L-315 antibodies therefore appear to recognize a conformational antigenic site that is concealed in the complete IgA myeloma protein, and thus distinguish native V_L-315 from unfolded and assembled forms of this domain. By contrast, free

TABLE 4

HELPER CELL RECOGNITION OF V_H-315 AND V_L-315 IS REGULATED BY H-2 LINKED GENES

Strain	H-2 haplotype	Anti-NIP responses in recipients of Th primed with	
		V_H	V_L
BALB/c	d	5.9 (2.4)	57.8 (1.0)
BALB.K	k	27.1 (3.0)	2.0 (0.6)
C3H	k	61.3 (0.7)	8.5 (1.1)
C3H·H-2^o	d*	0.4 (0.1)	35.2 (5.9)

Except for D region which is of k haplotype origin (12)

native and unfolded V_L-315 domain were both efficient inducers of helper cells that cross-reacted with the complete M315, demonstrating that the helper cells apparently do not discriminate between primary and higher order structure of L-315, and that the antigenic site is not hidden in the complete Ig molecule.

To further explore the nature of the antigenic determinant of the free V_L-315 we tested whether the antibodies and helper cells cross-reacted with the λ1 L-chain of myeloma protein J558, the V domain of which differs from that of V_L-315 by only 13 amino acids (10). Since anti-L-315 did not react with L-J558, and helper cells induced by L-315 did not recognize the complete IgA(λ1) myeloma protein J558, we concluded that the antigen was probably related to idiotypy.

Others (13, 14) have earlier reported similar differences between B and T cells in terms of recognition of native and denatured forms of the same protein antigen, however, to our knowledge our studies are the first to point out that this may also be true for antigens of V domains (idiotypes) of isologous immunoglobulins. Another similarity, not described before, between the V domain antigens of M315 and several conventional protein antigens is that T cell responses to the domains are controlled by H-2 linked IR genes. Thus, haplotype H-2^d mice are high responders to V_L while H-2^k mice are low responders. For responses to V_H the situation is opposite; haplotype H-2^k mice are high responders whereas mice with haplotype H-2^d are low responders. If one assumes that IR genes code for Ia antigens (15) this similarity suggests that antigens of the V domains are recognized by T cells in association with Ia antigens.

The major impetus for performing the studies presented here and elsewhere (1-3) was Jerne's idiotype network hypothesis (16). If T helper cells are active in the network, it must be important to learn more about their recognition of V regions. For this purpose we have employed V domains as carriers in a Mitchison type hapten-carrier system. This immediately poses the question whether the system provides information about cells that are relevant for the network. It may be argued that the carrier specific helper cells studies by us may not be able to function like the idiotype specific helpers reported by others (17, 18) which assist the maturation of B lymphocytes of a given idiotype into antibody secreting cells, and which may not be Ia restricted in their idiotype recognition judging from the depletion of these helpers on plastic dishes coated with the idiotype (17, 19). On the other hand, since B cells bear large amounts of Ia antigens on the surface, it is conceivable that conventional carrier specific helper cells that recognize Ig V domains, like those of the present study, may also detect B cells receptors.

An objection against this idea is that conventional helper cell may only be able to recognize processed antigen, and that such processing may not occur with the Ig receptors of B cells. We have previously suggested (3) on the basis of experimental data (1) that our conventional carrier-specific helper cells recognize V domains that are displayed in a structurally intact (i.e. unprocessed) form. However, since the mechanism and nature of processing is obscure, proof for this suggestion is lacking. Indeed, the cross-reactive recognition of unfolded, native and assembled forms of the same V domain by the helper cells of the present study may be interpreted as indirect evidence that processing has occurred. For example, a plasma membrane enzyme may cleave the three forms of the immunogen into fragments with identical antigenic determinants. It is also conceivable that the Ig receptor of B cells are processed in a similar manner. Thus, membrane bound IgD has been reported to be extremely susceptible to proteolysis, including its Fab portion which was degraded by trypsin into a fragment consisting of V_H and C_L (20). It is possible, therefore, that B lymphocytes display processed V-domains on their surface in association with Ia antigens, detectable as an immunogenic complex by conventional helper cells. This hypothesis differs from that of Bourgois et al. (20) by proposing that the processed V domains are presented to helper cells by B cells. Experiments in progress have been designed to explore whether the V_L-315 specific helpers are able to act directly on B cells.

ACKNOWLEGDMENT

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REFERENCES

1. Jørgensen, T., and Hannestad, K. (1977). Eur. J. Immunol. 7, 426.
2. Jørgensen, T., and Hannestad, K. (1979). Scand. J. Immunology 10, 317.
3. Jørgensen, T., and Hannestad, K. (1980). Nature (London) 288, 396.
4. Goetzel, E.J., and Metzger, H. (1980). Biochemistry 9, 1267.
5. Hochman, J., Inbar, D., and Givol, D. (1973). Biochemistry 12, 1130.
6. Brownstone, A., Mitchison, N.A., and Pitt-Rivers, R. (1966). Immunology 10, 465.
7. Jørgensen, T., Gaudernack, G., and Hannestad, K. (1977). Scand. J. Immunol. 6, 311.
8. Daugherty, H., Hopper, J.E., MacDonald, A.B., and Nisonoff, A. (1969). J. exp. Med. 130, 1047.
9. Hiramoto, R., Ghanta, V.H., McGhee, J.R. Schrohenloher, R. and Hamlin, N.M. (1972). Immunochemistry 9, 1251.
10. Tonegawa, S., Maxam, A.M., Tizard, R., Bernard, O., and Gilbert, W. (1978). Proc. Natl. Acad. Sci. USA 75, 1485.
11. Mitchison, N.A. (1971). Eur. J. Immunol. 1, 18.
12. Klein, J. (1975). Biology of the mouse histocompatibility-2 complex. Springer-Verlag, Berlin p. 37 and 211.
13. Thompson, K., Harris, M., Benjamini, E., Mitchell, G., and Noble, M. (1972). Nature New Biology 238, 20.
14. Schirrmacher, V., and Wigzell, H. (1972). J. exp. Med. 136, 1616.
15. Michaelides, M., Sandrin, M., Morgan, G., McKenzie, I.F.C., Ashman, R., and Melvold, R.W. (1981). J. exp. Med. 153, 464.
16. Jerne, N.-K. (1974). Ann. Immunol. (Paris) 125c, 373.
17. Woodland, R., and Cantor, H. (1978). Eur. J. Immunol. 8, 600.
18. Hetzelberger, D., and Eichmann, K. (1978). Eur. J. Immunol. 8, 846.
19. Adorini, L., Harvey, and Sercarz, E.E. (1979). Eur. J. Immunol. 9, 906.
20. Bourgois, A., Abney, E.R., and Parkhouse, R.M.E. (1977). Eur. J. Immunol. 7, 210.

ON THE PRIMACY OF ANTIGEN AS A PERTURBENT AND MEDIATOR OF IMMUNOLOGICAL HOMEOSTASIS: An anti-(anti-idiotypic) point of view

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> "An antigen particle carries several epitopes...Epitopes that are carried by components of one individual animal are idiotopes. The idiotypes to which they belong constitute the idium of the animal"...Niels K. Jerne

I. INTRODUCTION

In 1960 (1) Jerne coined the words idiotope and idiotype to help Burnet convey the following information: an animal can discriminate between "antigenic determinants not represented in its components" and "potential antigenic determinants which are inert because they are the same as determinants already present in its components" (2) in fewer words. It is unlikely that he conceived at that time, that 21 years later close to 500 immunologists would be meeting in Salt Lake City, Utah to discuss the implications of subdividing antigenic determinants into 2 universes; one self and one foreign.

By 1974 2 events had occurred that made such a possibility less remote. In that year 1) Jerne predicted that between 1970 and 1990 immunologists would be quite involved in studying network regulation (3) and 2) the Molecular Biology Institute of UCLA selected Immunology as a topic to be included in the annual series of symposia to be organized under the sponsorship of ICN Pharmaceuticals.

Thus, on Feb 8-15, 1981 the ICN-UCLA symposium on "Immunoglobulin Idiotypes and their Expression" acted as a testament to Jernes preception. The predominant focus in discussions of immunoregulation was on the network concept; even students of the MHC felt constrained to marry their I regions to members of the network, producing revisionist theories. This rapid embracement of the network concept made it a propitious time for reactionaries to surface and to ask the hard question. Is there sufficient experimental

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data to cause one to accept "networkism" as an existential theory or does it remain, as it was when first proposed in 1974, an elegant intellectual concept? What follows is the position of a reactionary who thinks that a) antigen is involved in all specific interactions between immunologically competent lymphocytes, and b) that presently available experimental evidence is insufficient to cause a modification of this orthodox position.

II WHAT IS A NETWORK

One problem in mounting a reaction to networkism stems from the difficulty in precisely defining it. This difficulty may actually be responsible, at least in part, for its present day popularity. Thus, some may consider that a system composed of helpful and suppressive interactions between cells and/or their soluble mediators constitutes a network. Although this may be true epistemologically (arguably) it is a scientific truism and useless as a definition. The nature of the interacting immunoregulatory moieties is essential to the concept. Although no clear cut network manifesto exists, one can piece together enough information from several publications to outline some of its basic rules. Essential to the theory is the notion that immunoregulation is brought about directly by anti-self recognizing molecules and only incidently by antigen. Thus, every antibody not only has an anti-antigen function but also an anti-idiotypic one. The anti-idiotypic function of the molecule is responsible for immunological homeostasis. In its simplest form the network theory assigns to antigen the function of removing anti-idiotypic or internal image molecules that act to suppress a system, which is otherwise poised to respond. In its broadest sense it implies that antibody molecules themselves act as antigens and immunoregulation is brought about by an immune response against a private portion of the immunological effector molecules variable region. Thus, the antigen recognizing units of the immune system have two functions; one is their effector function which stems from their ability to recognize a universe of foreign antigens (epitopes) and second is an immunoregulatory function stemming from their recognition of a second universe of antigens (idiotopes). The constellation of idiotopes (self epitopes) on any given molecule defines that molecule's idiotype.

III NETWORK REGULATION BROUGHT ABOUT BY IMMUNE RESPONSES AGAINST IDIOTYPES

One of the features of the network model that makes it so seductive is that it predicts directly that anti-idiotypic antibodies will produce profound alterations in immunoregulation. This allows the hypothesis to be tested (infinitely?) by experimentally altering immunoregulation with anti-idiotypically immune cells or molecules. Many such experiments have been done, many different outcomes have been achieved, and all of them have been interpreted to be consistent with the basic predictions stemming from the network theory. However, interesting as the results of these experiments have been, after some reflection one can see that similar outcomes would be predicted by all theories of immunoregulation, not only networkism. Thus, if one assumes that a) immunologically competent cells have receptors for antigen and b) that the cell surface receptor expresses the same variable region specificities as does the molecule the cell secretes, then it would be predicted that antibody which reacted with that receptor, could mimic the effect of stimulating the receptor with its specific ligand, which in the case of immunologically competent cells is antigen.

The demonstration that anti-receptor antibodies can trigger or inhibit cellular functions has not been confined to the immune system. For example, if one immunizes a rabbit with an electric eel, one can generate an antibody which acts as an antagonist of acetylcholine. This is not evidence that antibodies are involved in regulation of neuromuscular transmission. Similarly the observation that an autoantibody against the receptor for thyroid-stimulating hormone induces the thyroid gland to make thyroxin (leading to Graves disease) is not generally considered as evidence for network regulation of the endrocrine system. In short, regulating immune reactivity with antibodies to epitopes on receptors for antigen is not necessarily physiological anti-idiotypic regulation; it is autoimmunity.

Granted there are experiments that suggest that auto-anti-idiotypic antibodies can be generated during the course of an ongoing immune response and that these antibodies can have specific immunosuppressive effects. However most of these experiments were done with antigens that would be considered to be a) of the thymus independent type and b) difficult for enzymatic systems to degrade and remove. This commonality may not be totally fortuitous. One could suggest that the continuous bombardment of the immune system with certain types of antigen-antibody complexes can lead to the production of auto-antibodies to antibodies. The observation

that immunization regimes that favor the production of suppressive auto-anti-idiotypic antibodies also favor the production of rheumatoid antibodies (4) is consistent with this suggestion.

If one accepts this logic then one is not compelled to accept experiments in which antibodies are used as antigens as being relevant to networkism. Such experiments show what could happen if networkism existed but they do not establish the theory itself.

IV. REEXAMINATION OF THE BASIC LEVELS OF NETWORKISM

Given the available experimental evidence, what alternative to networkism is left to the reactionary who wants to believe that all immunologically competent cells express receptors for non self i.e. classical antigen?

In this analysis let us return to 1974 (3) and examine the 3 major areas Jerne considered in his formulation, "towards a network theory of the immune system" (3); A - repertoires; B - dualism; and C - suppression. How can these factors relate "towards an antigen theory of the immune system?"

A. Repertoires:

Jerne considered two separate repertoires. One was the repertoire of antibody combining sites which he referred to as "paratopes" and the other was the repertoire of "idiotypes" which he defined as a combination of epitopes displayed by the variable regions of a set of antibody molecules. These special epitopes combined to form the antibodies' idiotype.

There are two reactionary points of view concerning repertoires; one is that idiotopes, idiotypes, epitopes and paratopes are all part of a single repertoire. That is, they are just different types of antigens, and distinguishing between them in the repertoire is no different from distinguishing between the repertoires for albumins and globulins.

A second reactionary point of view is that the repertoires differ by several orders of magnitude; that is, the paratope (and paratype) repertoire is extremely large and the idiotype repertoire is considerably smaller. As I will develop below, these two reactionary positions are not necessarily different from one another and both are considerably different from the network theory position which requires the repertoires of paratopes and idiotypes to be of the same magnitude.

B. Dualism:

As Jerne pointed out the immune system displays a number of dualisms; the one relevant to the network theory is that antibody molecules can recognize as well as be recognized. I would like to stress a different dualism; one that has been put before us by many MHCologists over the past decade. The relevant dualism to an "antigenist" lies in the nature of the T cell receptor(s). T cells express a specificity for self markers encoded in the major histocompatibility complex (MHC) and a specificity for foreign antigenic determinants (X). The molecular basis for this dual specificity has not been completely resolved, but the reactionary interpretation is quite clear; that is T cells have a capacity similar to that of B cells in terms of germline determined antigen recognition. The major differential between these 2 cell classes are their requirements for triggering. The T cell must utilize two receptors to be triggered; one for antigen (X) and one for self.[1]

How does this dualism relate to idiotypy and networkism? A reactionaries position is one that is resistant to change. This being the case, he would say, that since a) all lymphocytes see antigen and since b) there clearly is experimental evidence for immunoglobulin dependent regulation, then it is equally clear that this phenomonology must have the same basis as the one previously described for the MHC; that is, some T cells have, in addition to their anti-(X) receptor, a receptor for self determinants encoded in or linked to one or more of the immunoglobulin gene complexes. Thus the reactionary point of view is the one first enunciated in 1977 by Janeway and his colleagues (5). I quote, "we contend that one helper T cell recognizes antigen in association with I region encoded structures and the other recognizes antigen in association with Ig."

Janeway et al came to this conclusion on the basis of

[1] *There is at least one exception to this rule, that being the response to the polymorphic determinants of the gene products of the MHC. These allo-antigens can react with self receptors in a fashion that leads to activation of the cell, without the need for an interaction with the second receptor for antigen (X). This is like a reaction to modified self. However, when the T cell reacts with MHC determinants that were present during the cell's ontogeny, activation does not occur unless self is modified or if the T cells second receptor, the one for antigen (X), is also engaged.*

experimental data. I came upon it independently, from a steadfast reactionary position of simplicity; that is, antigen is the force which drives the system and is the basis for immunological specificity, and therefore all lymphocytes should have clonally distributed antigen (X) specific receptors. If they also see something else it must be via another receptor.

AN ALTERNATIVE TO NETWORKISM

I offer the following phylogenetic scenario as the basis for an alternative to networkism. I should stress that most of the contentions that comprise this scenario are not novel; they have been pointed out previously. Hopefully their reiteration in the present context will be useful to other reactionaries who are enamored with the notion of antigen determined specificity.

Early in evolution it became important to protect the integrity of the cell or multicellular organisms from a hostile environment of predators. To do this the system learned to recognize self by virtue of having specific receptors for self. Anything which did not express self markers was considered foreign and attacked. This mode of recognition of foreigness did not allow the system to discriminate between foreigners and thus the system did not contain memory; that is, it could not learn from the first attack by a given enemy how to dispatch it more efficiently when a second attack was made. To do this the system had to learn to distinguish between foreigners. This was the evolutionary pressure that led to the immune response as we know it today in vertebrates.[2]

However, if the immune response of a shark is representative of the early phylogenetic immune response, one could see that a number of modifications needed to be made in order to achieve the level of immune responsiveness one finds today in mammals. The principal change in the humoral arm of the immune response that was required, was the production of antibody molecules which combined with the foreigners with greater tenacity. Thus, the earliest antibodies were equivalent to monomeric IgM i.e. antibodies of low affinity and low avidity. The simplest way to achieve greater stability in binding to antigen was to polymerize the low affinity monomeric unit and in so doing, increase avidity.

[2] *It is likely that modification of the original self recognizing molecule was an important strategy used in the development of molecules that recognize foreigners.*

This had the desired result of increasing binding capacity but had the deleterious side effect of decreasing mobility. Thus, these pentameric conglomerations of low affinity antibodies were very good for cleansing the blood but inefficient for attacking predators in tissue spaces. To do this a more mobile antibody with increased affinity had to evolve. This was a selectional pressure that led to the development of IgG. Over the course of time specialized subsets of IgG antibodies with specialized functions evolved as did other highly specialized antibody molecules such as IgA and IgE. The postulate is that along with the phylogenetic development of new classes of immunoglobulin new classes of helper T cells had to co-evolve.

The feature which makes helper T cells different from other classes of immunologically competent lymphocytes is the fact that at least some of these do not need helper cells to be triggered. Consequently, these cells can not afford to expose their receptors for antigen to the environment, for if they were to do so they could be activated by antigen when not in proximity to the cells they were supposed to help. If this were to occur, their helper capacities would be markedly diluted.. B cells have no such requirements because those that can be triggered directly by antigen can be triggered anywhere in the system. It doesn't matter because the B cell products are humoral and work at sites far distant from the cell that is actually secreting antibody. Other cells that need helper T cell signals in order to be activated would not be bothered by meeting antigen in an inappropriate place because antigen can not inappropriately activate them when the required helper signal is not present.

Thus, since the helper T cell could not afford to expose its receptor for antigen, except in specially organized lymphoid tissue where it performed its function, the requirement for a second receptor evolved. This receptor had to be present principally on cells which contained antigen. As it turned out, the self markers used were those that we now find encoded in the major histocompatibility complex.

It is postulated in this scenario that T cells find antigen containing cells by an anti-self Ia recognition mechanism and when in contact with the antigen bearing cells expose their epitope specific receptors (probably the specialized antigen bearing cell gives the T cell signal which causes it to expose its receptors). Since these specialized antigen presenting cells are properly placed anatomically, the T cell with exposed receptors that now encounters an antigen which fits its receptor, can perform its helper function efficiently. Thus the earliest phylogenetic helper T cells learned to react with self MHC in order

to have their clonally distributed receptors for antigen activated in the proper anatomical position.

During the course of the evolution of the humoral immune response from one of low affinity and low avidity, to one of high affinity, with many specialized subtypes of antibodies, concomitant evolution of T cell help was essential. Somewhere during the course of this evolution it became necessary for the T cell not only to find antigen bearing cells, but also the cells that secreted antibody. Perhaps it learned to do this, in part, by the recognition of MHC encoded antigens on B cells or even by recognition of Lyb-3. However, essential to this discussion is that along with the evolution of B cell products it became necessary for helper T cells to see B cell markers directly related to the immunoglobulins they were secreting, in order to optimally direct that B cell to perform its fully differentiated function. One could postulate that an evolutionary economic strategy would be to duplicate an MHC gene and transpose a homologue to an Ig locus.[3] Consequently, one of the self receptors that T cells use to help B cells is an MHC homologue encoded in, or closely linked to, one or more of the Ig loci. This type of self recognition leads to an outcome that can be perceived as being anti-idiotypically regulated whereas in fact, it is a modified MHC + (X)-like dual recognition in which a modern phylogenetic helper T cell sees a self marker that evolved from a precursor MHC-like molecule and which subserves a similar function to the molecule from which it evolved. Based on this analysis, let us now return to the question of repertoire raised earlier.

In terms of the ability of the system to see private immunoglobulin markers (i.e. idiotopes) this analysis predicts that the repertoire should be no different from that for any other epitope. However, in terms of those idiotypes that act as markers for self, the repertoire must be considerably smaller, as these markers must be shared by cells expressing different types of fine specificity.

PREDICTIONS OF THE ALTERNATE VIEW

What predictions does this view of the role of Ig recognition by T cells allow one to make that would distinguish it from networkism?

[3] *Although a divergent form of evolution is used as an example, a series of convergent evolutionary events would have the same logic and could produce the same outcome.*

1 - Repertoires:

Because of the significantly smaller repertoire of the self recognizing T cells, it predicts that the markers (?idiotypes) these cells see must be distributed on B cells which express different clonal specificites for antigen (X). Therefore, if one isolates an antibody by antigen binding and then makes an "anti-idiotype" antibody, (Ab2) this anti-idiotype antibody will in turn induce the production of many antibodies (Ab3) that have no ability to combine with the antigen used for the isolation of the idiotype positive molecules.

2 - By analogy with the MHC it predicts:

a) That Ir gene and relevant diseases will be linked to Ig polymorphisms;

b) That the relevant self recognition sites which are linked to Ig loci will act as transplantation antigens;

c) That studies with chimeric mice will show that self Ig recognizing cells will undergo "adaptive differentiation" (8). That is the ability of an Ig recognizing regulatory cell to interact appropriately with its target cell will be determined by the differentiative history of the cell rather than by its genetic constitution.

3 - That cell interactions which are restricted by polymorphic determinants linked to Ig loci will not be additionally restricted by polymorphisms linked to the MHC i.e. that the cell interactions governed by the two types of loci are likely to be alternative and not additive.

4 - That antigens under MHC linked Ir gene control will be able to interact normally with Ig dependent or recognizing T cells in non-responder mice.[4]

5 - That T cells have a dual specificity; some of them see antigen (X), plus something encoded in Ig. (Networkism, or at least its founder, has insisted upon unispecificity).

6 - Lastly, and perhaps most importantly, this model predicts that the idiotypic markers used in regulatory interactions need not be associated with the antibody combining sites.

There is at least some evidence that all of the above predictions are true (i.e. are really, at this stage, "postdictions!"). I will discuss some relevant findings that bear on these points that have not as yet been published.

EVIDENCE FOR A T CELL "FACTOR" THAT USES 2 DIFFERENT RECEPTORS: ONE TO SEE ANTIGEN (X) AND ANOTHER TO SEE A VH CONTROLLED GENE PRODUCT

Dr. Katsumi Yamauchi has shown that an $I\text{-}J^+$ Ly-1 cell makes a $I\text{-}J^+$ "factor" that induces suppressive activity in $Ly\text{-}2^+$ cells (10). The ability of this "factor" to induce its target cells to express suppressive activity requires that the "factor" producing cell(s) and its acceptor cell share genetic polymorphisms linked to the variable region of the Ig heavy chain gene complex (VH). The molecule used in the induction process is composed of two units; one of which binds antigen and the other of which bears an I-J determinant. The "factors" activity can be removed by absorbing it with antigen or by passing it through an anti-I-J column. However, mixing the material which is left after antigen absorption, with the material that comes through the anti-I-J column, reconstitutes suppressive activity. The material eluted from the I-J column, can substitute for the material which is left after antigen absorption. Thus, the $I\text{-}J^+$ unit does not see antigen.

[4] *The reason why this observation has generally eluded experimenters is because the Ig dependent T cell does not activate B cells directly. The MHC dependent T cell does. The Ig dependent one then interacts with the stimulated B cell to help direct its differentiation. Therefore it was not until the clever experiment of Bottomly (9), using an antigen not under Ir gene control to act as the stimulator for signal one and an Ir gene controlled antigen to provide the second signal, that this prediction could be fulfilled.*

Dr. Yamauchi is able to make hybrid molecules using antigen binding material from mouse strain A and $I\text{-}J^{+}$ material from mouse strain B. He can thus ask "which of the two units is responsible for the restriction linked to VH?" He has found that the VH restriction is imparted to the factor by macromolecule that is $I\text{-}J^{+}$ and which does not see antigen. As long as the $I\text{-}J^{+}$ chain comes from cells that share VH polymorphisms with the acceptor cells, the factors will function. The genetic constitution of the mouse that supplies the antigen binding macromolecule is irrelevant.

These findings strongly support the notion that the VH controlled interaction is not constrained by the antigen binding site, but rather by another molecule which seems to have the characteristic of a "classical" cell interaction type molecule (8) except that the so called "restriction site" on this molecule maps to VH and not to the MHC. Clearly the biologically activity of the molecule stems from a dual recognition - one of antigen and another of an as yet uncharacterized VH controlled determinant. This determinant may be an "idiotype" but the point is that an id anti-id reaction can not by itself activate the acceptor cell, an antigen recognizing product is also required.

THE NATURE OF A SRBC INDUCED "IDIOTYPE" SPECIFIC CELL THAT HELPS MYELOMA CELLS (MOPC 315) SECRETE TNP BINDING ANTIBODY

Another extremely important series of experiments that bears on the question at hand, has been done by Jim Rohrer and his colleagues (11). Their work jibes very well with that of Dr. Bottomly. They have shown that sheep red blood cell (SRBC immune Ly-1 cells of Balb/c mice can increase both the rate of division and the secretion of paraproteins by the TNP binding myeloma MOPC 315, if the myeloma cells come in contact with the helper cell product and TNP labelled SRBC. These two forms of helper activity, i.e. induction of increased proliferation and induction of increased secretion of paraprotein, are brought about by two different subsets of helper cells. The cells responsible for increasing proliferation express the phenotype, $Ly\text{-}1^{\pm},2^{-};Qa\text{-}1^{-}$, while the cells that help secretion are $Ly\text{-}1^{+},2^{-};Qa\text{-}1^{\pm}$. The surprising finding is that the $Ly\text{-}1;Qa\text{-}1^{+}$ helper cell, induced by SRBC immunization, sticks to the paraprotein of the 315 myeloma, but not to the paraprotein of a similar myeloma MOPC 460. A secretory helper cell with the same phenotype as the one raised by SRBC immunization can be produced by immunization with the 315 myeloma protein. Thus, this Ly-1;

Qa-1$^+$ cell has the characteristics of an idiotype specific helper cell.

The key question is why immunization with SRBC should activate an idiotype specific helper for a cell producing a TNP binding protein; particularly for an idiotypic specificity which is not ordinarily found in the pool of anti-TNP antibodies that Balb/c mice make after immunization with TNP protein conjugates. Further, why should this helper cell have both a specialized activity and a specialized phenotype? I submit that orthodox network theories do not predict these findings but that the hypothetical scenario I have outlined above does. It would explain Rohrers findings as follows; there are Qa-1$^+$ Ly-1 cells which see both SRBC and a marker on the 315 myeloma protein. The marker on 315 that the T cells see has nothing whatever to do with the idiotopes responsible for the TNP binding activity of the myeloma. Rather, Rohrer et al have fortuitously found a molecule expressing the antigen binding specificity of one of the molecules that would be made after immunization with an anti-antibody against a cross reactive idiotype marking an antibody with another antigen specificity; eg: the equivalent of one of the molecules which would be id$^+$ and antigen binding negative after immunization with, for example, an anti-A5A idiotype (12).

Thus, the product of MOPC 315 may be a classical Ab3 in Urbains network notion (6) and represents the first Ab3 for which an appropriately fitting epitope has been found. If this is true, the notion that some antibodies are "unspecific" (7) need not be entertained. It is the idiotype that is "unspecific" in terms of identifying antigen (X) specificities. Thus, 315 may express idiotopes relevant to TNP binding but the idiotype recognized by the regulatory T cell is more relevant to a different antigen specificity. This is why antibodies like 315 are not found after immunization by TNP; their cross reaction with TNP is incidental, their primary specificity is for another antigen.

Bottomly's work has suggested that there are anti-T15 (a dominant idiotype found in antiphosporylcholine antibodies of Balb/c mice) helper T cells that see, in addition to the T15 idiotype, a variety of different proteins (9). Rohrer's work suggests that a subset of Ly-1 cells that see SRBC also see an idiotype-like marker on the 315 myeloma protein. The way Rohrer's work extends the findings of Bottomly is that (a) it assigns a particular specialized function and phenotype to the "apparent" idiotype specific helper cell and (b) it shows that "apparent" idiotype specific helper T cells exist in mice that do not normally make proteins that have the characteristics of the assay mole-

cule i.e. the idiotype specificity along with the antigen binding characteristics. This finding is compatible with the notion that a) a subset of antigen specific T cells act only on B cells that have been previously "activated" by MHC restricted ($Qa\text{-}1^-$) helper cells,[5] and b) that this T cell subset sees both foreign epitope (X) and a marker on B cells and c) that this T cell subset exerts a very specialized regulatory function which, in Rohrer's system, is read out as increased secretion but which could be thought of more broadly as a signal that helps B cells differentiate.

The interpretations I have put forth are consistent with and, as far as I can tell not contradicted by, presently available experimental data. Of course this does not mean that they are correct. Right or wrong, they offer an alternative to a network theory in which unspecific (i.e. without antigen) anti-idiotypic physiological immunoregulation takes place. Hopefully these considerations may stimulate some alternative experimental approaches or interpretations that might help unravel the complex web of immunoregulatory cell interactions.

C. Suppression:

One of the key elements stressed by the founder of networkism is that, the essence of the immune system is the suppression of its lymphocytes. On this point I am sorely tempted to agree. What I have suggested is simply a more direct role for antigen in inducing "contrasuppression" (13).

[5] *The reason Rohrer et al can demonstrate the effect of the $Qa\text{-}1^+$ helper in the absence of $Qa\text{-}1^-$ Ly-1 cells is most likely due to the use of myeloma cells in lieu of B cells. Myeloma cells are activated by the oncogenes they carry and therefore do not need signal 1 to prepare them to accept signal 2.*

REFERENCES

1. Jerne, N.K. (1960) Ann. Rev. Microbiol. 14:341.
2. Burnet, F.M. (1959) The Clonal Selection Theory of Acquired Immunity, Cambridge Univ. Press, London, England.
3. Jerne, N.K. (1974) Ann. Immunol. (Inst. Pasteur) 125C:373.
4. Brown, J.C. and Rodkey, L.S. (1979) J. Exp. Med. 150:67.
5. Janeway, C.A., Murgita, R.A., Weinbaum, F.I., Asofsky, R. and Wigzell, H. (1977) Proc. Natl. Acad. Sci (USA) 74:4582.
6. Urbain, J., Cazenave, P.A., Winkler, M., Franssen, J.D., Mariame, B. and Leo, O. (1980) Prog. Immunol. IV (M. Fougereau and J. Dausset eds.) Academic Press, p. 81.
7. Oudin, J. and Cazenave, P.A. (1971) Proc. Natl. Acad. Sci (USA) 68:2616.
8. Katz, D.H. and Benacerraf, B. (1976) In: The Role of Products of the Histocompatibility Gene Complex in Immune Responses. (D.H. Katz and B. Benacerraf eds) Academic Press, N.Y. p 355.
9. Bottomly, K. (this book).
10. Yamauchi, K., Murphy, D.B., Cantor, H. and Gershon, R.K. (1981) Eur. J. Immunol., in press.
11. Rohrer, J., Lynch, R., Kemp, J. and Gershon, R.K. (in preparation).
12. Eichmann, K. (1979). Adv. Immunol., 25:195.
13. Gershon, R.K., Eardley, D.D., Durum, S., Green, D.R., Shen, F.W., Yamauchi, K., Cantor, H. and Murphy, D.B. (1981) J. Exp. Med., in press.

MYELOMA PROTEINS ARE TARGETS AND INDUCERS OF IMMUNOREGULATORY SIGNALS[1]

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The TNP-binding murine myeloma MOPC-315 is both a target and an inducer of specific immunoregulatory signals. As a target MOPC-315 is responsive to a multiplicity of: i) idiotype (Id^{315})-specific, and ii) TNP-antigen specific immunoregulatory signals from T and B cells. These signals can regulate the growth and differentiation of MOPC-315 cells. As an inducer the TNP-binding IgA protein (M315) released from MOPC-315 cells activates an extraordinary expansion of IgA-specific T cells that have many of the features of immunoregulatory cells. The data to be presented will focus on recent studies that have begun to identify the cellular and molecular mechanisms involved in two separate aspects of myeloma immunoregulation.

One form is a T cell mediated, Id^{315}-specific inhibition of M315 secretion from MOPC-315 cells. The T cells that inhibit secretion: i) are θ^+, $Lyt1^-2^+$; ii) have surface membrane structures that recognize M315 idiotypes but not idiotypes on M460, another IgA anti-TNP myeloma protein; iii) mediate inhibition via a diffusable, Id^{315}-specific T cell product; iv) effect a rapid, total and reversible inhibition of M315 synthesis without effecting other protein synthesis in the MOPC-315 cells, and v) do not require macrophages at the effector stage.

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The second form of immunoregulation involves IgA-binding T cells (Tα cells) that are expanded in mice with MOPC-315. We have observed that the Tα cells: i) are θ^+, $Ly_t1^-2^+$; ii) are adult thymectomy sensitive; iii) are present in mice with several other secretory IgA myelomas; iv) can be induced in normal mice by injection of large quantities of IgA myeloma protein and v) can be induced in vitro by treating nylon-wool purified T cells with purified, polymeric IgA. The in vitro induction of Tα cells is dependent on DNA and protein synthesis. A similar expansion of Tγ cells accompanies IgG myeloma, Tμ cells are increased in mice with IgM myeloma and Tε cells are elevated in IgE myeloma.

It is clear that the variable and constant regions of myeloma proteins play central but distinct roles in the immunobiology of myeloma. Since myeloma proteins have been extensively characterized in structural terms, myeloma provides a powerful experimental tool with which to explore fundamental immunoregulatory mechanisms.

INTRODUCTION

The development of circulating antibodies following immunization has been known since the beginning of this century when vonPirquet and Schick published their classic monograph on serum sickness (1). Although antibodies have been studied since that time it has only been in the last two decades that some understanding has developed about the events that occur during the latent period between immunization and the appearance of circulating antibodies. It is now established that the appearance of serum antibody is a late event that follows multiple interactions between distinct lymphoid and mononuclear cell populations that result in selection, expansion and differentiation of those B cell clones that eventually secrete the observed antibodies.

Although a great deal has been learned about some aspects of B cell development and the regulatory influences that operate on B cells during immune responses certain limitations have been imposed on this analysis because monoclonal popula-

tions of antigen-specific B cells have not been available for study. An analogous impasse existed in the study of antibody molecules until the discovery by Eisen *et al.* (2) that myeloma proteins were monoclonal antibodies. That observation was followed by a period of intense study of myeloma proteins (3) which established our present understanding of antibody structure and provided some of the first insights into the organization of immunoglobulin genes.

The major effort of our laboratory has been to develop hapten-specific myeloma cells as monoclonal models for the analysis of B cells. The rationale, strategy and advantages of this approach are similar to those that were applied when myeloma proteins were used as antibody models. The feasibility of using myeloma cells as B cell models has been established and the progress of this effort has recently been reviewed (4,5). These studies have clearly established that myeloma cells: i) are responsive to a multiplicity of specific immunoregulatory signals, and ii) are potent inducers of immunoregulatory cells. Thus myeloma cells have a dual relationship to the immunoregulatory system: they can *send* as well as *receive* immunoregulatory signals.

We selected for our studies murine myelomas whose antigen-binding properties were known because the extent of immunoglobulin expression by a single myeloma cell can then easily be determined. Myeloma cells that express surface immunoglobulin or secrete immunoglobulin can be visualized as rosette-forming cells or plaque-forming cells, respectively, using the appropriate haptenated erythrocytes. Most of the studies in which myeloma cells have been *targets* of immunoregulators have employed MOPC-315 which is a TNP-specific $IgA\lambda_2$ plasmacytoma of BALB/c mice. These studies have established that MOPC-315 cells: i) differentiate during *in vivo* growth, and ii) can be regulated by TNP-antigen-specific and

separate Id^{315}-(idiotype)-specific T cells. Those studies have recently been reviewed (4).

The studies which have established that myeloma cells induce immunoregulatory cells have employed a number of phosphorylcholine-binding, dextran-binding and nitrophenyl-binding myelomas that represent all the major heavy chain isotypes. These studies have established that myeloma proteins induce an extraordinary expansion of θ^+, Ly_t2^+ lymphocytes that express a surface membrane Fc receptor whose specificity is dictated by the inducing myeloma protein. These studies have also recently been reviewed (5).

The aims of this presentation are to: i) review and update studies that address the mechanism of idiotype-specific suppression of immunoglobulin secretion from myeloma cells; ii) summarize our present understanding of FcR^+ T cell induction by myeloma protein, and iii) present a conceptual framework in which to consider the relevance of these findings to the regulation of normal B cells.

METHODS

Id^{315}-specific Regulation of M315 Secretion

BALB/c mice were hyperimmunized with a mildly reduced and alkylated form of M315 in six weekly injections of 200μg each. The primary injection was in Freund's complete adjuvant, the second in Freund's incomplete adjuvant, and all subsequent injections were in PBS. The first and second injections were distributed over the hind foot pads and four dorsal subcutaneous sites. The subsequent injections were in the hind footpads and the peritoneal cavity. Three days prior to sacrifice the hyperimmunized mice were boosted with 50μg of M315 in PBS intravenously. Spleen cells were dispersed and collected

aseptically over Ficoll-hypaque and then were passed through a nylon-wool column. The recovered T cells were subjected to various specific cell separation protocols and finally added to an *in vitro* adapted line of MOPC-315 at a final T cell: MOPC-315 cell ratio of 100:1. Routinely, the cell mixtures were harvested after 24 hours of culture, MOPC-315 viability was determined, and the frequency of M315-secreting MOPC-315 cells was determined by an indirect Jerne plaque assay. In some experiments MOPC-315 cells were separated from the immune T cells by a 0.22μ pore membrane in the apparatus described by Ullrich and Zolla-Pazner (6). This configuration also permitted ^{3}H-leucine incorporation into M315 and total protein to be determined on purified MOPC-315 cells while they were being regulated by the Id^{315}-specific T cells (7).

Identification of T Cells with Fc Receptors in Myeloma

Spleen cell suspensions were prepared from mice with established subcutaneous plasmacytomas, and the lymphocytes with bound myeloma protein were quantitated using hapten-specific rosette assays as described (8). In some experiments the spleen cells were first cultured overnight, washed, exposed to various myeloma proteins or protein fragments and then the lymphocytes with bound myeloma protein were quantitated. Induction of Tα cells *in vivo* was achieved by twice daily injection of 15 mg of purified polymeric M315 i.p. in saline. Induction of Tα cells *in vitro* was achieved by culturing nylon-wool non-adherent lymphocytes (98% θ^{+}) with polymeric M315 as described (9).

RESULTS

Id^{315}-specific Inhibition of M315 Secretion

Before detailing our current understanding of the Id^{315}-specific T cell-mediated inhibition of M315 secretion, a summary will be presented of the more general issue of Id^{315}-specific regulation of MOPC-315 growth and differentiation.

When BALB/c mice are immunized with M315, three Id^{315}-specific effects are observed: i) the mice are rendered resistant to challenge with a lethal dose of MOPC-315 cells (10). The mechanism that mediates the transplantation resistance is still unclear but appears to involve short-lived suppressor T cells (11). ii) M315-immunized mice develop antibodies specific for M315 idiotypes (Id^{315})(12). The antibodies are predominantly of the IgG_1 subclass (13) and are directed to antigenic determinants located in or near the TNP-binding sites of M315 (14). Anti-Id^{315} antibodies bind *in vivo* and *in vitro* to the surface membrane M315 on MOPC-315 cells and mediate a reversible clearance of M315 from the cell surface (15). Affinity-purified isologous anti-Id^{315} antibodies do not affect M315 secretion by MOPC-315 cells *in vitro*, are not cytotoxic and do not influence the growth of MOPC-315 cells even when the antibodies are continuously present for up to three weeks. Serum containing anti-Id^{315} antibodies does not passively transfer MOPC-315 transplantation resistance if given at the time of MOPC-315 cell challenge (13). *In toto*, these findings suggest that the only consequence of engagement of surface M315 by anti-Id^{315} antibodies *per se* is a reversible clearance of the M315 from the cell surface membrane. iii) The third effect induced by immunization with M315 is a reversible inhibition of M315 secretion by MOPC-315 cells. *In vivo* experiments (16) have shown that the secretory inhibition is:

i) idiotype-specific; ii) mediated by, and adoptively transferred by immune T cells; iii) reversed by treatment of the inhibited MOPC-315 cells with pronase; and iv) effected across the 0.22μ pores of peritoneal diffusion chambers.

To better analyze the secretory blockade observed in idiotype-immune mice, an *in vitro* system was devised. Results from *in vivo* adoptive transfer experiments indicated that the effector cell was a T cell because treatment of the transferred spleen cells with anti-θ (rabbit anti-mouse brain associated) plus complement abrogated the transfer of inhibition of M315 secretion. Therefore, nylon-wool-purified Id^{315}-immune T cells were mixed directly with MOPC-315 cells. Following 24 hours of coculture, the cells were analyzed for viability, RFC and PFC frequency (7). Viability and RFC frequency were unaffected by the Id^{315}-immune splenic T cells compared to cultures containing normal splenic T cells or to MOPC-315 cells cultured alone. In contrast, the Id^{315}-immune splenic T cells inhibited the PFC frequency by as much as 80-90% when compared to controls. The secretory inhibition was shown to be T cell dose-dependent, with the maximum effect seen when the T cell:MOPC-315 cell ratio exceeded 100:1.

To determine whether adherent accessory cells (macrophages) played any role in the inhibition of M315 secretion depletion studies were performed. Nylon-wool-passed immune spleen cells were further adherent cell-depleted by two consecutive adherence steps on plastic petri dishes at 37°C for 60 minutes. Using the non-adherent cells as effectors, identical results were obtained. These data suggest that macrophages are not required at the effector stage of secretory inhibition.

We next determined the Lyt phenotype of the effector T cell. Using monoclonal anti-Ly_t-1 and anti-Ly_t-2 antibodies coupled to Sepharose-6MB columns specific cell depletion

studies were performed. We observed that only cells which passed through the anti-Ly_t-1 column were effective as secretory inhibitors, thus defining the phenotype of the regulatory T cell as $Ly_t\ 2^+$ (7).

To investigate the idiotypic-specificity of secretory inhibition, Sepharose 6MB columns were constructed using M315, or M460 (another murine IgA anti-TNP myeloma protein). M315 and M460 have dissimilar idiotypes. The data obtained from these experiments (Table 1) showed that the regulatory T cells responsible for secretory inhibition bound to the M315-Sepharose and not to the M460-Sepharose column. In addition, the T cells eluted from the M315-Sepharose column with 2 mg/ml free M315 were enriched for the specific regulatory cells.

Table 1

Specificity of Id^{315}-Immune T Cells that Suppress M315 Secretion

Effector:Target Cell Ratio	*Treatment of Id^{315}-Immune T Cells*	*% MOPC-315 Cells that Secrete M315*[a]
50:1	*No treatment*	*12 ± 6*
100:1	*"*	*8 ± 6*
200:1	*"*	*3 ± 1*
50:1	*Id^{460} Absorption*	*16 ± 7*
100:1	*"*	*10 ± 8*
200:1	*"*	*7 ± 4*
50:1	*Id^{315} Absorption*	*35 ± 6*
100:1	*"*	*36 ± 9*
200:1	*"*	*34 ± 11*
12:1	*Eluted from Id^{315} Absorbant*	*11 ± 8*
25:1	*"*	*4 ± 7*
50:1	*"*	*1 ± 2*

a) Means ± S.D. of PFC's measured after 24 hours of co-culture. MOPC-315 cells cultured alone had 43 ± 5% PFC.

A cell ratio of only 12:1 was now effective in mediating secretory inhibition and inhibition was virtually total at higher ratios. These observations indicate that the regulatory T cells have surface recognition structures specific for M315 idiotypes.

All of the previous experiments measured M315 secretion after 24 hours of co-culture with the regulatory T cells. To determine the kinetics of inhibition we harvested and assayed for PFC at 2 hour intervals following the addition of Id^{315}-immune splenic T cells to MOPC-315 cells. The M315 secretory inhibition was first observed after 6 hours and progressed until an inhibition of 80-90% of the control PFC was observed at 24 hours (7). This result suggests that the regulatory T cells are already programmed for the suppressive activity when added to the culture and probably require little further proliferation or differentiation.

Collectively, these data demonstrate that Id^{315}-specific T cells can specifically down-regulate the secretion of M315 from MOPC-315 cells that are already secreting M315. The regulatory T cell: i) acts independently of accessory macrophages; ii) is $Ly_t\ 2^+$; iii) recognizes and binds M315, and iv) is an example of a suppressor T cell that acts directly on the antibody-secreting cell.

Since the *in vivo* data indicated that secretory blockade was effected by a diffusable T cell product, *in vitro* experiments were carried out in which the T cells were separated from the MOPC-315 cells by a 0.22μ pore membrane. Id^{315}-specific inhibition of secretion was observed even though the target cells and regulatory cells were prevented from any direct contact. Interestingly, inhibition of secretion was reversed when the immune T cells were removed from the system (7).

Inhibition of M315 secretion by T cells is not simply due to engagement of the surface membrane M315 molecules because engagement with anti-idiotypic antibodies, TNP-hapten or TNP-antigens do not inhibit secretion. Furthermore, in previous studies we demonstrated that specific helper T cells could stimulate M315 secretion by MOPC-315 cells (17). One possible mechanism whereby secretion could be influenced is through regulation of synthesis. To evaluate this possibility we measured the incorporation of ^{3}H-leucine into the M315 and total proteins of MOPC-315 cells whose secretion had been inhibited by Id^{315}-specific T cells. The data in Table 2 show that inhibition of M315 secretion is accompanied by inhibition of M315 synthesis (7). Inhibition of M315 synthesis occurs without any apparent effect on other protein synthesis in the MOPC-315 cell since the decrement in total protein synthesis in inhibited cells can be accounted for entirely by the decrement in M315 synthesis. The molecular basis for this highly specific inhibition of synthesis is currently being studied.

Table 2

Inhibition of M315 Synthesis by Id^{315}-Immune T Cells

Contents of Lower Marbrook Chamber[a]	*^{3}H-Leucine Incorporation into MOPC-315 Cells*[b]	
	M315[c]	*Total Cell Protein*[d]
Media	*124936 ± 8934*	*236639 ± 10463*
Normal Spleen Cells	*107254 ± 4491*	*210733 ± 11843*
Id^{315}-Immune Spleen Cells	*9092 ± 2615*	*112213 ± 7762*

a) MOPC-315 cells added to upper chamber. Spleen cells present at 200-fold excess. Cultured for 48 hours prior to 24 hour pulse of ^{3}H-leucine.
b) Mean CPM ± S.D.
c) Immuno-precipitated IgA
d) TCA precipitated counts

In an effort to determine the fine specificity of the Id^{315}-specific T cell receptor, inhibition studies have been carried out using M315, its constitutive polypeptide chains, and enzymatically prepared fragments of M315 (7). We have observed that the Id^{315}-specific suppressor T cells recognize determinants located in $V_H{}^{315}$. Our observation is of particular interest because: i) Jorgensen and Hannestadt (18) have found that Id^{315}-specific helper-inducer T cells in BALB/c mice are specific for $V_L{}^{315}$, and ii) Odermatt *et al* (14) have shown that the anti-Id^{315} antibodies of BALB/c mice are directed to combinatorial idiotypes, i.e. determinants expressed on $V_{LH}{}^{315}$. It thus appears that in the Id^{315} system in BALB/c mice helper T cells recognize V_L, suppressor T cells recognize V_H and B cells recognize V_{LH}. Whether this pattern of molecular discrimination is fortuitous or has a more generalized significance awaits further investigation.

Induction of T cells with IgA Receptors (Tα cells) by IgA Myeloma

BALB/c mice with IgA myeloma develop extraordinary numbers of circulating and splenic lymphocytes with surface membrane localized myeloma protein (8). These lymphocytes are readily detected because they form rosettes with erythrocytes that have been conjugated with the hapten for which the myeloma protein has antibody activity. In mice with MOPC-315 (a-TNP) the lymphocytes form rosettes with TNP-SRBC while in mice with TEPC-15, McPC-603 or MOPC-167 (all a-PC) the lymphocytes form rosettes with phosphorylcholine-conjugated SRBC. The lymphocytes are θ^+, post-thymic cells that acquire the myeloma protein *in vivo* by adsorption to a surface membrane receptor for IgA. Specificity of these receptors for IgA was demonstrated in competitive binding studies. When cultured overnight the lymphocytes released the IgA that was bound *in vivo* and could

then bind the same or other IgA myeloma proteins. The lymphocytes appear to synthesize the IgA receptor since it reappears *in vitro* following its removal with pronase. IgA binding is not inhibited by IgG or IgM myeloma proteins or by F(ab)' prepared from IgA myeloma proteins. Thus the receptor binds IgA proteins and requires an intact Fc region for binding.

In previous studies Tα cells were not detected in mice with variant IgA myelomas that were poor IgA secretors or produced incomplete IgA molecules (8,19). This suggested that the secreted IgA protein played a central role in the expansion of Tα cells. To test this possibility we injected mice twice daily with large amounts of IgA myeloma protein and monitored Tα cell frequency. We observed a large Tα cell increase (9). As was shown for the Tα cells in mice with IgA myeloma the Tα cells induced by injection of IgA protein were predominantly Ly_t2^+ cells. Tα cells were not detected in nude or adult-thymectomized mice with IgA myeloma but were present in large numbers in myeloma-bearing nude mice that had been implanted with thymic grafts (20). Collectively, these observations suggest that the Tα cells are regulatory lymphocytes.

Tα cells can also be induced *in vitro* by exposing nylon wool-purified T cells to polymeric IgA (9). Induction is dependent on DNA and protein synthesis. The functional properties of Tα cells are presently under investigation.

Induction of T cells that express heavy chain specific receptors is not restricted to IgA myeloma. Tγ cells are increased in mice with IgG myeloma and Tε cells are increased in mice with IgE hybridoma (21). Tμ cell expansion has been observed in mice injected daily with large amounts of IgM myeloma protein (21). In the first six humans with untreated myeloma that we have examined two with IgA myeloma had increased Tα cells and four with IgG myeloma had increased Tγ cells (22).

DISCUSSION

The studies summarized above and others recently reviewed (4,5) demonstrate that myeloma cells can *induce* as well as *respond to* immunoregulatory signals. It is now clear that MOPC-315 responds to two major categories of regulatory T cells: i) antigen-specific, MHC-restricted, macrophage-dependent helper and suppressor T cells, and ii) idiotype-specific, macrophage-independent helper and suppressor T cells. It is also clear that distinctly separate T cells regulate clonal growth and clonal differentiation of MOPC-315.

We believe that MOPC-315 and other myelomas provide powerful and relevant monoclonal B cell models. The appropriateness of malignant B cells as models for normal B cells is a legitimate issue as was the questioned validity of myeloma proteins as antibody models. In retrospect the latter skepticism is noteworthy since the structural, functional and genetic studies of myeloma proteins have contributed so much to the development of modern immunology as well as to the emergence of the hybridoma era. Ironically, rather than being inappropriate models, myeloma proteins turned out to be better than "normal" antibodies because some myeloma proteins were molecular anomalies whose characterization provided useful information. For example, amino acid sequence analyses of immunoglobulin fragments provided insight into the arrangement and organization of immunoglobulin genes. Hopefully, analogous regulatory anomalies will be encountered during studies of myeloma cell regulation. Since such anomalies are permuted derivations of the initial existing order within the system, the analysis of anomalies can provide insight into that order.

The induction of FcR^+ T cell expansion by myeloma protein demonstrates that myeloma cells can elicit immunoregulatory

cells. Our bias is that this represents an exaggerated, but otherwise normal, immunoregulatory response. Although the functional properties of myeloma-associated FcR^+ T cells have yet to be identified it might be reasoned that an appropriate response to the surge of immunoglobulin output by the myeloma cells would be the expansion of a suppressor immunoregulatory cell population. Phenotypically, the Tα cells in myeloma are suppressor T cells (20). FcR^+ T cells could play several roles in immunoregulation because there are at least three ways in which they could directly interact with B cells. (Figure 1).

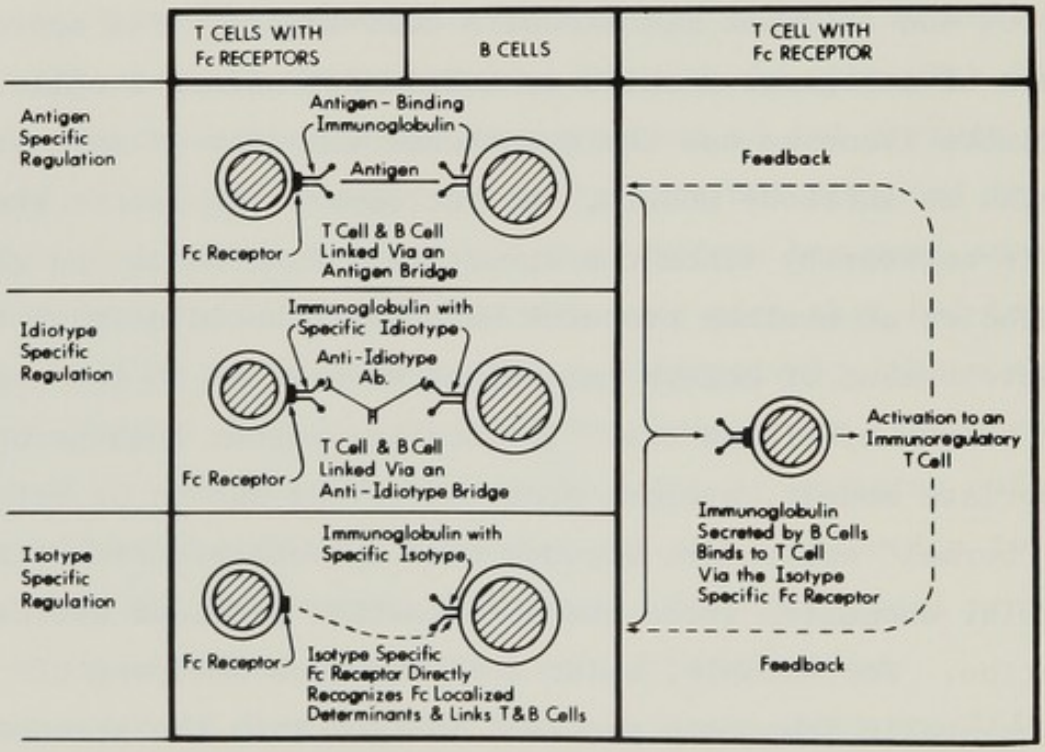

FIGURE 1. Model for FcR^+ T Cell Interactions with B Cells in Immunoregulation.

The demonstration of FcR^+ T cell expansion in myeloma is one more example where conspicuous alteration of a lymphoreticular cell population accompanies myeloma. Myeloma-induced suppressor macrophages have been demonstrated in humans (23)

and mice (24) with myeloma, and the severe osteolytic bone disease that accompanies myeloma results from activation of bone marrow macrophages (25). Thus there are at least three examples in which myeloma cells interact with host lymphoreticular cells in an effector cell fashion. It remains to be determined whether and how FcR^+ T cells contribute to the pathophysiology of myeloma.

Finally, the demonstration that FcR^+ T cells can be induced *in vitro* and *in vivo* with immunoglobulin emphasizes the need to distinguish between exogenous and endogenous idiotypes, variable regions and antigen receptors on T cells.

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REFERENCES

1. vonPirquet, C. and Schick, B. (1905). *Die Serumkrankheit. Leipzig: Franz Deuticke.*
2. Eisen, H.N., Little, J.R., Osterland, C.K., and Simms, E.S. (1967) *Cold Spring Harbor Symp. Quant. Biol. 32:*75.
3. Potter, M. (1973) *IN*: Multiple Myeloma and Related Disorders, p. 195, Harper and Row, Hagerstown, MD.
4. Lynch, R.G., Rohrer, J.W., Odermatt, B., Gebel, H.M., Autry, J.R. and Hoover, R.G. (1979). *Immunol. Rev. 48:*45.

5. Hoover, R.G., Gebel, H.M., Dieckegraefe, B.K., Hickman, S., Rebbe, N.F., Hirayama, N., Overy, Z. and Lynch, R.G. (1981). *Immunol. Rev. 56:*(in press).
6. Ullrich, S.E. and Zolla-Pazner, S. (1980). *J. Immunol. Meth. 39:*147.
7. Milburn, G.L. and Lynch, R.G. (1981). (manuscript submitted).
8. Hoover, R.G. and Lynch, R.G. (1980). *J. Immunol. 125:* 1280.
9. Hoover, R.G., Dieckegraefe, B.K. and Lynch, R.G. (1981). (manuscript submitted).
10. Lynch, R.G., Graff, R., Sirisinha, S., Simms, E.A. and Eisen, H.N. (1972). *Proc. Natl. Acad. Sci. 69:*1540.
11. Daley, M.J., Gebel, H.M. and Lynch, R.G. (1978). *J. Immunol. 120:*1620.
12. Sirisinha, S. and Eisen, H.N. (1971). *Proc. Natl. Acad. Sci. 68:*3130.
13. Frikke, M.J., Bridges, S.H. and Lynch, R.G. (1977). *J. Immunol. 118:*2206.
14. Odermatt, B., Perlmutter, R. and Lynch, R.G. (1978). *Eur. J. Immunol. 8:*858.
15. Milburn, G.L. and Lynch, R.G. (1981) (manuscript submitted).
16. Rohrer, J.W., Odermatt, B. and Lynch, R.G. (1979). *J. Immunol. 122:*2011.
17. Rohrer, J.W. and Lynch, R.G. (1978). *J. Immunol. 120:* 1066.
18. Jorgensen, T. and Hannestadt, K. (1980). *Nature 288:*396.
19. Gebel, H.M., Hoover, R.G. and Lynch, R.G. (1979). *J. Immunol. 123:*1110.
20. Hoover, R.G., Dieckegraefe, B.K., Lake, J., Kemp, J.D. and Lynch, R.G. (1981). (manuscript submitted).

21. Hoover, R.G., Dieckegraefe, B.K., Hirayama, N., Ovary, Z. and Lynch, R.G. (1981). (manuscript submitted).

22. Hoover, R.G., Hickman, S., Gebel, H.M., Rebbe, N. and Lynch, R.G. (1981). *J. Clin. Invest. 67:*308.

23. Broder, S. and Waldman, T. (1980). *IN:* Progress in Myeloma, p. 151, Elsevier/North-Holland, NY.

24. Zolla-Pazner, S. (1980). *IN:* Progress in Myeloma, p. 171, Elsevier/North-Holland, NY.

25. Schecter, G.P., Wahl, L.M. and Horton, J.E. (1980). *IN:* Progress in Myeloma, p. 67, Elsevier/North-Holland, NY.

SELECTIVE REGULATION OF IDIOTYPE PRODUCTION BY SUPPRESSOR T LYMPHOCYTES

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Antibody secretion by murine myeloma cells can be inhibited by idiotype reactive suppressor T lymphocytes (Ts) in vitro. The effect of such Ts is selective in that they block secretion of antibody without detectable suppression of surface receptor expression. Moreover, when cultured with a somatic cell hybrid that produces two idiotypically unrelated immunoglobulins, Ts specific for each myeloma protein inhibit secretion of only that antibody. The implications of these results for the molecular basis of T cell-mediated regulation are discussed.

INTRODUCTION

Recent advances in molecular biologic techniques and their application to studies of immunoglobulin (Ig) genes have provided major insights into the biochemical bases of lymphocyte differentiation. Rearrangements of Ig genes are now believed to be central events in the maturation of B lymphocytes, as well as the antigen-driven switching of heavy chain class (i.e. IgM to IgG, IgA, IgE). Moreover, DNA rearrangements and/or RNA splicing events may account for the change from predominantly membrane Ig expression to active Ig secretion that is seen when mature B cells are triggered by

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antigens or polyclonal activators. In addition to suggesting a number of mechanisms for the generation of antibody diversity, these investigations have raised critical questions about the manner in which ligand-receptor interactions at the lymphocyte surface generate signals that are translated into fundamental alterations at the level of the DNA and/or RNA. Clearly a major thrust of future immunological research will be an analysis of this "signal transduction" process, and the identification of relevant enzymes, promoters and regulators. It is anticipated that such studies will be extended to T lymphocyte-mediated regulation of B cell function, so that we can precisely define how interacting sets of immunocompetent cells communicate with one another. In order to achieve these goals, it is crucial to develop experimental systems in which cloned, homogeneous target cell populations can be regulated (induced or suppressed) by antigens, polyclonal activators or T lymphocytes.

During the last five years or so, we and others have shown that the growth and function of murine myeloma cells can be strikingly affected by a variety of immunoregulatory stimuli (1,2). Our earliest experiments demonstrated that antibody secretion by the 2,4-dinitrophenol (DNP) and 2,4,6-trinitrophenol (TNP) binding BALB/c myeloma, MOPC315, is specifically and reversibly suppressed by tolerogenic DNP-globulins and immune complexes (3,4). The work of Lynch and co-workers (2) has shown that the differentiation and function of MOPC315 cells *in vivo* can be regulated by carrier-specific helper and suppressor T lymphocytes in the presence of appropriate TNP-carrier conjugates. In this paper we will briefly review some of our recent experiments on regulation of myeloma cells by anti-idiotypic Ts, and attempt to

emphasize what we have already learned about the mechanisms of T cell mediated suppression as well as the potential importance of this and similar experimental systems. Details of this work have been published (5,6).

METHODS

The myeloma cells used in these experiments were tissue-culture adapted cloned lines of: MOPC315 (IgA,λ_2), MPC11 (IgG_{2b},κ) and a somatic cell hybrid between these two prepared by polyethylene glycol induced fusion in the laboratory of Dr. M.L. Gefter, Mass. Institute of Technology, Cambridge, MA, and selected and cloned as previously described (6,7). Suppressor T cells were induced by immunizing BALB/c mice intravenously (i.v.) with syngeneic splenocytes to which purified myeloma proteins were covalently coupled using the bifunctional coupling reagent, 1-ethyl 3-(3-diethylamino-propyl) carbodiimide (ECDI). Control animals were immunized with ECDI-treated cells (5). Seven days later, thymic or splenic nylon wool non-adherent T lymphocytes were prepared and cultured with the myeloma cells at suppressor:target cell ratios of 5-20:1(5,6). At the end of 3-5 days of co-culture at 37°C in 5% CO_2, cultures were harvested, washed and aliquots assayed for viable myeloma cell recovery and for numbers of antibody-secreting myeloma cells using a reverse hemolytic plaque assay with Staphylococcal protein A-coated erythrocytes as indicator cells (5). In some experiments, cultures of MOPC315 cells and Ts were also pulsed with ^{3}H-leucine or ^{3}H-glucosamine, and the amount of radio-activity incorporated into intracellular (synthesized) and supernatant (secreted) IgA measured after specific immunoprecipitation (3).

RESULTS

The initial series of experiments showed that subcutaneous immunization of BALB/c mice with myeloma-protein coupled cells primed animals for a myeloma idiotype-specific delayed hypersensitivity (DH) response, whereas transfer of T lymphocytes from i.v. immunized animals suppressed priming for DH (5). Moreover, Ts from mice immunized i.v. with M315-coupled cells also inhibited antibody secretion by MOPC315 targets following co-culture. A representative experiment illustrating this suppression is shown in Fig. 1. Several points are noteworthy. First, significant suppression requires 3-4 days of co-culture. Secondly, the maximum reduction of PFC we

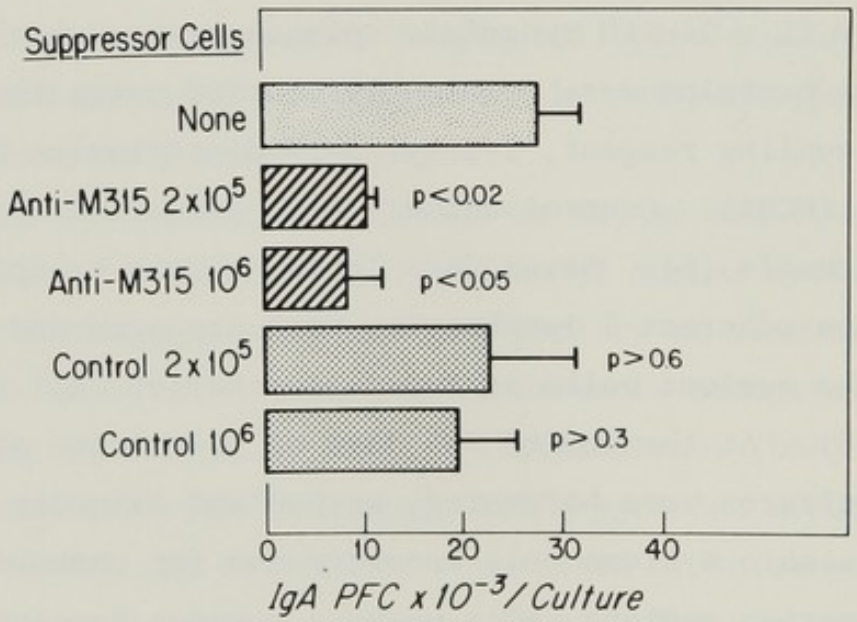

FIGURE 1. Suppression of MOPC315 cells by M315-reactive Ts. 10^5 MOPC315 cells were cultured in triplicate for 4 days with nylon wool non-adherent splenic T lymphocytes from BALB/c mice immunized i.v. with M315 coupled-or ECDI-treated syngeneic splenocytes (anti-M315 or control cells). Numbers of IgA plaque forming cells (PFC) were measured by reverse hemolytic plaque assay. Data adapted from Ref. 5, by permission.

have observed is about 60% at suppressor:target cell ratios of up to 20:1, and this is not accompanied by an effect on myeloma growth or viability. Finally, the Ts do not alter the proportion of viable MOPC315 cells expressing membrane receptors, as assayed by rosetting with TNP-modified sheep erythrocytes (5).

In a series of experiments (5) we have found that the Ts are Thy 1.2-bearing T cells that also express surface determinants encoded in the I-J subregion of the H-2 complex. They are idiotype-specific, since they can be depleted on and enriched 20-100 fold by binding to plastic dishes coated with M315 IgA protein but not dishes coated with mouse IgG or M460 IgA (MOPC460 being an IgA,κ myeloma that also binds DNP and TNP but by serology is idiotypically distinct from MOPC315). To date we have been unable to identify soluble mediators of suppression in cell extracts or culture supernatants of lymphocytes from mice immunized i.v. with M315-coupled cells. Moreover, M315-specific Ts can be induced in CAL20 mice which have a different IgV^h region from BALB/c (data not shown). This indicates that recognition of idiotype for generation of Ts is not V^h restricted, similar to results in other systems, (8). Ts can also be induced in (SJLxBALB/c) F_1 mice by i.v. immunization with M315-coupled BALB/c ($H\text{-}2^d$) or SJL ($H\text{-}2^s$) splenocytes. Thus, the induction of anti-idiotypic Ts does not show apparent H-2 restriction. Experiments are in progress to determine if the effector function of Ts involves recognition of H-2 encoded surface antigens on the myeloma target.

In order to further analyze the mechanism of action of Ts, we have examined their effect on a somatic cell hybrid prepared by fusing two idiotypically unrelated myelomas, MOPC315 and MPC11. Hybrids were selected on the basis of

their ability to secrete both Ig's (7). More than 90% of freshly cloned hybrid cells express both M315 IgA and MPC11 IgG on their surfaces by double immunofluorescence. Population assays (i.e. radioimmunoassay and biosynthetic labeling) confirmed that both Ig's are synthesized and secreted by the cells and expressed on their surface, and that >90% of λ_2 chains are IgA-associated and κ chains IgG_{2b} associated. Moreover, reverse hemolytic plaque assays using anti-IgA or anti-IgG sera separately or in combination as developing reagents indicated that in the hybrid population, essentially all secreting cells produced both Ig's (Table 1). Finally,

TABLE 1. Secretion of Ig by MOPC315, MPC11 and somatic cell hybrid lines.

MYELOMA CELLS	*DEVELOPING ANTISERA*	*PFC (Mean ± SE)*
$5x10^2$ MOPC315 + $5x10^2$ MPC11	*Anti-IgA*	*211 ± 3*
$5x10^2$ MOPC315 + $5x10^2$ MPC11	*Anti-IgA*	*214 ± 30*
$5x10^2$ MOPC315 + $5x10^2$ MPC11	*Anti-IgA + Anti-IgG*	*390 ± 34*
10^3 MOPC315-MPC11 Hybrid	*Anti-IgA*	*526 ± 18*
10^3 MOPC315-MPC11 Hybrid	*Anti-IgA*	*488 ± 40*
10^3 MOPC315-MPC11 Hybrid	*Anti-IgA + Anti-IgA*	*534 ± 30*

MOPC315-MPC11 hybrids or mixtures of parental cells were assayed for numbers of Ig-secreting cells using protein A-coated sheep erythrocytes as indicators and appropriate developing sera (5). When both developing sera are used, the numbers of PFC obtained with mixtures of MOPC315 and MPC11 are approximately equal to the sum of PFC obtained with each serum, whereas with the hybrid cell line the numbers of PFC are not significantly different using either developing serum separately or in combination. Previous experiments had established that MOPC315 cells did not produce plaques with the anti-MPC11 serum and vice versa.

the rate of spontaneous loss of Ig secreting cells per generation is <1 in 10^3 for IgA and <1 in 10^5 cells for IgG.

When this hybrid cell line is cultured with either M315- or MPC11-reactive Ts, each Ts inhibits secretion of only the Ig that is recognized on the cell surface (Fig. 2). This result has been confirmed in several experiments using splenic or thymic Ts. The specificity of the Ts is further demonstrated by their functional depletion on M315-or MPC11-coated

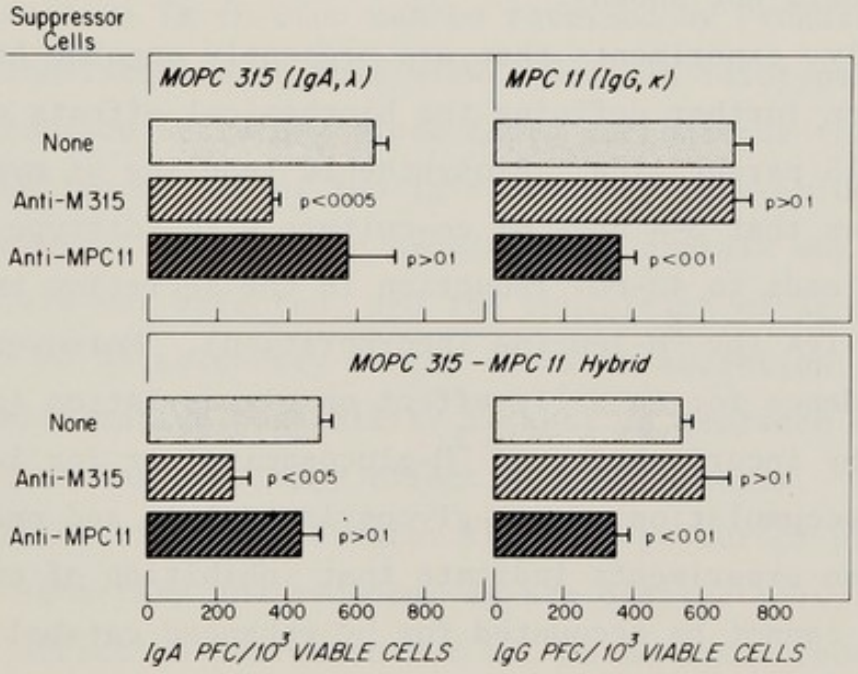

FIGURE 2. Effects of idiotype-reactive Ts on parent myeloma lines and somatic cell hybrids. 10^5 MOPC315, MPC11 or MOPC315-MPC11 hybrid cells were cultured for 4 days with 10^6 thymocytes from BALB/c mice immunized i.v. with M315-or MPC11-coupled syngeneic splenocytes, and numbers of IgA or IgG PFC measured. Data adapted from Ref. 6, by permissions.

dishes (6). This selective inhibition of Ig secretion cannot be explained by the presence of hybrid myeloma cells that secrete only one Ig, because: i) in the hybrid population, each secreting cell produces both antibodies, at least at culture initiation (Table 1), ii) the rate of spontaneous loss of Ig production is low, and iii) in these high density cultures ($5x10^5$ myeloma cells per ml) the myeloma targets double only once in the 3-4 days that they are co-cultured with Ts (data not shown).

Finally, experiments that are presently ongoing have been focussed on further defining the biochemical effects of Ts on the MOPC315 parent line. Biosynthetic labeling of myeloma cells shows that 3-4 days of co-culture with idiotype-reactive Ts leads to 40-60% reduction in the secretion and synthesis of IgA (by ^{3}H-leucine incorporation). Moreover, there is no evidence for an early effect on glycosylation (as measured by incorporation of ^{3}H-glucosamine) or for intracellular accumulation of non-glycosylated IgA, and preliminary pulse-chase experiments indicate that inhibition of antibody secretion cannot be accounted for by enhanced catabolism of synthesized IgA.

DISCUSSION

These experiments demonstrate that interaction of Ts with idiotypic determinants on cloned, homogeneous myeloma cells leads to inhibition of idiotype secretion and synthesis. The suppression exhibits two features that imply a highly selective action of Ts. First, the block in Ig secretion is not accompanied by any detectable effects on surface receptor expression (5). Secondly, when the myeloma target is a somatic cell hybrid producing two unrelated intact Ig's, Ts reactive

with each surface idiotype inhibit secretion of only that Ig (6).

On the basis of these results, it is possible to propose two alternative but not mutually exclusive mechanisms of action of anti-idiotypic Ts.

i) The secretory block is mediated at the myeloma cell surface by the Ts themselves or by secreted factors. Experiments of Rohrer et al. (9) showing that inhibition of MOPC315 cells by M315-reactive Ts *in vivo* can be reversed by Pronase treatment are consistent with this view. Such an idiotype-specific surface secretory block could account for the selective inhibition of one of two Ig's produced by hybrid myeloma cells. However, it would be necessary to explain why surface receptors are not affected, and the inhibition of Ig synthesis would have to be secondary to the block in secretion.

ii) The alternative possibility is that Ts generate a "signal" that has a primary effect on Ig synthesis. Since the available evidence indicates that the precursor protein pools for secretory and receptor Ig are distinct (10), a selective effect on secretion but not on membrane receptor expression is conceivable. However, it is unclear how such intracellular suppressive signals could affect the synthetic apparatus for only one of the two Ig's produced by the somatic cell hybrid myeloma line.

In the final analysis, it remains to be determined whether Ts affect transcription, translation or post-synthetic modification of Ig heavy or light chains. Resolution of these issues may require target cell populations in which essentially all the cells are functional and suppressible, and conditions for inducing suppression by soluble mediators. Once the experimental systems are optimized, the techniques are now available for defining the molecular basis of lymphocyte-mediated regulation.

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REFERENCES

1. Abbas, A.K., (1979) *Immunol. Rev. 48*, 245.
2. Lynch, R.G., Rohrer, J.W., Odermatt, B., Gebel, H.D., Autry, J.R., Hoover, R.G. (1979) *Immunol. Rev. 48*, 45.
3. Abbas, A.K. and Klaus, G.G.B. (1977) *Eur. J. Immunol. 7*, 667.
4. Abbas A.K. and Klaus, G.G.B., (1978) *Eur. J. Immunol. 8*, 217.
5. Abbas, A.K., Perry L.L., Bach, B.A. and Greene, M.I. (1980) *J. Immunol. 124*, 1160.
6. Abbas, A.K., Burakoff, S.J., Gefter, M.L. and Greene, M.I. (1980) *J. Exp. Med. 152*, 968.
7. Siebert, G.R., Harris, J.R. and Gefter, M.L. (1978) *J. Immunol. 121*, 1808.
8. Sy, M.-S., Dietz, M.H., Nisonoff, A., Germain, R.N. Benacerraf, B., and Greene, M.I. (1980) *J. Exp. Med. 152*, 1226.
9. Rohrer, J.W., Odermatt, B. and Lynch, R.G. (1979) *J. Immunol. 122*, 2011.
10. Sidman, C.L., et al. (1981) *Cell*, in press.

H-2 RESTRICTED CARRIER-SPECIFIC T CELL CLONES DO NOT SELECT IMMUNOGLOBULIN ISOTYPES

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ABSTRACT

We have obtained T cell clones specific for SRBC, HRBC and GAT antigen. The clones consist of helper cells which are carrier-specific and H-2 restricted. GAT-clones have no by-standard effect. All of them can induce antibody responses of various isotypes in adoptive transfer in vitro. Isotypic patterns of the responses obtained with clones were identical to those obtained with polyclonal T cells. The TH clones also require an hapten carrier bridge to provide help to B lymphocytes. They can be considered as TH1 cells. Results also indicate that the isotypic pattern of the response is only dependent on the memory B cell origin. The isotypes expressed on memory B lymphocytes may be selected by Ig-specific TH2 lymphocytes. The failure to generate Ig-specific TH2 cell clones may be accounted for by the immunization protocol or a T cell selection during the cloning procedure.

INTRODUCTION

Besides the antigen specific helper and suppressor cells T cells specific for Ig determinants have been described (1-3). Such Ig specific T cells may play an important role in antibody responses in terms of idiotype, allotype and isotype selection. Janeway has recently proposed a model which postulates the existence of two main types of helper T cells (4). According to this model the carrier-specific, H-2 restricted T helper cells (TH1) work in conjunction with T cells called TH2 that are involved in the regulation of idiotype, allotype or isotype expression. TH2 cells would have an antigen receptor and an Ig receptor specific for either idiotypic, allotypic or isotypic determinants. Contrary to the classical carrier-specific TH1 cells, TH2 cells would not require an hapten carrier bridge to deliver their signals to B cells.

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Thus, the isolation and characterization of antigen-specific T helper cell clones may help to solve the problem of the existence of different subsets of TH cells. We have recently developed a procedure which allows us to obtain such clones. In the present study we have investigated the ability of T cells from various clones to select immunoglobulin isotypes in an adoptive secondary response *in vitro*.

MATERIALS AND METHODS

Methods for the preparation of reagents, antigens, cultures and various cell types have been previously published in detail (5, 6).

Details concerning the cloning procedure have also been reported elsewhere (7). Briefly, activated T lymph node cells from antigen primed animals were allowed to expand *in vitro* for 5 to 10 days in the presence of antigen. Cells were then cloned in soft agar according to a modification of the method described by Sredni *et al.* (8). T cell colonies were then submitted to a second cloning step by limiting dilution, and the clones were cultured in the presence of antigen without filler cells and TCGF.

RESULTS

1. Experiments with RBC-specific clones

HRBC and SRBC-specific T cell clones were obtained using lymph node cells from NCS animals primed with 2×10^7 RBC. Cells were expanded and cloned using RBC ghosts as antigens. Figure 1 shows the anti-SRBC or -HRBC responses obtained by the addition of various numbers of T cells from 6 different clones to virgin anti Thy-1 + C' treated syngeneic spleen cells. These 6 clones were able to initiate a primary IgM anti-HRBC or -SRBC responses with as few as 10^3 cells per culture. 48 additional SRBC- or HRBC-specific clones have also been tested. All of them were able to stimulate a primary IgM antibody response to the corresponding antigen.

As shown in Fig. 1, HRBC-specific clones could not induce anti-SRBC responses and can therefore be regarded as antigen-specific helper cells. Contrary to this, all the SRBC-clones were capable of inducing a significant anti-HRBC response. This, could be explained by a non-symmetrical cross-reactivity between SRBC and HRBC antigens at the T cell level. Furthermore the addition of both HRBC and SRBC to the SRBC-clones increased their capacity to induce an anti-HRBC response (data not shown).This phenomenon resembles the by-standard

effect previously reported by Schreier et al. for similar clones (9). A similar by-standard effect was also observed with the HRBC-clones.

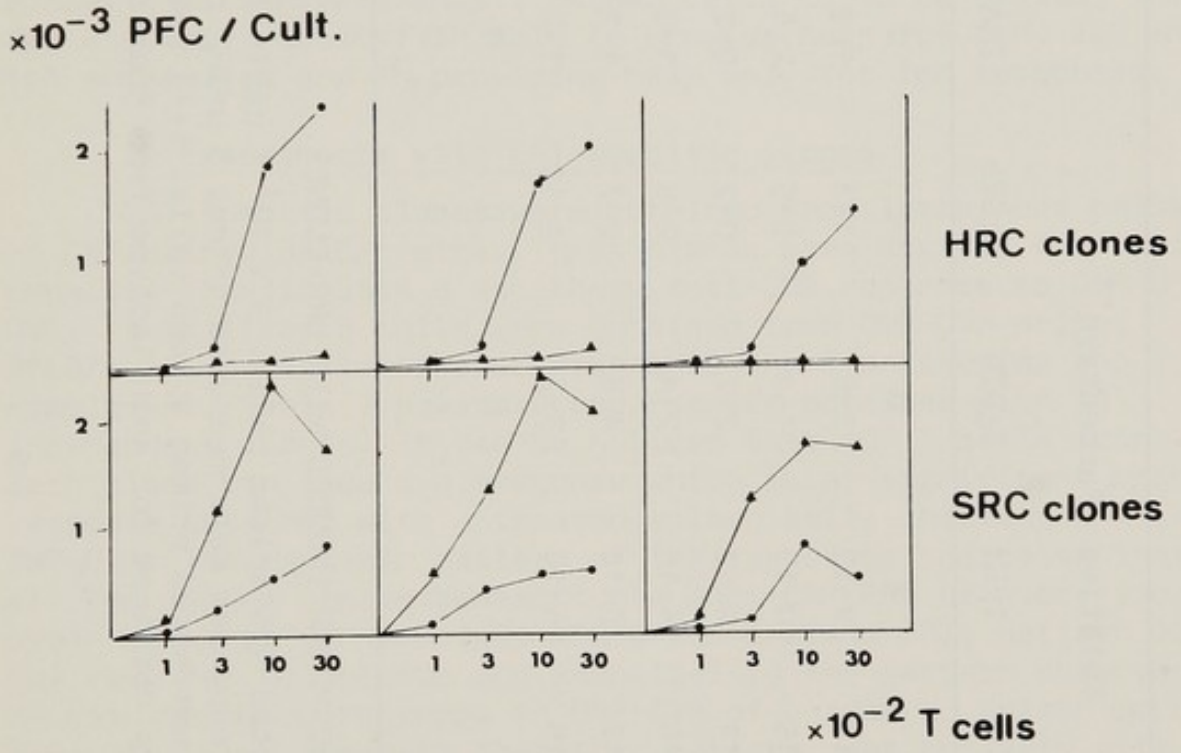

Figure 1. SPECIFIC RESPONSE TO SRBC (—▲—) and HRBC (—■—) INDUCED BY HRC- AND SRC-CLONES. Anti Thy-1 + C' treated virgin NCS-spleen cells (7.5 x 10^5 cells/well) were cultured in presence of 50 µl/ml of a 1 % suspension of SRBC and HRBC and various numbers of T cell clones. Direct plaques were measured on day 5.

RBC-clones have also been tested for their capacity to induce secondary responses in vitro. NCS mice were primed with 2 x 10^7 SRBC or HRBC i.p.. Spleen cells were collected on day 7 and treated with anti Thy-1 plus complement. 3 x 10^3 cloned T cells were added to 7.5 x 10^5 B cells and tested for their ability to stimulate secondary antibody responses of the various isotypes. Results obtained with several independent HRBC-clones are presented in Table 1. It can be seen that among the 7 clones tested 5 were able to induce IgM, IgG3, IgG1, IgG2a, IgG2b, and IgA antibodies. Table 1 also reveals that the isotypic patterns obtained with different clones are all identical to the pattern observed in a polyclonal situation i.e. secondary NCS anti-HRBC response in vitro. This demonstrates that the T helper cells which have been selected

	Total PFC response/ culture	Relative Percentage[++]					
		IgM	IgG3	IgG1	IgG2b	IgG2a	IgA
B cells	228 ± 145	100	0	0	0	0	0
Polyclonal T cells[+]	3841 ± 416	60-68	12-16	9-12	3-6	1-3	1-3
Clone : H8.1	2746 ± 381	65-72	12-15	10-13	1-4	2-5	2-5
H9.16	3117 ± 448	65-74	11-14	9-12	2-7	0-2	0-2
H9.20	3364 ± 517	58-65	12-16	14-18	2-4	2-6	1-3
H11.9	2938 ± 423	60-64	11-14	6-18	4-8	2-7	1-3
H12.4	3675 ± 485	51-59	12-15	15-18	3-7	4-10	1-3
H9.24	2716 ± 549	100	0	0	0	0	0
H18.5	3062 ± 421	100	0	0	0	0	0

Table 1. ISOTYPIC PATTERN OF THE SECONDARY HRBC RESPONSE INDUCED WITH HRC-CLONES.

B cells were from HRC primed anti Thy-1 + C' treated NCS spleen cells. 3×10^3 T cells from each clone were added to 7.5×10^5 B cells. PFC responses were measured on day 5 using rabbit class-specific antisera. (+) Response to HRBC of untreated primed spleen cells. (++) Results are expressed as the lowest and highest percentage obtained for each isotype from four replicates from two independent experiments.

during the cloning steps allow the expression of memory B cells whatever the isotype they are committed to. (Similar results were obtained using SRBC-clones in secondary anti-SRBC responses in vitro). Two HRBC-clones induce only an IgM response. This suggests that T helper cells could be divided into two distinct subsets: TH able to provide help for IgM, IgG and IgA antibodies and TH providing help only for IgM responses.

2. Experiments with GAT-specific clones

GAT-specific clones were obtained from lymph node cells of GAT primed BALB/c mice. These clones were assayed for their capacity to stimulate a secondary anti-DNP response to DNP-GAT. DNP sensitized B cells were obtained from DNP-OVA primed BALB/c spleen cells treated with anti Thy-1 antibodies and complement. Table 2 presents the results obtained with 10 independent clones. It can be noticed that 10^3 T cells from each clone can induce a response which is as significant as the response obtained with untreated spleen cells challenged with DNP-OVA. The isotypic pattern of the responses indicates that all the clones tested can activate anti-DNP PFC of every isotype, although IgM and IgG1 PFC predominated. This pattern is the same for all clones and identical to the pattern observed in the secondary response to DNP-OVA of untreated spleen cells. Data not shown indicate that this pattern does not vary with the number of T cells added to the cultures. These observations demonstrate again that these TH cells can provide help to any memory B cell irrespective of their membrane isotype.

Data presented in Table 3 indicate that the GAT-clones cannot induce an anti-DNP response in the presence of DNP sensitized B cells and DNP-KLH. This indicates that the T clones are carrier-specific. In addition, they were not able to activate anti-DNP PFC in cultures in the presence of DNP-KLH plus GAT. Thus, no by-standard effect can be demonstrated with these clones indicating that they require an hapten carrier bridge to provide help to B cells. Thus, according to Janeway's hypothesis, they should be therefore considered as TH1 cells. This interpretation has been confirmed by the demonstration that they cannot activate histoincompatible B cells. As shown in Fig. 2, the responses obtained by adding BALB/c ($H\text{-}2^d$) GAT cloned cells to AKR ($H\text{-}2^k$), B6 ($H\text{-}2^b$) or ATH ($H\text{-}2^s$) DNP-OVA primed B cells were not significantly different from responses obtained with B cells alone. The helper activity of the GAT-specific clones is therefore H-2 restricted.

	Total PFC response/ culture	Relative Percentage++				
		IgM	IgG3	IgG1	IgG2b	IgG2a
B cells	102 ± 56	45-50	—	38-46	1-3	4-9
Spleen DNP-OVA+	1841 ± 284	32-46	2-10	19-27	6-12	8-15
Clone : D6	712 ± 197	28-36	8-12	22-30	7-15	12-17
D14	1228 ± 321	30-38	3-7	24-34	5-12	15-21
D15	887 ± 216	35-42	4-9	22-29	8-11	11-17
D16	1294 ± 425	40-51	11-13	20-27	6-9	8-15
D19	1358 ± 383	30-37	8-12	28-34	4-9	16-22
D20	1018 ± 335	29-40	6-11	26-38	5-12	11-20
D22	994 ± 267	29-37	5-13	30-37	4-13	14-22
D24	1471 ± 356	32-44	3-10	31-39	6-10	15-19
D30	1166 ± 411	36-45	10-14	23-31	7-13	12-18
D46	1047 ± 309	40-45	8-11	22-31	4-10	16-23

TABLE 2. ISOTYPIC PATTERN OF THE SECONDARY RESPONSE TO DNP-GAT INDUCED WITH GAT-CLONES.

Anti Thy-1 + C' treated spleen cells from DNP-OVA primed BALB/c mice were used as B cell source. 10^3 cells from clones were added to 5×10^5 B cells. Cultures were stimulated with 5 µg/ml DNP-GAT. PFC responses were measured on day 5 using isotype-specific developing antisera. (+) Secondary response to DNP-OVA (5 µg/ml) of untreated spleen cells. (++) Results are expressed as the lowest and highest percentage of each isotype obtained from four replicates from two independent experiments.

T cell clones	ANTIGEN ADDED TO CULTURES DNP-GAT 5 µg/ml	DNP-KLH 0.25 µg/ml	DNP-KLH + GAT 0.25 µg/ml 5 µg/ml
—	92 ± 37	106 ± 41	94 ± 33
T KLH(o)	107 ± 44	1934 ± 216	ND
D6	910 ± 185	112 ± 58	118 ± 44
D14	1720 ± 246	123 ± 49	165 ± 51
D16	1034 ± 227	133 ± 64	111 ± 41
D19	1128 ± 172	199 ± 87	144 ± 76
D20	964 ± 135	146 ± 52	207 ± 89
D25	1264 ± 239	197 ± 91	158 ± 66
D30	1052 ± 208	153 ± 27	204 ± 82
D37	872 ± 184	107 ± 38	129 ± 44
D58	1159 ± 148	119 ± 48	179 ± 85

Table 3. SPECIFICITY OF THE HELPER ACTIVITY OF THE GAT-CLONES. 10^3 T cells from clones were added to 5 x 10^5 anti Thy-1 + C' treated spleen cells from DNP-OVA primed BALB/c mice.
(o) Nylon purified lymph node T cells from KLH primed animals.

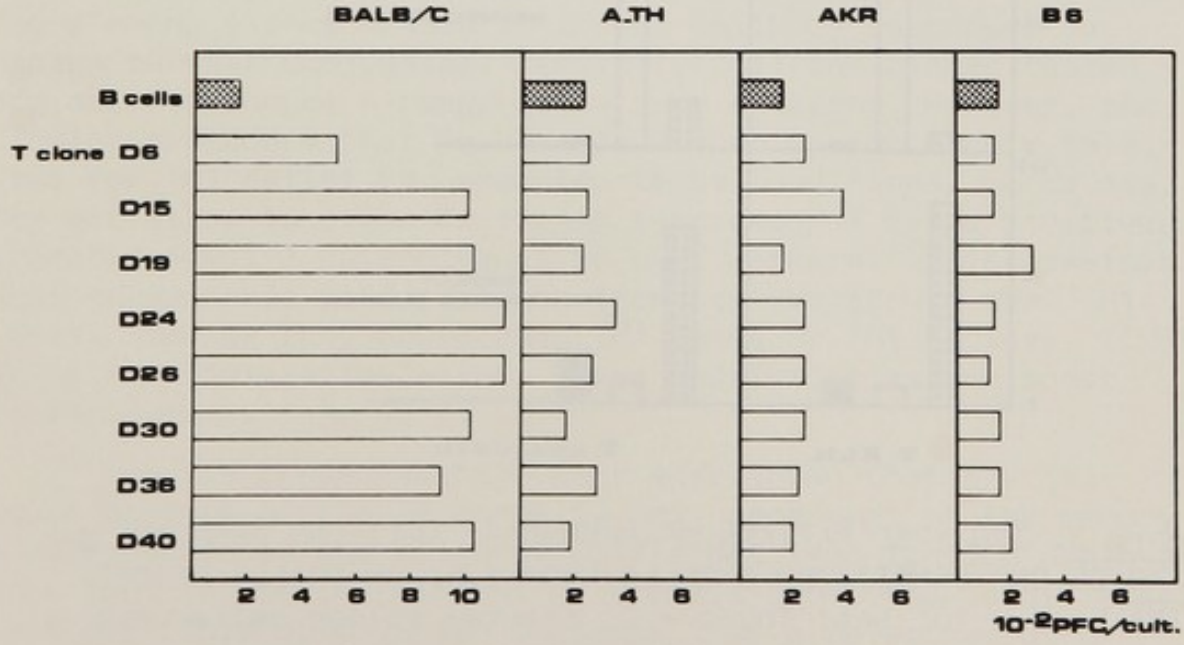

Figure 2. H-2 RESTRICTION OF THE GAT-CLONES. 2 x 10^3 T cells were added to anti Thy-1 + C' treated spleen cells from DNP-OVA primed BALB/c, B6, AKR or A.TH mice.

3. Influence of the priming route on the isotypic pattern.

BALB/c mice were primed with DNP-BSA either by the intraperitoneal route using Maalox and B. pertussis as adjuvant or by the oral route by the addition of 300 µg/ml DNP-BSA in the drinking water. Spleen cells were collected 30 days after priming i.p. or 10 days after the beginning of the diet. They were then treated with anti Thy-1 antisera and complement. BALB/c animals were also primed with KLH or diphteric toxoïd (DA) in CFA in the base of the tail. Seven days later, their lymph node cells were filtered through nylon wool columns and used as a source of TH cells. Mixtures of different T and B cells were challenged in vitro with DNP-KLH or DNP-OVA. Fig. 3 shows that the anti-DNP response of i.p. primed B cells consisted mostly of IgG antibodies. In the response of B cells from orally primed animals IgM and IgA antibodies were observed. These patterns were obtained using KLH or DA-specific T cells as well. These results suggest that the same T cell population can induce secondary responses of a different isotypic pattern. Thus, this pattern is only dependent upon the nature of the memory B cells recruited in the different immunization protocols.

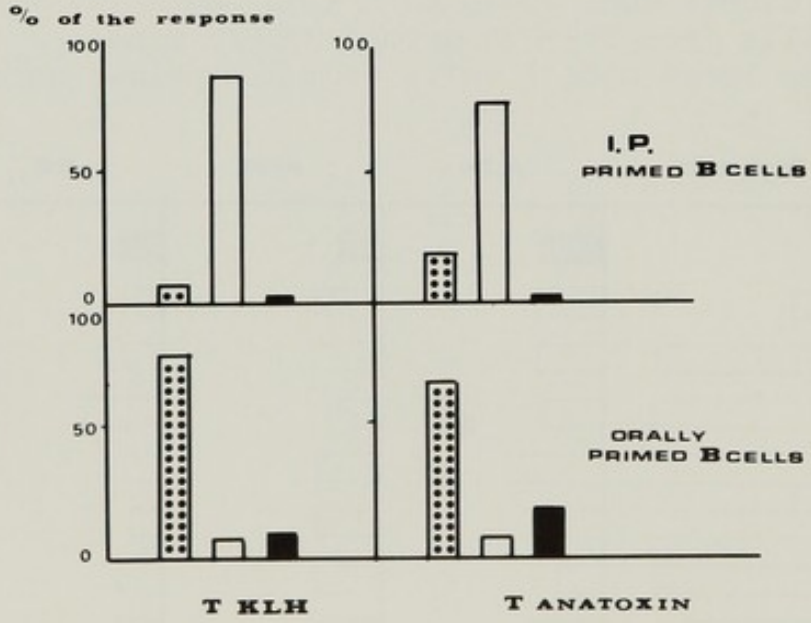

Figure 3. ISOTYPIC PATTERN OF SECONDARY ANTI-DNP RESPONSE IN VITRO OF DNP-SENSITIZED B CELLS FROM I.P. OR ORALLY PRIMED ANIMALS. 5×10^5 anti Thy-1 + C' treated spleen cells were cultured in presence of 1×10^5 nylon purified lymph node T cells from KLH or DA primed animals. 0.25 µg/ml DNP-KLH and 3 µg/ml DNP-OVA were respectively added to the cultures.

⊖ IgM ○ IgG ● IgA

DISCUSSION

The results presented here show that carrier-specific and H-2 restricted clones of T helper cells such as the GAT-clones cannot select immunoglobulin isotypes in an adoptive secondary response *in vitro*. This is in agreement with results previously reported by Pierce *et al*. (10). The carrier-specificity, the H-2 restriction and the requirement for an hapten carrier bridge of these clones allow their classification as TH1 cells. HRBC- and SRBC-clones are less strictly antigen-specific and have a by-standard effect. Their classification as TH1 is therefore questionable since TH2 cells have been defined as cells which do not require an hapten carrier bridge and have therefore a by-standard effect. Nevertheless, RBC-clones induce the highest PFC response to the homologous antigen. Data not presented here have also revealed that these clones can only help B cells of the same H-2 haplotype. It is therefore tempting to consider them as TH1 like the GAT-clones and we have shown that they do not select antibody classes. Consequently, our observations demonstrate that the classical TH1 cells can help responses of every isotype.

Our failure, up to now, to obtain antigen- and class-specific T cell clones does not necessarily mean that Ig-specific T cells do not exist. Since the capacity of the TH2 to activate B cells in the absence of TH1 is still controversial (11 - 13), one could assume that TH2 activities should be found among clones unable to induce antibody responses to hapten carrier conjugates. Presently, all the clones tested are able to induce a response to such antigens. However, the cloning procedure that we have developed may be highly selective for TH1 cells. For example, those conditions, up to now, did not allow to clone or expand suppressor T cells positively selected for I-J determinants or Ly-2 markers. It is possible that TH2 may have different requirements for growth than TH1 cells. Such as they could need TH1 cells or TH1 factors, TCGF or Ig for their stimulation. These possibilities are under investigation.

Results presented here have also shown that the isotypic pattern of the response is only dependent on the memory B cells added to the cultures. Different immunization protocols lead to the recruitment of B cells committed to different isotypes. This observation suggests that a selection of B cells does occur with respect to their surface Ig. This selection could be easily accounted for by the existence of Ig-specific T cells. Thus, it is conceivable that Ig-specific

T cells play a more crucial role in the induction of memory B cells than at the level of their expression in secondary responses.

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REFERENCES

(1) Kishimoto, T., and Ishizata, K. (1973). *J. Immunol.* 111,720.

(2) Herzenberg, L.A., Okumura, K., Cantor, H., Sato, U.L., Shen, F.W., Boyse, E.A., and Herzenberg, L.A. (1976). *J. Exp. Med.* 144,330.

(3) Seman, M., and Zilberfarb, V. (1979). *J. Immunol.* 122, 2534.

(4) Janeway, C.A. (1980). In *Regulation in the Immune System*, E. Sercarz and A. Cunningham (Eds).

(5) Seman, M., Chevalier, F., and Stanislawski, M. (1978). *Eur. J. Immunol.* 8,262.

(6) Mazié, J.C., Joskowicz, M., and Thèze, J. (1981). *Cell. Immunol.*, in press.

(7) Marchal, G., Seman, M., Milon, G., and Truffa-Bachi, P. (1981). Submitted.

(8) Sredni, B., Tse, H.Y., and Schwartz, R.H. (1980). *Nature* 283,581.

(9) Schreier, M.H., and Tees, R. (1981). *Int. Archs. Allergy appl. Immunol.*, in press.

(10) Pierce, S.K., Cancro, M.P., and Klinman, N.R. (1978). *J. Exp. Med.* 149,759.

(11) Woodland, R., and Cantor, H. (1978). *Eur. J. Immunol.* 8,600.

(12) Tada, T., Toshitada, J., Okumura, K., Nanaka, M., and Tokuhisa, J. (1978). *J. Exp. Med.* 147,446.

(13) Janeway, C.A., Bert, D.L., and Shen, F.W. (1980). *Eur. J. Immunol.* 10,231.

REGULATION OF ANTIBODY AFFINITY[1]

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One of the important qualitative aspects of the immune response, which has been subjected to considerable investigation, is the affinity of the antibody. In this discussion I would like to briefly summarize current understanding of the factors involved in regulating antibody affinity.

It is well known that the antibody produced, in response to most antigens, is highly heterogeneous with respect to affinity (1-3). As a general rule, the response to T-dependent antigens is more heterogeneous than is the response to T-independent antigens. It has also been reported, by numerour workers, that, in a variety of species, with a number of different antigens, there is a progressive increase in antibody affinity with time after immunization (1-5). This increase in affinity is more rapid after immunization with relatively low doses of antigen (2,6). Furthermore, this selective expansion of high affinity antibody-secreting cells is more pronounced after immunization with T-dependent than T-independent antigen, although it does occur in both cases (7). In general, however, the response to T-independent antigens and to haptens on T-independent carriers tends to be of low average affinity (7). A number of examples of a loss of high affinity antibody production, with a consequent

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decrease in average affinity, late after antigen injection have been reported (3-5,8,9). The mechanism of this late decrease in antibody affinity is not fully understood although specific suppressor cell activity and auto-anti-idiotype antibody production have been suggested as possible explanations. It should be noted that although there is a decrease in high affinity antibody secreting cells late after antigen injection, high affinity memory B cells persist. This persistance of high affinity B memory cells is responsible for one of the characteristic features of a secondary immune response: namely, the rapid production of large amounts of very high affinity antibody.

Several experimental modifications of the immune response have been shown to effect the selection for high affinity antibody production. Non-specific immunodepression (e.g., by use of cytotoxic drugs) tends to somewhat slow the rate of selection of high affinity antibodies (10-12). However, this effect is of a modest degree and the depression in the magnitude of the response tends to be more profound than the depression in antibody affinity. Similarly, in the absence of optimal T-cell help the response to a T-dependent antigen tends to be of decreased affinity (13-15). This effect has been observed under two circumstances: (a) specific helper T-cell tolerance (16-19); and (b) irradiated mice reconstituted with B cells and limiting numbers of T cells (13,14). While a reduction in helper T-cell activity does lead to a decrease in antibody affinity, the effect on the magnitude of the antibody response appears to be more profound. In contrast, two experimental procedures lead to a profound depression of high affinity antibody production: (a) specific B-cell tolerance (11,15,20,21); and (b) specific suppressor T-cell activity (11,22-24). In both of these situations the

depression in antibody affinity is marked even when the effect on the magnitude of the response is minimal.

In general the regulation of antibody affinity can be understood in terms of a selective proliferation of high affinity antibody producing cells. B cells capable of producing high affinity antibody, bear high affinity antigen receptors, preferentially capture antigen and are thereby preferentially stimulated to proliferate. In this way, high affinity antibody producing cells, which are initially relatively rare cells, came to predominate in the immune response. Factors which favor a vigorous immune response (e.g.: adjuvants (12) and helper T-cell activity) favor the selective expansion of high affinity clones and therefore favor high affinity antibody production. Factors which depress the immune response (e.g.: cytotoxic drugs, non-specific-suppressor cell activity) tend to result in inefficient selection of high affinity clones since such selection is dependent upon marked proliferation. Antigen-specific depression of the immune response (e.g.: B-cell tolerance or specific suppressor T-cell activity) predominantly affects high affinity antigen binding precursors of high affinity antibody secreting cells. In this way, it has a major effect on high affinity clones and results in a profound depression in antibody affinity, which can be out of proportion to the effect on the magnitude of the response.

Early studies indicated that passive antibody specifically depressed the immune response but led to an increase in antibody affinity (6). At the time, this was interpreted as an increase in selective pressure toward high affinity antibody production, an effect viewed as essentially equivalent to a reduction in antigen dose. However, in the light of the current network view of immune regulation

involving interactions between idiotypes and anti-idiotypes, this simple interpretation may have to be revised. It is likely that auto-anti-idiotype antibody production plays an important role in the regulation of antibody affinity. It has been suggested that the cyclic increases and decreases in antibody affinity sometimes observed during the immune response is the result of production of auto-anti-idiotype antibody. The role of auto-anti-idiotype antibody production and other network considerations on the regulation of antibody affinity have not yet been clearly delineated.

REFERENCES

1. Siskind, G.W. and Benacerraf, B. (1969) *Adv. Immunol. 10*,1.
2. Eisen, H.N. and Siskind, G.W. (1964) *Biochemistry 3*,996.
3. Werblin, T.P. and Siskind, G.W. (1972) *Immunochemistry 9*,987.
4. Goidl, E.A., Barondess, J.J. and Siskind, G.W. (1975) *Immunology 29*,629.
5. Werblin, T.P., Kim, Y.T., Quagliata, F. and Siskind, G.W. (1973) *Immunology 24*,477.
6. Siskind, G.W., Dunn, P. and Walker, J.G. (1968) *J. Exp. Med. 127*,55.
7. Goidl, E.A., Romano, T.J., Siskind, G.W. and Thorbecke, G.J. (1978). *Cell. Immunol. 35*,231.
8. Pini, C., Di Felice, G., Neri, R., Mancini, C., Vicari, G. and Doria, G. (1980) *J. Immunol. 125*,1349.
9. Neri, R., Pini, G., Vicari, G. and Doria, G. (1978) *Eur. J. Immunol. 8*,823.
10. Goidl, E.A., Cusano, A., Redner, R., Innes, J.B., Weksler, M.E. and Siskind, G.W. (1979) *Cell. Immunol. 47*,293.

11. DeKruyff, R.H. and Siskind, G.W. (1980) *Cell. Immunol. 49*,90.
12. Mond, J., Kim, Y.T. and Siskind, G.W. (1974) *J. Immunol. 112*,1255.
13. DeKruyff, R. and Siskind, G.W. (1979) *Cell. Immunol. 47*,134.
14. Gershon, R.K. and Paul, W.E. (1971) *J. Immunol. 106*,872.
15. Janeway, C.A., Jr. (1975) *J. Immunol. 114*,1394.
16. Andersson, B. and Wigzell, H. (1971) *Eur. J. Immunol. 1*,384.
17. Andersson, B., Celada, F. and Asjo, B. (1974) *Eur. J. Immunol. 4*,788.
18. Sanfilippo, F. and Scott, D.W. (1976) *Cell. Immunol. 21*, 112.
19. DeKruyff, R.H. and Siskind, G.W. (1979) *Cell. Immunol. 47*,134.
20. Theis, G.A. and Siskind, G.W. (1968) *J. Immunol. 100*, 138.
21. Szewczuk, M. and Siskind, G.W. (1977) *J. Exp. Med. 145*, 1590.
22. Takemori, T. and Tada, T. (1974) *J. Exp. Med. 140*,253.
23. Warren, R.W. and Davie, J.M. (1977) *J. Immunol. 119*, 1806.
24. Bell, E.B. and Shand, F.L. (1975) *Eur. J. Immunol. 5*, 481.

I. SUPPRESSION OF IgE ANTIBODY PRODUCTION IN SJL MICE BY INTERACTION OF PRIMED AND UNPRIMED T CELLS

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ABSTRACT

For selective anti-hapten IgE suppression in SJL mice, the interaction of two populations of T cells is necessary. One population is a hyperprimed T cell, the other an unprimed T cell. The population of hyperprimed T cells may be from animals immunized with a different protein to which the hapten is coupled. Injection at challenge of the antigen specific for the hyperprimed T cell is necessary. The induction and the expression of the hyperprimed T cell is antigen specific (the antigen must be the same for priming and challenge), but the action is not antigen specific.

INTRODUCTION

It has been shown that when inbred mice are primed with small amounts of hapten-carrier conjugate in $Al(OH)_3$, then infested with Nippostrongylus brasiliensis and taking advantage of the carrier effect (1-3), boosted with the same hapten coupled to an extract of Nippostrongylus brasiliensis, they produce high titer anti-hapten IgE antibody for several weeks. A notable exception was the SJL and to a lesser extent the AKR strain of mice. In these strains the IgE anti-hapten antibody production was of short duration, contrasting to the prolonged production of anti-hapten antibody of the IgG_1 and IgG_2 classes.

When these strains were irradiated with sublethal doses of X rays after challenge, anti-hapten IgE production persisted. However, if these immunized and irradiated mice were injected with large numbers of unprimed spleen cells, anti-hapten IgE production was abrogated. This class spe-

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cific anti-hapten IgE suppression was not produced when the unprimed spleen cells were treated by anti-Thy-1 and complement (4). Treatment of the unprimed spleen cell population with anti-Lyt-1 and complement, but not anti-Lyt-2 and complement, abrogated the capacity of these cells to produce this suppression (5). This was the first demonstration that Lyt-1 cells play a role in suppression of antibody production. The importance of Lyt-1 cells in feedback suppression of antibody production, or perhaps more correctly as inducers of suppressor cells, was confirmed in other systems (6-8).

To study the interaction of different populations of T cells in this class specific (IgE) suppression, adoptive transfer was used. Adoptive transfer has several great advantages, the two principal being that all recipient mice can be injected with cells from the same pool of donors and thus comparison of the experimental groups with control groups is more accurate and the other that injection of antibody-producing cells into irradiated recipients results in a much greater antibody production than what is observed in actively immunized animals.

METHODS

Immunization of donors, determination of antibodies of different classes (PCA reaction in rats for IgE, in mice for IgG_1, passive lysis for IgG_2), experimental irradiation of recipients and other details were published (9-12).

RESULTS

Only two experiments are reported here which illustrate the isotype specific (IgE) anti-hapten antibody suppression.

Tada had shown that induction of suppressor T cells is optimal in many cases with relatively large amounts of antigen (13). For this reason in the first experiment donor mice were primed with 1 microgram of DNP-KLH and either 0.2 microgram or 10 microgram of Ea and 1 mg $Al(OH)_3$. In addition to the primed cells, two groups were also injected

with large numbers of unprimed spleen cells. For challenge advantage was taken again of the carrier effect: the helper T cells were primed with the protein Ea, the B cells for production of anti-DNP antibody with DNP-KLH and the animals were challenged with DNP-Ea. Antibody titers 2 weeks after challenge are shown Table 1 .

ADOPTIVE TRANSFER

Effect of the priming method on the suppression mediated by unprimed spleen cells.

Group	Transferred spleen cells: 3×10^7 primed with*	Transferred spleen cells: 5×10^7 unprimed	Anti-DNP antibody**: IgE	Anti-DNP antibody**: IgG_1
1	DNP-KLH 1 µg & Ea 0.2 µg	-	1,280	320
2	+	+	1,280	320
3	DNP-KLH 1 µg & Ea 10 µg	-	640	320
4	+	+	20	160

*Priming of donors: 1 µg of DNP-KLH + 1 mg of $Al(OH)_3$ was injected i.p. 3 wk. later, 0.2 µg Ea + 1 mg $Al(OH)_3$ or 10 µg Ea + 1 mg $Al(OH)_3$ was also injected i.p. Spleen cells were harvested 1 wk. later. The recipients were challenged immediately after cell transfer with 10 µg DNP-Ea + 1 mg $Al(OH)_3$.

**Antibody titer was determined 2 wk. after transfer
- : no cells transferred
+ : cells transferred

(Itaya and Ovary, 1979)

Recipient mice injected with cells from donors primed with 0.2 microgram of Ea produced high titer of anti-DNP IgE antibody in both groups (those which were not injected with unprimed spleen cells and those which were injected with unprimed spleen cells in addition to the primed cells. On the contrary recipients which were injected with cells from donors primed with 10 µg of Ea and unprimed spleen cells had significantly less anti-DNP IgE antibody than those which did not receive unprimed spleen cells. In both groups the anti-DNP IgG_1 anti-DNP antibody was essentially the same.

In the second experiment all recipient mice were injected with cells from a pool of donors primed with 0.2 µg DNP-Ea and 1 mg $Al(OH)_3$. Other groups were injected with cells from donors hyperprimed with KLH. These donors were injected twice with 10 µg KLH and 1 mg $Al(OH)_3$ in two week intervals and the cells harvested two weeks after the second injection. Finally, some of the recipients were injected also with $5x10^7$ unprimed syngeneic spleen cells. Immediately after injection of the cells the mice were challenged either with 0.2 µg DNP-Ea and 1 mg $Al(OH)_3$ or with a mixture of 0.2 µg DNP-Ea, 10 µg KLH and 1 mg $Al(OH)_3$. Titers of anti-DNP IgE and IgG_1 two weeks after challenge are shown (Table 2).

If the recipients are injected with the three types of donor cells and challenged with the mixture of the immunizing antigen (DNP-Ea) and the protein (KLH) used for hyperpriming the second set of donors, the production of anti-DNP IgE antibody is significantly less than in the other groups. If the protein used to hyperprime the second set of donors is omitted, the anti-DNP IgE antibody is not suppressed. The IgG_1 titers in all groups are essentially the same. These experiments were repeated several times with the same results. Other antigenic combinations were also used several times (i.e. DNP-KLH and Ea). In several experiments the same protein was used as for the DNP carrier for hyperpriming. In this case it was not necessary to inject the free carrier to obtain suppression(12).

Table 2

Adoptive Transfer

Suppression of anti-hapten antibody response by injection of free carrier

Group	Transferred Spleen Cells: 1.5×10^7 primed with+ DNP-Ea	1×10^7 hyper-primed with++	5×10^7 un-primed	Challenge with respective antigens & 2mg $Al(OH)_3$	Anti-DNP// IgE	Anti-DNP// IgG_1
1	+	-	-	DNP-Ea 0.2 µg & KLH 10 µg	2,560	160
2	+	-	+		2,560	160
3	+	+	-		2,560	160
4	+	+	+		320	80
5	+	+	-	DNP-Ea 0.2 µg	1,280	160
6	+	+	+		1,280	160

+ Priming was done by ip injection of 0.2 µg DNP-Ea. The antigen was mixed with 1 mg $Al(OH)_3$ and injected ip.

++ Donors were hyperprimed ip with 10 µg KLH + 1 mg $Al(OH)_3$ two times with 2-wk intervals. Spleen cells were harvested 2 wk after the second injection.

// Antibody titer was determined 2 wk after transfer.

- : no cells injected

+ : cells injected

(Itaya and Ovary, 1979, modified)

If either the hyperprimed or the unprimed spleen cells were treated by anti-Thy-1 and complement, no suppression could be obtained. This fact indicates that both cells, i.e. the hyperprimed and the unprimed cells are T cells. Treatment of both populations of spleen cells with anti-Ly 11.2 serum and complement did not abrogate anti-hapten IgE suppression (14).

DISCUSSION

From Table 1, it can be seen that small amounts of the carrier protein (Ea) are sufficient to prime helper T cells for antibody production. This small amount was not sufficient in these experiments to prime suppressor T cells. When the hapten used for challenge was coupled to the protein used to prime the helper T cells, high titer antibody production was obtained. However, larger amounts of antigen were capable of priming not only helper but also suppressor T cells. That larger amounts of antigen did not prime only suppressor T cells is indicated by the fact that good antibody production resulted when the mice were challenged with the hapten-carrier conjugate in the group of mice which did not receive unprimed spleen cells.

The results in Table 2 indicate that it is possible to prime suppressor T cells with an unrelat- antigen (carrier). This second antigen must be injected together with the homologous carrier-hapten conjugate to obtain antibody suppression. Furthermore, two types of T cells are necessary for suppression. In this system the suppression is class specific, namely only the IgE anti-hapten antibody is suppressed; the IgG_1 is not. The anti-hapten IgG_2 class was also not suppressed. Very similar results were obtained by Kishimoto el al. using DNP-coupled to Tubercle bacillus (15). These authors were able to obtain suppressor factors and showed that although DNP specific stimulation was required for the induction of these factors, the action had an effect in an antigen nonspecific manner and that only the IgE class of antibodies

was suppressed (16).

It should be noted that IgE class specific suppression by suppressor T cells was first described in the rat by Okumura and Tada (17). It was also Tada who showed for the first time that several subsets of T cells are involed in suppression (18), and this phenomenon was then confirmed by many others (6,8). It is therefore possible that the two T cell populations interact and the result of this interaction is the production of the effector suppressor T cell or product. It is not possible to decide which cell becomes the effector suppressor or if even another cell is involved. This suppression is provoked by an action on the helper T cells or on the antibody producing B cells (?),it is isotype specific as only the IgE class is suppressed. It is also possible that this isotype specificity is not absolute, in any case, it is certainly more evident than suppression of other isotypes, which was not observed. The unprimed cells from earlier experiments were shown to be of the Lyt-1 class.

Another explanation might be that two effector T cells are necessary to produce this type of class specific suppression. One T cell recognizes the antigen (carrier) and perhaps also some other product on the cellular surface with which it interacts for suppression and a second T cell recognizes the constant portion of the H chain and perhaps also the antigen too. A similar mechanism was proposed for helper T cells by Bottomly (19).

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REFERENCES

1. Ovary, Z., and Benacerraf, B. (1963). Proc. Soc. Exp. Biol. & Med. 114, 72.

2. Mitchison, N.A. in Immunological Tolerance M. Landy and W. Braun eds., Academic Press, New York, 1969, pg. 149.

3. Raff, M.C. (1970). Nature, London 226, 1257.

4. Watanabe, N., Kojima, S., and Ovary, Z. (1976). J. Exp. Med. 143, 833.

5. Watanabe, N., Kojima, S., Shen, F.-W., and Ovary, Z. (1977). J. Immunol. 118, 485.

6. Eardley, D.D., Hugenberger, J., McVay-Boudreau, L., Shen, F.-W., Gershon, R.K., and Cantor, H. (1978). J. Exp. Med. 147, 1106.

7. Shen, F.W., McDougal, J.S., Bard, J., and Cort, S.P. (1980). J. Exp. Med. 151, 566.

8. Weinberger, J.Z., Germain, R.N., Benacerraf, B., and Dorf, M.E. (1980). 152, 161.

9. Itaya, T., and Ovary, Z.(1979).J.Exp. Med. 150, 507.

10. Ovary,Z.,Caiazza,S.S.,and Kojima,S.(1975). Int. Archs Allergy appl. Immun.48, 16.

11. Bloch,K.J.,Kourilsky,F.M.,Ovary, Z.,and Benacerraf,B.(1963).J.Exp.Med. 117, 965.

12. Ovary,Z., Itaya,T.,Watanabe,N.,and Kojima,S. (1978).Immunol.Rev.41, 26.

13. Tada,T.,Takemori,T.(1974).J.Exp.Med. 140, 239.

14. Meruelo,D.,Paolino,A.,Flieger,N.,Dworkin,J., Offer,M.,Hirayama,N.,and Ovary,Z.(1980). J. Immunol.125,2719.

15. Kishimoto,T.,Hirai,Y.,Suemura,M.,and Yamamura, Y. (1976).J. Immunol.117,396.

16. Kishimoto,T.,Hirai,Y.,Suemura,M.,Nakanishi,K., and Yamamura,Y. (1978).J.Immun.121,2106.

17. Okumura,K.,and Tada,T.(1971).J.Immunol.107,1682.

18. Tada,T. ICN-ICLA Symposia on Molecular and Cellular Biology,Vol.VI. (1977). Immune System: Genetics and Regulation. Eds. E.Sercarz & C.F. Fox. Academic Press Inc., New York, pg.345.

19. Bottomly,K, and Mosier,D.E. (1979).J.Exp. Med. 150,1399.

II. PCA REACTION FOR DEMONSTRATION OF IDIOTYPIC ANTIBODIES

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The presence of idiotypic antibodies was investigated in sera of C57BL/6 (B6) mice immunized with a monoclonal (MC) anti-dinitrophenyl (a-DNP) IgE from a BALB/c hybridoma. It was shown previously that these sera contain antibodies to allotype 7a; the preparation of these was described (1). Briefly, B6 mice were injected ip with 10 µg (equivalent to a PCA titer of 5000, since about 2 nanogram of IgE/ml give threshold PCA reactions) affinity column purified anti-DNP MC IgE from a BALB/c hybridoma (2) in Freund's complete adjuvant. Sera were tested by hemagglutination. Highest titers: (1/160) were found at 6 wks. Another MC antibody also of BALB/c origin with specificity for egg albumin (Ea) was a generous gift of Dr. I. Böttcher (3). Antisera containing IgE antibody to keyhole limpet hemocyanin (KLH) prepared in BALB/c or B6 mice were the same as used previously (1). The sera containing IgE antibody were diluted so as to give PCA reactions approximately of the same size and intensity (Exp. 1&2). These were mixed with a 1/10 dilution of either B6 a-allotype 7a or with B6 normal serum. The results are summarized in Table 1. When the MC a-Ea IgE was diluted 1/6000 and mixed with normal B6 serum, strong PCA reactions were obtained with the antigen (Ea). No PCA reaction could be obtained when a-allotype 7a was used. The same a-allotype 7a absorbed the PCA activity of conviently diluted a-KLH IgE antibodies of BALB/c mice, whereas normal B6 serum was ineffective. The a-allotype 7a was ineffective for absorption of PCA activity of a-KLH IgE of B6 mice. When a 1/200 dilution of the MC a-Ea IgE was mixed with the a-allotype 7a and the mixture injected into rats, strong PCA reactions were provoked with the antigen Ea. It was concluded that all the a-allotype 7a antibodies were saturated and enough free a-Ea was in the mixture to sensitize the rats for PCA reactions. If this mixture was then mixed with a-DNP MC IgE antibody diluted 1/1800 and injected into rats, no PCA reaction was observed with DNP-BSA. Normal B6 serum was ineffective to absorb the PCA activity of the a-DNP MC IgE antibody. As the mixture contained free a-Ea IgE and still could absorb the PCA activity of the immunizing a-DNP antibody it was concluded that our a-allotype 7a contained also anti-idiotypic antibodies.

REFERENCES

1) Sanches-Borges,M., Kumagai,Y., Okumura,K.,Hirayama,N., Ovary,Z.,Tada,T. (1981) Submitted to Immunogenetics.
2) Böttcher,I.,Ulrich,M.,Hirayama,N., Ovary,Z. (1980). Int. Archs Allergy appl. Immun.61, 248.
3) Böttcher, I.,Hämmerling,G.,Kapp,J.F. (1978). Nature (London): 275,761. (Grants NIH AI-03075 & N.C.I. CA-16247)

Table 1
PCA in rats with 2 hour sensitization period

Experiment	Sera injected intradermally	Antigen i.v.	PCA Result	Allotype
1) a)	BALB/c a-KLH 1/640 + B6 a-allotype^{7a}	KLH	0	7a absorbed
1) b)	BALB/c a-KLH 1/640 + B6 normal	KLH	+	7a present
1) a)	B6 a-KLH 1/160 + B6 a-allotype^{7a}	KLH	+	7b present
1) b)	B6 a-KLH 1/160 + B6 normal	KLH	+	7b present
2) a)	MC BALB/c IgE a-Ea 1/6000 + B6 a-allotype^{7a}	Ea	0	7a absorbed
2) b)	MC BALB/c IgE a-Ea 1/6000 + B6 normal	Ea	+	7a present
3) a)	MC BALB/c IgE a-Ea 1/200 + B6 a-allotype^{7a}	Ea	+	7a present in excess
3) b)	MC BALB/c IgE a-Ea 1/200 + B6 normal	Ea	+	7a present in excess
				Idiotype
4) c)	MC BALB/c IgE a-DNP 1/1800 + Mixture a) from experiment 3	DNP-BSA	0	absorbed
4) d)	MC BALB/c IgE a-DNP 1/1800 + Mixture b) from experiment 3	DNP-BSA	+	present

Abbreviations not in the text: BSA = bovine serum albumin; PCA = passive cutaneous anaphylaxis.

IMMUNOGLOBULIN-RELATED INDUCTION, RECOGNITION, OR RESTRICTION IN THE IMMUNOREGULATORY FUNCTION OF IN VITRO GENERATED Lyl CELLS

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I. ABSTRACT

Two functional Lyl T cells--an Lyl helper effector and an Lyl suppressor inducer--are generated by the in vitro culture of cortisone-resistant, nylon wool-purified thymocytes with antigen-pulsed macrophages. These cells can be phenotypically distinguished by additional immunogenetic markers, IJ and Qa-1. The differentiation sequences involved in their generation are reviewed, and experiments related to the role of immunoglobulin-related, idiotypic, or V_H-gene-restricted recognition in their induction and function are presented. Although Lyl suppressor inducers can be activated by immunoglobulin and are V_H-gene-restricted in their function, we could not find evidence of an immunoglobulin-recognizing, V_H-gene restricted, or idiotype-specific Lyl T helper effector in this system.

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II. INTRODUCTION

The advantages of in vitro systems with respect to experimental manipulation and intervention are obvious, but the in vivo relevance of such observations always needs to be questioned and, if possible, confirmed. The thymocyte-macrophage culture system is a case in point. We and others have found this system to be useful for probing the activation requirements, differentiation, phenotype and function of Lyl T cells (1-10). Nevertheless, curious results have been obtained. For instance, the same bulk population of antigen-primed Lyl cells may express quite opposite functions, depending on the mode in which it is assayed (7). This phenomenon has been largely resolved, and its resolution has been particularly helpful in revealing an Lyl helper effector and a separate Lyl suppressor inducer. However, when we examine the role of immunoglobulin (Ig) or Ig recognition in Lyl T cell function, we have found that the system may not be entirely relevant or, at least, that it does not have an important component which is present in in vivo-primed Lyl T cell populations. Here, we briefly present the system, a framework based on previously published work for understanding the experiments that follow, and the results of four experimental approaches we have taken with respect to the role (or lack thereof) of Ig, idiotype, or V_H genes in this system.

A. *The System*

In the thymocyte-macrophage (T-Mϕ) culture system, cortisone-resistant, nylon wool-purified thymocytes (CRNPT) are cultured with antigen-pulsed macrophages (4). In the experiments described below, the antigen is streptococcal group A vaccine (SAV) (7). This system generates two phenotypically and functionally distinct Lyl cells (7). One is an antigen-specific, MHC-restricted helper effector (Lyl:HE), having the cell surface phenotype $Qa\text{-}1^-:IJ^-$ (6,7,10). It is assayed in T-depleted spleen cell cultures for the enhancement of the anti-TNP plaque-forming cell (PFC) response to TNP-SAV. We also measure the PFC response to the A carbohydrate (ACHO) component of the SAV carrier and that portion of the anti-ACHO PFC response which has an idiotype which is serologically identical to that of the ACHO-binding S117 plasmacytoma protein (11,12). The second functional Lyl cell generated in this system is an Lyl suppressor inducer (Lyl:SI) having the $Qa\text{-}1^+:IJ^+$ phenotype. This cell is assayed in intact spleen cell cultures (not T-depleted), where it suppresses the expected primary PFC response to TNP-SAV or SAV. This cell interacts with a $Qa\text{-}1^+:IJ^+$:Lyl23 T cell in the assay

culture, resulting in suppression (7-10). The Lyl:SI and its interactions have been in most respects identical to the feedback suppression system described by Eardley and her colleagues (13,14), with the possible exception that we find that the suppression is potentiated if the assay culture also contains $Qa\text{-}1^-:IJ^+$:Ly23 T cells (9).

Our major effort in this study has focused on the afferent or differentiation pathway involved in the generation of functional Lyl cells. Using a variety of positively and negatively selected Lyt populations from Lyt-1 allotype congenic mice, we find that most, if not all, of the Lyl effectors are derived from precursor Lyl23 T cells (8,10). Lyl23 T cell differentiation is induced by the third functional Lyl cell described in this system, the Lyl helper inducer (Lyl:HI) (8,10). This cell is $Qa\text{-}1^+:IJ^+$, and we have not been able to phenotypically distinguish it from the Lyl:SI, which is also $Qa\text{-}1^+:IJ^+$. In fact, the $Qa\text{-}1^+:IJ^+$ Lyl:SI cell generated by the system will function in the assay for Lyl:HI and, on a per cell basis, is more potent than the usual source of Lyl:HI (nonimmune CRNPT) (10).

III. RESULTS AND DISCUSSION

A. Are There Two Helper Cells, One Of Which Requires Ig For Its Induction?

A major phenotypic discrepancy between our system and that described by Cantor et al (14,15) is that we have not been able to demonstrate much of a role, synergistic or otherwise, for $Qa\text{-}1^+$:Lyl cells in mediating the helper effect. This was true if we compared dose response curves of $Qa\text{-}1^-$:Lyl cells with Lyl cells unselected for Qa-1 phenotype or if we assayed a fixed number of Lyl cells containing differing proportions of $Qa\text{-}1^-$ and $Qa\text{-}1^+$ Lyl cells (10). The possibility that the reported synergy was masked in our B cell assay culture because of the presence of residual T cells with which the $Qa\text{-}1^+$:Lyl cells might interact to induce concomitant suppression and help is unlikely for two reasons. First, assay cultures constructed with B cells prepared by anti-Thy1.2 plus C treatment (the usual method), anti-Lyt1 and anti-Lyt2 plus C treatment, anti-Ig adherent B cells (16), or a sequential combination of the above gave similar results (10). Second, we have, in fact, demonstrated a synergy in our assay culture between $Qa\text{-}1^+$ and $Qa\text{-}1^-$ Lyl cells by using a different source of T cells. In vivo primed splenic Lyl T cells, on a per cell basis, induce substantially

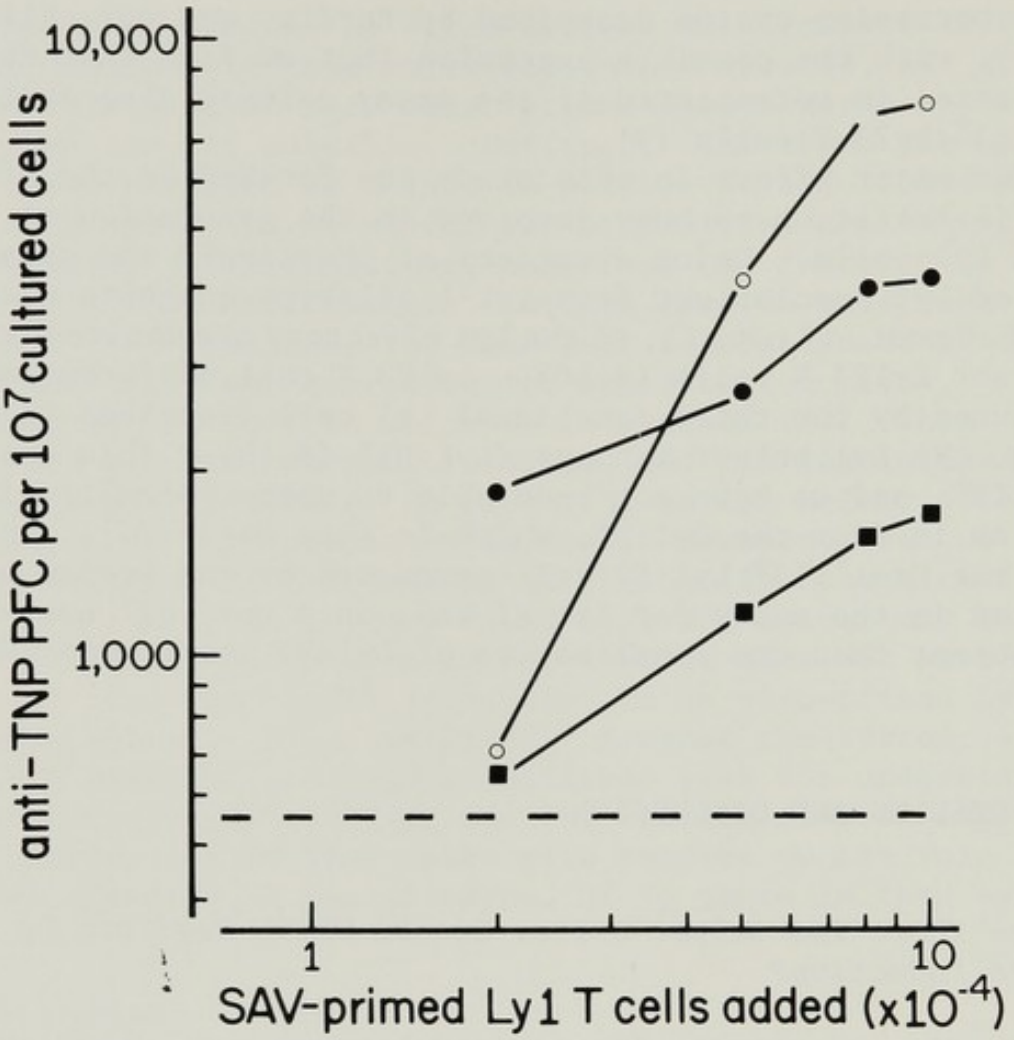

Figure 1. Lyl helper effector function of SAV-primed Lyl cells derived from: in vivo primed spleen cells, o——o; in vitro primed splenic T cells, ●——●; and in vitro primed CRNP T, ■——■. Dotted line represents background anti TNP PFC response to TNP-SAV of T-depleted assay cultures without Lyl:HE added.

higher PFC response than do in vitro primed Lyl cells derived either from CRNPT or from splenic T cells (Fig. 1).

To determine whether this difference was due to the presence of other potentially important cells in the in vivo priming environment which were missing in the in vitro priming environment or to trivial differences perhaps related to the vigor or efficiency, as it were, of in vivo versus in vitro primed cells, we tried to reproduce the finding totally in vitro. Cultures containing the whole spleen cell population were primed with antigen-pulsed macrophages. After 4 days,

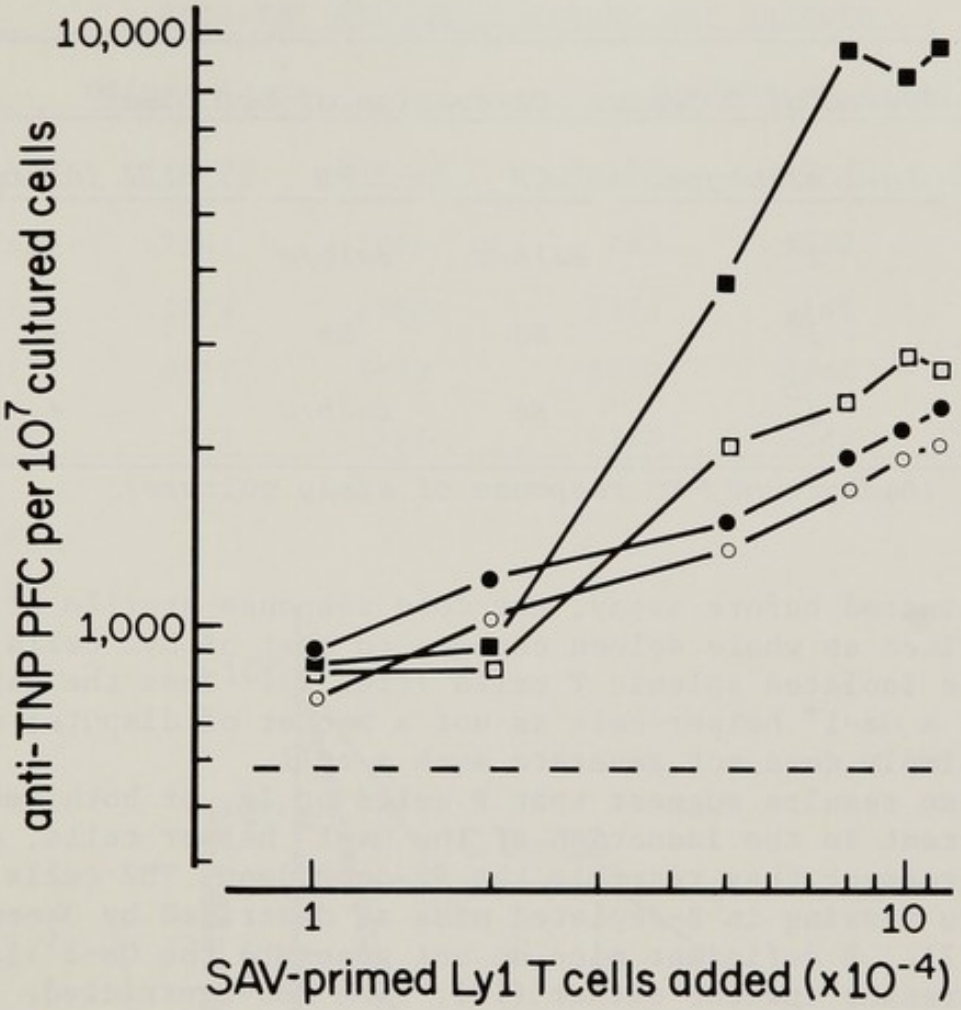

Figure 2. Helper effector function of SAV-primed Lyl cells from B6TL$^+$ mice derived from in vitro primed whole spleen (boxes) or from in vitro primed splenic T cells (circles). The assayed Lyl cells were either unselected for Qa-1 phenotype (closed circles and boxes) or selected for Qa-1$^-$:Lyl cells (open circles and boxes). Dotted line represents background anti TNP-PFC response to TNP-SAV of T-depleted assay cultures without Lyl:HE added.

the cells were nylon wool purified, selected for Lytl phenotype, and assayed for helper function. These responses were compared to those induced by Lyl cells derived from cultures containing nylon wool-purified splenic T cells rather than whole spleen (Fig. 2). Lyl cells derived from in vitro primed whole spleen were more efficient helpers than were Lyl cells derived from cultures in which splenic T cells were primed in the absence of B cells (Fig. 2). If Qa-1$^+$ cells

Table 1. V_H Gene Restrictions Between Lyl Cells

	Source of T Cells: Derivation of IgH Locus			
Strain	*Ig-1 allotype*	*CH*	*VH*	*S117 idiotype*
Balb/c	1^a	*Balb/c*	*Balb/c*	+
C.B-20	1^b	*B6*	*B6*	-
BAB.14	1^b	*B6*	*Balb/c*	+

(Background PFC response of assay culture)

are eliminated before assay, the dose response profile of Lyl cells primed as whole spleen changes to that of Lyl cells primed as isolated splenic T cells (Fig. 2). Thus the existance of a Qa-1^+ helper cell is not a matter of dispute; our system simply does not generate such a cell.

These results suggest that B cells or Ig, or both, may be important in the induction of the Qa-1^+ helper cells, and in this respect they resemble the Ig-dependent, TH2 cells, which are missing in B-depleted mice as described by Janeway et al (17). B deficient mice do not generate the Qa-1^+:IJ^+: Lyl suppressor inducer either (18). The MHC-restricted, carrier-specific TH1 and the carrier-specific, Ig-dependent TH2 cells in the system of Janeway et al have not, however, been characterized by Qa-1 phenotype (K. Bottomly, pers. comm.).

B. Can V_H Gene Restrictions Between Functional Lyl Cells and Their Targets Be Demonstrated?

Our initial approach to this question was to examine whether or not the effector cell activity of either of the two cells (Lyl:HE or Lyl:SI) is restricted in its interaction with spleen cells in the assay culture. To do this, we used three strains of mice which are congenic but differ at the IgH locus (Table 1). These strains included a particularly informative strain, the BAB.14, which has the C_H genes of B6 but most of the V_H genes of Balb/c, including the gene coding for the S117 idiotype. CRNPT from these three strains were primed with SAV-Mϕ and tested for Lyl:HE or Lyl:SI activity reciprocally in assay cultures from the three strains (Table 1).

Table 1 (con't). and Assayed Spleen Cells

Anti-TNP SAV PFC response per culture					
Helper Assay			Suppressor Assay		
Balb/c	C.B-20	BAB.14	Balb/c	C.B-20	BAB.14
7653	7891	2016	187	5022	127
5874	5724	2580	5373	2166	2455
6425	8517	5699	3720	4346	1052
807	945	1152	9368	5548	3880

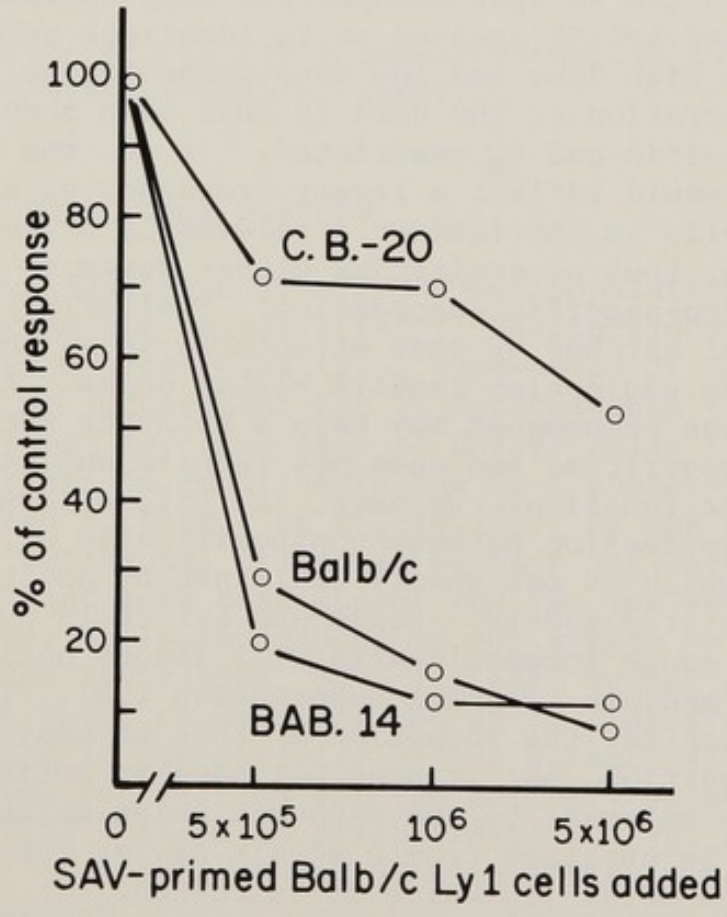

Figure 3. V_H gene restriction in the interaction of Ly1 suppressor inducers with recipient spleen cell cultures. SAV primed Ly1:SI from Balb/c mice were assayed for suppression of the anti-TNP PFC response to TNP-SAV in assay cultures containing C.B-20, Balb/c, or BAB.14 spleen cells.

For helper function we could not demonstrate a restriction. This was true for the anti-TNP PFC response to TNP-SAV, the anti-ACHO response to SAV, or the S117 idiotypic portion of the anti-ACHO PFC response. On the other hand, we demonstrated a V_H gene restriction between Lyl:SI and the assayed spleen cells (Table 1, Fig. 3). But unlike that described by Eardley et al (19), the restriction was not absolute. Some suppression was found in V_H incompatible strain combinations. The V_H gene restriction is most dramatic at lower doses of Lyl:SI in the assay culture (Fig. 3). This is the same dose range in which the suppression is antigen-specific (that is, homologous priming and assay antigens are required to suppress at this dose (7)). Both the V_H gene restriction and the antigen-specificity of the suppression can be overcome if higher doses of Lyl:SI are assayed (Fig. 3 and ref. 7). In fact, the high dose suppression, obtained with SAV-primed Lyl:SI assayed on V_H nonidentical spleen, is similar in magnitude to the antigen-nonspecific suppression obtained with unprimed Lyl:SI assayed on V_H identical spleen.

Can the high dose and low dose phenomena be reconciled? One interpretation of the data is that both phenomena are antigen-specific and V_H restricted. If so, the high dose phenomenon would reflect a lesser frequency of antigen-specific cells in unprimed or irrelevantly primed Lyl:SI populations, thus necessitating higher doses to obtain equivalent antigen-specific suppression. Similarly, a lesser frequency of matched V_H gene structures in V_H nonidentical combinations would also require higher doses. Alternatively, the high dose phenomenon may have a separate mechanism which is not V_H restricted and does not require antigen for its induction or function. We have, in fact, activated the feedback suppression pathway nonspecifically or, at least, in a mode which is not easily explained by antigen-specific or V_H restricted models. Nonimmune Lyl cells pulsed with aggregated mouse immunoglobulin will induce nonimmune Lyl23 T cells to mediate feedback suppression (20). The immunoglobulin used for the induction of this pathway must have an intact Fc portion, but it need not have an antigen specificity or idiotype relevant to the response being measured, nor does it need to be obtained from a strain having the same IgH gene locus as the strain in which it is tested.

In a separate approach, we have induced Lyl effectors with anti-idiotype antibodies and examined whether these cells are restricted to helping or suppressing the S117 idiotypic portion of the anti-ACHO response. The use of anti-S117 idiotypic antibodies rather than antigen readily induces antigen-specific Lyl:HE and Lyl:SI. When these are examined functionally, their activity does not appear to be confined

Table 2. Induction of Lyl:HE with anti-S117 idiotype antibodies

1st (induction T-MØ culture)	*2nd (assay) culture*		
	Anti-SAV response		
Source of Lyl helper cells	*Anti-ACHO PFC per culture*		
	Total	*id$^+$*	*id$^-$*
SAV-primed Lyl T cells	*1296*	*451*	*845*
Anti-id primed Lyl T cells	*808*	*395*	*413*
None added (background)	*19*	*0*	*19*

to the id$^+$ portion of the PFC response either in the helper (Table 2) or suppressor mode of assay.

C. Comparison With Other Systems

Others have described synergy between populations of T cells in mediating the helper effect. Distinctions on the basis of adherence properties (21), expression of Ia molecules (21-23), or requirement for Ig determinants (isotype, allotype, idiotype) for induction of helper cells (17,18) have been described in addition to the Qa-1 phenotypic distinction reported by Cantor et al (14,15). On the basis of phenotypic and functional criteria, we have not been able to demonstrate that more than one of the two (or more) putative T helper cells is induced in our system -- an antigen-specific, MHC restricted, Qa-1$^-$:IJ$^-$:Lyl helper cell. Indeed, we have found no evidence for an Ig (idiotype or V_H gene) restriction in the activity of our helper cells, but a relative idiotype restriction for the activity of the Lyl:SI (which is Qa-1$^+$:IJ$^+$) seems to exist (Table 1, Fig. 3, and ref. 19). Qa-1$^+$:Lyl cells have relatively little helper effect in our system (Fig. 1), but Qa-1$^+$:Lyl cells generated in vivo or in vitro from whole spleen cell cultures do provide some helper activity (Figs. 1 and 2). Therefore, our system does not dispute the existence of two helper cell sets; it simply generates only one of them.

A very reasonable speculation is that our helper cell corresponds to the so-called TH1 set described by several groups. This set includes the antigen-specific MHC-restricted,

I-A negative helper cell described by Swierkosz, Marrack, and Kappler (23), the IJ^-:Lyl, nylon wool-nonadherent helper cell described by Tada et al (21), the helper cell induced in Ig-deficient mice described by Janeway et al (17,18), and a large group of independently derived antigen-specific, MHC restricted helper cell clones or hybridomas (none of which, to date, are Ig or idiotype recognizing) (24-26). The $Qa\text{-}1^+$: IJ^+:Lyl cell, which in our system is a V_H-restricted suppressor inducer cell, but might conceivably function as a helper cell if induced in the presence of B cells or Ig (see above), may correspond, in terms of the helper function described by Cantor et al (14,15), to the helper cell requiring Ig for its induction described by Janeway et al (17,18), to the idiotype recognizing helper cell described by Sercarz et al (27), and to the nylon wool adherent helper cell described by Tada et al (21). Such a postulate, if true, would reconcile several diverse observations, but formal proof connecting function, cell-cell interactions in terms of MHC and Ig (idiotype) restrictions and phenotype (IJ, I-A, Lyt, and Qa-1) has not been obtained.

REFERENCES

1. Kontianen, S., and Feldmann, M. (1973). Nature (New Biol.) 245,285.
2. Feldmann, M., Beverly, P.C.L., Dunkley, M., and Kontianen, S. (1975). Nature 258,614.
3. Erb, P., and Feldmann, M. (1975). J. Exp. Med. 142,460.
4. McDougal, J.S., and Gordon, D.S. (1977). J. Exp. Med. 145,678.
5. Feldmann, M., Beverly, P.C.L., Woody, J., and McKenzie, I.F.C. (1977). J. Exp. Med. 145,793.
6. McDougal, J.S., and Cort, S.P. (1978). J. Immunol. 120, 445.
7. McDougal, J.S., Shen, F.W., and Elster, P. (1979). J. Immunol. 122,437.
8. Shen, F.W., McDougal, J.S., Bard, J., and Cort, S.P. (1980). J. Exp. Med. 151,566.
9. McDougal, J.S., Shen, F.W., Cort, S.P., and Bard, J. (1980). J. Immunol. 125,1157.
10. Shen, F.W., McDougal, J.S., Bard, J., and Cort, S.P. (1981). Submitted for publication.
11. Vicari, G., Sher, A., Cohn, M., and Kabat, E.A. (1970). Immunochem. 7,829.
12. Black, S.J., Hammerling, G.J., Berek, C., Rajewsky, K., and Eichman, K. (1976). J. Exp. Med. 143,846.

13. Eardley, D.D., Hugenberger, J., McVay-Boudreau, L., Shen, F.W., Gershon, R.K., and Cantor, H. (1978). J. Exp. Med. 147,1106.
14. Cantor, H., Hugenberger, J., McVay-Boudreau, L., Eardley, D.D., Kemp, J., Shen, F.W., and Gershon, R.K. (1978). J. Exp. Med. 148,871.
15. Stanton, T.H., Calkins, C.E., Jandinski, J., Shendel, D.J., Stutman, O., Cantor, H., and Boyse, E.A. (1978). J. Exp. Med. 148,963.
16. Wysocki, L., and Sato, V.L. (1978). Proc. Natl. Acad. Sci. U.S.A. 75:2844.
17. Janeway, C.A., Murgita, R.A., Weinbaum, F.I., Asofsky, R., and Wigzell, H. (1977). Proc. Natl. Acad. Sci. U.S.A. 74:4582.
18. Janeway, C.A., Bottomly, K., Dzierak, E.A., Eardley, D., Drum, S., and Gershon, R. (1981). J. Supramolec. Struct. and Cell. Biochem. Suppl 5,93.
19. Eardley, D.D., Shen, F.W., Cantor, H., Gershon, R.K. (1979). J. Exp. Med. 150:44.
20. Nicholson, J.K.A., and McDougal, J.S. (1981). J. Immunol. In Press.
21. Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. (1978). J. Exp. Med. 147:446.
22. Okumura, K., Herzenberg, L.A., Murphy, D., McDevitt, H.O., and Herzenberg, L.A. (1976). J. Exp. Med. 144: 685.
23. Swierkosz, J.E., Marrack, P., and Kappler, J.W. (1979). J. Exp. Med. 150:1293.
24. Kimoto, M., and Fathman, C.G. (1980). J. Exp. Med. 152,759.
25. Schwartz, R.H., Chen, C., Matis, L.A., Lerner, E.A., and Sredni, B. (1981). J. Supramolec. Struct. and Cell. Biochem. Suppl. 5,54.
26. Jones, B., and Janeway, C.A. (1981). J. Supramolec. Struct. and Cell. Biochem. Suppl. 5,55.
27. Sercarz, E., Adorini, L., Araneo, B., Benjamin, C., Harvey, M., Katz, M., Maizels, R., Metzger, D., Miller, A., Wicker, L., and Yowell, R. (1981). J. Supramolec. Struct. and Cell. Biochem. Suppl. 5,10.

STUDIES OF T LYMPHOCYTE FUNCTION IN B CELL DEPRIVED MICE

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Abstract: T cells obtained from B cell-deprived mice, prepared by suppression with heterologous anti-μ chain antibody, were tested for their functional activity. It was found that such cells contained essentially normal levels of mitogen and alloantigen reactive T cells, and normal levels of helper T cells restricted by MHC gene products. By contrast, such T cell populations lacked helper T cells required for dominant idiotype expression or found acting early in B cell antibody responses. These T cell populations also lacked T cells inducing various regulatory T cell interactions. Several deficiencies in immunoregulatory T cell functions were also noted. It is proposed that the primary effect of anti-μ chain antibody is on the B cell, while there is a secondary effect on an Lyt-1$^+$,2$^-$ T cell subpopulation with dual functions, namely help delivered to B cells via recognition of Ig on the B cell surface and the induction of immunoregulatory effects. This paper will summarize the experiments that lead to this interpretation.

It is well known that the maturation of T lymphocytes requires a functioning thymus (1). Furthermore, studies in many laboratories suggest that T cells are selected by thymic antigens for expression of particular anti-self major histocompatibility complex (MHC) antigen receptors that confer MHC restriction on these cells (2,3). Some studies suggest an effect of peripherally expressed MHC antigens in this process as well (4). Recent studies of helper T cells that selectively induce the expression of a particular idiotype have provided evidence that such cells do not recognize self MHC encoded determinants (5; Bottomly, this volume), but rather require immunoglobulin (Ig) or B cells expressing the idiotype for their development (6,7). We have noted in previous studies that the adoptive secondary antibody response to the hapten 2,4-dinitrophenyl (DNP) involves two distinct antigen-specific helper T cells (8,9). Such cells were most readily discriminated by the finding that only one of these cells could be detected in mice that had been depleted of B cells and Ig by treatment from birth with antibody to murine μ chains (8). Such mice also lack antigen-specific helper T

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cells specific for the TEPC-15 idiotype expressed in the response to phosphorylcholine (7). These experiments suggested that T cells might be divided into those that recognize MHC encoded determinants, either self or non-self, and those that recognize self Ig encoded determinants. The present experiments were undertaken to determine which immunoregulatory cells were deficient in anti-μ treated mice, using the experimental systems developed by Gershon and his co-workers (10; Gershon, this volume). The results suggest that an Lyt-1^{+}2^{-} subset of T cells mediating both helper function and the induction of regulatory T cell responses is the critical T cell affected by treatment with anti-μ chain antibody.

Materials and Methods: The basic design of these experiments was to isolate T cells from normal or age-matched anti-μ treated mice, and compare their activities in several different functional assays. Not all assays were run on each mouse. Anti-μ treated mice were prepared as follows: within 24 hours of birth, mice were injected with 50 ul of sterile anti-μ chain antibody prepared by immunization of rabbits with MOPC 104e protein after blocking with a large amount of normal mouse IgG. The antibody was enriched about two fold by precipitation three times with saturated ammonium sulfate, and dialized against phosphate-buffered saline. Mice received 200 ul per week in three injections until killed for use in the experiments (7). All of the biological assays have been described elsewhere, as referenced in the text.

Results: B Lymphocyte Function in Anti-μ Treated Mice: B lymphocyte function in anti-μ treated mice was assessed in various ways, as listed in Table 1. All mice are tested for Ig-bearing spleen cells; any found to have such cells are discarded as insufficiently suppressed.

TABLE 1

IMPACT OF ANTI-μ ANTIBODY SUPPRESSION ON B CELLS

1. Less than 1% of spleen cells sIg^{+} even after overnight incubation at 37^{o}.
2. No response to LPS; cell mixes with normal spleen cells show no suppression of normal LPS response by anti-μ treated spleen.
3. Serum Ig levels markedly reduced but not absent; serum idiotype (T15) absent.

These studies demonstrate that B cell function is essentially absent in anti-μ treated mice, as had been previously observed by other authors (11-13). Our goal was to use such mice to

determine the function of T cells in the absence of B cells, in order to determine the importance of B cells and/or Ig in T cell development.

Effect of anti-μ treatment on T cell functions: A great many different T cell functional assays were performed using T cells derived from anti-μ treated mice. The studies summarized in Table 2 show that the percent of T cells in anti-μ treated spleens is increased, and that their responses to mitogens and to non-self mixed lymphocyte stimulating determinants is normal or near normal.

TABLE 2

IMPACT OF ANTI-μ ANTIBODY SUPPRESSION ON T CELLS

1. Percent Thy-1 positive cells in spleen increased to about 65%.
2. Polyclonal responses to ConA and rabbit anti-mouse brain antibody are normal per T cell.
3. Proliferative response to non-self MHC or to Mls are about half the normal level per T cell.

Conventional MHC-restricted helper T cell function of T cells from anti-μ treated mice: Helper T cell function was assessed in three different experimental systems. In all three systems, the level of antibody produced when such cells from anti-μ treated mice were added to B cells was normal. Two of these results have been published elsewhere; the third is shown in Tables 3 and 5. That the helper T cells present in anti-μ treated mice are indeed MHC restricted has not been formally demonstrated. However, experiments by Bottomly (5; Bottomly, this volume) strongly suggest that this is so. Thus, Lyl cells responding to non-self MHC or Mls locus antigens, and Lyl cells specific for antigen and acting as MHC restricted helper cells are present at what appear to be normal levels in anti-μ treated mice.

TABLE 3

CONVENTIONAL (ThMHC) FUNCTION IN ANTI-μ SUPPRESSED MICE

1. No effect on plateau adoptive secondary anti-DNP response (8).
2. T15$^-$ anti-PC PFC response normal or increased using Th from anti-μ treated mice (7).
3. Educated Lyl cells from anti-μ treated mice give normal day 5 anti-SRBC PFC responses when added to B cells (Table 5).

Evidence for a second set of helper T cells in normal mice that is deficient in anti-μ treated mice: The same three assay systems have been tested for the presence of a second set of helper T cells by comparing the activity T cells from normal or anti-μ treated mice. These systems are listed in Table 4.

TABLE 4

EVIDENCE FOR A HELPER T CELL DEFICIT IN ANTI-μ TREATED MICE

1. One of two synergizing helper T cells detected at day 5 of the secondary adoptive anti-DNP antibody response is absent in anti-μ treated mice (8).
2. The T15$^+$ anti-PC PFC response is reduced about six fold using helper T cells from anti-μ treated mice (7).
3. The helper activity of Lyl T cells from anti-μ treated mice for the anti-SRBC response of B cells on day three of culture is essentially absent (Tables 5 and 6).

The first two systems have been published already, as referenced. In the first, it is the early secondary response that is deficient when helper T cells are derived from anti-μ treated mice. This has been shown to be due to the absence of an antigen specific, Thy1.2$^+$, Lyt-1$^+$2$^-$ T cell not requiring a hapten-carrier bridge in this system (9). We would postulate that such a cell is recognizing Ig, perhaps as idiotype, on B cell surfaces (see below). In the second system, it is clear that the T cell that is lacking in anti-μ treated mice is acting selectively on idiotype-bearing B cells, and may indeed recognize the idiotype (7). This cell has also been shown to be Thy1.2$^+$, Lyt-1$^+$,2$^-$, and antigen specific while not requiring a hapten-carrier bridge, and has furthermore been shown by Bottomly (this volume) not to be MHC restricted. The data for the third system are presented in Tables 5 and 6. Here, it is seen that Lyl cells activated with SRBC in vitro from normal mice induce strong day 3 anti-SRBC plaque forming cell (PFC) responses when added to normal B or B plus Lyl T cells while Lyl cells from anti-μ treated mice do not. However, day 5 responses are similar for the two groups. This suggests that there are two distinct helper T cells in the anti-sheep response, as had been previously suggested by experiments in which anti-Qa-1 and complement depleted some but not all helper function in this system (14). As in the anti-DNP system, the major impact of anti-μ antibody treatment on helper T cell function was observed shortly after induction of the response; peak antibody or PFC responses with the two different cell populations were similar.

TABLE 5

IMPACT OF ANTI-μ SUPPRESSION ON EARLY AND LATE ACTING Lyl HELPER T CELLS

Immune Lyl T Cells Added To ⟶		Lyl+B Day 3	Lyl+B Day 5	B, Day 5
No added cells		0	3834	0
Normal donors	$3x10^5$	4320	2997	2133
	10^5	5076	3321	1134
Anti-μ treated	$3x10^5$	1782	2916	2943
	10^5	540	1944	972

In vitro PFC responses to sheep red blood cells using cells from BALB/c mice.

TABLE 6

IMPACT OF ANTI-μ SUPPRESSION ON EARLY HELP AND FEEDBACK SUPPRESSION

		PFC Response to SRBC When Added To:		
Source of educated Lyl T cells		B Cells Day 3	Spleen Cells Day 5	(% Suppression)
No added cells		0	1132	(Control)
Normal donors	10^5	648	300	(74)
	$3x10^4$	1134	300	(74)
	10^4	108	666	(41)
Anti-μ treated	10^5	0	733	(35)
	$3x10^4$	0	2031	(0)
	10^4	0	932	(17)

In vitro anti-SRBC response using cells from C57BL/6J mice.

Effect of anti-μ treatment on T cells responsible for the induction of suppression: It has been previously demonstrated that Lyl T cells bearing Qa-1 antigenic determinants act on Lyl23 cells to induce feedback suppression (14), and that the Lyl and Lyl23 cells needed to share VH linked genes in order for this interaction to proceed (15). Suppressor inducer factors from such mice also require VH matching, and are not MHC-restricted in their function (22). These findings suggested that the helper T cells depleted by anti-μ treatment, which appear to act on B cells via B cell idiotypic determinants, and are not MHC restricted, might be similar or identical to the T cells inducing feedback suppression. When Lyl T cells from anti-μ treated mice were tested for their ability to induce feedback suppression, it was found that they were unable to do so, as shown in Tables 6 and 7.

TABLE 7

IMPACT OF ANTI-μ TREATMENT AND Igh-LINKED GENES ON FEEDBACK SUPPRESSION

Source of Educated BALB/c Lyl Cells	PFC per culture when added to spleen cells	
	BALB/c	C.B20
None	1546 (Control)	26
Normal	893 (43%)	533
Anti-μ Treated	1387 (10%)	493

		BALB/c Lyl+B	BALB/c T + B
None		3834	2808 (Control)
Normal donors	$3x10^5$	2997	513 (82%)
	10^5	3321	918 (67%)
Anti-μ	$3x10^5$	2916	1890 (33%)
	10^5	1944	2295 (18%)

Cultures performed with BALB/c Lyl T cells activated *in vitro* with SRBC PFC measured 5 days after initiation of cultures.

However, the same Lyl T cells from normal or anti-μ treated mice were able to help the responses of C.B20 spleen cells, in which induction of suppression is prevented by mismatching of VH linked genes. This suggests that anti-μ treatment leads to loss of the inducers of feedback suppression as well as of helper cells that recognize idiotype on B cell surfaces, but not of conventional helper T cells. It is not possible at the present time to discriminate Ig-recognizing helper T cells and inducers of feedback suppression; their close similarity is emphasized by sharing a number of characteristics, outlined in Table 8. At the present time, anti-I-J antibodies, which kill the inducer of feedback suppression (16), are being tested for their effect on helper T cells; such experiments may demonstrate differences between the two cell types. We have previously shown that a factor preparation induces both activities in Lyl cells (17), providing further evidence that this is a single cell, or that these cell types are progeny of a single precursor cell having similar but distinct functions.

TABLE 8

EVIDENCE FOR IDENTITY OR CLOSE RELATIONSHIP OF ThIg and IFBS

1. Both are decreased by treatment of the donor with anti-μ (Tables 5-7).
2. Anti-Qal treatment affects both helpers and inducers of feedback suppression (14).
3. Both interact with their target cells via VH linked structures (7,15).
4. Both have an independent antigen-specific component (7,22).
5. Both are activated by a T cell suppressor inducer factor (17).

Effects of anti-μ treatment on other immunoregulatory cell types: We have further examined anti-μ treated mice for other immunoregulatory cell functions. These findings are summarized in Figure 1, which represents a simplified immunoregulatory network; an arrow pointing down means that the cell or its product have been observed to be depleted in mice treated from birth with anti-μ antibody.

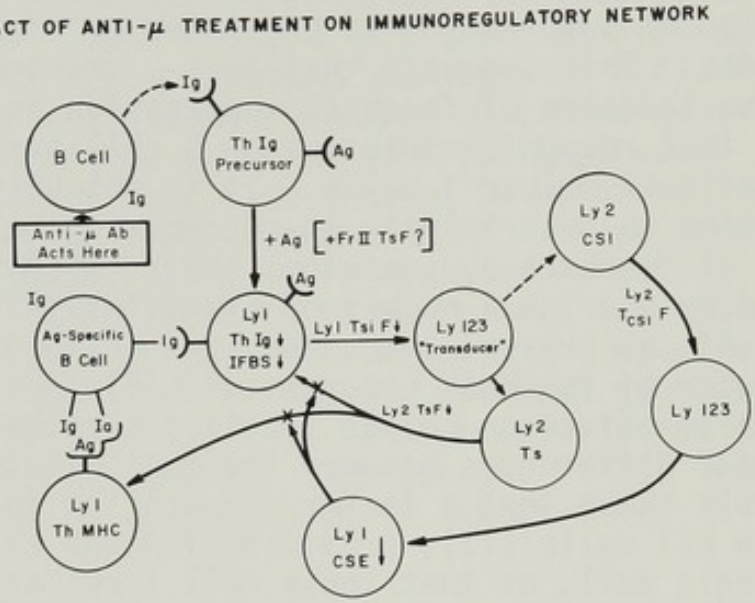

Figure 1. A hypothetical model of immunoregulatory pathways with the role of B cells included, and a possible interpretation of results of the present experiments included. A down arrow means that the cell or product is deficient or absent in anti-μ treated T cell populations. Abbreviations: ThIg: Ig-recognizing helper T cell; ThMHC: MHC-restricted helper T cell; IFBS: inducer of feedback suppression; Lyl-TsiF: Lyl derived inducer of suppression factor; Ts: suppressor effector T cell; TCSI*: contrasuppressor inducer T cell; TCS1F**: contrasuppressor inducer factor; Ly2-TsF: T suppressor effector factor; CSE: contrasuppressor effector T cell; CSF: contrasuppressor effector factor.

Effect of anti-μ treatment on lymphoproliferation in MRL-lpr/lpr mice: MRL-lpr/lpr mice have a lupus-like syndrome including the production of large amounts of anti-nuclear antibody. They die of immune complex nephritis between 4 and 6 months of age. They also manifest profound lymphoproliferation, especially of peripheral lymph nodes (18). The cells in these lymph nodes are 99% T cells, being Lyt-$1^{+}2^{-}$ or Ly null. Gershon and co-workers have shown that these lymph node cells stimulate intense feedback suppression, but do not themselves become suppressed by the Ly2 suppressor T cells they induce (19). Whether this is due to an inherent defect in the Lyl cells, or due to intense contrasuppressor activity, is not clear. Such mice also have hyperactive B cells and a

*subscript - T_{CSI} **subscript $T_{CS1}F$

polyclonal gammopathy. It seemed likely that the lymph node T cells of these mice would be helper T cells dependent on Ig and/or B cells for their proliferation. Thus, MRL-lpr/lpr mice were treated with anti-μ antibody from birth, with littermate controls remaining uninjected. Around four months, the lymph nodes of the controls began to enlarge, but those of the injected mice did not. Eventually, the anti-μ treated MRL-lpr/lpr mice did in fact develop large nodes, but this was significantly later than in the controls. Furthermore, we determined that anti-μ suppression was not fully effective in these mice, since they expressed auto-antibodies in their serum. We are thus repeating this experiment with a more potent antiserum, as well as using anti-μ treated MRL-lpr/lpr female mice as mothers for a second generation suppression experiment, in which the offspring will have received anti-μ trans-placentally from their mothers, in hopes of overcoming the early onset of B cell function in these mice. We believe that the retardation in lymph node hyperplasia observed does signify a role of B cells in the development of the proliferating T cells, but we can not yet conclude that such cells are indeed helper T cells involved in auto-antibody production.

Discussion: These studies have demonstrated that treatment of mice from birth with anti-μ chain antibody, as previously reported, essentially eliminates B cells and, to a lesser extent, Ig production (11-13). Furthermore, T cells responding to mitogens, to MHC antigens, and to nominal antigen in association with MHC gene products are essentially normal in such mice, a finding consistent with previous studies of anti-μ treated mice. However, we were able to demonstrate essentially total absence of a synergizing subset of Lyl helper T cells that acts early in an immune response, presumably by recognizing Ig-encoded determinants on B cell surfaces rather than MHC encoded determinants. Such cells appear to be identical to or closely related to T cells that induce immunoregulatory T cell networks, since these latter cells are also absent in anti-μ treated mice, and since both sets have a number of other properties in common. Whether this reflects similarity or identity can not be determined at the present time.

These studies would appear to define a role for Ig in the induction of two cell sets critical in regulating the expression of idiotype: namely, the Lyl helper cell that selectively activates B cells bearing idiotype, and the similar or identical Lyl, VH restricted cell that induces

suppression, ultimately perhaps idiotype-specific suppression. We would predict that the early helper T cell observed in anti-DNP antibody responses in vivo is in fact anti-idiotypic, and thus missing in anti-μ treated mice. Indeed, we have recently defined the genetics of what appears to be a dominant idiotype for the anti-DNP antibody response in BALB/c mice, the Id-460 (20). This idiotype is regulated as one would predict based on this hypothesis. That is, it is the dominant idiotype early (3-5 days after boosting) in the anti-DNP antibody response, after which its expression is strongly suppressed, although the response as a whole is continued (Dzierzak and Janeway, unpublished). Furthermore, we have identified a normal serum component bearing this idiotype, which does not bind to DNP (21). Thus, we would propose that this naturally occuring form of Id-460 is the true, biologically relevant form of the idiotype. We would propose that this idiotype positive, non-DNP binding material in fact induces helper T cells specific for the idiotype, and that the anti-DNP response makes use of such cells for selective expression of idiotype positive, DNP binding antibody. Thus, anti-μ suppression prevents the expression of this material, and of the second, anti-idiotypic helper cell in this system. Using monoclonal forms of this material, we are searching for the antigen recognized normally by Id-460, as well as to probe its structure and germ line expression.

In conclusion, it can be stated that subpopulations of T cells exist which are dependent on normal B cell function for their expression. Such T cells in turn would appear to regulate B cell function in synergy with MHC restricted T cells, and do so via recognition of Ig on the B cell surface. Such Ig-recognizing helper T cells may be important in the rapid activation of the most prevalent B cells, those bearing particularly common idiotypic determinants. The same or a closely related cell would also recognize VH-linked structures on Lyl23 cells, and induce regulatory T cells, including that which inactivates the helper/inducer T cell. This would lead to independent regulation of idiotype and antibody.

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References

1. Miller, J.F.A.P., and Osoba, D. (1967). Physiol. Rev. 47 437.

2. Zinkernagel, R.M. (1978). Immunol. Rev. 42, 224.
3. Bevan, M.J., and Fink, P.J. (1978). Immunol. Rev. 42, 3.
4. Zinkernagel, R.M. (1979) in "T and B Lymphocytes: Recognition and Function," F.H. Bach, B. Bonavida, E.S. Vitetta, and C.F. Fox, eds. Academic Press, New York, pg. 505.
5. Bottomly, K., and Maurer, P.H. (1980). J. Exp. Med. 152, 1571.
6. Bottomly, K., and Mosier, D.E. (1979). J. Exp. Med. 150, 1399.
7. Bottomly, K., Janeway, C.A., Mathieson, B.J., and Mosier, D.E. (1980). Eur. J. Immunol. 10, 159.
8. Janeway, C.A., Murgita, R.A., Weinbaum, F.I., Asofsky, R. and Wigzell, H. (1977). Proc. Nat. Acad. Sci U.S.A. 74, 4582.
9. Janeway, C.A., Bert, D.L., and Shen, F.-W. (1980). Eur. J. Immunol. 10, 231.
10. Gershon, R.K., and Cantor, H. (1980) in "Strategies in Immune Regulation", Sercarz, E.E., and Cunningham, A.J., eds. Academic Press, New York, pg. 43.
11. Lawton, A.R., Asofsky, R., Hylton, M.B., and Cooper, M.D. (1972). J. Exp. Med. 135, 277.
12. Manning, D.D. (1975). J. Reticuloendothelial Soc. 18, 63.
13. Murgita, R.A., Mattioli, C.A., and Tomasi, T.B., Jr. (1973). J. Exp. Med. 138, 209.
14. Cantor, H., Hugenberger, J., McVey-Boudreau, L., Eardley, D.D., Kemp, J., Shen, F.-W., and Gershon, R.K. (1978). J. Exp. Med. 148, 871.
15. Eardley, D.D., Shen, F.-W., Cantor, H., and Gershon, R.K. (1979). J. Exp. Med. 150, 44.
16. Eardley, D.D., Murphy, D.B., Kemp, J.D., Shen, F.-W., Cantor, H., and Gershon, R.K. (1980) Immunogenetics 11, 549.
17. Mattingly, J.A., Kaplan, J.M., and Janeway, C.A. (1980). J. Exp. Med. 152, 545.
18. Murphy, E.D., and Roths, J.B. (1978). Mouse Newsletter 58, 51
19. Gershon, R.K., Horowitz, M., Kemp, J.D., Murphy, D.B., and Murphy, E.D. (1978) in "Genetic Control of Autoimmune Disease", Rose, N., Bigazzi, P., and Warner, N. eds. Elsevier-North Holland, New York pg. 223.
20. Dzierzak, E.A., Janeway, C.A., Rosenstein, R.W., and Gottlieb, P.D. (1980). J. Exp. Med. 152, 720.
21. Dzierzak, E.A., Lerner, E.A., and Janeway, C.A. (1981). J. Supremol. Struct. Suppl. 5,99.
22. Yamauchi, K., Murphy, D.B., Cantor, H., and Gershon, R.K. (1981). Submitted for publication.

MODELING A CONTINUUM OF IMMUNE RESPONSIVENESS[1]

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Feedback suppression represents a model for the study of lymphocyte interactions which give rise to suppressed immune responses. Feedback suppression operates in a circuit such that the cell which induces it is one of the eventual targets of inactivation. Recently a second T cell circuit, termed "contrasuppression" has been described, and this second circuit has the capacity to interact with and regulate the first circuit. In this paper, we hypothesize that the feedback suppression and contrasuppression circuits represent opposing ends of a continuum of immune responsiveness which may result in tolerance and hyperimmunity, respectively.

Biologically active systems attempt to solve the problems of maintaining order in an entropic, antagonistic universe by manipulating information within regulatory networks. In the immune system, the problem is one of producing an effective response which maximally dispenses a foreign invader while only minimally damaging self tissues, and the regulatory phenomena that may be involved have become a topic of intense theoretical interest. A specific immune state is produced by the presentation of antigen into one or more available immunoregulatory pathways, and by the subsequent communication between immunologic cell subsets. This immune state can now modulate the presentation and regulation of responses to new antigens, which can in turn further modify the immune state.

The following is a review of how such cellular interactions can, at least in part, account for two such immune states which lie on distant ends of a continuum of immune responsiveness: tolerance and hyperimmunity. One of the T cell communicative networks to be discussed has served as a basis for current theories of regulatory circuits in the immune system - the feedback suppression circuit (1). The second has only recently been characterized by virtue of many of the same

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experimental strategies used in elucidating the first circuit, although this latter pathway, the contrasuppressor circuit, functions to counteract the effects of the former (2).

Dissecting an Immune Effect: An experimental approach which has been extremely valuable in elucidating regulatory pathways is the dissection of the T cell population into sets which perform various and discrete functions. By dissection, we can define three basic kinds of T cells: inducers, regulators or transducers, and effectors for a particular immunologic function.

For example, while cytotoxic T cells are the effector cells in T cell mediated destruction of cells which have been infected by virus, the generation of these effector cells requires the activity of an inducer cell which is probably specific for both the antigen and the effector (3). Another effector T cell is the suppressor cell which inhibits the generation and expression of immune responses and which requires a unique inducer cell to activate it (4). This will be discussed in considerably more detail in another section. Other kinds of inducer T cells function to activate non-T cell effectors. Examples are the helper T cells which assist B cells in the synthesis of antibody (5), and others which activate inflammatory cells to participate in delayed type hypersensitivity responses (6).

In each of the above cases, functions are ascribed to T cells which may correlate with discrete subpopulations of cells or which may represent multiple functions of a single set of cells. In order to distinguish between these possiblities, it is necessary to develop means to separate these populations of T cells and match each to its function.

A system which has proven very productive is the use of antisera which recognize polymorphic gene products on cell surfaces in the mouse (7) and in man (8). By determining a sufficiently large array of profiles of such markers on the surface of a cell with a particular function, this cell can be subsequently distinguished from cells with other profiles and other functions. It is both this profile and the function which defines a unique subset of cells. The isolation of any population of cells with a particular surface profile is not in itself a guarantee of the functional activity of that population.

The correspondence of Ly antigen expression and function has been studied extensively in the mouse and is often used in dissecting the components of T cell regulation (9). Other useful markers include the Qa system (10), specific lectin receptors (11), the products of the I region of the H-2 (particularly I-A (12) and I-J (13) and so on.

In the following sections, two regulatory circuits will be discussed in terms of the subpopulations of T cells which

interact to produce an immune effect. These subpopulations are in each case defined in terms of their function and their surface marker profile. The first of these pathways is the feedback suppression circuit which functions to inhibit immune responsiveness. The second is the contrasuppression circuit which interferes with the action of suppressor cells, such that a response proceeds undisturbed. Following discussion of each of these circuits and their components subsets, a system will be described for the reconstruction of cell interactions to mimic immune states in the animals.

The Feedback Suppression Circuit: Both suppressor cells and the components of the feedback suppression circuit have been described extensively (1). Basically a signal produced by an inducer cell is transduced and amplified by a second population of regulatory cells, resulting finally in the effector cells of T cell suppression (see Table I). The feedback inducer is defined functionally as having the capability to suppress immune responses provided that cells of the transducer subset are present. In the *in vitro* response to sheep red cells, for example, addition of feedback inducers will result in elimination of plaque forming cell generation (4). When added to cultures lacking the transducer cells of the inducer signal, no decrease in the amplitude of the response is seen. This functional activity of suppressor induction is correlated with a cell of the Ly-1; $I\text{-}J^+$, $Qa\text{-}1^+$ surface profile (14). The Ly-1 marker is also found on other inducer T cells, such as those which activate B cells to make antibody (9), activate macrophages in inflammation (9), activate cytotoxic T cells (3), and activate the burst forming unit of erythopoesis (15). In addition to Ly-1, the inducer of suppression also bears products of the I-J subregion and Qa-1 locus, markers which distinguish it from the T helper cell in the *in vitro* antibody response to sheep red cells (14).

The cell which transduces the induction signal into one of direct suppression is of the surface profile Ly-1,2; $I\text{-}J^+$ (14). It is functionally defined by the requirement for its presence for expression of suppression when feedback inducers are added. In the absence of suppressor induction, these cells have no apparent activity. While the mechanism by which these cells transduce the inducing signal into suppression is not completely understood, at least one mode of action may be the differentiation of these cells into mature suppressor effector cells in the presence of the inducing signal (16).

The effector cell in the feedback suppression system is functionally defined by its ability to suppress in the absence of transducing cells. These suppressor effectors bear the Ly-2 surface marker, but unlike the inducer and transducer subsets, do not carry the same I-J gene product, at least in the case of the suppressor of the *in vitro* response to sheep red cells (2).

TABLE I FEEDBACK SUPPRESSION CIRCUIT

In vitro educated cells	Assay culture	Response	Comments
—	normal T + B	+	Control
—	Ly-1 T + B	+	Control
Ly-1 (inducer)	normal T + B	—	In the presence of normal T cells (including transducers) Ly-1 inducer activates suppression.
Ly-1 (inducer)	Ly-1 T + B (absence of transducer)	+	In the presence of transducers, inducer cells produce no suppression.
Ly-1 (inducer)	Ly-1 T + Ly-2 T + B (absence of transducer)	+	Ly-1,2 transducer still absent, no effect of adding inducers.
Ly-2 (effector)	Ly-1 T + B	—	Ly-2 effector cell suppresses response in absence of transducer cells.

Thus a regulatory circuit is seen in which Ly-1: $I\text{-}J^+$ inducing cells produce a signal which is transduced via an Ly-1, 2; $I\text{-}J^+$ population to result in active Ly-2 suppressor effector cells. Studies which utilize these effector cells will be described following a discussion of a second circuit which functions to interfere with the activity of the one just described.

The Contrasuppression Circuit: Once again we return to our theme of defining cell sets and interactions in terms of both surface profiles and function. In this case, however, the function is one of blocking the action of suppressor cells, or "contrasuppression " of the immune response. To functionally define contrasuppressors, a response is generated which can be suppressed (usually by suppressor effectors), and cells are added which allow the response to return to original levels. The removal of a cell or cells responsible for this effect results in the reappearance of suppression. It is in this way that the surface phenotype of the cells involved in contrasuppression were determed (see Table 2).

The inducer of contrasuppression is, again, functionally defined by the requirement for a population of transducing cells for the appearance of the effect. Thus, if a suppressor effector cell (or its cell free product) is added with a contrasuppressor inducer (or its cell free product) to a culture containing no transducing cells, then suppression will dominate (recall that suppressor effectors require no transducing cells for the expression of their activity). In the presence of transducing cells, however, contrasuppression is produced and the suppression is diminished or even abolished, resulting in the restoration of the full response (2). In the absence of the contrasuppressor inducing cell, the suppressor effector dominates, regardless of the presence of the contrasuppressor transducers. The contrasuppressor inducer correlates with a cell surface profile of Ly-2; $I\text{-}J^+$ (to contrast with the $I\text{-}J^-$ suppressor effector in this system). The transducing cell is Ly-1, 2; $I\text{-}J^+$ (2,17), although evidence suggests that the product of the I-J subregion on this cell is actually distinguishable from that found on the surface of cells in the feedback suppression system (Donal Murphy, personal communication).

The effector of contrasuppression (again defined functionally by the absence of a requirement for transducer cells for expression of the effect), bears the profile Ly-1; $1\text{-}J^+$ (18). This cell functions to interfere with suppression in the in vitro response to sheep red cells and has more recently been shown to block suppression of contact sensitivity in vivo (19). In the in vitro system, this cell has been shown to reverse the suppression produced by specific and non-specific culture generated suppressors (18), ConA-activated

TABLE 2 CONTRASUPPRESSION CIRCUIT

In vitro educated cells	Assay culture	Response	Comments
Ly-2 (suppressor effector + contrasuppressor inducer)	Ly-1 T + B (absence of contrasuppressor transducer)	−	Ly-2 suppressor effector suppresses in absence of suppressor transducer. No effect of contrasuppressor inducer in absence of contrasuppressor transducer in assay culture.
Ly-2 (suppressor effector + contrasuppressor inducer)	Normal T + B	+	In presence of contrasuppressor transducer Ly-2 contrasuppressor inducer interferes with activity of suppressor effector.
Ly-2; $I\text{-}J^-$ (suppressor effector only)	Normal T + B	−	Removal of $I\text{-}J^+$ contrasuppressor inducer allows expression of suppressor effector activity, even in presence of contra-suppressor transducers.
Ly-2 (suppressor effector) + Ly-1 (contrasuppressor effector)*	Ly-1 T + B	+	Ly-1 contrasuppressor effector interferes with activity of suppressor effector even in absence of contrasuppressor transducers.

*Produced by preculture of neonatal cells (18)

suppressors (18), in vivo trypanisome activated suppressors (P. Diffley and D.R. Green, unpublished observation), several T cell derived soluble suppressor factors, (K. Yamauchi, G.M. Iverson, and D.R. Green, unpublished observation) and suppressors generated in vivo as a result of burn trauma (T. Kupper and D.R. Green, unpublished observation). The demonstration that these cells function to contrasuppress inhibitory factors indicates that the mechanism of action is not only removal or inactivation of the suppressor effector itself.

To further examine the action of the effector cells of both suppression and contrasupression it became necessary to design a system whereby these cells could operate upon their target cell(s) following which the effects upon the target(s) would be assayed in their absence. To do this we developed a system described in the following section. This system allowed us to demonstrate that a) T helper cells can be directly inactivated by suppressor effectors such that they fail to help B cell responses to sheep red cells even after the suppressors are removed, and b) that contrasuppressor effectors can block this inactivation of the helpers by the suppressors such that the helper cells remain resistant to suppressor cell signals even after the contrasuppressor effectors are removed. We believe these to be in vitro models for the immune states of tolerance and hyperimmunity respectively.

Rebuilding a Dissected Response - The Intermediate Culture System: The intermediate culture system allows the investigation of T cell interactions independent of assay conditions. Fractionated subpopulations of T cells are combined in 48 hour cultures, then refractionated so that each component of the culture can be examined by addition to fresh assay cultures.

An example of this technique demonstrated that the feedback inducer cell can be a target of the inhibitory activity of suppressor effector cells (20), (Table 3, a). Ly-1[2] inducer cells were placed in intermediate culture with mature Ly-2 supressor effector cells. Following 48 hour culture, the cells were treated with anti-Ly-2 antiserum plus complement to remove the effectors. The cells which remained, the inducers, were then added to cultures containing B cells, helper T cells

[2] *Abbreviations: Ly-1= Ly-1$^+$, Ly-2$^-$ T cell set; Ly-2= Ly-1$^-$, Ly-2$^+$ T cell set; Ly-1,2= Ly-1$^+$, Ly-2$^+$ T cell set; T_H= T helper cell; T_S= T suppressor effector cell; T_{FBI}= T-feedback inducer cell, T_{CS}= T contrasuppressor effector cell; T_{HI}= T cells from hyperimmune animals.*

TABLE 3 THE INTEREMEDIATE CULTURE SYSTEM

	Cells in intermediate culture	Antiserum Rx at 48 hrs	Cell type recovered	Added Assay to culture	Response (assay culture)	Comments
a.	T_{FBI} (Ly-1)	anti-Ly-2 + C	T_{FBI}	normal T + B	-	Ts deletes ability of T_{FBI} to induce suppression in an assay culture containing transducer cells (even after Ts is removed).
	T_{FBI} + Ts (Ly-2)	"	"	"	+	
b.	T_H (Ly-1)	"	T_H	B	++	Ts deletes ability of T_H to induce B cells to make antibody in assay culture (even after Ts is removed).
	T_H (Ly-1) + Ts (Ly-2)	"	"	"	—	
c.	T_H (Ly-1.1)	anti-Ly-2 + anti-1.2 + C	T_H	B	++	Presence of Tcs interferes with the effect of Ts on T_H, leaving full responsiveness.
	T_H + Ts (Ly-2)	"	"	"	—	
	T_H + Ts + Tcs (Ly-1.2)	"	"	"	++	
d.	T_H (Ly-1.1)	"	T_H	B + Ts	—	T_H, recovered from contrasupress culture, remains relatively resistant to suppression (even after Tcs is removed).
	T_H + Ts + Tcs	"	T_H	B + Ts	++	

Abbreviations: T_{FBI} = feedback suppressor inducer; Ts= suppressor effector; T_H= helper; T_{cs}= contrasuppressor effector

and transducer cells, as an assay of their activity. The feedback inducers exposed to suppressor effector cells showed a diminished ability to activate suppression as compared to cells cultured 48 hours without effectors.

Another use of this system involves inactivation of helper T cells by suppressor effectors (Table 3.b). Following 48 hours of culture the Ly-2 suppressor effectors were removed, leaving the Ly-1 helper T cells. These were assayed for helper cell activity by adding them to cultures of fresh B cells plus antigen. Five days later the activity of the helpers were reflected in the PFC response. Again, suppressor effector cells could directly inactivate the helper T cells such thatthey failed to help B cells even after removal of the suppressors.

In the presence of contrasuppressor effectors, however, the inactivation of T helper cells by suppressor effectors does not occur (Table 3.c). In this case the T helper cells were reisolated from the culture by virtue of their being from animals congenic at the Ly-1 locus. Thus, Ly-1.1 helpers, Ly-1.2 contrasuppressor effectors, and Ly-2 suppressor effectors were cultured together for 48 hours, then treated withboth anti-Ly-1.2 and anti-Ly-2 sera plus complement, leaving only the Ly-1.1 helpers. When these helpers were added to B cells under conditions for response to sheep red cells, the helpers showed restored activity as compared to those from intermediate cultures containing only suppressors. In addition, these contrasuppressed helper cells were now more resistant to suppressor cell signals than their unregulated counterparts (helpers cultured alone for 48 hours) (Table 3.d).

This effect mimics some of the *in vivo* aspect of hyperimmunity. A hyperimmune animal makes an active response to antigen even in the face of the highly potent immunosuppressive activity in the serum (21). This immunosuppressive activity has been attributed to both antibody and secreted T cell products (21 and G.M. Iverson, personal communication). That the ability of hyperimmune animals to resist the immunsuppressive products of hyperimmune serum might be due to contrasuppression was tested in the intermediate culture system (Table 4). In this case the interacting subsets were separated by nucleopore membranes in a double Marbrook set up. Naive T cells were placed in the outer chamber and suppressor cells with or without splenocytes from hyperimmune animals were placed in the inner chamber. Following two days of culture, the T cells in the outer chamber were recovered and assayed for activity on fresh B cells. Helper activity was inhibited by intermediate culturewith suppressor cells, an effect which was blocked by the presence of hyperimmune cells. In addition,

TABLE 4 HYPERIMMUNE SPLEEN CELLS HAVE CONTRASUPPRESSIVE ACTIVITY

Intermediate culture (double Marbrook) Inner chamber	Outer chamber	T_H cells recovered from outer chamber then added to: (assay culture)	Assay culture PFC response	Comments
-	T_H	B	++	Control
Ts	T_H	B	-	Ts deletes T_H activity across nucleopore membrane (even after Ts is removed)
Ts, T_{HI}	T_H	B	++	T_{HI} interfere with inhibitory activity of Ts such that T_H maintains activity.
-	T_H	Ts + B	-	Control T_H are suppressed by fresh Ts in assay culture.
Ts, T_{HI}	T_H	Ts + B	++	T_{HI} render T_H resistant to suppression (even after T_{HI} and Ts are removed).

T_H = T helper

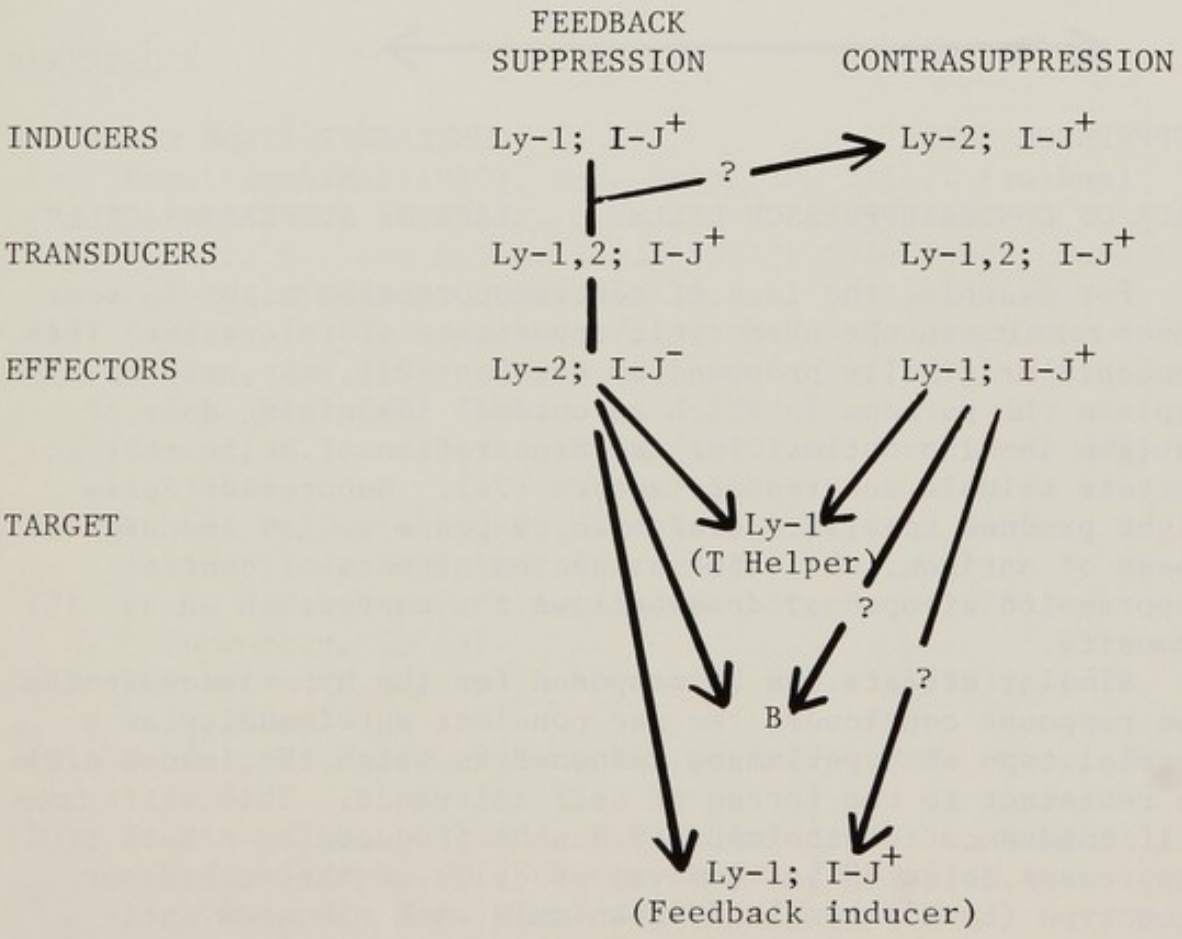
Ts = T suppressor effector

T_{HI} = T cells from hyperimmune

the T cells recovered from the outer chamber were resistant to further suppression following intermediate culture with both suppressors and hyperimmune cells. Thus, hyperimmune spleen cells were capable of conferring phenotypic resistance to suppression upon helper T cells across a nucleopore membrane.

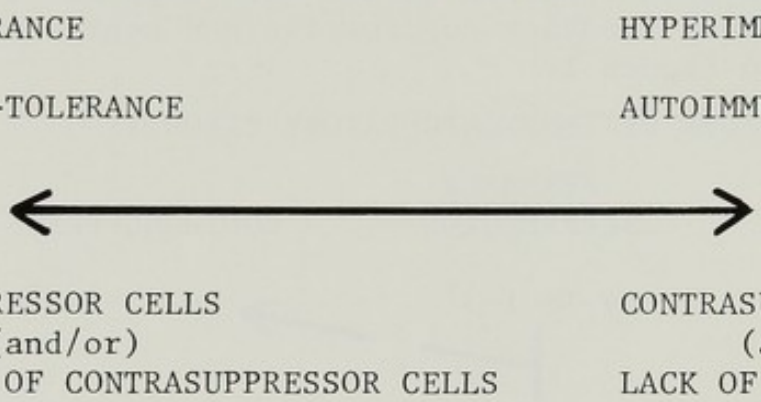
Thus, using the intermediate culture system, we have mimicked two types of natural immune states. Active tolerance was represented by the functional deletion of helper cell activity by suppressor cells, a deletion that persisted even after removal of the suppressors. Hyperimmunity was modeled as a contrasuppressive event with confers functional resistance to suppression upon T helper cells. The possible interrelationship(s) between feedback suppression and contrasuppression is shown in Figure 1.

FIGURE 1 THE STRUCTURE OF IMMUNOREGULATORY CIRCUITS



Tolerance, Hyperimmunity, Autoimmunity and the Continuum of Immune Responsiveness: It has often been observed that active tolerance can be produced by the action of suppressor T cells (22). The role of contrasuppressor T cells in maintaining a resistance to suppression in hyperimmune animals is currently under further investigation. As two ends of a continuum of immune responsiveness, however, these immune states might also be related to a lack of one or the other of these regulatory cells. Thus, it may be the ratio of suppressors to contrasuppressors that determines aspects of the state of responsiveness of the immune system (Figure 2).

FIGURE 2 A CONTINUUM OF IMMUNE RESPONSIVENESS



For example, the lack of contrasuppression might in some cases results in the phenotypic appearance of tolerance. This concept, originally proposed by Gershon (23), may help to explain the paradox in which an optimal immunizing dose of antigen is also optimal for the preparation of cells that secrete soluble suppressor factors (24). Suppressor cells might produce tolerant states in response to low and high doses of antigen, while the balancing effects of contrasuppression at optimal doses allows the expression of immunity.

Similar effects can be proposed for the hyperimmune end of the response continuum. We can consider autoimmunity as a special type of hyperimmune response in which the immune state is resistant to the forces of self tolerance. This shift from self tolerance to autoimmunity can be produced by a lack of suppressor cells (9). Removal of cells of the suppressor phenotype (Ly-2^+) resulted in animals with elevated anti-thyroglobulin, anti-thymocyte, and anti-erythrocyte (Coomb's test) levels. Animals restored with normal Ly-2 cells did not show these effects. In addition, the genetic propensity of the NZB mouse for autoimmunity has been associated with a deficiency in the suppressor circuit in these animals (25), although cause and effect is difficult to prove in this system.

On the other hand, the MRL/1 mouse presents with an autoimmune state accompanied by potent non-specific suppressor

cells, which can inhibit responses in the normal MRL/+ animals (26). It is possible that an imbalance in favor of contrasuppression in these animals produces these effects, which the animals suppressor cells cannot overcome. Preliminary evidence strongly suggests the presence of contrasuppressor activity in the lymph nodes of these animals (M. Horowitz, personal communication).

Thus, in considering a continuum of immune responsiveness, the immune state can be seen as a balance of opposing forces such as help, suppression, and contrasuppression. As we increase our understanding of the ways in which each of these regulatory pathways are themselves regulated we will perceive how a spectrum of immune states is achieved and how it can be manipulated. The prospect of such immunengineering has far reaching implications for the production of a more clinical spectrum of therapeutic techniques.

REFERENCES

(1) Eardley, D.D., (1980), *Fed. Proc. 39*, 3114.
(2) Gershon, R.K. (1981), *J. Exp. Med.* in press.
(3) Cantor, H., and Boyse, E.A., (1977), *Cold Spring Harbor Symp. of Quant. Biol. 41*, 23.
(4) Eardley, D.D., Hugenberger, J., McVay-Boudreau, L., Shen, F.W., Gershon, R.K., and Cantor, H., (1978), *J. Exp. Med. 147*, 1106.
(5) Cantor, H., and Boyse, E.A., (1975), *J. Exp. Med. 141*, 1376.
(6) Huber, B., Devinsky, O., Gershon, R.K., and Cantor, H., (1976), *J. Exp. Med. 143*, 1534.
(7) Shen, F.W., Boyse, E.A., and Cantor, H., (1975), *Immunogen. 2*, 591.
(8) Reinherz, E.L., and Schlossman, S.F., (1980), *Cell. 19*, 821,
(9) Cantor, H., and Gershon, R.K., (1979), *Fed. Proc., 38*, 2058.
(10) Stanton, T.H., Calkins, C.E., Jandinski, J., Schendel, D.J., Stutman, O., Cantor, H., and Boyse, E.A., (1978), *J. Exp. Med. 148*, 963.
(11) Kimura, A., Wigzell, H., Holmquist, G., Ersson, B., Carlsson, P., (1979), *J. Exp. Med. 145*, 473.
(12) Shreffler, D.C., and David, C.S., (1975), *Adv. Immunol. 20*, 125.
(13) Murphy, D.B., (1978), *Springer Sem. in Immunopath. 1*, 111.
(14) Eardley, D.D., Murphy, D.B., Kemp, J.D., Shen, F.W., Cantor, H., and Gershon, R.K., (1980), *Immunogen. 11*, 549.

(15) Glimcher, L., Shen, F.W., and Cantor, H. Manuscript in preparation.
(16) Shen, F.W., McDougal, J.S., Bard, S.F., and Cort, S.P., (1980), *J. Exp. Med. 151*, 566.
(17) Yamauchi, K., Green, D.R., Eardley, D.D., Murphy, D.B., and Gershon, R.K., (1981), *J. Exp. Med.* in press.
(18) Green, D.R., Eardley, D.D., Kimura, A., Murphy, D.B., Yamauchi, K., and Gershon, R.K. Submitted for publication.
(19) Ptak, W., Green, D.R., Durum, S.K., Kimura, A., Murphy, D.B., and Gershon, R.K. Submitted for publication.
(20) Green, D.R., Gershon, R.K., and Eardley, D.D. Submitted for publication.
(21) Haughton, G., and Adams, D.O., (1970), *J. Retic. Soc. 7*, 500.
(22) Gershon, R.K., and Kondo, K., (1971), *Immunol. 21*, 903.
(23) Gershon,R.K., In Immunological Tolerance (1974) D. Katz, and B. Benacerraf, eds., Academic Press, N.Y., p. 441.
(24) Asherson, G.L., Zembala, M., Thomas, W.R., and Perera, M.A.C.C., (1980), *Immunol. Rev. 50*, 3.
(25) Cantor, H., McVay-Boudreau, L., Hugenberger, L., Naidorf, F., Shen, F.W., and Gershon, R.K., (1978), *J. Exp. Med. 147*, 1116.
(26) Gershon, R.K., Horowitz, M., Kemp, J.D., Murphy, D.B., and Murphy, E.D. (Elsevier, N.Y., 1978) In Genetic Control of Autoimmunity (eds. Rose, N.R., Biguzzi, P.E., and Warner, N.L.), p. 223.

RECIRCULATION AND RADIOSENSITIVITY OF Lyt 1^+2^+ T CELLS

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ABSTRACT Evidence is presented that a large proportion of Lyt 1^+2^+ T cells are long-lived cells which reside in the recirculating lymphocyte pool. When transferred to irradiated mice, Lyt 1^+2^+ cells recirculate from blood to lymph more slowly than Lyt 1^+2^- cells but more rapidly than B lymphocytes. Lyt 1^+2^+ cells are more radiosensitive than Lyt1^+2^- cells but less radiosensitive than B cells.

INTRODUCTION

The discovery of Cantor and Boyse (1) that functionally distinct subsets of T cells can be distinguished on the basis of their Lyt phenotypes has proved of immense importance to the study of T cell biology. The Lyt 1^+2^- subgroup of cells comprises 50-70% of Thy 1-positive (T) cells and controls delayed-type hypersensitivity and collaborative interactions with B lymphocytes. Lyt 1^-2^+ cells are a minority population (2-5% of T cells) and control cell-mediated lympholysis and suppressor activity. A third subset of cells has the Lyt 1^+2^+ phenotype and accounts for 30-50% of T cells; the precise function of these latter cells is controversial (vide infra).

Although much progress has been made in defining the functions of these three T cell subsets, the ontogeny and differentiation of these cells is still far from clear. It was originally reported that nearly all T cells in very young mice have the Lyt 1^+2^+ phenotype (2). This finding led to the suggestion that Lyt 1^+2^+ cells differentiate into Lyt 1^-2^+ and Lyt 1^+2^- cells shortly after leaving the thymus. In a more recent study, however, it was reported that the proportion of Lyt 1^+2^+ cells is no higher in neonatal mice than in adult mice (3). This latter study is consistent with evidence from intrathymic labeling studies which suggests that the division of T cells into Lyt 1^+2^- and Lyt 1^+2^+ subgroups occurs in the thymus rather than in the periphery (4).

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Although Lyt 1^+2^- and Lyt 1^+2^+ cells appear to belong to quite separate lineages, the relationship between Lyt 1^+2^+ and Lyt 1^-2^+ cells has yet to be resolved. A point of particular interest here is that the proportion of Lyt 1^+2^+ T cells in the spleen falls sharply within a few weeks of adult thymectomy (2). This finding has led to the notion that most Lyt 1^+2^+ T cells are short-lived cells of recent thymic origin. In this article I will present evidence that a large proportion of Lyt 1^+2^+ cells are in fact long-lived cells which reside in the recirculating lymphocyte pool (RLP).

RESULTS

Proportions of Lyt 1^+2^+ T cells in spleen, lymph nodes, and thoracic duct lymph. Table 1 shows cytotoxic indices obtained on various sources of CBA/J lymphoid cells treated with monoclonal anti-Thy 1.2, anti-Lyt 1.1, anti-Lyt 2 (non-polymorphic), and anti-I-A^k (Ia.17) antibodies in the presence of complement. Lyt 1^-2^+ cells were virtually undetectable since anti-Thy 1.2 and anti-Lyt-1.1 antibodies lysed approximately the same proportion of cells. Lyt 1^+2^+ cells comprised approximately 30% of Thy 1.2^+ cells in normal or nylon-wool-purified spleen or lymph node cells. Thoracic duct lymphocytes (TDL) contained a similar proportion of Lyt 1^+2^+ cells. Significantly, adult thymectomy 6 weeks before did not reduce the content of Lyt 1^+2^+ cells in TDL; similar data were found in a previous study in

TABLE 1

PROPORTION OF Lyt 1^+2^+ T CELLS IN SPLEEN, LYMPH NODES, AND THORACIC DUCT LYMPH

Cells (CBA/J)	Cytotoxic index with:			
	anti-Thy 1.2	anti-Lyt 1.1	anti-Lyt 2 (% of θ^+ve)	anti-Ia.17
spleen	33	36	7 (21)	49
NW spleen	90	87	29 (33)	0
LN	68	69	22 (32)	30
NW LN	98	98	34 (35)	2
TDL	65	61	21 (32)	29
ATx TDL[a]	52	54	20 (39)	--

[a] ATx 6 wk before.

which TDL were examined at 1 year after thymectomy (unpublished data of P.C.L. Beverley and J. Sprent). Since most TDL form part of the RLP and are relatively homogeneous with respect to their lifespan (5), these data imply that a large proportion of Lyt 1^+2^+ T cells are long-lived recirculating lymphocytes.

Numbers of Lyt 1^+2^+ T cells mobilizable by thoracic cannulation. To quantitate the numbers of Lyt 1^+2^+ T cells entering the central lymph, normal adult CBA/J mice were subjected to continuous thoracic duct drainage for a period of three days. Over this period, 14×10^7 lymphocytes/mouse were collected, of which 8×10^7 were Thy 1.2^+ cells and 5×10^7 were Thy 1.2^-, Ia^k-positive (B) cells. Of the Thy 1.2^+ cells, 65% (5×10^7) were Lyt 1^+2^- and 35% (3×10^7) were Lyt 1^+2^+. From these data it can be calculated that of the total numbers of Lyt 1^+2^- and Lyt 1^+2^+ cells present in the spleen plus pooled LN of normal mice, 60-80% of these cells can be mobilized into the central lymph over a 3-day period. It should be mentioned that previous work has shown that the vast majority of lymphocytes entering the lymph over such a period are not the products of recent cell division (5).

Rate of recirculation and radiosensitivity of Lyt 1^+2^- cells. Finding cells in thoracic duct lymph is not proof that these cells reside in the RLP. For example, most plasmablasts in TDL are en route to the gut (5). To prove that cells recirculate, it is necessary to inject the cells intravenously and demonstrate that they appear in the central lymph. A convenient method here is to transfer lymphoid cells intravenously into irradiated mice and then monitor the appearance of donor-derived cells in the lymph of the host. Since small lymphocytes die rapidly in interphase after irradiation (6), the contribution of host lymphocytes to the cells entering the lymph is small. A priori, however, a large proportion of these residual radioresistant host cells might be of the Lyt 1^+2^+ phenotype and thus make it difficult to determine whether the Lyt 1^+2^+ T cells entering the lymph were of donor or host origin. In this respect it should be pointed out that the anti-Lyt 2 reagent used in this laboratory (which was kindly made available to us by Dr. Fitch and his collaborators of the University of Chicago) detects non-polymorphic determinants.

To examine the radiosensitivity of Lyt 1^+2^+ cells, normal mice were exposed to 300 Rads, 900 Rads, or no irradiation. The mice were cannulated 1 day later and TDL were collected over the following 15 hr. As shown in Table 2, irradiation caused a considerable reduction in TDL outputs,

TABLE 2

RADIOSENSITIVITY OF Lyt 1^+2^+ T CELLS

Treatment of TDL donors[a]	No. cells collected over 15 hr	Cytotoxic index with: anti-Thy 1.2	anti-Lyt 1.1	anti-Lyt 2	anti-Ia.17
0 R	56×10^6	82[b]	85	31(38)[c]	20
300 R	16×10^6	95	95	13(14)	3
900 R	7×10^6	99	99	4(4)	0

[a] Mice irradiated 0 hr; mice cannulated 24 hr; TDL collected 28-43 hr.
[b] Mean of 3 mice per group.
[c] Expressed as % of Thy 1.2^+ cells.

i.e., by 70% for 300 Rads and 90% for 900 Rads. This reduction was dramatic in the case of B cells, the proportion of Ia-positive cells falling from 20% to 3% for 300 Rads and to <1% for 900 Rads. The proportion of Thy 1.2^+ cells rose reciprocally, i.e., to 99% with 900 Rads. These data corroborate previous findings that B cells are more radiosensitive than T cells (6). Interestingly, as for B cells, irradiation caused a marked reduction in the proportion of Lyt 1^+2^+ cells. Thus, as a percentage of Thy 1.2^+ cells, the proportion of Lyt 1^+2^+ cells fell from 38% for unirradiated TDL to 14% for 300 Rads TDL and to 4% for 900 Rads TDL. These data imply that Lyt 1^+2^+ cells are more radiosensitive than Lyt 1^+2^- cells but less radiosensitive than Ia^+ (B) cells.

The finding that heavily irradiated mice were virtually devoid of Lyt 1^+2^+ T cells established the suitability of these mice as hosts for studying recirculation of donor Lyt 1^+2^+ cells. In the experiment shown in Table 3, CBA LN cells were transferred to H-2-compatible Lyt 1-incompatible irradiated B10.BR mice; the Lyt 1-difference between donor (Lyt 1.1) and host (Lyt 1.2) enabled direct detection of the donor Lyt 1-bearing T cells. The B10.BR hosts were exposed to 900 Rads and then cannulated 1 day later. TDL collected over the next 6 hr were very few in number (2×10^5/mouse) and consisted almost entirely of Thy 1.2^+ Lyt 2^- cells (Table 3). The cannulated mice were each injected with 8×10^7 CBA LN intravenously and TDL were collected

TABLE 3

DELAYED RECIRCULATION OF CBA/J Lyt 1^+2^+ T CELLS IN IRRADIATED B10.BR MICE

Time of TDL collection[a]	Cytotoxic index with: anti-Lyt 1.1	anti-Lyt 2	anti-Thy 1.2
(-6)-0 hr	0	1	99
0-14 hr	74	5	95
14-23 hr	92	12	97
23-37 hr	93	22	96
37-45 hr	92	26	95
45-64 hr	97	30	96

[a] B10.BR mice were given 900 R at -30 hr, cannulated at -6 hr, and given 8×10^7 CBA/J LN cells intravenously at 0 hr.

over the following 64 hr. During this period, 30% of the injected Lyt 1^+2^- cells and 15% of the Lyt 1^+2^+ cells were recovered; the recovery of Ia^+ cells was very low (<5%). Of particular interest was the finding that the first collection of TDL, which consisted of 74% donor (Lyt 1.1^+) cells, contained only 5% Lyt 2^+ cells. The proportion of Lyt 2^+ cells then rose progressively to reach 30% at 44-64 hr. These data indicate that although Lyt 1^+2^+ cells clearly show the capacity to recirculate, they do so at a slower rate than Lyt 1^+2^- cells but more rapidly than B cells.

DISCUSSION

The present finding that large numbers of Lyt 1^+2^+ cells reside in the RLP clearly has to be reconciled with the opposing evidence that Lyt1^+2^+ cells are short-lived cells of recent thymic origin. Perhaps the most likely explanation for this discrepancy is that there are in fact two distinct subsets of Lyt 1^+2^+ cells, one long-lived and the other short-lived. The data are quite compatible with this view since adult thymectomy does not lead to a total disappearance of Lyt 1^+2^+ cells: Cantor and Boyse (2) have emphasized that although the levels of Lyt 1^+2^+ cells decrease rapidly in the spleen after thymectomy, this level stabilizes at a plateau of about 50% of normal after four weeks.

One can only speculate as to the relationship and function of these two putative subsets of Lyt1$^+2^+$ cells. Although on a priori grounds it is possible that these cells are unrelated, perhaps the simplest view is that the short-lived cells are immature precursors of the long-lived set. If this were the case, one is still left with the problem of defining a function for the long-lived Lyt 1^+2^+ cells. To my mind the most attractive theory is that most Lyt1$^+2^+$ cells are the precursors of Lyt 1^-2^+ cytotoxic lymphocytes (CTL) (and other effector cells, e.g., suppressors (7)). Although there is firm evidence that Lyt 1^+2^+ cells can indeed give rise to Lyt 1^-2^+ cells in certain situations (reviewed in ref. 8), the precise relationship between these two sets of cells has yet to be resolved. Addressing this question experimentally is complicated by the fact that monoclonal anti-Lyt 1 reagents detect low but appreciable amounts of Lyt 1 antigen on "Lyt 1^-2^+" cells (9). Another problem is that the differentiation of CTL is controlled, albeit indirectly, by Lyt 1^+2^- helper cells (1).

In assessing the notion that CTL are derived from Lyt 1^+2^+ precursors, it is useful to consider the example of how B cells respond to antigen. In memory situations it is clear that antigen-reactive B cells reside in the RLP (reviewed in ref. 5). After contact with antigen, B cells differentiate in two directions. Some cells proliferate extensively, change their surface markers, and become terminally differentiated effector cells (plasma cells). In parallel, other cells clonally expand after responding to antigen but rejoin the RLP as memory cells with their surface markers unchanged. A similar scheme of asymmetric differentiation might apply to CTL precursors. Thus, contact of recirculating Lyt 1^+2^+ cells with antigen might induce some of these cells to lose (or lower their concentration of) the Lyt 1 antigen and become effector Lyt 1^-2^+ CTL. At the same time other cells would retain their Lyt 1^+2^+ phenotype and rejoin the RLP after proliferating.

The significance of the finding that Lyt 1^+2^+ cells recirculate at a slower rate than Lyt 1^+2^- cells is obscure. One possibility is that these cells have different routes of migration through the lymphoid tissues. In this respect it is of interest that Lyt 1^+2^+ cells have a greater tendency than Lyt 1^+2^- cells to localize in the splenic red pulp after intravenous injection (M. de Sousa, personal communication).

The observation that Lyt 1^+2^+ cells are appreciably more radiosensitive than Lyt 1^+2^- cells may account for the puzzling observation that low doses of irradiation often

enhance the immune response (6). The simplest interpretation of this finding is that low doses of irradiation selectively remove Lyt 2-bearing T suppressor cells.

REFERENCES

1. Cantor, H., Boyse, E.A. (1977) Immunol. Rev. 33, 105.
2. Cantor, H., Boyse, E.A. (1975) J. Exp. Med. 141, 1376.
3. Haaijman, J., Micklem, H.S., Ledbetter, J.A., Dangl, J. L., Herzenberg, L.A., Herzenberg, L.A. (1981) J. Exp. Med. 153, 605.
4. Scollay, R., Kochen, M., Butcher, E., Weissman, I.L. (1978) Nature 276, 79.
5. Sprent, J. (1977) In "The Lymphocyte: Structure and Function" (J.J. Marchalonis, ed.), Marcel Dekker, New York, p. 43.
6. Anderson, R.E., Warner, N.L. (1976) Adv. Immunol. 24, 216.
7. Eardley, D.D., Hugenberger, J., McVay-Boudreau, L., Shen, F.W., Gershon, R.K., Cantor, H. (1978) J. Exp. med. 147, 1106.
8. Roehm, N.W., Alter, B.J., Bach, F.H. (1981) J. Immunol. 126, 353.
9. Ledbetter, J.A., Rouse, R.V., Micklem, H.S., Herzenberg, L.A. (1980) J. Exp. Med. 152, 280.

CARRIER-SPECIFIC SUPPRESSION OPERATES THROUGH THE HAPTEN-SPECIFIC SYSTEM

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INTRODUCTION

Although Ig-oriented regulatory systems such as allotype and idiotype suppression have been well studied in their own right, they are usually treated as peripheral to regulation of the broadly heterogeneous antibody responses normally produced to hapten-carrier conjugates. Nevertheless, our recent studies show that a "hapten-specific" regulatory system which combines the properties of the allotype and idiotype systems plays a central role in controlling the amount, affinity and isotype representation in these kinds of antibody responses (1). In essence, the T cells and other cell populations active in this system selectively prevent or permit the expression of individual memory B cells according to the antibody combining site (variable region) and isotype (Igh constant region) commitment such B cells display.

Although the hapten-specific system has remained cryptic through roughly 10 years of intensive study of the carrier-specific regulatory systems commonly considered to be the principle mechanisms controlling heterogeneous antibody responses, its presence is clearly discernable in retrospect. That is, once the properties of the hapten-specific system are recognized, the data from these earlier studies become interpretable in terms of carrier-specific mechanisms which induce the hapten-specific system to support or suppress antibody production. Thus data presented here, which directly demonstrate that carrier-specific suppressor T cells (CTs) regulate antibody production in this way, introduce a substantially new perspective on how the immune system is organized and how "ordinary" antibody responses are controlled.

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We cannot present a full description of our studies within the space allotted in this volume. Thus we have chosen instead to outline our symposium presentation and hope that the reader will seek more detailed information from our recent publications (1-5).

I. OVERVIEW

Antigenic molecules have a series of structural determinants (haptens) to which B cells produce antibody (see Fig. 1). In addition, these molecules must have at least one (carrier) determinant used by "carrier-specific" regulatory T cells (CTs and CTh) to present the haptens on the antigen to B cells and other regulatory T cells.

The "hapten-specific" regulatory system (see Fig. 2) is composed of parallel elements that directly regulate the expression of memory B cells according to the structure of the antibodies such cells are committed to produce, i.e., according to the specificity and affinity the antibody displays for haptens on the antigenic molecule and the isotype and allotype of the antibody heavy chain constant region.

Each of the elements in the system can be independently induced to either suppress or support antibody production (depending on conditions under which "its" hapten is first introduced). Once induced to suppress antibody production, however, an element will resist subsequent induction (shifting) into a supportive (help) configuration and vice versa. Thus these elements provide the system with a selective bistable regulatory capability that allows the spectrum of antibodies produced in a given response to be defined initially and then maintained through subsequent antigenic stimulations unless conditions change dramatically.

Regulatory systems that more directly "sense" the immunologic environment (e.g., the carrier-specific suppression and allotype suppression systems) induce the hapten-specific elements to suppress or support antibody production. Signals from these systems appear to be independently integrated within each of the elements to yield a final set of decisions defining the character of the antibody response generated under a given set of conditions. Thus the hapten-specific system offers a common channel through wich a combination of diverse regulatory influences can be expressed in an orderly fashion.

Carrier-specific suppressor T cells (our primary concern here) induce hapten-specific elements to suppress production of the antibodies they regulate. Carrier-specific helper

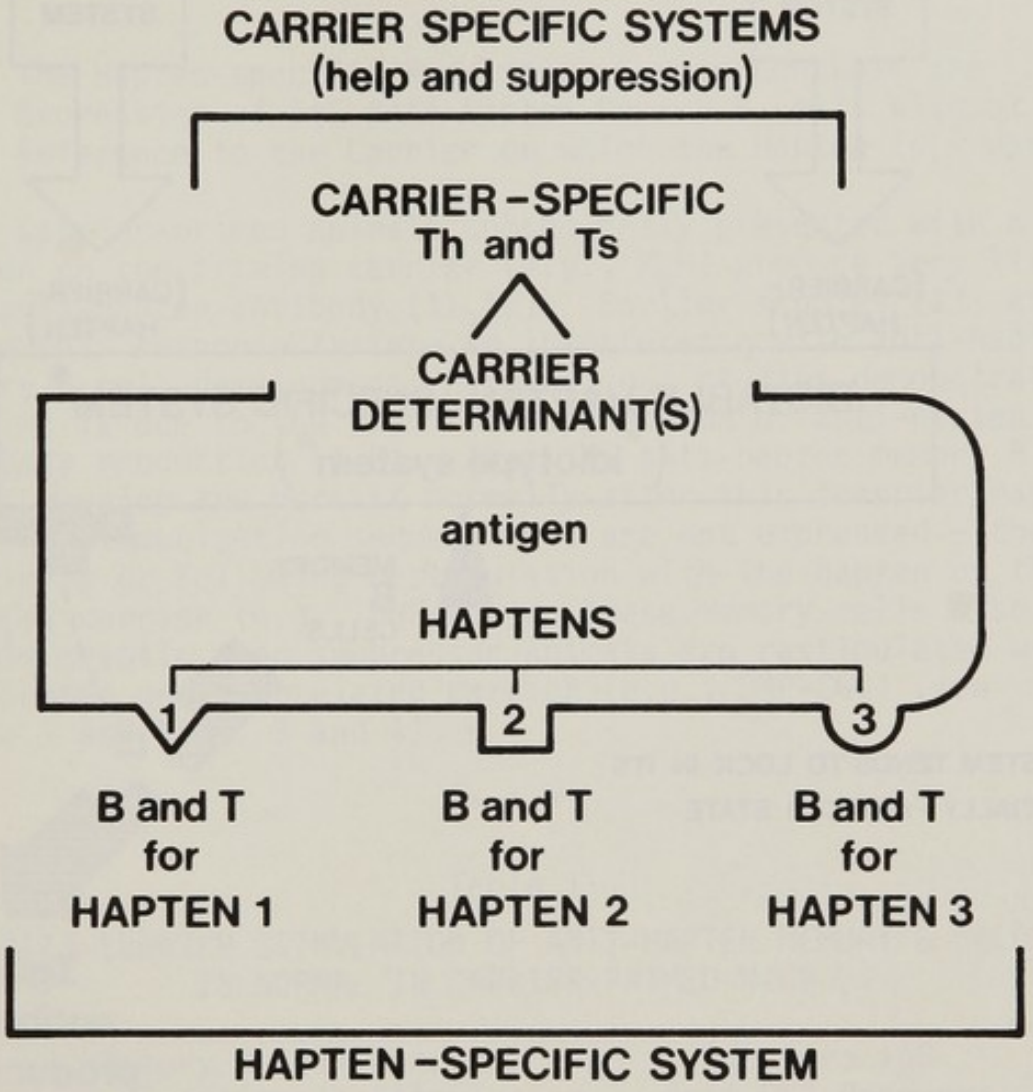


Figure 1. Schematic of an Antigenic Molecule.

Haptenic and carrier determinants are functionally distinct; however, the same structure could, in principle, serve as a haptenic determinant in one instance and a carrier determinant in another.

* * *

T cells appear to provide pressure in the opposite direction, i.e., to induce hapten-specific elements to support antibody production. Which of these activities prevails depends largely on whether the more slowly maturing CTs population has reached full functionality before or after a given hapten is presented on the priming carrier, i.e., whether antibody production to the hapten become established before the CTs population becomes capable of inducing suppression for it.

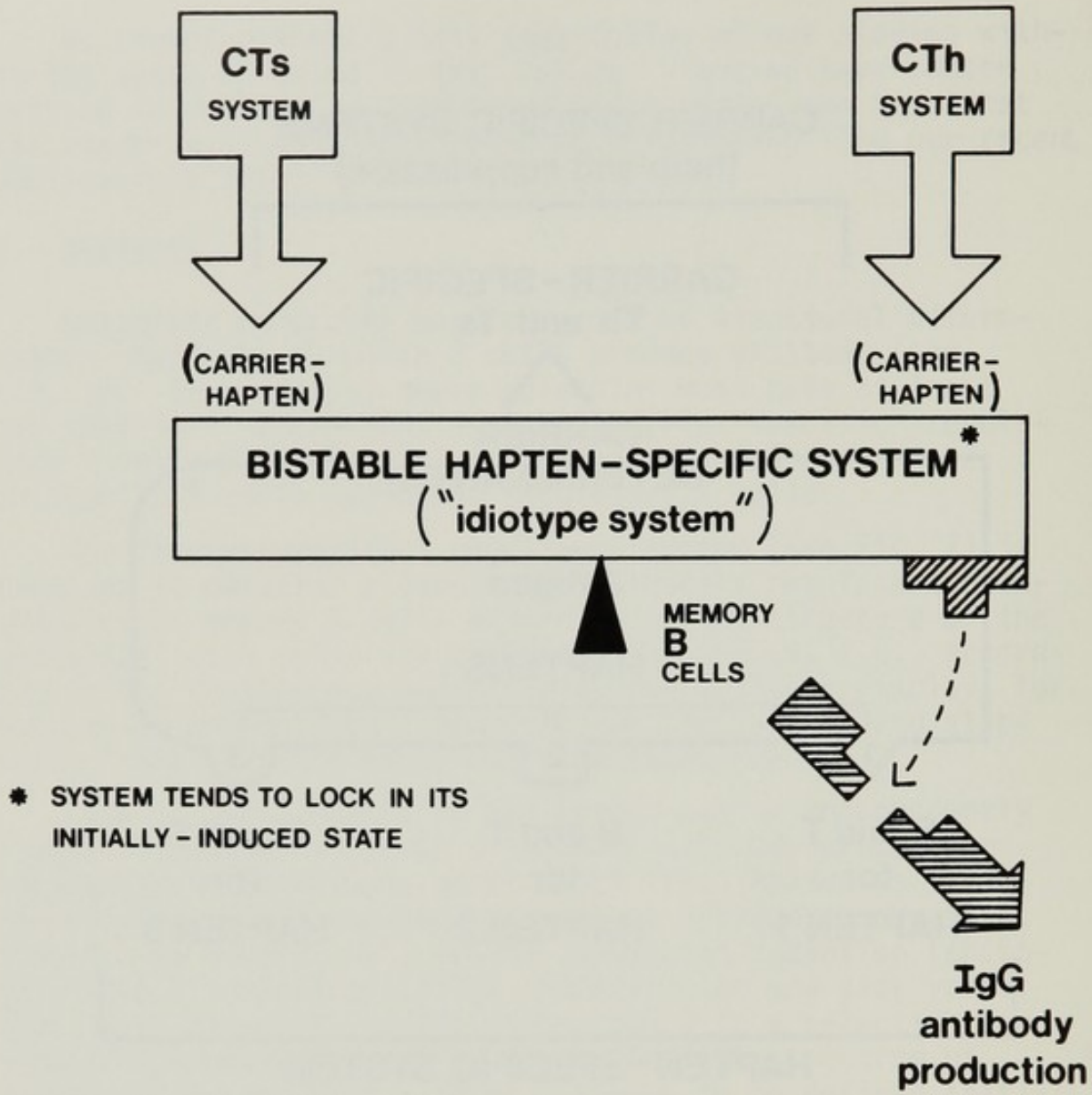


Figure 2. Simplified Schematic for Antibody Response Regulation.
CTs = carrier-specific suppressor T cells
CTh = carrier-specific helper T cells
See Overview in text for the rationale.

* * *

Contrary to previous belief, carrier-specific populations show no evidence of regulating each other's activity in the KLH-specific systems where they have been most extensively studied (e.g., 6-10). That is, CTs do not interfere with delivery or maintenance of carrier-specific help and CTh do not interfere with CTs activity. Thus the precedent for "feedback" suppression loops and other regulatory mechanisms that ostensibly rely on interactions between carrier-specific populations is severely undermined. The mechanism operant in these systems therefore requires re-examination with appropri-

ate diagnostic methods to determine whether they also operate by inducing hapten-specific suppression.

II. The Hapten-specific Regulatory System Controls the Expression of IgG Anti-hapten Memory B cells Without Reference to the Carrier on which the Hapten is Presented

Carrier-primed animals subsequently presented with a new hapten on the priming carrier (e.g., KLH) produce very little IgG anti-hapten antibody (11,12). Earlier studies (13) attributed this response failure to interference with anti-hapten memory B cell development; however, our studies demonstrate that it is due to the specific suppression of anti-hapten antibody production (1-5). That is, anti-hapten memory B cells develop and persist normally after this "carrier/hapten-carrier" immunization sequence but are not expressed either initially or following restimulation with the hapten on the priming carrier (e.g., DNP-KLH). These memory cells also remain cryptic when suppressed animals are restimulated with the hapten on an unrelated carrier (e.g., DNP-CGG) (see Table 1 and Figs. 3 and 4).

* * *

Table 1

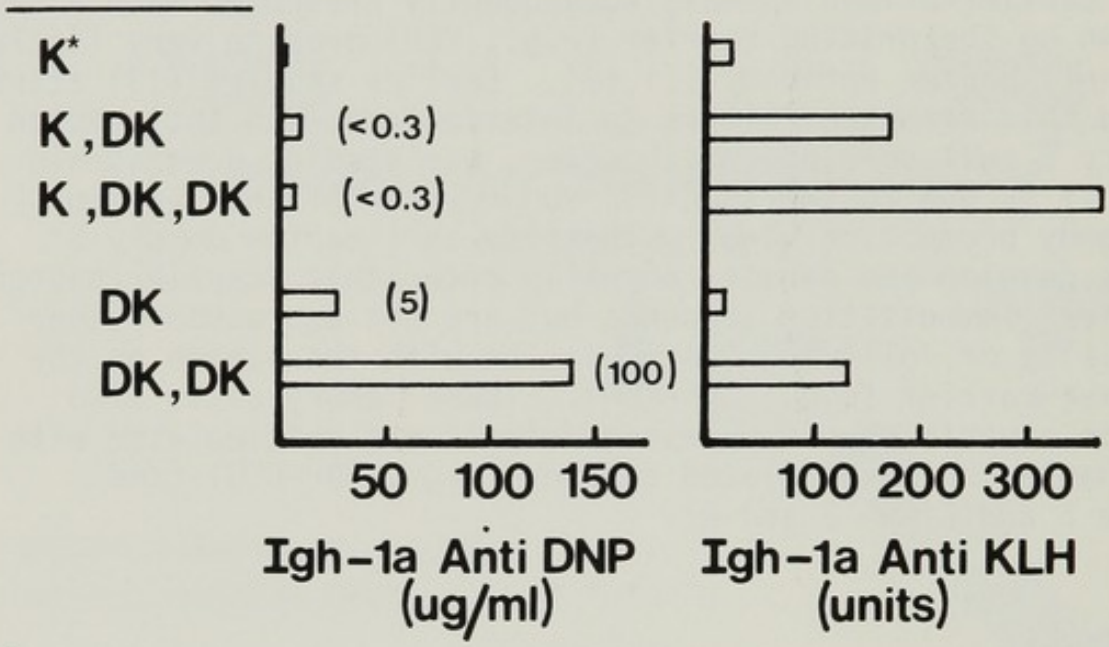
HAPTEN-CARRIER STIMULATION OF ANTI-HAPTEN MEMORY B CELLS IS NORMAL IN CARRIER-PRIMED MICE

Memory B Cell Donors Form of Antigenic Stimulation			Adoptive IgG Anti-DNP Response	
Group	KLH	DNP-KLH	Units	Mean K_a ($\times 10^6$)
I	*	Alum	73	10
II	Alum	Alum	73	8
III	*	Aqueous	18	0.7
IV	Alum	Aqueous	50	8

Donors (BALB/c x SJL) were killed 3 weeks after the first indicated stimulation with DNP-KLH. Splenic B cells were prepared by cytotoxic depletion of T cells using monoclonal anti-Thy-1.2 (30-H12, J. Ledbetter). Cells obtained from 10^7 spleen cells were co-transferred with 2×10^6 nylon-passed syngeneic T cells from KLH-primed donors (100 µg on alum + 10^9 *Bordetella pertussis* at least 6 weeks before transfer).

Recipients (650 rad irradiated BALB/c) were stimulated with 1 μg aqueous DNP-KLH (intravenous) at time of transfer and bled 1 week later for assay. Assay details are as described in Ref. 14. Igh-1b (IgG_{2a}) response data are shown here. Igh-1a (IgG_{2a}) and IgG_1 response data were similar.

* * *



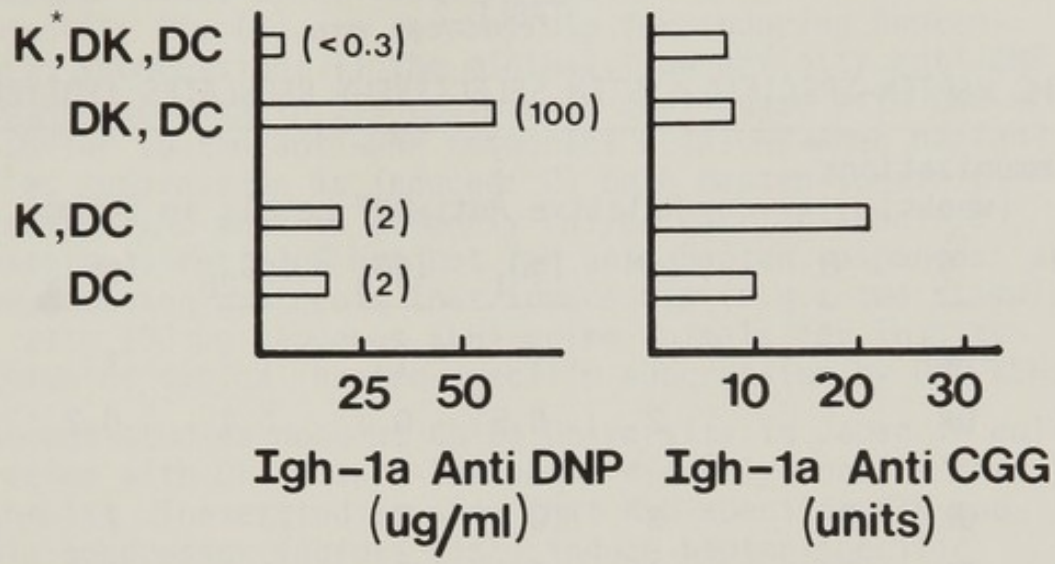
*K = KLH; DK = DNP-KLH

Figure 3. Anti-DNP Responses are Suppressed in KLH-Primed Mice Exposed to DNP-KLH.

Each indicated antigenic stimulation was given as 100 μg of antigen on alum, i.p., at approximately 6 week intervals. Responses were measured 2 weeks after last indicated stimulation; radioimmunoassay (RIA) units shown are relative to "standard" secondary response antiserum pools. Mean K_a (in parentheses, x 10^6) was determined from RIA binding. Assay details are described in Ref. 14.

* * *

This suppression does not interfere with antibody production to either the priming carrier or the unrelated (second) carrier molecule. Such interference should certainly have been detectable in the primary response mounted to the unrelated carrier (even if the secondary and tertiary responses to the initial priming were sufficiently strong to mask



*K = KLH; DK = DNP-KLH; DC = DNP-CGG

Figure 4. The carrier/hapten-carrier exposure sequence induces hapten-specific suppression.

See Legend to Figure 3.

* * *

adverse effects). Thus data from these experiments clearly demonstrate that the suppressive mechanism is "hapten-specific" in that it suppresses responses to one haptenic determinant on an antigenic molecule without impairing antibody production to other determinants on the same molecule (See Fig. 4).

These data also demonstrate that the suppressive mechanism does not deplete carrier-specific help (since depletion of such help would impair anti-carrier as well as anti-hapten responses). Therefore, the failure of the IgG anti-hapten antibody response in carrier/hapten-carrier immunized animals is clearly ascribable to a suppression-effector mechanism that operates independently of carrier-specific interactions.

In sum, suppression occurs in carrier/hapten-carrier immunized animals that have normal anti-hapten memory B cell populations and normal carrier-specific helper T cell populations. In this sense, it mimics a concerted idiotype suppression directed at preventing the expression of memory B cells capable of producing antibodies with combining sites for the "new" hapten introduced in the carrier/hapten-carrier immunization sequence. However, the ability to selectively suppress production of individual isotypes with such combining sites (see Table 2) distinguishes the hapten-specific system from currently known idiotype-suppression systems. Thus these data

TABLE 2

THE HAPTEN-SPECIFIC SYSTEM SELECTIVELY REGULATES ISOTYPE

Immunizations* (weeks)			Relative Anti-DNP Levels in Serum†				
0	4	8	IgM	IgG_1	IgG_3	IgG_{2b}	IgG_{2a}
-	DK§		1	1	1	1	1
K	DK		2	0.5Ψ	0.3	0.4	0.2
-	DK	DK	3	10	8	13	7
K	DK	DK	4	8	2	1	0.3

* >10 BALB/c x SJL mice per group; 100 μg each antigen on alum

† Normalized means of responses measured by RIA two weeks after last immunization. See Fig. 2 for representative IgG_1 and IgG_{2a} responses in individual animals.

§ K = KLH; DK = DNP-KLH

Ψ Broadly distributed

* * *

define a novel regulatory mechanism of prime importance for determining the composition of heterogeneous antibody responses.

III. Carrier-specific Suppressor T Cells Induce Hapten-specific Suppression

As indicated above, DNP-KLH induces suppression in KLH-primed mice (see Figs. 3 and 4). Similarly, DNP-CGG induces suppression in CGG-primed mice. Nevertheless, KLH-primed mice produce normal anti-DNP responses to DNP-CGG and vice versa (except when suppression has already been induced by previous exposure to DNP on the initial carrier). Thus hapten-specific suppression is induced only when carrier-primed animals are immunized with (a new) hapten on the priming carrier. In other words, although the suppression-effector mechanism operates independently of carrier-specific interactions, suppression is induced by a carrier-specific mechanism.

Several lines of evidence suggest that the "traditional" KLH-sepcific CTs (6) are responsible for inducing hapten-specific suppression: 1) the minimal, low affinity anti-DNP responses reported in CTs recipients challenged with DNP-KLH are similar to the anti-DNP responses obtained when hapten-specific suppression is induced: 2) both hapten-specific suppression and what is commonly called carrier-specific suppression affect IgG but not IgM anti-hapten responses; and, 3) immunization protocols that induce CTs (e.g., two stimulations with 100 μg aqueous KLH) prime animals for in situ induction of typical hapten-specific suppression by DNP-KLH.

Recent studies done at Chiba University in Japan in collaboration with Dr. Masaru Taniguchi directly confirm this hypothesis. These studies show that KLH-specific CTs and soluble suppressor factors (TsF) induce hapten-specific rather than carrier-specific suppression in "traditional" in vivo CTs assays. That is, IgG anti-DNP responses are sepcifically suppressed when unprimed (non-irradiated) recipients of these cells and factors are challenged with DNP-KLH and remain suppressed when recipients are subsequently challenged with DNP-CGG (see Tables 3 and 4). Thus although matching between carrier and hapten-carrier conjugate is clearly required for suppression induction, once suppression is induced it suppresses antibody production to the hapten on an unrelated carrier.

* * *

TABLE 3

CTs FACTORS (TsF) INDUCE HAPTEN-SPECIFIC SUPPRESSION

KLH TsF*	Antigenic Stimulation†		IgG_{2a}
day 0	day 0	4 weeks	Anti-DNP
no	DK	--	100
yes	DK	--	38
no	DK	DC	295
yes	DK	DC	70

*Tada protocol: sonicated extract from 5 x 10^7 KLH-primed spleen (aqueous, 2X)

†100 μg each antigen on alum; responses measured 2 weeks after last stimulation.

TABLE 4

CARRIER-SPECIFIC CARRIER CELLS INDUCE HAPTEN-HAPTEN SUPPRESSION

KLH 2^0 Cell Transferred*	Recipient Immunizations			IgG_{2a} Antibody Responses (RIA)†		
	0	4	8	Anti-DNP (μg/ml)	Anti-KLH (Units)	Anti-CGG (Units)
Spleen	DK	DK		20	8	n.t.
"	DK	DK	DC	10	n.t.	35
Spleen (T-depleted)	DK	DK		140	11	n.t.
"	DK	DK	DC	125	n.t.	20
None	DK	DK		120	9	n.t.
"	DK	DK	DC	100	n.t.	39

*Tada protocol: 100 μg aqueous KLH at -4 and -2 weeks; 5×10^7 BALB/c spleen (or remainder after Thy-1 + C').

†100 μg each antigen on alum; response measured 2 weeks after last stimulation

* * *

Anti-KLH and anti-CGG responses in CTs recipients proceed normally, again indicating that hapten-specific suppression has been induced (see Table 3). Similarly, anti-CGG responses proceed normally in TsF recipients subsequently immunized with DNP-CGG despite pronounced suppression of the anti-DNP response to this antigen. Thus, in both kinds of recipients, the data clearly demonstrate that hapten-specific suppression is induced by the initial exposure to DNP-KLH.

Anti-KLH responses, however, are impaired in TsF recipients immunized with DNP-KLH but not in similarly immunized CTs recipients. This impairment apparently occurs in these mice but not in recipients of KLH-primed spleen cell populations containing CTs because these populations also contain the hapten-specific cell populations that support antibody production to the native determinants on the KLH molecule and hence prevent suppression-induction for these determinants (but not for DNP on KLH). The soluble TsF preparation, in contrast, lacks

these cell populations. Thus when TsF recipients are challenged with DNP-KLH, all of the determinants on the KLH molecule are treated as "new" haptenic determinants and the bistable hapten-specific system is induced to suppress rather than support antibody production to these determinants.

Similar results are obtained by priming animals with KLH or DNP-KLH under conditions where the animal is prevented from producing an antibody response to the antigen. For example, when young allotype (Igh-1b) suppressed mice are primed with DNP-KLH, they fail to produce Igh-1b antibodies to either DNP or KLH. When these animals go into remission (about 12 weeks of age), they develop the ability to produce 1b antibodies to any subsequently introduced antigen but nonetheless fail to produce antibodies to KLH or to DNP introduced on either KLH or CGG. Since these DNP-KLH-primed animals show normal 1b anti-DNP and 1b anti-KLH memory B cells and mount a normal primary response to CGG, their inability to produce antibodies to DNP and KLH determinants clearly is ascribable to hapten-specific suppression. We have suggested that this suppression is induced because the cell populations that normally support 1b production and thereby prevent suppression induction are depleted (by allotype Ts). Thus CTs, once mature, can induce hapten-specific suppression for the 1b antibody responses.

The evidence we have obtained contradicts the common belief that CTs regulate antibody production by depleting CTh. That is, we have shown that CTs induce hapten-specific suppression, that the hapten-specific suppression mechanism operates independently of the carrier on which the hapten is presented, and that CTh are present and functional in suppressed animals. Thus earlier evidence based only on measurement of anti-hapten responses and only on measurement of responses following a stimulation with a single hapten-carrier conjugate (rather than sequential stimulation with two different conjugates) yielded ambiguous evidence with respect to whether CTs depleted CTh or induced hapten-specific suppression. (It is curious that although those of us interested in regulatory immunology reviewed this evidence extensively and repeatedly over the years, we nonetheless failed collectively to recognize its potential ambiguity.)

In sum, studies discussed here create a substantially different picture of the mechanisms involved in carrier-specific regulation. Our data indicate the following:

1) Carrier-priming generates both carrier-specific suppressor T cells (CTs) and carrier-specific helper (CTh) T cells in the same animal.

2) CTs do not deplete CTh.

3) CTs suppress by inducing specific suppression for IgG anti-hapten antibody production when confronted with "new" hapten on the priming carrier

4) CTs do not generally induce suppression for determinants on the priming carrier because the bistable properties of the hapten-specific system premit the establishment and maintenance of antibody production to initially introduced determinants despite the subsequent maturation of the CTs population.

The generality of these conclusions requires testing since our analysis has mainly been restricted to antibody responses stimulated by hapten-carrier conjugates. The idea that CTs deplete CTh, however, derives largely from these kinds of studies. Thus, there is ample reason to suspect that many if not all antigen-induced suppressions currently under study are based on mechanisms similar to the carrier-specific induction of hapten-specific suppression described here.

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REFERENCES

1. Herzenberg, L. A., Tokuhisa, T., and Herzenberg, L. A. (1980) Nature 285, 664.
2. Herzenberg, L. A., Tokuhisa, T., Parks, D. B., and Herzenberg, L. A. (1981) In: The Immune System, S. Karger AG Pub., Basel, Switzerland (in press).
3. Herzenberg, L, A., Tokuhisa, T., and Herzenberg, L. A. (1981) Immunology Today (in press).
4. Herzenberg, L. A., Tokuhisa, T., and Herzenberg, L. A. (1981) (submitted)
5. Herzenberg, L. A., Tokuhisa, T., Parks, D. R. and Herzenberg, L. A. (1981) (manuscript in preparation)
6. Tada, T. (1974) In: Immunological Tolerance: Mechanisms and Possible Therapeutic Applications, (eds) Katz, D. H. and Benacerraf, B., Academic Press, New York, pp. 471-492.

7. Tada, T., Okumura, K. (1979) Advances in Immunol. 28, 1.
8. Tada, T., Taniguchi, M., and Tokuhisa, T. (1977) In: Ir Genes and Ia Antigens, (ed) McDevitt, H. O., Academic Press, New York, pp. 517-527.
9. Eardley, D. and Gershon, R. K. (1975) J. Exp. Med. 142, 524.
10. Eardley, D. D. and Sercarz, E. E. (1976) J. Immunol. 116, 600.
11. Mitchison, N. A. (1971) Eur. J. Immunol. 1, 10.
12. Rajewsky, K., Schirrmacher, V., Nase, S., and Jerne, N. K. (1969) J. Exp. Med. 129, 1131.
13. Katz, D., Paul, W. E., Goidl, E. A. and Benacerraf, B. (1970) J. Exp. Med. 132, 261.
14. Herzenberg, L. A., Black, S. J., Tokuhisa, T. and Herzenberg, L. A. (1980) J. Exp. Med. 151, 1071.

THE ROLE OF IDIOTYPE AND THE MHC IN SUPPRESSOR T CELL PATHWAYS[1]

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INTRODUCTION

In the decade since Gershon and Kondo (1) first demonstrated the existence of suppressor T cells (Ts) in a model of 'infectious tolerance,' a great deal of attention has been focused on how Ts are induced and on how they carry out their regulatory activity. Beginning with the observation by Kapp et al. (2) that genetic nonresponder mice produced a predominant Ts response when primed with the synthetic polypeptide antigen L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), our laboratories have studied in depth several model suppressor systems involving both humoral and cell mediated immune responses. The two major themes which have emerged from this work are: 1) the requirement for interactions between multiple distinct T cell subsets for the efficient action of the suppressor regulatory pathway and 2) the importance, respectively, of genes in the mouse major histocompatibility complex (H-2) on chromosome 17, and of genes linked to the Igh complex on chromosome 12 in the control of these interactions. This paper will summarize the experimental models employed in our studies, the key experiments underlying the main findings, and the overall scheme involved in Ts generation and activity, as we now see it. These results will be briefly compared to other well studied suppressor models and some important unresolved issues discussed.

THE EXPERIMENTAL MODELS

Several different experimental systems have been employed to study Ts, in part for technical reasons, and in

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part to permit comparison of results in models of cell-mediated vs humoral immune responses. The following section summarizes the various systems according to the antigen used.

L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT): GAT is a random linear synthetic polypeptide, the response to which is under dominant H-2 linked immune response (Ir) gene control (3). H-$2^{a,b,d,k}$ are responding haplotypes. H-$2^{p,q,s}$ are nonresponding haplotypes. Administration in vivo of alum-pertussis and GAT electrostatically complexed to the carrier methylated bovine serum albumin (MBSA) elicits IgG GAT specific plaque-forming cells (PFC) in nonresponder mice (4). Spleen cells from nonresponder mice will also make in vitro PFC responses to GAT-MBSA (5). Administration of GAT in alum elicits a Ts response in nonresponder strains, as measured by the ability of T cells from such mice to suppress the response of syngeneic animals to GAT-MBSA (7). Analysis of anti-GAT antibodies in responder and nonresponder strains has revealed a major set of idiotypic determinants called CGAT which are present on the majority of anti-GAT antibodies of all tested mouse strains, irrespective of Igh allotype markers (6-8).

L-glutamic acid^{50}-L-tyrosine^{50} (GT): This model is identical to the GAT model with the following exceptions: 1) no otherwise immunologically normal inbred mice respond to GT, 2) nonresponders can be divided into those showing Ts activity after GT administration, and those failing to do so (9,10). A minimum of two genes, located in the I-A and I-C subregions, control the ability to show GT-specific Ts activity (11-13). These genes interact in a complex complementation pattern.

Azobenzenearsonate (ABA): The cell-mediated aspects of ABA immunity and suppression have been studied using an in vivo model of delayed type hypersensitivity. ABA-conjugated spleen cells (ABA-SC) are injected subcutaneously into syngeneic mice, which are challenged in the footpad with the active ABA diazonium salt 4-5 days later, and DTH read as swelling after 24 hr (14). Ts are induced by i.v. injection of such ABA-SC, and can be detected in the spleens of such treated mice 6-7 days later (15). Most studies have been carried out in A/J mice, which possess a major cross-reactive idiotype (CRI) on a large fraction of their anti-ABA antibodies, a trait shown to be linked to the Igh-1 locus (16).

Some recent studies have utilized Ts induced by injection into A/J mice of SC conjugated with CRI^+ immunoglobulin (17). Further, both ABA-SC and CRI-SC induced Ts have been tested in in vivo and in vitro anti-ABA humoral response systems, involving the serum antibody response to ABA-KLH in the former case (18), and the PFC response to ABA-brucella in the latter (Rock et al., unpublished observations).

4-hydroxy-3-nitrophenyl-acetyl (NP): This cell-mediated model is similar to the ABA system, in that Ts are induced by NP-SC given i.v. (19). However, immunity is established by giving 1) cyclophosphamide pretreated C57BL/6 mice NP-BGG in CFA s.c., followed 6 days later by challenge with NP-BSA (20) or 2) NP-O-succinimide s.c. followed 6 days later by footpad challenge with the same reactive compound (21). The B6 mice used for most of these studies produce anti-NP antibodies bearing a major allotype linked idiotype termed NP^b (22). Further, Ts generated by the i.v. administration of NP-coupled syngeneic cells can suppress the in vivo and in vitro anti-NP humoral response in an idiotype-specific fashion (23).

THE SUPPRESSOR PATHWAY

Although minor differences in the time of appearance of the various Ts, and the requirements for additional antigen stimulation in eliciting different steps in the pathway exist, the scheme outlined below, and the characteristics of the T cells and subcellular factors that are given, are derived from concordant data obtained in a variety of the experimental systems outlined above. Where one model has been particularly important in establishing a given point, this fact will be mentioned. Each identified T cell and factor will be reviewed in outline, and the entire pathway summarized in Fig. 1.

Ts_1 (Table 1): The first identified T cell in the suppressor pathway has been termed Ts_1. This cell is probably equivalent to the 'inducer' of feedback suppression described by Eardley et al. (24) for the in vitro SRBC response. Apparently derived from a precursor cell sensitive to low (<20 mg/kg) dose cyclophosphamide treatment (25), the Ts_1 is an Lyt 1^+2^-, $I\text{-}J^+$, Thy 1^+ cell (26, Sy et al., manuscript in preparation). It can first be identified functionally in the spleen of primed animals 3-7 days after antigen administration . Studies in both the ABA and NP models have shown that this cell bears antigen receptors

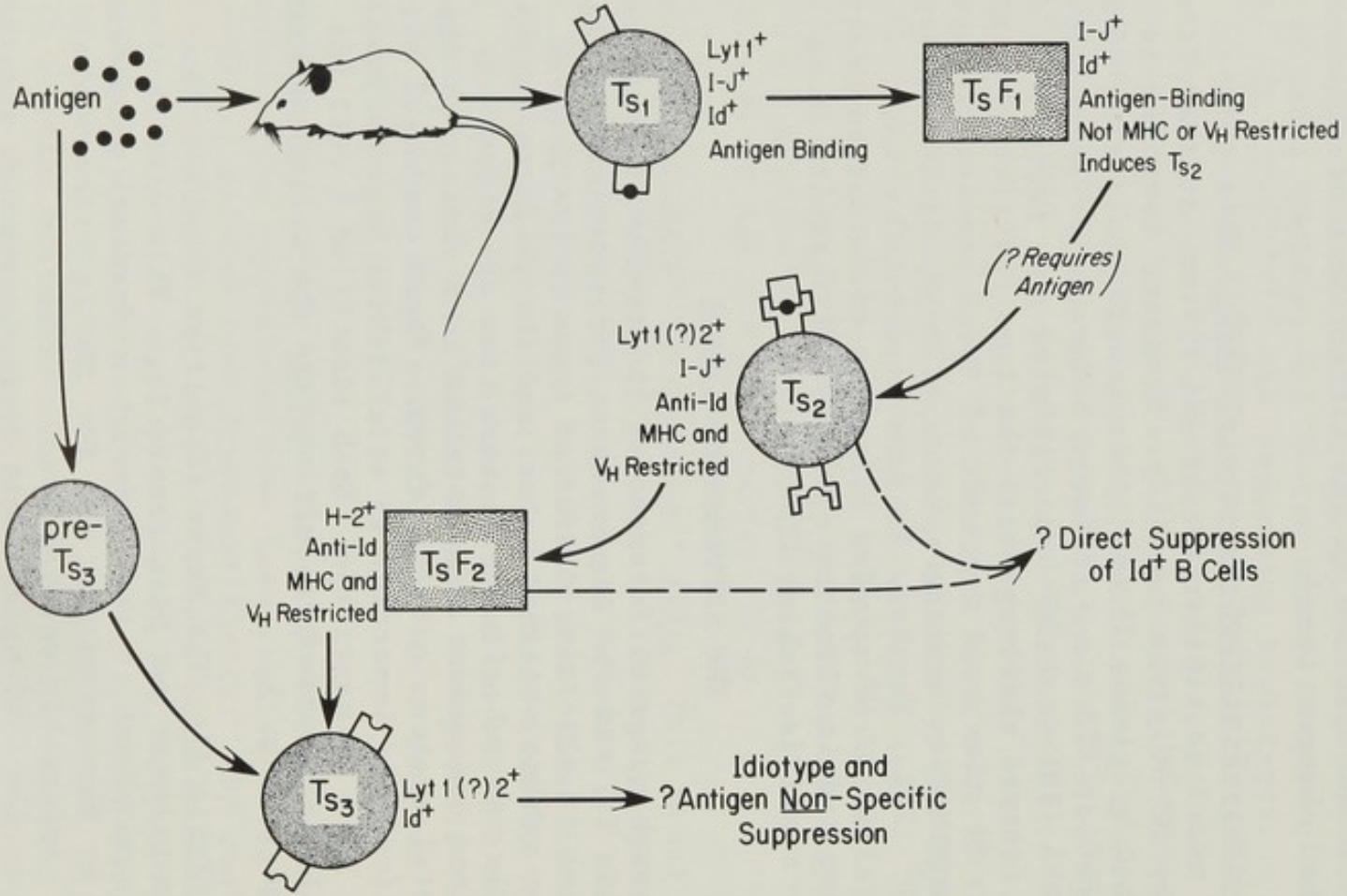

Fig. 1. Generalized T Suppressor Pathway

enabling it to bind to hapten coated plastic surfaces without the involvement of MHC gene products (27,28). Further, Ts_1 can be killed by treatment with anti-idiotypic antibodies (anti-CRI in the A/J-ABA model (29), anti-NP^b in the B6-NP model (19)) and complement. The appearance of the serological determinants detected by these antiidiotypic antisera on Ts_1 is controlled by genes linked to the Igh complex. Ts_1 are suppressive only when given at the time of antigen priming, and not when given to previously primed recipients, thus characterizing them as 'afferent' suppressors (30). For this reason, complications of 'allogeneic effects' prevent direct assessment of whether or not the activity of Ts_1 is H-2 restricted (but see section on TsF_1 below). Transfers between H-2 identical animals differing in background genetic constitution, including congenic pairs differing only with respect to genes linked to Igh-1, have failed to demonstrate <u>true</u> 'V_H' restriction of Ts_1 function. However, Ts_1 in the ABA (31) models will not suppress Igh-1 different mice directly. Despite this, it can be shown that these cells, or their functional product TsF_1, do act appropriately in such recipients, and the failure to observe suppression is due to the role of Igh-linked genes in controlling idiotypic interactions further along the suppressor pathway, as shown in the ABA model (see below). To date, the only activity of Ts_1 observed is the stimulation, presumably via a subcellular soluble product termed TsF_1, of a second set of Ts called Ts_2.

TABLE 1
Ts_1 SUMMARY

1.	Lyt 1^+
2.	$I\text{-}J^+$
3.	Antigen binding and idiotypic (CRI for ABA; NP^b for NP)
4.	Induced by tolerizing regimes (e.g. haptenated cells i.v.)
5.	Precursor is cyclophosphamide sensitive
6.	H-2 restriction untested
7.	Not V_H restricted
8.	Afferent suppressor
9.	Produce TsF_1

TsF_1 (Table 2): Based on the report by Tada and colleagues of a soluble suppressor factor derived from disrupted protein-specific Ts (32), Kapp et al. (33) examined GAT-specific Ts for such material, and found an antigen-specific suppressor material in the crude cell-free sonicate of spleens containing GAT-Ts. Subsequent study in the GAT (34-36), GT (37), and ABA (38) systems has shown the active principle of such soluble preparations to be a protein-containing structure of ~40-60,000 dalton MW. Immunochemical studies using solid phase adsorption and functional bioassay have demonstrated that a single molecule (or molecular complex) has specific antigen binding activity, bears I-J subregion controlled serologic determinants, and, like the cell from which it is derived, shares (Igh-1 controlled) idiotypic determinants with antibody of the same specificity. Again, like the Ts_1 cell source, these TsF_1 are afferent suppressive materials. They show no qualitative or quantitative H-2 restrictions when tested in a variety of H-2 haplotypes (39,40, Sy et al., manuscript in preparation). ABA-TsF_1 does not directly suppress Igh-1 different recipients, but it functions apparently normally in such recipients, eliciting a second set of suppressors termed Ts_2 which can be detected by transfer into recipients of appropriate Igh-1 type (Sy, unpublished observations). Thus, the Igh-restriction of TsF_1 can be termed a 'pseudorestriction', not related to the intrinsic activity of TsF_1 across Igh-1 differences, but to features of the distal Ts pathway, as will be indicated below.

TABLE 2
TsF_1 SUMMARY

1.	$I\text{-}J^+$
2.	Antigen-binding and idiotypic (CRI for ABA, CGAT for GAT)
3.	Not H-2 restricted in activity
4.	Not V_H restricted in activity (Note: may show pseudorestriction with respect to V_H)
5.	Afferent suppressor factor
6.	Induces, together with antigen, Ts_2

Ts_2 (Table 3): As first clearly demonstrated in the GAT and GT models, the only detectable activity of TsF_1 is the triggering of normal, nonprimed T cells to become suppressive (41). These new Ts (referred to as Ts_2) are induced in vivo or in vitro in 2-5 days and derive from precursors resistant to low dose cyclophosphamide treatment (42). This induction process in the GAT and GT models has been explicitly shown to require both TsF_1 and specific antigen (43,44). The active Ts_2 is an Lyt 2^+ cell (the presence or level of Lyt 1^+ on these cells is unresolved) which also bears I-J subregion encoded determinants (Sy et al., in preparation, Germain and Cantor, unpublished observations). This cell population, which can also be induced with spleen cells bearing idiotypic antibody (17), as well as by idiotypic TsF_1, is distinguished from Ts_1 not only by being Lyt 2^+, but by its receptor specificity. Ts_2 in the ABA and NP models have been shown to bind to idiotype coated plastic surfaces, i.e., they are anti-idiotypic (27,28). Further, the Ts_2 are efferent suppressors, able to act when given as late as the day of challenge for a DTH response (30,43). When tested in this mode to avoid as far as possible allogeneic effects, the activity of Ts_2 shows H-2 restriction, which has been mapped in the B6 NP model to the I subregion (43). Similar studies have also revealed Igh-1 linked restriction on activity, which, as will be detailed below, is related to the Igh-1linked control of idiotypic determinants on a third T cell, the Ts_3, which is the target of Ts_2 activity. The function of Ts_2 can be replicated by a soluble product termed TsF_2.

TABLE 3
Ts_2 SUMMARY

1.	Lyt 1(?)2^+
2.	$I\text{-}J^+$
3.	Anti-idiotypic (αCRI for ABA, αNP^b for NP)
4.	Induced by TsF_1 or idiotype-coupled spleen cells
5.	Precursor not cyclophosphamide sensitive
6.	Shows functional H-2 restriction
7.	V_H (Igh-1) restricted
8.	Efferent suppressor
9.	Produces TsF_2
10.	Requires Ts_3 for suppression of cell mediated responses; may directly suppress idiotypic B cells

TABLE 4
TsF_2 SUMMARY

1. $H\text{-}2^+$
2. Anti-idiotypic
3. H-2 restricted in activity
4. V_H (Igh-1) restricted in activity
5. Efferent suppressor factor
6. Target undetermined (Ts_3; Id^+ B cell?)

TsF_2 (Table 4): This product, studied primarily in the ABA model (30), bears H-2 encoded determinants, is anti-idiotypic, and H-2 and Igh-1 restricted in activity, as would be expected from the properties of the parent Ts_2 cell. It is an efferent acting suppressor material. Direct tests of its interaction with Ts_3 target cells have not yet been performed.

Ts_3 (Table 5): When it became clear that Ts were anti-idiotypic, and acted in an antigen specific manner, yet required Igh-1 matched recipients for function, the obvious explanation was that they acted on idiotypic target cells. However, attempts to demonstrate serologically detectable idiotypic determinants on a significant fraction of DTH effector cells were consistently negative (46). This raised the possibility that an additional cell population, bearing idiotypic determinants, was required for Ts_2 action, but was not necessary for DTH effector action. This hypothesis was tested in both the ABA and NP systems. In the ABA model the

TABLE 5
Ts_3 SUMMARY

1. Lyt $1(?)2^+$
2. $I\text{-}J^+$
3. Idiotypic
4. Precursor cyclophosphamide sensitive
5. Induced during immunogenic priming regimen
6. Activated by anti-idiotypic Ts_2
7. Suppresses in idiotype and(?) antigen non-specific manner once specifically triggered.

experiments utilized the allotype congeneic pair BALB/c (which is CRI^-) and C.AL-20 (which is CRI^+). These studies demonstrated that BALB/c recipients containing anti-idiotypic Ts_2 generated by CRI-SC injection did not permit expression of ABA specific DTH by transferred ABA-immune C.AL-20 lymphocytes, although control normal recipients did show transfer of immunity. If the transferred C.AL-20 cells were treated with anti-CRI + C prior to transfer, they still could transfer immunity to normal recipients, showing that CRI^+ DTH effectors were not required, but now such CRI^- cell populations transferred immunity to Ts_2 containing recipients. Thus, elimination of a CRI^+ cell from the immune lymph node population prevented Ts_2 induced suppression of ABA immune T cells. Further studies showed that the CRI^+ cell responsible is a T cell (termed Ts_3, and probably equivalent to the previously described T_{aux} cell in the DNP model (47)), which must come from a primed animal, and which, once triggered by Ts_2 and antigen, can suppress in an idiotype--and at least in certain circumstances, antigen-nonspecific manner (48). In addition, studies in the NP system established that the Ts_3 is an immune $I\text{-}J^+$ cell derived from cyclophosphamide sensitive precursors, which must be both H-2 and Igh compatible with the Ts_2 population to obtain immune suppression (26).

Overall Pathway: The general scheme that emerges from the studies summarized above is shown diagrammatically in Fig. 1. In this proposed pathway, appropriate antigen administration elicits idiotypic Ts_1, which via idiotypic, antigen-specific TsF_1, in the presence of antigen, trigger anti-idiotypic Ts_2. These Ts_2 in turn, via TsF_2, act on a third, idiotypic antigen primed T cell, the Ts_3, to generate effector suppressor activity which once evoked can be non-specific both with respect to idiotype and antigen.

DISCUSSION

The general pattern of T cell mediated suppression has proved remarkably constant among the various model systems explored in our laboratories. In addition, much of the data available from studies on Ts carried out by other investigators can be fit into the framework revealed by our studies (for a review of this issue, see 49). Thus, KLH-specific TsF studied by Tada, Taniguchi and coworkers acts by triggering a second population of Ts (50). $Lyt\ 1^+$ cells produce an $I\text{-}J^+$ antigen binding TsF in the SRBC feedback suppression model which acts across H-2 differences to trigger resting T cells to transmit activation signals to

effector suppressor cells (51). In the C57BL/10 ($H\text{-}2^b$) genetic nonresponder, administration of hen egg lysozyme elicits suppression involving at least two interacting T cell subpopulations bearing idiotypic and anti-idiotypic receptors (52). Hence, it would appear that the pathway shown in Fig. 1 may serve as a useful general model for antigen-specific T cell mediated suppression in the broad sense.

However, this scheme can by no means account for all the well-documented findings concerning Ts activity, nor does it allow us to provide answers to a whole series of additional questions raised by the work to date. The differences between our results and those of other laboratories include but are not limited to: 1) the report by Okumura et al. that the cell source of KLH specific inducing TsF is an Lyt 2^+ cell (53), and the findings by Tada et al. (54) that this material is restricted in its function by genes in the I-J subregion. Although this could most easily be accommodated in our scheme by considering the KLH-TsF producer a Ts_2, this would require that not all Ts_2 be anti-idiotypic, an issue discussed again below; 2) the demonstration by Eardley et al. (55) of an apparent V_H linked restriction on the action of Lyt 1^+ inducer Ts. This restriction was reported to involve what we would term Ts_1-Ts_2 interaction in clear contrast to the ABA results. However, the 'pseudorestriction' phenomena described above may explain this discrepancy. In the experiments of Eardley et al., no cultures were performed in which the acceptor Lyt 123^+ cell was added back to Lyt 1^+ T cells and B cells of the same V_H type as the original inducer cell. Our results would predict suppression would be observed in such a case, on the basis of idiotype-anti-idiotype interactions, revealing the true V_H restriction in the SRBC model to be between Ts_2 and effector Ts or target T or B cells bearing idiotype, and <u>not</u> between inducer and acceptor Ts. Tests of this prediction are currently in progress (Eardley and Gershon, personal communication).

Several extremely intriguing and important questions remain unanswered by the experimental work to date. Although Ts_1 and Ts_2 have been shown to bind to antigen coated dishes without apparent MHC involvement (27,28), it is not clear that these cells have no H-2 recognition capacity, or that such H-2 recogition is not critical to their function. More direct tests of H-2 recognition, using antigen pulsed cell monolayers for binding experiments (56,57) or antisera blocking experiments during in vitro

induction protocols, are needed. If H-2 recognition by these cells is demonstrated, then one must address the question of why these cells easily show binding to free antigen while proliferating, DTH or cytolytic T cells fail to do so.

A second related question is the H-2 restricted function of Ts_2 (and TsF_2). If these cells see free antigen (idiotype), then what accounts for the H-2 restriction? It may very well be that the recognition event involving H-2 is directed toward the Ts_2, (perhaps by the antigen primed Ts_3), and not mediated by the Ts_2 itself.

As concerns the anti-idiotypic nature of Ts_2--are all cells of this functional type anti-idiotypic? If so, how is this peculiar repertoire limitation dictated? Conversely, if as seems likely, there is no such repertoire limitation, then what accounts for the predominant role of anti-idiotypic Ts_2 in the models we have studied? One possibility, raised by M. Dietz, is that both antigen-specific and idiotype specific Ts_2 are simultaneously generated during the induction of suppression in an individual mouse. In those cases in which the Ts_1 bear a predominant idiotype shared by members of the strain, anti-idiotypic Ts_2 will be easily revealed by the adoptive transfer techniques used to study suppression, since all idiotypic partner cells needed for efficient suppression will be present in each recipient. However, for cases in which the Ts_1 populatin does not use a major, shared set of idiotypic receptors, the anti-idiotypic Ts_2 will be essentially anti-private idiotype. Such Ts_2 will not find appropriate targets upon transfer, and hence, not be revealed by our assay methods. In such a case, anti-antigen Ts_2, perhaps generated by antigen-TsF_1 complexes, will be the major functional Ts type, acting by antigen--rather than receptor bridging mechanisms. The 'individual network' experiments of Gorczynski et al. (59) lend support to this possibility.

Some remaining issues concern the biochemistry, genetics, and cell biology of Ts and their products. The precise molecular characteristics of TsF_1 and TsF_2 are not known at present. Hopefully, the approach of using monoclonal cell lines or hybridomas, as employed so elegantly by Cantor and associates to study an effector TsF (60), will soon be revealing in this regard. It will be of particular importance to determine if $I\text{-}J^+$, id^+ TsF have one (61) or two (62) polypeptide chains, because of the

relevance of this to the question of the protein vs. carbohydrate nature of the I-J controlled determinants and its implications for the genetics of TsF production. This latter subject is a most interesting one. The data on shared serologically detectable idiotypic determinants on Ts, TsF and antibodies of similar specificity and, in particular, the control of the presence of such serologic determinants on T cells and their products by genes linked to the Igh-1 complex on chromosome 12 have raised the issue of whether T cells use the same V_H gene segments as B cells do for immunoglobulin, but with different D, J and C gene segments. This question remains unresolved. The demonstration of the critical role of idiotype-anti-idiotype interactions in Ts activity and the recent suggestions of regulatory, rather than structural gene control of idiotypy (63), raise the possibility that the observed idiotypic sharing is a serologic cross-reaction of similar, but distinct gene products, selected both in evolution, and in ontogeny to fit into a network predominated by true Ig V_H gene products, thus accounting for the observed linkage. It will require data at the RNA/DNA level to finally resolve this issue. Lastly, we have virtually no information on the way in which the various TsF carry out their effector function. It is presumed that they possess 'Fc' regions analogous to the biologically active portions of antibodies (64), but it remains to be seen how such structures cause activation of particular target cells in the case of TsF_1, and suppression in the case of the product of Ts_3. Exploration of these many intriguing questions should occupy many of us profitably for some time to come.

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REFERENCES

1. Gershon, R. K., and Kondo, K. (1971). Immunol. 21, 903.
2. Kapp, J. A., Pierce, C. W., Schlossman, S., and Benacerraf, B. (1974). J. Exp. Med. 140, 648.
3. Dunham, E. K., Dorf, M. E., Shreffler, D. C., and Benacerraf, B. (1973). J. Immunol. 111, 1621.
4. Gershon, R. K., Maurer, P. H., and Merryman, C. F., (1973). Proc. Natl. Acad. Sci. USA. 70, 250.

5. Kapp, J. A., Pierce, C. W., and Benacerraf, B. (1973). J. Exp. Med. 138, 1107.
6. Ju, S-T., Benacerraf, B., and Dorf, M. E. (1978). Proc. Natl. Acad. Sci. 75, 6192.
7. Ju, S-T., Kipps, T. J., Theze, J., Benacerraf, B., and Dorf, M. E. (1978). J. Immunol. 121, 1034.
8. Theze, J., and Somme, G. (1979). Eur. J. Immunol. 9, 294.
9. Debre, P., Kapp, J. A., and Benacerraf, B. (1975). J. Exp. Med. 142, 1436.
10. Debre, P., Kapp, J. A., Dorf, M. E., and Benacerraf, B. (1975). J. Exp. Med. 142, 1447.
11. Debre, P., Waltenbaugh, C., Dorf, M. E., and Benacerraf, B. (1976). J. Exp. Med. 144, 272.
12. Benacerraf, B., and Dorf, M. E. (1976). Cold Spring Harbor Symp. Quant. Biol. 41, 465.
13. Germain, R. N., Waltenbaugh, C., and Benacerraf, B. (1980). J. Exp. Med. 151, 1245.
14. Bach, B. A., Sherman, L., Benacerraf, B., and Greene, M. I. (1978). J. Immunol. 121, 460.
15. Greene, M. I., Bach, B. A., and Benacerraf, B. (1979). J. Exp. Med. 149, 1069.
16. Nisonoff, A., Ju, S-T., and Owen, F. L. (1977). Immunol. Rev. 34, 89.
17. Sy, M-S., Bach, B. A., Brown, A., Nisonoff, A., Benacerraf, B., and Greene, M. I. (1979). J. Exp. Med. 150, 1229.
18. Dohi, Y., and Nisonoff, A. (1979). J. Exp. Med. 150, 909.
19. Weinberger, J. Z., Germain, R. N., Ju, S-T., Greene, M. I., Benacerraf, B., and Dorf, M. E. (1979). J. Exp. Med. 150, 761.
20. Weinberger, J. Z., Greene, M. I., Benacerraf, B., and Dorf, M. E. (1979). J. Exp. Med. 149, 1336.
21. Sunday, M. E., Weinberger, J. Z., Benacerraf, B, and Dorf, M. E. (1980). J. Immunol. 125, 1601.
22. Makela, O., and Karjalainen, K. (1977). Immunol. Rev. 34, 119.
23. Sherr, D. H., Ju, S-T., Weinberger, J. Z., Benacerraf, B., and Dorf, M. E. (1981). J. Exp. Med. 153, 640.
24. Eardley, D. D., Hugenberger, J., McVay-Boudreau, L., Shen, F. W., Gershon, R. K., and Cantor, H. (1978). J. Exp. Med. 147, 1106.
25. Debre, P., Waltenbaugh, C., Dorf, M. E., and Benacerraf, B. (1976). J. Exp. Med. 144, 277.
26. Sunday, M. E., Benacerraf, B., and Dorf, M. E. (1981). J. Exp. Med. In press.

27. Sy, M-S., Dietz, M. H., Germain, R. N., Benacerraf, B., and Greene, M. I. (1980). J. Exp. Med. 151, 1183.
28. Weinberger, J. Z., Germain, R. N., Benacerraf, B., and Dorf, M. E. (1980). J. Exp. Med. 152, 161.
29. Dietz, M. H., Sy, M-S., Greene, M. I., Nisonoff, A., Benacerraf, B., and Germain, R. N. (1980). J. Immunol. 125, 2374.
30. Dietz, M. H., Sy, M-S., Nisonoff, A., Greene, M. I., Benacerraf, B., and Germain, R. N. (1981). J. Exp. Med. 153, 450.
31. Sy, M-S., Dietz, M. H., Nisonoff, A., Germain, R. N., Benacerraf, B., and Greene, M. I. (1980). J. Exp. Med. 152, 1226.
32. Takemori, T., and Tada, T. (1975). J. Exp. Med. 142, 1241.
33. Kapp, J. A., Pierce, C. W., DelaCroix, F., and Benacerraf, B. (1976). J. Immunol. 116, 305.
34. Kapp, J. A., Pierce, C. W., and Benacerraf, B. (1977). J. Exp. Med. 145, 828.
35. Theze, J., Kapp, J. A., and Benacerraf, B. (1977). J. Exp. Med. 145, 839.
36. Germain, R. N., Ju, S-T., Kipps, T. J., Benacerraf, B., and Dorf, M. E. (1979). J. Exp. Med. 149, 613.
37. Waltenbaugh, C., Debre, P., Theze, J., and Benacerraf, B. (1977). J. Immunol. 118, 2073.
38. Bach, B. A., Greene, M. I., Benacerraf, B., and Nisonoff, A. (1979). J. Exp. Med. 149, 1084.
39. Kapp, J. A. (1978). J. Exp. Med. 147, 997.
40. Germain, R. N., and Benacerraf, B. (1978). J. Immunol. 121, 608.
41. Waltenbaugh, C., Theze, J., Kapp, J. A., and Benacerraf, B. (1977). J. Exp. Med. 146, 970.
42. Waltenbaugh, C., and Benacerraf, B. (1978). In: 'Ir Genes and Ia Antigens', H.O. McDevitt, ed. p. 549.
43. Germain, R. N., Theze, J., Kapp, J. A., and Benacerraf, B. (1978). J. Exp. Med. 147, 123.
44. Germain, R. N., Theze, J., Waltenbaugh, C., Dorf, M. E., and Benacerraf, B. (1978). J. Immunol. 121, 602.
45. Weinberger, J. Z., Benacerraf, B., and Dorf, M. E. (1980). J. Exp. Med. 151, 1413.
46. Sy, M. S., Brown, A. R., Benacerraf, B., and Greene, M. I. (1980). J. Exp. Med. 151, 896.
47. Sy, M-S., Miller, S. D., Moorhead, J. W., and Claman, H. N. (1979). J. Exp. Med. 149, 1197.
48. Sy, M. S., Nisonoff, A., Germain, R. N., Benacerraf, B., and Greene, M. I. (1981). J. Exp. Med. In press.
49. Germain, R. N., and Benacerraf, B. (1981). Scand. J. Immunol. 13, 1.

50. Tada, T. (1977). In: The immune system: Genetics and regulation, E. Sercarz, L. A. Herzenberg and C. F. Fox, Eds., Academic Press, New York p. 345.
51. Yamauchi, K., Murphy, D. B., and Gershon, R. K. (1981). J. Supramol. Struct. Supp. 5, 75.
52. Sercarz, E. E., and Metzger, D. W. (1980). Springer Sem. Immunopath. 3, 145.
53. Okumura, K, Takemori, T., Tokuhisa, T., and Tada, T. (1977). J. Exp. Med. 146, 1234.
54. Tada, T., Taniguchi, M., and David, C. S. (1976). J. Exp. Med. 144, 713.
55. Eardley, D. D., Shen, F. W., Cantor, H., and Gershon, R. K. (1979). J. Exp. Med. 150, 44.
56. Swierkosz, J. E., Rock, K., Marrack, P. C., and Kappler, J. W. (1978). J. Exp. Med. 147, 554.
57. Araneo, B. A., Yowell, R. L., Metzger, D. W., and Sercarz, E. E. (1981). Proc. Natl. Acad. Sci. USA. 78, 499.
58. Gorczynski, R. M., Khomasurya, B., Kennedy, M., MacRae, S., and Cunningham, E. A. J. (1980). Eur. J. Immunol. 10, 788.
59. Fresno, M., Mcvay-Boudreau, L., Nabel, G., and Cantor, H. (1981). J. Exp. Med. In press.
60. Pacifico. A., and Capra, J. D. (1980). J. Exp. Med. 152, 1289
61. Taniguchi, M., Takei, I., and Tada, T. (1980). Nature 283, 227.
62. Nadler, P. I., Miller, G., Sachs, D. H., and Hodes, R. J. (1981). J. Supramol. Struct. Supp. 5, 69.
63. Owen, F. (1981). J. Supramolec. Struct. Supp. 5, 30.

THE ROLE OF IDIOTYPY IN GUIDING CELLULAR RESPONSES [1]

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The structure of genes which encode variable region domains relevant to the immune response has been clarified to a great extent. Amino acid and nucleic acid sequencing have revealed that assembly of immunoglobulin (Ig) molecules involves the participation of V, J, D and C region DNA sequences (1-4). V-J joining, closer juxtaposition of V to C, and heavy chain switching require rearrangements and splicing at the level of the DNA, while intervening sequences are removed from the primary RNA transcript, permitting faithful translation of intact Ig molecules. The Ig heavy chain gene arrangement is 5′, μ, γ_3, γ_1, γ_{2b}, γ_{2a}, and α 3′ (2). Yet, while this genetic structure is certainly relevant for B cells, the role of V, J, and C regions and their assortment and splicing is not known for T cells.

Our studies discussed below will relate to the relative role of two gene clusters. The murine major histocompatibility complex (MHC) on chromosome XVII has been shown to influence T cell receptor restriction and repertoire diversity, and certain MHC molecules may be integral to the T cell receptor itself. On the XIIth chromosome reside the genes for the Ig heavy chain. The demonstration of serologically shared idiotypes between B and T cells and their products and the linkage of idiotypes to V_H region genes underscores the role of these gene loci in T cell recognition apparatus. The structure of soluble T cell derived products and how they might possibly be analyzed at the DNA, RNA, and peptide levels, as well as their functional assessment in regulating immunity will be addressed.

The system we are studying involves the hapten azobenzenearsonate (ABA). Antibody responses to ABA are dominated by a cross-reactive idiotype (CRI). The ability to mount a CRI predominant response has been found to be linked to the IgC_H loci on chromosome 12, and to the Igk-Trp locus on

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chromosome 6. Inbred strains of mice such as A/J ($H\text{-}2^a$, $Igh\text{-}1^e$) make an anti-ABA antibody response, roughly half of which has CRI determinants. C.AL-20 ($H\text{-}2^d$, $Igh\text{-}1^d$) mice (which have the A/N allotype on the BALB/c [$H\text{-}2^d$, $Igh\text{-}1^a$] background) make a similar CRI positive response, but BALB/c mice do not. These observations link idiotype expression to allotype genes. The light chain (encoded on chromosome VI) also plays a role in the production of CRI structures. Strains of mice which have an inappropriate light chain gene but appropriate heavy chain gene cannot make a set of CRI bearing antibodies. The F1 between this strain and one which possesses relevant light and irrelevant heavy chain genes is able to make a CRI positive antibody response. Thus, at the level of the B cell, both H and L genes are required for CRI expression.

T cell responses to ABA are elicited by derivitizing the cell surface of spleen cells (ABA-SC) with ABA and using such hapten coupled cells to induce T cell subsets of the following sorts:

Lyt 1+ (T_{DH}: T cells mediating delayed type hypersensitivity)--subcutaneous immunization.
Lyt 2,3+ (Tc: T cells mediating cytotoxicity in vitro) --s.c. or i.v. immunization.
Lyt 1+, I-J+ Ts (Ts: T suppressor cells capable of initiating a suppressor pathway) (6)--i.v. immunization.

These subsets are related by their specificity for the hapten, ABA. In some cases the ABA hapten is clearly recognized only when associated with defined H-2 encoded elements. For example, Tc lyse ABA coupled cells in an H-2 restricted manner. The H-2 restriction for cytolytic T cells maps to the K and D regions. The activation of T_{DH} requires I-A subregion homology between splenic adherent cells which can present antigen and T_{DH} which respond to that hapten. The role of I-A region encoded structures was further shown by passive administration of anti-I-A antibodies which blocked immunity *in vivo*. The use of agents such as ultraviolet radiation (UV), which selectively impairs I-A+ APC function, also limits the capacity to sensitize mice with ABA (7). The activation of Lyt 1+ Ts has recently been studied in great detail. Early in the generation of suppressor cell function two independent types of signals are necessary for complete activation. The first requires the interaction of a ligand binding ABA-specific Ts precursor with ABA-SC; ABA-protein is incapable of mediating such an interaction. This by itself cannot activate/ differentiate

the Ts until another, H-2 related, event also occurs. This event can be mediated by an I-J specific allogeneic effect directed solely against the precursor Ts which has previously interacted with antigen. The allogeneic effect can be replaced with a soluble factor generated across the I-J subregion of the MHC. Since allogeneic effects are presumed to be a nonphysiologic reflection of normal signaling, then Ts activated by antigen alone must also experience an H-2 related event to achieve full activation. So in the case of all known types of T cells active in responding directly to ABA, H-2 elements play a determinative role (7).

It was possible to show through a large series of immunochemical procedures that Ts produced a factor capable of inhibiting Lyt 1+ T_{DH} function and dampening cytolytic activity. This 68 K MW antigen binding factor was retained by affinity columns composed of anti-CRI or anti-I-J antibodies coupled to Sepharose. This molecule or molecular complex did not directly suppress DTH or CML but rather induced a second order cell. Thus, the first order, ligand activated I-J+, CRI+, Lyt 1+, Ts_1 induced a second order Ts_2 via TsF_1. Ts_2 was shown to be I-J+, Ly 1,2,3, and to possess anti-idiotypic structures. Thus, Ts_2 could be enriched on CRI+ antibody coated plastic dishes. The interaction between TsF_1 and Ts_2 is unrestricted, e.g., TsF_1 from A/J can induce Ts_2 in A/J, A.BY ($H\text{-}2^b$, $Igh\text{-}1^e$), C.AL-20 ($H\text{-}2^d$, $Igh\text{-}1^d$), or BALB/c ($H\text{-}2^d$, $Igh\text{-}1^a$). While TsF_1 can induce Ts_2 in *any* strain this is not always apparent because Ts_2 release a soluble, I-J+, anti-idiotypic factor (TsF_2) which is restricted by both H-2 and Igh-1 genes. Thus A/J TsF_1 BALB/c induces Ts_2 which can function in C.AL-20 but not BALB/c (i.e., it is restricted to $H\text{-}2^d$ and $Igh\text{-}1^d$). TsF_1 also has the remarkable property of being produced by strains which lack the necessary light chain genes for production of CRI+ antibody. One can conclude that regulatory elements of chromosome VI do not influence the structure of at least some T cell receptors. It is known that Ts_2 induced by TsF_1 are apparently restricted by the H-2 gene complex. In order to explain the induction of restriction we envision that TsF_1 could be presented to Ts_2 by a set of specialized antigen presenting cells (APC). Restriction would be effected by the endogenous MHC molecules of the APC, not by the I-J+ TsF_1 since TsF_1 works in all strains. Therefore, antigen on cell membranes activates idiotypic Ts_1; the product of Ts_1, TsF_1, is perhaps carried on another APC to the complementary Ts_2. The H-2 restricted Ts_2 then elaborates TsF_2. It might be considered that TsF_2 is not a single molecule but really two or more molecules which act together to effect H-2 and Igh-1 restricted suppression. Alternatively, the H-2 restriction

is related not to the induction of Ts_2 but rather solely to the interaction of this cell with other cells further down the suppressor pathway.

As mentioned, the T_{DH} cell itself is insensitive to lysis by anti-CRI antibody. If so, in what manner can TsF_2, with its anti-idiotypic receptor, regulate the Lyt 1 subset? Recently, we have found that in the animals immunized with ABA-SC subcutaneously there coexists with T_{DH} a subset of cells with CRI structures on its cell surface. These cyclophosphamide sensitive cells are not Lyt 1+ Ts_1, but rather an Lyt 2,3+ set. It appears that this idiotype bearing cell, termed Ts_3, is the direct target of Ts_2. At present it can be stated that anti-idiotypic Lyt 1,2,3 Ts_2 will cause idiotypic Ts_3 (9) to nonspecifically suppress Lyt 1+ cells. We are presently determining if Ts_3 are activated by I-A+ ABA coupled APC or are stimulated by less well understood means. It can be readily seen that apparent H-2 restriction of Ts_2 could really reflect interactions between Ts_3 and T_{DH}. A summary of the different characteristics of Ts cells is presented in Table 1 below.

TABLE 1
CHARACTERISTICS OF SUPPRESSOR CELLS

	Ts_1	Ts_2	Ts_3
Ligand binding	+	–	+
Idiotype binding	–	+	–
Lyt phenotype	Lyt 1+	Lyt 1,2,3	Lyt 2,3
Produce factors	+	+	?

It is clear that the major principle in regulating ABA reactivity relates to CRI bearing elements. These moieties seem to determine and guide the activation and control of many diverse subsets of cells, leading to the dampening of activity of even non-CRI bearing cells. It seems likely that these rules of

$$\text{Ag + self H-2} \xrightarrow{induce} \text{idiotypic } Ts_1 \xrightarrow{stimulate} Ts_2 \xrightarrow{trigger} Ts_3$$

regulation will also apply to the understanding of regulation of other immune responses (10).

At present, we are refocusing our attention on the rules governing the structure of TsF_1. Although we know that

minimally TsF_1 has an idiotypic polypeptide and an I-J (? polypeptide), we have no clear data pertaining to whether this is a single chained or two-chained structure. If it is a single chained structure we cannot readily explain how genes on distinct chromosomes can create it. We could pose the unlikely possibility that a structural locus analogous to V_H occurs somewhere on chromosome XVII, or vice versa. Moreover, how do we explain idiotypy together with fine specificity for the ABA without a light chain contribution? To resolve these questions, we have created ABA specific T cell lines, and have also begun to develop V_H gene probes. Hopefully these answers will be forthcoming within the next while. At present we are also evaluating whether idiotypes are also involved in the recognition of antigens in nonimmune somatic cells. These studies have already shed some light on the evolution, development, and maintenance of V regions in the genome.

REFERENCES

1. Seidman, J. G., Leder, A., Mau, M., Norman, B., and Leder, P. (1978). *Science* 202, 11.
2. Honjo, T., and Kataoka, T. (1978). *Proc. Natl. Acad. Sci. USA* 75, 2140.
3. Sakano, H., Huppa, K., Heinrich, G., and Tonegawa, S. (1979). *Nature* 280, 288.
4. Early, P., Huong, H., Davis, M., Calame, K., and Hood, L. (1980). *Cell* 19, 981.
5. Nisonoff, A., and Greene, M. I. *Proc. 4th Int. Cong. Immunol.*, in press.
6. Greene, M. I., and Benacerraf, B. (1980). *Trans. Rev.* 50, 163.
7. Greene, M. I., Sy, M-S., Perry, L. L., and Bromberg, J. (1981). *Immunol. Today*
8. Greene, M. I., and Sy, M-S. (1981) *Fed. Proc.*, in press.
9. Sy, M-S., Nisonoff, A., Germain, R. N., Benacerraf, B., and Greene, M. I. (1981). *J. Exp. Med.*, in press.
10. Herzenberg, L. A., Black, S. J., and Herzenberg, L. A. (1980). *Eur. J. Immunol.* 10, 1.

SPECIFIC ACTIVATION OF SILENT CLONES THROUGH THE IMMUNE NETWORK AS MODEL TO STUDY THE FLEXIBILITY OF IMMUNE SYSTEM

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There are numerous observations which show that by using various extrinsic manipulations one is able to activate clonotypes which are not normally activated by conventional immunization with a particular antigen. One of the most striking examples was provided by the activation of silent allotypes in rabbits and mice (1,2,3). Other examples were provided by studies in which bacterial immunostimulants were used in order to amplify the antibody response. In various mammalian species it is very difficult to detect antibodies against self-antigens. However, the bacterial immunostimulants such as LPS or MDP can stimulate in mice the appearance of PFC against syngeneic bromelein treated RBC or thymocytes (4). Another striking example was provided by the analysis of the IEF patterns of the IgG fraction in an anti-inulin response. The BALB/c mice developed a homogeneous response subsequent to immunization with bacterial levan (BL) or Inulin-BA,(In-BA) which was represented by a group of three prominent bands in the pH 6.0 to 6.5 range (5). However, a heterogeneous response was observed after immunization with inulin coupled to KLH in FCA or In-NWSM. This indicates that inulin coupled with various adjuvants can cause the activation of several clonotypes (5).

It was because of these findings that we explored the specific pathways of activation of the silent repertoire; namely, through the various members which compose the immune network.

For this purpose, we chose to study the effects of anti-anti-idiotypic antibodies on A48Id$^+$ anti-BL clones, since it was shown that A48Id of ABPC48 myeloma protein is not expressed on anti-BL antibodies produced by IghC^a mice in response to immunization with BL or BL-SRBC conjugates (6).

We observed the appearance of A48Id$^+$ anti-BL antibodies produced by BALB/c mice in response to immunization with BL in different experimental systems

1. Idiotype suppressed nude BALB/c mice.

BALB/c mice in response to immunization with bacterial levan, which is a β(2-6) polyfructosan with β(2-1) branch points, develop two families of antibodies in response to immunization with BL.

The first category binds to inulin which is a β(2-1) polyfructosan. These antibodies share the cross-reactive

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idiotypes (IdX) of In-binding myeloma proteins. Among various idiotypes identified on In-binding myelomas, the IdX A and B were most estensively studied (6).

The second category binds to BL and do not express IdX of In-binding myeloma proteins nor the idiotypes of two BL-binding myeloma proteins (i.e. ABPC48 (A48) and UPC10 (U10) (6).

In a previous work we have shown that administration of homologous anti-IdX antibodies led to a long lasting suppression of an anti-In response and of IdX's (7). The suppression of the IdX^+ component of the anti-In response was never accompanied by an activation of the $A48Id^+$ component in various strains of mice studied (Table 1).

Table 1. Occurrence of A48Id in IdX suppressed mice

Strain	IghC	Normal mice					IdX suppressed mice				
		In	IdXA	-B	-G	A48	In	IdXA	-B	-G	A48
BALB/c	a	++++	++	++	++	-	+	-	-	-	-
Nude BALB/c	a	++	+	+	+	-	-	-	-	-	+
C58/J	a	++++	++	++	++	-	+	-	-	-	-
BAB.14	a	++	+	++	+	-	-	-	-	-	-
RICxBJ	a	++++	++	++	++	-	+	-	-	-	±
RICxBG	a	++++	+++	+++	+++	-	+	-	-	-	-
CAL.20	e	+++	+	+	+	-	-	-	-	-	-
B.C 8	a	++++	++	++	++	-	+	-	-	-	-

Responses measured are in vivo responses to BL. The degree of response is designated in a - to ++++ scale.

By contrast, the idiotype suppression induced in nude BALB/c mice by homologous anti-IdX antibodies was characterized by three important features compared with that observed in normal mice.

a) The suppression of inulin antibody response by anti-IdX A and B antibodies led to an unusual increase of the total anti-BL response.

b) The administration of anti-A48Id antibodies in nude mice caused a slight suppression of the total anti-BL response.

c) The IdX suppressed nude mice developed an $A48Id^+$ response after subsequent immunization with BL (7).

These observations indicated that the suppression of the dominant clones led to the activation of $A48Id^+$ silent clone (s), and the T cells probably played an important role in this process. This hypothesis was verified in further experiments in which T cells from a normal BALB/c mouse were infused on the day of immunization with BL in IdX suppressed nude mice. Nude mice infused with syngeneic T cells failed to develop an $A48Id^+$ response, indicating that T cells pre-

vent the activation of silent clones (8) (Table 2).

Table 2. Infusion of T cells prevents the expression of A48Id in IdX suppressed nude BALB/c mice

Pretreatment with Anti IdX antibodies	Immunization with 20 µg BL	Infusion with T cells	A48Id*
-	-	-	-
-	+	-	-
-	+	+	-
+	+	-	+
+	+	+	-

* A48Id was detected by PFC assay

A putative A48Id specific suppressor T cell similar with the 460Id specific suppressor T cell that we have previously described in the trinitrophenyl system (9) could be involved in the regulation of expression of A48Id clones.

2. Activation of A48Id clones by neonatal pretreatment with low doses of anti A48Id antibodies.

Recently, we have shown that injection of 0.01 µg of anti-A48Id antibodies after birth followed by immunization with BL one month later, led to a substantial activation of the $A48Id^+$ component of the anti-BL response as assessed by PFC and RIA assays (10). A similar increase of the A48Id response was observed in 7 week old mice despite the activation at this age of the anti-In response. We have previously shown that there is a substantial ontogenetic delay in the appearance of the IdX response which is expressed maximally at 4 to 6 weeks (11). The activation of A48Id clones was specific since: a) the pretreatment after birth with anti-348Id antibodies did not lead to activation of the A48Id response (MOPC384 binds LPS of S. tranaroa and expresses an unrelated idiotype).

b) the pretreatment after birth with anti-A48Id antibodies followed by immunization one month later with TNP-Ficoll did not alter the 460Id component of the anti-TNP response. (Table 3).

Table 3. Appearance of A48Id in BALB/c mice pretreated at birth with 0.01 µg anti A48Id antibodies

Pretreatment	Immunization	A48Id*	460Id*
-	BL (20µg)	-	-
Anti A48Id	BL (20µg)	+	-
Anti A48Id	TNP-Ficoll(10 µg)	-	+
Anti 384Id	BL (20µg)	-	-

* A48Id and 460Id were detected by PFC and RIA assays

I would like to mention that we verified the existence of A48Id$^+$ anti-BL antibody forming cells in these animals by immortalizing them in hybridomas. These hybrids were obtained by fusion of SP2/0 cells with spleen cells from BALB/c mice treated at birth with 0.01 µg of anti-A48Id antibodies and immunized one month later with BL (Table 4).

Table 4. Hybridomas obtained by fusion of SP2/0 myeloma cells with spleen cells of BALB/c mice pretreated at birth with 0.01 µg anti A48Id antibodies and immunized 1 month later with 20µg bacterial levan

Monoclonal Antibody	Binding* to BL	Expression of ** A48Id	Isotype ***
MSBL 76	+	+	IgM
MSBL 97	+	+	IgG_3
MSBL 36	+	-	IgG_3
MSBL 24	+	-	IgG_3

* Ability of monoclonal antibody to bind BL was studied by RIA as follows: microplates were coated with BL and then with 50% FCS. After 3 x washings, the plate were incubated for 3 hrs with monoclonal antibodies, washed 3 x and then incubated with ^{125}I-goat antimouse antibodies.

** Expression of A48Id was studied by the ability of A48I bearing molecules to compete with the binding of alkaline phosphatase labeled - anti A48Id antibodies to A48 coated plates.

*** Isotypic specificities of monoclonal antibodies was studied by direct binding of 3H-goat antiIg of IgG_1, IgG_{2a}, IgG_{26} and IgG_3 to plates coated with monoclonal antibodies and MOPC-104E, MOPC11, MPC10, MOPC195 and aLPS7 monoclonal antibody of IgG_3 class, respectively.

Several conclusions emerge from this study:

a) the low doses of antibodies administered after birth have a profound effect on the precursor of anti-A48Id anti-BL antibody forming cells leading to a selective activation of A48Id$^+$ clones without alteration of the magnitude of the total anti-BL

b) the effect of anti-A48Id antibodies is very specific. No polyclonal effect mediated through a mitogenic receptor,which displays a hypothetical Vh-like receptor, was observed.

c) the expansion of A48Id precursors requires the antigenic stimulation.

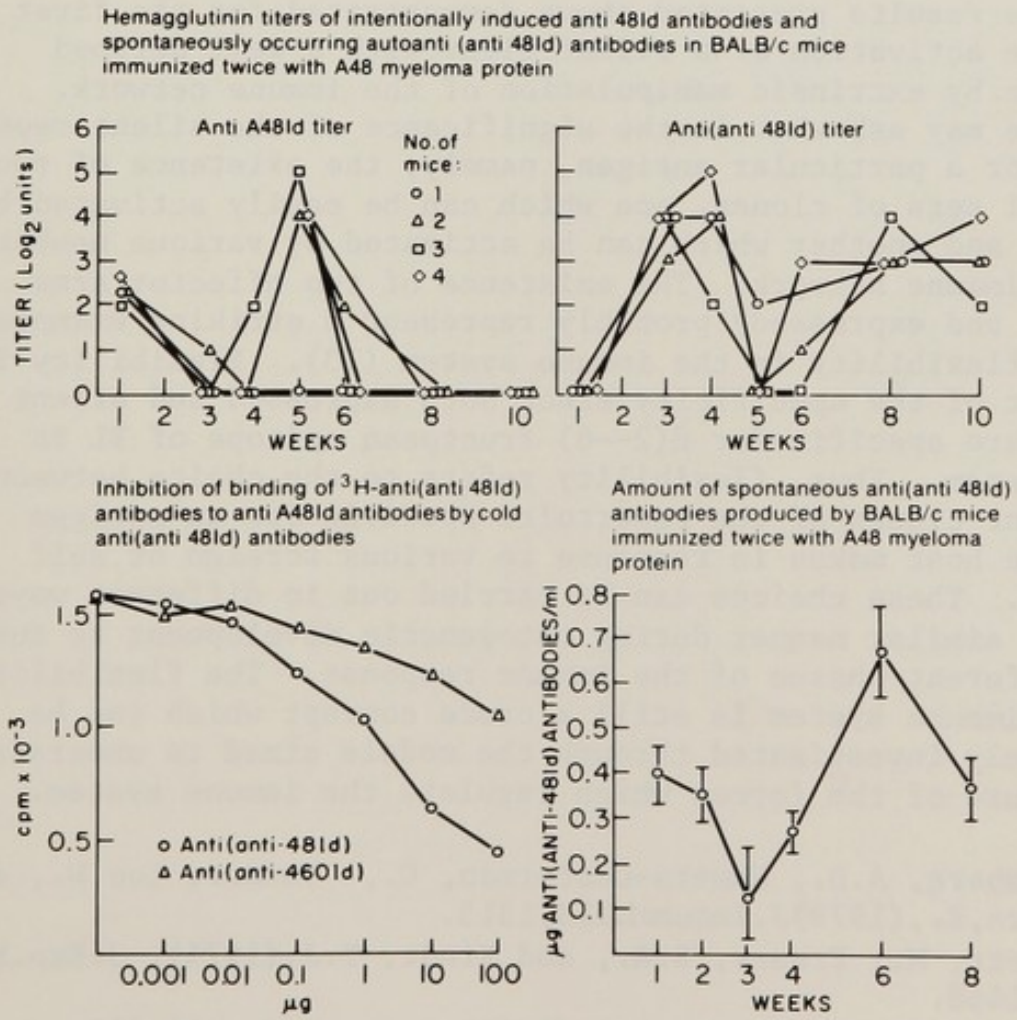
3. Activation of A48Id clones in BALB/c mice producing anti-anti-A48Id antibodies.

Anti-anti-A48Id antibodies (Ab_3) were obtained in adult BALB/c mice following two different experimental situations as follows:

in mice immunized with anti-A48Id-KLH conjugates in FCA

in mice immunized only two times with A48 Protein.

By using this protocol, we observed for the first time the appearance of spontaneous auto anti-anti-Id antibodies. The experimentally induced as well as auto-Ab_3 exhibited the same properties; namely, the ability to interact with both Ab_2 and Ab_4, and to share, at least, some A48 idiotopes (12). In the mice producing auto-Ab_3, we observed an inverse fluctuation between Ab_2 and Ab_3 indicating very clearly that a chain of complementary anti-idiotypic reactions can take place in a single individual. Indeed, the kinetic studies in these animals have shown that after completion of immunization with A48 the Ab_2 titer increased and had fallen by 3 weeks, at this time each of these mice displayed a significant Ab_3 titer. By 5 weeks, the Ab_3 titer had fallen and was replaced by Ab_2, but by 8 weeks Ab3 was found in the absence of detectable Ab_3 titer (Fig.1).



Interestingly, mice which produced Ab_3 developed a considerable amount of $A48Id^+$ anti-BL response upon immunization with BL (Table 5).

Table 5. Appearance of A48Id in BALB/c mice producing anti (anti A48Id) antibodies (Ab_3)

Pretreatment	Presence of Ab_3	Immunization with 20µg BL	A48Id*
-	-	-	-
-	-	+	-
A48 x 2 times	+	+	+
Ab_2 x 8 times	+	-	-
Ab_2 x 8 times	+	+	+

* A48Id was detected by PFC and RIA assays.

We explained this increase of A48Id anti-BL antibody response by elimination of $A48Id^+$ specific suppressor cells or that Ab_3 can represent a large and heterogeneous population of antibodies which can include a set of Ab_1(12).

The results presented above demonstrated for the first time the activation of a silent clone with a well defined idiotype by extrinsic manipulation of the immune network.

One may ask what is the significance of the silent repertoire for a particular antigen, namely, the existence of two parallel sets of clones, one which can be easily activated by antigen and another which can be activated by various members of the immune network. The existence of two effector arms (silent and expressed) probably represent a striking example of the flexibility in the immune system (13). Flexibility is distinct of the specificity since both expressed and silent clones are specific for β(2--6) fructosan epitope of BL in this system. Thus, flexibility refers to the choice between different clones of the repertoire specific for an antigen that the host makes in response to various foreign or self signals. These choices can be carried out in different ways or in a similar manner during ontogenetic development or during different phases of the immune response. The flexibility of the immune system is still a crude concept which can be adequately investigated through the models aimed to understand the nature of the forces which regulate the immune system.

1. Strosberg, A.D., Hamers-Casterman, C., Vander, Loo W., and Hamers,R.,(1979)J.Immunol.113,1313.
2. Mudgett, M., Fraser, B.A., and Kindt, T.J.(1975). J.Exp.Med. 141,1468.
3. Bosma, M.J. and Bosma, G.C.(1979).J.Exp.Med. 134,512.
4. Lowy, I., Bona, C., Ciorbarw, R. and Chedid, L.(1977). Immunology 32,975.

5. Stein, K.E., Bona, C., Lieberman, R., Chien,C.C. and Paul,W.E.(1981). J.Exp.Med. 151, 1088.
6. Lieberman,R., Potter, M., Humphrey, W. and Chien, C.C. (1974). J.Immunol. 117, 2105.
7. Bona, C., Lieberman, R., House,S., Green,I., and Paul,W.E. (1979). J. Immunol. 133,1614.
8. Lieberman,R., Bona,C., Chien,C.C., Stein,K.E. and Paul,W.E. (1979). Ann.Imm.(Paris).130C,247.
9. Bona, C. and Paul,W.E.(1979).J.Exp.Med. 149,592.
10. Hiernaux,J., Bona,C. and Baker,Ph. J.Exp.Med.(in press).
11. Bona,C., Mond,J.J., Stein,K.E., House,S., Lieberman,R. and Paul,W.E. (1979). J.Immunol. 123,1484.
12. Bona,C., Heber-Katz,E. and Paul,W.E. J.Exp.Med.(in press).
13. Mitchison,N.A. in Strategy of Immune regulation(E.Sercaz) and Cunningham,A., eds.) Academic Press, New York,London, Toronto, Sydney, San Francisco.(1980);135.

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SPECIFICITY OF IDIOTYPE SUPPRESSION IN THE A/J ANTI-AZOPHENYLARSONATE SYSTEM[1]

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A number of arsonate-binding hybridoma proteins, derived from immune A/J mice, were shown to express the major cross-reactive idiotype characteristic of the A/J anti-arsonate response. These proteins constitute a relatively homogeneous antibody population although they differ from one another at occasional residues in both the heavy and light chains. As predicted by their minor structural differences, it is possible to make heterologous antisera and monoclonal antibodies which recognize some but not all of these hybridoma proteins. Such restricted, or anti-"private", reagents recognize a portion of the major idiotype-bearing antibody molecules produced in the course of a normal A/J anti-arsonate response. The capacity of the anti-private reagents to trigger the suppression of the entire cross-reactive idiotype family via idiotype → anti-idiotype → idiotype interactions was examined. It was found that the restricted antisera described in this report only suppressed the appearance of those antibody molecules to which they could bind directly.

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INTRODUCTION

The analysis of hybridoma proteins derived from fusions between immune B lymphocytes and myeloma cell lines has contributed greatly to the understanding of the structural diversity associated with the A/J anti-azophenylarsonate (Ars) antibody response and the major cross-reactive idiotype (Id^{CR}) characteristic of this system (1). Various Id^{CR+} monoclonal antibodies identified on the basis of their extensive serological cross-reactivity in assay systems designed to detect the major idiotype have been examined. Amino acid sequence studies have shown that the Id^{CR+} molecules constitute a family of structurally related but nonidentical proteins (2-7). As predicted by the occasional differences found in both the heavy and light chains, it was possible to prepare antisera which recognized "private" determinants associated with individual Id^{CR+} hybridoma proteins. The subsequent detection of these private determinants in Id^{CR+} immune sera confirmed that the Id^{CR+} hybridoma proteins were, in fact, representative of the normal *in vivo* Id^{CR+} antibody response (3,8). Hybridoma proteins have also proved useful in the analysis of the Id^{CR-} portion of the anti-Ars response. In this case, amino acid sequence studies of randomly chosen Id^{CR-} monoclonal antibodies revealed "minor" cross-reactive idiotype families which were structurally unrelated to the major idiotype family (7). Again, antisera directed against representative hybridoma proteins indicated that the minor idiotype families also recur consistently among individual A/J mice (8-10).

Reagents specific for the major, private and minor determinants have been applied to the study of various network interactions. One particularly intriguing aspect of the Ars antibody response is the sequence of cellular events which can result in idiotype suppression (11). Under the appropriate conditions, both Id^{CR+} antibody molecules and antisera directed against Id^{CR} have been shown to trigger Id^{CR} specific suppressor cell activity (12-14). The purpose of the present report is to examine the specificity and amplification capacity of suppressor cell networks induced by the various types of antisera.

RESULTS AND DISCUSSION

"Public" vs. "Private" Id^{CR} Determinants. A rabbit antiserum specific for the major ("public") cross-reactive idiotype (anti-Id^{CR}) was prepared by immunizing first with a pool of A/J anti-Ars serum antibody and then boosting with an Id^{CR+} hybridoma protein, 16-46 (2). A panel of 9 hybridoma

proteins recognized by this reagent were subsequently isolated and classified as Id^{CR+} on the basis of their ability to inhibit greater than 80% of the binding of ^{125}I-16-46 to rabbit anti-Id^{CR} (reference 3 and Fig. 1a).

These 9 hybridoma proteins could be divided into two distinct subsets on the basis of their reactivity with two additional reagents. A monoclonal BALB/c anti-idiotype (MB-anti-Id^{cr}), prepared by fusing the spleen of a BALB/c mouse immunized with the Id^{CR+} hybridoma protein 36-65 (8), reacted with most of the Id^{CR+} proteins but failed to bind the Id^{CR+} hybridoma proteins 44-10 and 45-208 (Fig. 1B). In contrast, 44-10 and 45-208, were recognized by a heterologous antiserum, (anti-Id^{44-10}) obtained from a rabbit immunized only with the hybridoma protein 44-10 (reference 3 and Fig. 1C). Anti-Id^{44-10} failed to recognize any of the hybridoma proteins detected by MB-anti-Id^{cr}. The determinants associated with MB-Id^{cr} and Id^{44-10} were found only in A/J serum samples which contained Id^{CR}. As shown in Table 1, both MB-anti-Id^{cr} and anti-Id^{44-10} failed to react significantly with: 1) Ars-KLH immune A/J sera absorbed with Ars-BGG; 2) Ars-KLH immune sera from A/J mice neonatally suppressed with rabbit anti-Id^{CR}; 3) Ars-KLH immune BALB/c or B10.A sera, or 4) nonimmune A/J sera. Furthermore, the determinants detected by these two reagents were present in almost every individual A/J Ars-immune serum tested (8). Thus these two reagents recognized non-overlapping sets of Id^{CR+} hybridoma proteins as well as certain serum antibodies which displayed the same overall characteristics as the entire Id^{CR+} response.

To further test the association between the expression of Id^{CR} (determinants recognized by rabbit anti-Id^{CR}) and MB-Id^{cr} and Id^{44-10} (determinants recognized by MB-anti-Id^{cr} and anti-Id^{44-10}), 15 Igh Recombinant strains derived from matings between either A/He and BALB/c or BAB/14 and C.AL-9 and selected on the basis of intra-Igh-V region recombination events were examined (15). A/He and C.AL-9 Ars-immune sera are Id^{CR} positive, BALB/c and BAB/14 are not. In all cases, immune sera from strains which were Id^{CR} positive on the basis of reactivity with the rabbit anti-Id^{CR} antiserum also reacted with the monoclonal BALB/c anti-idiotype; those that were not recognized by the rabbit antiserum failed to react with the monoclonal reagent. Preliminary results obtained with the rabbit anti-Id^{44-10} antiserum indicate a similar trend. All of the strains that failed to express Id^{CR} also lack Id^{44-10} determinants, but as yet only 7 out of 10 of the Id^{CR+} positive strains have been shown to express Id^{44-10}. The discrepancy may be due to the relatively low titers of anti-Ars antibody obtained from the 3 inconsistent strains. Alternatively, anti-Id^{44-10} may recognize a determinant

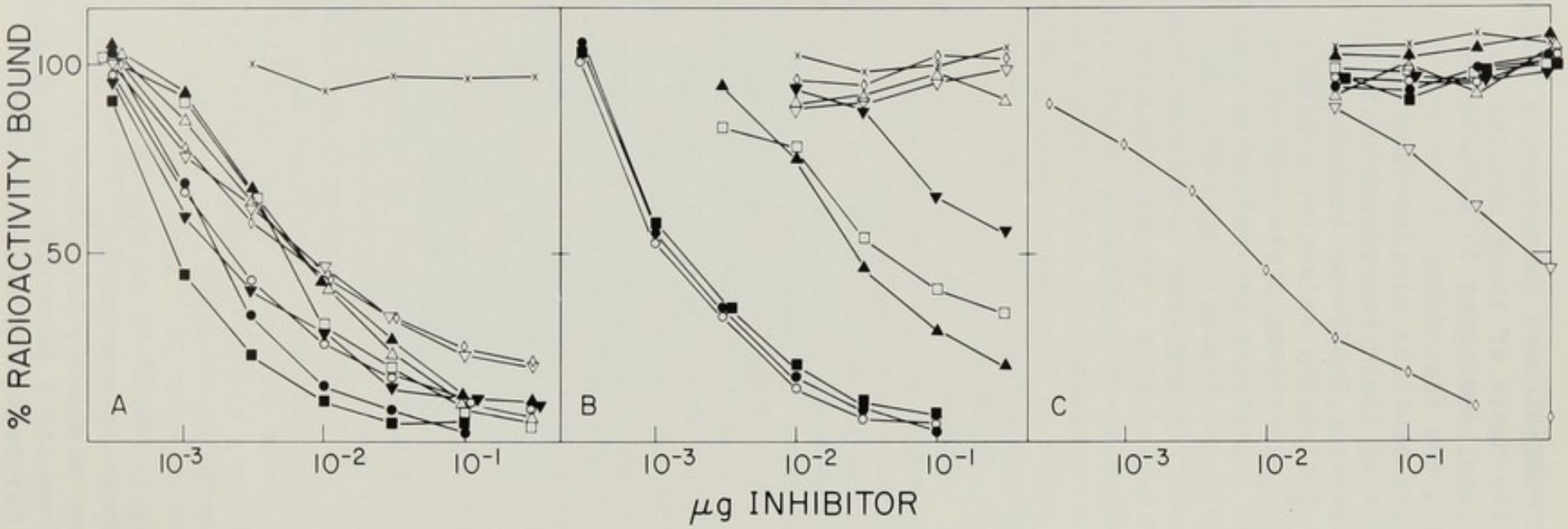

Figure 1. Specificity of rabbit anti-Id^{CR} (A), BALB/c monoclonal anti-Id^{cr} (B), and rabbit anti-Id$^{44-10}$ (C). 3 ng of ^{125}I-36-65 (A and B) or ^{125}I-44-10 (C) were added to polyvinyl microtiter dishes precoated with the appropriate antisera along with dilutions of the following unlabeled Ars-binding hybridoma proteins: 16-46-4-8 (■ , IgG2a); 31-62 (○, IgG2a); 36-65 (●, IgG1); 36-71 (△ , IgG1); 44-10 (◇ , IgG1); 45-223 (▲ , IgG1); 45-248 (□ , IgM); 45-273 (▼ , IgG3); 45-208 (▽ , IgG1); and 36-60 (X, IgG2a).

TABLE 1

Id^{44-10} and MB-Id^{cr} are present only in sera containing Id^{CR}

Serum sample	Anti-Ars antibody	Id^{CR}	Id^{44-10}	MB-Id^{cr}
Immune A/J	2920	1920	76	1150
Immune A/J-adsorbed with Ars-BGG	<3.5	<2	6	<3
Immune A/J-suppressed with anti-Id^{CR}	3570	<2	0	<3
Immune BALB/c	1110	<2	0	N.T.
Immune B10.A	2380	<2	0	<3
Normal A/J	N.T.	3	1	<3

The various pools of immune sera were obtained from mice immunized at least twice with Ars-KLH as described previously (2). Suppression of Id^{CR} was induced by neonatal injection of rabbit anti-Id^{CR}. Id^{CR}, MB-Id^{cr} and anti-Ars antibody titers are expressed in terms of μg equivalents of 36-65/ml as determined by solid phase radioimmunoassay (2,8). Id^{44-10} is expressed in terms of the percent inhibition of binding of ^{125}I-44-10 by rabbit anti-Id^{44-10} by 1 μl of immune sera in the radioimmunassay described in reference 3.

dependent on a combinantion of V and D region gene segments which were separated by recombination in the construction of the strains. The correlation between Id^{CR} expression and the expression of the "private" determinants (MB-Id^{cr} and Id^{44-10}) suggests that the entire Id^{CR} family is derived from a single germ line V_H gene. Alternatively, if every antibody sequence were encoded in the germ line then one might expect that the phenotypes could be separated by recombination.

A "Minor" Cross-reactive Idiotype. Serological evidence for minor cross-reactive idiotypes associated with the A/J anti-Ars response emerged from the study of Ars-binding

hybridoma proteins which failed to express the major cross-reactive idiotype (8,9). The structural basis for one such a minor idiotype family became apparent when the N-terminal amino acid sequences of 8 Id^{CR-} A/J Ars-binding hybridoma protein heavy and light chains were determined (7). These 8 monoclonal antibodies had been selected solely on the basis of their inability to react with rabbit anti-Id^{CR}, yet 3 of the 8 proteins were remarkably similar; only a few minor differences were found in the framework and CDR1 regions of both the heavy and light chains (8). All three hybridoma proteins reacted with a rabbit antiserum prepared against the single protein 36-60 (rabbit anti-Id^{36-60}). Comparison of the Id^{36-60+} sequences to the Id^{CR+} sequences showed that they differed by as many as 29 out of the first 48 N-terminal heavy chain residues and by 31 out of the first 47 light chain residues, and were therefore probably encoded by separate germline genes (7,8). Further evidence for the relatively high frequency of this minor idiotype is that Id^{CR-} protein sequences homologous to the Id^{36-60+} group have also been reported by others (5,16). It was therefore not surprising that rabbit anti-Id^{36-60} reacted with almost all the A/J anti-Ars immune sera we examined. Unexpectedly, rabbit anti-Id^{36-60} also reacted with all BALB/c anti-Ars immune sera although it failed to react with Ars-immune sera from B10.A (8). A similar inter-strain cross-idiotype has been found by Brown et al. (17).

Independent Regulation of Major and Minor Idiotype Expression. It has previously been reported that expression of the major idiotype could be suppressed by injection of the appropriate heterologous antisera without affecting the overall anti-Ars antibody titer (11). It was of interest to establish whether the minor idiotype family, Id^{36-60}, could be similarly suppressed. In addition, the suppressive capacity of the BALB/c monoclonal anti-Id^{cr} was examined. As shown in Table 2, neonatal injection of MB-anti-Id^{cr} prior to Ars-KLH immunization as adults, resulted in a profound decrease in the level of Id^{CR} (the idiotype defined by the rabbit antiserum), but did not alter Id^{36-60} expression. Determinants recognized by MB-anti-Id^{cr} were totally absent in these suppressed sera (data not presented). Similarly, the rabbit anti-Id^{36-60} suppressed the expression of Id^{36-60} but not Id^{CR}. The independent regulation of the major and minor idiotype families further supported the finding that they were structurally distinct.

TABLE 2

Independent regulation of major and minor idiotype

Antiserum used to induce suppression	*anti-Ars antibody*	*Id^{CR}*	*Id^{36-60}*
none[a]	*1860*	*520*	*81*
MB-anti-Id^{cr}	*600*	*10*	*85*
none[b]	*2630*	*1394*	*56*
rabbit anti-Id^{36-60}	*1428*	*894*	*8*

A/J mice were suppressed by neonatal injection of either MB-anti-Id^{cr} or rabbit anti-Id^{36-60} and then immunized twice with Ars-KLH as adults. The results are summarized as the median values of 10[(a)] or 4[(b)] mice per group. Id^{CR} and anti-Ars antibody titers were determined in solid phase radioimmunoassays and are presented in terms of μg equivalents of 36-65/ml. Id^{36-60} is expressed in terms of percent inhibition of binding of ^{125}I-36-60 by rabbit anti-Id^{36-60} as described in Table 1 (reference 8).

Extent of MB-anti-Id^{cr}-induced suppression. Careful examination of the data in Table 2 shows that the relative proportion of Id^{CR} in the group of mice suppressed with MB-anti-Id^{cr} is approximately 20-fold lower than the control group. The extent of this suppression was somewhat greater than expected considering that MB-anti-Id^{cr+} reacted strongly with only 3 out of 9 of our Id^{CR+} hybridoma proteins (Fig. 1B). These results suggest that if suppression were induced with a reagent that recognized private determinants present on a subset of Id^{CR+} molecules, then Id^{CR+} molecules that expressed the major crossreacting determinants but lacked the original more restricted determinants would also be suppressed. In order to address this question more directly, suppressed and nonsuppressed sera were compared for their content of Id^{44-10}, since MB-anti-Id^{cr} and rabbit anti-Id^{44-10} recognized nonoverlapping subsets of Id^{CR+} hybridoma proteins (Fig. 1). The data presented in Table 3 demonstrates that the injection of MB-anti-Id^{cr} into either neonatal or adult mice prior to antigen challenge results in suppression of both MB-Id^{cr} and the majority of Id^{44-10}.

TABLE 3

Suppression induced by BALB/c monoclonal anti-Id^{cr}

Serum sample	*Suppression regimen*	*MB-Id^{cr}*	*Id$^{44-10}$*
D117-1	*none*	*680*	*17*
D117-2		*450*	*30*
D140-1	*MB-anti-Id^{cr} ascites 10 µl as neonates*	*<1*	*2*
D140-2		*<1*	*<1*
D140-5		*<1*	*<1*
D140-6		*<1*	*10*
D112-1	*Sp2 ascites 10 µl as adults*	*1560*	*26*
D112-2		*925*	*38*
D112-3		*333*	*2*
D112-4		*300*	*10*
D114-1	*MB-anti-Id^{cr} ascites 10 µl as adults*	*<1*	*2*
D114-2		*<1*	*<.5*
D114-3		*<1*	*5*
D114-4		*<1*	*<.5*

A/J mice were suppressed as neonates or adults by injection with MB-anti-Id^{cr} and subsequently immunized twice with Ars-KLH. MB-Id^{cr} titers are expressed as µg equivalents of 36-65/ml. Id$^{44-10}$ titers are expressed as µg equivalents of 44-10/ml.

This result would seem to be in keeping with expected "network"-related interactions.

Two alternative explanations for the results presented in Table 3 were immediately apparent: 1) The specificity of the suppressor effector cells induced with MB-anti-Id^{cr} was broader than the original specificity of the MB-anti-Id^{cr} antibody due to sequential anti-idiotype → idiotype → anti-

idiotype T cell interactions (13) which resulted in the recognition of cross-reactive determinants; or 2) The subsets serologically defined on the basis of reactivity with our panel of hybridoma proteins did not reflect subsets normally occurring in immune sera. In other words, typical antibodies in immune sera express determinants recognized by both MB-anti-Id^{cr} and anti-Id^{44-10}, such that suppression with one reagent leads to the loss of the other determinant. To test the latter possibility, several pools of immune serum were absorbed with MB-anti-Id^{cr}-coupled Sepharose 4B. As shown in Table 4, this procedure removed all antibodies reactive with MB-anti-Id^{cr} and reduced the amount of Id^{CR} by greater than 95%. In addition, the level of Id^{44-10} was markedly reduced. These results could not be attributed to nonspecific effects, since the level of Id^{36-60} remained unchanged. Furthermore, affinity purified 44-10 protein, even when mixed with immune serum, did not bind to the column, indicating that the retention of Id^{44-10} molecules in the immune sera was not due to either a low affinity interaction between MB-anti-Id^{cr} and 44-10 protein or idiotype-anti-idiotype complexes which might be present in immune serum. Rather, immune sera must contain molecules which express both Id^{44-10} and MB-Id^{cr} determinants.

In summary, the extensive suppression of Id^{CR} induced by the injection of MB-anti-Id^{cr} appears to reflect the actual specificity of MB-anti-Id^{cr} for antibodies found in Ars-immune sera. Presumably, MB-anti-Id^{cr} reacts with approximately 90% of the Id^{CR+} molecules found in immune sera. These results strongly suggest that the microheterogeneity of the idiotype response as seen in the hybridoma sequences is an underestimate of the total heterogeneity characteristic of the Id^{CR+} serum response since some molecules in serum are composites of the sequences studied so far. It is noteworthy that not all Id^{44-10} associated-determinants present in immune serum can be removed by adsorption on an MB-anti-Id^{cr} column. This indicates that there are Id^{CR+} molecules in serum which are similar to 44-10 in that they fail to bind to MB-anti-Id^{cr}. Combined with the fact that small amounts of Id^{44-10}-like molecules are present in immune sera when suppression is induced with MB-anti-Id^{cr}, these results strongly suggest that specificity does not degenerate as the various elements of the regulatory network operate to suppress idiotype production.

TABLE 4

Activity remaining after absorption with monoclonal anti-idiotype

serum sample	anti-Ars antibody	MB-Id^{cr}	Id^{CR}	Id^{44-10}	Id^{36-60}
D112-Pre	3850	1280	2970	10.0	4.9
-Post	625	<2	210	0.8	6.7
D118-Pre	3940	1442	2500	28.6	8.8
-Post	1330	<2	105	.8	10.7
D119-Pre	4760	870	3800	33.3	13.3
-Post	770	<2	190	1.2	14.6
XD43 -Pre	3615	1052	1250	25.1	N.T.
+ -Post	1875	<2	32	17.8	
Prot.44-10					

Three pools of Ars-immune sera (D112, D118, D119) were selected on the basis of relatively high percentages of Id^{CR} and Id^{44-10}. Serum sample XD43 contained a low level of Id^{44-10} but was mixed with affinity purified 44-10 hybridoma protein. Aliquots of each serum sample were diluted in PBS containing 50% fetal calf serum, and a portion of each sample was passed through a monoclonal anti-Id^{CR}-coupled Sepharose column. Pre- and post-column samples were adjusted to equal protein concentrations and compared by radioimmunoassay (2,3,8). MB-Id^{CR}, Id^{CR}, and anti-Ars antibody titers are presented in terms of µg equivalents 36-65 per ml; Id^{44-10} and Id^{36-60} in terms of µg equivalents of 44-10 and 36-60 per ml respectively.

REFERENCES

1. Kuettner, M.G., Wang, A.L., and Nisonoff, A. (1972) J. Exp. Med. 135, 179.
2. Marshak-Rothstein, A., Siekevitz, M., Margolies, M.N., Mudgett-Hunter, M. and Gefter, M.L. (1980) Proc. Natl. Acad. Sci. 77, 1120.
3. Marshak-Rothstein, A., Benedetto, J., Kirsch, R.L., and Gefter, M.L. (1980) J. Immunol. 125, 1981.
4. Estess, P., Nisonoff, A., and Capra, J.D. (1979) Mol. Immunol. 16, 1111.
5. Estess, P., Lamoyi, E., Nisonoff, A., and Capra, J.D. (1980) J. Exp. Med. 151, 863.
6. Lamoyi, E., Estess, P., Capra, J.D., and Nisonoff, A. (1980) J. Immunol. 124, 2834.
7. Margolies, M.N., Marshak-Rothstein, A., and Gefter, M.L. (1981) Mol. Immunol., in press.
8. Marshak-Rothstein, A., Margolies, M.N., Benedetto, J.D., and Gefter, M.L. (1981) Eur. J. Immunol., in press.
9. Gill-Pazaris, L.A., Brown, A.R., and Nisonoff, A. (1979) Ann. Immunol. (Inst. Pasteur) 130C, 199.
10. Gill-Pazaris, L.A., Lamoyi, E., Brown, A.R., and Nisonoff, A. (1981) J. Immunol. 126, 75.
11. Hart, D.A., Pawlak, L.L., and Nisonoff, A. (1972) J. Exp. Med. 135, 1293.
12. Owen, F.L., Ju, S.-T. and Nisonoff, A. (1972) J. Exp. Med. 135, 1293.
13. Hirai, Y., and Nisonoff, A. (1980) J. Exp. Med. 151, 1213.
14. Hirai, Y., Lamoyi, E., Dohi, Y., and Nisonoff, A. (1981) J. Immunol. 126, 71.
15. Riblet, R. (1977) in Immune System: Genetics and Regulation. Sercarz, E., Herzenberg, L.A., and Fox, F., eds. Academic Press, New York. p.83.
16. Alkan, S.S., Knecht, R., and Braun, D.G. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 191.
17. Brown, A.R., Lamoyi, E., and Nisonoff, A. (1981) J. Immunol., in press.

IDIOTYPES OF ANTI-MHC MONOCLONAL ANTIBODIES

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ABSTRACT Anti-idiotypic antibodies to several anti-H-2 and anti-Ia hybridoma antibodies have been used as probes of anti-major histocompatibility complex (MHC) receptors. Each anti-H-2 idiotype studied was readily detected only on the monoclonal antibody used as immunogen, and not on numerous other monoclonal antibodies of similar H-2 specificity, nor in conventional anti-H-2 antisera. In contrast, one anti-Ia idiotype examined was readily detected on conventional anti-Ia antibodies of appropriate allotype and specificity. Despite failure to detect the anti-H-2 idiotypes in immune sera, these idiotypes could be induced by treatment with purified anti-idiotypic antibodies in mice of the same Igh-C allotype as that of the idiotype. Thus, the genetic information required for both anti-H-2 and anti-Ia idiotype expression appeared to be present in these mice. The relative amount of each idiotype produced in response to antigens and/or anti-idiotype administration may reflect either the size of the antibody repertoire reactive with the corresponding antigens, or the regulatory mechanism controlling anti-MHC gene expression.

INTRODUCTION

Anti-MHC receptors appear to be involved in a large number of important immune interactions. In addition to their role in responses to alloantigens such as the MLR and the transplant rejection reaction, they appear to play a physiologic role in the recognition of foreign antigens in the context of self determinants (1,2). Antibodies to idiotypic determinants on anti-MHC receptors may therefore be useful probes for investigating and perhaps for modifying these immune interactions.

Approaches to the production of such anti-idiotypic reagents have previously involved immunizations with immunocompetent cells (3,4) or with heterogeneous alloantibodies

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(3,5,6), but these approaches have proved difficult to reproduce. The recent advent of hybridoma technology has made available a reproducible source of anti-MHC antibodies which can be used to prepare anti-idiotypic reagents. We have used several anti-H-2 and anti-Ia monoclonal antibodies to produce such reagents in heterologous animals. We shall summarize here some of the data we have so far obtained concerning the expression of these idiotypes in the antibody repertoire of mice treated with antigen and/or with anti-idiotype.

METHODS

Animals and Alloantisera. CWB mice were kindly provided by Dr. M. Bosma, ICR, Philadelphia, PA. All other mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or were raised in our own animal colonies. New Zealand white rabbits were obtained from Animal Production, NIH. Miniature swine were from our partially inbred herd and were housed at the NIH Animal Center, Poolesville, Maryland. Alloantisera were produced by immunization with skin grafts and lymphoid cells as previously described (7).

Monoclonal Antibodies. Clone 11-4.1 (8) was provided through The Salk Institute. All other hybridomas used in this study were developed and produced in our laboratory as previously described (9). Monoclonal antibodies were purified from culture supernatant by affinity chromatography on protein A Sepharose (Pharmacia) or by ion exchange chromatography.

Anti-Idiotypic Antibodies. Miniature swine and rabbits were immunized with hybridoma antibodies by intramuscular injection of antibody in complete Freund's adjuvant (200 μg and 50 μg per injection for pigs and rabbits, respectively). Purified anti-idiotypic antibodies were obtained from hyperimmune sera by adsorption with insolubilized normal mouse immunoglobulins or myeloma proteins of appropriate class, followed by adsorption to and elution from idiotype-bearing Sepharose columns. Detailed methodology for the preparation and purification of these anti-idiotypes has been published elsewhere (10,11).

Assays. Hemagglutination and hemagglutination inhibition assays were performed as previously described (10). The enzyme-linked immunoabsorption assay (ELISA) has also been described elsewhere (11). Briefly, ELISA plates were coated with hybridoma antibodies in PBS at 1-2 μg/ml. Inhibitors

were serially diluted, mixed with an appropriate dilution of purified anti-idiotype, and added to sequential wells of ELISA plates. Plates were developed with a peroxidase-conjugated rabbit anti-pig immunoglobulin (Miles Yeda), followed by an orthophenylene diamine substrate solution (Aldrich Chemical Co.).

Cell sorter analyses were performed on lymph node cells treated with test sera, stained with a mixture of fluoresceinated anti-mouse IgG_1 and anti-mouse IgG_2 and analyzed on a fluorescence activated cell sorter (FACS II). Data were collected on $5x10^4$ viable lymph node cells and displayed as a 1000 channel cell frequency histogram in which fluorescence intensity was plotted on the x axis and cell number on the y axis.

RESULTS

Several of the hybridomas against which we have so far prepared heterologous anti-idiotypic antibodies are shown in Table I. Yields of purified anti-idiotypic antibodies were approximately 0.1-0.5 mg/ml of hyperimmune pig or rabbit serum. Specificity of the antibodies was determined by both hemagglutination and ELISA assays (10,11).

The presence of idiotypic determinants detected by these reagents was assessed in a panel of hybridomas and in a variety of conventional immune sera. By both the hemagglutination inhibition assay and the ELISA inhibition assay, all

TABLE I
HYBRIDOMA ANTIBODIES USED TO PRODUCE ANTI-IDIOTYPES

Hybridoma Designation	Immune Cells Fused	Specificity	Known Cross-reactions	Reference
11-4.1	BALB/c α CKB	K^k	K^q,p,r	8
3-83P	BALB/c α C3H	K^k	D^k,b,p,q,r,s	9
16-3-22S	C3H.SW α C3H	K^k	none	9
23-10-1S	BALB/cH-2^{dm2} α BALB/c	L^d	L^q, D^q	12
14-4-4S	C3H.SW α C3H	I-E^k	d,p,r	9

four anti-H-2 anti-idiotypic reagents examined were inhibited specifically only by the hybridoma monoclonal antibody against which they were prepared and not by numerous other monoclonal antibodies of similar H-2 specificity, nor by conventional alloantisera directed against the same H-2 antigens (10, and unpublished information). In contrast, one of the anti-Ia anti-idiotypic reagents examined (anti-14-4-4S) was inhibited by C3H.SW anti-C3H immune sera, the strain combination from which this hybridoma was derived.

In order to determine whether the cross-reactive idiotype in C3H.SW anti-C3H alloantisera was carried by antibodies of the same anti-Ia specificity as 14-4-4S, absorptions of this alloantiserum in appropriate strains were performed. As seen in Table II, B10.A(5R) and B10.A(2R) were capable of absorbing the idiotype from this alloantiserum, while strains B10 and B10.A(4R) were not. Since only the I-A and I-E subregions have been shown to encode classical Ia antigens (13), this data strongly suggests that anti-I-E^k antibodies in this alloantiserum were responsible for the idiotype detected.

TABLE II
EFFECT OF IN VIVO ABSORPTION OF C3H.SW ANTI-C3H ALLOANTIBODY ON IDIOTYPE LEVELS

In Vivo Absorption[a] in	H-2 Haplotype[b]								Residual % ELISA Inhibition[c]
Strain	K	A	B	J	E	C	S	D	
B10	b	b	b	b	b	b	b	b	27
B10.A(4R)	k	k	b	b	b	b	b	b	43
B10.A(2R)	k	k	k	k	k	d	d	b	$\leq$ 0
B10.A(5R)	b	b	b	k	k	d	d	d	$\leq$ 0

[a]0.5 ml of antiserum injected ip in mice of the indicated strains. Mice exsanguinated 4 hr later.

[b]Small letters designate haplotype of origin of H-2 regions and subregions (capital letters). Arrows show inferred position of genes coding for antigens responsible for absorption of idiotype.

[c]Maximum percent inhibition of binding of anti-idiotype to plate-bound idiotype as determined by assay of 2-fold dilutions of serum in ELISA inhibition assay.

Additional studies have shown that C3H.SW animals immunized with B10.A(2R) cells develop serum idiotype, while C3H.SW animals immunized with B10.A(4R) cells do not, confirming these absorption results. CWB mice, a strain congenic with C3H.SW but possessing a different Igh allotype locus, did not produce significant levels of idiotype when immunized with C3H lymphoid cells, indicating the importance of Igh-linked genes in the expression of this idiotype.

Although, as mentioned above, the anti-H-2 idiotypes could not be detected in conventional alloantisera by either the hemagglutination inhibition assay or the ELISA inhibition assay, several of these idiotypes were readily detected in the sera of animals treated with anti-idiotypic antibodies. For example, BALB/c mice treated with 40 μg of purified pig or rabbit anti-3-83P antibodies uniformly produced serum idiotype detectable by both of these serologic assays. The idiotype levels rose over the first 2 weeks following treatment and remained elevated for at least 6 weeks thereafter. In addition, the sera from some of these anti-idiotype treated mice contained anti-H-$2K^k$ reactivity as detectable by a binding assay using flow microfluorometry on the fluorescence activated cell sorter (FACS) (Fig. 1). As seen in this figure,

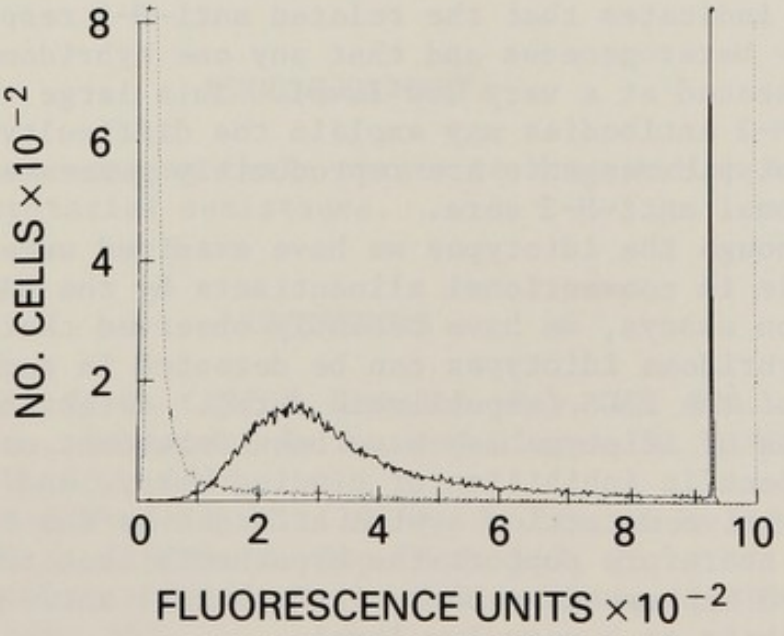

FIGURE 1. Serum from Mouse 1529, a BALB/c animal treated with rabbit anti-3-83P anti-idiotypic antibodies, was tested for binding to C3H (————) and C3H.OH (--------) lymph node cells on the FACS.

serum from rabbit anti-3-83P treated BALB/c animal No. 1529 showed significant binding to C3H lymphoid cells, but not to C3H.OH lymphoid cells. This binding could be specifically inhibited by the pig or rabbit anti-3-83P anti-idiotypes, as could the binding of 3-83P itself (14), indicating that the binding activity was indeed present on idiotype-bearing molecules. Induction of idiotype by such treatment was observed in animals of the same Igh linkage group as the strains of origin of the idiotype (in this case, BALB/c), but not in mice of several other Igh types. Appearance of serum idiotype has been observed following anti-idiotype treatment for all of the anti-idiotypes listed in Table I.

DISCUSSION

Hybridoma antibodies appear to provide a window among clones normally produced in conventional immune responses to a variety of antigens, including alloantigens (15). Anti-idiotypic reagents detect unique antigenic determinants, or idiotopes, on such hybridoma antibodies, and such idiotopes presumably reflect the source of individuality of antibody molecules, the V_H and V_L gene repertoires. Thus, the "private" nature of the anti-H-2 idiotypes we have examined probably indicates that the related anti-H-2 response is extremely heterogeneous and that any one hybridoma idiotype is represented at a very low level. This large repertoire of anti-H-2 antibodies may explain the difficulty with which anti-idiotypic reagents are reproducibly generated against conventional anti-H-2 sera.

Although the idiotypes we have examined were not readily detectable in conventional alloantisera by the HAI or ELISA inhibition assays, we have recently observed that low levels of the hybridoma idiotypes can be detected in such sera by the use of the FACS (unpublished data). Detection of these low levels of idiotype may have been dependent on the use of a site-specific inhibition of binding assay, and/or on the very sensitive detection system afforded by the FACS. These findings therefore support the hypothesis that these idiotopes are indeed expressed as part of the normal anti-H-2 repertoire, albeit at a very low level.

On the other hand, the anti-Ia idiotype we have examined, anti-14-4-4S, was readily detectable in conventional immune sera. These findings suggest that this idiotype may be a common component of the immune response of appropriate animals. However, the particular anti-Ia hybridoma studied detects Ia.7, which is probably not a true alloantigen, but rather a common determinant of all I-E molecules (16). Thus,

the ease of detection of idiotype in this system could be peculiar to the anti-Ia.7 repertoire, rather than a general characteristic of anti-Ia responses. Alternatively, anti-Ia antibody responses may generally be less heterogeneous than anti-H-2 antibody responses, perhaps reflecting a smaller V gene repertoire or less somatic diversification.

The fact that anti-H-2 idiotypes could be induced by anti-idiotypic reagents, even though these idiotypes were rare in the normal response, indicates that the relevant V genes must be present in all mice of appropriate Igh type. This finding does not, however, indicate whether such V genes are of germ line origin or arise by somatic diversification. Some, but not all, of the idiotype-bearing molecules induced by anti-idiotype treatment appear to bind antigen. It is our current hypothesis that the induced idiotype-bearing molecules represent a scrambling of idiotopes on different antibody molecules, only some of which have a combining site suitable for the binding of antigen (17). By this hypothesis, the same hypervariable and/or J regions may be used in antibodies of different specificity. In any case, the production of anti-H-2 antibodies in the absence of exposure to conventional antigen may indicate that treatment with anti-idiotypes will be an effective means of dissecting and manipulating immune responses to MHC determinants.

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REFERENCES

1. Zinkernagel, R. M. (1978). Immunol. Rev. 42, 224.
2. Hodes, R. J., Hathcock, K. S., and Singer, A. (1980). J. Exp. Med. 152, 1779.
3. Binz, H., and Wigzell, H. (1975). J. Exp. Med. 142, 197.
4. Krammer, P. N. (1978). J. Exp. Med. 147, 25.
5. Rubin, B., Hortel-Wulff, B., and Kimura, A. (1979). J. Exp. Med. 150, 307.
6. Binz, H., Ramseier, H., and Lindenmann, J. (1973). J. Immunol. 111, 1108.
7. Sachs, D. H., and Cone, J. L. (1973). J. Exp. Med. 138, 1289.
8. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A., and Herzenberg, L. A. (1978). Curr. Top. Microbiol. Immunol. 81, 115.

9. Ozato, K., Mayer, N., and Sachs, D. H. (1980). J. Immunol. 124, 533.
10. Sachs, D. H., Bluestone, J. A., Epstein, S. L., and Ozato, K. (1981). Transplant. Proc. 13, 953.
11. Epstein, S. L., Ozato, K., Bluestone, J. A., and Sachs, D. H. (1981)., manuscript submitted.
12. Ozato, K., Hansen, T. H., and Sachs, D. H. (1980). J. Immunol. 125, 2473.
13. Shinohara, N., and Sachs, D. H. (1981). J. Immunol. 126, 934.
14. Bluestone, J. A., Sharrow, S. O., Epstein, S. L., Ozato, K., and Sachs, D. H. (1981). Nature, in press.
15. Ozato, K., and Sachs, D. H. (1981). J. Immunol. 126, 317.
16. Ozato, K., Lunney, J. K., El-Gamil, M., and Sachs, D. H. (1980). J. Immunol. 125, 940.
17. Sachs, D. H., El-Gamil, M., and Miller, G. (1981). Eur. J. Immunol., in press.

IDIOTYPIC INTERACTIONS OF ANTIBODIES AND CELLULAR RECEPTORS THAT PROVIDE AND REGULATE IMMUNE ACTIVITY

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Introduction

The diversity of antibodies, in respect to both the combining site and the idiotypic repertoires, makes it inescapable that the immune system is embodied by a network of complementarities amongst its own elements (1). While this postulate has been extensively confirmed along the last few years (reviewed in 2), the precise definition of the relevance of such internal recognition to the development and physiology of the immune system is, yet, far from complete. Questions can still be raised concerning the most central point in these concepts, namely whether a network is a necessary consequence of diversity, devoid of functional significance, or whether immune systems cannot develop and function but for the properties derived from idiotypic recognition.

It is clear that a complete description of an immune system is an impossible task. Given the clonal markers of individuality of lymphocytes, their wide heterogeneity as to the differentiated structures and functions they express, and the enormous activity characteristic of the "closed" immune system, that description would be equivalent to a detailed analysis of each of the inhabitants in a town 100,000 times bigger than Paris: who is talking to whom, who has died and who was born, where and when is everything happening, the dominant families and the simple citizens that are, nonetheless, unique. One might be tempted to declare it incomprehensible or else, to immortalize, one by one, each of those inhabitants by somatic cell hybridization. Two points should be made clear, however : the uniqueness of each moment in the life of each immune system, and the legitimacy of our attempts to understand the general laws of that life and its politics.

We will attempt here to briefly comment on the importance of idiotypic interactions in determining the selection of available antibody repertoires, and in

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maintaining the activity in the system by driving precursor cells into the immunocompetent cell pool and by stimulating the latter into effector functions. We will also discuss some implications of the general phenomenon of idiotypic mimicry (internal images) for the evolution of other components in the immune system.

The selection of available, mature repertoire by idiotypic manipulation of adult animals

Several experiments have clearly shown the relevance of functional idiotypic network interactions in the expression of potential repertoires.

As suggested more than 10 years ago (3) let us suppose that the potential repertoire is similar in all rabbits of the same allotypic immunoglobulin genotype. Normal rabbit X and rabbit I produce different idiotypes AbX and Ab1 when immunized against the same antigen. Since Ab1 is not expressed in rabbit X, it could be possible that the rabbit X immune system contains an auto-antiidiotypic activity (Ab2) against Ab1 which suppress the synthesis of Ab1. If we suppress Ab2 activity by raising Ab3 antibodies against Ab2, we can expect to favour the synthesis of Ab1 (or Ab1') in rabbit X. This hypothesis is testable if one assumes that Ab2 antibodies directed against a given Ab1 synthetized by different rabbits are idiotypically similar.

The following experimental scheme was therefore tried. A first antibody (Ab1) synthetized by rabbit I against a given antigen was isolated. Anti-idiotypic antibodies (Ab2) were raised against Ab1 in allotype - matched rabbit II. Isolated Ab2 were used as immunogen to induce anti-(anti)-idiotypic antibodies (Ab3) in allotype matched rabbit III. Antigen was then given for the first time in rabbit III which had previously synthetized Ab3, this animal produced antibody specific for the antigen (Ab1').

The main results of these experiments (4, 5) can be summarized as follows: rabbit III produced anti-(anti)-idiotypic antibodies Ab3 without detectable antibody function for the antigen, Ab1' antibodies are idiotypically similar, and in some cases identical, to Ab1.

Similar results were obtained in mice. The BALB/c anti-DNP M460 idiotype which is recurrent in the anti-DNP response of BALB/c strain and of strains with the $Igh C^a$ allotypic haplotype but not expressed in the other strains can be induced in $Igh C^c$ DBA/2 (6) and $Igh C^e$ NZB (7) mice immunized against DNP after they have previously produced

Ab3 antibodies either against polyclonal or against monoclonal anti-M460 Ab2.

These idiotypic "manipulations" of the immune systems clearly show that the repertoire available to antigens in one individual is smaller than the potential individual repertoire and that the potential idiotypic repertoire is more or less the same in different individuals of the same species.

Thus what one observes is the selection of possibilities that are almost infinite. If the potential repertoire is germline encoded, its expression is under the control of complex regulatory mechanisms involving idiotypes. These mechanisms drive the potential repertoire into different available repertoire following the genotype of the individual (in terms of immunoglobulin allotypy). In a somatic view of diversification, there is a constant selection of the available repertoire from limited number of germ-line genes. Manipulations of the idiotypic network can change the mechanisms of selection so much so that very rare somatic variants are selected and could be expanded in modified conditions of selection.But whatever the origin of antibody diversification, the results of idiotypic manipulations show that the potential repertoire is enormous.

The idiotypic network is probably not open-ended but it rather turns back onto itself and presents a high degree of connectance:

The frequence of LPS reactive B lymphocytes able to express the anti-DNP idiotype M460 after polyclonal activation is around 1:1500 (8). This frequency estimated using a syngeneic monoclonal anti-idiotypic antibody can only be understood, according to clonal selection concepts, if one immunoglobulin can in fact bind a very large number of other immunoglobulins. This would mean that the connectance within the functional idiotypic network is high.

Anti-idiotypic antibodies have been prepared against Ab3 (Ab4 antibodies). A part of Ab4 cross-reacts with Ab1, the other part does not (9, 10). These observations suggests that the idiotypic network is not a juxtaposition of single chains Ab1-Ab2-Ab3-Ab4 --- but in some way circular inside a given idiotypic system with some degree of connectance with other idiotypic systems.

Antibodies to recurrent idiotypes against alloantigens are the internal images of the alloantigens

In species in which inbred strains are not available, the synthesis of private idiotypes particular to each individual (or a very small number of individuals) seems to be the rule (11). Important exceptions are the idiotypes directed against alloantigens:

It has been demonstrated that rabbit idiotypes against rabbit allotypes are recurrent (12,13,14). Similar data have been also obtained in the mouse (15). The observation that idiotypes against allotypes, and probably against all alloantigens, are highly conserved during evolution suggest that they play a central role in the regulation of the immune system and in the selection of available repertoire.

Moreover, it has been shown that anti-idiotypic Ab2 against anti-allotypic Ab1 are the "internal images" (1) of the immunizing allotype. For instance, when rabbits are immunized to Ab2 directed against anti-b6 Ab1, they produce Ab3 and all these Ab3 exhibit anti-b6 activity (16). These results together with those obtained in the mouse showing that anti-H2 antibodies can be induced by injection of Ab2 antibodies directed against anti-H2 (17), suggest that, in their available repertoire, all individuals of a given species possess internal images of the alloantigens of that species.

Structural components of the idiotypic network that are not antibody molecules: the expansion of immune networks to functionally relevant areas

The original form of the network hypothesis does not consider in detail two important points of immune physiology, namely the basis forself-nonself discrimination and the ontogenic aspects upon which expansion of precursors and selection of available repertoires must be considered. The former, however, is a fundamental property of immune systems, while very postulates of the network hypothesis identify self and nonself in the same universe of recognizable molecular patterns. It follows that self-nonself discrimination in network perspectives cannot be based on antibody recognition as it had been current to do in immunological theories (e.g. clonal abortion and two signals hypothesis). Immune reactivity by competent cells must necessarily be distinguished from antibody receptor recognition, although the latter has to

provide the selective properties of clonal responses.

Cell surface recognition structures ensuring self-non-self discrimination, that is those controling activation must, therefore, be distinct from antibody receptors on B lymphocytes and, on simplistic basis, lie outside the immune network. It is clear, however, that the completeness of antibody repertoires provides the system with complementary molecules to such activating receptors, either in the form of combining sites or of idiotopes on antibody molecules. Those discriminatory, functionally relevant receptors are then included in the general idiotypic network via cross-reactivity with antibody idiotypes or combining sites.

These considerations have now received experimental demonstration: a number of idiotypes were found to either "cross react" with polyclonally expressed B cell surface structures that are involved in cell triggering (18,19), or to be recognized by similar structures on B cells (20).

The common property to all such antibody idiotypes, that are either similar or complementary to activating receptors, is their "recurrent" nature in the normal immune system. This suggests the possibility that such antibodies are of "germ-line" type and that the idiotopes on activating receptors have played a fundamental evolutionary role determining the accumulation of germ-line antibody genes (21). Furthermore, that internal complementarity provides the immune system with inbuilt capabilities to auto-stimulation, ensuring the levels of autonomous activity and "natural" antibody production, characteristic of the normal "steady state".

The finding that such receptors are already expressed at the precursor cell level (22) also indicates that idiotypic interactions play an important role in the expansion of the precursor cell pools (19) and, by cross-reactivity between activating and antibody receptors at the single cell level, in the selection of available repertoires in the immunocompetent cell pool. Part of these possibilities have recently been demonstrated *in vivo* by studying the progeny of females actively immunized against a recurrent idiotype (8).

The development of these experiments will have to consider the extend of diversity of such idiotypic receptors, as well as their molecular nature and genetic determination, in order to derive the detailed mechanisms by which available antibody repertoires are selected.

The role of T lymphocytes in the maintenance of available repertoires

Practically no information is available on the antibody repertoires of T cell-deprimed mice. It is reasonable to assume, however, that the general characteristics of the B cell compartment are maintained in the absence of T cells, in particular those discussed above: antibody driven expansion of precursors, turn-over of competent cells, and a primary selection of available repertoires in the bone marrow. On the other hand, a number of observations have recently accumulated indicating that the peripheral expression of the antibody repertoire is modulated by the activity of idiotype-specific T cells, that can either suppress or expand antibody clones. We arrive, therefore, at the discussion of clonal dominance and of available repertoire expression. Most such mechanisms appear to operate in the normal steady state, before introduction of external antigen, since it can be demonstrated that normal mice contain T cells that diminish the quantitative levels of idiotype expression (23) as well as helper cells that are specific for internal idiotypes and control the levels of expression of such idiotypes (24). Here again, it appears that only "recurrent" idiotypes find the respective complementary specificities in the normal T cell repertoires (25) and the question arises on the definition of the *primus movens* in these interactions: is the available antibody repertoire, primarily selected on a T cell independent basis, that modulates peripheral T cell repertoires, primarily selected in the thymus on antibody independent basis? Or, is the available T cell repertoire that draws the fine anatomy of the antibody repertoire, by selecting for persistance in the "steady state" the appropriate specificities amongst the overwhelming diversity continously produced in the marrow? Or, most likely, are both of these not alternatives but different, partial ways of describing reality?

Conclusion

With the extremely limited amount of structural and functionnal information we have available at present, and the problems inherent to the analysis of networks, it is astonishing that so much can be understood, or at least discussed about the immune system. It is all the more remarkable that Niels Jerne perceived the essence of a system turned to the inside, reading the lead of perspective of Paul

Ehrlich who considered the generality of immune responses to the outside world.

References

1 - Jerne, N.K. (1974). *Ann. Immunol. (Inst.Pasteur, 125C,* 373.
2 - Urbain, J., Wuilmart, C., and Cazenave, P.-A. (1980). *Cont. Topics in Mol. Immunol. 8* (in press).
3 - Oudin, J., and Michel, M. (1969). *J. Exp. Med. 130,* 619.
4 - Cazenave, P.-A. (1977).*Proc.Nat.Acad.Sci. (Wash.) 74,*5122.
5 - Urbain, J., Wikler, M., Franssen, J.D., and Collignon, C. (1977). *Proc.Nat.Acad.Sci. (Wash.) 74,* 5126.
6 - Le Guern, C., Ben Aïssa, F., Juy, D., Mariamé, B.Buttin,G. and Cazenave, P.-A. (1979). *Ann. Immunol. (Inst.Pasteur), 130C,* 293.
7 - Le Guern, C. and Cazenave, P.-A., submitted.
8 - Bernabé, R.R., Coutinho, A., Cazenave, P.-A., and Forni,L. (1981), submitted.
9 - Wikler, M., Franssen, J.D.., Collignon, C., Leo, O., Mariamé, B., Van de Walle, P., De Groote, D. and Urbain, J. (1979). *J.Exp.Med. 150,* 184.
10 - Cazenave, P.-A. and Voegtlé, D. (1981), submitted.
11 - Oudin, J. (1974) in *The Antigens 2,* 277.
12 - Roland, J. and Cazenave, P.-A. (1979). *C.R. Acad. Sci. (Paris). 288,* 571.
13 - Gilman-Sachs, A., Dray, S., and Horng, W.J. (1980). *J. Immunol. 125,* 96.
14 - Roland, J. and Cazenave, P.-A. (1981).*Eur.J. Immunol.,* in press.
15 - Bona, C., Mongini, P.K.A., Stein, K.E. and Paul, W.E. (1980). *J. Exp. Med., 151,* 1334.
16 - Roland, J. and Cazenave, P.-A. (1981) submitted.
17 - Ramseier, H. (1979). *Expl. Cell Biol. 47,* 107.
18 - Coutinho, A., Forni, L., and Blomberg, B. (1978). *J. Exp. Med. 148,* 862.
19 - Coutinho, A., Forni, L., and Bernabé, R.R. (1980). *Springer Sem. Immunopath. 3,* 171.
20 - Juy, D., Primi, D. and Cazenave, P.-A. (1981).*J. Supra. Struct. and Cell. Bioch.Supp.5,* 80.
21 - Coutinho, A. (1980). *Ann. Immunol. (Inst.Pasteur),131 D,* 235.
22 - Forni, L., Cazenave, P.-A., Cosenza, H., Forsbeck, K. and Coutinho, A. (1979). *Nature, 280.* 241.

23 - Bona, C., Hooghe, R., Cazenave, P.-A., Le Guern, C., and Paul, W.E. (1979). *J. Exp. Med. 149*, 815.
24 - Forni, L., Coutinho, A., Köhler, G., Jerne, N.K.(1980). *Proc. Nat. Acad. Sci. (Wash.) 77*, 1125.
25 - Ivars, F., Holmberg, D., Cazenave, P.-A. and Coutinho, A. (1981) submitted.

CORRELATION OF ENDOGENEOUS AUTO-ANTI-IDIOTYPIC ANTIBODY SYNTHESIS WITH IDIOTYPIC REGULATION IN OUTBRED RABBITS[1]

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ABSTRACT Studies in this laboratory are aimed at assessing the importance of the idiotype network for regulating immune responses in outbred rabbits. Initial studies showed that outbred rabbits could mount auto-anti-idiotype responses specific for antibody idiotypes synthesized earlier by the same individual. Purified anti-hapten antibodies elicited auto-anti-idiotypic antibodies when injected back into the individual that synthesized the anti-hapten antibodies. Auto-anti-idiotypic antibodies were compared with isologous anti-idiotypic antibodies. In each case, the autologous anti-idiotypic antibodies recognized a smaller percentage of the antibodies than did the isologous anti-idiotypic antibodies, yet in each case, the smaller percentage recognized by autologous antibodies was contained within the larger percentage recognized by isologous antibodies. An individual rabbit that synthesized antibodies specific for *Micrococcus lysodeikticus* later synthesized auto-anti-idiotypic antibodies. The naturally elicited auto-anti-idiotypic antibodies were present when antibody clonotype distribution, as assayed by isoelectric focusing analysis, shifted substantially. Recent studies showed that clonotype shifts, presumably the result of idiotype regulation, are common in immunized rabbits.

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INTRODUCTION

Idiotypic markers are epitopes on immunoglobulin (Ig) molecules or T-cell receptors that can be used to distinguish between subsets of antibodies or T-cells. Since the first descriptions of idiotypes (1,2) these markers have been used to study diverse molecular and cellular interactions. Some, but not all, idiotypes are in or near the antigen binding site of the antibody molecule (3), and available evidence indicates no compulsory association of a given idiotype with antibodies of a particular specificity (4). The concept of the idiotype network, first described by Jerne (5), is one of the most intensely studied aspects of idiotypy. The network concept in simplest terms suggests that the paratope and idiotype of Ig molecules are distinct sites and that paratopes can recognize idiotopes of other molecules. Such recognition can lead to suppressing synthesis of the idiotype by the cells carrying the idiotype as a receptor.

Much has been learned about the functional elements of the network from studies in inbred mice and rats. Partial clarification of the roles of helper T-cells, suppressor T-cells, idiotype specific helper T-cells, and soluble T-cell factors has been gained. For a recent review, see (6). This paper describes our network studies with normal outbred rabbits as a model system.

MATERIALS AND METHODS

All materials and methods are described in detail in earlier papers. Methods of hapten conjugation to KLH, antihapten antibody purification, $F(ab')_2$ preparation, radioiodination of $F(ab')_2$ fragments, radioimmunoassay techniques, competitive radioimmunoassay inhibition with free hapten, and auto-anti-idiotype antibody production are detailed in reference 7. Sequential radioimmunoassays are described in reference 8.

Methods for autoradiographic isoelectric focusing with radioactive micrococcal carbohydrate, vaccination of rabbits with *Micrococcus lysodeikticus*, purification of antimicrococcal carbohydrate antibodies, and methods for dilution-induced precipitin assays are described in reference 9.

RESULTS AND DISCUSSION

Our initial studies were designed to determine whether it is possible for a normal outbred rabbit to mount an antibody response specific for its own previously synthesized antibody idiotypes. Rabbits were immunized with the hapten TMA (p-aminophenyl-N-trimethylammonium chloride) coupled to KLH. The anti-TMA antibodies were specifically purified, pepsin digested, and one aliquot was polymerized with glutaraldenyde. The polymer was injected back into the same individual that originally synthesized the antibody in an attempt to elicit auto-anti-idiotypic antibodies. Monomeric anti-TMA $F(ab')_2$ fragments were iodinated and mixed with these antisera, and antigen-antibody complexes were precipitated with monospecific goat anti-rabbit IgG Fc antiserum. The results (Table 1) show that each rabbit made auto-anti-idiotypic antibodies specific for its own anti-TMA antibodies but nonreactive with the anti-TMA antibodies from other individuals.

Further studies showed that observed idiotype-anti-idiotype reactions were hapten inhibitable. Free TMA, DMA (N, N-dimethyl-P-phenylenediamine hydrochloride), and PNBA (p-nitrobenzoic acid) were used in attempts to inhibit the reaction of idiotypic antibodies with anti-idiotypic antibodies. The results (8) showed that the homologous epitope TMA was the best inhibitor, while the structurally similar inhibitor DMA was equally inhibitory only at much higher molar excesses of free epitope. PNBA was not inhibitory.

TABLE 1
REACTIONS OF AUTO-ANTI-IDIOTYPIC ANTISERA WITH ^{125}I-LABELED F(ab')$_2$ FRAGMENTS OF ANTI-TMA ANTIBODIES[a]

Antiserum	Reaction with ^{125}I-Labeled F(ab')$_2$ Fragment:[b]				
	B-18	B-19	B-21	B-22	B-26
Preinoculation[c]	0.8	1.3	1.5	2.3	2.3
Pooled Normal Rabbit Serum	1.1	1.5	2.0	2.3	1.2
B-18 anti B-18	17.0	2.3	0.6	2.3	2.7
B-19 anti B-19	0.9	39.3	1.6	2.0	2.4
B-21 anti B-21	0.6	3.4	40.7	1.3	2.3
B-22 anti B-22	0.1	0.7	0.3	23.3	0.7
B-26 anti B-26	0.2	1.6	0.1	0.3	23.3

[a] *From reference 7, with the permission of the Rockefeller University Press.*
[b] *Percentage of labeled F(ab')$_2$ bound in each reaction.*
[c] *Preinoculation (day 0) serum from each individual.*

These results clearly show that normal outbred rabbits can successfully mount auto-anti-idiotypic antibody responses directed against autologous idiotypes. But in no instance could auto-anti-idiotypic antibodies be elicited that would react with more than 40% of the anti-TMA antibody idiotypes. Even with repeated immunizations, 3 of the rabbits responded to less than 25% of their own antibody idiotypes, suggesting that some idiotypes may be involved in autoregulation, while others are not.

Our next concern was whether or not auto-anti-idiotypic antibodies and isologous anti-idiotypic antibodies recognize the same idiotypes. If only selected idiotypes induce auto-anti-idiotypic antibodies (or effector T-cells) that are regulatory, then the network might not be effective as a regulatory mechanism for all immune responses. For these studies, rabbits were immunized with TMA coupled to KLH, and the anti-TMA antibodies were purified. The F(ab')$_2$ fragments of the anti-TMA antibodies were polymerized with glutaraldehyde and injected back into: a) the same rabbit that synthesized the

antibodies and b) an allotype-matched recipient. Labeled anti-TMA $F(ab')_2$ was used in sequential radioimmunoassays (RIA) in which the labeled $F(ab')_2$ was first reacted with auto-anti-idiotypic antiserum and the supernatant of that reaction was then reacted with the isologous anti-idiotypic antiserum. The opposite order of reaction also was done for each antibody $F(ab')_2$ preparation, with the results shown in Table 2. The results showed that, in each case, when isologous anti-idiotype was added first, no idiotypic molecules remained in the supernatant to react with auto-anti-idiotypic antibodies that were then added. But, when the auto-anti-idiotypic antibodies were added first, substantial numbers of molecules did not react with auto-anti-idiotype but reacted with the isologous anti-idiotypic antibodies subsequently added.

These data are open to several interpretations. First, perhaps the fewer clonotypes recognized by auto-anti-idiotypic antiserum were a function of individuals tolerant of some

TABLE 2
SUCCESSIVE PRECIPITATION OF IDIOTYPE WITH AUTO-ANTI-IDIOTYPE PRECIPITATION FOLLOWED BY ISOLOGOUS ANTI-IDIOTYPE PRECIPITATION OR ISOLOGOUS ANTI-IDIOTYPE PRECIPITATION FOLLOWED BY AUTO-ANTI-IDIOTYPE PRECIPITATION[a]

^{125}I $F(ab')_2$	First Antiserum	% Counts PPTED	Second Antiserum	% Counts PPTED
E-5	E-5 AAI[b]	12	E-9 IAI	9
E-5	E-9 IAI[c]	25	E-5 AAI	3
E-10	E-10 AAI	18	E-12 IAI	45
E-10	E-12 IAI	67	E-10 AAI	0
E-11	E-11 AAI	28	E-13 IAI	35
E-11	E-13 IAI	61	E-11 AAI	1

[a] *From reference 8, used with permission of Williams and Wilkins Co.*
[b] *Auto-anti-idiotypic antiserum*
[c] *Isologous anti-idiotypic antiserum.*

clonotypes but losing tolerance to other clonotypes. This is not a strong argument because it is common practice to break self-tolerance by incorporating antigen into complete Freund's adjuvant and giving several injections of the emulsion as we did. A more reasonable argument is that the individual can respond to only certain idiotype subsets and not to others. The data show that even with isologous allotype-matched recipients, not all antibodies will elicit anti-idiotype responses, indicating a minimum of three groups of antibody idiotypes exist: 1) a group that does not elicit anti-idiotype in the species, 2) a group that elicits anti-idiotype production only in other members of the species, and 3) a group that elicits auto-anti-idiotypic antibodies (or T-cells) within the individual.

We have made a numerous attempts to identify individuals in which auto-anti-idiotypic antibody responses occurred as a normal consequence of inducing an antibody response. To date, one such individual has been found (9), a rabbit given a series of IV injections of *M. lysodeikticus* vaccine and was rested a long period while the antibody content of the serum dropped to nearly undetectable levels. The rabbit was then given a second-round series of injections of the same vaccine and rested a second time. This schedule was followed a total of 4 times. After this rabbit was injected 3 consecutive weeks in the second round, the sera then taken showed the peculiar property of forming large amounts of precipitate when it was diluted 1:10 to 1:30 in neutral buffer. Rheumatoid antibodies were also found in second-round sera but were absent after dilution-induced precipitation. The washed precipitate did not dissolve upon heating to 40°C and consisted solely of IgG molecules.

Table 3 shows the results of attempts to inhibit dilution-induced precipitation of the interacting IgG molecules. The most effective inhibitors of the dilution-induced precipitation reaction were glucose and mannuronic acid; a combination of these sugars produced maximum inhibition of precipitate formation. The same sugars did not inhibit rheumatoid factor activity. These results show an active, specific involvement in the dilution-induced precipitation process of micrococcal carbohydrate reactive antibodies, particularly those reactive with sugar ligands homologous with the immunodominant carbohydrate epitopes of the micrococcal cell wall, glucose and mannuronic acid (10). RIA showed antibodies in second-round sera reactive with antibodies specific for micrococcal carbohydrate from the first-round antisera. The auto-anti-idiotypic antibodies specific for first-round anti-micrococcal carbohydrate antibodies in secound-round antisera were confirmed by affinity chromatography.

The dilution-induced precipitation was found only in second- and fourth-round sera, not in first- or third-round sera. Rheumatoid antibodies also were found only in second- and fourth-round sera. The results showed that the dilution-induced precipitation correlated with the presence of idiotype, auto-anti-idiotypic antibodies, and rheumatoid factor.

TABLE 3
EFFECT OF INHIBITORS ON DILUTION-INDUCED PRECIPITATION OF SERUM 102

Inhibitor(s)	*% Inhibition*
Phosphate buffer	*0*
NaCl (0.3 M)	*0*
Glycine (0.25 M)	*0*
D-Glucose	*18*
D-Mannuronic Acid	*37*
D-Glucose + D-Mannuronic Acid	*47*

From reference 9, with the permission of the Rockefeller University Press.

Sera from all 4 rounds were analyzed for clonotype distribution by analytical isoelectric focusing, followed by radioactive antigen localization. These experiments were an attempt to determine if natural auto-anti-idiotype synthesis had suppressive effects on the clonotypes bearing the idiotypes. Samples of two different first-, second-, third-, and fourth-round sera were electrofocused, fixed in the gel, exposed to ^{125}I-labeled micrococcal carbohydrate, washed, dried, and exposed to X-ray film. The resulting autoradiograph (Fig. 1) shows substantial clonotype distribution differences among the different rounds. Significant differences are obvious on the autoradiograph but not on the stained plate, which demonstrates the great differences in sensitivity between protein staining and autoradiography. The two first-round (1°) samples contained antibody clonotypes focusing at pH 7-8. Second-round (2°) samples showed fewer or no pH 7-8 clonotypes and an increase or first emergence of clonotypes in the range of pH 6.5-7.4. Third-round (3°) sera showed first-round clonotypes reappearing along with second-round clonotypes, so clonotypes absent in the second-round response were reexpressed in the third-round response in the absence of auto-anti-idiotypic antibodies. The fourth-round sera (4°) again showed dilution-induced precipitation as in 2° sera, and clonotypes in the pH 7-8 range were deleted. Also, the fourth-round dilution-induced precipitation showed 3-fold more precipitate than 2° sera did, suggesting an anamnestic auto-anti-idiotype response.

These data are consistent with data from other laboratories showing apparent network-mediated regulation of immune responses both in outbred (11,12) and inbred (13,14,15, 16,17,18,19) animals. The data presented here show that outbred rabbits can respond to idiotype epitopes by mounting

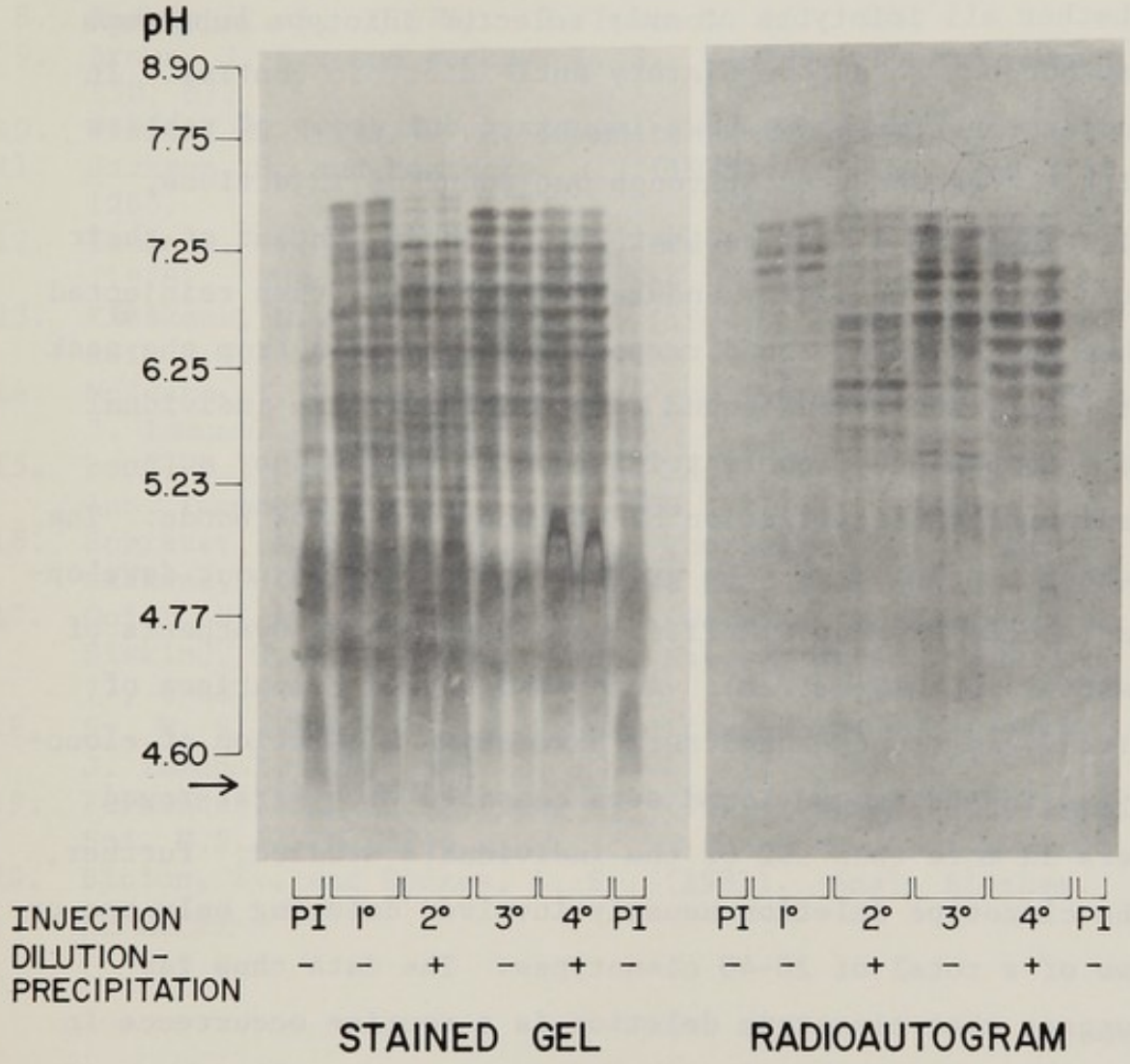

Figure 1. *Stained gel and radioautogram of electrofocused samples of serum from rabbit 102 taken from the 4 rounds of injections and preinoculation (PI).*

auto-anti-idiotype responses. This seems to be the case for some but not all idiotypes. This point requires further study. In addition, the system has been shown to operate in one individual as a natural consequence of immunization with a bacterial cell wall vaccine.

We are continuing to study the two related questions of whether network regulation occurs naturally as a part of a normal immune response in a high percentage of cases and

whether all idiotypes or only selected idiotype subgroups are subject to autoregulatory anti-idiotypic control. In recent experiments we have immunized one group of rabbits with *M. lysodeikticus* through one round of injections, allowed them to rest so that the antibody content of their sera dropped to nearly undetectable levels, then reinjected them for a second-round response. Sera taken from the peak of first- and second-round responses from each individual were compared by isoelectric focusing followed by autoradiographic localization of antibody clonotype bands. The recent studies have been greatly facilitated by our developing technology for simplified and inexpensive synthesis of carrier ampholytes (20). Autoradiographic comparison of first- and second-round sera have shown a deletion of clonotypes in the second-round sera compared with first-round sera in more than 90% of the individuals studied. Further, the clonotype deletion usually involved deleting only one or two of a total of 20-40 clonotypes. The data thus far suggest that clonotype deletion is a routine occurrence in this system and that it usually involves a small subset of the total clonotype response. Studies are in progress to attempt to identify either a humoral or cellular mediator of the deletions.

REFERENCES

1. Oudin, J., and Michel, M. (1963). C. R. Acad. Sci. 275, 805.
2. Kunkel, H. G., Mannik, M., and Williams, R. C. (1963). Science 140, 1218.
3. Brient, B. W., and Nisonoff, A. (1970). J. Exp. Med. 132, 951.
4. Oudin, J., and Cazenave, P. A. (1971). Proc. Nat. Acad. Sci. U.S.A. 68, 2616.
5. Jerne, N. K. (1974). Ann. Immunol. Inst. Pasteur. 125c, 373.
6. Rodkey, L. S. (1980). Microbiol. Rev. 44, 631.
7. Rodkey, L. S. (1974). J. Exp. Med. 139, 712.

8. Rodkey, L. S. (1976). J. Immunol. 117, 986.
9. Brown, J. C., and Rodkey, L. S. (1979). J. Exp. Med. 150, 67.
10. Wikler, M. (1975). Z. Immunitaets-forsh. 149, 193.
11. Jackson, S. and Mestecky, J. (1979). J. Exp. Med. 150, 1265.
12. Tasiaux, N., Leuwenkroon, R., Bruyns, C., and Urbain, J. (1978). Eur. J. Immunol. 8, 464.
13. Kluskens, L., and Köhler, H. (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 5083.
14. McKearn, T. J., Stuart, F. P., and Fitch, F. W. (1974). J. Immunol. 113, 1876.
15. Bona, C., Cazenave, P. A., and Paul, W. E. (1979). Ann. Immunol. (Paris). 130c, 303.
16. Schrater, A. F., Goidl, E. A., Thorbecke, G. J., and Siskind, G. W. (1979). J. Exp. Med. 150, 808.
17. Goidl, E. A., Thorbecke, G. J., Weksler, M. E., and Siskind, G. W. (1980). Proc. Nat. Acad. Sci. U.S.A. 77, 6788.
18. Sy, M. S., Moorhead, J. W., and Claman, H. N. (1979). J. Immunol. 123, 2593.
19. Fernandez, C., and Möller, G. (1979). Proc. Nat. Acad. Sci. U.S.A. 76, 5944.
20. Binion, S., and Rodkey, L. S. (1981). Anal. Biochem. in press.

DECREASED PRODUCTION OF AUTO-ANTI-IDIOTYPIC ANTIBODY BY THE NZB STRAIN OF MICE[1]

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The heterogeneity of affinity of anti-hapten plaque-forming cells (PFC) following immunization with thymic-dependent (TD) and thymic-independent (TI) antigens was determined in the NZB mouse. Following the administration of a TD antigen in NZB mice, the heterogeneity of the affinity of anti-hapten PFC is relatively restricted and of high average affinity, as compared to that of non-autoimmune prone strains. Finally, transfer of immune NZB spleen cells plus antigen failed to suppress the anti-TNP PFC response of the recipients and failed to result in detectable auto-anti-idiotypic antibody blocked PFC. The results thus suggest defective regulation of the immune response by NZB mice.

INTRODUCTION

The NZB strain of mice is a classical murine model for the study of autoimmune disorders. This strain and certain related strains, in particular the (NZBxNZW)F_1, produce a spectrum of auto-antibodies (1-7) directed to several autologous antigenic determinants. I report here the results of a series of experiments designed to characterize the heterogeneity of the affinity of the anti-hapten PFC responses TI or TD antigens in autoimmune strains. Further experiments compared the mechanisms down-regulating the immune response of the NZB and of non-autoimmune prone strains of mice. I present evidence that the auto-anti-idiotypic antibody response, which is one of the mechanisms responsible for the down-regulation of the immune

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response is decreased or absent in the response of the NZB mouse. (Goidl, E.A. and Fernandes, G., manuscript in preparation).

MATERIALS AND METHODS

Materials and methods have been extensively described in previous publications (7-8).

RESULTS

Heterogeneity of affinity of anti-hapten PFC response of of NZB mice following immunization with TD antigens

Male NZB mice of different ages were immunized with trinitrophenylated-bovine gamma globulin (TNP-BGG) or dansylated (1-dimethylamino-naphtalene-5-sulphonyl-(DAN))-keyhole limpet hemocyanin. Two weeks after immunization, the splenic anti-TNP or anti-DAN PFC responses were assayed. For both antigens the anti-hapten PFC response is of relatively high average affinity and of unusual restricted heterogeneity when compared to that of non-autoimmune prone strains of mice. In NZB mice ranging in age from one to nine months, there are relatively few of the low affinity indirect splenic PFC, which usually comprise a high proportion of the PFC response of non-autoimmune prone strains of mice.

Kinetics of changes in the magnitude and the heterogeneity of the PFC response of NZB mice following immunization with TI antigens

The immune response of NZB mice, of non-autoimmune prone mouse strains and of athymic mice to relatively thymus independent antigens were compared. The magnitude of the PFC response at different times following immunization with

trinitrophenylated-lysyl-Ficoll (TNP-F) or TNP-*Brucella abortus* was measured. The peak magnitudes of the anti-TNP PFC responses of the NZB and of Nu/Nu mice following immunization with TNP-F were comparable, in contrast to that of euthymic non-autoimmune prone strains of mice. Furthermore, the downward slope of the decreasing PFC response to TI antigens has been shown to be relatively abrupt in non-autoimmune prone strains of mice (9), in contrast these slopes are shallower in both NZB and Nu/Nu strains. With respect to time after immunization, the anti-hapten PFC responses of NZB and Nu/Nu mice to TI antigens tend to be of greater heterogeneity of affinity than are the responses of non-autoimmune prone mouse strains.

Genetic influences on the heterogeneity of antibody affinity in autoimmune prone strains of mice

The heterogeneity of affinity of anti-TNP PFC response to TNP-BGG was measured in the following autoimmune prone strains: NZB (H-2^d), NZW (H-2^z) and the hybrid (NZBxNZW)F_1 and in the following non-autoimmune prone strains: the CBA/H (H-2^k) and in the (NZBxCBA/H)F_1 as well as DBA/2 (H-2^d) and (NZBxDBA/2)F_1. The autoimmune prone strains produced immune responses of restricted heterogeneity of affinity and of high average affinity. In contrast, the non-autoimmune prone strains produced highly heterogeneous PFC responses comprising a major low-affinity component. These results imply that factors distinct from the H-2 haplotype are involved in regulating the heterogeneity of the affinity of the antibody response.

Failure of immune spleen cells from NZB mice to suppress the anti-TNP response or to cause the appearance of anti-idiotype antibody blocked PFC in syngeneic recipients immunized with TNP-F

In view of the kinetics of the changes in the magnitude and

heterogeneity of affinity of the anti-hapten PFC response following immunization with TI antigens, transfer of day 7 immune spleen cells from TNP-F immunized NZB mice into syngeneic normal non-irradiated recipients along with antigen was performed. We have shown that one striking manifestation of the regulation by auto-anti-idiotypic antibody can be observed after cell transfers of immune donor cells into normal recipients (7). Transfers of 7 day anti-TNP-F immune spleen cells suppress the immune response when transferred into normal non-irradiated syngeneic recipients immunized with TNP-F. Such apparent suppression has been shown to be hapten-reversible when assayed in the presence of free hapten. We have also shown that this suppression is mediated by auto-anti-idiotypic antibody (8). Immune spleen cells from NZB donors were shown to fail to suppress the anti-TNP response and to cause the appearance of auto-anti-idiotypic antibody blocked PFC, in recipients. In contrast, immune spleen cells from AKR/J or (NZBxDBA/2)F_1 in syngeneic transfers were fully able to cause suppression of the anti-TNP PFC response and to cause the appearance of auto-anti-idiotypic antibody blocked PFC.

DISCUSSION

Several interesting features of the immune responses of autoimmune prone mouse strains have been described:

(1) the heterogeneity of affinity of the anti-hapten PFC responses to TD antigens is relatively restricted and of high average affinity as compared to that of non-autoimmune prone strains. (2) the heterogeneity of affinity of the anti-hapten PFC responses to TI antigens is greater than that of non-autoimmune prone strains. It is worth noting that the heterogeneity of affinity and the downward regulation of

immune response to TI antigens is similar in NZB and Nu/Nu mice (10). (3) Finally, immune spleen cells from NZB mice fail to suppress the anti-TNP response or cause the appearance of auto-anti-idiotypic antibody blocked (hapten-augmentable) PFC in recipients immunized with TNP-F.

One role of auto-anti-idiotypic antibody *in vivo* is the regulation of the magnitude and of the duration of the immune response as originally hypothesized by Jerne (11). Wigzell has suggested (12) that this "positive autoimmunity" and other immune elements directed against self-determinants could be viewed as beneficial to the individual. It is tempting to propose that the decreased auto-anti-idiotypic antibody production seen in the NZB mouse is related in some manner to the etiology of autoimmune diseases.

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REFERENCES

1. Howie, J.B. and Helyer, R.J. (1968) *Adv. Immunol. 9*,215.
2. Talal, N. (1976) *Transplant. Rev. 31*,240.
3. Rowley, M.J., Buchanan, H. and Mackay, J.R. (1968) *Lancet 2*,24.

4. Hallgren, H.M., Buckley, C.E. III, Gilbertsen, V.A. and Yunis, E.J. (1973) *J. Immunol. 3*,1101.
5. Good, R.A. and Yunis, E.J. (1974) *Fed. Proc. 33*,2040.
6. Fernandes, G., Yunis, E.J., and Good, R.A. (1976) *Clin. Immunol. Immunopathol. 6*,318.
7. Schrater, A.F., Goidl, E.A., Thorbecke, G.J. and Siskind, G.W. (1979) *J. Exp. Med. 150*,138.
8. Goidl, E.A., Schrater, A.F., Siskind, G.W. and Thorbecke, G.J. (1979) *J. Exp. Med. 150*,154.
9. Goidl, E.A., Romano, T.J., Siskind, G.W. and Thorbecke, G.J. (1978) *Cell. Immunol. 35*,231.
10. Schrater, A.F., Goidl, E.A., Thorbecke, G.J. and Siskind, G.W. (1979) *J. Exp. Med. 150*,808.
11. Jerne, N.K. (1974) *Ann. Immunol. (Paris) 125(C)*,373.
12. Wigzell, H. (1977) in *Autoimmunity* (Talal, N., ed.) pp. 693-707. Academic Press. New York.

REGULATION OF A MOUSE V_H IDIOTYPE

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This paper deals with the production of a cross-specific V_H idiotype (U10-173) in two allotype-congenic strains of mice. One strain (CWB) normally produces detectable U10-173, the other (CWA) does not. We report two unexpected results: antigen-dependent stimulation in CWB mice of U10-173$^+$ molecules that cannot be shown to bind the immunizing antigen, and stimulation in x-irradiated CWA mice of latent U10-173$^+$ molecules that bind the immunizing antigen.

INTRODUCTION

Distinct antigens (idiotypes) on immunoglobulin (Ig) variable (V) regions have provided a serological basis for identifying related or unique V domains apart from their antigen binding specificity. Two categories of idiotypes (Id) may be defined: those that are individually specific (Idi) to a given subpopulation of Igs and those that are cross-specific (Idx) and shared by more than one population of Igs (reviewed in 1).

This report concerns a previously described mouse Idx (U10-173) (2,3). Briefly, U10-173 is an antigenic marker that identifies a specific V_H isotype (V_H-1) (4) found on Igs of different classes (IgM, IgG, IgA) and on Igs known to bind different carbohydrate antigens (e.g., 2-6 levan, 1-6-D-galactan); it represents 0.5-2.0% of the serum Ig in U10-173$^+$ strains. Five inbred mouse strains lack the U10-173 marker: these are C3H, C3H.SW, CBA, PL/J and AKR.

We have studied antigen dependent stimulation of U10-173 in two allotype-congenic mouse strains: C3H.SW (CWA) and C3H.SW.Igh^b (CWB). U10-173 is detectable in CWB but not in CWA. When such mice were repeatedly injected with bacterial levan two unexpected results were noted: 1) levan induced many CWB mice to produce more U10-173 than anti-levan antibody, and 2) levan could stimulate transient production of U10-173$^+$ anti-levan in a variable percentage (8-22%) of CWA

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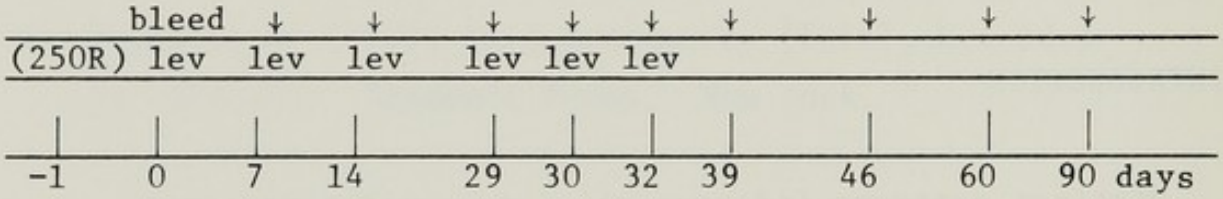
mice provided they received a sublethal dose of x-irradiation (250R) prior to being immunized. Possible explanations for these results are discussed.

MATERIALS AND METHODS

The CWB mice used in this study were derived after 6 backcrosses of the Igh-1^b locus onto the C3H.SW (CWA) genetic background; these mice originated from the laboratory of L. Herzenberg (Stanford University School of Medicine, Palo Alto, CA 91125).

CWB and CWA mice were repeatedly immunized (IP with 15 µg of bacterial levan) and bled according to the schedule depicted below. In some experiments CWA mice were x-irradiated (250R) 1 day prior to the first immunization. Quantitation of U10-173 and anti-levan was done by radioimmune assay as described elsewhere (5).



RESULTS

Levan-dependent stimulation of U10-173 in CWB mice

The injection of 12 CWB mice with levan resulted in a 20-40 fold increase in serum U10-173, from ∿40 µg/ml to 900-1600 µg/ml (Table 1). In half of the mice (arbitrarily designated as group 1), virtually all of the U10-173 corresponded to specific antibody. This was evident from the near matching quantities of anti-levan and U10-173 at days 30, 60 and 90 and from the immunoabsorption of $\geq$ 90% of the U10-173 onto levan-Sepharose beads (group 1, Table 2). However, with time CWB mice of group 1 appeared to favor production of U10-173 over that of anti-levan as indicated by the decreasing ratios of the quantity of anti-levan/U10-173. This tendency was in fact clearly demonstrated in the remaining CWB mice (group 2). In these mice, disproportionate production of U10-173 and anti-levan was already apparent at day 30 and became more pronounced at days 60 and 90. At the latter intervals, we detected approximately 2X more U10-173 than anti-levan.

Table 1

Levan dependent stimulation of U10-173 in CWB mice

Day	[anti-levan]†	[U10-173]†	[anti-levan]/[U10-173]*
CWB (group 1)**			
0	< 1	45 ± 4	-
7	1738 ± 462	882 ± 164	1.97 ± 0.28
30	1156 ± 151	1087 ± 144	1.30 ± 0.24
60	1306 ± 339	1326 ± 306	0.97 ± 0.06
90	1461 ± 305	1677 ± 260	0.80 ± 0.08
CWB (group 2)**			
0	< 1	37 ± 6	-
7	997 ± 194	884 ± 74	1.10 ± 0.15
30	699 ± 147	1248 ± 509	0.74 ± 0.16
60	528 ± 56	957 ± 110	0.59 ± 0.05
90	728 ± 76	1366 ± 191	0.56 ± 0.05
CWA			
0	< 1	< 1	
7	1659 ± 500	"	
30	971 ± 330	"	
60	571 ± 105	"	
90	1273 ± 217	"	

†mean serum concentrations (μg/ml ± SEM) of anti-levan and U10-173 at day 0, 7, 30, 60 and 90 in immunization regimen.
*mean ratios (± SEM) of individual values.
**CWB mice were arbitrarily divided into groups 1 and 2 on the basis of their serum anti-levan concentrations.

Consistent with this, only about 60% of the serum U10-173 in these mice could be adsorbed onto levan-Sepharose beads (group 2, Table 2).

As shown earlier (5), CWB 7S antibody specific for levan (at days 7, 30, 60 and 90) shows only 4-5 major bands upon isoelectric focusing; the bands focus in the pH range 6.9-7.7 and they are U10-173+. In view of this kind of restriction and concordance between anti-levan and U10-173, it is puzzling that half of the CWB mice should also produce substantial U10-173 without demonstrable anti-levan specificity.

Table 2

Concentration of anti-levan and U10-173 in CWB serum after absorption with levan-Sepharose*

	absorbed with BSA-Sepharose		absorbed with Levan-Sepharose	
Day	[anti-levan]	[U10-173]	[anti-levan]	[U10-173]
Group 1				
1	0	26	<1	32
7	1625	821	"	39
30	1090	981	"	28
60	1210	1250	"	59
90	1400	1593	"	131
Group 2				
0	0	17	<1	22
7	890	803	"	37
30	678	1170	"	403
60	487	890	"	364
90	664	1210	"	511

*pooled serum samples taken from mice of groups 1 and 2 of Table 1; concentrations of anti-levan and U10-173 given in µg/ml.

Levan-dependent stimulation of latent U10-173 in CWA mice

Unlike CWB, CWA mice lack detectable U10-173 (4). This is also true for CWA mice injected with levan (Table 1); such mice produced large quantities of anti-levan but no U10-173. However, when CWA were exposed to a sublethal dose of x-irradiation (250R) prior to being injected with levan, a small proportion of the mice (22%) became U10-173$^+$ on day 39 of the immunization regimen (Table 3). The response was very transient as the same mice lacked detectable U10-173 on days 14 and 46. Anti-levan antibody, on the other hand, was detected throughout the time interval examined with the highest levels of antibody occurring at day 39. Interestingly, all of the U10-173 produced was associated with Ig having anti-levan specificity (Table 4). As U10-173 is latent in normal CWA mice, its detection in x-irradiated CWA mice will be referred to as latent U10-173 (U10-173').

Table 3

Levan-dependent stimulation of latent U10-173 (U10-173') in x-irradiated CWA mice

mouse	[anti-levan]* d14	d39	d46	[U10-173']* d14	d39	d46
14	37	50	21	1.0	20	<1.0
89	44	79	53	"	35	"
18	31	90	64	"	44	"
33	43	139	88	"	50	"
07	50	179	105	"	56	"
31	65	220	192	"	69	"
90	79	165	100	"	103	23
27	167	572	492	"	141	<1.0

*serum concentrations (μg/ml) of anti-levan and U10-173' at day (d) 14, 39 and 46 in the immunization regimen.

We have repeatedly used the above procedure to elicit transient production of U10-173' in a variable percentage (8-17%) of CWA mice; x-irradiation alone is without effect. It should be noted that we have been unable to obtain similar results in another U10-173$^-$ strain (AKR). No x-irradiated AKR mice have been found to produce U10-173' in response to levan.

Serological equivalence of U10-173' and U10-173

To test whether U10-173' displayed antigenic determinants identical to those of a reference myeloma protein (U10), we carried out noncompetitive and competitive binding assays of the kind described earlier (6). Pooled serum Ig from the day 39 bleeds of CWA mice in Table 4 was radiolabeled with ^{131}I ([^{131}I]U10-173'); similarly, a 7S Ig preparation of U10 was radiolabeled with ^{125}I ([^{125}I]U10-173). The simultaneous addition of both labeled preparations to U10-173-reactive wells enabled us to measure their binding under conditions in which each would have to compete with the other. The quantity of reactive [^{131}I]U10-173' and [^{125}I]U10-173 added was determined by noncompetitive binding assays carried out in antibody excess and in parallel to the above.

Table 4

Adsorption of latent U10-173 (U10-173') on levan-Sepharose

Immunoabsorbent	[anti-levan]*	[U10-173']*
None	151	92
BSA-Sepharose	131	83
levan-Sepharose	<1	<1

*concentration of anti-levan and U10-173 (μg/ml) after immunoabsorption of a pooled sample of sera from day 39 bleeds of CWA mice in Table 3.

The results of one of three competitive assays are shown in Table 5. As can be seen, a decreasing proportion of each antigen was bound with increasing total antigen added, demonstrating that both antigens had to compete for antibody. More importantly, the ratio of [^{131}I]U10-173'/[^{125}I]U10-173 bound reflected the ratio of [^{131}I]U10-173'/[^{125}I]U10-173 added as would be expected of identical antigens. We conclude that U10-173' is serologically equivalent to U10-173.

Table 5

Radioimmune analysis of latent U10-173 (U10-173'); competitive binding of [^{131}I]U10-173' and [^{125}I]U10-173 to U10-173-reactive wells

Nanograms* [^{131}I]U10-173'		Nanograms* [^{125}I]U10-173		Ratio of [^{131}I]U10-173'/[^{125}I]U10-173	
Added	% Bound	Added	% Bound	Added	Bound
0.249	92	0.48	88	0.518	0.711
0.497	65	0.95	68	0.523	0.555
0.996	39	1.95	53	0.510	0.564
1.992	22	3.90	31	0.510	0.543

DISCUSSION

Our studies of antigen-dependent stimulation of a mouse V_H marker (U10-173) as reported here and elsewhere (4,5) illustrate different levels of control of V_H gene expression; namely, control by C_H-linked genes, specific antigen and possible immune elements specific for V_H framework determinants.

The production of U10-173 is controlled by genes closely linked to the C_H allotype locus. This was evident from our earlier analysis of recombinant inbred (RI) lines derived from crosses of the F_2 generation of ($U10\text{-}173^-$ x $U10\text{-}173^+$) parents (4). No genetic recombinants between the C_H locus and that controlling U10-173 were found in a total of 33 RI lines examined. In view of this close linkage it is not surprising that crossing a C_H locus from a $U10\text{-}173^+$ strain (C57BL/10) onto the genetic background of a $U10\text{-}173^-$ strain (C3H.SW) confers a $U10\text{-}173^+$ phenotype to the congenic partner strain (CWB). However, unlike C57BL/10 mice, CWB show an enormous U10-173 response to levan. U10-173 levels rise 20-40 fold. C57BL mice, on the other hand, increase their U10-173 levels only 3-4 fold (4). The basis for this difference is not clear as CWB and C57BL/10 mice are not known to differ at their MHC locus ($H\text{-}2^b$).

Virtually all of the anti-levan antibody produced in CWB mice was $U10\text{-}173^+$; moreover, that (IgG) which could be isofocused on 5% polyacrylamide gels showed only 4-5 bands (5). However, after repeated immunization, many CWB mice produced more U10-173 than anti-levan and isoelectric focusing of their serum revealed $U10\text{-}173^+$ bands which were distinct from those of $U10\text{-}173^+$ anti-levan (5). How do we explain these results? If levan, in addition to being an immunogen, were to act as a strong B cell mitogen it might nonspecifically stimulate $U10\text{-}173^+$ B cells having unrelated antigen binding specificities. However, we have no evidence that levan can induce nonspecific U10-173 production in other mouse strains. Thus, another explanation is to ascribe the apparent extra U10-173 to very low affinity anti-levan. The multivalent binding of levan to many such U10-173 molecules on the surface of a given cell might trigger production of U10-173 molecules that individually cannot be shown to bind levan. Possibly, these U10-173 molecules represent somatic variants of a single V_H-1 gene for the CWB anti-levan response; alternatively the light chains of these molecules may be heterogeneous. Variations in the L chains or in the D_H or J_H regions of $U10\text{-}173^+$ molecules could presumably occur without altering the display of U10-173 determinants since the U10-173 marker can be detected on isolated H chains (2) and

on myeloma proteins that differ in their D_H or J_H regions (7).

A third explanation to account for extra U10-173 is to postulate something in addition to levan as responsible for stimulating increased U10-173 production. One might presume the added stimulus to be trace amounts of B cell mitogen (LPS) in the levan preparation although we have not been able to increase U10-173 production in CWB mice by injecting small amounts of LPS (5). This leaves the possibility that the constant presence of a large monoclonal population of U10-173^+ B cells in levan immune CWB mice may stimulate cells and molecules with anti-U10-173 specificity. The net effect may be stimulation of U10-173^+ B cells having unrelated antigen-binding specificities. This recalls the original findings of Oudin and Cazenave (8) who observed Id^+ molecules of unrelated binding specificity in the same rabbit as a result of immunization against a single protein antigen. Viewed in this context, Jerne's network concept of immune regulation is applicable (9).

In sharp contrast to CWB, CWA mice lack detectable U10-173 and failed to produce even small quantities of U10-173 in response to levan: all of the anti-levan antibody was U10-173^-. However, sublethal x-irradiation of CWA mice followed by multiple injections of levan resulted in transient production of U10-173. This latent U10-173 (U10-173') was serologically indistinguishable from U10, a reference myeloma protein, and could be entirely adsorbed onto levan-Sepharose beads. The above argues for the presence of U10-173^+ B cells in CWA mice. Such cells may be relatively rare and their stimulation by levan favored only when other cells (specific for levan or U10-173) are temporarily depleted by x-irradiation. This would explain the transient nature of U10-173' production. As CWA and CWB mice are only known to differ at their C_H locus, the genetic implication of U10-173' is that C_H-linked genes (regulatory genes?) must influence or control the expression of the V_H-1 isotype.

The detection of U10-173' is relevant to the transient production of latent a allotypes in rabbits (reviewed in 10 and 11). Similar to U10-173, rabbit a allotypes are V_H markers found on Igs of different classes and on Igs having unrelated antigen-binding specificities. The a allotypes appear to consist of several subspecificities (12,13) and this may explain in part their presence on most serum Ig molecules, unlike U10-173 which represents only 1-2% of normal serum Ig. Both U10-173 and rabbit a allotypes segregate as autosomal dominant genes linked to the C_H locus.

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REFERENCES

1. Weigert, M. and Riblet, R. (1978) Springer Seminars in Immunopathology 1, 133.
2. Bosma, M. J., DeWitt, C., Hausman, S. J., Marks, R., and Potter, M. (1978) J. Exp. Med. 146, 1041.
3. Bosma, M. J., DeWitt, C., Potter, M., Owen, J., and Taylor, B. (1977) In ICN-UCLA Symp. on Mol. and Cell. Biol. (Eds. Sercarz, E., Herzenberg, L., and Fox, C. F.) 6, 99 (Academic Press, N. Y.).
4. Potter, M. (1977) Adv. in Immunol. 25, 141.
5. Enghofer, E., Glaudemans, C. P. J., and Bosma, M. J. (1979) Mol. Immunol. 16, 1103.
6. DeWitt, C. and Bosma, M. (1979) J. Exp. Med. 150, 351.
7. Potter, M., Mushinski, E. B., Rudikoff, S., Glaudemans, C. P. J., Padlan, E. A., and Davies, D. R. (1979) Ann. Immunol. (Inst. Pasteur) 130C, 263.
8. Oudin, J. and Cazenave, P. A. (1971) PNAS 68, 2616.
9. Jerne, N. K. (1974) Annls. Immunol. Inst. Pasteur, Paris 125C, 373.
10. Strossberg, D. A. (1977) Immunogenetics 4, 499.
11. Kindt, T. J. and Yarmush, M. W. CRC Crit. Rev. Immunol., in press.
12. Horng, W. J., Knight, K. U., and Dray, S. (1976) J. Immunol. 116, 117.
13. Brezin, C. and Cazenave, P. A. (1976) Ann. Inst. Pasteur 127C, 333.

RESCUE OF A CLONE SUPPRESSED BY AUTOGENOUS IDIOTYPE. Donald A. Rowley, La Rabida-University of Chicago Institute and Departments of Pathology and Pediatrics, University of Chicago, Chicago, IL 60637.

Unequivocally, endogenous idiotypes regulate: A/He mice immunized with phosphorylcholine (Pc) binding Ig (H8) of T15 idiotype are unresponsive to immunization with Pc; conversely, mice immunized with Pc are unresponsive to immunization with H8 (1,2). These studies which established the principal of "the priority of the first response" provide a model to test a key challenge: How can a prior response be suppressed and a complementary response be rescued

A procedure for rescue evolved from two observations. First, a prior response eliminated or made tolerant mature B lymphocytes of the complementary clone, suggesting that rescue would require regeneration of responsive cells from immature or stem cells (3). Second, neither response was diminished when mice were immunized to have simultaneous complementary responses, suggesting that complementary antibodies produced simultaneously protect the clones producing them (1,2). If this is the case, then treatment with Ig of the same idiotype as that produced by the suppressed clone might promote regeneration and rescue.

These possibilities were tested in a series of experiments using mice given sublethal irradiation (300R total body) and αPc passively. Irradiation alone eliminates recognizable mature lymphocytes; recovery accompanying regeneration of lymphoid tissue is about 50% at 3 weeks and is complete by 4 to 5 weeks. αH8 given at the time of sublethal irradiation specifically suppresses recovery of responsivity to Pc. Mice suppressed in this way and treated with αPc during the regeneration of lymphoid tissue regained responsivity to immunizaion with Pc, Table 1.

Rescue or partial rescue was similarly obtained in mice made unresponsive by active immunizaton with H8. Such mice have moderately high serum levels of αH8 and the mice (or their splenic B lymphocytes in culture) are unresponsive to immunization with Pc (1,3). The variables tested were: irradiation alone, treatment with αPc alone, and combined treatment. Rescue was observed only in mice which were both irradiated and treated with large amounts of αPc. Rescue was slow, being only partial 4 to 5 weeks after irradiation (3); however, complete rescue was observed in mice immunized both 1 and 2 months after irradiation. Furthermore, treatment with αPc (T15 idiotype) permitted recovery of the T15 idiotype, Table 2.

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Table 1
Treatment With αPc Promotes Recovery From Suppression Caused by Irradiation and Complementary Idiotype

Day 0		Day 7 to 11	Day 35	Day 39
300R	αH8	αPc	Challenge Immunization	αPc PFC Per Spleen
+	+	0	Pc-TNP	8,000
+	+	+	Pc-TNP	28,000
+	0	0	Pc-TNP	28,000

Each group contained 5 or 6 mice. αH8 was 0.2 ml of a 1:4 dilution of an A/He antiserum having a Log_2 titer of 11 against H8 coated SRBC. αPc was a total of 0.6 ml ascites having a titer of 11 against Pc coated SRBC; αPc was IgM, T15 idiotype, secreted by a hybridoma designated M2, produced and kindly supplied by Patricia Gearhart. All injections were I.V. The mean numbers of αTNP PFC/spleen for the groups were 74,000; 156,000, and 62,000 respectively.

Table 2
Rescue of a Clone Suppressed by Autogenous Complementary Idiotype

Active Immunization	Treatment		Challenge Immunization	αPc PFC/spleen T15 Id/total
	300R	αPc		
H8	+	0	Pc-TNP	0/ 1,200
H8	+	+	Pc-TNP	3,200/ 8,000
none	+	0	Pc-TNP	3,500/12,600

Each group contained 4 mice. Irradiation was given 2 weeks after mice received the last of 3 injections of H8 in Freund's adjuvant. αPc (see Table 1) was 0.5 ml given i.p. every other day beginning 2 days after irradiation for a total of 5 injections; αPc was diluted 1:4 for the first injection, 1:2 for the second injection and was given whole for the remaining injections. All mice were immunized with Pc one month and with Pc-TNP two months after irradiation. PFC against Pc-SRBC were assayed in the presence and absence of a monoclonal αT15 reagent kindly provided by Dr. John Kearney, University of Alabama. The mean number of αTNP PFC/spleen for the groups were 85,000; 105,000, and 93,000 respectively.

The observed rescue is most readily rationalized by assuming that x-irradiation temporarily suppresses the suppressor system by eliminating mature lymphocytes and in this way reducing or eliminating suppressive antibody and/or factors. X-irradiaton may have the added non-specific effect of stimulating proliferation of suppressed as well as non-suppressed lymphocytes during regeneration. Treatment with αPc presumably neutralizes complementary antibody and regenerating lymphocytes which would perpetuate unresponsiveness, and αPc in sufficient amounts may have the added effect of eliminating or tolerizing regenerating complmentary lymphocytes as occurs following primary immunization.

Admittedly the experimental base is thin for these speculations. But even partial answers may help provide a conceptual basis for manipulating a complex network where we can expect that: to rescue may require suppression (suppression of a previously induced suppressor response) and to suppress may require rescue (rescue of a suppressed suppressor response). The challenge is further complicated by the expectation that cells in the various subpopulations of amplifier and suppressor cells may be in different phases of maturation and have different susceptibilities to regulation. But the present studies do indicate that passively given idiotype may be used as a specific reactant for aiding rescue of clones having the same specificity as the passively given idiotype. Furthermore, it seems likely that sublethal irradiation or other procedures which destroy mature lymphocytes will be useful or necessary adjuncts.

1. Rowley, D.A., Kohler, H., Schreiber, H., Kaye, S.T., and Lorbach, I. (1976). Suppression by autologous complementary idiotypes: The priority of the first response. J. Exp. Med. 144, 946.
2. Rowley, D.A., Miller, G.W., and Lorbach, I. (1978). Simultaneous complementary idiotypic responses: Absence of reciprocal regulation. J. Exp. Med. 148, 148.
3. Rowley, D.A., Griffith, P., and Lorbach, I. Regulation by complementary idiotypes: Ig protects the clone producing it. J. Exp. Med. In press.

WORKSHOP SUMMARY: Network Control of B cell Activity

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The purpose of this workshop was to summarise, discuss, and evaluate current concepts concerning the immunoregulatory control of B cell activity by the idiotypic network. Unlike earlier workshops at this meeting, its purpose was not to present new information, but was rather to evaluate information already available, including that presented during the previous main, poster, and workshop session. The proceedings were conversational and socratic in tone rather than formal, and in keeping with this spirit many useful ideas were contributed by individuals which cannot be acknowledged here in detail. Nor would individuals necessarily acknowledge their ideas in the guise in which they here appear.

There were three major themes. First, the major lines of evidence for network control were summarised, and the outstanding gaps in information, objections, and queries were specified. Next, the question of what major function the network performs was raised: what immunoregulatory function can idiotypes serve which cannot be better served by antigen? Finally, the special features of the recognition of idiotypes on B cells by regulatory T cells were explored. It was agreed that in the recognition of exogenous antigens, the receptors of B and T cells have strikingly different properties, in that antibodies (the B cell receptors) characteristically recognise conformational determinants whereas T cells recognise sequences (implying that B cells recognise native proteins whereas T cells recognise denatured fragments). In the recognition of idiotypes, paradoxically, T cells seem to recognise the same sort of features which B cells would do. Does this imply that idiotype-recognising T cells belong to a category separate from other cells, and thereby to an extent partake of the nature of B cells?

The Evidence for Network Control; also gaps and queries

The workshop list of the major lines of evidence runs as follows (the names mentioned in this list do not necessarily indicate historical priorities or give proper scholarly credit).

(i) The presence of idiotypes in the parallel set

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(Oudin and Casanave). Idiotypes present in an antibody response may also be present at the same time in 'normal' immunoglobulin or in antibodies produced in parallel but directed at other antigens.

(ii) T cells, as well as antibodies, may both express a shared idiotype at surprisingly high frequency. This is particularly striking in the response of mice to azo-benzene-arsonic acid (anti-ARS).

(iii) The production of anti-idiotype antibody turns on spontaneously in at least some immune responses.

(iv) Passively administered anti-idiotype antibody can exert an immunoregulatory effect in the sense of either helping or suppressing production of the idiotype in question during a subsequent immune response. Furthermore, there is a strong immunoglobulin class effect, in the sense that certain classes of anti-id tend to help while others tend to suppress.

(v) Not only can anti-id exert an immunoregulatory effect, but so also can passively administered idiotypic antibody (Kelsoe and Rajewski). As with item (iv) on this list, the quantity of material needed is remarkably small - 1 μg of immunoglobulin can produce a dramatic effect. The symmetry between id and anti-id in producing these effects is striking.

(vi) Depletion of id-specific T cells (specific for one particular id) prior to administration of antigen can markedly decrease the content of that idiotype in an immune response (Woodward and Cantor). This is a somewhat isolated observation, at present in need of confirmation and further exploration.

(vii) The previous item did however receive strong support from the evidence presented at this meeting that the entire class of id-specific T cells can be depleted by anti-μ treatment (Bottomly, Janeway).

(viii) As judged by the effect of administering anti-id, the network is particularly important in control of the expression of dominant and silent idiotypes. Dominant idiotypes are those idiotypes which regularly comprise a major fraction of a particular immune response. Silent idiotypes are idiotypes which do not normally form an appreciable fraction of the response in question, but which do so after

suppression of the dominant idiotype or idiotypes.

(ix) The idiotype cascade ($Ab_1 \rightarrow Ab_2 \rightarrow Ab_3$ ....,) (Bona, Urbain). Administration of anti-id can generate anti-anti-id, and so on. What is particularly striking is that administration of Ab_2 generates an Ab_3 population which tends to have a high content of Ab_1 (or at least of Ab_1', i.e. of immunoglobulin which shares idiotypic determinants with Ab_1 without necessarily being able to combine with the original antigen). W. Paul suggested that this indicates that the network consists predominantly of recursive id - anti-id pairs, and that it therefore tends not to extend indefinitely. K. Rajawski on the other hand attributed this simply to the fact that in the presumably heterogeneous Ab_2 population, the only common structure is the combining site for Ab_1.

Our corresponding list of gaps and queries was briefer, and run thus

(i) The studies which have generated the foregoing list have been carried out mainly in systems selected to show dominant idiotypes. To what extent they apply to more heterogeneous systems is therefore open to question. Put another way, the question is whether restricted or heterogeneous responses are the more typical. Is it possible, for example, that restricted responses predominate in immunopathology, while heterogeneous responses predominate in resistance to infection?

In this context M. Zauderer raised the important question whether the route of entry of an antigen into the immune system may effect the extent to which it triggers network controls. Immune complexes, for example, may be particularly prone to do so.

(ii) Are the cells which participate in the idiotype network essentially the same as those which respond to foreign antigens? The problem is particularly acute for T-cells, where the view that the cell which recognises idiotype is distinct and has a distinct receptor has been vigorously supported and equally vigorously challenged.

(iii) The redundancy of the network. The network may be seen as doing little or nothing which cannot be equally well done by antigen - but see the following discussion. The redundancy is all the more objectionable if new sets of T cells have to be introduced to operate the network. In this connection the point was made that cloning is extremely impor-

tant. Now that so much progress is being made with cloning T cells, thanks largely to the use of Interleukin-2, it is reasonable to expect that the truth about hypothetical T cell sets will emerge from examining libraries of clones.

Closely related to this question is the question whether the network has a physiological function. This forms the subject of our next theme.

The Physiological Role of the Network

(i) K. Rajewski proposes that the network does for the immune system what dreams do for the mind. If only we knew more about what dreams do in fact do, this suggestion would be more helpful. Some other suggestions were as follows.

(ii) Affinity control. This clearly appealed to some of the more mathematically minded networkers, but there seems to be a dearth of direct evidence.

(iii) Long-term immunological memory, proposed by G. Siskind. An appealing idea, for there are obvious needs for long-term memory in maintaining resistance to infection and also in maintaining tolerance of self. These needs are met, it has been thought previously, by a combination of mechanisms which include antigen-harbouring, long-lived lymphocytes and long-lived clones. The question is whether the network has anything significant to add.

(vi) Fine tuning, particularly early in the immune response. This applies, as J. Goodman pointed out, for example, in the anti-ARS response where the cross-reactive idiotype(s) occur characteristically early on, and are later replaced by more heterogeneous antibody.

(v) J. Ivanyi proposed that the network provides a particular instance of certain more wide-spread, and phylogenetically more primitive, homeostatic control mechanisms. These are exemplified by the differentiation which occurs in the bone marrow from a common haemopoietic stem cell, under the influence of varied micro-environments.

(vi) The idiotype network is clearly related to immunoglobulin class-control, in the sense that both involve regulatory cells which look at immunoglobulin determinant on B cells. Class control is obviously important physiologically, in enabling the immune system to make appropriate responses to diverse forms of infection. Conceivably the network may be a mere epiphenomenon of class-control. Alternatively there

may be important connections between the two, which are as yet ill-understood, but which are hinted at by the importance of the anti-id immunoglobulin class which has already been mentioned.

(vii) Finally, one of us (B. Pernis) has proposed that one very important aspect of idiotype controls is that of preventing the simultaneous synthesis and secretion of appreciable levels of mutually interacting immunoglobulin idiotypes.

This event would entail immune-complex pathology. In this context it is immaterial to establish which of the interacting molecules would be the antigen and which would be the antibody. It is in fact pertinent to point out that it has been possible (Iverson) to suppress the production of an anti-idiotype with the prior administration of an idiotype, in symmetry with the suppression of an idiotype by the prior administration of an anti-idiotype.

This symmetrical inhibition would tend to favour the dominance of some sets of B clones on the basis of their concentration and/or time of emergence in a system and would tend to minimize the extension of a network of interactions within the immune system.

This part of the discussion was inconclusive but optimistic; the feeling was that important functions are there but remain to be defined.

Anti-id Receptors, and the Determinants which they recognize

This discussion took place against a background of the revolution which is occurring in our understanding of the functions of regulatory T cells. Put in its extreme form, ideas which have been accepted for a decade will have to be rejected. Gone is the antigen bridge joining a T cell to a B cell via carrier-hapten determinants. Gone is any antigen-specific helper factor which recognises antigen but not Ia molecules (that the factor may itself bear Ia determinants is not relevant to this argument). In their stead, the proposal is that both dendritic cells and B cells take up antigens and cleave them - the only difference being that B cells take up antigen via their SIg receptors. After cleavage, fragments return to the outside of the cell, where they are recognized by regulatory T cells in association with Ia molecules. This proposal is strongly supported by the work of K. Hannestad and H. Grey (cited, but not present in person) showing that T cells, unlike B cells, cannot distinguish between native and denatured proteins; Grey now has supporting evidence for antigen presentation by B cells.

A mechanism of this sort, as W. Paul argued, can hardly apply to T cells which recognize B cell receptors. Accordingly, there must be two distinct types of receptor, one carried by 'conventional' helper T cells which recognize extrinsic antigens in association with Ia molecules, and the other carried by idiotype-recognizing T cells (and presumably also by class-regulating T cells). This fits well with the evidence of K. Bottomly and others that Id-specific T helper cells are not MHC-restricted. The notion was discussed that the immunoglobulin V-region might function for these cells as a restriction element, analogously to the MHC for conventional helper cells, or even (R. Gershon) that there may exist yet another restriction element which maps near but is distinct from immunoglobulin. From the point of view of the obligatory antigen-cleavage theory (the Grey-Hannestad-Paul doctrine) this notion is anathema.

A further question arises in the context of the information presented separately at this meeting by H. Cantor, R. Cohn, and M. Cramer, concerning the antigen-receptor on MHC-unrestricted T cells. Whenever this receptor has been isolated, and this has now been done in these three laboratories by different methods, it turns out to be a 68K molecule produced by suppressor T cells and not by helpers. How then does this division between helper and suppressor T cells relate to the division between anti-antigen and anti-id cells? Is it possible that these two divisions can in some ways be aligned, possibly by sub-dividing both helper and suppressor categories? If so, we can expect to see a radical change in our thinking about regulatory T cells. We may also have the option of revising the nomenclature: we may be able to kick some or all suppressor T cells out of the camp altogether and leave helper T cells on their own with their cytotoxic cell allies, immune for yet awhile to the attentions of molecular biology.

ANTIBODY AND MHC GENES[1]

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I. INTRODUCTION

The last year has marked an incredible expansion of information concerning the molecular biology of immunity. We now understand general features of the organization, rearrangements, and diversification of antibody genes, and, in the last year, several groups have isolated the genes encoding transplantation antigens. This paper will summarize recent developments reported at this meeting concerning our current knowledge of organization, expression, and diversification of antibody genes as well as the organization of genes encoding transplantation antigens in both mouse and human.

II. ORGANIZATION OF ANTIBODY GENES

Antibody genes have been most extensively studied in the BALB/c mouse. Three unlinked families of genes encode the antibodies—two for light chains (λ and κ) and one for heavy (Fig. 1). The mouse λ, κ, and heavy chain gene families have been localized on chromosomes 16, 6, and 12, respectively (1, C. Croce, personal communication).

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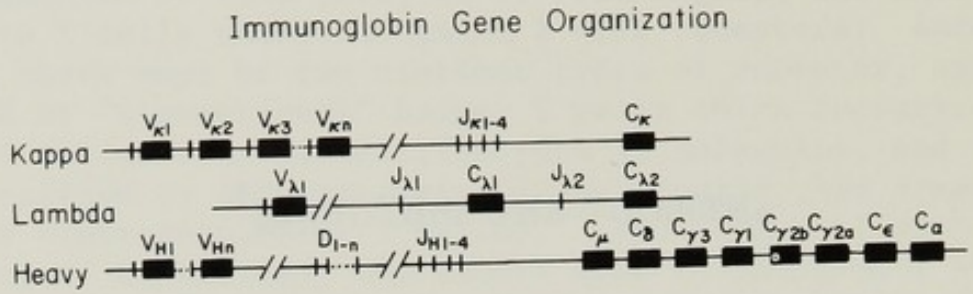

FIGURE 1. A model for the organization of the three antibody gene families. The gene families are on separate chromosomes. The 5' to 3' order of the C_H genes is given. The membrane exons are not depicted. Adapted from (2).

Light chains are encoded by three distinct gene segments—V, J, and C, whereas the heavy chains are encoded by four—V, D, J, and C. The C_H genes encode the various classes and subclasses of immunoglobulins (e.g., C_μ-IgM, C_α-IgA, etc.).

The V_H gene family appears to be divided into clusters of closely related V_H gene segments (Adams, Bothwell, Hood, and Riblet).[2] For example, the seven closely related members of the V_H NP^b family all appear tandemly linked in two gene clusters which are probably linked to each other (Bothwell). Furthermore, deletional mapping has determined the relative order of several of these V_H gene families with respect to one another (Adams, Riblet). Thus the V_H gene family appears to be composed of clusters of closely related V_H genes that are linked to one another and to the C_H genes on chromosome 12.

The existence of a D gene segment was initially suggested by protein sequence data (3). The existence of this gene segment was demonstrated at the DNA level by studies on expressed V_H genes encoding the immune response to phosphorylcholine (4). At this meeting, Tonegawa and his co-workers, as well as Early and Hood, reported the isolation of germline D gene segments and formally demonstrated that they were a third element coding for the V_H gene in the heavy chain gene family. Moreover, Tonegawa and his co-workers have demonstrated that 1) one D gene segment is 700 nucleotides to the upstream side of the J_H gene segments (5,6) and 2) a second cluster of eight D gene segments has been isolated and the D gene segments sequenced. These data suggest that the D gene segments are organized in tandemly arrayed clusters and are, as initially predicted, distributed between the V_H gene segments on one side and the J_H gene segments on the other (Fig. 1).

[2] *Single names refer to articles by the named authors in this volume.*

Honjo and his co-workers have carried out an extensive series of studies that have determined the tandem gene linkage of most of the eight C_H genes in the heavy chain family. In addition, these investigators have determined the distances between the C_H genes in all but a single case—the C_δ and the $C_{\gamma 3}$ genes (7). The entire heavy chain C_H gene cluster includes about 150 kilobases of DNA (Fig. 1).

The organization of the C_λ genes appears distinct from those of the kappa and heavy chain gene families in two regards. First, one J_λ gene segment appears to be associated with the $C_{\lambda 1}$ gene and a second with the $C_{\lambda 2}$ gene (Fig. 1) (Storb). Second, protein sequence data suggest that a single V_λ gene may be associated with the $C_{\lambda 1}$ and $C_{\lambda 3}$ genes (Herman Eisen, personal communication). Additional amino acid sequence data suggests that there is a second V_λ gene that may be associated with one or more additional C_λ genes (8).

The organization of κ light chain genes in human DNA has recently been studied by Leder and his co-workers (9). The κ family includes a single C_κ gene segment and a cluster of four functional J_κ gene segments, just as in the mouse. In contrast, there are six C_λ gene segments and the corresponding J_λ locations have not yet been determined (Hieter).

A. Variable Gene Formation

The joining of V_L and J_L as well as V_H, D, and J_H gene segments to generate, respectively, the V_L and V_H genes is probably mediated by recognition sequences which lie to the 3' side of the V gene segments and the 5' side of the J gene segments (4,10,11). Early et al. noted that these recognition elements had striking organizational features (Fig. 2) (4). Two highly conserved inverted repeats (boxes), a 7 mer and a 10 mer, are separated by variable spacer sequences of 11 nucleotides and 22 nucleotides for the V_κ and J_κ gene segments, respectively. Precisely the same recognition elements are noted in the λ gene family except for the inversion of the spacer lengths (Fig. 2). Early et al. postulated that in light chains variable region formation occurred by virtue of two distinct joining proteins which could bind to the "one-turn" (11 nucleotides is approximately a single turn of the DNA helix) and "two-turn" recognition sequences. After binding, the joining proteins would then form heterodimers thus juxtaposing one-turn and two-turn recognition elements and their associated V and J gene segments so that a recombinational event could join the gene segments. This model is consistent with the distribution of recognition sequences in the λ and κ gene families. In addition, the "one-turn"-"two-turn" joining model made a striking prediction about the structure of recognition elements for the, as of then, unisolated germline D gene segments. This model suggested that one-turn

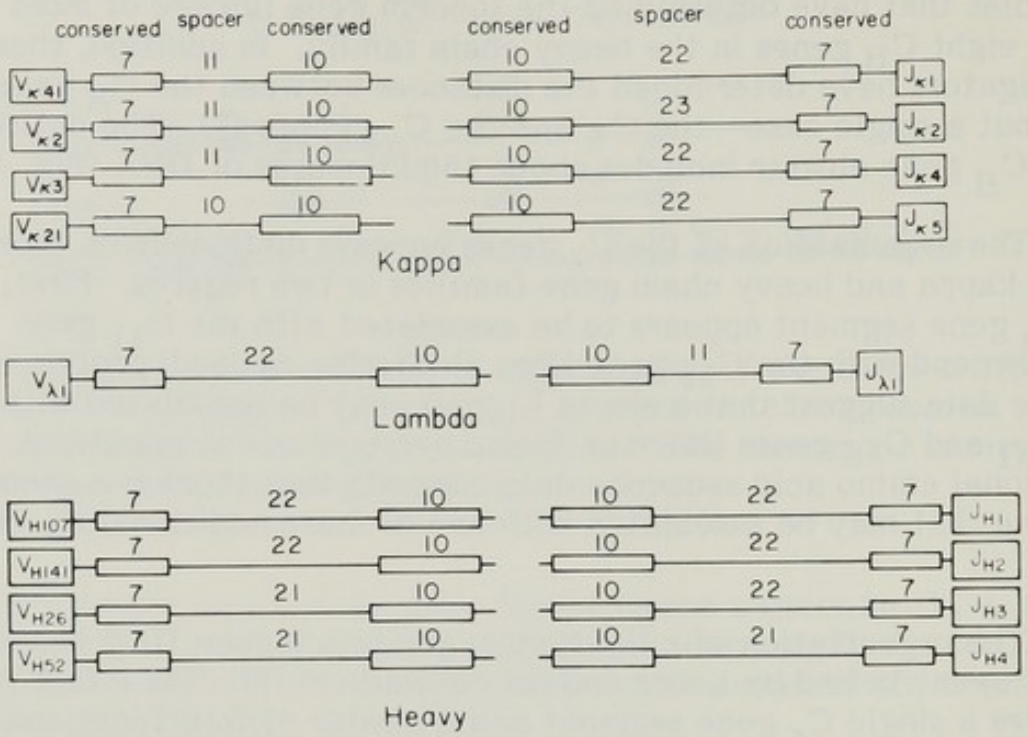

FIGURE 2. A diagrammatic model of the recognition elements for variable gene formation. The numbers represent lengths of nucleotides. Boxes indicate inverted repeats. From (12).

recognition sequences would lie on both the 3' and 5' side of the D gene segments since the heavy chain V_H and J_H gene segments each had to their corresponding 3' and 5' sides the two-turn recognition elements (Fig. 2). Recently Tonegawa and his co-workers (5,6), as well as Early and Hood (13), have isolated germline and D-J joined D gene segments and confirmed this hypothesis. These observations provide strong support for the model of variable gene formation presented by Early et al. (4).

The joining together of V_H, D, and J_H or V_L and J_L gene segments may occur productively or nonproductively (13). Nonproductive rearrangement leads to the joining of antibody gene segments in a configuration that is not consistent with expression of the variable gene at the protein level. For example, nonproductive rearrangements might arise as a consequence of joining events that leave the D or the J gene segments in a different reading frame than the V gene segment. Alternatively, nonproductive rearrangements in heavy chains may arise from D to J_H joining without an associated V_H to D joining (13) (Tonegawa). Nonproductive rearrangements of both types have been noted by several different investigators (13-15). With the delineation of nonproductive rearrangements, it was attractive to postulate that allelic exclusion occurred because of a nonproductive

rearrangement in the nonexpressed chromosome which would, accordingly, be rendered incapable of being expressed. This hypothesis is incorrect since both normal B cells and myeloma cells may contain light or heavy chain-encoding chromosomes that are in the germline configuration (16,17). A number of different workers have attempted to quantitate the fraction of nonexpressed κ and heavy chain gene chromosomes that remain in the germline configuration in normal B cells (18) (Coleclough). Most recent data suggest that about 70% of the unexpressed κ chromosomes are in the germline configuration and 10% or less of the unexpressed heavy chain chromosomes are in the germline configuration (16). From these observations a stochastic model for variable gene formation has been postulated in which immunoglobulin expression is a consequence of the random joining together of antibody gene segments, which may have a certain probability of leading to nonproductive rearrangements (19, see 13).

The simple stochastic model of immunoglobulin gene formation is insufficient since there appears to be a developmentally regulated order of activation of variable gene formation in each of the three immunoglobulin gene families. In an early stage of differentiation, the pre-B cell initially expresses cytoplasmic μ chains without any light chains (20). Thus variable gene formation is initially activated in the heavy chain gene family. Recent evidence provided by Hieter and his co-workers suggest that the κ gene family is activated next. They observed that eight human diploid B-cell lines producing κ chains have their C_λ genes in the germline configuration, whereas 10 human diploid B-cell lines producing λ chains have all of their C_κ genes either rearranged or deleted. These observations suggest that 1) the κ gene family is activated before the λ gene family to undergo variable region formation. If a productive V_κ gene is generated, then the corresponding κ IgM molecule is expressed as a cell-surface receptor molecule. 2) Once a successful light chain has been produced there is a positive signal, possibly in the form of the antibody molecule on the cell surface (21), that shuts down the enzymatic machinery of variable region formation so that when a successful V_κ rearrangement occurs no λ gene segment rearrangement can occur. On the other hand, if both κ chromosomes undergo nonproductive rearrangements, then the λ gene family is activated and V_λ and J_λ gene segments may be joined to generate a productively rearranged V_λ gene which can be expressed as the functional λ IgM molecule.

There are many questions that remain unanswered with regard to the DNA rearrangements leading to variable gene formation. How are the gene families progressively activated to rearrange first heavy, then κ, and finally λ gene segments? What enzymes and mechanisms are involved in these DNA rearrangement processes? What is the mechanism of allelic exclusion? Is the expression of V genes within a family random, or programmed and developmentally regulated as might be suggested by data from Klinman's laboratory which

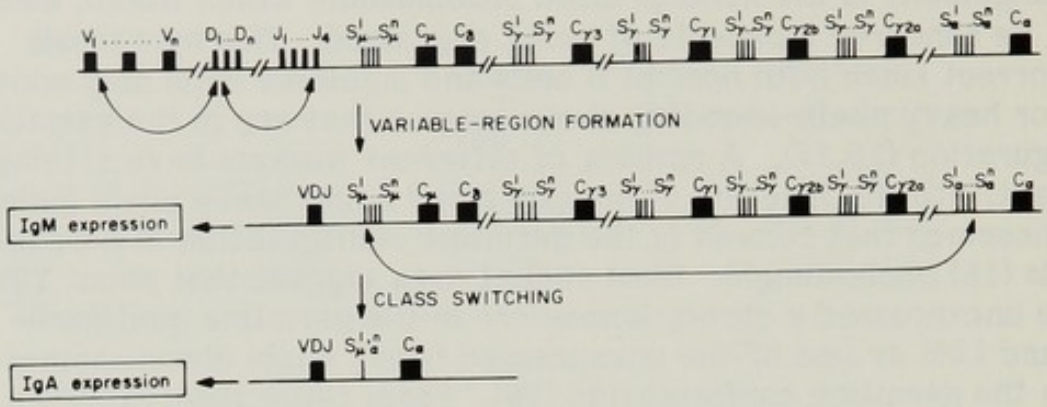

FIGURE 3. Two types of DNA rearrangements in B-cell differentiation. S denotes switch sequences. From (12).

demonstrates that the ability to respond to particular types of antigens is a precisely regulated developmental event in inbred strains of mice (22).

B. Class Switching

Variable gene formation or V_H-D-J_H joining initially places the V_H gene to the 5' side of the C_μ gene, the C_H gene most upstream in the heavy chain gene cluster (23) (Fig. 3). Accordingly, the B cell initially expresses μ chains and IgM molecules. At a later stage in B-cell differentiation, a second type of DNA rearrangement can occur in which the C_μ gene is replaced by any one of the other seven C_H genes. This DNA rearrangement event, the class switch, is mediated by repetitive sequences, denoted switch (S) sequences, that exist to the 5' sides of the C_H genes (Fig. 3). The switch sequences are made up of tandemly arrayed homologous repeat sequences which are different for each C_H gene. For example, the homology units for the switch sequences for the C_α and C_γ genes are 80 and 49 nucleotides in length, respectively (24) (Honjo). All C_H genes examined to date appear to have these switch sequences, although the putative switch sequences for the μ, δ and ε genes have not yet been characterized (Fig. 3).

Two general models have been proposed to explain the recombination events in class switching (25). The first model proposes that switch recombination may be precisely developmentally regulated through the expression of two of eight distinct switch proteins. Since the switch sequences for each C_H gene appear to be distinct, this model postulates that the eight switch proteins each bind distinct switch sequences (Fig. 3). Thus switch recombination may be regulated

by activating a pair of appropriate switch proteins. For example, in Figure 3, switch proteins for the S_μ and S_α sequence mediate the switch recombination event in a manner similar to that postulated above for variable region formation. Thus the cell may determine the direction of switch recombination. The second model proposes that the switch recombination event occurs via a generalized recombinational mechanism mediated by relatively short homologous sequences that are interspersed throughout all of the switch sites (Honjo). By this model switch recombination would be a random process and could not be developmentally regulated. This model obviously would require fewer enzymes and recognition proteins to mediate the switch recombination event. It is currently impossible to distinguish between these two alternative models.

A second major point must be considered with regard to models for switch recombination. Is the switch recombination event generally intra- or interchromosomal in nature. Davis et al. recently pointed out that the intervening sequence between the joined V-D-J and the $C_{\gamma 1}$ genes contains a 500 nucleotide stretch from the switch region of the C_α gene. This observation has two important implications. First, two or more switch recombination events may have occurred—in this case $C_\mu \rightarrow C_\alpha$ and then $C_\alpha \rightarrow C_{\gamma 1}$. Second, the switch recombination process can actually occur in an upstream fashion in that the switch from C_α to $C_{\gamma 1}$ must occur in a 3' to a 5' direction (see Fig. 3). If switch recombination is intrachromosomal, then the intervening sequences between the S_μ and S_α should be lost when the S_μ and S_α sequences are joined (Fig. 3). The simplest way to explain this upstream switch is to suggest that this particular switch recombination event occurred in a chromosome that had previously undergone sister chromatid exchange so that two copies of the appropriate C_H genes were present on this recombined chromosome. With a chromosome containing two copies of the appropriate C_H genes almost any order of C_H switching can be explained (see 25). Thus a major question is whether sister chromatid exchange, that is interchromosomal recombination, is a relatively frequent or a relatively infrequent event. To date only one example in about 15 switch sequences examined appears to require upstream switching. Rajewsky reported a hybridoma cell line which appears to switch spontaneously to both upstream and downstream loci at very low frequencies ($\sim 10^{-6}$/cell/generation) (26). That these cells which have been isolated by antibody selection are partially tetraploid may mean that they are capable of genetic behavior which is not common to B lymphocytes. Thus we believe that a majority of the class switching events will be mediated by intrachromosomal recombination, although occasional interchromosomal recombination may occur—thus generating the potential for upstream switching. The analysis of additional examples both in myeloma and normal B-cell populations of the switch recombination event will determine just how frequently interchromosomal recombination must be invoked.

A variety of interesting questions concerning switch recombination remain. What are the enzymes that mediate this complicated recombination process? How often are multiple switch recombination events employed and indeed how often do upstream switch recombination events occur? Is the interchromosomal recombination event relatively infrequent? Answers to many of these questions will only be obtained after cell lines can be identified which are poised at an early stage of B-cell differentiation so that they can be triggered to undergo switch recombination. Obviously it will be difficult to obtain such cells, although there are certain B-cell lines that may be undergoing the class switch spontaneously (Hammerling). In these cells both the enzymes mediating these events and the specific nature of the DNA rearrangements could be studied.

C. RNA Splicing of μ and δ Transcripts

The membrane (μ_m) and secreted (μ_s) forms of the μ chain appear to be encoded by a single C_μ gene which has two alternative modes of RNA splicing (27-29). The μ_m and μ_s RNAs are identical but for their 3' regions—the μ_s RNA encodes a C-terminal tail which is 20 residues in length and highly hydrophilic whereas the μ_m RNA encodes a hydrophobic tail of 41 residues (Fig. 4). The coding region for the μ_s tail is directly contiguous with the fourth domain of the μ chain, whereas the hydrophobic tail of the μ_m is encoded by two separate exons which are 1850 nucleotides to the 3' side of the fourth domain of the C_μ gene. Thus the μ_s mRNA is generated by an RNA splicing pattern which retains several hundred nucleotides directly to the 3' side of the $C_\mu 4$ domain, whereas the μ_m RNA is generated

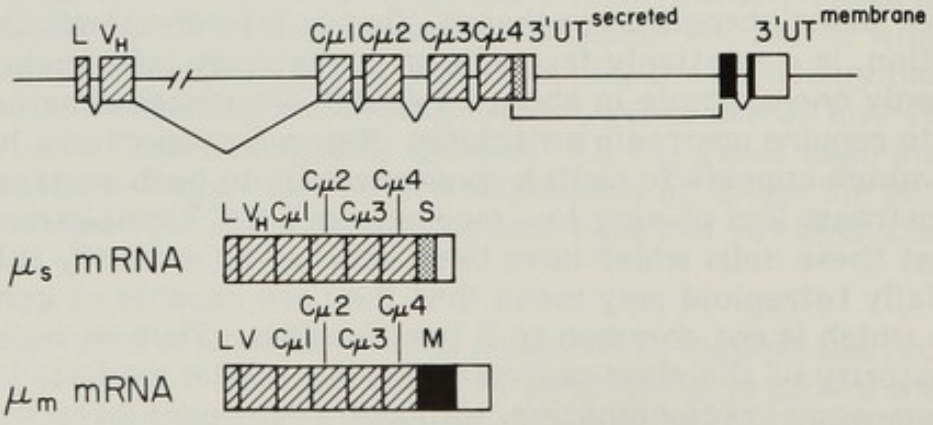

FIGURE 4. A model of the RNA splices necessary to produce μ_m and μ_s mRNAs. The top line depicts the organization of the C_μ gene.

by two RNA splicing events which joins the $C_\mu 4$ exon with the two downstream membrane exons (Fig. 4). The amino acid sequence properties of the secreted and membrane tails correlate beautifully with the respective functions of the secreted and membrane IgM molecules (Fig. 5). The μ_s tail is hydrophilic and has a penultimate cysteine residue which engages in disulfide bridging with other μ chains to generate the pentameric structure of the secreted IgM molecule. In contrast, the μ_m tail has a stretch of 26 uncharged (boxed) residues, just enough to span the lipid bilayer as an α helix. This putative transmembrane stretch is punctuated on either side by acidic and basic residues—a characteristic common to many transmembrane regions (see 28). The two C_γ genes studied in this regard to date also have membrane and secreted forms which are encoded by distinct exons in a manner analogous to that discussed above for the μ gene (Wall).

During an intermediate stage of B-cell differentiation, individual cells simultaneously express IgM and IgD molecules employing the same V domain (Fig. 6). Indeed, the majority of the B cells in the spleen of a mouse express both IgM and IgD molecules. Some of these B cells may differentiate to become plasma cells expressing only IgD molecules. Thus B cells can express IgD molecules in two alternative states of differentiation. An analysis by Hood and Wall and their co-workers of the C_μ and C_δ genes in these two stages of B-cell differentiation revealed that the C_δ gene was in two distinct organizational states (30) (Wall). In B cells simultaneously expressing the μ and δ genes the V_H gene has been placed to the 5' side of the C_μ gene. The C_δ gene and the DNA which intervenes between the C_μ and C_δ genes is in a germline configuration (Fig. 7) (Blattner, Tonegawa, Wall). The C_μ and C_δ genes are only 2.5 kb apart in contrast to the 15 kb or greater distance separating all other tandemly linked pairs of C_H genes (31) (Blattner) (Fig. 7). This organization is consistent with the postulate that a single nuclear transcript is synthesized which includes the V_H, the C_μ, and the C_δ genes and with alternative

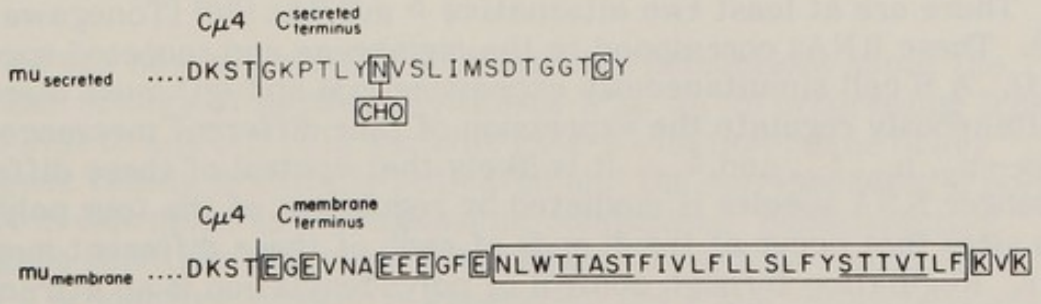

FIGURE 5. The amino acid sequences of the tail regions for μ_s and μ_m polypeptides. From (28).

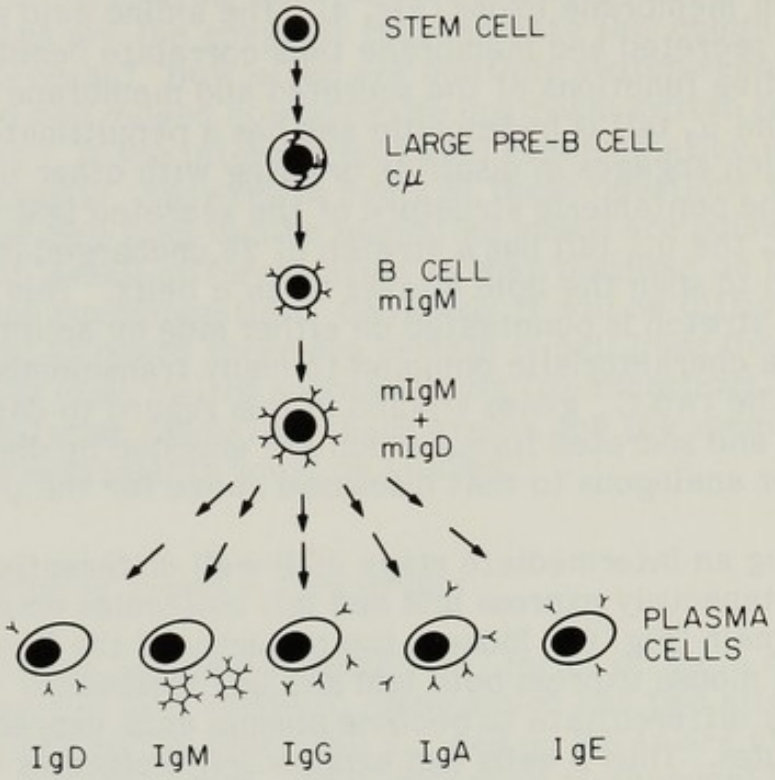

FIGURE 6. A model of B-cell differentiation. From (12).

pathways of RNA splicing generate the δ mRNAs. Indeed, Wall and his co-workers have recently demonstrated the existence of an RNA transcript of >20 kb pairs containing both C_δ and C_μ sequences (Wall). In contrast, the plasma cell which expresses only IgD molecules has undergone a classic switch recombination event in which the V_H gene is placed to the 5' side of the C_δ gene with the deletion of the C_μ gene (26,30) (Fig. 7). Thus δ mRNA may be synthesized by one of two mechanisms—RNA splicing in early B cells and switch recombination in terminally differentiated plasma cells.

There are at least two alternative δ mRNAs (30) (Tonegawa, Wall). These RNAs correspond to the membrane and secreted species of IgD. A B cell simultaneously expressing IgM and IgD must therefore simultaneously regulate the expression of four different messenger RNAs—μ_s, μ_m, δ_s, and δ_m. It is likely that control of these different messenger RNA species is mediated by regulation of the four polyadenylation sites that occur at the 3' ends of each of these different messenger RNAs. Regulation through control of polyadenylation is an attractive hypothesis because polyadenylation apparently precedes RNA splicing. Thus the nuclear transcript for μ and δ genes may be polyadenylated at one of four sites corresponding to the 3' ends of the respective mRNAs. The assumption is that the site of polyadenylation would mark the exons to be included in the final mRNA. This hypothesis

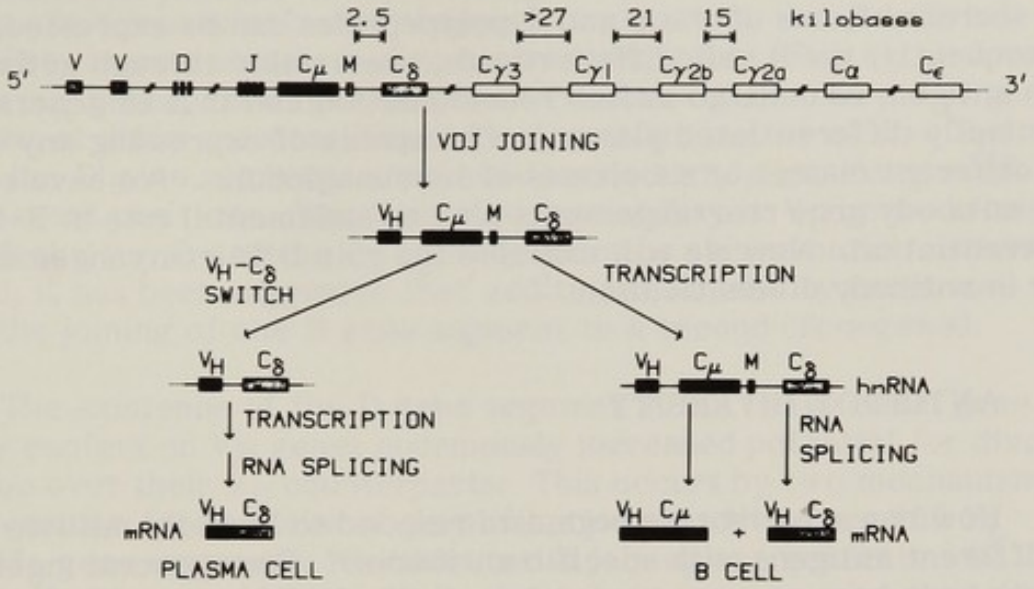

FIGURE 7. A model for alternative mechanisms of δ expression. In $\mu^+\delta^+$ B cells, δ and μ expression occurs by alternative RNA splicing choices. In δ^+ plasma cells, δ expression occurs by switch recombination. Adapted from (29).

obviously requires the RNA splicing enzymes to disregard perfectly good pairs of upstream and downstream splice signals while linking, for example, the V_H, C_δ, and δ membrane exons. Perhaps this exclusion of good pairs of RNA splicing signals is mediated through the secondary structure of the nuclear transcripts juxtaposing the appropriate exons for splicing. Once an in vitro RNA splicing system is available, it should be possible to work out the enzymology of RNA splicing in this system.

D. B-Cell Differentiation

We can now make fairly sophisticated predictions about the molecular biology of antibody synthesis (Fig. 6). In progressing from a stem cell to a pre-B cell, variable gene formation in the heavy chain gene family is initially activated and the corresponding μ polypeptide is expressed as a cytoplasmic component. Subsequently, the κ gene family is activated with regard to variable region formation and, if a productive V_κ gene is generated, the κ IgM receptor is expressed on the cell surface. If, however, both κ family chromosomes are nonproductively rearranged, then variable region formation is initiated in the λ gene family which, if successful, then leads to a λ IgM that is placed on the cell surface. Thus there is the sequential and developmentally regulated order of antibody gene family

activation—heavy, κ, and λ. At a later stage in B-cell differentiation, alternative modes of RNA splicing must operate so that the membrane and secreted forms of the μ and δ polypeptides can be expressed. Subsequently, the B cell differentiates, presumably through activation with antigen, to undergo switch recombination and thus to generate terminally differentiated plasma cells capable of expressing any of the different classes or subclasses of immunoglobulin. We have seen how antibody gene rearrangements play a fundamental role in B-cell differentiation. Now we will examine the role DNA rearrangements play in antibody diversification.

III. ANTIBODY DIVERSITY

How can a vertebrate organism respond to literally millions of different antigens with specific antibodies? Four general mechanisms have been proposed to explain antibody diversity. First, there is a large number of distinct germline antibody gene segments. This extensive germline diversity encodes significant antibody diversity. Second, antibody gene segments may undergo somatic diversification. By this mechanism, each individual expands its antibody repertoire by somatic variation during differentiation. Third, the existence of multiple gene segments encoding the V genes generates diversity through the combinatorial joining of antibody gene segments. Finally, any light chain can associate with any heavy chain to add another parameter to the variability of antibodies. Each of these mechanisms appears to contribute significantly to antibody diversification.

In the mouse it is possible to make estimates about the sizes of the antibody gene families. The κ and heavy chain gene families each have 100 to 300 germline V gene segments (Adams and Cory) and four functional J gene segments (Fig. 1) (32-34). There are 10 or more germline D gene segments (Tonegawa). The mouse λ family has 2-3 V_λ and 3-4 J_λ gene segments (35) (Storb).

Analyses of V regions at the protein level from several different systems suggests that a V gene segment may be joined to any J of the same gene family (3,36). Thus the combinatorial joining of antibody gene segments appears capable of generating V gene diversity. Moreover, the junction regions for V_L and V_H genes encode the third hypervariable region, an important region for antigen binding. Thus diversity in this region should lead to antibody molecules with distinct antigen-binding properties.

A somatic mechanism which operates in the third hypervariable regions of the V_L and V_H genes arises as a consequence of the mechanism for variable gene formation (37,38). In light chains the recombinational event joins the V and J gene segments at nucleotides other

than those directly at the end of the V and the beginning of the J gene segment then hybrid codons would be generated in this junctional region. This is precisely what happens, and indeed, this junctional variability is seen clearly in five heavy chain genes (Fig. 8). In four of five V_H genes, the D gene segment is joined to the same J_H1 gene segment at different sites (Fig. 8A). Likewise, in four of five V_H genes the D gene segments are joined to V_H and J_H gene segments at different positions. Thus enormous variability in V_H genes arises at both the V_H-D and the D-J_H boundaries by this somatic mechanism. Indeed, it has been suggested that additional variability can arise from the joining of one D gene segment to a second (Tonegawa).

The existence of the D gene segment in the heavy chain gene family confers on V_H genes enormously increased potential for diversification over their V_L counterparts. This occurs by two mechanisms. First, assume for the sake of simplification that there are 100 V_L and 4 J_L gene segments. Combinatorial joining can generate 100 V_L genes. If there are 100 V_H, 10 D, and 4 J_H gene segments, then 4000

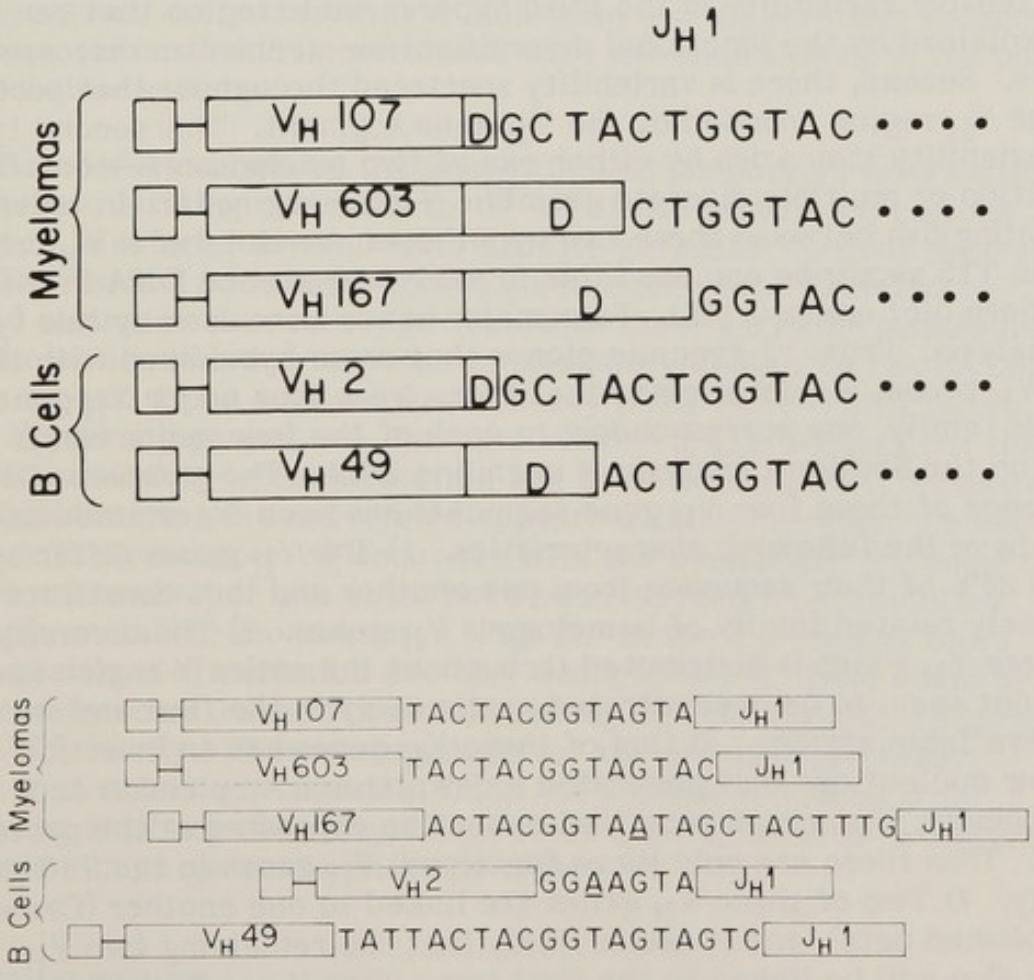

FIGURE 8. Junction diversity at J(A) and D(B) gene segment boundaries. Figure 8A depicts the J_H1 gene segment sequence and Figure 8B the D gene segment sequence. From (39).

V_H genes can be generated. Thus the addition of a third gene element in the formation of variable regions increases exponentially the potential of diversification through the combinatorial joining mechanism. Second, the existence of two boundaries (V-D and D-J_H vs. V_L-J_L) at which segmental joining occurs provides an additional site for junctional variability in V_H genes of the type discussed above. Thus through combinatorial joining and through the existence of a second junctional site for diversification, the D gene segment increases enormously the extent of diversity in V_H as compared with V_L genes.

A second somatic mechanism appears necessary to explain diversity that occurs in the V segments to the N-terminal side of the third hypervariable region. Over the past eight years our laboratory has studied the immune response to phosphorylcholine. The amino acid sequences of V_H regions from nine myeloma proteins binding phosphorylcholine are revealing. Four of these proteins are identical in sequence and have been denoted the T15 prototype sequence. The remaining five proteins differ from the T15 prototypes by 1 to 13 substitutions. Two types of variability are present. First, there is extensive variability in the third hypervariable region that can be explained by the junctional diversification mechanism discussed above. Second, there is variability scattered throughout that portion of the V_H region encoded by the V_H gene segment. This second type of variability may arise by either one of two mechanisms—somatic mutation or multiple discrete germline V gene segments. In order to distinguish between these two hypotheses, we isolated a V_H probe to the T15 sequence and used this to analyze germline DNA in the Southern blot analysis (40). Four major bands were seen by this type of analysis. From 15 genomic clones that cross-hybridized with the T15 V_H probe, we determined that there were four major V_H genes in this family, one corresponding to each of the four major bands seen on the Southern analysis of germline DNA. The complete DNA sequence of these four V_H gene segments has been determined and they have the following characteristics. 1) The V_H genes differ by 86 to 96% of their sequence from one another and thus constitute a closely related family of homologous V_H genes. 2) The diversity in these V_H genes is distributed throughout the entire V region and does not seem to be concentrated particularly in the first and second hypervariable regions. 3) One of these V_H genes has an insertion of four nucleotides that generated a downstream stop codon and, accordingly, is a pseudogene that cannot be expressed at the protein level. Thus there are only three functional V_H genes in the T15 gene family. 4) Two of these V_H genes are linked to one another (Crews, unpublished data), and it seems likely that the remaining two V_H genes also will be linked to the first two. Thus it seems probable that the T15 gene family will be a cluster of tandemly linked, homologous V_H genes. Baltimore and his colleagues have come to similar conclusions through an analysis of germline V_H gene segments in the NP^b gene family (Bothwell).

We were in a unique position to analyze the nature of antibody diversity in that we could compare the genotypic information in the T15 gene family with the corresponding phenotypic information derived from myeloma and hybridoma proteins derived from these V_H genes. We collaborated with Pat Gearhart (Carnegie Institution, Baltimore, MD) who generated 19 hybridomas directed against phosphorylcholine. We determined the complete V_H sequences for ten of these V_H regions and for the V_H regions of nine myeloma proteins. Thus we were in a position to compare the complete sequences of 19 V_H regions against the corresponding germline V_H gene segments to determine what types of mechanisms might be necessary for generating the corresponding V_H genes. Two striking observations arose from the V_H region sequences (Fig. 9). 1) There are nine variant proteins in the 19 examined (Gearhart) (41). This means that somatic mutation must occur since there are, at most, three distinct germline T15 V_H genes that can be expressed. 2) The variants are observed only in antibodies which have undergone class switching. All of the V_H regions derived from IgG molecules are variants and about half the V_H regions derived from IgA molecules are variants. In contrast, the V_H regions derived from IgM molecules are all germline sequences. Thus somatic mutation is correlated with the class switch in the phosphorylcholine system.

A careful analysis of the genotypic and phenotypic information leads to two striking conclusions. 1) All of the variant V_H regions appear to have been derived from a single germline V_H gene segment—T15. The variant sequences all are much more closely related to the T15 sequence than to any of the other three germline sequences. Thus the entire immune response to phosphorylcholine in the BALB/c mouse appears to be encoded in the heavy chain gene family by a single V_H gene segment. 2) Two mechanisms might account for this somatic variability—recombination and somatic mutation. Somatic recombination or gene conversion appears unlikely because one can compare the genotypic V_H sequence with the phenotypic V_H sequences to determine whether one could have been derived from the other by simple somatic recombination (Fig. 9). In the V_H regions there are 24 positions of variation and only a single one of these could be explained by somatic recombination among the closely related genes of the T15 family. Thus we conclude for the phosphorylcholine response that somatic recombination is not a major mechanism for generating antibody diversity. Thus some type of somatic mutational mechanism must generate antibody diversity.

IV. MHC GENES

The major histocompatibility complex is a chromosomal region in vertebrates that is composed of a series of multigene families

FIGURE 9. Sequences of the V_H gene segments (DNA) and hybridoma (IgM and IgG) and myeloma (IgA) V_H segments belonging to the T15 family. The V_H gene segments are translated into protein sequences. From (40).

whose products are responsible for a variety of different immune functions. One of these gene families encodes the transplantation antigens. Transplantation antigens are unusual among eukaryotic systems analyzed to date in the extensive nature of their genetic polymorphism. The transplantation antigens are present on all of the somatic cells of an organism and appear to play an important role in T-cell surveillance and destruction of neoplastically transformed or virally infected cells. The transplantation antigen is a 45,000 dalton integral membrane protein which is noncovalently associated with β_2-microglobulin. Amino acid sequence studies on the transplantation antigens from mouse and man have suggested that they may be divided into three external domains, a transmembrane portion, and an internal cytoplasmic domain (42,43). Amino acid sequence comparisons of the third external domain of the transplantation antigens with immunoglobulin domains suggest that there may be a distant evolutionary relationship between these two gene products (44). This idea of a homology relationship also is supported by the close association of transplantation antigens with β_2-microglobulin which is very similar to a free immunoglobulin domain.

cDNA clones for the transplantation antigens of man (HLA) (45,46) and mouse (H-2) (47) have been isolated and characterized (Strominger, S. Weissman, Hood). The analysis of these clones revealed several interesting features about the corresponding messenger RNAs for transplantation antigens. 1) The messenger RNAs have highly and moderately repetitive sequences in their 3' untranslated

regions (47). 2) Sequence analysis of these clones reveals that there is a striking homology between the coding regions for the third domain of the transplantation antigen and the constant region domains of immunoglobulins (47). 3) Southern blot analysis of germline and somatic DNA with these cDNA clones reveals two surprising features. First, there are 10 to 15 DNA fragments that cross-hybridize with these clones (47). This observation suggests that there may be far more than the three to four genes for transplantation antigens that have been defined in mouse and man by serologic analyses. Second, the germline and somatic DNA both demonstrate identical banding patterns and there appears to be no rearrangement in the DNAs from cells that do not express endogenous transplantation antigens (sperm) and those which do (somatic cells) (47).

A mouse germline genomic library has been screened with transplantation antigen cDNA clones and more than 200 clones have been isolated which appear to represent 40 to 50 distinct cross-hybridizing germline sequences for transplantation antigens (K. Moore, unpublished data). Several of these clones appear to contain two or more genes for transplantation antigens. Thus the genes encoding transplantation antigens appear to represent, at least in part, a cluster of tandemly linked and closely homologous genes. The 40 or so distinct DNA fragments that cross-hybridize with DNA clones to transplantation antigens might be explained in two ways. First, all 40 may encode distinct transplantation antigens that may be expressed at different developmental stages or in different tissues. Of course, some of these cross-hybridizing DNA fragments also may be pseudogenes. Second, a fraction of the 40 cross-hybridizing clones may represent T-cell differentiation antigen such as Qa-1, Qa-2, Qa-3, Qa-4, Qa-5 or the TL antigen. These differentiation antigens also are integral membrane proteins which appear to be 45,000 daltons in size and associated with β_2-microglobulin. Since no amino acid sequence data exists on any of these T-cell differentiation antigens, it appears that the technique of DNA transformation, recently employed in Axel's laboratory to express H-2 genes, may be very useful in defining the nature of gene products encoded in this multigene family (Sim).

A. β_2-Microglobulin

A cDNA clone of β_2-microglobulin has recently been isolated from a cDNA library constructed from a mouse cell line (Parnes and Seidman). DNA sequence analysis of this cDNA clone suggests that the general organization of this gene resembles that of V genes in immunoglobulins. The cDNA probe to the β_2-microglobulin has been used in Southern analysis to demonstrate that this gene is present in a single copy and corresponding genomic clones have been isolated and are now in the process of being characterized.

V. SUMMARY

The striking homology relationships, both in sequence and in general organizational features, among the genes encoding antibodies, transplantation antigens, and β_2-microglobulin suggest that these genes are members of a supergene family. It will be interesting to determine whether any of the regulatory strategies displayed by the antibody gene families will also be employed by the MHC gene families. Indeed, the recombinant DNA approach has given us profound insights into the organization and expression of antibody genes. These same techniques are now beginning to unravel the mysteries of the MHC complex. It is clear that the future will require a much closer collaboration between molecular biologists, cellular immunologists, and immunogeneticists to obtain the appropriate reagents and cell lines necessary to expand our understanding of the molecular biology and enzymology of gene expression in these fascinating systems.

REFERENCES

1. Hengartner, H., Meo, T., and Müller, E. (1978). Proc. Nat. Acad. Sci. USA 75, 4494.
2. Early, P., and Hood, L. (1981). in "Genetic Engineering" (J. Setlow and A. Hollaender, eds.), vol. 3, in press.
3. Schilling, J., Clevinger, B., Davie, J. M., and Hood, L. (1980). Nature 283, 35.
4. Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). Cell 19, 981.
5. Sakano, H., Kurosawa, Y., Weigert, M., and Tonegawa, S. (1981). Nature 290, 562.
6. Kurosawa, Y., von Boehmer, H., Haas, W., Sakano, H., Trauneker, A., and Tonegawa, S. (1981). Nature 290, 565.
7. Nishida, Y., Kataoka, T., Ishida, N., Nakai, S., Kishimoto, T., Bottcher, I., and Honjo, T. (1981). Proc. Nat. Acad. Sci. USA 78, 1581.
8. Francis, S. H., Leslie, R. G. Q., Hood, L., and Eisen, H. (1974). Proc. Nat. Acad. Sci. USA 71, 1123.
9. Hieter, P. A., Max, E. E., Seidman, J. G., Maizel, J. V., and Leder, P. (1980). Cell 22, 197.
10. Seidman, J. G., Nau, M. M., Norman, B., Kwan, S.-P., Scharff, M., and Leder, P. (1980). Proc. Nat. Acad. Sci. USA 77, 6022.
11. Sakano, H., Hüppi, K., Heinrich, G., and Tonegawa, S. (1979). Nature 280, 288.
12. Hood, L. Science, in preparation.
13. Early, P., and Hood, L. (1981). Cell 24, 1.
14. Steinmetz, M., and Zachau, H. G. (1980). Nucleic Acids Res. 8, 1693.

15. Hieter, P. A., Korsmeyer, S. J., Waldmann, T. A., and Leder, P. (1981). Nature 290, 368.
16. Joho, R., and Weissman, I. L. (1980). Nature 284, 179.
17. Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. (1978). Cell 15, 1.
18. Coleclough, R., Perry, R. P., Karjalainen, K., and Weigert, M. (1981). Nature 290, 372.
19. Perry, R. P., Kelley, D. E., Coleclough, C., and Kearney, J. F. (1981). Proc. Nat. Acad. Sci. USA 78, 247.
20. Levitt, D., and Cooper, M. D. (1980). Cell 19, 617.
21. Alt, F. W., Vincenzo, E., Bothwell, A. L. M., and Baltimore, D. (1980). Cell 21, 1.
22. Klinman, N. R., and Press, J. L. (1975). J. Exp. Med. 141, 1133.
23. Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R., Weissman, I. L., and Hood, L. (1980). Nature 283, 733.
24. Davis, M. M., Kim, S. K., and Hood, L. E. (1980). Science 209, 1360.
25. Davis, M. M., Kim, S. K., and Hood, L. (1980). Cell 22, 1.
26. Neuberger, M. S., and Rajewsky, K. (1981). Proc. Nat. Acad. Sci. USA 78, 1138.
27. Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980). Cell 20, 293.
28. Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L., and Wall, R. (1980). Cell 20, 303.
29. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). Cell 20, 313.
30. Moore, K. W., Rogers, J., Hunkapiller, T., Early, P., Nottenburg, C., Weissman, I., Bazin, H., Wall, R., and Hood, L. (1981). Proc. Nat. Acad. Sci. USA 78, 1800.
31. Liu, C.-P., Tucker, P. W., Mushinski, J. F., and Blattner, F. R. (1980). Science 209, 1348.
32. Max, E. E., Seidman, J. G., and Leder, P. (1979). Proc. Nat. Acad. Sci. USA 76, 3450.
33. Sakano, H., Hüppi, K., Heinrich, G., and Tonegawa, S. (1979). Nature 280, 288.
34. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Nature 286, 676.
35. Tonegawa, S. (1976). Proc. Nat. Acad. Sci. USA 73, 203.
36. Weigert, M., Gatmaitan, L., Loh, E., Schilling, J., and Hood, L. (1978). Nature 276, 785.
37. Seidman, J. G., Max, E. E., and Leder, P. (1979). Nature 280, 370.
38. Weigert, M., Perry, R., Kelley, D., Hunkapiller, T., Schilling, J., and Hood, L. (1980). Nature 283, 497.
39. Hood, L., Davis, M., Early, P., Calame, K., Kim, S., and Crews, S. (1980). Cold Spring Harbor Symp. Quant. Biol. 45, in press.
40. Crews, S., Griffin, J., Huang, H., Calame, K., and Hood, L. (1981). Cell, in press.
41. Gearhart, P. J., Johnson, N. D., Douglas, R., and Hood, L. (1981). Nature, in press.

42. Martinko, J. M., Uehara, H., Ewenstein, B. M., Kindt, T. J., Coligan, J. E., and Nathenson, S. G. (1980). Biochemistry 19, 6188.
43. Uehara, H., Coligan, J. E., and Nathenson, S. G. (1981). Biochemistry, in press.
44. Nathenson, S. G., Uehara, H., Ewenstein, B. M., Kindt, T. J., and Coligan, J. E. (1981). Ann. Rev. Biochem., in press.
45. Ploegh, H. L., Orr, H. T., and Strominger, J. L. (1980). Proc. Nat. Acad. Sci. USA 77, 6081.
46. Sood, A. K., Pereira, D., and Weissman, S. M. (1981). Proc. Nat. Acad. Sci. USA 78, 616.
47. Steinmetz, M., Frelinger, J. G., Fisher, D., Hunkapiller, T., Pereira, D., Weissman, S. M., Uehara, H., Nathenson, S., and Hood, L. (1981). Cell 24, 125.

SUMMARY SESSION: ANTIBODY DIVERSITY - A SOMATIC MODEL

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A year and a half ago when I was asked to speak in this summary session, Charlie and Eli chose as my title "Antibody Diversity - A Somatic Model". At the time, I expected people would be throwing eggs at me for continuing to suggest such an absurdity. However, as the meeting approached and as we've heard this week, SOMATIC THEORIES ARE BACK IN VOGUE.

In the last decade, Lee, Mel and I have often shared the same podium debating "the" theory of antibody diversity. Truly an amazing and amusing turn of events has occurred in the antibody diversity issue. Practically everyone in the past decade who advanced a theory was at the same time right and wrong; right because all major theories of diversification appear to operate, but wrong in that none is the exclusive mechanism.

For example, there are certainly far more germ-line genes than the twenty-five genes predicted by those who championed somatic diversification in the early 1970's. On the other hand, there do not appear to be anywhere near the 50,000 genes advocated by early proponents of the germ-line theory. In addition, recent discoveries at both the DNA and protein level indicate the importance of V-region gene interactions in the generation of diversity and the discovery of the D segment fulfills the "minigene" view.

THE GERM-LINE IS EXTENSIVE, NON-CONTIGUOUS AND COMPOSED OF INTERACTING GENE SEGMENTS

Understanding of the genetic mechanisms operative in antibody synthesis has been greatly advanced by recent discoveries at the DNA level. It is now well established that an immunoglobulin variable region gene is composed of a V segment (residues 1 to 95 in mouse V_k), and a J segment (residues 96 to 108 in mouse J_k) which "interact"

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to form a complete V gene. The DNA sequence analysis of J segments indicates that there are four functional J_K sequences. Recent data suggest a similar number of J_H segments. Thus, we know with precision the extent of the germ-line repertoire that encodes the C terminal 10% of both heavy and light polypeptide chains.

There is still controversy concerning the exact number of "V" genes, but most view this number to be under 1,000 and probably under 500. These estimates are based on the number of genes encountered in embryonic DNA utilizing cDNA probes for mouse myeloma heavy and light chains of various subgroups. In the systems most carefully studied (V_{PC}, $V\lambda$, V_K21) (Hood, Weigert, Huppi) less than 10 genes have been detected by each probe. Since each probe is relatively subgroup specific, and amino acid sequence data argue that there are around 50 subgroups of V_K and 5-10 of V_H in the mouse, the likelihood is that there are in the order of 500 V genes.

Thus, studies at both the DNA and protein level argue that the V region germ-line is extensive and composed of interacting genes.

SOMATIC EVENTS GENERATE ADDITIONAL DIVERSITY IN THE V GENE SEGMENT

Evidence presented at this meeting indicates that there are relatively few germ-line genes in embryonic DNA. However, myeloma and hybridoma protein sequences in each case have revealed more variation in the V segment than is evident from the estimated number of germ-line genes. This argues that somatic events must operate on this gene segment.

In the mouse lambda system, all the evidence points to one $V\lambda1$ gene in embroyonic DNA and, yet, Weigert's early studies indicate that more than 10 $V\lambda1$ protein phenotypes are expressed. The amino acid sequence variation is exclusively in the hypervariable regions but the fact remains that this $V\lambda1$ segment must undergo somatic diversification in order to generate the observed number of protein sequences. Matty Scharff came out of the cold and showed us antigen binding variants one in the V segment which affected binding.

Gearhart and Hood showed that the number of expressed protein sequences for phosphoryl choline is greater than the number of observed germ-line genes. Here the protein sequence variations are not limited to the hypervariable regions. Bothwell has similar results in the NP system.

Thus, studies both at the protein and DNA level imply that somatic processes play a significant role in the generation of V gene segment diversity.

SOMATIC MUTATION OCCURS IN THE J SEGMENT

There are a limited number of J segments in both mouse and man. Sequence studies of the entire murine J_K region have revealed only four functional segments. The protein sequence data indicate that several more "germ-line" J segments should be required. However, since most of this variation exists in the first residue of J, V-J recombination could, and probably does, explain this variation.

In man the situation is complicated because of the outbred nature of the population. However, the amino acid sequence data cannot be easily explained unless one invokes somatic mutation. At the protein level, the heavy chain J segments are especially diversified and it would require over 20 germ-line J segments to explain the amino acid sequence data, even if junctional diversity explained all position one variations.

Somatic variation in J_H likely explains one of Scharff's antigen binding variants and the published Dex J_H segment similarly shows what is likely somatic variation.

J segment diversity in man may be attributable, at least in part, to polymorphism of the J genes. Whether such polymorphism exists in the mouse is presently unknown. We presented some data here in the A/J anti-arsonate system that could be interpreted in that way. It is tempting to speculate that J region genetic diversity could give rise to differences in immune responsiveness that map to Ig rather than MHC genes. Further influences on immune regulation and total antibody diversity including selective presence or absence of idiotypes may result from these polymorphisms in J. This is clearly an issue that awaits resolution in the 80's.

I would like to comment briefly on the prevailing notions of framework and CDR's in this context. While these may be operationally correct, I would predict that many more framework variations, especially in V_H/V_L interactional areas will affect binding - even subtly - thus the requirement of selection on variants outside HVR might be met.

Another point to keep in mind is 1) why did the first proteins sequenced show major variability in HVR while 2) now, we see about 50/50 variation in FW vs HVR. I believe the reason is that the first group of immunoglobulins studied was randomly chosen myelomas, and now most of us are selecting for Ag binding and often idiotype sharing - almost dictating similar HVR.

Returning for a moment to Scharff's results, recall he showed by fluctation analysis that somatic mutation was random, occurred $1x10^{-4}$ per cell per generation and occurred in V segment, J segment and C segment.

IDIOTYPES

The J segment in conjunction with the D segment may be responsible for most individual idiotypes. While there are persuasive data from Shilling that the D segment alone is responsible for the individual idiotype, and that the J segment is not involved in either IdX or IdI in the dextran system, this may represent a special case. In certain circumstances, V-D interactions may generate the idiotypic determinants. A more general situation may be that V-D and J interactions in concert are responsible for the idiotype.

The T cell receptor could be encompassed within the gene interaction principle. This would accommodate "specificity" and "idiotype" similarity between B and T cell receptors and not require classical V regions on T cells. This construct would not require separately evolved recognition mechanisms but would allow the utilization of the same system, albeit in different "frameworks". That is, the D or J segments of Ig genes could be used by the T cell receptor system in concert with a different set of V genes and C genes (V_T and C_T) to generate a molecule with similar specificity and idiotype. This would allow the unigenic inheritance of the T cell idiotype and would explain its linkage to the Ig constant region locus.

Having the D and/or J gene segments encode the idiotype could help explain the "Oudin-Casenave enigma" in which antibodies of diverse specificity, or with no known specificity can arise during an immune response to a particular antigen but can often share idiotype with the antibodies directed against that antigen. It would be required only that the J segments be drawn from the same pool for the two sets of antibodies. Thus, for example, if the J segment were intimately involved in the "regulation"

of a particular idiotypic response, molecules with the same J segment, but different V regions, could share idiotype and either share or not share antigenic specificity.

Thus, the data lead to a new synthesis: A moderate number of germ-line V gene segments undergo a modest amount of somatic diversification. These V gene segments freely combine with a limited number of D and/or J segments, which themselves may undergo somatic diversification. The points at which these gene segments interact are sites of additional diversity (junctional diversity) that account for the single most hypervariable positions in both heavy and light chains as well as the observed length variation in the third hypervariable region of the heavy chain. As powerful as this latter mechanism may be, it should be emphasized that it can account for only the diversity of part of one hypervariable region of each chain.

Finally, I would stress that diversity and idiotype depend on the system:

1) In dextran system, the major mechanism is combinatorial and the IdX is V segment while the IdI is in the D segment - only because everything else is constant.
2) In the PC system, some form of V segment mutation is the major form of diversity and the idiotypes may be scattered.
3) In the Ars system, V/J joining are thought to be critical.

Thus, at this time, we can still not provide a general structural correlate of the idiotype, and it is likely we never will as the correlate may differ in each system. The operationally important heterologous idiotypic reagents of the 60's and 70's will gradually give rise to the monoclonal anti idiotypes of the 80's.

THE FUTURE

In my view, the immunology of the 80's will be heavily focused on regulation:

1) Regulation of class switching.
2) Regulation of secreted vs membrane molecules.
3) Regulation between the germ-line and the expressed repertoire.
4) Regulation by anti-idiotype

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Many of the ideas incorporated into this summary/ synthesis were developed with Tom Kindt.

PINCH HITTING FOR HANS WIGZELL

Melvin Cohn

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"Why would Casey bunt to Everts, Tinker and Chance?"
Mechkonik

There are three interrelated conclusions that have dominated thinking at this meeting. It is generally accepted that 1) recognition of self-idiotypes, i.e. an idiotypic network, contributes significantly to regulating the normal immune response to a reference antigen; 2) the T-cell receptor which mediates MHC specific, restrictive recognition of antigen, is V_H-encoded; and 3) somatic selection for anti-self reactivity in a given affinity range generates the anti-nonself repertoire by unselected heterocliticity.

I. CONCEPTUAL CONSIDERATIONS

A. Does network regulation contribute in a primary way to antigen-specific immune responsiveness?

In the extreme there are two views as to how the immune system is regulated; either it is reference antigen-driven or it is idiotype-driven. These two views lead to fundamentally different models of responsiveness. The view that the immune system is reference antigen-driven leads to associative recognition models whereas the view that the immune system is idiotype-driven leads to network models. The latter have reigned supreme at this meeting.

Since we wish to explain antigen-specific responsiveness, I will begin by stating the two postulates required of all models.

1. Antigen-sensitive cells of anti-self and anti-nonself specificities are generated continuously throughout life.
2. Each antigen-sensitive cell has two pathways open to it,

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inactivation or induction.

Since no mechanism for generating combining sites can determine a priori whether a specificity is directed toward a self or nonself antigen, the first assumption is a fundamental law of immune responsiveness. From this is derived the second assumption which implies that the self-nonself discrimination must be made at the level of the combining specificity of the receptors on antigen-sensitive cells (1).

What is the mechanism by which this decision is arrived at by each antigen-sensitive cell which encounters antigen? One explanation (the associative recognition model) is that the interaction of antigen with receptor leads to inactivation of that cell (Signal (1)) whereas the associative (or linked) recognition of the antigen by a cooperating ("helper") system

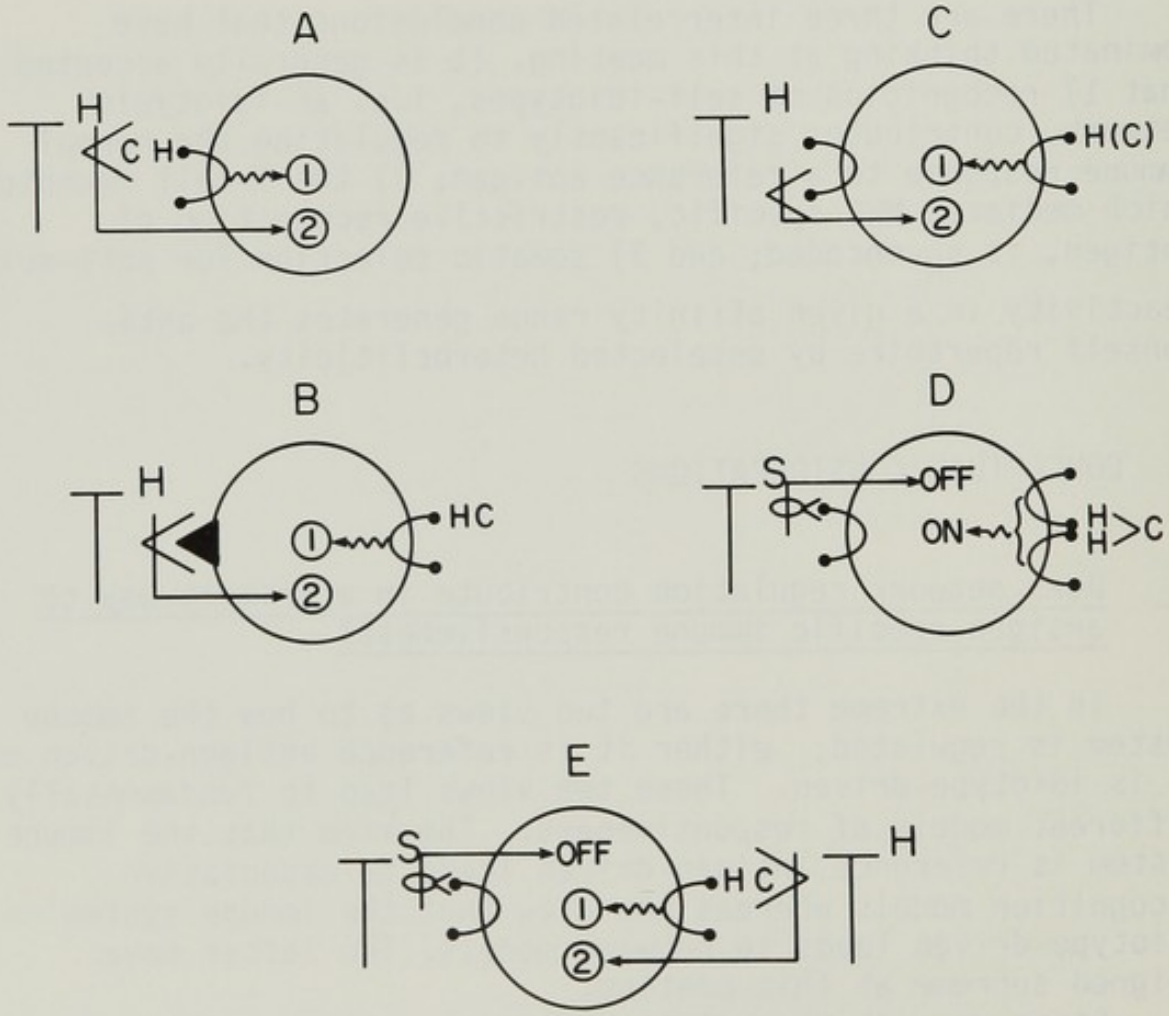

FIGURE 1. Associative recognition and Network Models.
● = Id
◀ = Surface determinant
HC = Two determinants on a reference antigen

(T^H) leads to induction (Signals ① + ②) (Fig. 1A). It is the presence or absence of the effector antigen-recognizing cooperating system (T^H) which determines whether an antigen-sensitive cell interacting with antigen (HC) will be inactivated or induced. Under this theory, inactivation is due to paralysis (negative unresponsiveness) not to "suppression" (positive unresponsiveness). The self-nonself discrimination is a paralysis/induction decision.

This model predicts the phenomenon of nonassociative recognition of antigen. The antigen-sensitive cell cannot tell whether another determinant on the reference antigen is being recognized by the cooperating system. It is only apprised of a second interaction by the signal it receives (Signal ②). Consequently, an interaction of the cooperating ("helper") system (T^H) with any determinant on the surface of an antigen-sensitive cell receiving Signal ① should lead to induction. Under associative recognition theory these nonassociative interactions are abnormal because they cannot be used to make the self-nonself discrimination. An antigen-sensitive cell receiving Signal ① by interaction with either a self or nonself component (HC) would be induced if Signal ② were delivered via nonassociative recognition of an unrelated component on the surface of the antigen-sensitive cell (Fig. 1B).

The idiotype network is an attempt to solve this problem of the self-nonself discrimination under a framework of nonassociative recognition. The solution is based on two assumptions.

1. Different rules govern the immune responsiveness to self-idiotopes distinguishing them from all other self-epitopes (non-idiotopes).
2. The recognition of the idiotype has a 1:1 (or close to it) relationship to the recognition of the reference antigen.

If we accept these two assumptions provisionally and consider a system regulated uniquely by nonassociative recognition of antigen via the idiotype then the essence of any network model is that the self-nonself discrimination must be regulated by a suppressive system recognizing the idiotype. It cannot be regulated by a paralysis/induction decision. Why?

Consider the relationship between a hapten and a carrier. A hapten (H) is a nonimmunogenic molecule which upon linkage to an immunogenic carrier molecule (C) permits induction of antigen-sensitive cells anti-H. Under a nonassociative recognition model, the only reason that H could be nonimmunogenic is because it cannot initiate Signal ① to the

antigen-sensitive cell (Fig. 1B). The prediction of nonassociative recognition models that a nonimmunogenic substance cannot be paralytic runs counter to fact. In any case, if H could initiate Signal ①, nonassociative recognition of a surface component (e.g. an idiotype) by a cooperating T^H system would induce a response. Linking H to C should have no effect on the response (Fig. 1C). Under an associative recognition model H could be nonimmunogenic because it is too small to be recognized associatively and delivers a paralytic Signal ① only. Coupling H to C provides a source of inductive Signal ② (Fig. 1A). Under a nonassociative idiotype network model only one solution to the requirement for hapten-carrier linkage is possible. The signal delivered via the receptor (Signal ①) must be inductive (ON), not paralytic. An inductive Signal ① (ON) also runs counter to fact but for the sake of argument let us assume that H is nonimmunogenic because it is incapable of interacting to deliver an ON signal. Upon coupling it to a carrier, H may be visualized to be capable of delivering its ON signal either because it is converted to a site filling ligand (H'C) capable of inducing a conformational Signal ON or because it is polymerized (H_nC) and therefore capable of delivering an aggregation Signal ON (Fig. 1D). The requirement that the signal via the combining site of the receptor be inductive (ON) places the self-nonself discrimination uniquely in the hands of suppression. Paralysis plays no role under idiotype network models. Under an associative recognition model suppression (positive unresponsiveness) cannot determine the self-nonself discrimination because the suppressive mechanism interacting with self-epitopes on antigen-sensitive cells themselves would turn off the immune system. Suppression plays a role in determining the class of the response, cell-mediated or humoral.

A system regulated uniquely by nonassociative recognition of idiotype and antigen thus becomes very unsatisfying because it cannot be mapped onto the known cell-cell interactions, e.g. the requirement for a cooperating ("helper") system for induction is obviated by the inductive ON Signal ①. Consequently most network formulations have been compromise versions which invoke associative recognition of antigen modulated by idiotypic network regulation (Fig. 1E). Whatever form this cybrid model takes, network regulation is relegated to a secondary or parasitic role.

In order that a cybrid model be internally consistent, a mechanism must be provided by which the immune system 1)

distinguishes self-idiotopes as a class from self-epitopes (non-idiotopes) as a class and at the next level, 2) distinguishes idiotopes which are promiscuously distributed relative to paratopes from idiotopes which are selectively distributed relative to paratopes. Since under the associative recognition model, the existence of such a mechanism is ruled out, all cybrid theories are self-contradictory. Further, in cybrid formulations, there is no need to invoke network regulation because the associative recognition model, itself, adequately explains all of the experimental findings on manipulation of the immune system via the idiotype as examples of breaking tolerance (autoimmunity) or of rendering immunogenic, antigens present at such low concentrations that, in essence, they "escape" the immune system (i.e. do not maintain tolerance effectively or are not regulated by the network). We will deal with examples of this after considering the next question (Section II).

B. Is the T-cell receptor which mediates MHC restricted recognition of antigen, V_H-encoded?

The general tenor of this meeting has been that two T-cell worlds exist; one is restricted and the other unrestricted by MHC. As a first approximation, the unrestricted effector T-cell is pictured to act by secreting a monovalent humoral antigen-specific antibody which is not immunoglobulin but has a combining site encoded by the identical v_H-locus expressed by cells of the B-lineage. These humoral antibody factors might arm third party effectors and appear able to induce ("amplify") or suppress a specific response to antigen. Only the divine prankster could invent an unrestricted T-cell system which is involved in an auto-regulated circuit of exquisitely specific, alternating antigen- and idiotype-driven "amplifier" and "suppressor" antibodies, the goal of which is to produce a nonspecific factor delegated the role of regulating the biologically active effectors of the immune system. Warming to his task, he then fashioned the factors with v_H-encoded combining sites knowing that no aspect of the known chemistry of the V_H domain would have permitted any predictions concerning the observed idiotypic or combining site properties of the factors.

If two parallel and independent universes of regulatory T-cells exist, the question of the reason for this dualism of function should be raised. However, before considering such a

question, we must await a better characterization of the apparently unrestricted system and its antigen-specific humoral factors. In the meantime, I will assume that the normal effector function of all T-cells is restricted and reference antigen-specific. The existence of T-cells anti-idiotype is a second order phenomenon (Sections IA and II).

The MHC restricted T-cell universe is involved in cell-cell interactions. Regulatory restrictive recognition is a detail of the mechanism of associative recognition. There is no evidence that an MHC restricted T-cell uses the v_H-locus to encode the combining site of its receptor. Nevertheless, I would like to deal with the general consequences of this widely accepted assumption.

The v_H-locus used to encode the B-cell receptor is selected upon, in complementation with the v_L-locus, to encode combining specificities ($V_L V_H$) which are directed at targets on pathogens which cannot be eliminated by cell-mediated systems. Protection against them requires a functioning humoral antibody system which uses immunoglobulin molecules as antigen-specific mediators. The germline encoded combining specificities are largely anti-carbohydrate. Since almost everyone extrapolates from the putative unrestricted T-cell world to the restricted T-cell world, the receptors used by the latter are assumed to be v_H-encoded. Further, since it is improbable that the germline encoded V_H complemented with the germline encoded V_L specifies anti-bacterial, -fungal or -helminth combining activity while the germline encoded V_H alone specifies anti-MHC combining activity almost everyone has assumed that the germline v_H-encoded combining repertoire is random. It is from this assumption that one is forced to the conclusion that somatic selection for low affinity anti-self (MHC, Id, etc. or combinations of self-X+MHC, +Id, +etc.), entrains, by heteroclitic reactivity, high affinity anti-nonself-X in various combinations with MHC, Id or other (Section III).

Competing with this assumption is one in which the germline encoded combining repertoire is neither v_H-encoded nor random but is selected for its anti-MHC specificities. These are largely anti-allele specific determinants on the restricting elements (R) K/D or I. I would argue that no somatic selection process acting on a germline encoded random repertoire could have the prescience to decide which

determinants on self-R are allele specific. The recognition of this specificity must be germline encoded (7).

As first seen by Langman (2), all models of T-cell restriction must start with the assumption that R is special because it is an acceptor of the signal delivered by the effector T-cell. The two opposing views of restriction place its origin either in an [R+X→RX] interaction or in a receptor made up of two linked subunits, one specifying anti-R and the other anti-X.

The [R+X→RX] hypothesis *per se* says nothing about the receptor. As long as R is assumed to be a unique signal acceptor, the recognition of any X determinant alone on the RX complex will have a functional consequence. However,

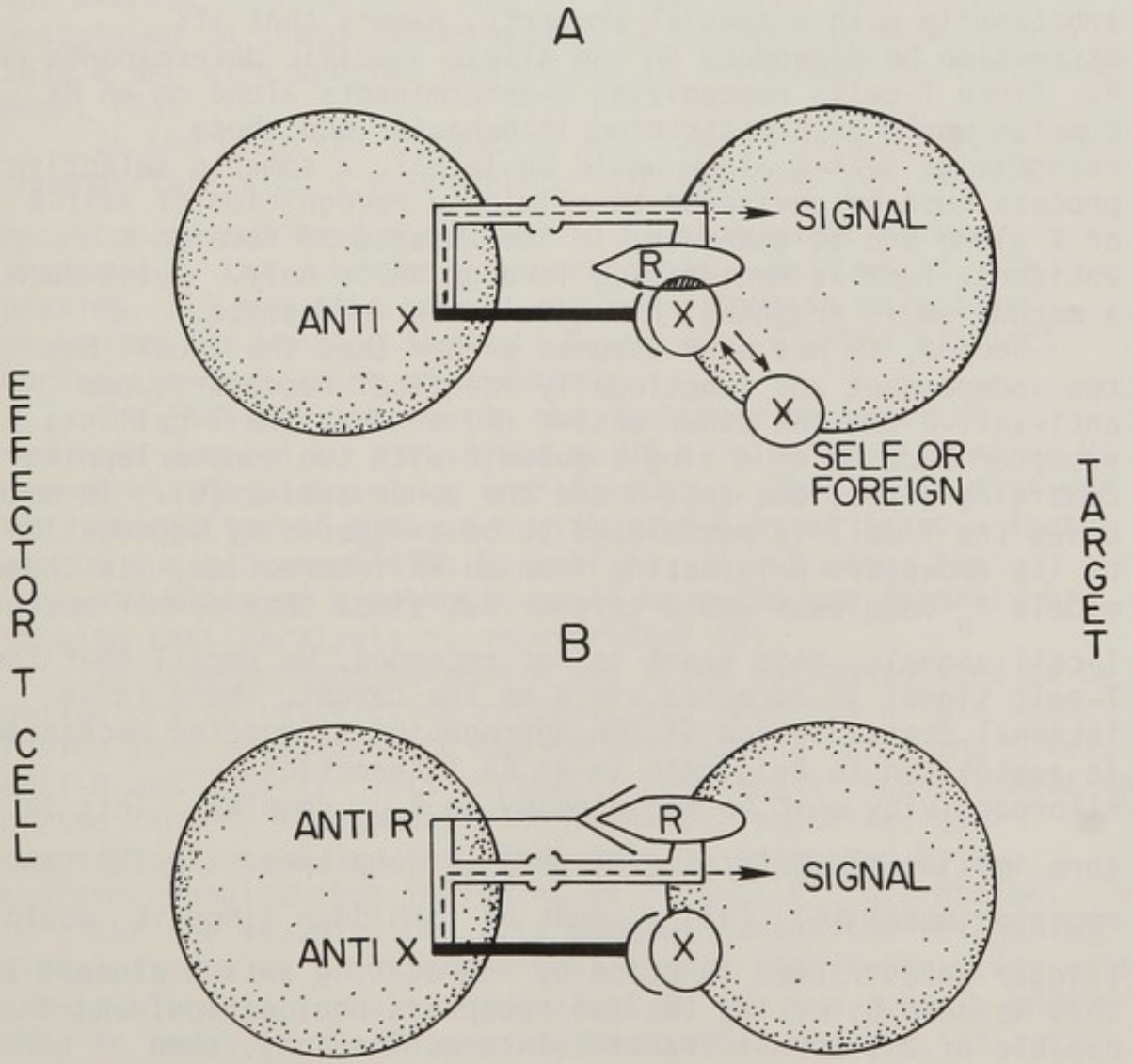

FIGURE 2. Models of the restricted T-cell receptor illustrated with the cytotoxic T^K-cell.

recognition of unbound X by the same receptor cannot lead to a functional signal (Fig. 2A). This basic model of T-cell restrictive function faces difficulties in trying to answer two questions.

1. Why should the restriction specificity be largely allele-specific (as can be defined by the use of allo antisera)?

2. Why should the frequency of alloreactive cells be high?

Two answers to these questions in the [R+X→RX] framework have been proposed.

First, it has been assumed that RX creates a new antigenic determinant (NAD) recognized by the T-cell (3). In order to answer the two questions, the NAD is endowed arbitrarily with a special property, namely that its expression be dependent on the allele-specific determinants on R. Since T-cells recognizing X-determinants alone on an RX complex would be unrestricted in behavior and those recognizing self-R alone would be lethal, a somatic selective process must be envisaged to eliminate recognition of self-R or X alone and to generate, in the absence of foreign-X antigens, T-cells recognizing foreign NAD's only. Until such a mechanism is proposed, NAD models are incomplete.

Second, it has been assumed either that the T-cell has two independent and functionally identical receptors, one anti-self-R and the other anti-X (4) or that the T-cell has a receptor made up of a single subunit with two nonoverlapping combining sites, one anti-R and the other anti-X (5). In both cases the T-cell is postulated to be triggered by aggregation of its receptors originating from an RX interaction. In these models X_n complexes would trigger but since they cannot accept T-cell signals, this event is not recorded. We recall that the T-cell signal is accepted via R on the target. There is an internal contradiction if the aggregation triggering mechanism is postulated to originate in an RX interaction. Alloreactivity must be mediated by an $R_{n\geq 2}$ complex. This in turn implies the existence of $(RX)_{n\geq 2}$ complexes. In the two receptor model (4), $(RX)_{n\geq 2}$ must be forbidden since it would trigger unrestricted function by aggregating anti-X alone. If this is done by making the two receptors nonidentical and capable of a 1:1 anti-R+anti-X interaction only, then alloreactivity cannot be explained as this would require an anti-R+anti-R interaction in response to allo-$R_{n\geq 2}$. In the one subunit, two site model (5) [or NAD model (3)], $(RX)_{n=1}$ cannot

deliver an aggregation signal and $(RX)_{n\geq 2}$ would lead to unrestricted activity. Therefore, aggregation models of triggering are untenable if they are derived from RX interactions. Only conformational signals are tenable in the RX framework and this is compatible with NAD models only.

However, even were we to put these considerations aside, we cannot avoid the question as to why an anti-R site need be postulated at all under these formulations (4,5, Fig. 2A). Clearly, anti-R is introduced and endowed in an ancillary way with special properties to account for allele-specific restriction and high frequency alloreactivity. These properties are not derived from a solution to the problem of restricted T-cell function which only requires special rules for R+X→RX. Anti-R with special properties (Section III) is postulated in these latter models (4,5) for the same reason that a NAD with special properties is introduced in the former model (3).

In the [R+X→RX] framework, one subunit models (3,5) are incompatible with a v_H-encoded, idiotype positive, anti-X receptor of the kind described to be on humoral factors produced by the putatively unrestricted T-cell world. In passing, all two site models require a somatic selective mechanism for keeping the anti-R and anti-X sites distinct during the generation of diversity.

The [R+X→RX] hypotheses seem unlikely because the membrane protein R, postulated to be a "glue" for X and/or R, cannot distinguish self-X from foreign-X. This would disfavor the interaction of foreign-X with self-R by at least a factor of 100 due to competition by self-X. In any case, the [R+X→RX] class of hypothesis could be ruled out formally by showing that paralysis is unrestricted (6).

If interactions between R and X are not postulated to be obligatory to restrictive recognition, then its origin must reside in a receptor with two nonoverlapping combining sites, anti-R and anti-X. This virtually requires a receptor made up of two linked, functionally nonidentical subunits, one specifying anti-R and the other anti-X, triggered by a conformational change following an "X anti-X" interaction.

The one subunit, two site model in which the triggering signal is due to a conformational change upon interaction with anti-X alone is formally equivalent to the two subunit model when it comes to the triggering mechanism. However, if anti-species-R is germline encoded, as I would expect, a one subunit model would require such a baroque mechanism of v_T-gene expression that it would have little likelihood of

being correct.

In summary, we are left only with models in which the triggering event is due to a conformational signal in a single receptor (2,6,7). A signalling system dependent on conformational changes is incompatible with an idiotype-driven nonassociative recognition model. It is this and the role of suppression which makes restrictive recognition difficult to reconcile with network recognition (1,7, Section IA).

In the [R+X→RX] framework we need consider only NAD formulations which are incomplete (Section III). In the non[R+X→RX] framework, we need consider only a receptor comprised of two nonidentical subunits, physically linked, one specifying anti-R and the other anti-X (Fig. 2B). This latter model developed in several publications (2,6,7) may be summarized as follows:

The effector T cell receptor consists of 3 physically linked elements, anti-R, anti-X and a signal donor. The anti-R and the anti-X sites must be nonoverlapping and coupled to different constant regions since the separation of their functions must be maintained. These 3 elements could be linked either before or after interaction with X; the former seems more likely.

The target or antigen-sensitive cell must possess 2 elements which are physically independent, an R element linked to a signal acceptor and an antigen-X bound to the target cell membrane directly or to the receptor on the antigen-sensitive cell.

The function of anti-R is to bring R coupled to its acceptor into a signalling channel or synapse. The function of anti-X is to activate the signal donor as the consequence of a conformational change when it interacts with antigen-X.

This model (7) has the following consequences:

1. Alloreactivity must be mediated via interaction with the anti-X subunit. Allo-R is a special case of a foreign-X.

2. The germline encoded combining specificity is anti-species-R (expressed initially by both the anti-R and anti-X subunits). The v_T-genes encoding the T-cell receptor cannot be the v_H-locus encoding an idiotype positive, anti-X receptor believed to be expressed by unrestricted T-cells and their humoral factors.

3. Haplotype inclusion must operate at the v_T-locus and at the DNA level, one subunit (anti-R) being encoded by $v_R c_R$ and the other (anti-X) by $v_X c_X$.

An experiment testing the two subunit structure of the

receptor has been presented by Marrack and coworkers at this meeting. They fused two distinguishably restricted T-cells, and selected a hybrid which carried out the restricted activity of each parent. No unselected complementation of restriction and antigen specificity was observed. At face value, this finding argues against all two subunit models of the T-cell receptor. In order to save any two subunit model of the receptor in the [R+X→RX] framework, special rules must be invoked as to which RX complexes can form. However, the very assumption that, as a general mechanism, RX interactions are obligatory to restrictive recognition, is already so unlikely that adding special rules for RX interaction is an

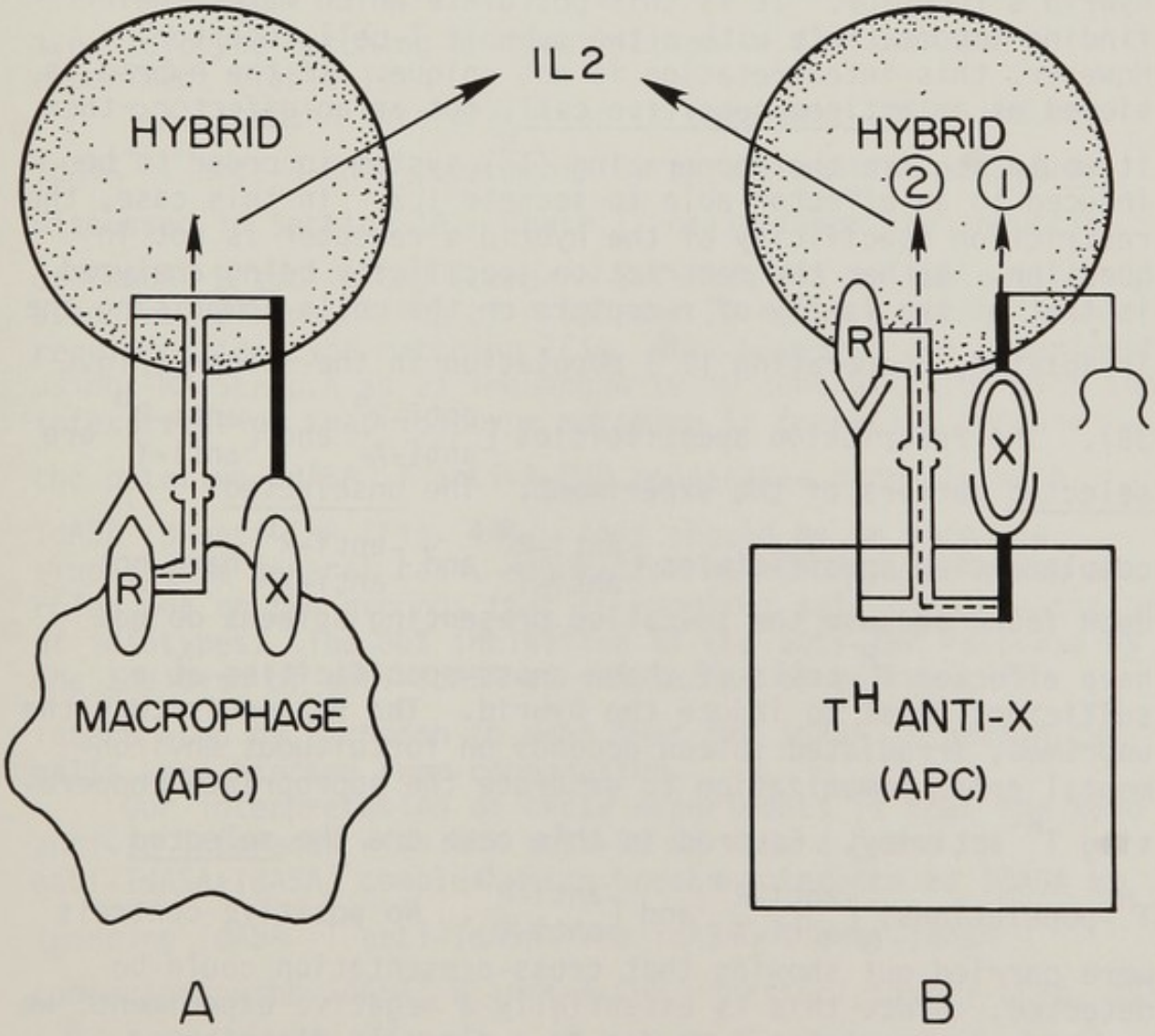

FIGURE 3. Two interpretations of the Marrack et al. experiment.
APC = Antigen-presenting cell.

unreasonable interpretation of their experiment. In the non[R+X→RX] framework, two subunit models of the receptor can be saved only by challenging the concept of antigen-presentation. Marrack et al. assume that the hybrid is an effector T-cell activated to secrete IL2 by interaction with an antigen-presenting cell (APC) usually referred to as a macrophage. This concept requires that the hybrid's receptor see both R and X on the APC. The APC triggers the hybrid to secrete IL2 by running the signal backwards from the APC to the hybrid, i.e. via the R acceptor (now a donor) on the APC to the T-cell donor (now an acceptor) (compare Figs. 2B and 3A). The restrictive specificity of the triggered secretion of IL2 is postulated to be due to recognition of the APC by the hybrid's receptor. It is this postulate which makes their finding incompatible with a two subunit T-cell receptor. However, this interpretation is not unique. If the hybrid is viewed as an antigen-sensitive cell, not as an effector, then it would require the cooperating (T^H) system in order to be induced to an effector able to secrete IL2. In this case, the restriction specificity of the hybrid's receptor is not in question. Rather the restriction specificity being analyzed is that of the family of receptors on the cells comprising the irradiated, cooperating (T^H) population in the spleen (Fig. 3B). The restriction specificities $[^{\text{anti-R}^a}_{\text{anti-X}}$ and $[^{\text{anti-R}^b}_{\text{anti-Y}}$ are selected markers of the experiment. The unselected complementing specificities $[^{\text{anti-R}^a}_{\text{anti-Y}}$ and $[^{\text{anti-R}^b}_{\text{anti-X}}$ have not been found because the so-called presenting spleens do not have effector T^H cells of these cross-specificities at a sufficient level to induce the hybrid. The reason is that the unprimed, irradiated spleen depends on fortuituous environmental cross immunization to generate the appropriate cooperating T^H activity. Favored in this case are the selected T^H-populations, $[^{\text{anti-R}^a}_{\text{anti-X}}$ and $[^{\text{anti-R}^b}_{\text{anti-Y}}$. No positive controls were carried out showing that cross-presentation could be detected. Since this is essentially a negative experiment, we can view "presentation" as due to a clonally distributed effector cell in the spleen e.g. T^H, and no contradiction with two subunit models of the restricted T-cell receptor need be implied.

II. SOME EXPERIMENTAL DATA

Here we will deal with some examples of the use of manipulations of the idiotype to argue that a network functions in normal responsiveness and that the T-cell uses a v_H-encoded receptor.

Eichmann and his coworkers have manipulated the streptococcal A carbohydrate (Strep. A-CHO) system by using guinea pig IgG_1 anti-IdA5A. From these studies they conclude that the "helper" T-cell expressing the v_H-encoded IdA5A cooperates only with B-cells expressing the $v_L v_H$ encoded-IdA5A i.e. idiotype matching or "restriction". Consider one experiment (Table I).

The Eichmann interpretation is that anti-IdA5A induces $IdA5A^+$ T^Hanti-A-CHO, presumably by an aggregation Signal ON discussed in Section IA. These T^H cells favor the induction of $IdA5A^+$ B anti-A-CHO (compare 35% with 74% in Table I) by 1) associative recognition of A-CHO and 2) network recognition requiring idiotype matching (Fig. 4A). However, the experiment using TNP-Strep.A as an immunogen is in contradiction with the interpretation that idiotype matching is operating because the putative $IdA5A^+$ T^Hanti-A-CHO cooperates normally with $IdA5A^-$ B anti-TNP (Fig. 4A). This should be no surprise since, as a general case, regulation by idiotype matching is ruled out on the grounds that it requires self-complementation of idiotypes. The 86% inhibition of the anti-TNP response by the addition of anti-IdA5A at initiation of culture, is interpreted by Eichmann to mean that the $IdA5A^+$ T^Hanti-A-CHO cells were blocked from cooperating.

Our interpretation of these experiments is that the xeno anti-Id interacts with the self-Id to form a [xeno anti-IdA5A+IdA5A] complex which breaks tolerance to IdA5A by inducing $IdA5A^-$ T^Hanti-IdA5A. The $IdA5A^-$ T^Hanti-IdA5A cooperates with $IdA5A^+$ B anti-A-CHO nonassociatively (Fig. 4B) accounting for the favoring of an $IdA5A^+$ anti-A-CHO response (compare 35% to 74% in Table I). This same T^Hanti-IdA5A cooperates with B anti-TNP when TNP-Strep.A is the immunogen because humoral $IdA5A^+$ anti-A-CHO produced by the induced

Table I. A basic experiment on idiotype-matching and T-cell idiotypes

Priming of mouse spleen in vivo	In vitro response to Strep.A.		TNP-Strep.A.	
	Anti-A.CHO	IdA5A$^+$ PFC	Anti-TNP	Inhibition of anti-TNP response by anti-IdA5A added at start of culture.
	IgM PFC/10^6	%	IgM PFC/10^6	%
Unprimed	2	-	38	-
Strep.A	83	35	258	6
G.P.anti-IdA5A	52	74	277	86

Data from Table 2 in Ref. 8

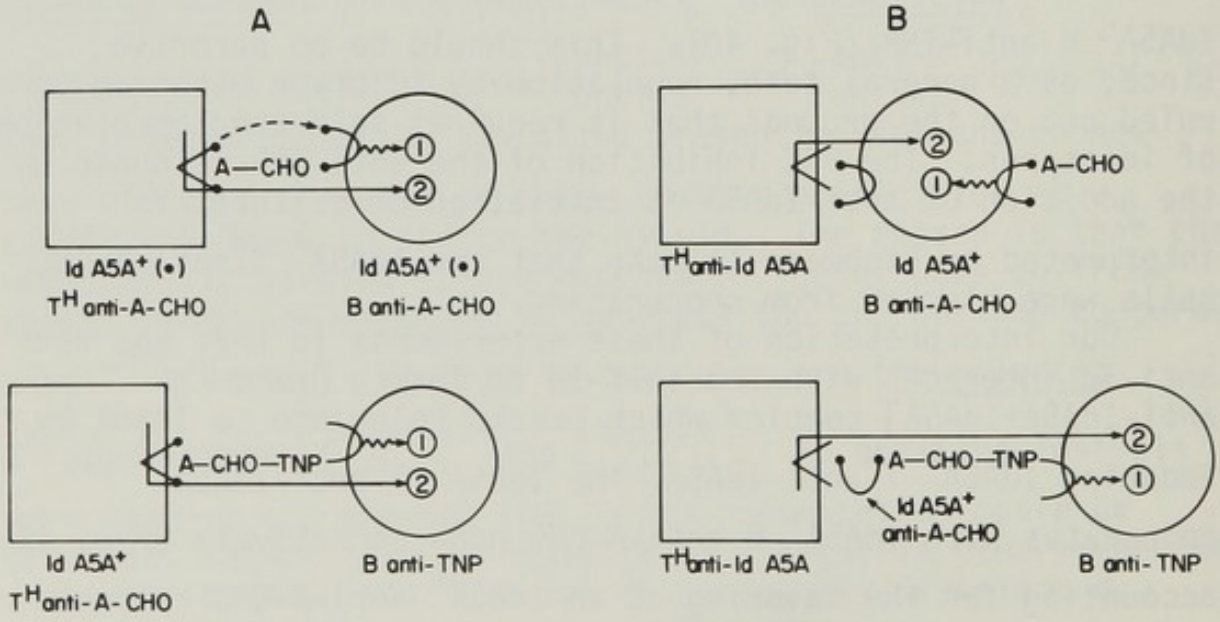

FIGURE 4. Two interpretations of the effects of anti-idiotype.
(-----→) indicates idiotype matching.

IdA5A$^+$ B anti-A-CHO cell in the spleen population binds to the TNP-Strep.A to provide a carrier determinant for the T^Hanti-IdA5A (Fig. 4B). The 86% inhibition of the anti-TNP response by anti-IdA5A (Table I) is due to the blocking of the interaction between this humoral IdA5A$^+$ anti-A-CHO and TNP-Strep.A-CHO.

A missing control would test the two interpretations. In the above protocol, an MHC matched B-cell population genetically incapable of expressing IdA5A should be inhibitable in its anti-TNP response by anti-IdA5A if the Eichmann interpretation were correct and not be inhibitable if our interpretation were correct.

The Eichmann interpretation is argued to be confirmed by an experiment presented here by Rajewsky. His reasoning is as follows: the foreign (xeno or allo) anti-Id might act as a carrier to break tolerance to the self-Id by inducing T^Hanti-Id. However, one would not expect to break tolerance to the self-Id if a syngeneic anti-Id were used and one does.

The experiment is to make a monoclonal anti-IdNP (AC-38). This monoclonal AC-38 anti-IdNP reacts with a very small proportion of the idiotopes comprising a normal anti-NP response to NP-carrier. It sees essentially the idiotope present on a monoclonal anti-NP referred to as carrying the B1-8 IdNP. If AC-38 anti-IdNP is injected into syngeneic mice, the total anti-NP response upon challenge with NP-carrier is unchanged but the proportion of the anti-NP expressing the B1-8 IdNP is dramatically increased.

Our interpretation is that B1-8 IdNP is at such a low concentration normally that it escapes recognition as an effective tolerogen by the immune system. This assumption also places it outside of any possible regulation by a putative network. Complex formation between of the B1-8 IdNP and the AC-38 anti-IdNP so increases its avidity that it is rendered immunogenic, inducing T^Hanti-IdNP. Complex formation might also create conformational changes which introduce foreign determinants. In this case, the findings in the IdNP and IdA5A systems would be explained identically. These T^Hanti-IdNP cells favor the induction of IdNP$^+$ B anti-NP by nonassociative recognition as in the IdA5A case above.

If, under the Eichmann-Rajewsky interpretation, B1-8 IdNP$^+$ T^Hanti-NP had been induced by an aggregation Signal ON (Section IA), then the preferential induction of the minor B1-8 IdNP$^+$ B anti-NP class would have required idiotype

matching (or restriction). This we argue is untenable.

Rajewky confirms our interpretation by showing that an animal immunized with B1-8 IdNP$^+$ anti-NP, behaves like one treated with AC-38 anti-IdNP in that the anti-NP response to NP-carrier is comprised of an increased proportion of B1-8 IdNP$^+$ anti-NP. In this case, no question of the induction of IdNP$^+$ T^{H}anti-NP is involved. The B1-8 IdNP$^+$ acting as an immunogen (high dose in adjuvant) induces IdNP$^-$ T^{H}anti-IdNP.

There is no published result involving manipulation of the immune response by treatment with anti-Id or immunization with Id itself which warrants the interpretation that the network operates as a regulatory mechanism primary to associative recognition or that Id$^+$ T anti-X are induced, i.e. the v_H-locus encodes the T-cell receptor. Further, there is no reason to believe that the T cells anti-Id which are induced, play a functionally useful role, qualitatively distinct from T cells anti-X. First, in the above experiments, the total anti-A-CHO or anti-NP response was effectively the same independent of the manipulation via the idiotype. The only thing that changed was the relative contribution of a reference V-framework to the total anti-X activity. Second, there are many interpretations at the moment of the data presented at this meeting by Goodman and Bottomly who concluded that there exists two <u>qualitatively</u> distinct T^H populations, one anti-X and the other anti-IdX, which act <u>obligatorily</u> synergistic in the induction of an immune response. An argument of synergy cannot be made from an experiment showing that a given mixture induces a greater response than the sum of its constituents, without first demonstrating that the response to each constituent alone either is a linear function of the dose or is null. T^H-populations, anti-X or anti-IdX, are expected to show an exponential dose-response relationship.

Maybe it would be salutary to consider how we would deal with this vast literature on the "idiotypes" of effector T-cells if it were demonstrated that v_H-genes are not rearranged in them? Would the emperor have any clothes?

III. A PHILOSOPHICAL REVOLUTION OR CONCEPTUAL TINKERING?

Most workers extrapolate from Jerne's model of diversification (9) to the revolutionary generalization that somatic selection for antigen-sensitive cells reactive to self in some given range of affinities, unselectedly entrains antigen-sensitive cells comprising the total repertoire to nonself by heteroclitic reactivity. The most sophisticated form of this generalization was developed here by Dröge. This concept implies a germline encoded repertoire which is most easily visualized as random. Since reactivity-to-self is postulated to drive combining site diversification, the self-nonself discrimination resides in the nonoverlapping affinity distributions of the selected anti-self and the unselected anti-nonself.

There is a wide range of variations of this assumption.

1. The selection for reactivity to self-NAD's at "intermediate" affinity co-opts, as unselected, reactivity to nonself-NAD's at "high" affinity.

2. Selection for anti-self-R at "intermediate" affinity co-opts, as unselected, reactivity to allo-R at "high" affinity and at high frequency.

3. Selection for reactivity to self-Id ± self-R at "intermediate" affinity determines the initial conditions of a functional network.

These selection procedures for anti-self reactivity in a given affinity range have a common denominator.

The precursors of the antigen-sensitive cells which express receptors and upon which the affinity selection will operate fall into three categories. Those with "high" anti-self affinity are eliminated by the equivalent of a paralytic Signal (1); those with "intermediate" anti-self affinity are induced to proliferate but somehow are not allowed to become effectors; those with "low" anti-self affinity are not self-reactive and are therefore eliminated by turnover. There is no operational difference in the fates of "high" and "low" affinity anti-self cells. The "low" affinity anti-self class comprises most of the "high" affinity anti-nonself cells under this view but these are eliminated as useless by this theory of anti-self selection because they would be restricted to allo-R if the selection specificity were self-R or specific for a nonself network if the selection specificity were self-Id.

The positive selection for "intermediate" affinity anti-self is key to the generation of the "high" affinity anti-nonself which functions in the restricted and network

worlds. Having selected for the precursors passing an "intermediate" affinity window, they differentiate to become antigen-sensitive cells inducible to effectors. This selection does not solve the problems discussed earlier as to how the immune system distinguishes either allele-specific from common determinants on self-R or self-idiotopes from all other self-epitopes. Further, for network function, this self-selective process is self-defeating because the selection for "intermediate" affinity anti-self-Id is equivalent to selection against an optimally functional network. It might be worth stressing that "high", "intermediate" and "low" are relative terms. The "intermediate" affinity range is so low that the anti-self-R receptor on an effector T-cell cannot interact with self-R in the absence of an interaction with foreign-X via anti-X (Section IB). Yet in the thymus, the affinity is high enough to trigger proliferation.

In contrast, under the associative recognition model the "high" and "intermediate" self-reactive cells are eliminated by tolerance (Signal ①) and the repertoire is derived from the "low" affinity or "unreactive-to-self" cells. The self-nonself discrimination applies to that universe of antigens indistinguishable as self or nonself on the basis of a physical property. This discrimination must be based on the immunological history of the antigen. Only under one special circumstance is it permissible to view the selection for a restricting specificity as part of the problem of the self-nonself discrimination. If it is assumed that induction and paralysis are obligatorily restricted as in NAD models (3), then these two processes are identical (discussed in ref. 6). In this case, all that need be considered is the reasonability and logic of the argument that selection for "intermediate" affinity anti-self-R (NAD) entrains, as unselected, "high" affinity anti-allo-R (NAD) at high frequency. If induction and paralysis are not obligatorily restricted as in the two subunit model (Fig. 2B), no question of self-nonself discrimination is involved in the selection for self-restriction specificity (6).

Come what may, given its present momentum, thinking about T-cells, in the coming year, will be dominated by network circuitry to which is coupled a somatic selection for self-reactivity that generates an anti-nonself repertoire by unselected heterocliticity all of which is v_H-encoded.

Alas! Alas! Philosophical revolutions should be made of sterner stuff.

ACKNOWLEDGMENTS

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REFERENCES

1. Cohn, M. Cellular Immunol., in press (1981).
2. Langman, R. In "Rev. Physiol. Biochem. Pharmac." 81, 1 (1978).
3. Matzinger, P. and Bevan, M. Cellular Immunol. 29, 1 (1977).
4. Janeway, C.A., Wigzell, H. and Binz, H. Scand. J. Immunol. 5, 993 (1976).
5. Hoffmann, G.W. In "Contemp.Topics in Immunobiol." 11,185 (1980).
6. Cohn, M. and Epstein, R. Cellular Immunol. 39, 125 (1978).
7. Cohn, M., Langman, R. and Geckeler, W. In "Progress in Immunol. IV", 153 (1980).
8. Hetzelberger, D., Eichmann, K. Eur. J. Immunol. 8, 846 (1978).
9. Jerne, N. K. Eur. J. Immunol. 1, 1 (1971).

IMMUNOREGULATION AND THE CONTROL OF IDIOTYPE EXPRESSION BY INTERACTING CELL SETS

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This year's ICN-UCLA Conference on Immunoglobulin Idiotypes and Their Expression has clearly indicated the two major themes which have come to dominate experimental immunological science. The first of these, which has been considered in detail in Areas I, II and III of this meeting, is concerned with evaluation of the molecular genetics, cell biology, membrane chemistry, and soluble products of individual immunocompetent cells. It is clear that this theme will continue to be a center of attention of many immunologists both because lymphocytes provide an unparalleled cell type in which to characterize the processes determining cellular proliferation and differentiation and because the detailed understanding of the chemical events which determine responsiveness of individual cells will provide the insights necessary for the design of pharmacologic agents which can be used to enhance or depress specific immune responses in a precisely controlled way.

The second major theme of contemporary immunology is the consideration of the immune system as a complex set of interacting cells. Indeed, one of the most remarkable aspects of the immune system is that it contains so many distinct members whose activities must be subjected to some type of coherent regulation if a useful response is to occur. The fourth area of this meeting has largely been devoted to a consideration of the impact of idiotypy on cellular interactions; it is this portion of the meeting that I have been asked to comment upon.

The concept that the immune response is a highly regulated process is relatively new. Previously, immunologists were quite content to consider antigen as the only stimulatory element in the immune system. This did not reflect naivete so much as it did the sensible concern of wishing to deal with first things first. Nonetheless, this approach led to a model of the immune system in which the principal perturbing element was the stimulatory capacity of

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antigen. Such a system, if not subject to other control mechanisms, could lead to a continued antigen-driven expansion of a limited set of clones until these cells dominated the system and, indeed, displayed the features of a lymphoid malignancy. Considerations of this type forced immunologists to realize that regulatory processes must play an important role in the economy of the system. Although this was initially a painful process, the interest in regulation of immune reactivity has now grown to the point that it is, as already noted, one of the major themes of immunology.

We may divide cells of regulatory systems into two general types, based on their specificity. One group of regulatory cells recognizes antigen, and the specific effects of these cells are mediated by an antigen-bridge between the interacting cells or through an antigen-specific factor, produced by the regulatory cell and focused on its target by antigen.

The second general type of regulatory cell is specific for the receptor of the cell upon which it acts. In such cases, the regulatory process may proceed in the absence of antigen itself. Furthermore, such receptor-based regulatory cells can be thought of as dictating the expressed repertoire through a complex process which begins prior to exposure of the system to antigen and which continually refines that repertoire as a result of the immunization history of the individual. A point I wish to make now and to return to later is that fully developed regulatory processes often involve the sequential action of antigen-specific and receptor-specific cells

During this meeting, considerable attention has been devoted to both types of regulatory cells. It would not be possible in the short space of these comments to meaningfully summarize the large body of interesting work which has been presented here. Rather, I will attempt to discuss some of the key elements of these regulatory processes in the light of the material discussed at this meeting and to comment, where possible, on some of the major porblems which require solution.

In beginning a consideration of the antigen-specific regulatory cells, we must initially deal with the classical major histocompatibility complex (MHC) restricted, antigen-specific helper cell. This cell displays the still difficult to explain property of specificity both for a nominal antigen and for an MHC encoded restriction element. In order to become activated, this cell must be presented with antigen in

the context of an Ia-bearing, specialized antigen-presenting cell. In some instances, this cell mediates its helper function by the production of a lymphokine, such as interleukin-2 (IL-2) or a closely related molecule. However, in many instances, these MHC restricted helper cells appear to be able to help only those B cells which express the Ia molecule for which the helper is restricted and which have bound the nominal antigen for which the helper is specific. In these instances, the molecular basis of help is not clear but is probably not simply limited to the local production of IL-2. The nature of the receptors of this cell type and of its specific products, if any, have been subjects of great controversy. Indeed, this cell has been considered to a relatively limited extent in this meeting, precisely because it has not been amenable to idiotypic analysis. Nonetheless, we must not underestimate its potentially central role in all regulatory processes because it may very well be the only authentic, independently acting helper cell which can be brought to bear for the majority of thymus-dependent antigens. If current approaches to understanding regulation are to make progress in providing a coherent picture of the behavior of the immune system, they must provide a means through which the level of activity, or the numbers of the MHC-restricted helper T cells are detected by the regulatory system and determine the action of the regulatory cells. To my knowledge, no such direct linkage between MHC restricted helpers and the regulatory pathway has yet been achieved.

In contrast to the antigen-specific MHC-restricted helper T cell, which has been quite resistant to idiotypic analysis, antigen-specific suppressor cells have provided a very productive area for the study of idiotype expression on cellular receptors. It is now clear that antigen-specific suppressor cells often bear idiotypic determinants which cross-react with idiotypic determinants found on antibodies specific for the same antigens. Furthermore, Frances Owen (these proceedings) has shown that suppressor cells express a membrane antigen controlled by genes linked to the Igh-C gene complex; this antigen may be a marker of the "constant" region of the receptor of these suppressor cells.

The idiotypic determinants found on receptors of cells of suppressor pathways provide a focal point for the intertwining of the antigen-based and the receptor-based recognition systems. For example, there is increasing evidence from the work of several laboratories that the suppressor system is in reality a cellular pathway comprised

of a series of types of lymphocytes which act in a linear fashion to finally generate the so-called "suppressor-effector" cell or the "Ts_3" cell. The initially recognized cell in the system studied by Gershon, and his colleagues (these proceedings) is the suppressor-inducer, which has the properties that it is an antigen-specific Lyt 1^+, $I\text{-}J^+$ cell. This cell appears to be analogous to the Ts_1 cell described by Germain, Benacerraf and their associates (these proceedings), which expresses many of the same properties and which has been shown to express idiotypic determinants. In both cases, this cell appears to act on an intermediate cell, termed the suppressor-transducer or the Ts_2 cell. Where investigated, the intermediate cell is specific for the idiotype of the suppressor-inducer or Ts_1 cell. One mode of action of the idiotype-specific (i.e., "anti-idiotype") intermediate cell is to act upon a third T cell, the suppressor-effector or Ts_3 cell. Strikingly, the anti-idiotype specific Ts_2 cell acts upon idiotype positive (and antigen-binding) effector cells. In this it displays what I believe to be a fundamental principle of receptor or idiotype based regulation. This principle is that the idiotype-specific cell, which is itself induced by an idiotype-bearing cell functions through the induction of an idiotype-bearing cell, rather than an anti- (anti-idiotype) specific cell.

The suppressor-effector cell appears to act, through an antigen-bridge mechanism, to inactivate the helper cell (Cantor, these proceedings) and, in a similar fashion, the Ts_3 cell inactivates the MHC-restricted antigen-specific T cell which mediates delayed hypersensitivity.

The question of whether cells of the suppressor pathway act directly upon B cells also needs consideration. The evidence currently available strongly suggests that suppressor-effector cells and Ts_3 cells act mainly, if not exclusively, upon helper T cells and on suppressor-inducer cells. However, there are several systems which have been described in which idiotype-specific suppressor cells appear to regulate the production of antibodies bearing that idiotype. In instances in which the properties of these idiotype-specific suppressors have been carefully evaluated, they appear to have some properties in common with the suppressor-transducer or Ts_2 cells (1), (Rohrer, et. al., these proceedings). Thus, as a working hypothesis, we may propose that the Ts_2 cell may act either to activate Ts_3 cells, which suppress MHC restricted antigen-specific helper cells, or to differentiate into cells which directly suppress-idiotype specific B cells.

I would now like to consider the issue of idiotype-specific (or receptor-specific) helper cells. This is, perhaps, the most fascinating and still controversial aspect of the regulatory process. There is mounting evidence that T cells can regulate the class, allotype and idiotype of antibody. This process is believed to represent the action of a specialized subset of T lymphocytes which act through selective amplification of the carrier-specific helper process. There has been great interest in the possibility that these regulatory cells are directly specific for class, allotype or idiotype-specific antigenic determinants expressed on B cell receptors.

Indeed, one particularly provocative set of experiments on the regulation of idiotype expression has been interpreted to suggest the existence of idiotype-specific regulatory T cells which are also antigen-specific. This concept has been proposed by Bottomly and her colleagues (these proceedings) based on studies of the role of T lymphocytes in regulating clonal dominance in the anti-phosphorylcholine system. Bottomly has suggested that these postulated antigen-specific, receptor-specific T cells constitute a set which parallels the antigen-specific, MHC-specific T cells. To put this differently, this concept holds that this set of T cells use immunoglobulin determinants as restriction elements, in much the same way that MHC gene products are restriction elements for carrier-specific T cells. The simple elegance of this symmetrical relation should not prevent us from noting certain important difficulties. First, the postulated antigen-specific, Ig-restricted helper cell appears to bind antigen directly (it has not been clearly shown to bind Ig determinants) and is postulated to react with these two types of determinants sequentially, rather than simultaneously. By contrast, the MHC-restricted T cell almost uniformly fails to bind either antigen or MHC gene product alone; its action is dependent upon both molecules being present upon individual antigen-presenting cells or B cells, and it appears to recognize the two determinants as one unit. Indeed, current ideas concerning the selective forces which have led to the appearance of MHC-restricted T cells are based on the need for the T cell to see antigen on the cell with which it interacts and not to be distracted by "free" antigen.

Secondly, the number of distinct MHC restriction elements, although not small, is still quite manageable, and allows the existence of a reasonably large precursor

population which can recognize any individual MHC restriction element and any given antigen. By contrast, the set of idiotypic determinants is extremely large. If all could act as restriction elements, the number of distinct T cell types required to allow the expression of each antigen-specific receptor with each idiotype-specific receptor would be truly astronomical. Of course, this difficulty could be partially solved by introducing the assumption that only a very limited set of idiotypic determinants may act as restriction elements. I will develop the notion of specialized "regulatory idiotopes" from a quite different basis at the end of this paper.

In my opinion, it would be prudent to consider that the antigen-specific T cell which regulates Ig expression be considered to be quite different from the MHC-restricted, carrier-specific helper cell. Indeed, in most instances, it has not been directly demonstrated that the Ig regulatory cell actually bears receptors for Ig determinants. It is conceivable that these cells mediate their apparently Ig-specific effects by other means.

An additional issue to be considered at this time is the nature of the regulatory pathways and their true roles in regulatory processes. What has been elegantly demonstrated is the existence of linear pathways, perhaps allowing amplification of the regulatory process. These pathways lead to one or more effector cells which can interact with and thus regulate the helper pathways. In some cases, the regulatory pathways appear to contain internal feedback loops or amplification loops; for example, the suppressor-effector cell can act to inhibit the suppressor-inducer cell. However, the overall activity of the suppressor pathway has not yet been decisively linked to the level of activity of the helper pathway. Without such linkage, the suppressor process cannot be regarded as a very effective regulatory mechanism. I suspect, of course, that such linkage must exist. That is, that the activation of suppressor-inducers must be determined by the action of an MHC-restricted helper cell. Indeed, since long-term lines of some cells in the suppressor pathway appear to be dependent upon IL-2, IL-2-producing helper cells might determine the level of activity of the suppressor pathway.

Let us now turn to a further consideration of the interrelationship of the immune system through idiotype-anti-idiotype interaction and, particularly, to the question of whether the immune system should be likened to an

enormous three-dimensional spider web, in which the "plucking" of a lymphocyte in any region of the web leads to detectable perturbations flowing throughout the entire system. It has been proposed initially by Jerne (2) that such an extended network truly exists and that it is a key element in the maintenance of the immune system itself because the number and level of activation of any individual lymphocyte clone can be most efficiently regulated, in the absence of antigen, by the action of receptor or idiotype-specific cells. In a sense, this notion can be restated to say that not only are receptor-based regulatory processes formally possible but they exist in an effective fashion prior to the introduction of antigen. I think it more likely that the immune system can be imagined to consist of two types of clones. One type are those whose receptors bear idiotypic determinants (idiotopes) found on few other clones. Thus, the effective concentration of the idiotopes they bear is very low and, in all likelihood, so is the effective concentration of clones specific for these idiotopes. I postulate this because it seems most likely that idiotope-bearing clones induce idiotope-specific clones. Since both the idiotope-bearing and idiotope-specific cells are represented in very low frequency, effective interactions between them are very unlikely events. For such clones, then, the "network" does not exist, in a meaningful sense, prior to immunization. However, a second group of clones appears to exist which express idiotopes which are shared by a reasonably large number of other clones. Much of this sharing may involve cells which are not specific for the same antigen. Since the concentration of these idiotopes is reasonably high, they should induce an expansion in those clones which are specific for such idiotopes. For these clones, an effective idiotope- anti-idiotope regulation could exist prior to immunization. Furthermore, the existence of such interaction might provide an advantage for B cells (or T cells) bearing such idiotopes when antigen is introduced and might lead to a further relative expansion of these clones. Because these idiotopes have the potential for being particularly critical in regulatory processes, I would suggest they be termed "regulatory idiotopes." Among obvious candidates for classification as regulatory idiotopes are the T15 idiotypic determinants (3), the Ars cross-reactive idiotype, (Capra, these proceedings), the A5A idiotype of anti- A carbohydrate (4), the IdX in the anti- α(1-3) dextran system (Davie, these proceedings), and the idiotypic determinant(s) of MOPC-460 (5). We might further use the regulatory idiotope concept to return to the proposal of Bottomly that Ig

determinants function as restriction elements. If that idea has merit, I would propose that it would hold only for the regulatory idiotopes.

The regulatory idiotope concept has certain interesting implications for networks. For example, consider the consequence of the production of an antibody expressing a regulatory idiotope. Such an antibody should lead to the activation of those clones of B and T cells which are idiotope-specific. Since we have already assumed that these "anti-idiotope" clones were quit highly represented, the appearance of cells and antibodies specific for regulatory idiotopes should be a quite regular consequence of the introduction of antibody-beaing a regulatory idiotope. Using terminology introduced by Cazenave (6), immunization with an Ab_1 (antigen-specific antibody bearing the Id_1 regulatory idiotope) should lead to the appearance of antibodies specific for Id_1. These antibodies (anti-Ab_1 or anti-Id_1) are generally designated Ab_2's. As noted above, the frequency of precursors of Ab_2 producing cells is high because they have been induced by the highly represented regulatory idiotope of Ab_1. However, there is no particular reason to suggest that the Ab_2 molecules also bear regulatory idiotopes; their expanded numbers are due to their specificity for Id_1, the regulatory idiotope of Ab_1. If these Ab_2 molecules do not express regulatory idiotopes, then immunization of animals with Ab_2 should have a very interesting consequence. Precursors of cells secreting antibodies specific for idiotopes of Ab_2 should be relatively infrequent, since they would not have been previously expanded. On the other hand, precursors bearing the Id_1 idiotope are relatively common since Ab_1 bears a regulatory (e.g. cross-reactive) idiotope. Since Ab_2 can interact with B (or T) cells bearing Id_1 as well as with B (or T) cells expressing anti-Ab_2 receptors (i.e., Ab_3), the outcome of immunization with Ab_2 should be determined by the relative frequency of Id_1- and Ab_3-bearing cells. It seems clear that the frequency of Id_1-bearing cells should be much higher than that of Ab_3-bearing cells. Thus, immunization with Ab_2 should predominantly lead to the appearance of antibodies expressing Id_1. In the past, it had been considered that this did not, in fact, occur, since immunization with Ab_2 rarely leads to the appearance of molecules which bound the antigen for which Ab_1 was specific. The definition of Ab_1 (or "Ab_1'") as a molecule capable of binding the antigen for which the prototypic Ab_1 molecule was specific as well as expressing Id_1 imposed an artificial limitation, since we initially defined a regulatory idiotope as widely shared among

clones and not necessarily restricted to antibodies of a single specificity. However, if we do not require antigen-binding as a marker of the set of Id_1 bearing antibodies (i.e., the "Ab_1 family of antibodies") how can we distinguish Ab_1 from Ab_3 (i.e. anti-Ab_2), since both interact with Ab_2. One way is based on an immunization test. Immunization with Ab_1 should elicit Ab_2, whereas immunization with Ab_3 could elicit anti-Ab_3 (Ab_4), as well as Ab_2. In two recent attempts to construct Ab_1 - Ab_2 - Ab_3 - Ab_4 systems by intentional immunization of allotype matched rabbits (7) or inbred mice (8), the result observed was that the majority of Ab_4 molecules bound to Ab_1; that is, most Ab_3 molecules expressed the Id_1 idiotope. By my definition, those "Ab_3" molecules were not true Ab_3's (i.e., anti-Ab_2's) but rather Ab_1 (or more properly Ab_1') molecules, since they shared the Id_1 regulatory idiotope and most likely arose because Ab_2 (anti-Ab_1) bound to Id_1 and led to the activation of precursors bearing this determinant on their receptors.

If this notion of the interconnections of the immune system is correct, it suggests that idiotope- anti-idiotope interactions are mainly reflective, not expanding. In turn, this might limit the degree of interconnectiveness of the system. Thus, the regulatory consequences of perturbing any individual Ab_1 clone would be largely limited to the anti-Ab_1 clones and to the Ab_1' clones [i.e., the clones which shared the Id_1 regulatory idiotope(s)]. The network might then be thought of as a series of relatively small families of interconnected cells (mini-networks) with only very fragile connections between distinct families.

I would like to close this discussion on regulation with a brief comment on future approaches to the study of cellular interactions in the immune system. Immunologists have made rather remarkable progress in uncovering much of the phenomenology of regulation. On the other hand, the definition of the molecular basis of regulatory processes has progressed relatively slowly because of the great difficulty involved in preparing pure populations of the interacting cells. It is now clear that this difficulty can largely be solved by the use either of cloned lines of the interacting cells, as so elegantly illustrated in in the work on suppression in the sheep erythrocyte system reported here by Cantor and his colleagues (these proceedings), or by the creation of somatic cell hybrids which continue to express regulatory phenotypes. These approaches, used together with monoclonal antibodies and molecular genetic probes as analytic tools, promise to revolutionize our understanding of cellular

processes. They should make it possible for us to consider moving from the description and characterization of the regulatory pathways to the next level, which will involve a quantitative measure of the number of interacting cells and the rate at which the interactions occur. Indeed, until we can apply such quantitative approaches to regulation, we will not have a true picture of the economy of the immune system nor will we be able to reliably predict the outcome of any given perturbation. With such information, it should be possible to rationally undertake manipulation of the immune system for therapeutic or prophylactic purposes.

REFERENCES

1. Bona, C., and Paul, W. E. (1980). In Regulatory T Cells. (B. Pernis and H. Vogel, eds.) Academic Press, Inc., New York, p. 21.
2. Jerne, N. K. (1974). *Ann. Immunol.* (Paris) 125C, 373.
3. Potter, M., and Lieberman, R. (1970). *J. Exp. Med.* 132, 737.
4. Eichmann, K. (1972). *Eur. J. Immunol.* 2, 301.
5. Bona, C., and Paul, W. E. (1979). *J. Exp. Med.* 149, 592.
6. Cazenave, P. A. (1977). *Proc. Natl. Acad. Sci. USA* 74, 5122.
7. Wikler, M., Franssen, J. D., Collignon, C., Leo, O., Mariame, B., van Wolle, P., de Groote, D., and Urbain, J. (1979). *J. Exp. Med.* 150, 184.
8. Bona, C., Heber-Katz, E., and Paul, W. E. (1981). *J. Exp. Med.* 153, 951.

Changing Paradigms in Immunology

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I wish to begin by making the prediction that this conference will be looked back upon in years to come as a milestone in the development of immunological thought. I say this because I feel, and will attempt to document the feeling in what follows, that this is one of the first times that a full fledged international meeting is devoted to a new paradigm in immunological thinking. As my close friends know, I have been interested in the paradigmatic structure of scientific change for the past few years (I am sure that some of them have felt that this is an obsession and others that it is a sign of precocious middle age). My main interest has been in attempting to reconstruct the history of ideas leading to one of the two central paradigms of immunology, clonal selection and the newly emergent paradigm which I will discuss in this paper, autoreactivity.

Before launching into the discussion, however, I would like briefly to summarize some of the thinking which has been going on in recent years in the history and philosophy of science. For several years these otherwise staid areas of intellectual endeavor have been a fascinating, often acrimonious battleground not too unlike the often acrimonious, never dull battleground of immunology. The conflict has been primarily between followers of two philosophers of science, Sir Karl Popper and Thomas Kuhn. Popper, who is one of the modern masters of philosophy, had developed the theory of falsification as the means by which science functions. The traditional mode of inductive reasoning in which data was accumulated to the point where one would draw general conclusions from a large enough pile had been discredited by Hume. Hume had pointed out that because an event has occurred in the past does not mean that we can say that it will continue to occur in the future. The often quoted example is that because all of the swans that one sees are white it does not follow that all swans are in fact since the very next swan which one sees may be black. Popper made the logical extension of this by saying that even though one can not verify by the accumulation of data, one can falsify. Thus, counting yet another white swan is of no interest because it tells us nothing about the general color of swans, but counting one black swan does give us something upon which to generalize, i.e. all not swans are not white. This being the case,

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Popper argued, the only thing that a scientist can do is to falsify a notion because no matter how many experiments are done demonstrating a phenomenon they fall prey to Hume's criticism. However, once one does an experiment which falsifies a hypothesis then one has done what the scientist is meant to do and that is to describe something about how the world works. Of course, it follows from this that scientists can only figure out how the world works by describing how it does not work.

Kuhn, on the other hand, argued that while all of this is undoubtedly true in the purely philosophical sense it is not how scientists actually function. He argued that scientists examine the world by establishing a set of paradigms and designing their experiments within the limits of these paradigms. The paradigm, as defined by Kuhn, is a rather nebulous idea but we can consider it to be a series of beliefs which are accepted by a body of workers within a field. The paradigm then serves as a framework within which experiments are designed and that corner of the world which a given field is attempting to understand becomes understood in terms of these paradigms. According to this notion scientific progress occurs in two ways. The first being a description and understanding of the world within the paradigm, i.e. using the paradigm to design experiments and to gain as much of an understanding of the world as we can from those experiments. The second way in which progress occurs is when anomulous results begin to accumulate to the point where they challenge the existing paradigm. When that occurs, the anomulous observations are explained by the establishment of a new paradigm. Thus change occurs in two ways, one utilizing the paradigm to its fullest and the other the change of the paradigm. Adherants of both schools would probably by now admit that neither falsification nor paradigms can explain everything but it is a rare scientist who doesn't agree that the notion of the paradigm more aptly describes how science actually functions.

One of the fundamental paradigms in immunology has been that the body does not react against itself and every theory which has attempted to explain the immune response has had to explain the lack of autoreactivity. In those cases where there have been clearly demonstrated responses to self these have been termed autoimmune diseases. Yet with 75 years of this paradigm behind us we have slipped into a new era in which the paradigm has been reversed and reactions against self are used to explain both the initiation and regulation of the immune response. I have gone through the sequence of experiments and their interpretations which argue that autoreactivity to MHC products is necessary to generate an immune

response (the Zinkernagel-Doherty-Shearer phenomenon) and that auto-responses to idiotypes may play a crucial role in the regulation of immune responses (the Jerne network hypothesis) in two minireviews. I find it interesting that this major change has slipped into our consciousness without the disruption which large paradigmatic changes usually cause (remember the title of Kuhn's book is "The Structure of Scientific Revolution" and even bloodless revolutions do not go unremarked upon). Contrast this with the other paradigmatic change which we have just undergone in immunology, i.e. the idea that immune reactions not only have a positive effect but that they may have a negative effect and that there are cells which suppress immune responses. This change came about with great pain and was accepted (with religious zeal when the conversion finally came) only with great reluctance on the part of most immunologists. I don't give this recounting of recent history smugly as if to say "some of us knew what was really happening." Even though the man who instituted the paradigmatic change and bore the brunt of the resistance, Dick Gershon, is one of my closest friends, I felt in the early 1970's that I could not let even so great a friendship as this stand in the way of the progress of science and designed a series of experiments called the "Richard K. Gershon Memorial Experiments" to once and for all put to rest this silly notion of the suppressor cell. Unfortunately something went wrong each time we carried out the experiments because we found suppression where it clearly should not have been since the paradigm we were functioning within (pure clonal deletion) had no room for suppression. So we had to leave the experiments and go on to other things. One would have thought that the resistance to the idea of the T-cell bearing a receptor for self and recognizing and reacting with self in order to react with something foreign would have caused at least as much outrage as the idea of a suppressor cell, but it did not. Perhaps what we might learn from this is that scientific revolutions become less painful when the participants are exhausted from an earlier revolution. But it seems fitting that two of the three topics of this current meeting deal with those new paradigms, namely auto-anti-idiotype and their role in regulation and suppressor cells and their role in regulation. My firmly held conviction is that these paradigm shifts coming so close, one upon the other, will continue to be treated together and the immunology of the 80's will be devoted in large part to working out the mechanism by which suppressor cells regulate autoreactive cells.

The dominant themes of this meeting are the mechanisms involved in the acquisition and expression of specificity in

the immune response. The evolution of our thinking about specificity and its mechanisms is interesting and merits a few moments of consideration. We all know that long before there were any scientific basis for medicine it was known in folk medicine that recovery from a disease conferred immunity to that disease in later years. With the discovery of the methods of active and passive immunization the idea that the body was actively producing something which physically combated disease grew. The first attempt to explain the basis of this specificity of the product in terms which we would consider modern is Erhlich's side chain theory. As you know, Erhlich visualized the array of specificities which an animal could generate as being displayed on the surface of certain cells. These side chains serve the dual function of being detoxifying agents (toxophore) as well as feeding the cell (haptophore). Erhlich saw the array of these reactivities as being due to chance since an individual living in a temperate climate could produce antibodies against toxins found only in the tropic (his example). So the cell was seen as the seat of specificity in this first model which we may call a selection model. The cell still remains at the center of specificity as ideas about antibody change with the introduction of template notions of immunity. Breinl, Haurowitz, Mudd, Alexander, and Pauling all had models in which the specificity of the antibody was not inherent in the information of the cell (even by chance) but rather the information was imposed upon the antibody molecule as it was being synthesized in the cell. In these instructional models there is no need for the cell to have displayed at the surface a specificity element nor to have the genes for these elements. With the advent of molecular biology and understanding of how the gene functions Burnet introduced a "half-way house" theory called the indirect template. In this model Burnet used what was known about inducible enzyme systems and visualized the antigen acting as a form of inducer, but in those pre-trinity days he had the induction acting upon RNA in the cell. Niels Jerne made the movement back to a form of selection theories by postulating that cells have the ability to be genetically determined to synthesize an antibody of a given specificity. He postulated that antigen reacts with the antibody not at cell surface but rather in the circulation and the complex comes back to the cell surface where the antibody induces the cell to proliferate and synthesize more antibody. Both Sir McFarland Burnet and David Talmage independently modified that theory to have the recognition element and unit of specificity on the cell surface in what we now recognize as clonal selection theory of antibody formation. In this

theory the antigen reacts with the cell which is pre-programmed to produce a different specificity so that we now have the cell with the specificity element, the recognition unit, at its cell surface, and this is a product of the genetic information within the cell. The beauty of the intertwining of the discoveries in modern immunology are seen in this meeting because such a large portion of the meeting is devoted to the genetic mechanisms by which that specificity displayed at the cell surface is derived and also the nature of the cell surface receptors.

It is interesting that the path to understanding the organization of the immunoglobulin gene has been so different. This depended first upon determining that antibodies were protein and then using the methods of protein chemistry determining the chain structure. When the chain structure was worked out, one could begin to postulate a strategy for the function of the molecule but not until it was possible to sequence amino acids and variable and constant portions of the molecule became revealed. At that point the mechanism seemed obvious since diversity could be generated by creating variable or hypervariable regions. When the not insignificant problem of determining the genetic basis for this variability becomes available (and once again just to show that nothing involved in the immune response is simple) a surprising new phenomenon of gene movement during differentiation was discovered, the two gene-one polypeptide theory was proven and we have for the moment at least a satisfying picture of the face of G.O.D.

The purpose of studying history and of toying with philosophy should be to see if one can predict the course of events by examining the patterns which have emerged. If all of the above is not too far off base one would predict then from the patterns which have evolved that we would spend the next few years consolidating and chiseling into soft stone the rules which are emerging about the nature of receptors and the genes which code for them. Even more autoreactivity should be discovered and found to be necessary for generation and regulation of a wide array of responses. Attention will then begin to focus very strongly on the regulation of autoreactivity. What is the nature, for example, of the autoreactive receptor? What prevents destructive reactions during coordinated autoreactivity with foreign antigen? Who suppresses the suppressor? I feel that this meeting will be a landmark meeting just as the Squaw Valley Meeting of 1975 was. That meeting had an aura of excitement about it (which in retrospect we may blushingly think of as smugness) because the existing paradigms of cell cooperation had apparently solved all the problems of cellular immunology.

The topics to be covered at this meeting will be landmarks because I see this meeting as the first clear bringing together of the newly formed paradigms in immunology and the thinking of the participants at this meeting will to a great degree define the limits of the experiments which are carried out over the next half-dozen years.

REFERENCES

Burnet, E.M. (1959) The Clonal Selection Theory of Immunity. Nashville, Tenn., Vanderbilt University Press.
Golub, E.S. (1980) Immunology Today 1,v.
Golub, E.S. (1981) Immunology Today 2,v.
Golub, E.S. (1980) Cell 21,603.
Golub, E.S. (1980) Cell 22,641.
Golub, E.S. (1981) Cell, in press.
Jerne, N.K. (1955) Proc. Nat. Acad. Sci. USA 41,849.
Kuhn, T. (1970). The Structure of Scientific Revolutions. 2nd Edition. University of Chicago Press.
Ontai, S. (1981) Theories of Antibody Formation. Senior Thesis, Harvard University.
Talmage, D.W. (1959) Science 129,1643.

WORKSHOP SUMMARY: PC-T15 SYSTEM

Conveners: J. Cerny & H. Köhler

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Heinz Köhler opened the workshop on the PC system by remembering the beginning of experimental studies on the response against phosphorylcholine in BALB/c mice. The PC system can celebrate its 10 year anniversary now since in 1971 Humberto Cosenza and Heinz Köhler had done the first successful experiments suppressing the T15 idiotype in BALB/c mice. Much credit for using PC as antigens to induce the T15 idiotype goes to Michael Potter, who made the suggestion to use the bacterial vaccine R36A as antigen.

As first speaker, Jan Cerny summarized his findings on regulation of T15 idiotype-positive ($T15^+$) B cells reactive to a T-independent (TI) form of PC (R36A vaccine). Incubation of normal T cells with the vaccine generates specific suppressor cells (T_S) that inhibit B cells directly and that bind to antigen-coated dish but not to T15-coated dish. The T_S express T15 detectable by both homologous and heterologous anti-T15 antibody, however, preliminary studies using a panel of monoclonal anti-T15 antibody indicate that T_S carry only a few of the idiotopes of the TEPC-15/HOPC-8 family expressed on PC-reactive B cells. Furthermore, T helper cells (T_H) specific for $T15^+$, R36A-reactive B cells have been identified in both normal and antigen-primed spleen cell populations. T_H bind to T15-coated dish, and they trigger the T15 production upon adoptive transfer into nude mice without a concomittant antigen stimulation provided that the recipients were primed ten to twenty days before the transfer. Dr. Moisier commented that bacterial polysaccharides are know to persist in the reticuloendothermal system, for a long time and the apparent "antigen-independent" triggering of $T15^+$ by anti-T15 T cells may, in fact, involve a synergistic effect of antigen-reactive T cells activated by the residual antigen in the primed recipient. Dr. Cerny replied that this is indeed possible, however, he considers it unlikely because he has recently succeeded in repeating the results *in vitro* whereby purified B cells from antigen-primed mice were triggered to produce $T15^+$ antibody by addition of T cells,without the antigen.

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Then Günter Hämmerling reported some new data from experiments on limiting dilution analysis of PC-specific T helper cells (T_H) that have been carried out jointly with K. Eichmann and J. Cerny. Normal T cells are stimulated in vitro with ConA-, cloned in the presence of TCGF and then used as helper cells for anti-PC response induced microtiter wells containing PC-primed B cells without any antigen. If increasing numbers of T cells were added to the cultures and the anti-PC-PFC responses were measured as the fraction of responding wells a biphasic cell dose response cruve was obtained. This can be interpreted to indicate that two different antigen-independent T helper cell populations exist which differ in frequencies and which are controlled by a suppressor T cell.

Next Dr. B. Kim discussed his finding on the analysis of suppressor T cells (T_S) for the T15 idiotype. Such suppressors are induced in neonatal BALB/c mice by two different methods: (1) administration of anti-idiotypic antibodies and(2) injection of a tolerogenic dose of the C-polysaccharide. Anti-idiotypic-induced suppressors can be removed either by panning on T15 coated dishes or by incubation with IgM T15 idiotype and complement. In contrast, C-polysaccharide-induced suppressors adhere to PC-coated dishes. This indicates that the 2 neonatal treatments induce different T suppressor cell populations which work with different effector mechanism. A 3rd type of suppressor cell or both previous types, perhaps, seems to be generated by simultaneous administration of anti-idiotypic antibodies and C-polysaccharide. Here, only about 50% of the suppressive activity is removed by panning on T15 coated dishes. The 2 types of cells interact with each other in a mixed culture,namely the anti-T15 T_S inactivates the PC-specific T_S.

Next Kim Botomly reviewed her data on the role of idiotype (Id) specific T helper cells in the dominant expression of the T15 Id. In a T-dependent (TD)anti-PC-PFC response, 2 T cells seem to be involved: one is the conventional T_H which is carrier-specific, induced by carrier priming, and is also MHC-restricted. The other T cell is needed to induce a dominant expression of the T15 idiotype in the TD anti-PC response. This T_H is not MHC-restricted and can be induced by priming with several different antigens, however, it is not required for the $T15^+$ response to T Id antigens. The Id-specific T_H is lacking in μ-suppressed mice. The question raised by Dr. Köhler whether the Id-specific T_H is required for primary B cells i.e. using B cells from BALB/c mice not primed with PC. Dr. Botomly answered she had not data on this point. She then discussed the possibility that the Id-specific T_H recognizes both the Id and the priming antigen. The cells can be removed by incubation with Id^+IgM plus complement, however, the results of panning on antigen-coated dishes have not been clear-cut. It would seem that the concept of a T_H with dual recognition of Id & AG should be regarded with caution.

The discussion moved then more deeply into the issue of T15 clonal dominance. Dr. Köhler remarked that one way of gaining better understanding of the T15 dominance would be to investigate the ontogeny of the T15 clone(s). He summarized his findings on the maturation of T15 dominance. Contrary to earlier data by Klinman and colleagues, anti-PC responses and PC specific precursors can be demonstrated before day 5 after birth. Using PC-LPS as antigen in newborn BALB/c mice, anti-PC PFC response can be induced that is T15 non-dominant until day 5 or 7 after birth; later the response becomes T15 dominant. Similarly, B cell precursors taken from neonatal livers before day 4 or 5 consist of $T15^+$ and $T15^-$ clones when analyzed in splenic fragment cultures. Dr. Köhler offered 2 possible mechanisms to explain the late expression of T15 dominance: (1) T15 dominance is achieved by idiotype-specific regulatory interaction, occurring very early in ontogeny or (2) $T15^+$ precursor clones mature earlier and/or faster than non-T15 precursors. This would establish a developmental advantage of T15 positive B cells over non T15 expressing B cells and could explain the establishment of T15 dominance in the anti-PC response.

José Quintans then summarized his experiments in (CBA/n x BALB/c) F_1 mice (NBF_1) suggesting that $T15^+$ B cells can dominate the primary anti-P response without a T help, even when the stem cell pool is removed. Adoptive transfer of splenic B cells from BALB/c mice into immunodeficient NBF_1 male recipients restored the responsiveness to PC antigens (both TI and TD). The PFC are of the donor origin, and the response is dominated by T15 Id. Furthermore, the transplantation of BALB/c fetal liver cells (containing progenitors of PC-specific B cells) into irradiated NBF_1 males results in emergence of reactivity to PC antigens that are dominated initially by $T15^+$ clones which are later superseded by $T15^-$ clones.

The last topic of the workshop was the structure of T15 idiotype and its expression by lymphocyte clones. The antibody responses to PC have been regarded as highly restricted, involving a very small number of B cell clones; the response in BALB/c strains appeared to be almost monoclonal as judged by T15 Id expression. However, the analysis with monoclonal anti-T15 antibodies reveals that T15 is a family of distinct idiotopes. Gunter Hammerline briefly eluded to his findings that while all of those idiotopes are presented on anti-PC serum antibody from BALB/c mice only some of them are found on the antibody from other strains. Latham Claflin said that the analysis of sera from CBA mice shows that in an individual mouse there are distinct groups of $T15^+$ and $T15^-$ antibodies. Moreover, Claudia Berek reported on a monoclonal anti-T15 antibody that reveals an idiotopic diversity even in BALB/c mice: in a given animal, there are plasma cells secreting T15-like antibody lacking the specific idiotope. According

to Patricia Gearheart, there may be idiotypic differences between isotypes of anti-PC antibody in BALB/c mice since she finds that sequences of hybridoma antibodies of IgG class are more diverse than those of IgM. Overall, it seems that the expression of T15 shows greater diversity than has been considered previously.

Dr. Cerny concluded the workshop and thanked all participants for the lively discussion.

SUMMARY OF SYSTEM WORKSHOP ON NITROPHENYLS

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The purpose of this summary will be do date information on the genetic control of the immunoresponse to nitrophenyl haptens (NP, DNP) discussed in the workshop and during the course of the meeting. The immune response to nitrophenyl hapten is being analysed at several levels and the direction that studies in the mouse anti-hapten response have taken were set by the concept of idiotype or antigenic determinants on the V region of the antibody molecule.

V-REGION DIVERSITY IN THE ANTI-HAPTEN RESPONSE

Strains of mice have been identified that respond to NP groups on protein by producing anti-NP antibodies having higher affinities for NP analogues, NNP, NIP and NBrP than for NP itself. This pattern of binding is described as heteroclicity. The immune response of C57BL/6 mice to this hapten has been studied in detail and the following characteristics have been established: a) the primary response consists of lambda light chain-bearing antibodies, b) the primary response is restricted in heterogeneity as judged by isoelectrofocussing (IEF), c) the anti-NP antibodies can be idiotypically defined as the NP^b idiotype, d) every individual of the strain invariably express the NP^b idiotype with its full set of idiotypic determinants, e) heteroclicity is a characteristic of the NP^b idiotype which is inherited as a single genetic unit in close linkage to the $Ig.1^b$ heavy chain allotype. Therefore, it has been assumed that NP^b idiotype is the phenotypic expression of germ line heavy chain variable region gene(s) (1-5).

Until recently little has been known about the structure of and heterogeneity among individual antibody species contributing to the NP idiotype. Serological analysis of anti-NP immune response at the level of individual antibody species using hybridoma proteins (M. Reth and Imanishi-Kari, discussed in this conference and (6).) strongly suggests that the inheritable NP^b idiotype-positive response is composed of a heterogeneous family of interrelated antibodies whereby the cross-reacting determinants of non identical Ig molecules are inherited.

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An attractive explanation for the inheritable heterogeneity of NP^b idiotype would be that the immune response of inbred strains of mice to NP antigen is controlled by few related V_H germ line genes. The heterogeneity is then created by somatic mutations and somatic rearrangements of V_H with different D and J_H. Thus the detailed biochemical analysis of the members of the NP^b idiotype will provide means to distinguish what are the inheritable NP^b determinants and will tell whether several different V_H D and J_H segments are expressed. As described in this volume (Bothwell et al.) diversification of V_H NP^b germ line gene seem to occur by somatic mutation and somatic rearrangements of V ,D and J H genes. Seven different but closely related V_H NP^b germ line genes were sequenced by Bothwell et al., from which one was found to be expressed in one of the two hybridoma DNA sequenced. The sequence of the expressed DNA in the second hybridoma did not correspond to any of the sequenced seven germ line genes, but was very similar to one of them. What remain to be seen however is if only one of these or if other germ line genes are expressed in the anti-NP response. The former possibility seem to be the case in the anti-PC response described by Hood et al. (in this volume).

DO T CELLS EXPRESS THE SAME POOL OF V_H GENES AS B CELLS?

What T cells express in its functional receptors has been for long a controversional subject. The possibility that T cells use V gene products has been suggested originally by the work described by Binz and Wigzell (7) and were followed by several other groups using different or similar systems which are discussed in this volume. The serological data presented by Cramer et al. in this workshop indicate that some of the NP^b determinants are expressed in the hapten specific T cell receptors. These results and several others presented during the conference on T suppressor cells could be interpreted in two different ways a) T and B cells express the same V_H and V_L genes or b) that T cells express different gene(s) which share determinant(s) with B cell Ig receptors. Recent data from S. Tonegawa's Lab. indicate that in citotoxic killer T cells rearrangements of V ,D and J may not be the same as in B cells (8).

IDIOTYPE-ANTI-IDIOTYPE INTERACTIONS IN T CELL ACTIVATION AND SUPPRESSION.

This portion of the workshop dealt with the role of idiotype directed interactions in both help and suppression of immune responses. Janeway described a system wherein T helper function were studied in mice deprived of B cells by repeated injections of antibody to u chains. These mice contain normal levels of T helper activity delivered to B cells via a hapten-carrier

bridge, but a second idiotype-specific helper T cell important in the early phases of an antibody response is deficient. This Lyt-1 $^{+}$, 2 $^{-}$ is specific for antigen, but does not require a hapten-carrier bridge. As anti-u treated mice are deficient in Lyt-1^{+} ,2^{-} cells necessary for induction of feedback suppression, this data suggested a relationship between the idiotype specific helper T cell and the inducer cell involved in feedback suppression.

The demonstration that idiotypic manipulation of the immune system can result in the expression of normally silent idiotypes, and allotype linkage of idiotype expression, indicates that normal idiotype expression is most likely controlled by specific regulatory systems. This question was discussed by D.Primi. Using an in vitro culture system where LPS was used to reveal the total V gene repertoire of B cells, it was shown that B cells from all strains of mice cultured in the absence of T cells synthesized anti-TNP PFC, a portion of which carried M460 idiotype (9).B cells themselves were shown to exert an important regulatory influence on idiotype expression as co-culture of B cells from BALB/c or DBA/2 with anti-M460 hybridoma cells showed a decrease of M460 positive PFC while the total anti-TNP response was unaffected. K. Denis described a system where anti-idiotype reagents made against neonatal anti-DNP antibodies were used to examine responses in neonatal and adult mice. It was shown that the neonatal response was of a restricted nature, reproducible among individuals of a strain, influenced in expression by the Ig allotype of the responding cell, and most interestingly these idiotypes were not demonstrable in adults of the same strain suggesting regulation of their expresssion.

The next question discussed was the regulation of immune responses by naturally occuring auto-anti-idiotypic (AAI) antibodies. This type of regulation has been shown for both antibody and cell-mediated immune responses. A system wherein the antibody response to both thymic-independent (TNP-Ficoll) and thymic-dependent (TNP-BGG) antigens is regulated by AAI antibodies suggesting even highly heterogeneous responses can be affected was described by G.Siskind. Studies of secondary responses suggested an anmestic AAI antibody response and that secretion of both IgM and IgG are regulated. Most interestingly, AAI antibodies from 6-week-old mice were found to have no effect on responses of 6 month old mice and vice-versa suggesting age related changes in idiotype expression. A system wherein AAI antibodies regulate the T cell-mediated contact sensitivity (CS) response to DNFB by two distinct mechanisms was discussed by J. Moorhed. Contact sensitivity to DNFB in mice is maximal at 6 days post sensitization and declines rapidly thereafter. This decline is due to the appearance of AAI antibodies in the serum 9-12 days post sensitization. These antibodies inhibit the passive transfer

of CS by DNFB-immune lymph node cells with or without complement . Complement dependent inhibition of transfer was shown to be due to elimination of Ia^- effector delayed-hypersensitivity T cells (T_{DH}), while complement independent inhibition was mediated via the activation of an Ia^+ Ts-auxiliary cell which in turn delivers a suppressive signal to the effector T_{DH} cell.

Finally, the role of idiotype-anti-idiotype interactions in suppressor T cell (Ts) pathways was discussed by D.Sherr and by S.D.Miller. David Sherr discussed about Ts pathways in regulation of the DTH response to the hapten NP in C57BL/6 mice. Intravenous injection of NP-coupled syngeneic spleen cells leads to the induction of an NP^b positive, MHC unrestricted Ts-1 (afferent suppressor)which in turn leads to the induction of an anti-idiotypic, MHC and V_H restricted Ts-2 which in conjunction with an idiotype positive Ts-3 (raised by immunization) suppresses the passive transfer of DTH to NP (efferent suppression). A similar system was discussed by S.Miller in the regulation of CS to DNFB. Intravenous injection of syngeneic DNP-modified spleen cells leads to the induction of an antigen-binding , MHC unrestricted Ts-1 which in turn leads to the induction of an anti-idiotypic, MHC and V_H restricted Ts-2. Ts-1 in the DNP system can act as an efferent suppressor via an antigen-activated, anti-idiotype positive, I-J positive Ts-auxiliary cell provided the effector T_{DH} cell carries surface-associated DNP-membrane fragments to serve as a focus for the idiotype positive Ts-1 cell. The Ts-1 -Ts-auxiliary cell interaction thus appears to be restricted by genes linked to the Igh-1 locus. Ts-1 also induces an anti-idiotype positive Ts-2 cell which in the DNP system seems to be an afferent Ts as it only inhibits the induction of CS.

Thus, it appears that idiotype-anti-idiotype interactions may play some role in the regulation of both antibody and T cell-mediated immune responses . However no direct straightforward experiments have been presented to show how cells are really communicating with each other.The development of new techniques such as cell hybridization, development of continuously growing clones of T and B cells and recombinant DNA technologies will make possible the study of B and T receptors and antigens at different stages of the immune response.This will allow us to know more about the structure and interactions of cells for the expression and suppression of the immune response.

REFERENCES

1. Imanishi, T. and Makela, O. (1973). Eur. J. Immunol.2,323.
2. Jack, R.S., Imanishi-Kari, T. and Rajewsky, K. (1977). Eur. J. Immunol. 7,559.

3. McMichael, A.J., Phillips, J.M., Williamson, R.R., Imanishi, T. and Makela, O. (1975). Immunogenetics 2, 161.
4. Karjalainen, K. and Makela, O. (1978). Eur. J. Immunol.8, 105.
5. Imanishi-Kari, T. , Rajnavolgyi, E., Takemori, T., Jack, R. S.,and Rajewsky, K. (1979). Eur. J. Immunol. 9.324.
6. Reth, M., Imanishi-Kari, T. and Rajewsky , K. (1979). Eur. J. Immunol. 9, 1004.
7. Binz, H. and Wigzell , H., (1977). Contemporary Topics Immunol. 7 ,113.
8. Kurosawa, Y., von Boehmer, H., Haas, W., Sakano, H., Trauneker A., and Tonegawa S. (1981).Nature 290,565.
9. Rosenstein, R. W., Zeldis, J. B., Richards F. I. (1978).In Springer seminars in Immunopathol. 1,133 (144).

WORKSHOP SUMMARY: Ars-CRI system

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A variety of topics revolving around the use of hybridoma proteins to analyze both structural and regulatory aspects of the anti-arsonate response were discussed. Dr. M. Margolies compared the N-terminal amino acid sequences of 6 Ars-binding hybridoma proteins expressing the major cross-reactive idiotype to 8 randomly chosen Ars-binding proteins which lacked the major idiotypic determinants. Three of the CRI negative proteins were closely related both by structural and serological criteria; they could be detected by rabbit anti-CRI antisera in a direct binding assay but not in a conventional inhibition assay and thus constituted the type of "minor" crossreactive idiotype previously described by Nisonoff. Results from both the Gefter and Nisonoff labs indicated that at least one minor idiotype family was expressed by almost all BALB/c and A/J mice and could be regulated independently of the major idiotype family.

One potential problem which emerged during the workshop was the specificity of the idiotype assays used by various laboratories studying the Ars-CRI. This situation reflects the lack of a myeloma protein bearing Ars-CRI determinants which can serve as a reference standard. Given the availability of a number of hybridomas with known amino acid sequences it was suggested that one mutually accepted protein could be made available for distribution to all interested laboratories through the Salk Cell Distribution Center. It was also suggested that monoclonal anti-idiotopic reagents useful for radioimmunoassays be similarly distributed. While some workers enthusiastically endorsed this concept, others felt that anti-idiotypic reagents prepared from induced anti-arsonate antibodies were still the most reliable probes for the major Ars-CRI. The final questions of distribution and standardization were left for further discussion and arrangements between interested workshop participants.

L. Wysocki and V. Sato reported the isolation of CRI^+ non-antigen binding hybridoma proteins from A/J mice immunized with a monoclonal rat anti-CRI. These molecules

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resembled the CRI^+ Ars-binding proteins both with respect to their reactivity with other anti-CRI reagents and by preliminary N-terminal sequence analysis of the heavy and light chains. Such molecules did not appear to be a major component of the normal A/J anti-Ars response.

The role of carrier proteins in effecting the expression of the Ars-CRI was briefly addressed. P. Hornbeck discussed preliminary experiments which showed that the amount of Ars-CRI^+ antibody elicited with various Ars-protein conjugates varied directly with the protein carrier employed, and independtly of the total amount of Ars specific antibody elicited with these conjugates. These data are reminiscent of results obtained by Wang and Nisonoff in their analysis of Ars-CRI^+ antibody found in A/J mice immunized with Ars-BGG or Ars-KLH. R. Woodland also discussed similar findings in (CBA/N x A/J) F_1 mice expressing the X id defect where immunization with Ars-BGG elicited no Ars-CRI^+ antibody in 60% of the mice tested but normal amounts in the others. Cell mixture experiments between phenotypically Ars-CRI^+ and Ars-CRI^- donors showed no overt idiotype specific T suppressor cell activity but did show an absence of stimulatable Ars-CRI^+ memory B cells in cell populations prepared from phenotypically Ars-CRI^- donors.

The analysis of regulatory mechanisms involved in DTH response to ABA was presented by Sy. He described a suppressive circuit including Ts1 cells (Lyt 1^+2^-, CRI^+, $I\text{-}J^+$, antigen-binding), Ts2 cells (Lyt 2^+, $I\text{-}J^+$, anti-CRI^+, not antigen binding) and Ts3 cells (Lyt 2^+, $I\text{-}J^+$, CRI^+). Detection of Ts3 cells has been accomplished by treating DTH effector T cells with anti-CRI and Complement. After this treatment effector DTH cells become refractory to Ts2-induced suppression, indicating that a CRI^+ T cell (Ts3) present in the immune lymph node population is required for expression of Ts2 function. However, the suppression mediated by such cell interactions appears to be idiotype nonspecific. Futhermore, anti-CRI Ts can be generated in BALB/c mice although this strain does not express the A/J major CRI. Therefore generation of CRI and anti-CRI do not seem to be totally interdependent nor absolutely genetically linked and the absence of certain VH genes apparently does not always restrict the ability to develop anti-CRI receptors.

Lewis outlined some characteristics of the primary anti-ABA response to ABA-Brucella abortus in A/J mice. This

response is partially T cell dependent and approximately 50% of the anti-ABA primary PFC are inhibited by anti-CRI. Moreover, CRI-specific suppressor T cells can be induced *in vitro* by culture of normal A/J spleen cells with ABA-BA plus anti-CRI.

Bellone described the presence of two distinct Lyt 1^+ helper T cells which act synergistically in the activation of memory B cells to produce an idiotype positive PFC response to TMA *in vitro*. One of these helper T cells (Th1) is carrier-speific and acts via antigen-bridge, the other (Th2) has specificity for both idiotype and carrier and does not require an hapten-carrier and does not require an hapten-carrier conjugate to activate B cells. Similarly, Doria described the induction by ABA-KLH of two ABA-specific helper T cells which were revealed *in vitro* by anti-TNP PFC response to TNP-ABA-KLH or TNP-HRBC and ABA-BGG. These helper T cells can be distinguished by their mode of interaction (cognate or polyclonal) with B cells.

The various points discussed in this workshop have invariably demonstrated a previously unsuspected complexity in the multiple aspects of the ABA system. Nevertheless, it was generally felt that anlaysis of the anti-ABA response respresents a very powerfull approach to antigen- and idiotype-mediated regulatory interactions.

WORKSHOP SUMMARY :

IMMUNE RESPONSE TO PROTEINS

AND POLYPEPTIDE

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The immune response to the terpolymer poly $Glu^{60}Ala^{30}Tyr^{10}$ (GAT) has been studied both at the antibody and cellular level. Ju et al. and Thèze et al. have described the idiotype of the anti-GAT antibodies (C.GAT). This idiotype is broadly distributed and found in anti-GAT sera of all strains of mouse whatever their allotypic markers. Idiotypic specificities cross-reacting with C.GAT are also present in anti-GAT sera from rat and guinea pig. Thèze et al. and Ju et al. have dissected this idiotype in various specificities. Allotypically linked idiotypic specificities have been described: s.r.GAT is linked with Igl^a, Igl^c and Igl^eallotype while Gte is linked to Igl^b and Igl^e allotypic marker. The GA-1 specificity is expressed on a minor fraction of the anti-GAT antibodies and "GA" related antigenic determinants are responsible for the induction of GA-1 idiotype (Ju et al.). A public idiotypic specificity expressed in an identical form by all strains of mice and by a serie of hybridoma products (HP) from BALB/c, DBA/2 and B6/D2 origin has also been described (Thèze et al.), Fougereau et al. have determined the sequence of the first 35 to 40 amino acid residue of the heavy chain of HP from DBA/2 and BALB/c origin and the sequences are very similar; in addition sequences up to residue 35 to 40 of three κ light chain from BALB/c HP are almost identical.

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Kapp et al. reported on the idiotype of a GAT specific suppressor factor produced by a T cell hybridoma.The molecular weight of this factor appears to be 25 000 after purification. This factor binds preferential to GA and bears GA-1 and C.GAT idiotypic determinants. A similar factor specific for poly $Glu^{50}Tyr^{50}$ (GT) bears C.GAT idiotypic determinants but not the GA-1 idiotypic specificity. Waltenbaugh have shown data indicating that a GT specific suppressor factor bears both I-J determinants defined by monoclonal anti-IJ antibodies and C.GAT idiotypic determinants. Thèze et al.have obtained GAT specific T cell lines and T cell clones. These cells exhibit a very specific helper activity. The activity of the T cell lines can be selectively block by a syngeneic anti-idiotypic anti-serum made in BALB/c mice. This serum does not block the helper activity of OVA specific T cell lines.

M. Seman has performed experiments showing that macrophages from GAT non-responder animals can present DNP or Ars to T cells when these haptens are coupled to GAT as DNP-GAT or Ars-GAT. Therefore T cells from non-responder animals primed to DNP-GAT for example can proliferate specifically to DNP-OVA. On the contrary in GAT responder animal the hapten is not seen and priming with DNP-GAT or Ars-GAT does not lead to the induction of DNP or Ars T cells able to proliferate. These results were discussed in term of control of the molecular orientation of the GAT molecule by macrophages.

In the immune response to the staphylococcal nuclease (Sachs et al.) the expression of nuclease idiotype on T helper cells was demonstrated by the specific elimination of TH activity by anti-idiotypic antibodies + C' (Hodes et al.). The susceptibility of nuclease primed TH to elimination by such treatment was idiotype specific in that anti-idiotype affected only TH from strains normally expressing the same idiotype in the humoral response to immunization with nuclease. Furthermore anti-idiotype, in the absence of C', suppressed the response to TNP-nuclease in an antigen-specific manner; this inhibition occurredat the level of TH cells and is independent of B cell idiotype. Anti-idiotype antibodies are able to induce nuclease specific TH cells regardless of the idiotype normally present on the anti-nuclease antibodies of the strain tested. These TH cells induced by anti-idiotype expressed the idiotype corresponding to the specificity of the anti-ID used in priming

(Nadler et al.). These finding demonstrate that anti-Id may mimic antigen in its ability to induce a population of antigen-specific ID-bearing TH cells.

The main results obtained in the response to hen lysosyme (HEL) were reported by E. Sercarz . The cross-reactive idiotype IDX-HEL found in all antibody molecules produced in secondary response to HEL has also been found on T suppressor cells specific for HEL. Interestingly the IDX-HEL determinant(s) can appear also on hybridoma antibodies specific for different, non-cross-reactive portion of HEL. Two different, TH are involved in the maturation of the anti-HEL response. One of these cells is antigen specific while the other is IdX specific and exert a positive selection among B cells first activated by the other TH. This would explain why the anti-HEL antibodies recovered in early primary response do not display IdX while antibodies recovered in the late primary response an secondary response bear this idiotypic determinants because of the activity of IdX-HEL specific TH.

Berkower reported on shared idiotypic determinants among monoclonal antibodies to distinct determinants of sperm whale myoglobulin. For example HP from two clones that bind simultaneously to monomeric myoglobin and must therefore bind to distant sites on the antigen molecules shared idiotypic specificities. Therefore as with the anti-HEL HP, monoclonal antibodies having different combining sites specific for different antigenic determinants share idiotopes. The explanation might lie in some idiotypic network regulation of the overall response to antigen.

Corradin described his results on the immune response of BALB/c mice to apocytochrome-c. He isolated an hybridoma bearing the major idiotype of the BALB/c anti-apocytochrome-c antibodies. At the T cell level both idiotypic and antigen specific T cells have been found.

Adorini described the protocole he used to produce a suppressor T cell line specific for HEL and transformed by radiation leukemia virus. A specific factor able to suppress a T cell-dependent proliferation assay was extracted from these cells.

WORKSHOP SUMMARY: Carbohydrates

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The workshop on idiotypes in immune responses to carbohydrate antigens was divided into two main subjects: what is idiotype? and, regulation of expression of idiotype. Idiotype has been suggested to be the focal point in the regulation of the immune response. The term idiotype, however, has been used to describe many different types of antigenic determinants. Some of the questions considered were: a) the relationship of idiotypic determinants to complementarity determining residues; b) the association of idiotype with antibody specificity; and c) should hapten inhibitable and hapten non-inhibitable idiotypes be considered equivalent, particularly in an immune network. The fundamental problem remains the relationship between idiotypic differences and antibody diversity. Should IdX always be considered to be associated with germline genes, and might it be generated in different ways? Should IdI be considered an indication of somatic diversity, either by D segment assembly or somatic mutation? In either case, what is the importance of IdI in modifying the affinity and specificity of the molecule?

B. Clevinger presented recent data using chemical as well as serological means to define the IdI of J558 (anti-$\alpha(1\rightarrow3)$ dextran (Dex) binding myeloma). Although heterologous antisera had localized the IdI to the D segment, a monoclonal anti-J558 IdI (prepared by J. Kearney) showed broader specificity in that it reacted with some Dex-binding hybridomas that have a different D segment from J558. Clevinger used selective diazotization of tyrosine residues to compare the effects on J558 and one of the hybridomas, H Dex-24. Modification of tyrosines on both molecules abolished the reactivity with anti-IdI. When diazotization was carried out in the presence of hapten the J558 IdI was protected, whereas the H Dex-24 IdI was not. These results demonstrate that the critical tyrosine on J558 is directly in the hapten binding site, and the reactive tyrosine on H Dex-24 is not. They further indicate that IdI's defined by

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heterologous and homologous anti-IdI reagents can have different specificities.

B. Pernis presented some recent experiments performed by C. Victor. They showed that in animals suppressed for J558 a significant percentage of T cells could be stained with the Kearney anti-J558 IdI. Furthermore, the receptor for anti-IdI could be capped off the cells and shown to be resynthesized by them. M. Koshland reported that anti-IdI to MOPC104E (D segment), but not anti-IdX (hv2) led to complement fixation.

In the α(1→3) Dex system, the IdX is located in hv2, and, as mentioned, the IdI's have been localized to the D segment. This may not be a generalized feature of anticarbohydrate antibodies. M. Pawlita has characterized 11 BALB/c hybridomas that bind galactan. Measurements of the Ka's showed that the hybridomas had the same range of affinities for antigen as the previously characterized myeloma proteins. In these galactan binding proteins, both the IdI and the IdX could be localized to hv2.

M. Nahm described a new anti-Id prepared against an anti-group A streptococcal carbohydrate (GAC) hybridoma. The anti-Id reacts with a determinant present on the light (L) chain, but requires a heavy (H) chain for expression. H chains from Id negative proteins reconstituted the Id as well as H chains from Id positive proteins. In addition, he reported that L chains bearing this Id were not always associated with anti-GAC antibodies.

Two presentations on anti-DNA antibodies were included in this workshop. T. Marion has extensively analyzed anti-DNA hybridomas made from unimmunized (NZBxNZW)F_1 mice. He summarized the results on those of the γ2a isotype (comprising 9 of 13 hybridomas). All but two of them were different by isoelectric focusing (IEF), but by idiotypic and antigen binding characteristics they fell into four groups. Marion concluded that the repertoire of anti-DNA antibodies in NZB mice consists of a large number of different but related clonotypes. J. Rauch presented her studies of anti-DNA antibodies in MRL/lpr and MRL/++ mice. She described a germline Id that is present in both mouse strains, but that is always associated with anti-DNA antibodies in MRL/lpr mice; in MRL/++ mice a significant proportion of serum Id is not associated with anti-DNA antibodies.

G. Leslie presented data demonstrating that the response to GAC in the rat is restricted to IgM and γ2c (equivalent to the mouse γ3 isotype) and is characterized by a public idiotype, Id-1. He has defined at least two γ2c allotypes in the rat, and has shown that Id-1 can be associated with either of the γ2c allotypes.

D. Briles discussed the anti-GAC response in the mouse. He had previously shown that the IEF spectrotype of anti-GAC antibodies is highly restricted in individual mice, but that two individuals of the same strain are rarely identical. This indicates that each animal is expressing only a fraction of the repertoire available in the strain. Recent adoptive transfer experiments indicate that the B cells are clonally committed prior to immunization. The level at which this commitment occurs and whether it is modulated with time remains to be delineated.

M. Bosma presented data on the U10-173 Id which is found on a number of myeloma proteins of different classes that bind different carbohydrates, levan and galactan. Studies of an allotype congenic pair, C3H·SW·Ig^a (CWA) and C3H·SW·Ig^b (CWB) revealed that both strains responded well to immunization with levan, but only CWB produced U10-173$^+$ antibodies. Interestingly, half of the U10-173$^+$ molecules in the sera of CWB mice did not bind levan. Furthermore, mild x-irradiation of CWA mice prior to immunization with levan resulted in 25-30% of the mice producing U10-173, all of which bound levan. The latter result points out the fallacy of using the expression of an idiotype as a marker for the presence of a structural gene. K. Stein mentioned that DBA/2 mice which do not respond to B512 (α1→6) Dex can be induced to make high titer anti-Dex by immunization with a thymus dependent oligosaccharide protein conjugate.

R. Ward continued the discussion on thymus dependent (TD) vs thymus independent (TI) immunogens with his data on the B1355 (α1→3) Dex system. Using the Klinman splenic focus assay he examined the Dex precursor frequencies and idiotype and light chain expression following Dex or Dex-hemocyanin (Hy) immunization. He found that the precursor frequency was three times higher following Dex-Hy than Dex immunization. The frequency of IdX was 24% of the Dex-Hy precursors, and 71% of the Dex precursors. In addition, the frequency of Kappa bearing antibodies, while only 7% of Dex precursors, comprised 54% of the Dex-Hy population. The data suggest that TI and TD antigens stimulate B cells that express different subsets of the available repertoire. Another possible interpretation is that the additional precursors seen in the Dex-Hy immunized group represented additional specificities.

J. Hiernaux presented the results of experiments performed with C. Bona in which the repertoire has been altered by the use of anti-Id antibodies. He showed that low doses of anti-A48 antibody given to neonatal mice led to expression of A48 on 50% of the levan plaque forming cells (PFC) following immunization with levan, in contrast to untreated mice that do not express A48 following immunization. In contrast, high

doses of anti-A48 suppressed not only the expression of A48, but the entire response to levan. These results, priming with low doses of anti-Id and suppression with high doses have been observed in other systems. The cellular mechanisms by which they occur are now beginning to be investigated.

The final presentation by P. Brodeur concerned the studies of V region gene order. Brodeur reported that the S117 gene is 5' to the Dex gene, in agreement with the order, with respect to the C region, reported by studies of recombinant mice. These studies are at an early stage, but progress will no doubt be rapid and we look forward to future reports.

Several general conclusions can be drawn from the presented data and discussion. All of the studies indicate in one way or another that responses to polysaccharides are highly regulated. One cannot equate idiotype with antigen binding specificity and lack of expression of idiotype cannot be equated with lack of a particular V gene. Many questions were raised for which we have no answers. Several examples of Id^+ non-antigen binding molecules were presented. Are these simply very low affinity antibodies, or do they bind other antigens? Do environmental antigens, such as levan, prime for IdX dominant responses to other antigens, such as aromatic haptens? What is the function of such molecules in the idiotype network? What are the mechanisms of clonal and isotype restriction? What regulatory role does the T cell play in responses to "T-independent" antigens? Elucidation of the number and site of action of regulatory genes will constitute an important area in future investigations of the immune response to polysaccharide antigens.

WORKSHOP SUMMARY: CELL SURFACE MOLECULES AND MHC

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Three general questions concerning T cells were addressed at this workshop. They were: 1) What types of molecules, besides antigen, do T cells recognize? 2) What types of receptors are used by T cells? and 3) Are the receptors on different functional T cells the same or different? A brief summary of the approaches to these questions presented at the workshop are given below. For more details on each, consult the authors abstracts published in the Journal of Supramolecular Structures and Cellular Biochemistry, Supplement 5, 1981.

G. Fathman described his experiments showing that a anti-(B6A)F_1 alloreactive T cell clones recognize unique 1-A hybrid MLR stimulating determinants present on (B6A)F_1 but absent on parental A and B6 cells. Using antigen (GAT or (TG)-A-L) reactive cloned T cells from B6AF_1 immune mice he showed that certain clones recognized antigen presented uniquely by F_1 antigen presenting cells. Genetic mapping studies suggested that the hybrid determinants recognized by alloreactive and antigen reactive T cell clones were identical. This is compatible with the notion that Ir gene phenomena and I-A encoded MLR stimulating determinants are defined by the same cell surface determinants.

S.Rich described her studies directed at determining which subregion of the MHC are required (or sufficient) for triggering suppressor T cell activity. She found that suppressor T cells could respond to each of the subregions examined. MLR-Ts cells were primed against full MHC differences and restimulated with cells sharing K,D,D only or I

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region haplotypes with the priming stimulator cells. Each of the region specific stimulators triggered partial, but significant, MLR-TsF activity. Third party stimulation was ineffective.

Two different authors, Kees and Krammer, presented serological evidence demonstrating the presence of idiotypic determinants on cytotoxic T lymphocytes (CTLs). This would indicate that the antigen specific receptor on CTLs is sufficiently restricted to allow the detection of such determinants. Such anti-idiotypic sera should now make it possible to isolate and chemically characterize this receptor.

U. Kees described her elegant experiments which demonstrates that cytotoxic T lymphocytes (CTL) bear idiotypic determinants. CBA or B10 mice were immunized with syngeneic Newcastle disease virus (NDV) immune CTL. Sera from mice given four to six injections were screened in a RIA using CTLs as targets. Three of the antisera recognized idiotypic determinants on NDV-immune CTLs used for immunization. These antisera did not block the lytic activity of the CTLs but pretreatment of the CTLs with the anti-idiotype, plus C', consistently reduced the cytotoxicity of the CTLs. On the other hand, the antisera did not react with influenza-immune CTLs, syngeneic alloreactive CTLs or allogeneic NDV-immune CTLs.

P. Kramm described his experiments which also demonstrate that CTLs bear idiotypic determinants. In this system, AKR mice were immunized with syngeneic CTLs that were specific for TNP and were H-2 ($H\text{-}2^k$) restricted in their cytolysis. The antisera were reactive with TNP specific, H-2 restricted CTLs from AKR mice but not with TNP specific H-2 restricted CTLs from C57BL/6 mice. Furthermore, the antisera did not react with FITC specific, H-2 restricted CTLs from AKR mice. From these experiments they concluded that the antisera reacted with TNP-specific associated determinants, idiotypes, on H-2 restricted CTLs from AKR mice.

The experiments described by both Kees or Krammer indicate that the antisera they raised recognized either; 1) a receptor for antigen (NDV in Kees experiments as TNP in Krammer's experiments) if the two receptor model is correct; or a receptor for antigen plus self. If the one receptor model is correct. Both experiments excluded reactivity of the antisera with anti-self receptors.

J. Levy described experiments indicating that the antigen specific receptors on suppressor and cytotoxic T cells are distinct from each other. She raised syngeneic (DBA/2) and allogeneic (C57BL/6) antisera specific for tumor specific (P815) suppressor factors. These antisera were

capable, in the presence of complement, of eliminating suppressor cells from suppressive spleen cell populations. However, the antisera did not eliminate *in vitro* generated tumor specific killer cells. These results are consistent with the hypothesis that different functional sets of T cells use different kinds of receptors.

THE IMMUNOLOGICAL ORCHESTRA, CIRCA 1981

Douglas R. Green
Richard K. Gershon

In previous publications stemming from the ICN-UCLA symposia dealing with the subject of Immunology, various renditions of the Immunological Orchestra have been presented to illustrate the changes in our understanding of the complex pathways of immunoregulation that have occurred between meetings (see The Immune System: Genes, Receptors and Signals, edited by Eli E. Sercarz, A. R. Williamson and C. Fred Fox) Academic Press, New York, 1974, p xxii; and Immune System: Genetics and Regulation, (edited by Eli E. Sercarz, Leonard A. Herzenberg, and C. Fred Fox), Academic Press, New York, 1977, p xvii).

The Immunological Orchestra, circa 1981 includes some new faces in light of the fact that our interest in the Orchestra includes not only the conductor (discussed below) but the music as well. The notion that an antigen presenting cell is involved in Ir gene function suggests a major role for this cell as composer of the score (right). Idiotype-anti-idiotype interactions add to the flavor of such immunoregulatory compositions (left). Indeed, T cell recognition of musical scores by the composer (iR) and the theoretician (iD) was a major theme of the 1981 meeting.

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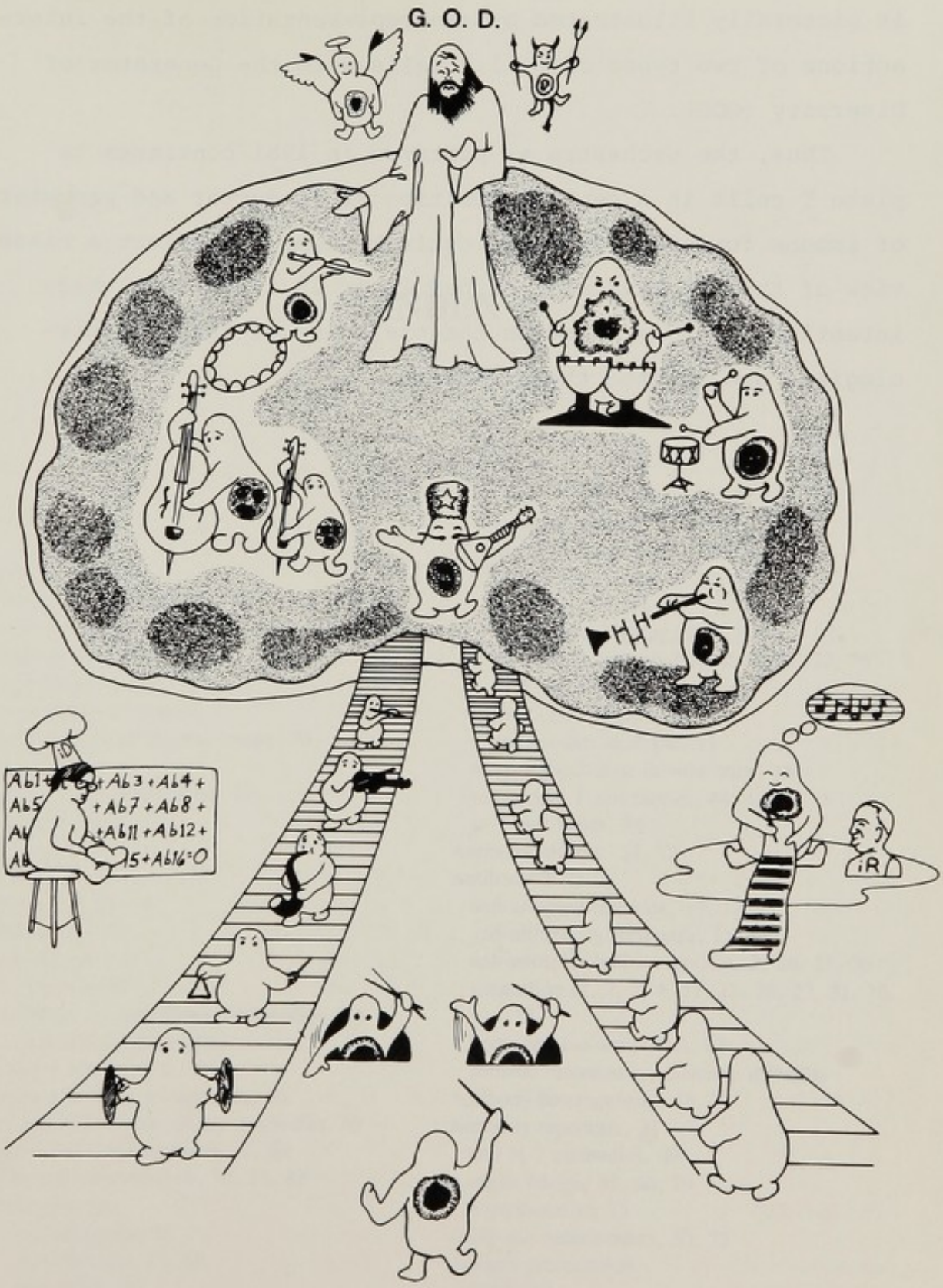
In the 1977 rendition of the orchestra, it was held that the notion of a single conductor was no longer tenable because of the complexity and number of feedback interactions that take place between the three major T cell sets; the Ly-1 T cell set, the Ly-c T cell set and the Ly-1,2 T cell set. Thus, in the 1977 rendition of the orchestra (opposed to those of 1968 and 1974) there were two conductors; and Ly-1 conductor which turned the music on and an Ly-2 conductor which turned the music off. In the cue box signalling the two conductors was the "prompter", and Ly-1,2 cell. Interestingly, as we have acquired further knowledge of the immunoregulatory apparatus the notion of a single conductor has not only become tenable again, but perhaps also more accurate. Thus, in 1981 one perceives and Ly-1 inducer T cell as the conductor. The conductors activity is controlled by two "prompter" cells; and Ly-2 suppressor T cell which tells the conductor to stop and a countermanding Ly-1 contrasuppressor T cell that tells the conductor not to follow the signals given by the T suppressor cell. Thus, the conductors activity is regulated by two cell subsets with opposing activity.

The implication of this version of the orchestra is that when the suppressor cell manages to convince the conductor cell to stop conducting, the members of the orchestra also stop playing. This reflects the notion that the activity of the effector cells in the immunological orchestra is not autonomous; in the absence of a signal from an inducer cell (here represented by the Ly-1 conductor) these effector cells will not perform their immunological chores in an optimal fashion.

Little has changed since 1977 in our understanding of the mechanism by which Ly-1,2 cells control the immunoligcal music. Nevertheless, it remains clear that their participation is extremely important in regulating the activity of the


G. O. D.
iD
iR

immunological prompters, again in two opposing fashions. This is pictorally illustrated by the representation of the interactions of two types of Ly-1,2 cells with the Generator of Diversity (GOD).

Thus, the orchestra as pictured in 1981 continues to place T cells in a central position as conductor and prompter of immune function. If this outlook seems to reflect a biased view of the importance of either iD or iR it is completely intentional and is based on the prejudice of the metaphorologists who created the orchestra.

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