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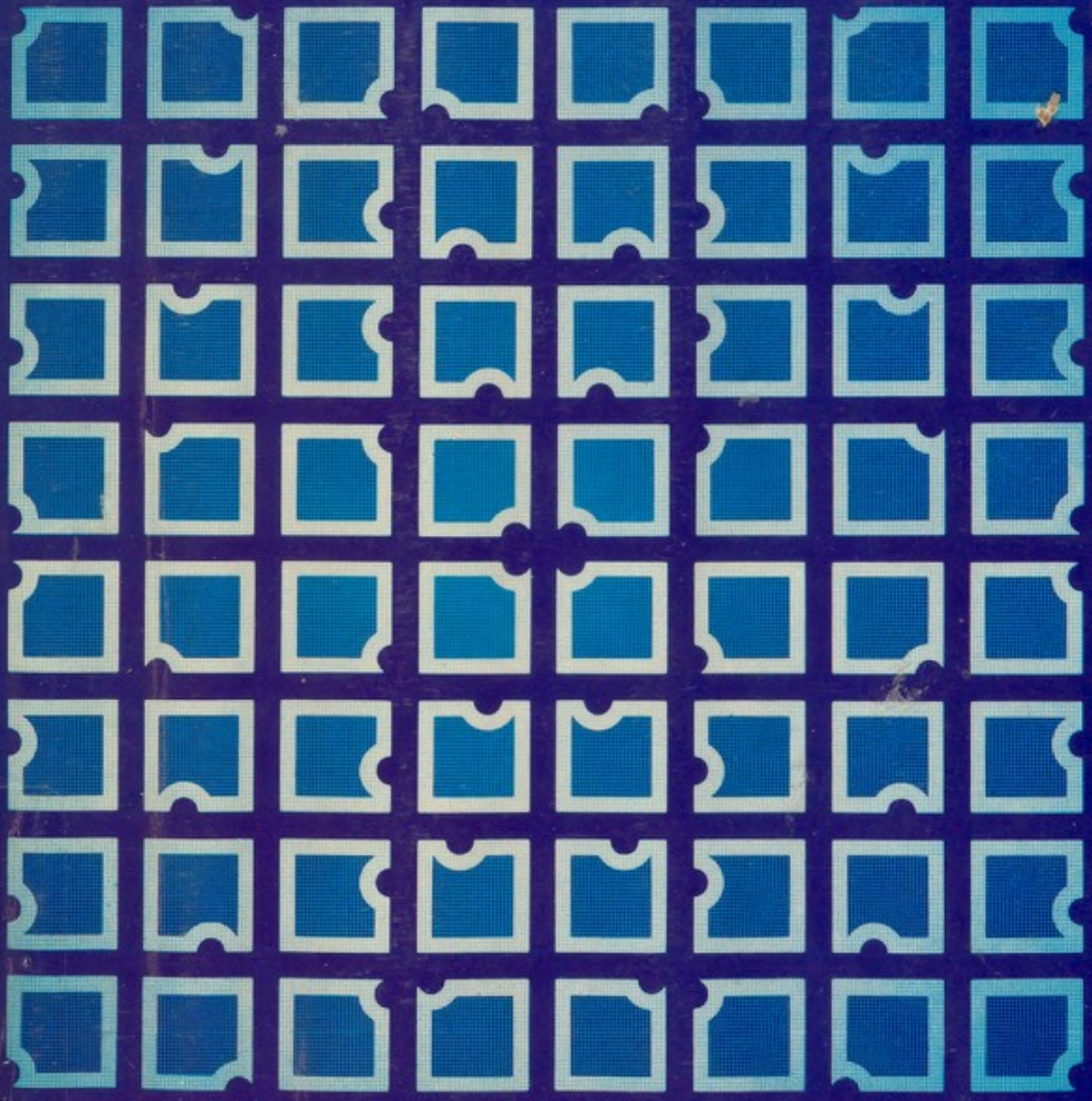
GENETIC CONTROL OF IMMUNE RESPONSIVENESS

EDITED BY
HUGH O. McDEVITT
& MAURICE LANDY



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Preceding volumes in the PERSPECTIVES IN IMMUNOLOGY Series

IMMUNE SURVEILLANCE

Edited by R. T. SMITH and M. LANDY

1970, 550 pp.

Continued progress in the advancement of tumor immunology, the central position of the surveillance concept in immunologic thinking and the lack of any prior comprehensive evaluation of this subject led to the organization of this conference. The thirty-one participants represented a broad spectrum of experience in carcinogenesis, developmental biology and cellular and tumor immunology. Three days of intensive discussions centered on basic issues such as whether vertebrates are indeed equipped with surveillance mechanisms to detect and destroy spontaneously arising neoplastic cells; the nature of the relationship between histocompatibility and tumor antigens; whether surveillance is mediated principally by cellular immune mechanisms; and whether the immune apparatus is amenable to interference with or augmentation of its tumor surveillance efficacy.

This volume records an edited version of these extensive and probing discussions, and presents an authoritative exposition of the immunobiologic areas relevant to the surveillance concept, a weighted assessment of the evidence pro and con for immune surveillance and a delineation of the prospects for dealing with neoplasia by immunologic means.

IMMUNOLOGIC INTERVENTION

Edited by J. W. UHR and M. LANDY

1971, 337 pp.

Several decades of successful research into the mechanisms underlying the immune response and immunologically induced tissue damage have yielded an enormous amount of basic information. The implications of this knowledge for the eventual design of new therapeutic approaches to coping with problems of human disease are immense, but there is still a large gap between such studies and their clinical application. This conference was organized to sift through the ideas, models and immunological reagents that have emanated from these studies and to determine which may already be appropriate to the need and those worthy of further exploration. The thirty-three participants representing both basic and clinical aspects of immunology engaged in three days of extensive discussions that encompassed virtually all elements of the multi-component nature of the immune response and focussed on the unusual array of opportunities it offers for selective manipulation.

As the edited product of this conference, this volume presents a major overview of notable developments in contemporary immunology and identifies a remarkable array of approaches by which the immune response can be manipulated to achieve therapeutic effects. These developments, and the ideas and suggestions that emerge, presage new directions for the clinical application of immunologic knowledge.

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"GENETIC CONTROL OF IMMUNE RESPONSIVENESS"

Relationship to Disease Susceptibility

In recent years, studies in many animal species, and with many antigens, have shown that specific immune responses are under strict genetic control. In retrospect, the older immunological literature contains many examples of such control mechanisms, and it is now established that these genetic controls operate at several discrete steps in the immune response. The class of immune response gene most thoroughly studied is that linked to the major histocompatibility gene complex. Indeed, it was the finding of *this* linkage that really launched the present era. The most striking development to emerge from these studies is that the major histocompatibility-linked immune response genes seem to control antigen recognition by thymus-derived, antigen-reactive cells, and may represent the structural genes for the T-cell receptor.

The numerous and far-reaching implications of these developments for all of biology and medicine prompted the National Institute of Allergy and Infectious Diseases to organize the Fifth Brook Lodge Conference around the issues in "Genetic Control of Immune Responsiveness," emphasizing particularly the relationship to disease susceptibility. On May 8-10, 1972, thirty-six leading investigators in immunology, genetics, cell biology, infectious diseases, virology, and oncology met at Brook Lodge for three days of intensive discussion to organize and evaluate these concurrent developments and to project the needs and the opportunities in this emerging area joining immunology and genetics. This conference was the first occasion on which many new and exciting developments in our understanding of the histocompatibility genes and their linked genetic control of specific immune responses, expressed in T cells, have been compared and contrasted with the even more recent discovery of immunoglobulin allotype-linked specific immune response genes which are expressed as receptors on B cells. The two types of genes thus play a symmetrical role in determining the specificity of antigen recognition by each cell type. Still other categories of immune response genes are those linked neither to histocompatibility nor to allotype genes; *these* immune response genes may be expressed in other cell types and could be involved in immune cell collaboration.

[Continued on back flap]

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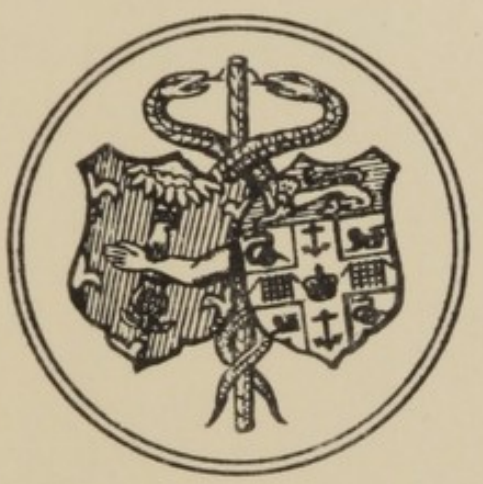

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Relationship to Disease Susceptibility
1972

Genetic Control of Immune Responsiveness

Relationship to Disease Susceptibility

Edited by
Hugh O. McDevitt Stanford University School of Medicine

and

Maurice Landy National Institute of Allergy and Infectious Diseases

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This volume is dedicated to Werner Braun whose untimely death, at 58, is a great loss to the scientific community generally and to immunology in particular.

His career spanned more than three decades and was almost equally divided between microbial genetics and immunology. In both disciplines his scientific accomplishments were remarkable in number, timeliness, originality, and in their impact.

As experimentalist, editor, administrator, and counsellor to professional organizations, and most of all, as a catalyst for his colleagues, he exerted a profound influence on the course of biologic science.

His views were influential in the discussions and reflections that eventually led to the organization of the Brook Lodge Immunology Conferences; he was co-editor of the first volume of this series, and an important participant and contributor to several of these volumes, including the present one.

Apart from his own researches he brought to contemporary immunology wisdom, experience, insight, and a generosity of spirit and helpfulness which will have a lasting effect on the accomplishments of many of his colleagues.

He will be sorely missed by the entire immunology community.

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FOREWORD

With the completion of the planned cycle of five Brook Lodge Immunology Conferences, it seems appropriate to reflect on the original motivation that led in 1966 to the organization and planning of these immunology colloquia. They grew out of the belief that as a dynamic, productive, and exciting biological discipline, immunology would benefit from a series of specialized conferences in which the data and information being generated at an accelerating pace were assessed and integrated in a manner that would help guide and determine further development. This seemed, moreover, to be an altogether proper role for an NIH Institute that had been historically associated with the major research support for this field.

Six years and five Brook Lodge Conferences later, it is clear that the communication problem has indeed become difficult and complex. In immunology, new findings and new research areas are appearing at a rate and volume well beyond our ability to assimilate and evaluate them in ways that make for rational progress. Despite a general awareness of this tidal wave, and the threat it constitutes to the development of immunologic knowledge, most efforts in communication continue to involve evermore massive reporting of data and findings. Few indeed are the meetings or conferences directed towards sorting, analyzing, and seeking to ascertain which elements of this mountain of information are relevant and significant.

Judgments as to the merit and value of these five conferences and their published volumes are best left to the scientific community they are intended to serve. However, it seems clear even now that their distinctive format has given scope to the remarkable generalists and specialists in this discipline, and enabled them to employ their talents in an imaginative and constructive fashion. As this fifth volume attests, the character and scope of the concepts that can be formulated in 1972 are both altered and much more precise and defined than the issues and questions as we saw them a scant five years ago.

Many individuals contributed importantly to the effectiveness and the accomplishments of this conference series, and it is impossible to acknowledge each. Particularly noteworthy have been the generous funding provided by the Extramural Programs, NIAID; the progressively more efficient retrieval of the animated discussions by stenotypist, Eugene Wallach; the warm cooperation of the Upjohn Company in offering the facilities and ambiance of Brook Lodge for

these conferences; and the commitment in every way of the Academic Press organization to the rapid and efficient transition from typescripts to published volumes.

I owe a great debt to the five colleagues with whom I planned and organized these conferences who were so diligent and imaginative in the laborious editorial process of converting a mass of transcript into readable and interpretive volumes. This form of close collaboration proved to have rewards for me far beyond the completion of the immediate task. Most of all, however, I want to acknowledge the unstinting cooperation of the many colleagues in immunology and allied fields who gave so generously of their knowledge, and so freely offered their findings and views to the test of peer group judgment.

Maurice Landy
Chief, Allergy and Immunology Branch
Extramural Programs, National Institute of
Allergy and Infectious Diseases

PREFACE

A few years ago, proposals to study the inheritance of specific immune responses in normal animals would have met with scepticism, if not derision. Fortunately, a number of chance observations led to a deliberate effort on the part of several investigators to study the genetic control of specific immune responses, and it is now clear that there are a large number of specific immune response genes. Information in this field is accumulating rapidly and has already shown us that there are several different types of immune response genes. These Ir genes seem to fall into two major classes. The histocompatibility-linked specific immune response genes were the first to be placed in a definite linkage group, and appear to code for the T cell antigen receptor. The close association between H-linked Ir genes and the genes coding for the major histocompatibility antigens suggests an important structural and functional interrelationship between histocompatibility antigens and specific antigen receptors on immunocompetent T cells. The second major class of Ir genes to be placed in a definite linkage group are the immunoglobulin allotype-linked immune response genes, which appear to be expressed in bone marrow-derived immunocompetent lymphocytes where they determine the structure of the B cell antigen receptor. There is thus a striking symmetry: two separate cell lines, each possessing antigen-specific clonality, each possessing its distinct dictionary of antigenic specificities, and each with its distinctive mechanism of antigen recognition. T cell antigen recognition appears to be under the control of the histocompatibility-linked immune response genes, which may code for the structure of the T cell antigen receptor. B cells, on the other hand, recognize antigen through the expression on their surface of immunoglobulin receptors, which are coded for by immunoglobulin allotype-linked genes.

The function of immunoglobulins as antigen receptors on B cells is acceptable to most immunologists, and is supported by an impressive body of experimental evidence. However, the hypothesis that the T cell antigen receptor is *not* one of the known immunoglobulin classes is heretical in terms of present immunologic dogma. The evidence for and against the hypothesis that the H-linked Ir genes control the structure of the T cell antigen receptor, and speculations concerning the nature of such a receptor, comprise a major part of this volume. The question is not yet settled and it is for the reader to decide how well the case is put. It should be noted in passing that the existence of a dual

system of interacting cell types, each with a separate mechanism for antigen recognition, permits a much more precise, multi-level regulation of antibody synthesis, which may be an important safeguard against the too frequent development of autoimmunity.

It is likely that the histocompatibility-linked and immunoglobulin allotype-linked specific immune response genes have great survival value, and reflect the end result of a long process of selection during the course of evolution. This speculation has already found support in the numerous instances of association between histocompatibility type and specific disease entities, in both animals and man. The causal mechanisms underlying these relationships are not yet clear and will undoubtedly require the development of new techniques for determining immune response genotypes in animals and man, and methods for measuring cell-mediated immunity to pathogenic agents such as leukemogenic viruses, before these mechanisms can be worked out. This field of investigation is presently in its infancy, but there is every reason to believe that these techniques can be developed and that correlations between immune response genes and a variety of disease states will be uncovered, and ultimately will have great value in understanding pathogenesis, and in predicting disease susceptibility.

Although progress has been rapid, particularly in the past few years, the increasing frequency of reports of new immune response genes, and new associations between histocompatibility type and specific disease entities, suggests there is much more to be done. In this volume we have sought to bring together the detailed experimental evidence on the mechanism of action of specific immune response genes, and to show how the two major classes of immune response genes affect our understanding of basic immunology and antibody formation on the one hand, and of host factors determining disease susceptibility on the other. It is our hope that in this volume the presentations and the uninhibited discussions that ensued will succeed in clarifying the significance of the findings already made, and help point the way to future studies.

Hugh O. McDevitt
Maurice Landy

Genetic Control of Immune Responsiveness

Relationship to Disease Susceptibility

SESSION I

THE CELLULAR LOCUS OF ACTION OF HISTOCOMPATIBILITY-LINKED Ir GENES

Ir gene control of pure T cell functions – Analogous control of carrier recognition – Blocking of Ir gene function by H alloantisera – Ir gene regulation of IgM to IgG switch – Expression of Ir genes in T *and* B cells – Specificity of antibody produced in responders and nonresponders – Production of nonresponder type antibody in tetraparental mice.

Glossary of Terms and Definitions for Session I

Because of the complexity of a number of the antigens referred to in this session, and because several "shorthand" terms have crept into usage in the discussions, the editors considered it desirable to include a glossary defining these antigens and terms for the benefit of the reader.

Several shorthand designations and terms are used liberally throughout the discussions and need some general definition. These are:

1. T cell. This is taken to be a thymus-derived, antigen-reactive, immunocompetent lymphocyte.
2. B cell. This designation is used for bone marrow-derived, immunocompetent lymphocytes bearing surface immunoglobulin, functioning as the precursors of antibody-producing lymphocytes and plasma cells.
3. V-gene. This is the designation used for the structural gene coding for the variable region of a particular immunoglobulin, or for immunoglobulins of a particular class.
4. C-gene. This is the designation used for the structural gene coding for the constant region of immunoglobulin heavy and light chains.

The following antigens are referred to several times in this session and are given abbreviated designations therein. Rather than redefine each of them throughout the text, we have chosen to define them in this glossary.

PLL	A linear polymer composed entirely of poly-L-lysine.
DNP-PLL	Poly-L-lysine substituted with dinitrophenyl groups on the epsilon amino groups of lysine.
GA	A linear polymer of glutamic acid and alanine.
GL	A linear polymer of glutamic acid and lysine.
DNP-GL	A linear copolymer of glutamic acid and lysine substituted with dinitrophenyl groups on the epsilon amino groups of lysine.
GT	A linear copolymer of glutamic acid and tyrosine.
GAT_{10}	A linear copolymer of glutamic acid, alanine and tyrosine in which tyrosine makes up 10% of the amino acid residues.
BSA	Bovine serum albumin.
DNP-BSA	Bovine serum albumin substituted with dinitrophenyl groups, the number of dinitrophenyl groups per mole-

	cule of BSA being indicated by a subscript following the DNP.
GPA	Guinea pig albumin.
DNP-GPA	Guinea pig albumin substituted with dinitrophenyl groups.
HSA	Human serum albumin.
(T,G)-A--L	A branched, multichain copolymer of the general structure shown in Fig. 20 of Session II. The copolymer has the structural formula: poly-L- (tyrosine, glutamic acid)-poly-D,L-alanine--,poly-L-lysine. The structural formula is written this way to indicate that the amino acids in parentheses are in random sequence, the dash indicated a long stretch of poly-D,L-alanine, and the double dash indicated a branch point where the alanine joins the epsilon amino groups of lysine which forms the backbone of the molecule. Thus, the synthetic polypeptide is built on a chain of poly-L-lysine with each lysine being substituted in its epsilon amino group by a long side chain of alanine, attached to the amino terminus of which is a short random sequence of tyrosine and glutamic acid.
(H,G)-A--L	Poly-L(histidine, glutamic acid)-poly-D, L-alanine-poly-L-lysine. Structural analog of (T,G)-A--L in which the tyrosine is replaced with histidine.
(Phe,G)-A--L	Poly-L(phenylalanine, glutamic acid)-poly-D, L-alanine--poly-L-lysine. A structural analog of (T,G)-A--L in which the tyrosine is replaced by phenylalanine.
(T,G)-Pro--L	Poly-L(tyrosine, glutamic acid)-poly-L-proline--poly-L-lysine. This synthetic polypeptide has the same general structure as (T,G)-A--L and its congeners, but in this case, the side chains of poly-D, L-alanine are replaced with poly-L-proline.
(Phe,G)-Pro--L	Poly-L(phenylalanine, glutamic acid)-poly-L-proline--poly-L-lysine. A structural analog of (T,G)-Pro--L in which the tyrosine is replaced with phenylalanine.

CHAIRMAN SIMONSEN: The subject of this conference could as well be described as T cell immunology at the crossroads. Probably it is the feeling of all of us that we have come to a very exciting and critical stage in the development of immunology, where that half of the immune apparatus which we call T cells is up for review with respect to the very way they go about recognizing foreign antigens. In other words, what is the nature of that most elusive entity, the Tcell receptor?

It is only fair to say at the outset that none of us know the answer just yet; accordingly, the actual state of the game calls for an almost painful open-mindedness towards the various possibilities. It is against this background that I call on Benacerraf to develop for us just how study of the genetic control of immune responses in guinea pigs and mice could have done so much to lead T cell immunology to the present state of affairs.

BENACERRAF: The series of Brook Lodge immunology conferences have abundantly manifested the virtue of being provocative and stating the issues clearly, so that the thesis presented may be accepted or discarded on the basis of the evidence available. Such a distinctive approach is especially critical in order to deal effectively with our present complex subject.

I propose first to define the different classes of specific immune response genes that have been identified and to describe how they have been discovered. I shall also discuss the properties of these Ir genes and the cells of the immune system where they are expressed. I will then give the evidence from our and other laboratories on which our conclusions are based.

Historically, the discovery of specific immune response genes (Ir genes) has required the immunization of random bred animals or of inbred strains with antigens of restricted structural heterogeneity, which present the immune system with a restricted challenge (Table 1) such as: 1. synthetic polypeptides, 2. isoantigens which differ little from autologous components and, 3. limiting immunizing doses of native antigens, as originally shown by Vaz and Levine, and by Green and ourselves. Such challenges permit the observation of clear individual differences in response. Contrary to our expectation, based on the recognized extensive heterogeneity of immunoglobulin specificities, we were surprised that the immune responses to many of the antigens of the types referred to were found to be under the control of individual dominant autosomal genes. Such unigenic control was particularly evident for the type of immune responses which are at-

TABLE 1

Antigens Used to Identify Histocompatibility-Linked Ir Genes

I.	Synthetic polypeptides with limited number of L-amino acids.
II.	Limiting immunizing doses of complex protein antigens.
III.	Weak native isologous antigens.

tributable to thymus-derived cells, namely, delayed hypersensitivity, carrier recognition in antihapten antibody responses, and also the cellular immune reactions elicited *in vitro* such as macrophage inhibitory factor (MIF) production and specific blast formation. With some systems studied by McDevitt, Sela, and Grumet, in the mouse, the differences in antibody response controlled by specific Ir genes are quantitative, and reflect primarily the absence of IgG and secondary responses in animals lacking the specific Ir genes, a behavior which characterizes thymus-independent antigens.

These various findings suggest, therefore, that the first class of specific Ir genes which were discovered is necessarily expressed in thymus-derived cells. We must emphasize in addition that these Ir genes are both distinct and specific, since they control the specific recognition of different antigens.

A major breakthrough in this system has been the recognition by McDevitt and Tyan that this particular class of genes exemplified by the *Ir-1* locus was intimately linked to the major histocompatibility region in the mouse. This was confirmed for a variety of mouse immune response genes, and for specific immune response genes with identical properties in guinea pigs, and more recently in rats.

We feel that the linkage to the major histocompatibility region is not fortuitous, but meaningful, and that this property classifies this type of Ir genes as a unique class. We feel also that it is related to the cell type where the genes are expressed and to the function which they subserve.

One of the most important properties of histocompatibility linked Ir genes (H-linked), recognized early, was that the antibody responses under unigenic control were highly heterogeneous with respect to class, specificity, and affinity of the antibodies produced. It was also realized that since the H-linked Ir genes have been localized within the H-2 region, they cannot be linked to the known immunoglobulin structural genes of either the heavy or the light chains or to immunoglobulin allotypes, since both in man and mouse respectively the H-2 and HL-A regions are on different linkage groups from the immunoglobulin allotypes. We recognize that in these species the immunoglobulin allotypes are C region markers, but there is every reason to expect, from the rabbit allotype data that the immunoglobulin V and C genes are coded on the same chromosome. The conclusion can be safely made, therefore, that the H-linked Ir genes are distinct from the known immunoglobulin V genes. Nevertheless, the possibility that a new set of reduplicated V region genes exist with the H-complex, cannot be excluded.

I would like next to bring to your attention that the discovery of H-linked Ir genes required little effort, once antigens with limited heterogeneity or limiting doses of native antigens were used. The differences related to the possession or lack of the gene are absolute and qualitative, particularly with respect to cellular immunity. These same antigens did not permit the identification of allotype-linked Ir genes.

It was soon recognized, however, that allotype linked specific Ir genes exist and should control immunoglobulin V regions. However, the type of antigen which must be used to identify these genes and the methodology is different from the one used to detect H-linked Ir genes. The identification of allotype linked Ir genes requires the use of antigens which induce the production of antibody responses with clonal properties, and unique homogeneity, such as streptococci and pneumococcal polysaccharides. Furthermore, the genetically controlled differences observed with these antigens are not usually all or none in terms of the response, but are concerned rather with the quality or specificity of the antibody produced. These differences therefore require rather sophisticated analysis which permit the distinction of shades of specificity or antibody populations with defined idiotypic specificities. The allotype-linked specific Ir genes will determine, therefore, whether antibodies with a particular specificity or idiotype may be detected in the population of antibody produced. These genes must, therefore, directly control the structure of the variable region of the immunoglobulins.

There are, therefore, two distinct classes of specific Ir genes, the H-linked Ir genes necessarily expressed in T cells, and the allotype-linked Ir genes concerned with immunoglobulin structure, necessarily expressed in B cells. The H-linked Ir genes appear to be concerned with the recognition of a much more restricted class of antigens or range of specificities than the V region immunoglobulin structural genes, which explains why the methodology required for the recognition of the two types of Ir genes is so different.

Thus, the discovery of the H-linked Ir genes, and the speculation concerning the specific process which they control in T cells raises some doubts against the established dogma: that there exists only one type of molecule responsible for immunological specificity, namely the immunoglobulins, through the V regions of their H and L chains.

This dogma has led some immunologists to the conclusion that since there are two classes of lymphocytes, T and B, concerned with the two types of immune responses, cellular and humoral respectively, and since both T and B cells are specific and bind antigens, the T cell receptor must necessarily be an immunoglobulin, as it was established for the B cell receptor. This is a broad issue which is therefore germane to our discussions and which will be dealt with at length in Session IV. However, concerning this session's subject, if we reject the dogma (which we are inclined to do on the basis of our experience with the H-linked Ir genes), we propose that the main receptors for specificity on T cells are not immunoglobulins, but are the product of the H-linked Ir genes. If indeed the main T cell receptors were immunoglobulins, there should not be any difference in the specificities of cellular immune reactions and of antibodies, because basically the same dictionary of specificities should be expressed in both T and

B cells. There is, however, considerable evidence from many laboratories that operational differences exist between the specificity of cellular immune reactions and the specificity of humoral antibodies. I will not digress further on these points because I know that these are some of the major issues to be debated when all the data have been presented. Nevertheless, I want from the start to state clearly our present position, perhaps intentionally in radical terms, to clarify the issues before we present some of the data which have led us to our conclusions.

In guinea pigs, inbred strains 2 and 13, as well as random bred lines have been used to study the genetic control of specific immune responsiveness. As shown in Table 2, the abilities of these inbred guinea pigs to form immune responses to the synthetic polypeptide antigens, 2,4-dinitrophenyl-poly-L-lysine (DNP-PLL), poly-glutamyl, alanine (GA) and poly-glutamyl, tyrosine (GT) copolymers and to limiting doses of native antigens and their hapten conjugates, bovine serum albumin (BSA), DNP-BSA, DNP-GPA, are inherited according to strict Mendelian genetics, indicating that the immune responses to these antigens are controlled by distinct dominant Ir genes. Thus, strain 2 but not strain 13 animals respond to DPN-PLL, GL, GA, and to low doses of BSA, HSA, and DPN-BSA, whereas strain 13 and not strain 2 respond to GT and to limiting doses of DNP-GPA. All (2 × 13)F_1 guinea pigs are responders of each of these antigens, illustrating the dominant character of these responses. Responsiveness to DNP-

TABLE 2

Inheritance of Specific Ir Genes and of the Major Histocompatibility Locus of Strain 2 and Strain 13 Guinea Pigs by (2 × 13)F_1 and Backcross Animals

Antigens	Strain		(2 × 13)F_1	(2 × 13)F_1 × 13		(2 × 13)F_1 × 2	
	2	13		50%[a]	50%	50%	50%
DNP-PLL-GL	+[b]	−[b]	+	+	−		
GA	+	−	+	+	−		
GT	−	+	+			+	−
BSA 0.1 μg	+	−	+	+	−		
HSA 1 μg	+	−	+				
DNP-BSA 1 μg	+	−	+	+	−		
DNP-GPA 1 μg	−	+	+			+	−
Major H Locus							
Strain 2	+[b]		+	+	−		
Strain 13		+	+			+	−

[a] Column identifies the same group of backcross animals.

[b] Plus indicates responsiveness and presence of major histocompatibility specificities; minus indicates nonresponsiveness and absence of major histocompatibility specificities of the inbred strains.

TABLE 3

Responses of Random Bred Hartley Guinea Pigs to Immunization with Several Poly-L-Lysine Conjugates of Noncrossreacting Haptens

Guinea pig no.	Immune responses at 21 Days			
	5-Dimethylamino 1-napthalene PLL	p-Toluene-sulfonyl-PLL	2,4-Dinitro-phenyl-PLL	Benzyl-penicilloyl-PLL
1-11	positive[a]	positive	positive	positive
12-33	negative	negative	negative	negative

[a] A positive response is characterized by delayed sensitivity to 10 μg hapten-PLL conjugates and by the production of antihapten antibodies. *Nature,* **200,** 544, 1963.

PLL, GA, and to low doses of BSA and DNP-BSA segregate together in 50% of $(2 \times 13)F_1 \times 2$ back-cross offspring.

Let us consider now the cells where the class of Ir genes under discussion are expressed. In the two systems most extensively studied, the PLL gene in guinea pigs and genes at the *Ir-1* locus in mice, responsiveness can be passively transferred to irradiated nonresponder recipient strains with immunocompetent cells from animals possessing the Ir genes, demonstrating that the genes are indeed expressed in cells which participate in the immune response. The adoptive transfer of responder $(2 \times 13)F_1$ spleen and lymph node cells into irradiated strain 13 guinea pigs (protected by strain 13 bone-marrow) transfers the ability to mount both cellular (delayed hypersensitivity) and humoral immune responses to DNP-PLL. It should be noted that in these chimeras, the responding cells have been found to be of donor origin. Thus, the Ir genes are expressed in immunocompetent cells.

The next question is: In what immunocompetent cells are the H-linked genes necessarily expressed? In guinea pigs, those functions which are attributed essentially to the activity of "thymus-derived" cells, such as cellular immunity and carrier function, depend exclusively upon the presence of the relevant Ir gene. Thus, the reactions of cellular immunity to PLL, DNP-PLL, GA, and GT are totally under the control of the corresponding specific immune response genes. They are never observed in animals lacking the genes.

In addition, as we observed with Levine, responsiveness to antigens under control of specific immune response genes is accompanied by antibody production to the haptens they bear. As illustrated in Table 3, guinea pigs possessing the PLL gene produce anti-DNP antibodies in the course of this response. These animals also respond to immunization with PLL conjugates of other noncross-reacting haptens, with vigorous antihapten antibody synthesis. Guinea pigs lacking the PLL gene and incapable of responding to DNP-PLL do not respond to benzyl-penicilloyl-PLL or to other unrelated hapten PLL conjugates. This experiment indicates that the PLL gene is concerned with the specific recognition of the car-

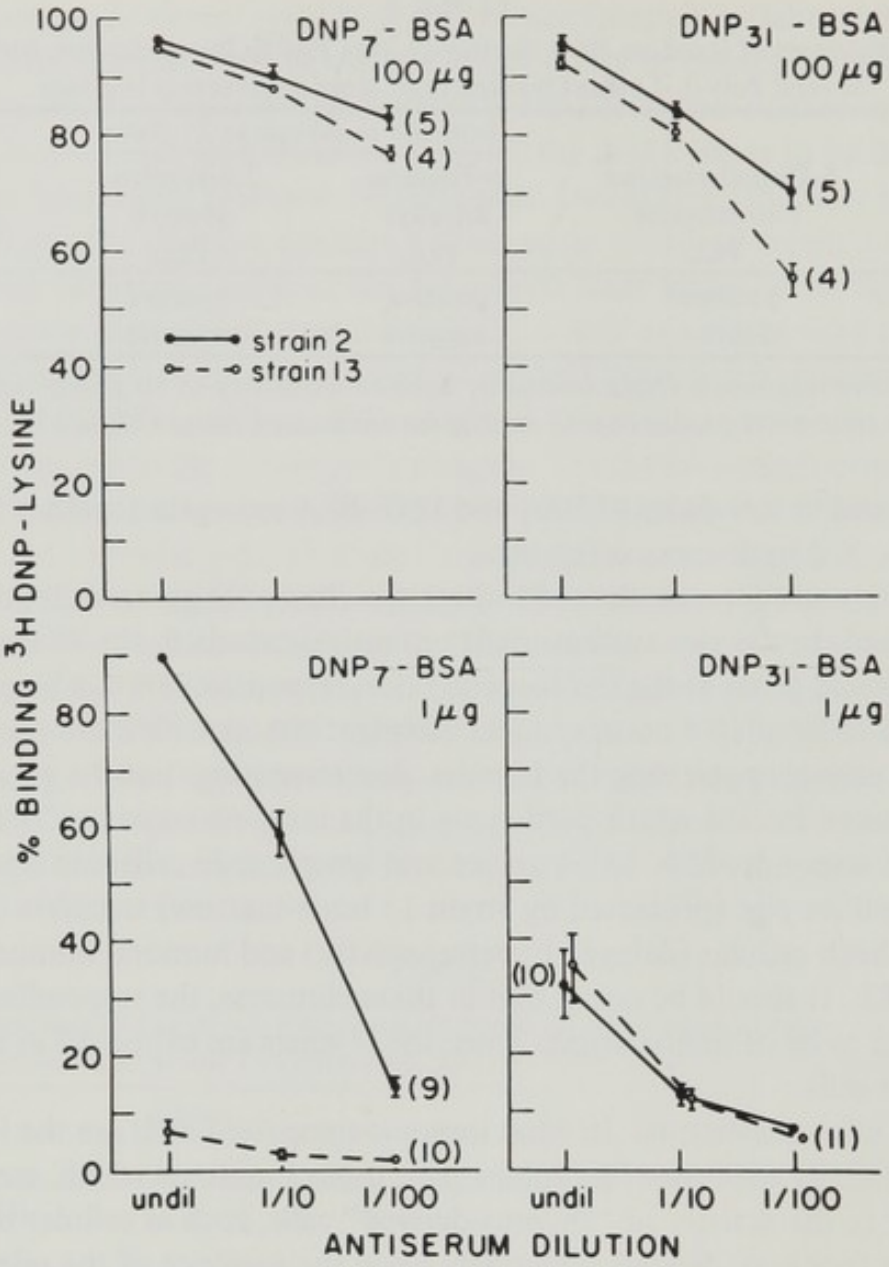

Fig. 1. Anti-DNP antibodies in sera of strain 2 and strain 13 guinea pigs, 3 to 4 wk following immunization with 100 or 1 μg of DNP_7-BSA or DNP_{31}-BSA in CFA. Data are expressed as percent binding of 0.1 ml 10^{-5} M ^{3}H-DNP-lysine by globulin fraction of 0.1 ml of antiserum. Number of animals/group is shown in parentheses. *J. Immunol.*, **107**, 378, 1971.

rier molecules which is known to be the function of thymus-derived cells. When sensitized T cells respond to the PLL carrier, the production of antibodies to the attached haptens, by specific B cells, is observed.

A similar situation has been demonstrated, in collaboration with Green, in the genetic controls of the anti-DNP antibody responses to limiting doses of DNP_7-BSA or DNP_6-GPA (Figs. 1 and 2). As mentioned earlier (Table 2), strain 2 but not strain 13 synthesize anti-DNP antibodies when immunized with 1 μg DNP_7-BSA, whereas strain 13 but not strain 2 produce anti-DNP antibodies in

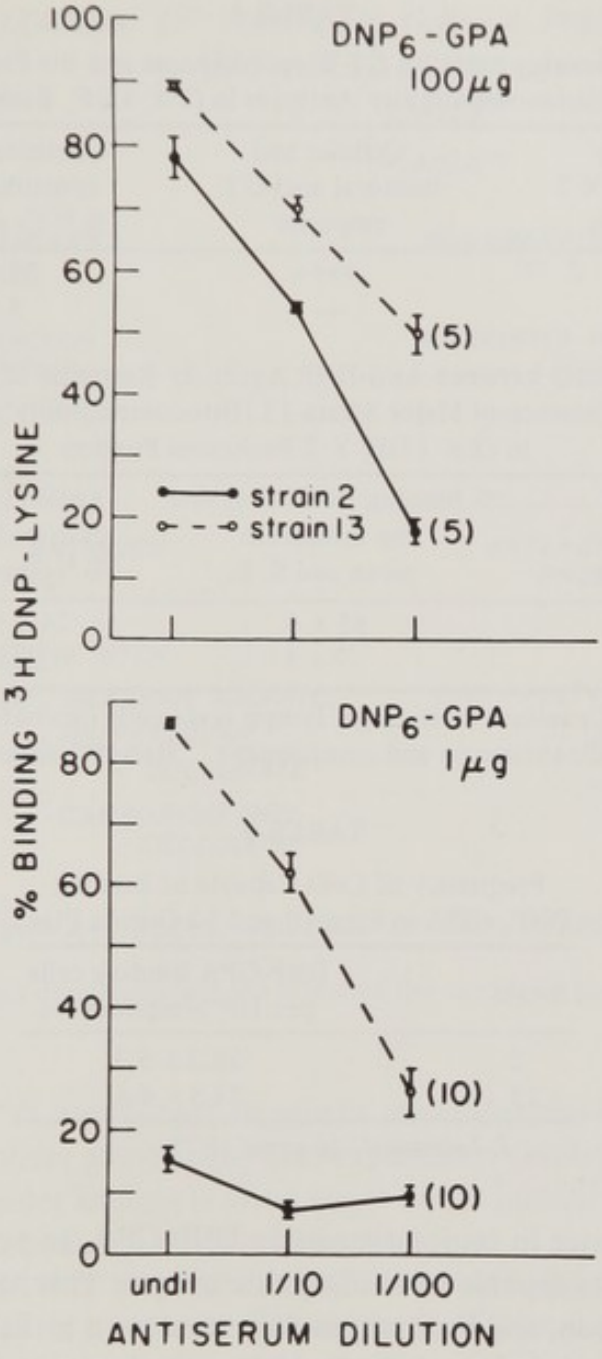

Fig. 2. Anti-DNP antibodies in sera of strain 2 and strain 13 guinea pigs, 3 to 4 wk after immunization with 100 or 1 μg of DNP_6-GPA in CFA. *J. Immunol.*, **107**, 328, 1971.

response to 1 μg DNP_6-GPA. Furthermore, these anti-DNP responses are controlled by specific Ir genes linked to strain 2 H-specificity in the case of DNP_7-BSA and to strain 13 H-specificity in the case of DNP_6-GPA (Table 4). The capacity to form anti-DNP antibody exists equally in both inbred strains, but it is determined by the genetically controlled recognition of the specificity of the carrier, again a function of thymus-derived cells. According to these results and our interpretation, both strain 2 and strain 13 guinea pigs would be expected, in

TABLE 4

A. The Relationship between GT Responsiveness and the Presence of Major Strain 13 Histocompatibility Antigens in (2 × 13)F_1 Backcross Progeny

Number (2 × 13)F_1 × 2 backcross	Cellular and humoral anti-GT response	Antistrain 13 cytotoxicity[a] % ^{51}Cr release
9	++++	32.8
8	—	3.5

B. The Relationship between Anti-DNP Antibody Response to 1 μg DNP_6-GPA and the Presence of Major Strain 13 Histocompatibility Antigens in (2 × 13)F_1 × 2 Backcross Progeny

Number (2 × 13)F_1 × 2 backcross	% Binding ^{3}H-DNP-lysine by antiserum mean and S. E.	Antistrain 13 cytotoxicity[a] % ^{51}Cr release
7	85 ± 4	24.4 ± 2
7	7 ± 3	1.2 ± .6

[a] Specific ^{51}Cr release from target lymph node cells incubated with strain 2 antistrain 13 alloantiserum and complement. "Harvey Lectures," in press 1972.

TABLE 5

Frequency of Cells Capable of Binding DNP_6-GPA in Strain 2 and 13 Guinea Pigs

Strain	DNP-GPA Binding cells per 10^5 lymphocytes
2	26.2 ± 5.1
13	24.5 ± 4.6

J. Immunol., in press 1972.

spite of their difference in responsiveness to DNP-GPA, to possess a similar number of B lymphocytes capable of binding this antigen. This has been verified recently by Davie, Green, and Paul whose data are shown in Table 5. Furthermore, the data presented and the conclusion which we have reached of the necessary expression of H-linked Ir genes in T cells concerned with carrier specificity, would lead one to predict that if a nonimmunogenic molecule such as DNP-PLL is administered to a nonresponder guinea pig complexed with an immunogenic carrier which is able to stimulate thymus-derived cells specific for this carrier, an antibody response should be induced against DNP-PLL. This is precisely what happens, as we observed many years ago with Green and Paul. When strain 13 or Hartley guinea pigs lacking the PLL gene are immunized with DNP-PLL complexed with an immunogenic albumin, as shown in Fig. 3, high amounts of anti-DNP antibodies are produced in the absence of cellular immunity. Thus,

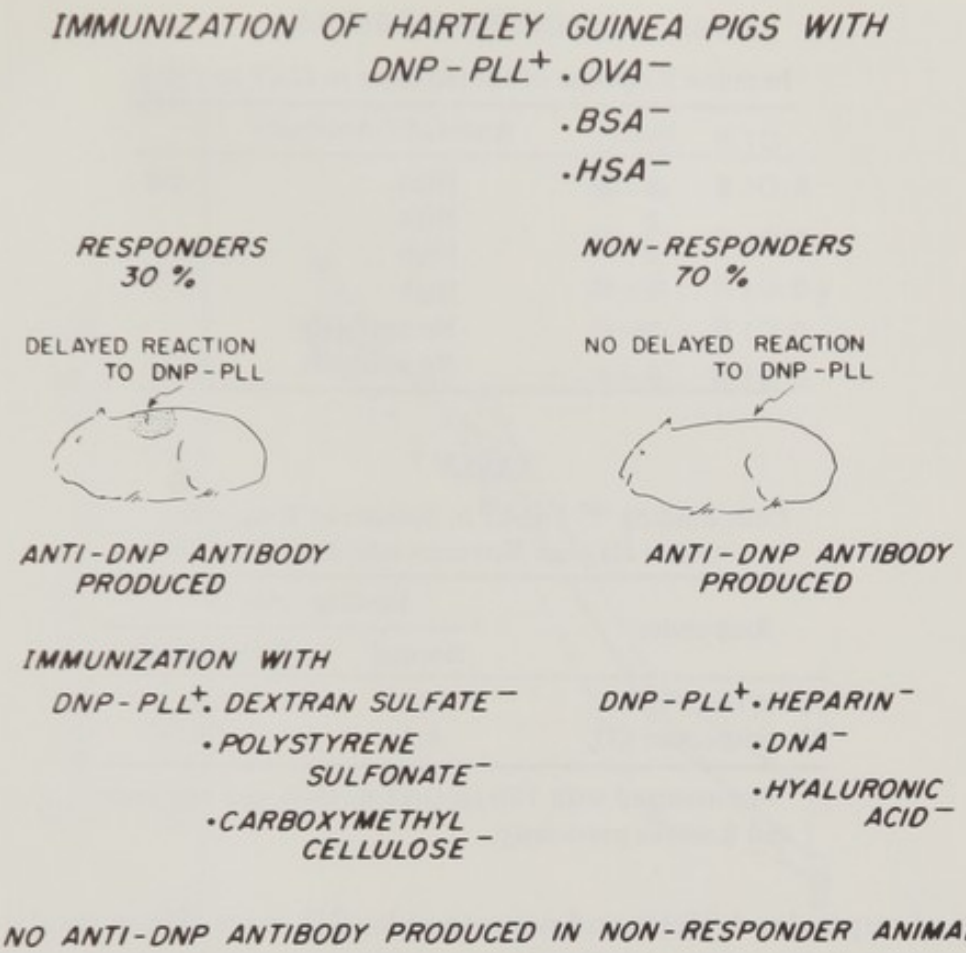

Fig. 3. Procedure for demonstrating the effect of the carrier on the immune response to DNP-PLL.

DNP-PLL which is an immunogen in genetic responder animals, may behave as a hapten in nonresponder guinea pigs. This experiment confirms that the genetic defect in nonresponder animals is not a result of the inability to synthesize antibodies to some determinants on the molecule.

Similar results were also obtained in the mouse using a system developed with Martin and Maurer a year ago. The response of inbred mice to the terpolymer of L-glutamic acid, L-alanine and L-tyrosine, GAT_{10}, is under H-linked genetic control. This polymer is of importance because contrary to the ones studied by McDevitt and associates, the genetic control of the antibody response to GAT_{10} is all or none. Mice with $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$ are high responders whereas mice with $H\text{-}2^p$ and $H\text{-}2^s$ do not produce antibodies to GAT_{10} (Table 6). As shown in Fig. 4, this has been verified with mice differing only at H-2 with a B10 background. Considering the fact that $H\text{-}2^s$ mice such as SJL are absolute nonresponders to GAT_{10}, it was of considerable interest to verify whether GAT_{10}-binding cells could be detected in these mice and whether SJL mice could be induced to form anti-GAT_{10} antibodies by immunization with GAT_{10} on an antigenic carrier. Table 7 compares the number of GAT_{10}-binding cells in the

TABLE 6

Immune Response of Inbred Mice to GAT and H-2

H-2	Anti-GAT Antibody
a	High
b	High
d	High
k	High
p	No antibody
s	No antibody

TABLE 7

Cells Binding ^{125} I-GAT in Spleens of Responder (A/He Ha) and Nonresponder SJL Mice

Responder	Binding cells/10^4	
	Normal	Immunized[a]
A/He/Ha	3.2	8.9
Nonresponder SJL	8.3	7.9

[a]Immunized with 100 μg GAT in alum and pertussis 7 and 8 weeks previously.

spleens of responder A/HeHa and nonresponder SJL mice. These results were obtained in collaboration with Dunham and Unanue. Similar numbers of antigen-binding cells are present in both strains. Furthermore, the number of such cells is increased by immunization only in the spleen of responder animals. I would also add that the cells which bound GAT_{10} in both responder and nonresponder animals showed typical cap formation when exposed to antigens at room temperature or 37° C. Nonresponder SJL mice could also be induced to synthesize anti-GAT antibodies following immunization with GAT_{10} complexed with methylated albumin by Maurer and Merryman, an observation which was confirmed in our laboratory.

A most interesting property of guinea pig H-linked Ir genes, which we have studied is their expression both in inbred strains and also in a variable percentage of random bred animals. This permits an evaluation of the degree to which the linkage between an individual Ir gene and the major histocompatibility specificities of an inbred strain is preserved in random-bred guinea pigs, or in other words, the degree to which histocompatibility typing may permit the prediction of the responsiveness of individual random bred animals to an antigen. Undoubtedly this will depend upon how close the linkage is between the Ir gene studied and the locus controlling the histocompatibility specificities.

The result of such a study is given in Table 8. Seventy-eight random bred Hartley guinea pigs (42 PLL responders and 36 nonresponders) have been tested

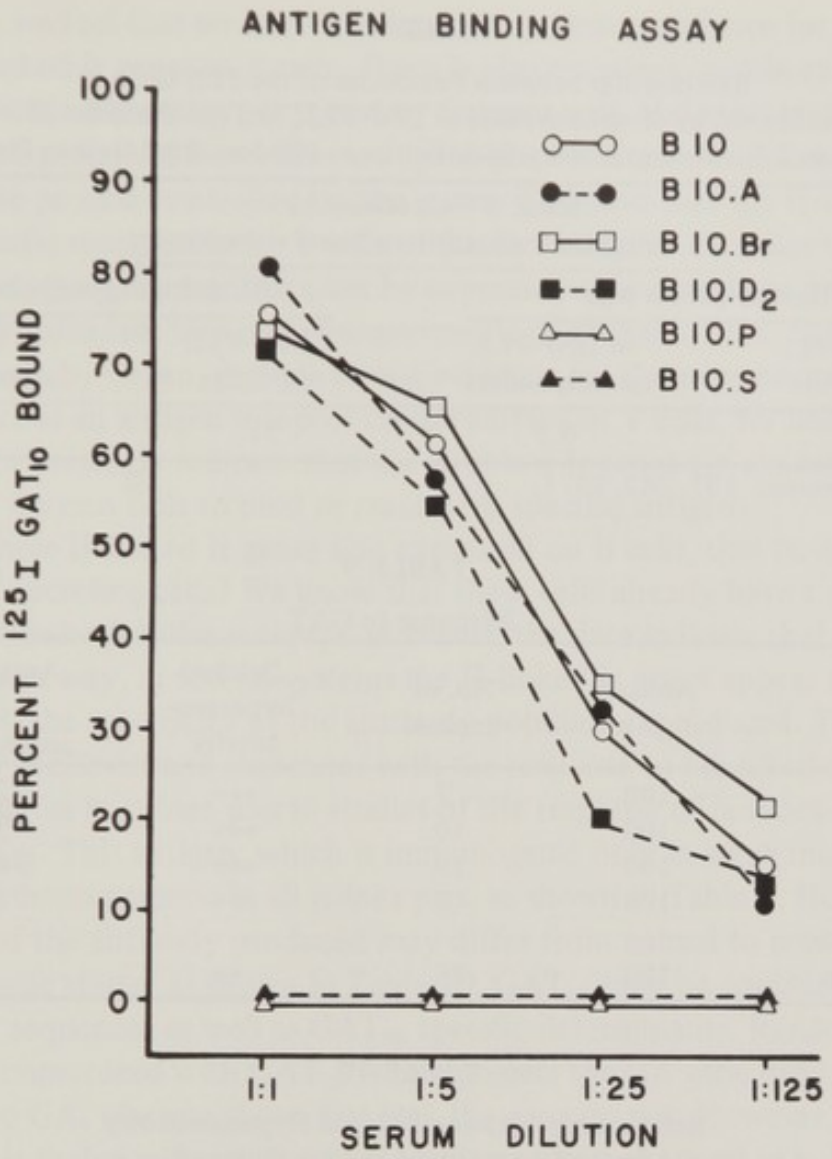

Fig. 4. Percentage of ^{125}I-labeled GAT$_{10}$ found by various dilutions of pooled sera obtained from congenic mice three weeks after immunization.

for the presence of strain 2 specificities on their lymphocytes with antistrain 2 alloantisera prepared in strain 13 guinea pigs. In each of these animals, without exception, possession of the PLL gene was always associated with the susceptibility to lysis of the cells by antistrain 2 antisera and complement. In contrast, the cells from PLL negative animals were not susceptible to lysis by these antisera. Identical results were observed with random-bred NIH multipurpose guinea pigs. There is therefore an absolute association between the PLL gene and strain 2 H-specificities in random-bred animals. Such an absolute association is not reserved for the GA gene which is only found associated 90% of the time with the PLL gene and thus with strain 2 specificities or with the HSA gene, which has a much lower frequency of association.

TABLE 8

Relationship between Possession of the PLL Gene, as Shown by Responsiveness to DNP-PLL, and the Presence of Strain 2 Histocompatibility Specificities in Random Bred Guinea Pigs

Mean % ^{51}Cr release by 1/5 antistrain 2 alloantiserum and complement			
Hartley guinea pigs		NIH Multipurpose guinea pigs	
42 DNP-PLL responders	36 DNP-PLL nonresponders	1 DNP-PLL responder	14 DNP-PLL nonresponders
27.2	0.3	51	0.2

J. Immunol. **107**, 382, 1971.

TABLE 9

Response to GAT

Strain	Antigen dose μg	No. of animals	Delayed hypersensitivity	Antigen bound % mean and (S.E.)
2	100	7	+++	72.6 (5.7)
13	100	10	+++	49.0 (4.6)
Hartley	100	19	+++	76.5 (2.2)
Coulson	100	8	+++	72.8 (3.9)
NIH multi-purpose	100	26	++	

TABLE 10

Relationship between Delayed Hypersensitivity to GA in GAT Immunized Hartley Guinea Pigs and Anti-GA Antibody Specificity in Their Sera

Cellular Immunity to GA	No. of Animals	Anti-GA ABC in %	Anti-Gat Antigen bound %[a]
+	9	22.9 (6.8)	79.2 (2.8)
-	10	4.7 (1.7)	74 (3.2)

[a]Mean value and standard error in parenthesis.

TABLE 11

Alternative Hypotheses Concerning Nature of H-linked Ir Gene Product

1. The products of these Ir genes constitute another essential recognition system of thymus-derived cells distinct from immunoglobulins.
2. They indirectly affect the interaction between antigen and immunoglobulin receptors on immunocompetent cells.

Thus, we feel that we have developed very strong evidence for the expression of H-linked Ir genes in T cells. There is also excellent corroborative evidence from McDevitt's laboratory, obtained by Grumet with McDevitt and Mitchell, which will be presented later. The next problem which merits discussion is the nature of the process controlled by the genes. We belive that the H-linked Ir genes control specific recognition by T cells of distinct antigens. In order to perform this function, the gene product must be expressed either inside or on the surface of T cells. We also feel that recent experiments of Shevach, Paul, and Green, to be presented next by Green, provide strong evidence for the expression of the specific gene product as an antigen receptor on the surface of T cells. We believe, therefore, that the data presently indicate that the H-linked Ir genes are concerned with the capacity of thymus cells to bind or react with specific antigens.

Are these H-linked Ir genes also expressed on B cells, that is, on precursors of antibody secreting cells? We know that these cells already have a perfectly adequate immunoglobulin receptor. However, the data indicate that in some as yet mysterious way, in several systems the H-linked Ir genes appear to effect, to some extent, the specificity of the antibody population produced. This has been observed by McDevitt and associates with the response to branched copolymers and by ourselves in guinea pigs in studies of the response of random-bred guinea pigs to GAT_{10}. This antigen, which is immunogenic only in some mouse strains, is uniformly immunogenic in all guinea pigs, as shown in Table 9. However, the specificity of the antibody produced may differ from animal to animal according to their genetic status, as shown in Table 10. GAT_{10} may be considered to have GA and GT sequences as well as GAT_{10} specific determinants. Random-bred guinea pigs immunized with GAT_{10} which possess the GA gene display delayed sensitivity to GA, whereas those that lack the gene do not. However, what is more remarkable is that significant anti-GA binding is only observed in guinea pigs possessing the GA gene irrespective of the level of anti-GAT antibodies produced. We must also stress that such an influence of the H-linked Ir genes on the specificity of the antibody produced is not observed in every system. For instance, identical binding for GT is observed in GT responders and nonresponders immunized with GAT.

There are several possible explanations for the observed effect of H-linked Ir genes on the specificity of the antibody population produced: 1. These genes may also be expressed on the surface of B cells and affect the specificity of antigen binding by the immunoglobulin receptors to some extent. 2. Alternatively, the genes may have an effect on antibody specificity by effecting, in some unknown way, the efficiency of specific T cell-B cell interactions.

As a final analysis we present in Table 11 the two major hypotheses which, in our opinion, may explain the process coded for by the H-linked specific Ir genes.

1. The product of these genes constitutes another essential recognition system of T cells distinct from immunoglobulins-a view which we presently favor.

2. The product of the genes indirectly effects the interaction of immunoglobulin receptors which antigen or immunocompetent cells of both classes. It is, therefore, very relevant if T cells are indeed shown to bind antigen through immunoglobulin receptors, an issue still unresolved.

Naturally it is my hope that our discussions will permit a choice to be made.

COHN: Did you ever assay for antibody to PLL itself?

BENACERRAF: Yes, we prevailed upon Lawrence Levine, who has developed an exquisitely sensitive complement fixation technique, to do this. By means of his procedure it was ascertained that animals immunized with poly-lysine itself (which produced delayed sensitivity to poly-lysine) never formed antibodies to poly-lysine. I might add that this result has also been obtained by other means as well.

COHN: I would like to ask Benacerraf whether these animals ever produced antibodies to PLL when it was complexed to a carrier-protein?

BENACERRAF: I don't remember whether we explored that possibility, but Green may know.

GREEN: My recollection is that we did not do the experiment Cohn refers to; we only tested by complement fixation sera of animals immunized to PLL alone.

BODMER: Did the guinea pigs you used in these investigations come from widely different sources? In other words, how many different types of animals did you use?

BENACERRAF: The animals we used were an entirely different, nonwhite strain of guinea pigs. I don't know their precise origin except that they derive from a large breeding colony.

BODMER: The critical matter, though, is the actual size of the founding stock.

BENACERRAF: I have no particular knowledge of that. All domesticated guinea pigs have been derived from relatively small numbers of founding animals. I will say, however, that the incidence of the PLL gene in the Hartley animals is about 30%, so that they are likely to be heterozygous, which gives a very good chance for crossing over, but that has not been observed for the PLL gene and the strain 2 "H" specificities.

This does not hold for every antigen. For example, responsiveness to GA and to low dose BSA are properties of strain 2 animals, as is PLL responsiveness, but the former are not linked with PLL responsiveness or strain 2 "H" specificities in *every* random-bred animal. So there is some recombination between the GA gene and strain 2 "H" specificities.

CEPPELLINI: But Benacerraf has just mentioned that these genes are not always associated at the population level.

BENACERRAF: Two of them do show association in outbred population, but the association is not absolute.

CEPPELLINI: If I understand Benacerraf correctly, he insists that some of these immune responses to different antigens are inherited as a unit in inbred strains but may be found to be dissociated in individual outbred animals.

My point is that while association, positive or negative, between two traits at the population level is suggestive (but no proof) of close linkage, lack of association is by no means evidence *against* linkage. The only sound test of linkage is segregation analysis.

Moreover, if the different immune responses will be shown in segregation to be occasionally separated by crossing over, that will be the *only* conclusive proof that they are controlled by different, although adjacent, nucleotide sequences, against the other alternative that they are pleiotropic expressions of the same Ir allele

CHAIRMAN SIMONSEN: Benacerraf has chosen to have Green complete the general introduction to this session and we will now hear from him.

GREEN: I would like to bring up for our consideration one of the ways of looking at how histocompatibility antigens may operate on the surface of cells. The relevant experiments I will present were conducted in cooperation with Shevach and Paul at the NIH. The model that we chose to examine is the effect of anti-histocompatibility antibodies (alloantisera) on an aspect of cellular immunity commonly studied by immunologists, i.e., the *in vitro* blast transformation of lymphocytes by antigen. The two antigens we selected for use were DNP-GL and GT. Response to these antigens is under genetic control as shown by Benacerraf earlier in this session. Table 12 shows the histocompatibility antigen linkages to the immune response to DNP-GL and GT. It is evident that strain 2 responds to DNP-GL, strain 13 responds to GT; the F_1 animal, carrying both histocompatibility antigens, responds to both antigens. By back-cross analysis, discussed previously, we have shown that the strain 2 histocompatibility antigen is linked to the DNP-GL response and the strain 13 histocompatibility antigen is linked to response to GT.

TABLE 12

Relationship between Histocompatibility Type and Immune Response to DNP-GL and GT in Inbred Guinea Pigs

Strain	Histo-compatibility type	DNP-GL response	GT response
2	2/2	+	—
13	13/13	—	+
F_1	2/13	+	+
$F_1 \times 2$	2/2	+	—
	2/13	+	+
$F_1 \times 13$	2/13	+	+
	13/13	—	+

The outline of the experimental design is shown in Fig. 5. At this point I should, in all candor, tell you that this type of experiment has been tried previously by other investigators, unfortunately without much success. In retrospect, I believe that we were successful because of information derived only a few months ago at the NIH by our colleagues, Rosenstreich and Rosenthal. They demonstrated a very interesting characteristic of the lymphocytes of peritoneal exudates of guinea pigs, viz., they are more responsive to antigen stimulation *in vitro*. These exudates are composed of approximately 70% monocytes and 30% lymphocytes.

In studies on these peritoneal cells, separation of the lymphocytes from the monocytes by means of the fiber column technique yields a lymphocyte population that proves far more sensitive to antigen stimulation *in vitro* than cells obtained from lymph nodes. Consequently greater antigen stimulation can be obtained with these particular cells as opposed to the lymph node lymphocytes that had been used previously in this type of study.

In the present experiments we obtained these peritoneal exudate lymphocytes, exposed them either to antigen or PHA at 37° C for 30 minutes, and then washed off unattached antigen or mitogen. I should also mention at this point that these suspensions of peritoneal exudate lymphocytes still contain on the order of 2 or 3% of monocytes and that these monocytes appear to be necessary for the reaction to proceed. However, the great preponderance of cells are lymphocytes.

At this point, we add either normal guinea pig serum or alloantisera, heated to eliminate functional complement. The sera are anti-2 or anti-13 produced, for example, by immunizing strain 2 animals with strain 13 spleen or lymph nodes in complete Freund's adjuvant. The animals are then boosted with lymph node or spleen cells, mixed with incomplete adjuvant, and the antisera obtained 4 to 6

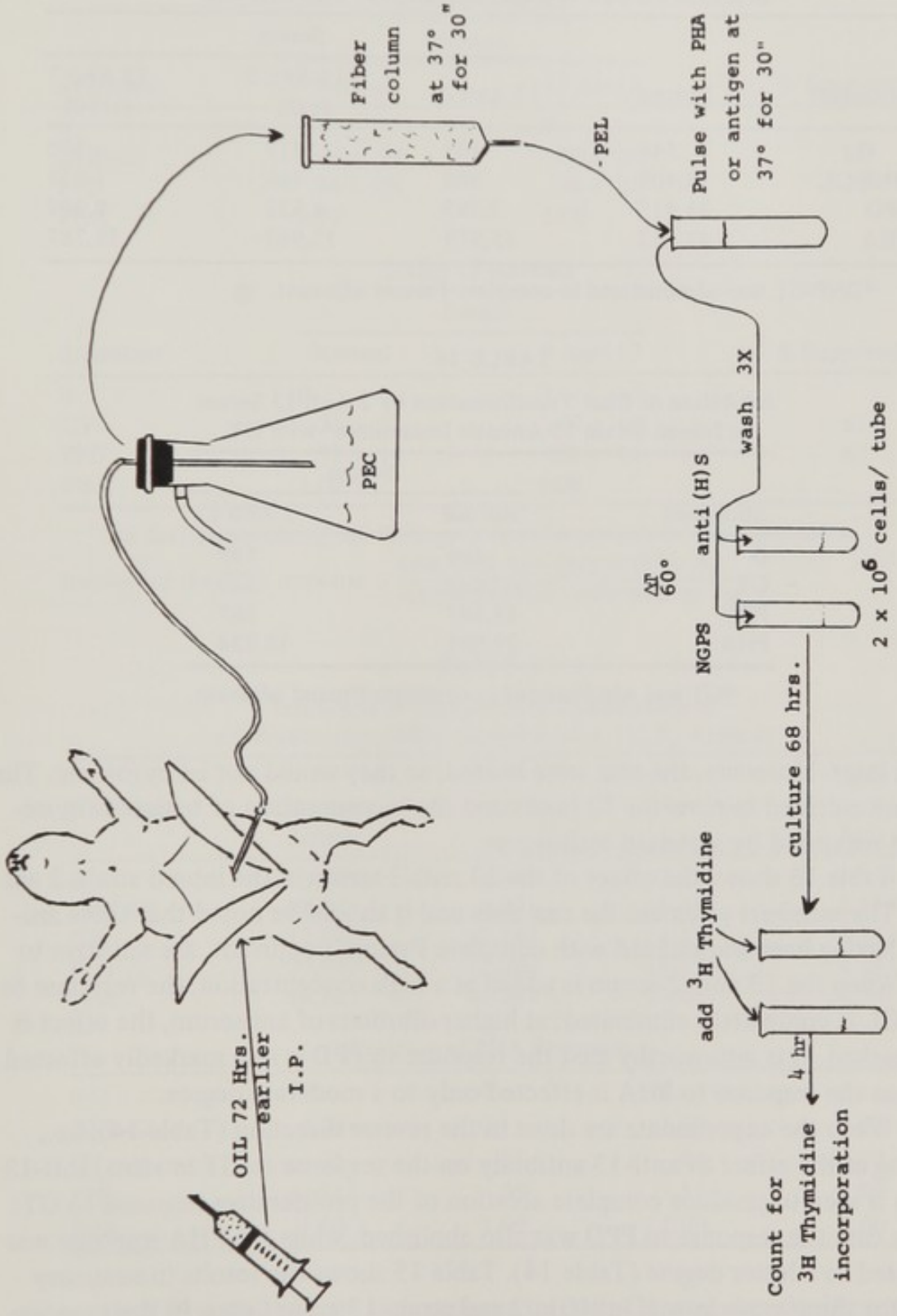

Fig. 5. Overall schema for obtaining and culturing peritoneal exudate lymphocytes.

TABLE 13

Inhibition of Blast Transformation by 13 Anti 2 Serum in Inbred Strain 2 Animals Immunized[a] with DNP-GL

Stimulant	Normal	Serum		
		13 Anti 2	13 Anti 2 (1:2)	13 Anti 2 (1:10)
O	546	489	217	300
DNP-GL	13,409	380	466	1,937
PPD	31,917	2,383	6,532	8,904
PHA	37,152	15,979	13,967	18,787

[a]DNP-GL was administered in complete Freund adjuvant.

TABLE 14

Inhibition of Blast Transformation by 2 Anti 13 Serum in Inbred Strain 13 Animals Immunized[a] with GT

Stimulant	Serum	
	Normal	2 Anti 13
O	466	599
GT	3,612	320
PPD	14,807	387
PHA	29,001	10,934

[a]GT was administered in complete Freund adjuvant.

weeks later. Moreover, the sera were heated, so they would *not* be cytotoxic. The cells are cultured *in vitro* for 72 hours and the incorporation of tritiated thymidine is measured by standard techniques.

Table 13 shows the effect of the 13 anti-2 serum in the inbred strain 2 animals. The numbers given are the raw data and it should be noted that these animals, having been immunized with complete Freund's adjuvant, are sensitive to PPD. When the 13 anti-2 serum is added at a high concentration, the response to DNP-GL is completely eliminated; at higher dilutions of antiserum, the effect is less marked. It is noteworthy that the response to PPD is also markedly effected, whereas the response to PHA is effected only to a moderate degree.

When the experiments are done in the reverse direction (Table 14), i.e., looking at the effect of anti-13 antibody on the response to GT *in vitro*, anti-13 serum is seen to produce complete ablation of the proliferative response to GT; in this case the response to PPD was also abolished, while the PHA response was inhibited to a lesser degree (Table 14). Table 15 shows the results in summary form for three experiments in strain 2 and strain 13 guinea pigs. In this case we have chosen to normalize the response to PHA, i.e., the PHA response is considered as unity and all other numbers are normalized to this value of one. It is

TABLE 15

Inhibition of Lymphocyte Proliferation by Antihistocompatibility Serum

	Strain 2 Animals		
	Serum		
Stimulant	Normal	13 Anti 2	% Suppression
0	0	0	
DNP-GL	.63 ± .14	.08 ± .05	87
PPD	.94 ± .08	.46 ± .18	51
PHA	1.00	1.00	

	Strain 13 Animals		
	Serum		
Stimulant	Normal	2 Anti 13	% Suppression
0	0	0	
GT	.21 ± .05	.07 ± .04	67
PPD	.72 ± .14	.11 ± .11	85
PHA	1.00	1.00	

The data summarizing the findings in three experiments, are expressed as

$$\text{fraction of the PHA response} = \frac{\text{cpm with Ag} - \text{cpm with no Ag}}{\text{cpm with PHA} - \text{cpm with no Ag}}$$

TABLE 16

Inhibition of Lymphocyte Proliferation by Antihistocompatibility Serum in (2 × 13) F_1 Animals

Stimulant	Normal Serum	Anti 2	Anti 13	Anti 2 + Anti 13
0	3,499	1,479	1,484	1,494
DNP-GL	68,361	1,231	46,385	2,178
GT	17,070	12,147	1,770	3,793
PPD	20,049	12,086	15,159	4,344
PHA	56,887	44,281	28,094	34,943
	Fraction of PHA Response			
0	0	0	0	0
DNP-GL	1.21	0	1.69	.02
GT	.25	.25	.01	.07
PPD	.31	.25	.51	.09
PHA	1.00	1.00	1.00	1.00

evident that 13 anti-2 ablates the response to DNP-GL and also significantly reduces the response to PPD. In the reverse experiment with 2 anti-13 sera, the GT response is very largely eliminated and PPD response is diminished even more

than the GT response. However, the most impressive experiments were those performed in F_1 animals immunized to both DNP-GL and GT. Table 16 shows the results of an individual experiment in F_1 animals; here it is seen that the anti-2 antiserum completely inhibits the response to DNP-GL, but affects the response to GT very little. The reverse is seen with the anti-13 serum, i.e., the DNP-GL response is only slightly diminished—but the GT response is markedly reduced. Of particular interest is the finding in F_1 animals, that the PPD response is not diminished to the same extent as when strain 2 and 13 animals were used. In other words, the PPD responses are considerably less affected in F_1 animals than in either the strain 2 or the 13 animals.

In the lower part of the table the responses are expressed as a fraction of the PHA response, and here it is apparent that the anti-2 serum completely ablates the DNP-GL response, while the GT response is completely eliminated by the anti-13 antibody, and in this instance the PPD response is affected hardly at all. However, when a mixture of both anti-2 and anti-13 are used, the GT, DNP-GL and PPD responses all proved to be inhibited.

Figure 6 gives a summary of four experiments in F_1 animals, again showing the proliferative responses in the presence of either normal sera or alloantisera. The anti-2 ablates the response to the DNP-GL, leaving intact the response to GT and PPD. Conversely the 2 anti-13 serum has no effect on the DNP-GL response, but eliminates the GT response, and here it has hardly any effect on the PPD response.

Prior work in our laboratory had demonstrated that in these systems, rabbit antiguinea pig immunoglobulin serum does not at all affect the *in vitro* response to DNP-GPA. Furthermore, I would emphasize that these antihistocompatibility sera were absorbed over columns containing immunoglobulin as well as columns containing the antigen DNP-GL; these treatments did not at all affect the ability of these alloantisera to block the specific immune response *in vitro*. Controls such as these give us confidence that we are not likely to be dealing either with some kind of cross-reacting antibody to the specific antigen or with antibodies which cross-react with guinea pig immunoglobulin.

I would conclude by saying that in our view these experiments indicate that the Ir gene product is on the surface of thymus-derived cells and is located near the sites occupied by histocompatibility antigens.

BENACERRAF: In coming to this conclusion, Green has been very cautious, as he usually is. There is, however, an alternative interpretation of this experiment. It could be that the alloantisera, which have been able to suppress the *in vitro* responses in the F_1 animals are in fact directed to the product of the Ir gene itself, rather than only to the histocompatibility specificities. I particularly stress this possibility since these antisera were raised with lymphocytes.

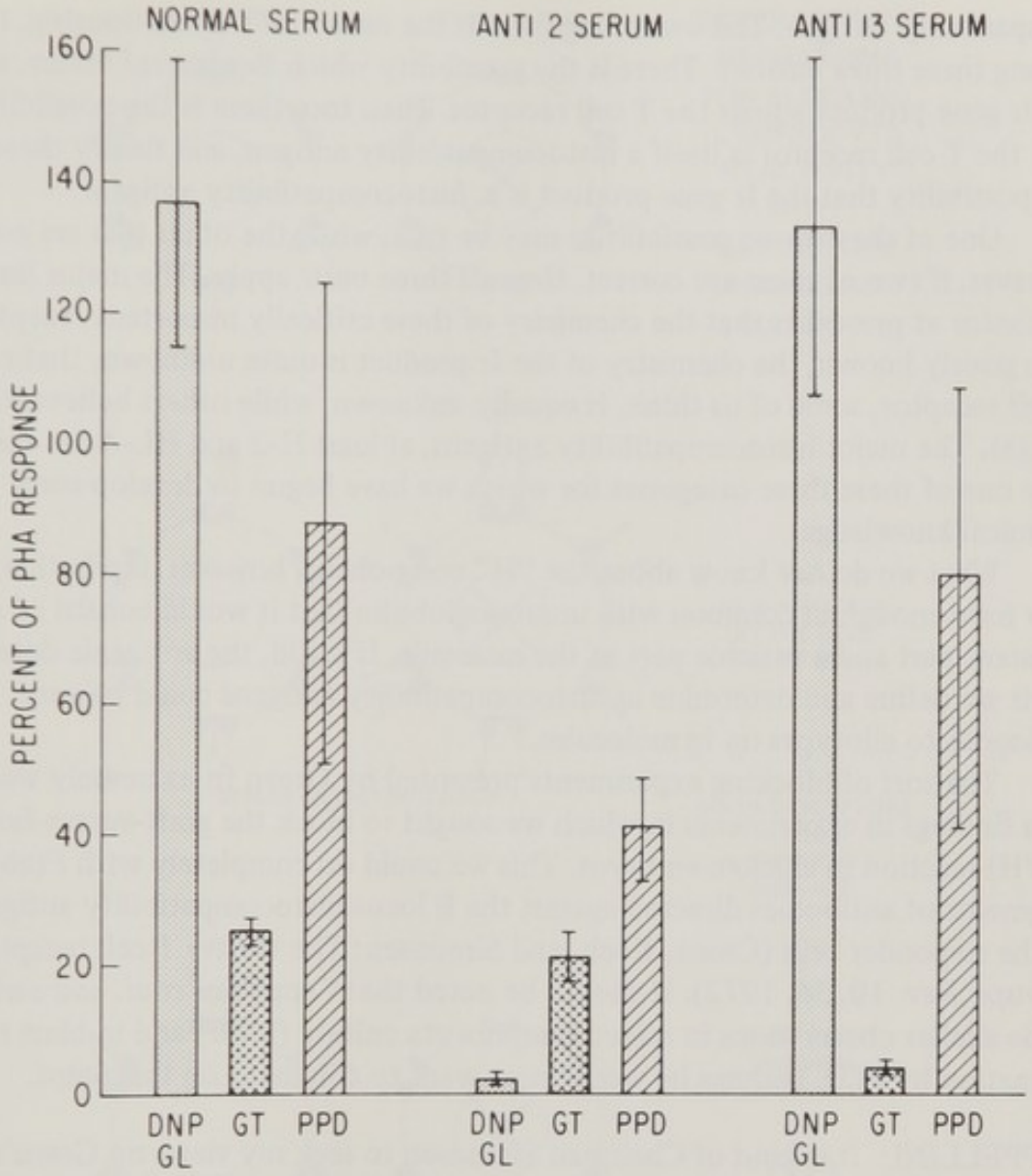

Fig. 6. Inhibition of antigen-induced proliferation of F_1 peritoneal exudate lymphocytes by antihistocompatibility serum. Results are expressed as the fraction of the PHA response. Each bar represents the arithmetic mean of four experiments ± one standard error of the mean.

Alternatively, they may function precisely as Green has mentioned, if one were bold enough to consider the possibility that the receptors on the thymocytes bear a relationship to the histocompatibility antigens that is analogous to the relationship the variable regions of immunoglobulin bears to the constant region. That is, they may be part of the same molecule.

CHAIRMAN SIMONSEN: Now that we have been exposed to Green's findings as well as Benacerraf's exposition of the issues, we have no less than three entities to discuss, namely the Ir gene product, the T cell receptor, and the histo-

compatibility antigen. The overriding issue is the nature of the relationship, if any, among these three entities. There is the possibility which Benacerraf favors, that the Ir gene product equals the T cell receptor. Then too, there is the possibility that the T cell receptor is itself a histocompatibility antigen, and finally there is the possibility that the Ir gene product is a histocompatibility antigen.

One of these three possibilities may be true, while the other two are not; however, if two of them are correct, then all three *must* apply. The major limiting factor at present is that the chemistry of these critically important receptors is so poorly known; the chemistry of the Ir product is quite unknown, that of the T cell receptor, some of us think, is equally unknown, while others believe it to be IgM. The major histocompatibility antigens, at least H-2 and HL-A are the only one of these three categories for which we have begun to develop some chemical knowledge.

What we do *not* know about the "H" component, however, is whether it may have enough in common with immunoglobulin that it would consist of a constant part and a variable part of the molecule. If it did, the antigenic determinants we define and determine as histocompatibility antigens could be very analogous to allotypes on Ig molecules.

The sort of blocking experiments presented by Green fit extremely well our own findings in experiments in which we sought to block the graft-versus-host (GVH) reaction in chicken embryos. This we could do completely with F(ab) fragments of antibodies directed against the B locus histocompatibility antigens of the responder cells (Crone, Koch, and Simonsen: The elusive T cell receptor; *Transpl. Rev.* **10**, 36, 1972). It should be noted that Ceppellini *et al.* had earlier made similar observations in mixed lymphocyte culture (MLC) and in blast transformation by PPD. Perhaps he would now want to comment on this point.

CEPPELLINI: It is kind of Chairman Simonsen to seek my views on Green's experiments; I consider this work to be of the greatest importance. As a matter of fact, we had already reported similar results two years ago. From the beginning, we became aware of their relevance for a possible new interpretation of the nature of the Ir genes. (Ceppellini, Miggiano, Curtoni, and Pellegrino, *Transplant. Proc.*, **3**, 63, 1971).

We observed that allogeneic human antisera obtained by repeated blood transfusions were able (in the absence of complement) to inhibit activation of the MLC when they were directed against either the stimulator cells (mitomycin-C treated in the one way MLC) *or* the responder cells. Blocking of the lymphocyte response was also observed with respect to tuberculin, tetanus toxoid, and even to suboptimal doses of PHA. This blocking was not accompanied by impairment of all viability as judged by dye exclusion and by ^{3}H thymidine incorporation after washing and stimulation with optimal doses of PHA.

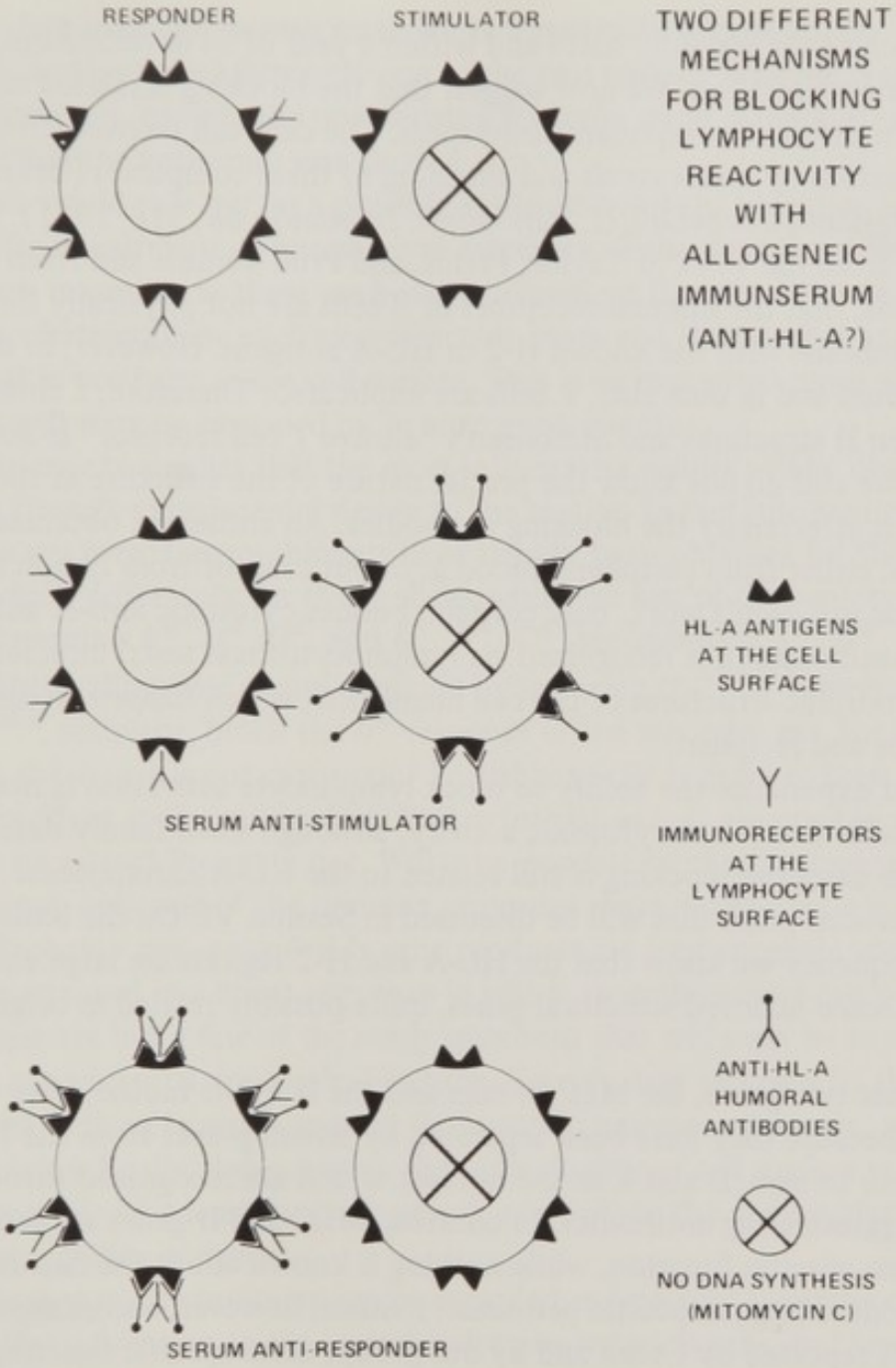

Fig. 7. Two postulated mechanisms by which anti-HL-A blocks the MLC. *Prog. in Immunol.*, 1, 1013, 1971.

Our interpretation of the inhibition in the two situations (antisera against stimulator or against responder) are depicted in Fig. 7: In the first instance the allogeneic antibody blankets the antigenic structures on the target cells which are responsible for activation of responder cells; in the second, the antibody interferes, by steric hindrance, with the receptor structures by which responder cells recognize the stimulating antigen.

The schema is certainly naive and within a year of its development already requires some corrections. We now suggest that the blocking is related to aggregation of cell membrane components into spots. The cells can recover from the effect of antiserum by endocytosis and shedding of these complexes (Bernoco, Mattiuz, Miggiano, Ceppellini, *G. Batt. Virol. Immunol.* **66**, 316, 1971). We now know, from the work of Taylor, Pernis, and Prud'homme and from our own investigations, that the immunoreceptors of B cells are not physically linked on the cell membrane with the known H-2 or HL-A antigens. However, in the system Green reported and in ours also, T cells are implicated. Therefore, a close relationship between H structures and Simonsen's "elusive T cell receptor" is not yet disproved. We still do not know the precise nature of the structure at the cell surface which is affected by the blocking antibodies. An antiserum obtained by injecting cells, either from peripheral blood as in our case, or from lymph nodes and spleen as in Green's work, may contain, besides cytotoxic anti-H antibodies, allogeneic antibodies (not recognized via lymphocytotoxic tests) directed against other polymorphic structures of the cell membrane, which *happen* to differ between donor and recipient.

In our experience the ability to block lymphocyte activation is not always correlated with anti HL-A cytotoxic activity, although some family data suggest that in these cases the blocking is still related to the HL-A chromosome. Here we come to a crucial point that will be discussed in Session VI. On the basis of crossing-over frequency we know that the HL-A and H-2 regions are large enough to encompass some hundred structural genes, quite possibly related in origin and function.

At least two genes, the MLC in man and the Ir in the mouse, have been identified, because they have been separated by crossing-over from the H antigens (LA and Four in man, D and K in the mouse), which are recognized through cytotoxic hemagglutinating antibodies. In contrast, MLC and Ir genes are recognized through their specific function, while nothing is known about the nature of their primary product (e.g., a specific permease?). Since, however, according to the experiments reported by Green and by our group, these specific functions can be blocked by allogeneic antibodies, two main alternatives must now be considered:

a. The antibodies (even if not cytotoxic) react with H antigens. Hence H antigens, although controlled by different genes, are essential for the functional expression of MLC and Ir genes.
b. The antibodies react with specific products of MLC and Ir genes, which are expressed on the cell membrane along with H and other membrane components such as "blood groups" (although they are not recognized via cytotoxic tests and are not necessarily important for histocompatibility).

I admit that occasionally man has some limitation as an experimental animal, and therefore, the beautiful experiment of Green is more informative in some respects

than our data based on human material. It is particularly noteworthy that each allogeneic antiserum inhibits only one of the two specific immune responses of F_1 immunocytes, namely the one deriving from the same parental strain, 2 or 13, against which the antiserum was elicited.

This would rule out, as a possible explanation of the blocking, cell death or nonspecific impairment of lymphocyte reactivity, if the blocking were through H antigens, inasmuch as these are both expressed on F_1 cells. It is not known, however, whether allelic exclusion affects Ir genes and the possible expression of their specific products at the cell surface. This is an important piece of information that will soon be obtained by *in vitro* experiments.

It is easy to predict that the most informative results will be obtained by adapting Green's experimental design to the mouse. In fact, through two successive crossover events, recombinants have already been obtained by Shreffler and others, which differ only for the Ir allele, while the rest of the haplotype is identical (e.g. parentals K^d, Ir^d, D^d, and K^k, Ir^k, D^k; recombinant K^d, Ir^k, D^d).*

Will the allogeneic serum obtained by injecting $H\text{-}2^{k,k,k}$ lymphocytes into an $H\text{-}2^{d,d,d}$ recipient, block the Ir^k response of the recombinant, for which, however, the serum is not cytotoxic? Probably yes, if Ir genes express an immunogenic product at the cell surface. Will cytotoxic anti-D obtained in an $H\text{-}2^{k,k,k}$ recipient be active? Probably not. Will it be possible to obtain allogeneic antisera able to block only one of the immune responses characteristic for a given Ir haplotype? Probably yes—provided Ir gene products are immunogenic and the Ir genes are arranged in a linear sequence in which recombinations can occur.

These are but a few of the many questions that will soon be answered by this new experimental approach. For now, Green's data and ours (if the MLC can be equated with an Ir gene), suggest that specific immune responses, at least the ones involving T cells, depend upon the individuality of cell surface structures which are related (at least for their genetic control) to the main systems of histocompatability antigens.

Whether these structures can be regarded as (specific?) immunoreceptors, comparable in function to the Ig molecules present on B cells, and how similar to Ig or to H antigens they may prove to be, I am not prepared to guess. Actually, despite all the accumulated experimental data, we still have not achieved an understanding of the mechanism of MLC activation.

GREEN: I would like to make two points in responding to Ceppellini. First of all, I did not mention that if one used a heterologous anti-T cell antibody specific only to guinea pig T cells, in this type of system, one completely eliminated response to all antigens such as PPD, DNP-GL, and GT.

*See Session II for full details.

Secondly, I want to state that I was deliberately cautious as regards precisely what these antibodies were detecting. This is because we are now in the process of absorbing these antisera with guinea pig hepatoma cells and with a pure population of the L_2C leukemia, a B cell line. This should enable us to determine whether we can remove the cytotoxic properties of the serum without eliminating their inhibiting activities. Until these studies are complete, it is best that I remain cautious.

However, it is indeed possible that what we are seeing is not really an anti-histocompatibility antibody, but rather an antireceptor antibody.

CEPPELLINI: I would add one point to Green's comments, with which I warmly concur. In our system, by absorbing an antiserum obtained by repeated transfusions from the same donor to the same recipient with the lymphocytes of HL-A identical genotype siblings or the donor, we remove completely their ability to block the donor's lymphocyte responsiveness. By absorbing this serum with the lymphocytes of an unrelated individual phenotypically identical to the donor for the four HL-A antigens, we removed *all* cytotoxic effect, but were *not* able to annul the blocking of the immune response.

CHAIRMAN SIMONSEN: Does that suggest that this receptor is on a different molecule from that carrying HL-A specificity? I suppose it does.

BODMER: My comment relates to the possibility that the "H" antigen system is the *same* as the T cell receptor and Ir gene product. We must not for a moment forget that "H" antigens are found on *all* cells. Therefore, in Benacerraf's equation, if the "H" antigen becomes the constant region of a molecule which has a variable region, as has been suggested, then the constant part, which is what is measured with "H" antiseria—appears on all types of cells. Thus, one can't really equate "H" antigens with the Ir product and the T cell receptor. What we call "H" antigen must be more than just the constant part, which is what we recognize serologically.

CHAIRMAN SIMONSEN: Bodmer is correct. However, I would point out that T cell receptors could in fact be on *other* cell types as well. What is so special about the T cell might not be recognition as such, but rather the reaction into which the recognition event triggers the cell.

HERZENBERG: I have a question of fact. I wonder in terms of Green's remarks, whether he has any indication of what proportion of the cells are actually taking up the tritiated thymidine, with either the antigen PPD or the mitogen PHA? I am particularly intrigued by the fact that PPD stimulation is inhibited markedly in both situations, whereas this does not apply to PHA.

GREEN: We did not make autoradiographic studies of the number of cells incorporating ^{3}H thymidine. The point I sought to make was that the PPD responses were affected much more in the strain 2 and 13 guinea pigs, but not in the F_1 animals. This is *probably* so because the PPD response, although not directly shown by other means, is also under genetic control linked to, let us say, both the strain 2 and strain 13 histocompatibility antigens. Thus, when one blocks with either anti-2 or anti-13 serum in strain 2 or 13 animals, one blocks *all* the histocompatibility-linked Ir receptors of the inbred parental animal. In the F_1, on the other hand, exposure to each separate antihistocompatibility antibody may leave the alternate receptor open for the PPD response. This could account for the finding that singly the histocompatibility antibodies do not inhibit the response very well in F_1 animals. However, a mixture of the two antisera reduces to base line the response in the F_1 to DNP-GL and PPD, so the interpretation can be made that PPD may also be under this type of genetic control.

As regards the PHA response, this seems to be an entirely different situation. I have already alluded to the fact that purification of the lymphocyte population eliminates reactions to antigens. Here one needs in the lymphocytic cell population at least 1 to 2% monocytes, whereas for a complete PHA response, the elimination of as many monocytes as is technically possible *still* results in an intact PHA response. Thus, it may be that the PHA response is *entirely* outside the histocompatibility-linked response system.

HERZENBERG: The PPD response is particularly interesting in that Green can inhibit the F_1 cells almost completely with the mixture of the two antibodies. Therefore, determining by autoradiography the proportion of the cells stimulated in the F_1, I think, would be especially interesting.

GREEN: I agree, the experiment is certainly worth doing.

WARNER: I would like to return to Benacerraf's first explanation, and question whether he distinguishes the alternative that there may be a distinct immunoglobulin component which is different from known immunoglobulins, and is not expressed in B cells; such as a variable region gene, which is different from V λ, V κ, V_H but which may antigenically cross-react with other antivariable region antibodies? Shouldn't such a possibility be kept in mind so that we do *not* limit ourselves to the notion that we *have* to find a classical Ig?

BENACERRAF: The answer to Warner has to be made on logical grounds, because the data are lacking. The issue raised by Warner is whether it is possible to have on T cells a dictionary of specificities distinct from that on B cells? Naturally, that is a strong possibility.

In addition, I would like to make clear that there is nothing which has emerged during this session that in any way commits the immune response genes to a clonal expression.

RAFF: I have a question for Bernacerraf. He didn't say whether he had looked at the capacity of thymus cells to bind any of the antigens he discussed.

BENACERRAF: We have looked into this matter and are not yet prepared to make a statement other than to say it seems to be interesting.

RAFF: One of the possibilities that Benacerraf did not mention is that these antigens may be tolerizing T cells in nonresponder animals. In view of Gershon's recent observations, wouldn't it perhaps be unwise to exclude this possibility?

BENACERRAF: Raff is referring to the data presented recently by Gershon (*Fed. Proc.* **31**, 778, 1972) which tend to demonstrate that T cells from nonresponder animals make an abortive response to GAT_{10} which is no longer observed on secondary challenge, whereas responder T cells make *both* a primary and second-response as measured by the stimulated incorporation of ^{3}H uridine. I must say that having been present when Gershon presented his report and having looked at the data, I am not convinced by the data as such, nor its solidity. Accordingly, I have not yet found it necessary to explain it. In my view there is as yet no clear evidence that the lack of response to GAT_{10} is caused by tolerance induction.

CHAIRMAN SIMONSEN: As Benacerraf's criticism is a very important one, I would invite Raff to reply.

RAFF: The fact is that the system Gershon has used involves the transfer of thymus cells into lethally irradiated syngeneic recipients, which then receive the antigen. The proliferative response of the adoptively transferred thymus cells is assayed by the incorporation of radiolabeled IUdR. He found that both responder and nonresponder thymus cells proliferated in response to GAT, but that nonresponder thymus cells did not respond to a second challenge of GAT, whereas responder thymus cells did respond. Moreover, if he first primed the mice with GAT before harvesting their thymus cells and transferring them, cells from nonresponders did not proliferate when challenged with GAT in the adoptive host, whereas the responder cells did proliferate. Gershon's interpretation of these data was that nonresponder thymus cells could recognize GAT but are readily rendered tolerant. While recognizing that these experiments are preliminary, it would seem to me to be unwise at this stage to exclude the possibility that nonresponder cells may actually be able to "recognize" the antigen.

BENACERRAF: In my opinion there are several criticisms which can be addressed to Gershon's experiments: 1. the differences themselves were not impressive; 2. results varied from experiment to experiment, and 3. most important, the experiments were not adequately controlled with any nonimmunogenic molecules. To my way of thinking, it is conceivable that GAT_{10} could cause nonspecific stimulation of nucleic acid synthesis for nonimmunologic reasons in both unimmunized responder and nonresponder animals.

WILSON: I have a suspicion that Grumet has data that bear on this and ask that he be invited to comment.

GRUMET: We do in fact have some preliminary data concerning the concept of tolerance in nonresponders. In a system such as that shown in Fig. 8, using only aqueous antigen with no adjuvants, after primary challenge with antigen both responder and nonresponder animals make an equal IgM response to (T, G)-A--L.

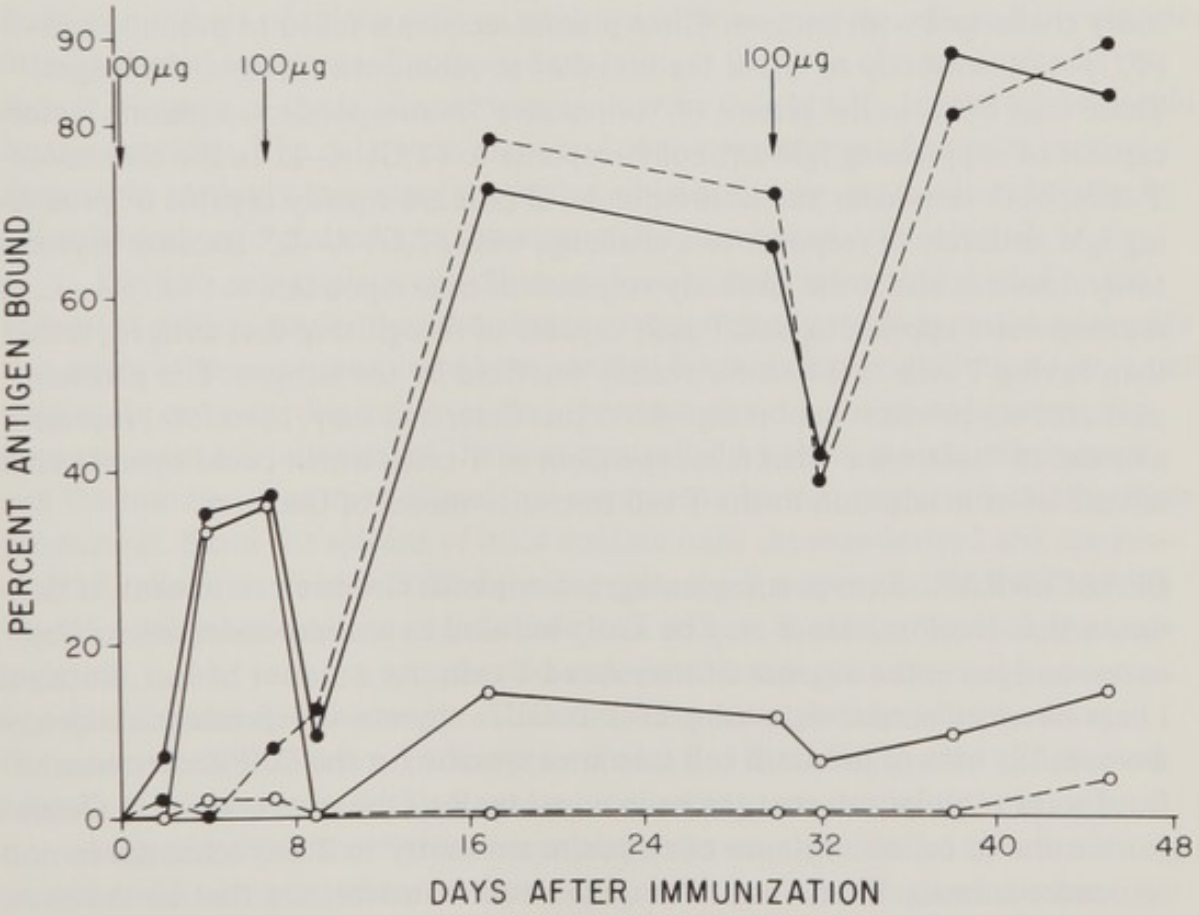

Fig. 8. Antibody production between responder and nonresponder mice following multiple challenge with (T,G)-A--L. Total (——) and MeR(-----)antibody response of C3H.SW responder (–•–) and C3H nonresponder (–○–) mice to primary (day zero), secondary (day 7), and tertiary (day 30) challenge with (T,G)-A--L 100μg. Each point represents a plasma pool of five mice. *Ann. N. Y. Acad. Sci.*, **190**, 170, 1971.

Although each strain develops a primary response, only the responder animal will switch over to an IgG response after secondary or tertiary antigen challenge.

The nonresponder animal, on the other hand, is essentially unreactive to a subsequent antigen challenge, with only a very transient dip in his antibody titer (presumably due to complexing of antibody by the small amount of antigen given). Nonresponder total anti-(T,G)-A--L antibody is all IgM and remains stable for at least a month after immunization. There is *no* new peak of response and *no* switch-over to IgG antibody after secondary or tertiary antigen challenge. challenge.

Spleen and lymph node cells from one of these unreactive nonresponder animals were removed and transferred into an x-irradiated syngeneic host, and that animal was then challenged with (T,G)-A--L. The transferred cells, which were formerly unreactive, will now produce a new burst of IgM antibody (just as they had done in their original host on initial antigen challenge).

Plasma from the original unreactive, immunized nonresponder animals was also transferred to intact, nonirradiated, syngeneic hosts, who were simultaneously challenged with antigen. These plasma recipients failed to produce anti-(T,G)-A--L antibody to either the initial or to subsequent antigen challenges. There thus exists in the plasma of "unreactive" nonresponders, a plasma factor capable of suppressing IgM antibody response to (T,G)-A--L. In the absence of T cells, both responder and nonresponder B cells are equally capable of producing IgM antibody in response to a challenge with (T,G)-A--L.[a] Because thymectomy does not affect the antibody response of nonresponders to (T,G)-A--L, the nonresponder appears to lack T cells capable of recognizing that antigen, rather than having T cells that become readily tolerized by the antigen. The plasma-mediated suppression in nonresponders just described may, therefore, represent a model of "tolerance" that is independent of T cells, which could operate either instead of or in addition to the T cell tolerance model of Gershon.

BENACERRAF: I am in complete agreement with Grumet's statement, if he means that B cell tolerance may be easily induced in nonresponder animals by excess antigen in the absence of stimulated T cells. As a matter of fact, Katz and I have obtained similar data using DNP-D-GL, a thymus-independent antigen; we were readily able to induce B cell tolerance specific for the DNP determinant. But I want to point out that the issue raised by Raff in connection with Gershon's work is the so-called existence of exquisite sensitivity to T cell tolerance in nonresponder animals. That, I regard as quite another matter, one that on the basis of the evidence thus far presented, I don't believe in.

[a] EDITORS' COMMENT: The evidence in support of this statement is given in subsequent comments by Grumet and is documented in Figs. 11 and 12.

CHAIRMAN SIMONSEN: We are now going to move on and hear from Shearer on the question of expression of the Ir genes in B cells.

SHEARER: I propose to summarize findings that represent the combined efforts of Edna Mozes, Sela, and myself, and also the contribution of Lois Lichtenberg for more recent data. These studies have been designed to determine whether the expression of Ir genes at the cellular level could be detected in marrow-derived cells and/or in relevant thymus-derived cells. This particular approach has involved a limiting dilution cell titration technique, and it employs the transfer of immunocompetent cells into syngeneic, irradiated recipient animals. Thus, we were able to avoid the potential complication of interpreting data in animals in which the injected cells and the recipients differed at H-2.

The immunogens we employed are the synthetic polypeptides built on multichain poly-L-proline and on multichain poly-D-alanine, namely (T,G)-Pro--L, (Phe, G)-Pro--L, (Phe, G)-A--L, and (T, G)-A--L.

The limiting dilution technique we employed involves injecting graded and limiting numbers of spleen cells or mixtures of bone-marrow cells and thymocytes into irradiated recipients. Fourteen days later, at the time of peak antibody response, the sera of the irradiated recipients were individually titered for hemagglutinating activity. The data were expressed as the percentage of animals making a detectable antibody response (above that obtained in proper irradiated control animals) and were plotted as a function of the number of cells injected.

The data using spleen cell dilutions have already been published. For the present it suffices to say that the observations originally made in intact mice with this series of immunogens, have been verified by these limiting dilution spleen cell studies. In my view this indicates that there is a direct correlation between the immune response potential of the host controlled by these genetic phenomena, and the number of antigen-stimulated response units that one can detect by these procedures. Since the spleens of mice contain *both* thymus-derived and marrow-derived cells, it was not possible by using spleen cell suspensions to demonstrate whether the genetic defect might reside in T cells, in B cells, or in both. Accordingly, we turned to more complicated experiments using thymus and bone-marrow-limiting cell dilution assays. These transfers were performed in two ways: the marrow cell-dilution experiments were carried out using graded and limited inocula of marrow cells with an excess, nonlimiting, number of thymus cells. Conversely the thymus cell-dilution experiments were performed by transferring graded and limiting numbers of thymocytes with a nonlimiting and constant number of syngeneic marrow cells.

The strains of mice which are important in this work are the SJL and the DBA/1 strains and their response to (Phe, G)-Pro--L. This immunogen consists of two immunopotent regions. As was shown by Mozes, McDevitt, Jaton, and Sela, the SJL strain is a high responder to the Pro--L portion of this immunogen,

whereas the DBA/1 strain is a low responder to Pro--L. Conversely, DBA/1 mice are high responders to Phe, G, whereas SJL mice are low responders. As a matter of information, the Phe, G response is controlled by the *Ir-1* gene and is H-2 linked, but the immune response to the Pro--L portion of this immunogen is controlled by *Ir-3* and is not H-2 linked.

Limiting dilutions of thymus and marrow cells in the SJL strain indicate that there is a statistically significant five-fold greater number of SJL marrow precursor cells for the Pro--L determinant than for the Phe, G determinant. This is in agreement with the fact that SJL mice are low responders to Phe, G but high responders to Pro--L. When the number of thymocytes was the limiting factor, no differences were detected in Pro--L versus Phe, G precursors, even though SJL mice are low responders to Phe, G.

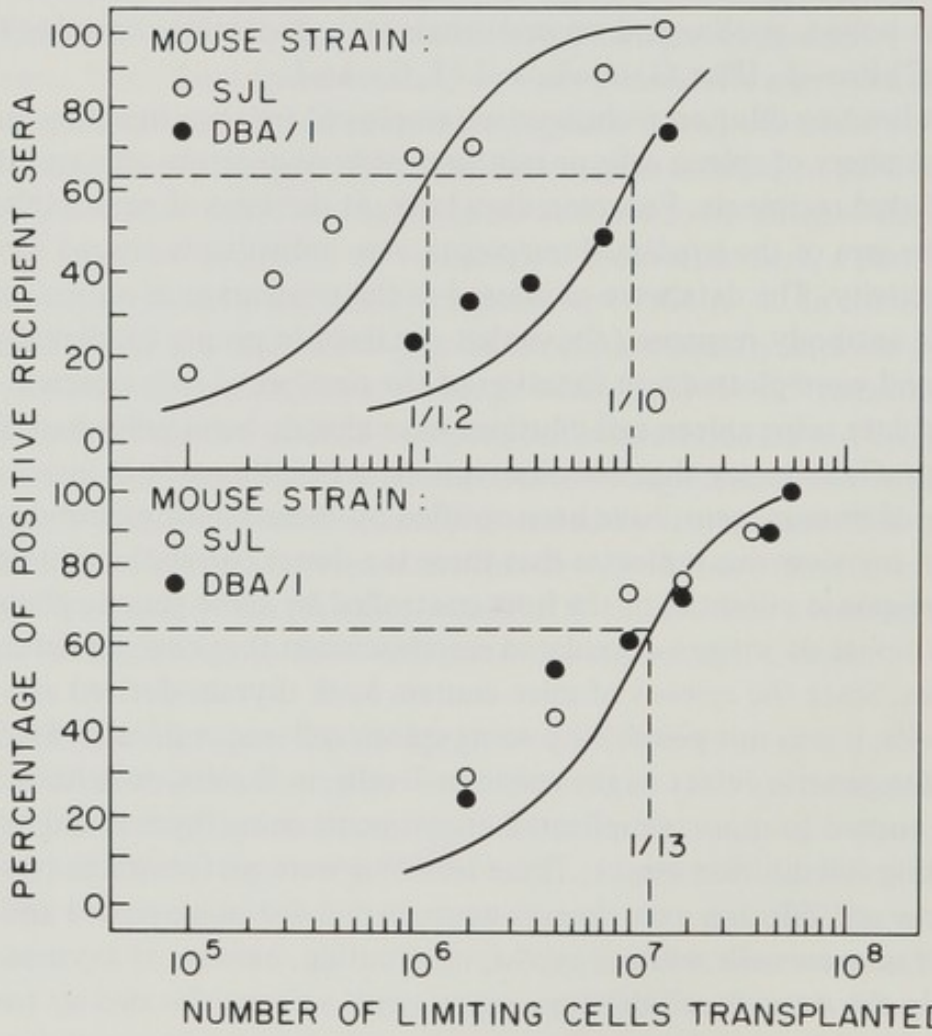

Fig. 9. Effect of graded and limiting numbers of bone-marrow or thymus cells on the antibody response to (T,G)-Pro--L. Percentage of anti-(T,G)-Pro--L positive sera in SJL (symbol ○) and DBA/1 (symbol •) recipients after irradiation and injection of (T,G)-Pro--L and: upper, 10^8 thymocytes with graded numbers of marrow cells; or lower, 2×10^7 marrow cells with graded numbers of thymocytes. All cell combinations were injected into syngeneic hosts. *J. Exp. Med.*, **135**, 1009, 1972.

When we turned to the DBA/1 strain, we saw the exact reverse pattern in the marrow dilutions, namely, that the marrow cell frequency for the high responding Phe,G determinant was five times greater than the frequency estimated for the low responding Pro--L specificity.

Again the thymocyte dilution curves were identical in the DBA/1 strain, for both the Phe,G and the Pro--L specific responses. These data indicate that the cellular expression of the genetic defect of immune responses to the Phe,G and Pro--L portions of this immunogenic macromolecule are not reflected in the numbers of thymocytes, although they are reflected in the number of B cells required for a detectable response.

It could be argued that (Phe,G)-Pro--L is a complicated immunogen in the sense that it contains two immunopotent determinants. Therefore, we switched from (Phe,G)-Pro--L to (T,G)-Pro--L, in which the antibody response is directed mainly to the Pro--L portion, and to (Phe,G)-A--L in which the immune response for these two mouse strains is directed mainly to the Phe, G portion.

The data for limiting dilution experiments with thymus and bone-marrow cells for (T,G)-Pro--L are summarized in Fig. 9. An eight-fold greater frequency of precursors was detected in the marrow for the high responder SJL strain than for the low responder DBA/1 strain (Fig. 9, top). In contrast, no differences were detected between these two strains when the immune response depended on the limiting number of thymocytes injected (Fig. 9, bottom). Thus, for the family of immunogens built on multichain Poly-L-proline, we have not seen any difference reflected in the thymus cell dilutions, irrespective of whether the gene involved was *Ir-1* or *Ir-3*.

We then changed to (Phe,G)-A--L, and again obtained the same pattern in the marrow dilutions as we had before. In this case, the DBA/1 strain was the high responder, and the SJL the low responder. A distinct difference was obtained in the number of marrow cell precursors estimated (Fig. 10, top). However, in contrast to the results obtained for the Pro--L series of immunogens, we found a marked difference in the dilution patterns of thymocytes for high and low responders to (Phe,G)-A--L. Thus, DBA/1 mice gave a thymocyte dilution pattern which not only showed a higher frequency than the SJL low responder, but the dilution of high responder thymocytes did not, moreover, follow Poisson statistics (Fig. 10, bottom). This suggests that there is a defect in the SJL mouse T cell population for (Phe,G)-A--L, and that the thymocyte population in DBA/1 mice is not only quantitatively different, but there may be a qualitative distinction between high and low responder T cells, inasmuch as the dilution of DBA/1 thymocytes did not conform to the Poisson model.

I particularly want to make the point that for the Phe,G-specific response, SJL mice given (Phe,G)-Pro--L did not show a dilution pattern reflected by differences in the thymocytes; but for Phe,G on the A--L backbone thymocytes from the same low responder strain *did* reflect a defect in T cells. In other words,

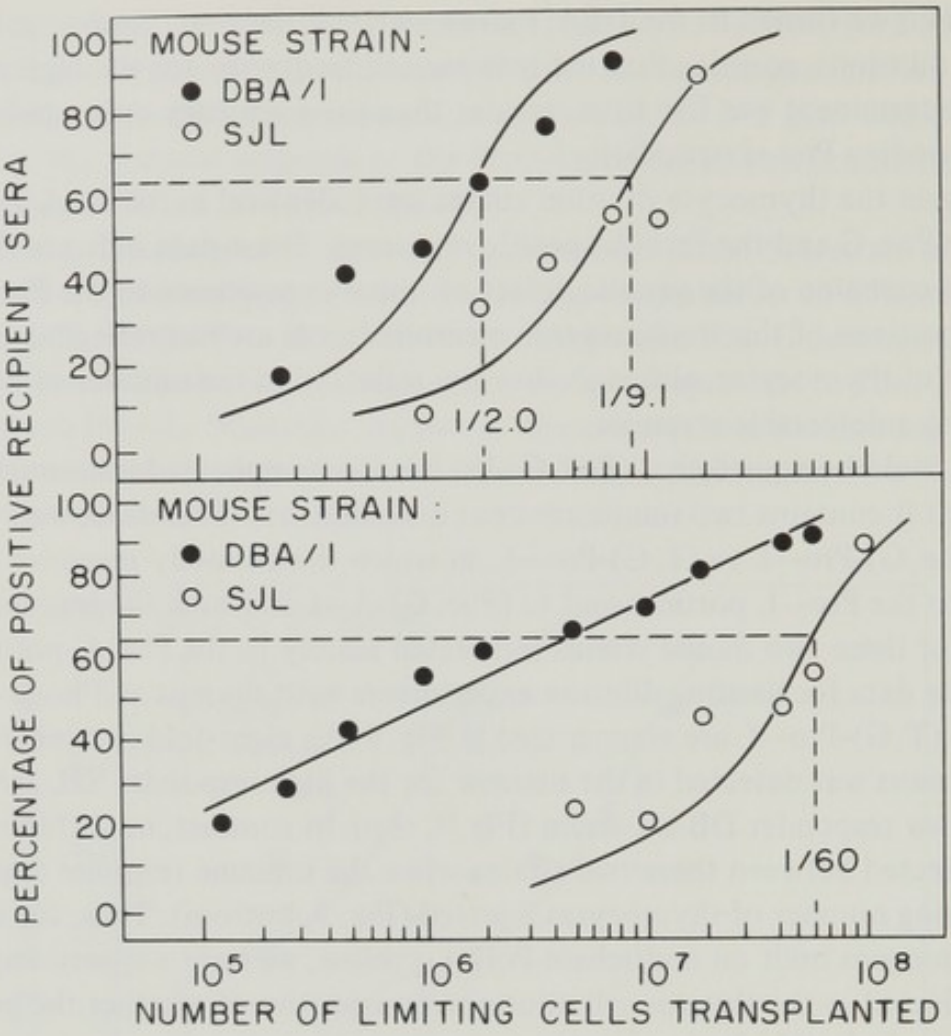

Fig. 10. Effect of graded and limiting numbers of bone-marrow or thymus cells on the antibody response to(Phe,G)-A--L. Percentage of anti-(Phe,G)-A--L positive sera in SJL (symbol ○) and DBA/1 (symbol •) recipients after irradiation and injection of (Phe,G)-A--L and: upper, 10^8 thymocytes with graded numbers of marrow cells; or lower, 2×10^7 marrow cells with graded numbers of thymocytes. All cell combinations were injected into syngeneic hosts. *J. Exp. Med.*, **135**, 1009, 1972.

the chemistry of the structure of the immunogenic macromolecule is very important in determining whether or not the *Ir-1* gene appears to be expressed in thymocytes as detected by this technique. Consequently, in my view, it is not possible to predict *a priori* whether or not a given genetic defect will be detected at the thymocyte level.

More recently, we have used (T,G)-A--L, since this is the antigen in which most of the mouse genetic information has been obtained. We have studied three mouse strains with respect to this immunogen: C57BL/6, the high responder–C3H/HeJ, a low responder–and SJL, also a low responder. The limiting dilution patterns are not yet complete, but they appear to be conforming to the following patterns: for the bone-marrow-limiting dilution, the same pattern was seen as in all previous cases. This is shown in Table 17. High responder C57BL/6 marrow

TABLE 17

Contribution of Bone Marrow Cells in the Response to (T,G)-A--L as a Function of the Genetic Constitution of the Mouse Strain

Number of cells transplanted		Percentage of recipient mice giving a posivitve response		
Thymus ($\times 10^6$)	Marrow ($\times 10^6$)	C57BL/6 (high)	C3H/HeJ (low)	SJL/J (low)
100	0.5	28	-	14
100	2	50	22	36
100	4	81	42	27
100	8	90	43	36
100	20	100	67	36

TABLE 18

Contribution of Thymus Cells in the Response to (T,G)-A--L as a Function of the Genetic Constitution of the Mouse Strain

Number of cells transplanted		Percentage of recipient mice giving a positive response		
Thymus ($\times 10^6$)	Marrow ($\times 10^6$)	C57BL/6 (high)	C3H/HeJ (low)	SJL/J (low)
0.25	20	53	-	16
2.5	20	60	56	14
5	20	62	67	0
10	20	67	64	15
20	20	86	-	14
100	20	100	67	36
200	20	-	-	36

cell precursor frequencies were higher than those obtained for low responder C3H/HeJ, which in turn were higher than the frequencies obtained for low responder SJL marrow cells. It is important to note that the SJL marrow dilution curve never rose above a 36% positive response.

In the thymocyte dilution experiment seen in Table 18, we found that the C57BL/6 high responder curve followed a non-Poisson pattern similar to that obtained using thymocytes from DBA/1 high responders to (Phe,G)-A--L. We found, moreover, that the low responder C3H/HeJ thymocyte dilution followed precisely the same pattern, even though this strain is a low responder. Conversely, the SJL low responder strain did not yield more than a 36% positive response. So not only does the expression of immune response genes at the cellular level vary as a function of the chemistry of the immunogen, but the genetics of the mouse

TABLE 19
The Antibody Response to Synthetic Polypeptides Built on poly DL Ala--polyLys by Mice of Different H-2 Types

		% antigen bound		
Mouse strain	H-2 type	(T,G)-A--L	(H,G)-A--L	(Phe,G)-A--L
C57BL/6	b	42	-	-
C3H·SW	b	79	5	73
AKR	k	-	70	73
C3H/HeJ	k	17	71	74
SJL/J	s	5	5	13
A·SW	s	0	0	15

Science, **163**, 1207, 1969.

also constitutes a variable, even between two mouse strains which in the intact state appear to be low responders.

Now, let us for a moment go back to some data published about three years ago by McDevitt and Chinitz. In Table 19 I show some excerpts from one of their tables in which they compared the responses of strains carrying the $H\text{-}2^b$, $H\text{-}2^k$ and $H\text{-}2^s$ of the H-2 complex for their antibody responses to (T, G)-A--L, (H, G)-A--L and (Phe, G)-A--L. I direct your attention to the fact that C57BL/6 is a high responder to (T, G)-A--L, a low responder to (H, G)-A--L, a high responder to (H, G)-A--L, and a high responder to (Phe, G)-A--L. In contrast, C3H/HeJ mice are poor responders to (T, G)-A--L, high responders to (H, G)-A--L, and high responders to (Phe, G)-A--L. The compelling point in these published data is the fact that SJL did not respond to any of these three polypeptides. In fact, recent data by Mozes indicates that SJL mice are nonresponders, even to the loop peptide lysozyme on A--L.

In view of these particular patterns of responses in intact SJL mice and their thymocyte dilution curves, it may be worth suggesting that for this particular series of polypeptides on A--L, the SJL strain may be the only true nonresponder we have thus far dealt with.

I do not want at this juncture to get involved in the matter of which portion of these molecules one might describe as haptens or carriers. However, if we feel that it is important to use the PLL system as a yardstick, and it may be, I suggest that we use A--L and Pro--L, and attach to the amino terminal ends of the side chains a hapten such as DNP. It would be interesting to see how immune response patterns to the hapten on such carriers behave as judged by limiting dilution studies.

In my opinion, the PLL gene in guinea pigs may be the equivalent or counterpart of the gene in SJL mice controlling responses to the A--L copolymers.

CHAIRMAN SIMONSEN: Would McDevitt like to speak to this?

McDEVITT: I think there are a number of points where we are inclined to take a somewhat different approach.

To come back to some of the data Shearer took up earlier, SJL is not the only strain that is a low responder to (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L. The A.SW strain is also a low responder for all three antigens, so that this is not a function of SJL as such, but rather a function of the $H\text{-}2^s$ allele.

Then, too, the data concerning the different results of limiting dilution studies with (T,G)-A--L in C57 and C3H mice, and (Phe,G)-A--L in DBA/1 and SJL mice are confusing to me. SJL has a great many other genetic differences from either the C57 or C3H strains, and I think one has to decide whether he wants to study what may be other genetic differences or those that are associated with H-2.

From my point of view, the crosses Shearer wants to test would be precisely the wrong ones. If one wants to study the H-linked immune response genes, I would suggest he use congenic strains such as C57BL/10 and B10.BR, or C3H and C3H.SW; in other words, strains that differ *only* at H-2 and therefore at Ir as well. The divergent results Shearer has found in his different strain combinations may very well reflect other genes controlling thymic cell numbers, or proliferation rates, or still other factors we know very little about.

In order to carry out limiting dilution assays in a way that could yield a clear answer as to which cell type was limiting (assuming the method is entirely valid—something I am not yet completely convinced of)—it would be necessary to use strains that are congenic and that differ *only* at the H-2 locus. By mixing strains that have radically different genotypes, one may very well be bringing in other genetic effects which have nothing to do with the H-2 linked immune response genes.

SHEARER: McDevitt's point is well taken and studies are now in progress to include C3H.SW in these studies. If the SJL is in fact such a peculiar strain, perhaps we should use the $H\text{-}2^p$ strain of mouse that Benacerraf has used in the GAT system. $H\text{-}2^p$ mice also appear to be poor responders to the A--L series of polypeptides.

BENACERRAF: If we look at the data and focus on the limiting dilution assay, Shearer has basically three patterns for nonresponders; one which shows primarily a B cell defect but no T cell defect, another which shows a marked T cell defect, and another which shows an intermediate pattern.

SHEARER: The first of these patterns is the one we detect with the Pro--L immunogens, which gives a Poisson distribution and a frequency estimate of $1\text{-}2 \times 10^{-7}$. The other two patterns are seen with the A--L immunogens. In SJL mice, we have been able to get only about 36% responses; in the case of the C57BL/6 and C3H/HeJ mice, their thymocytes generated flat dilution curves.

BENACERRAF: I want to emphasize that the strains that yield these patterns as recapitulated by Shearer are extremely interesting and worthy of further study.

HERZENBERG: I concur with Benacerraf's point and feel there is still another consideration. Has Shearer considered making mixtures of thymus cells to ascertain whether the phenomena which he is observing are due to a single cell type as opposed to the possibility of two cell types interacting? Perhaps not all of the conferees are aware that in SJL hybrids there is a situation in which one can get T cell suppression of B cells. Preliminary evidence suggests there are two types of T cells, one of which cooperates in, and the other which suppresses certain immune responses, particularly in the allotype suppression system. Some of these phenomena may be showing up in Shearer's cooperation curves.

SHEARER: Is Herzenberg referring to the SJL curve or the flat curve?

HERZENBERG: Well, actually to both cases. The flat curve could reflect a two-cell system, one inhibiting and the other enhancing whatever response one is looking at with SJL. The fact that Shearer gets practically no response at all with T cell addition could also be accounted for in that way by considering the predominant cell type to be the suppressor type rather than the activating type.

SHEARER: I cannot disagree with Herzenberg's suggestion, although this phenomenon might be due to some degree of thymus independence under certain conditions, and/or to nonspecific soluble factors which might be released by thymocytes. This would then make the immune response not necessarily dependent precisely on the number of thymus cells injected.

My particular prejudices concerning the SJL curve, are simply that we may have both a T cell and a B cell defect here. When we say we are giving an excess, nonlimiting number of marrow cells, probably in fact we are not. Consequently it is not possible to get above 30 or 40% response. The same thing can be argued for the B cell dilution, that if the thymocyte defect for SJL mice is so severe, then simply by increasing the number of thymocytes we inject, it is impossible to attain a situation where we have this response limited only by the number of marrow cells injected.

Finally, I would add that many transfer experiments, particularly those using (Phe,G)-Pro--L and (Phe,G)-A--L have been carried out using allogeneic cell mixtures, namely SJL and DBA/1 cells in one or another of the irradiated parental hosts. In this type of system, if one is not careful, he is liable to misinterpret the data, because if one gets a low response for a particular cell combination of low and high responders, one cannot determine whether that low response is due to a genetic phenomenon or to inefficient cell-to-cell interaction across an H-2 barrier. However, with the (Phe,G)-Pro--L immunogen which

consists of two immunopotent determinants, we can control that factor in our experiments. So if we immunize with (Phe,G)-Pro--L and measure the Phe,G responses and the Pro--L responses, we always have a built-in control to test for efficient cell-to-cell interactions. Accordingly, we have done these allogeneic transfer combinations. I will conclude by saying that they verify the results which we have obtained using syngeneic cell mixtures.

WARNER: There are three points I would like to make which support what McDevitt was saying about the importance of using congenic strains. The first point concerning low response is that SJL is a strain in which malignancies eventually develop in many of the mice. There may thus be a premalignant condition in the mice which could be reflected in the existence of a population of cells that are not immunocompetent. McDevitt mentioned that A.SW *in vivo* studies do show the same pattern as SJL and that would be an argument against the aforementioned explanation, but I think it nonetheless would be important to show with a limiting dilution assay that A.SW mice behave in the same way as do SJL mice.

Secondly, in our experience, if one compares 129/J with C57BL, he in fact finds that the difference in response to (T,G)-A--L between them is almost as great as that between C57BL and CBA even though both are simply termed responder-type strains. This may indicate that there are other genetic factors that also control the degree of anti-(T,G)-A--L responses. I would, therefore, strongly emphasize that Shearer must use congenic strains in these limiting dilution studies. I am sure he may find there are examples of other genetic controls that affect the rate of antibody production, perhaps at the allotype level. In fact, 129/J and C57BL mice do carry different immunoglobulin allotypes.

A third point I would like to make concerns results from Benacerraf's work and also in our own studies with (T,G)-A--L, namely that antigen-binding cells are present in similar numbers in the spleens of both unimmunized responder and nonresponder type mice. In our studies there were also similar numbers in the spleens of athymic nude mice; we therefore believe that the majority of these cells are B cells. The antigen-binding studies concern peripheral B cells in the spleen, whereas Shearer's data with the same antigen infers that there is a smaller number of antibody-forming precursors in the bone-marrow. I would, therefore, like to ask whether he could conclude that there is a deficiency in the proliferation of antigenically stimulated B cells rather than an actual reduction in the number of such cells bearing receptors for (T,G)-A--L?

SHEARER: My comments are on the first and last points raised by Warner. His first question concerning possible complications using the SJL mouse because of the malignancies involved, can only be answered using the (Phe,G)-Pro--L immunogen which is really where we derived most of our experience. In the same mice,

there are responses to Phe,G and to Pro--L in the same antisera, and we get high responses to Pro--L. If this malignancy phenomenon has something to do with abrogation of the immune system, I would expect it to be broader than just for the Phe,G specificity, yet we get perfectly normal high responses to Pro--L.

Concerning the antigen-binding results, there appears to be some discrepancy between what Warner has found and what I find. I would point out that in intact mice, McDevitt, Sela, Grumet, and coworkers were not able to find significant differences in the immune responses of high and low responders in the primary response. I presume that Warner's antigen-binding system is done in intact animals, not preimmunized.

WARNER: Yes, that is the case.

SHEARER: So I am not surprised that antigen-binding cells in nonprimed animals don't show any difference. This is what one would predict from the antibody titers that McDevitt, Sela, and Grumet have obtained in the primary response. For limiting dilution transfers of spleen cells, which presumably contain peripheral B cells, we see five to six-fold significant differences in the primary response. This is a discrepancy which I cannot explain other than by suggesting that maybe the limiting dilution transfers are at least as sensitive as the experiments in intact mice.

BRAUN: My comment is based on Herzenberg's suggestion that there may be two cell types involved in the Shearer experiment, one that stimulates and one that inhibits. I would suggest on the basis of available experimental data, that it is entirely feasible to assume that there is only one T cell type involved, which depending on its intensity of triggering regulatory events in antigen-reactive B cells, may either enhance or inhibit B cell functions. I would like to review the evidence for such biphasic responses in B cells, particularly since this sort of explanation, namely, that depending on the intensity of the T cell's effect on the B cell, one may get either enhancement of B cell function, or alternatively, inhibition, which might also explain Shearer's Poisson-distribution versus non-Poisson distribution data.

Recent data on the effect of allogeneic interactions on B cell performance *in vitro* show that certain allogeneic interactions can result only in stimulation of B cell performance, whereas, depending on the frequency of the allogeneic cells in the mixture, other allogeneic interactions can result either in enhancement or inhibition (Adler and Braun, unpublished data).

If one mixes C57 and CBA spleen cells in the Mishell-Dutton system, enhanced responses are observed regardless of whether the proportion of allogeneic cells is 1:1, 1:2, 3:1, etc. However, if one mixes CBA and DBA spleen cells, 1:1, 1:2, 2:1, etc., an inhibition of antibody formation ensues. Since we were aware that an excessive stimulation of cAMP levels in B cells inhibits B cell functions

(Braun, *PNAS*, 68, 114, 1971) whereas modest stimulation enhances B cell performance, and since we also knew that MLC reactions can elevate cAMP levels (Shiotawa and Braun, unpublished data), we naturally wondered whether CBA-DBA interactions, at high proportions of each cell type, may be inhibitory for B cells due to excessive stimulation. If this were so, the prediction was that by diluting out the proportion of either CBA or DBA cells in the mixed spleen cell culture, there should result a stimulated rather than an inhibited response. This turned out to be correct—when the proportion of DBA cells was reduced to 2%, stimulated antibody formation occurred compared to responses in pure CBA cultures. Incidentally, the addition of a cAMP stimulator, such as poly A:U, to the 98% CBA + 2% DBA cultures shifted the response to inhibition.

It may thus be that in Shearer's interactions between activated T cells and antigen-reactive cells, one may get both enhancement and inhibition, comparable to the allogeneic interactions between CBA and DBA in our tests and this may account for the non-Poisson distribution in his limiting dilution tests. In contrast, in his other systems, he might have a situation comparable to our C57 × CBA data, where one gets only enhancement from the T-B cell interactions.

I am really saying that what controls the response by B cells and the nature of the response of B cells, whether their functions are enhanced or inhibited, may be a function of the extent of membrane interactions, or humoral factor-mediated interactions, between antigen-activated T cells and the antigen-activated B cells. In certain genetic situations one may only obtain enhancement, whereas in other genetic situations one could obtain either enhancement or inhibition, depending on the degree of stimulation of the regulatory system in B cells (a system that we believe to be cyclic AMP mediated). I am thus, in effect, suggesting that the sort of interactions which obtain under conditions where Shearer gets a non-Poisson distribution may occur in genotypes in which there are interactions that can be either enhancing or inhibitory to B cell functions. On the other hand, Poisson distribution of responses in Shearer's assay may occur in those genotypes where the interaction between activated T cell and antigen-reactive B cell can result only in enhancement of B cells.

SHEARER: I would not rule this out. I think it is more likely to occur in the *Ir-3* system, in which we have enhancement by poly A:poly U of the low responder to the level of the high responders. Poly A:poly U does not in any way alter the responsiveness of these animals to the antibody response controlled by *Ir-1*.

BRAUN: I didn't intend to limit my remarks to that particular situation.

UHR: On behalf of the nongeneticists, I would like to ask for clarification of some of the discussion that has taken place.

If I understand Shearer's interpretation of his data, it is that there are H-2 linked Ir genes which operate at the level of the B cell but not the T cell. The counter-arguments are that he is not using congenic strains and that one is looking at genes and their products beyond the system of immediate interest. I have not yet heard a discussion, however, of the fact that T cells of H-2 linked nonresponders can give an identical limiting dilution curve as the responder cells. It seems to me that this is a very important point, and that those who believe that the *Ir-1* gene acts at the T cell level should comment on the validity of the methodology used.

CHAIRMAN SIMONSEN: That is just what McDevitt was trying to do.

McDEVITT: Yes indeed. First, I suspect that the limiting dilution curves may be misleading because they are identical for responder and nonresponder T cells, in spite of independent evidence, cited by Benacerraf, that Ir genes affect pure T cell functions. Second, it is conceivable that a large difference in the efficiency of antigen recognition by T cells, requiring a larger number of interactions between T and B cells for a detectable effect, would give the appearance of a difference in the numbers of B cell precursors.

BENACERRAF: I would not comment on the methodology, but take whatever information we can get out of it. I would, however, like to address myself to another point. Since the SJL mouse is so important in this system, and there has been inferred a defect in the rate of differentiation or proliferation of specific B cell precursors in this nonresponder strain, I would like to recall that although SJL are nonresponder to GAT_{10}, we and Maurer were able to stimulate adequate anti-GAT_{10} antibody responses in SJL mice by immunization with GAT_{10} complexed with methylated serum albumin.

SHEARER: In responding to Benacerraf, I would say that what we see as differences in the limiting dilutions of B cells, or bone-marrow cells, does not necessarily of itself prove that the defect resides in the B cells. We all know, or we think we know, that in terms of the immunological process, one of the later steps is the stimulation of B cells. It is entirely possible that the actual defect may have occurred at some stage before the bone-marrow dependent step, and we just see the differences detected at that level. What could those stages be? In my opinion they could either be the B cell alone, or cell-to-cell interaction. The fact is that when we use immunogens built on poly-L-proline, we don't see the differences in the thymus. We do see differences in the thymus dilutions for SJL with immunogens built on poly-D,L-alanine. In my opinion, that validates the system considerably. Had we consistently found no differences in the limiting dilution of thymocytes irrespective of the mouse strain, or irrespective of the immunogen,

then I think we would have been doubtful about the approach. We do know that the proliferation of T cells for (Phe,G)-Pro--L is not defective in the low responder animals. However, we do not know this about the immunogens built on multichain poly-D,L-alanine.

SELA: At a time when detailed studies are being made of the genetic control of the ability to respond to complex antigens, or to individual determinants of simple antigens, it is important to note that antigenic competition may give rise to effects superficially similar to those of genetic control. A genetically controlled ability to respond to certain dominant antigens on a multideterminant molecule can influence, for better or worse, the response to other determinants on the same molecule. It is easy to see that under some circumstances this could give rise to false conclusions. For example, if the ability to respond to the Phe,G determinant had been studied in different strains of mice using (Phe,G)-Pro--L as the *only* immunogen, it would be found that C3H/HeJ were low responders while DBA/1 were high responders to Phe,G. In fact, both strains are capable of a high response to Phe,G, determined in tests using (Phe,G)-A--L as the immunogen.

The genetic difference which *does* indeed exist between these strains, controls the ability to respond to Pro--L, rather than the response to Phe,G. Presumably it is the level of anti-Pro--L response which in turn influences the response to Phe,G, being inhibitory in C3H/HeJ, but not in DBA/1. Alternatively one could ask whether the C3H makes a *low* response to Phe,G in (Phe,G)-Pro-L because it makes a *high* response to Pro-L—this seems rather unlikely.

COHN: I don't think it is paradoxical that one should find a difference in a B cell population and not a difference in a T cell population, and yet have the receptor identical in both.

I just want to make that clear by pointing out an asymmetry in the relationship between T and B cells. Consider 100 T cells and 100 B cells with specificity for (Phe,G)-Pro--L. Suppose that in DBA/1, (the high responder) B cells there are 95 anti-(Phe,G) and 5 anti-Pro--L cells, and that there was the same ratio in the T cell population. If I assayed the B cell population for each specificity by the limiting dilution technique, I would find 95 anti-(Phe,G) and 5 anti-Pro--L, a difference of 20 to 1 to the antigen (Phe,G)-Pro--L. I would define this as one unit of T cell cooperation activity.

If, on the other hand, I had an SJL mouse which possessed 5 anti-(Phe,G) and 95 anti-Pro--L B and T cells, then I would find exactly the same 1 unit of T cell cooperating activity, but I would find 5 anti-(Phe,G) for 95 anti-Pro--L in the B cell population. So because of this asymmetry, the fact that Shearer finds identical T cell levels and different B cell levels in the two mice says that the B cells have two populations in different ratios in the two strains, but the T cells

may or may not be in different ratios. The result does not rule out T cell differences, as such differences were not assayed for.

SHEARER: I want to clarify one aspect concerning the points Cohn raised. We should realize that if we have a limiting dilution curve in which the Phe,G and the Pro--L responses are coincident in a single strain of mouse, one can subject this data to a 2×2 Chi square test for association of Phe,G and Pro--L responses. In other words, we can ask the question whether there are two populations of T cells stimulated by (Phe,G)-Pro--L or if only a single population is triggered? The Chi square values obtained ranged between 22 and 45, well above the critical value of 3.8. In other words, there was strong statistical evidence for association of the response for Phe,G and Pro--L in the thymus cell limiting dilutions. This is statistical evidence supporting the hypothesis that we are dealing here with a single population of T cells which is responsible.

CHAIRMAN SIMONSEN: I think that turns on the specificity. Would Cohn agree?

COHN: I don't understand it. I am saying that a Phe,G cell and a Pro--L *T* cell are functionally identical as far as cooperating activity is concerned. Therefore, the test Shearer proposes is not relevant.

SHEARER: This is one reason we went to the (Phe,G)-A--L and (T,G)-Pro--L immunogens.

COHN: Yes, but what I was trying to explain is that Shearer could have identical levels of thymus cells, assayed by cooperating activity for (Phe,G)-Pro--L in a high and low responder. Yet the two T cell populations in high and low responders could have different ratios of allotypic specificities to Phe,G and Pro--L, just as to the B cell populations of the two strains. The Ir gene could be expressed both in B and T cells, and he would see no difference at the T cell level. He would, on the other hand, see it at the B cell level because *that* is the property of his assay. I am trying to make a point on principle.

BENACERRAF: I cannot let Cohn's statement go unchallenged. As his comments indicate, Cohn assumes that all antigenic determinants which can induce the formation of antibody can function equally well as haptens and as carriers. This is clearly not the case. Polysaccharides and poly D amino acids, for instance, are adequate as haptenic determinants but cannot function as carrier determinants.

I know that Grumet has some data relevant to this issue and might also speak to this point.

CHAIRMAN SIMONSEN: Would Grumet like to speak?

GRUMET: First, I would like to ask Shearer a couple of questions he and I have already discussed informally. Specifically, I would like to go over the (T,G)-A-L model he has used. If one takes the extreme example of transferring a large number of B cells and a very large number of T cells, he is saying that there is no difference between a C57 and C3H animal in terms of percent responders, so by his definition, one has no difference between responder and nonresponder.

SHEARER: No, that is not quite true. What the dilution curves say is that we have injected a large number of T cells with a very small number of B cells. We are in the range of 1 to 8 million thymocytes per recipient. Then, there appears a difference between responders and nonresponders.

GRUMET: But if one has an animal given a large number of B cells and gives him the highest numbers of T cells, he gets 100% responders for either C3H or C57. Therefore, at the upper range of his cell transfers, he is saying there is no difference between responder and nonresponder.

I think, therefore, that the definition of responder and nonresponder Shearer is using makes it very difficult for me to accept some of these critical data.

SELA: Could Grumet reword his statement? I just don't understand what he infers.

GRUMET: If one takes an entire thymus and bone-marrow from a C57 and transfers it to an irradiated C57 and the entire marrow and thymus from a C3H animal, and transfers that to an irradiated C3H, then in challenge of each of those, by the stated definition, each will be a responder. Therefore, when Shearer looks at his data that way, one would conclude that there is no difference between C3H and C57 animals in response to (T,G)-A--L.

SHEARER: Yes, when one injects high numbers of marrow and thymus cells, but not in the range of limiting numbers.

GRUMET: The criteria Shearer has selected for cutoff to distinguish responder from nonresponder may simply be too low. It may be that he would have to select a much higher antibody titer, or he may have to look at the Ig class of his hemagglutinating antibody in order to detect differences between responder and nonresponders. It is a question of methodology that makes it difficult for me to reconcile the data he has and some of the data we have.

I would like to present this data because I think there is a very basic conflict between the conclusions Shearer's data would arrive at and those ours would

arrive at. From Shearer's data, he would say there is no difference in T cells, and therefore the *Ir-1* gene is expressed in B and not in T cells.

SHEARER: That is so, as far as C3H/HeJ and C57BL/6 is concerned. Grumet also raised a point that, by these techniques, we don't see any difference in C3H/HeJ and C57BL/6; therefore, the approach is not valid. The bone-marrow curves are quite different. It is only in the thymus curves that we don't see a difference.

Now, as I said previously, I do not necessarily accept this to mean the defect has to be due to a B cell. I am perfectly willing to accept at least two possibilities for *Ir-1* concerning C3H/HeJ and C57BL/6. It might be a B cell difference, or it might be a difference in cell-to-cell interaction. The alternative that I see we can both live with, is that it could be a defect at the level of cell-to-cell interaction.

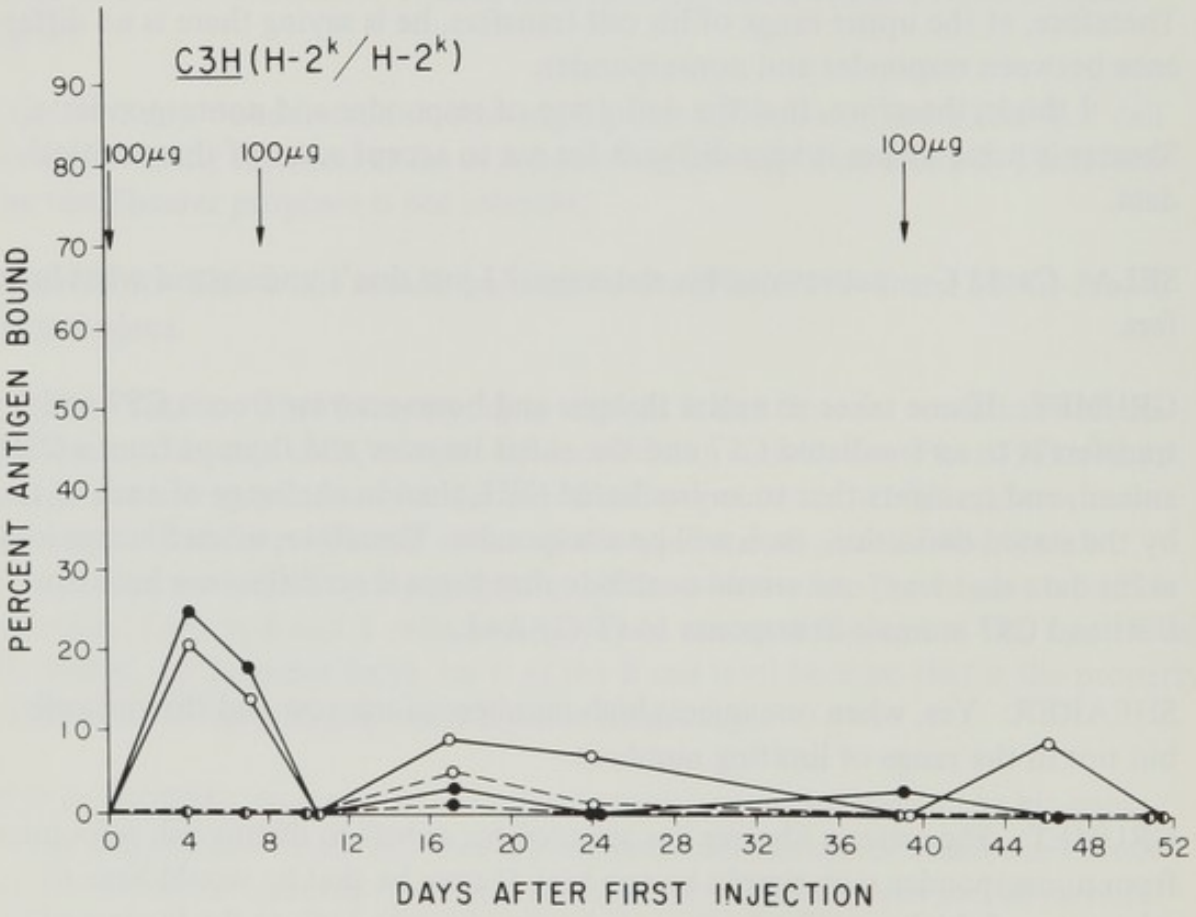

Fig. 11. Effect of thymectomy on nonresponder antibody production to (T,G)-A--L. The response of adult thymectomized or sham-thymectomized C3H nonresponder mice to 100 μg of aqueous (T,G)-A--L given intraperitoneally on days 0, 8, and 39. Sham-thymectomized total antibody, —○—; sham-thymectomized 2-Me-resistant antibody, --○--; thymectomized total antibody, —●—; thymectomized 2-Me-resistant antibody, --●--. *Ann. N. Y. Acad. Sci.*, **190**, 170, 1971.

GRUMET: Work done with McDevitt and Mitchell would arrive at a different conclusion, i.e., *Ir-1* is expressed in T cells rather than in B cells. We used congenic C3H and C3H.SW mice, differing only at the H-2 locus. The former is an $H\text{-}2^k$ nonresponder, the latter an $H\text{-}2^b$ responder. Aqueous (T,G)-A--L antigen was given at day 0, day 7, and again at day 30, and antibody determined by a modified Farr assay. The results are seen in Fig. 8.

These data show that the initial primary response is the same in responders and nonresponders. If one looks at each animal prior to giving the secondary antigen challenge, you would say they are both the same, that there is no difference. All of the antibody of each strain in the primary response is IgM. Following the secondary stimulus the responder animal produces a typical secondary or memory type of response, with a much higher titer, and it all becomes, or shifts over to IgG. The nonresponder is basically nonreactive to the secondary or tertiary antigen challenge, so that there is a marked difference here between responder

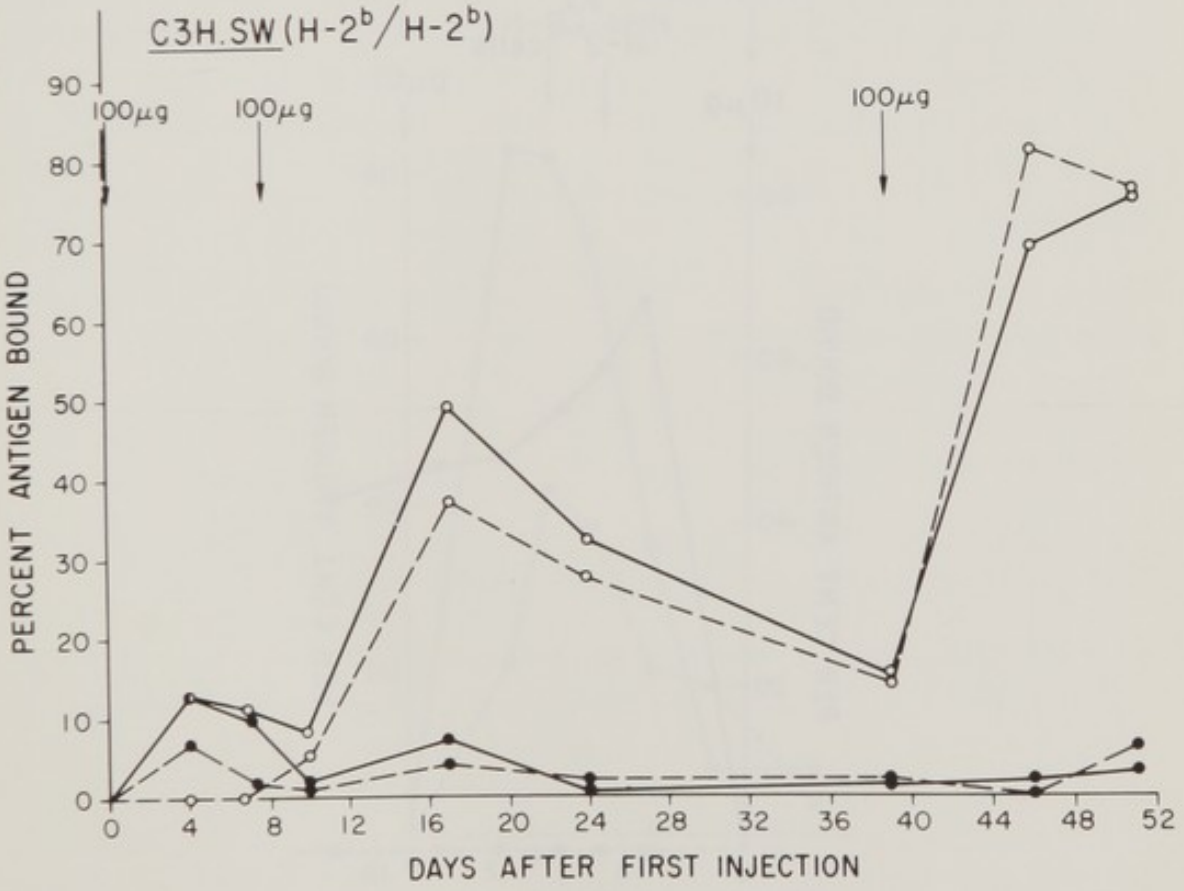

Fig. 12. Effect of thymectomy on responder antibody production to (T,G)-A--L. The response of adult thymectomized or sham-thymectomized C3H.SW responder mice to 100 μg of aqueous (T,G)-A--L given intraperitoneally on days 0, 8, and 39. Sham-thymectomized total antibody —○—; sham-thymectomized 2-Me-resistant antibody, --○--; thymectomized, total antibody —●—; thymectomized, 2-Me-resistant antibody --●--. *Ann. N. Y. Acad. Sci.*, **190**, 170, 1971.

and nonresponder in their ability to switch over to IgG antibody formation following secondary antigen challenge.

We took both responder and nonresponder animals and thymectomized them (adult thymectomized, irradiated, bone-marrow rescued animals). Fig. 11 shows the results with nonresponders (C3H) and Fig. 12 shows the results with responder animals (C3H.SW). Thymectomy caused essentially no change in the immune response pattern of the nonresponders (Fig. 11). In Fig. 12 it can be seen that thymectomy obliterated the IgG switchover of responders, i.e., the ability of these animals to develop 7S IgG immune memory, and so from this data we would conclude that thymus cells are essential for showing the difference between responders and nonresponders, and therefore, that the Ir gene is expressed in thymus cells. In contrast, thymectomized animals basically respond in the same manner whether they are responder or nonresponder.

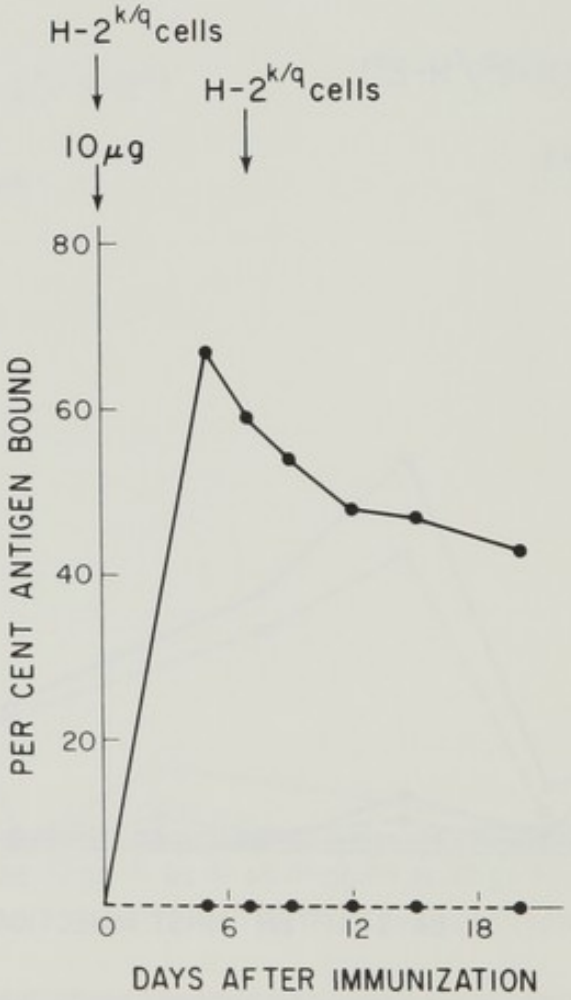

Fig. 13. Effect of syngeneic cell transfer on nonresponder antibody production to (T,G)-A--L. Total (——) and MeR (----) antibody response of (C3H.Q × C3H/DiSn)F_1 ($H\text{-}2^{k/q}$) mice given 88 × 10^6 $H\text{-}2^{k/q}$ lymphoid cells intravenously (i.v.) on day 0 and primary (day 0) challenge with 10 μg (T,G)-A--L i.p. Each point represents a plasma pool of five mice. *J. Exp. Med.* in press 1972.

A second point I want to make is that Shearer published some very fascinating experiments with allogeneic transfers, but I think there is a difficulty with allogeneic transfers, which require that one must be very cautious.

We have taken nonresponder animals, C3H.Q ($H\text{-}2^q$) and congenic C3H ($H\text{-}2^k$) animals, each nonresponders to (T,G)-A--L. Both strains make only an IgM response with no IgG switchover. The F_1 animal is the same as either parent, i.e., a nonresponder. One half to one spleen equivalent of either parental or syngeneic F_1 cells were transfused into intact F_1 recipients. Figure 13 represents the controls given syngeneic F_1 cells. All mice received 10μg of (T,G)-A--L on day 0. This curve is the same as for either parent or for the F_1 animal given no cells, with only the pure IgM response appearing.

Figure 14 shows the F_1 host animal given *parental* cells. Again, these are all nonresponder cells with no responder genes for (T,G)-A--L in donor or recip-

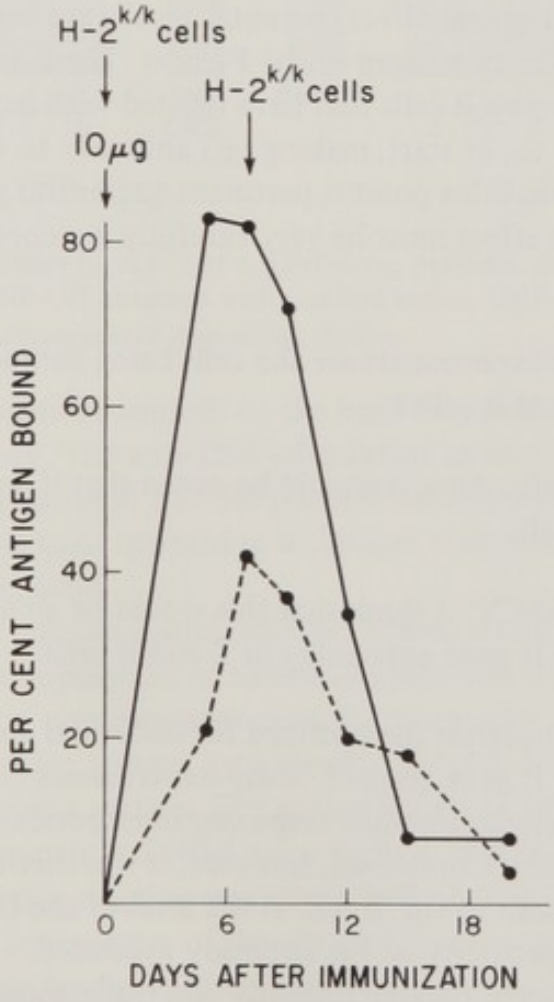

Fig. 14. Effect of allogeneic cell transfer on nonresponder antibody production to (T,G)-A--L. Total (——) and MeR (----) antibody response of (C3H.Q × C3H/DiSn)F_1 ($H\text{-}2^{k/q}$) mice given 150 × 10^6 $H\text{-}2^{k/k}$ lymphoid cells i.v. on day 0 and 208 × 10^6 $H\text{-}2^{k/k}$ lymphoid cells i.v. on day 7 and primary (day 0) challenge with 10 μg (T,G)-A--L i.p. Each point represents a plasma pool of three to five mice. *J. Exp. Med.*, in press 1972.

ient. If the parental cells are given and the GVH is thus started at the same time antigen is given, these F_1 hosts do switch over to IgG antibody (Fig. 14). The allogeneic effect has been described in detail by Benacerraf and his colleagues, and this is the way it operates in the (T,G)-A--L system. It appears that a GVH induced at the proper time can induce a switchover to IgG. This is also dose-related, so that if fewer cells are given, less IgG appears. We have not gone up to much higher doses of cells yet, but in any case, as soon as an allogeneic cell transfer is encountered (as in Shearer's studies) one must be extremely cautious in interpreting the result of who is the responder, or why the animal is responding.

From this and from the previous data, I believe that the *Ir-1* gene is expressed in the T cells and not in B cells. It *could also* be expressed in B cells. I don't think my data would support that, but they certainly would not refute it completely. But I believe these data do show that *Ir-1* is expressed in T cells, and that by means of nonspecific T cell activation, (which is my interpretation of the mechanism of the allogeneic effect) parental T cells are being turned on by the foreign histocompatibility antigen of the F_1 host. These activated T cells then exert their effect on nearby B cells that have reacted with haptens and induce those B cells to switchover to, or start, making IgG antibody to the hapten.

Again I think that this point is pertinent to Shearer's data, in that the problem of the allogeneic effect must be very carefully watched for in cell transfer experiments.

RAFF: In Grumet's experiments are the cells being stimulated by the allogeneic effect, virgin or primed B cells?

GRUMET: Virgin cells. Also, it should be noted that if antigen is not given, no antibody is made at all.

CHAIRMAN SIMONSEN: I think that this would be an appropriate time to hear from Schlossman on Ir gene expression in T and B cells.

SCHLOSSMAN: Benacerraf has outlined for us several cogent arguments favoring a T cell locus for Ir gene control. Many experiments demonstrate the presence of T cell mediated cellular immune responses in responder animals and their absence in nonresponders. Unresolved, however, is whether the Ir gene can also express itself, independent of the T cell, at the level of the B cell, i.e., does the Ir gene influence the specificity of the antibody produced.

We thought that responder antibody and nonresponder antibody, i.e., antibodies made in Ir gene negative animals, might be different, but it has only been in the past year or so that techniques have become sufficiently sensitive to detect these differences (Levin *et al.*, *J. Exp. Med.* **133**, 1199, 1971). Guinea pigs were immunized with defined peptides in a variety of adjuvants and separated into re-

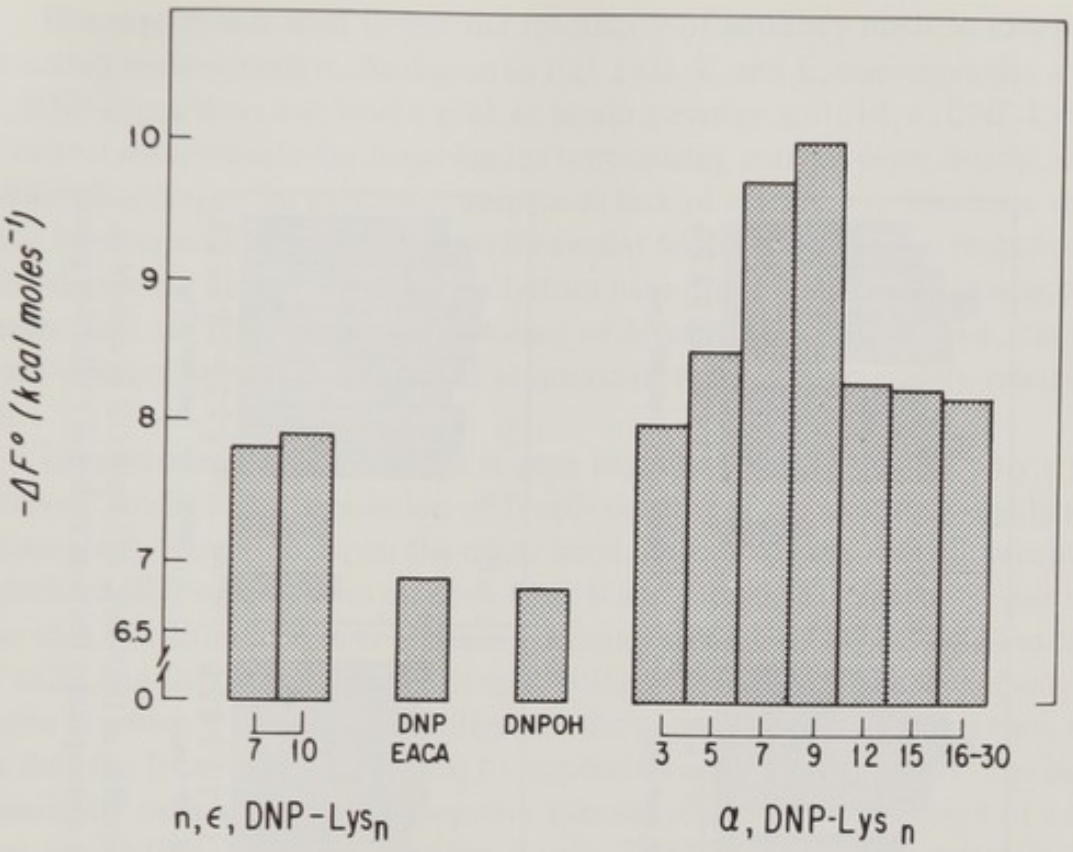

Fig. 15. Binding energies ($-\Delta F^{\circ}$) of α, DNP-Lys_n peptides, n, ϵ, DNP-Lys_n peptides, DNP, EACA, and DNP-OH obtained with purified anti-α, DNP-Lys_{7-10} antibody from a responder animal in fluorescence quenching studies.

sponder and nonresponder animals on the basis of positive or negative delayed skin tests, respectively. The anti-DNP-oligolysine antibody was purified on a DNP-BSA immunoabsorbent and the purified antibody investigated for specificity utilizing the fluorescent quenching technique with a large series of closely related DNP-oligopeptides.

All responder animals tested, whether inbred strain 2 or outbred Hartley animals, produced a population of antibody molecules which differed from the antibody produced in nonresponder animals. Responder antibody recognized the homologous immunizing antigen by a specific boost in binding energy with the homologous immunizing antigen. For example, anti-α, DNP-Lys_{7-10} antibody could readily discriminate α, DNP-Lys_{7-9} from α, DNP-Lys_5 or α, DNP-Lys_{12}. The overall $^{\Delta}F^{\circ}$ for this reaction was about 10Kcal (Fig. 15). This extraordinary specificity is found irrespective of the adjuvant used to generate the response and is characteristic of antibodies made in immune response gene positive animals. Responder antibody discriminates not only the position of the hapten but the precise chain length of the lysines used to induce the immune response. As shown in Fig. 16A, B, and C, responder antibody to 14, ϵ, DNP-Lys_{14} is very specific for this compound. The $^{\Delta}F^{\circ}$ for 14, ϵ, DNP-Lys_{14} is at least 550 cal/m greater than that obtained with either 13, ϵ, DNP-Lys_{13} or 15, ϵ, DNP-Lys_{15}.

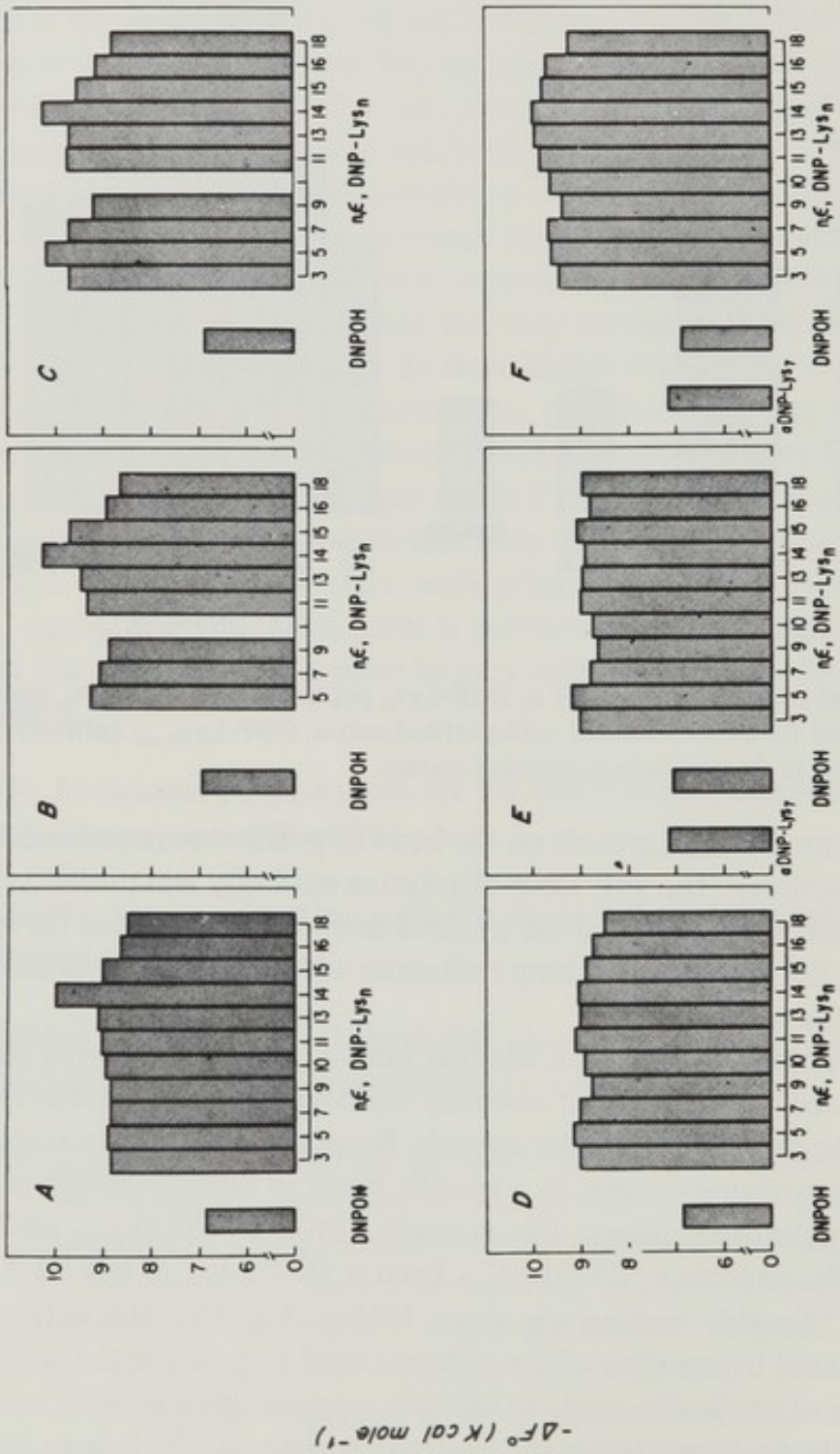

Fig. 16. The energy of binding ($-\Delta F^\circ$) of n, ϵ, DNP-Lys$_n$ peptides, α, DNP-Lys$_7$ and DNP-OH to anti-14, ϵ, DNP-Lys$_{14}$ antibody raised in responder (top A, B and C) and nonresponder (bottom D, E and F) Hartley guinea pigs.

This system was used to test the specificity of antibody made in identically immunized nonresponders. As shown in Fig. 16D, E, and F, nonresponder anti-14ϵ, DNP-Lys$_{14}$ does not have a peak in binding energy with 14, ϵ, DNP-Lys$_{14}$ and cannot discriminate the homologous immunizing antigen from closely related peptides. Nonresponder antibody, despite its lack of specificity, does have an overall binding energy for DNP-peptides similar to that produced in responders. Antibodies to a number of DNP-oligolysines have now been studied in nonresponders and all lack the specificity found with responder antibody, i.e., they cannot discriminate the homologous immunizing antigen from closely related ones.

These studies suggest that the Ir gene negative animal lacks not only a population of T cells but a population of B cells capable of elaborating a highly specific antibody. Responders, on the other hand, appear to have specific receptors for defined DNP-oligolysines on *both* their B and T cells. Alternatively, one could argue that the difference in Ir + and Ir - animals (for the PLL gene) resides only in T cells, and that the difference in specificity of the antibody seen, reflects the manner in which T cells present antigen to the appropriate B cell. This view argues that the B cell pool is identical in responders and nonresponders. The lack of specific T cells in the Ir gene negative animals results in the selection of a different population of antigen-sensitive B cells. Thus, with the appropriate T cell, one selects a specific population of B cells, not selected for in the absence of specific T cells.

Recently Williamson and I have attempted to define the B cell pool in both responder and nonresponder animals. The extraordinary specificity of antibody made to defined DNP-oligolysines suggested that it would be of limited heterogeneity. We have now done isoelectric focusing on a large number of antisera to defined DNP-oligolysines, and as expected they are remarkable for their limited heterogeneity (Fig. 17). Approximately 50 guinea pigs have been immunized to α, DNP-Lys$_{10}$ and a smaller number to other defined antigens. On the average, each animal produces 2 clones of antibody as measured by isoelectric focusing, development with ^{131}I DNP-lysine and radioautography. The number of antibody clones to α, DNP-Lys$_{10}$ per animal varies from 1 to 3 and is, in general, independent of the adjuvant used. Complex antigens such as DNP-BGG or DNP-BSA, on the other hand, evoke a heterogeneous response characterized by a very large number of clones.

McDEVITT: What does Schlossman mean by qualitatively different antibody clones?

SCHLOSSMAN: We have looked at antibody formed as early as ten days after immunization and have followed the response for upwards of four months. Preliminary results suggest that the clonal pattern remains relatively constant for

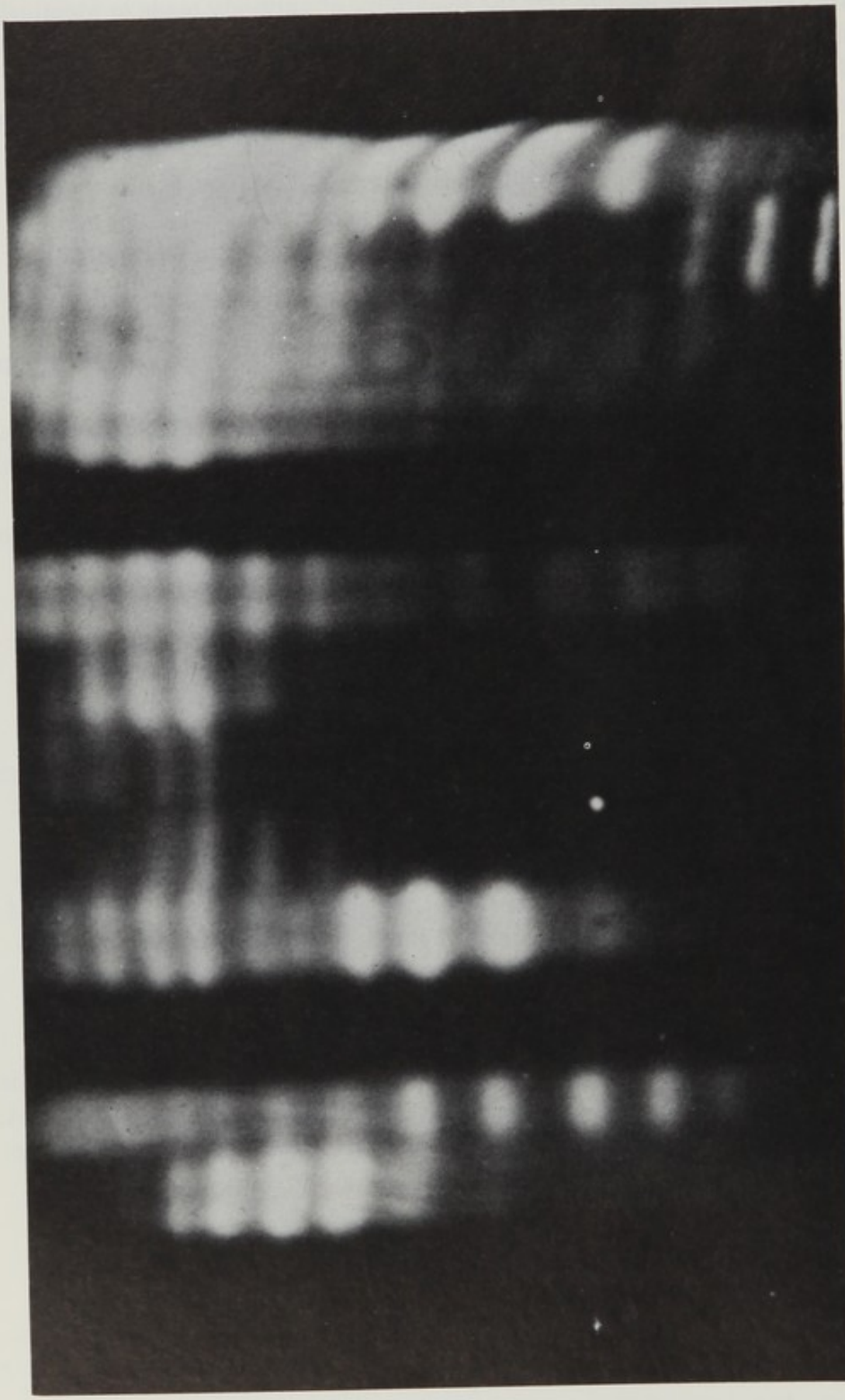

Fig. 17. Isoelectric spectrum of anti-α, DNP-Lys_{10} antibody. Each vertical pattern shows the antibody produced by a single animal. Note the repeat frequency of some clones and the homogeneity of the antibody response.

long periods of time following immunization. Most of our effort has been directed at defining the clonal pattern of antibody raised to α, DNP-Lys_{10} in responder animals with the view to determining the size of the V-gene pool.

At the same time we are looking at the clonal pattern formed in nonresponder animals to identical antigens. One of the problems we have encountered has been the lack of inbred guinea pigs at Mill Hill. We rely on the presence of delayed skin tests to defined DNP-oligolysines to assess responder status. The Mill Hill strain 2 guinea pigs are actually the Heston 2 line, whereas the strain 13 animals were derived from the NIH line. Despite the confusion in guinea pig pedigrees and the finding that some strain 13 respond, the results are very impressive.

Although we have immunized a large number of animals, only a small number have been analyzed carefully. Guinea pigs were injected with α, DNP-Lys_{10} in complete Freund's adjuvant containing *Mycobacterium butyricum, M. tuberculosis* H37 Ra (2 mg/ml), *M. tuberculosis* H37 Ra (10 mg/ml) and *M. tuberculosis* H37 Rv. Despite the variation in the amount of antibody produced, for the most part the number of clones induced is unrelated to the adjuvant used. At best H37 Rv, the strongest adjuvant used, increases the heterogeneity of the clonal response only slightly.

The overall data on the clonal character of these responses thus far obtained is summarized in Table 20. In one group of 12 responder guinea pigs immunized to α, DNP-Lys_{10} we have seen a total of 24 clones. In this group, one clone was seen in seven different animals. Others have been seen four, three and two times, respectively. A number of clones have only been seen once. We obviously need more data to determine the size of the V-gene pool in these animals but the frequency of identical repeats suggests that it will be small and in keeping with a germ-line mechanism.

WILSON: How many clones were present in the nonresponders?

SCHLOSSMAN: Fewer nonresponders have been analyzed. In fact we have good data on only five animals where a total of nine clones were seen (again averaging 2 per animal). More importantly in the nonresponder group of five animals, two different clones were repeated three times (Table 20). The frequency of repeats

TABLE 20

Isoelectric Focusing of Anti-α DNP Lys_{10}, Antibody Raised in Guinea Pigs

Immune status of guinea pigs	No. of clones-n times						
	1	2	3	4	5	6	7
All guinea pigs tested (17)	14	1	2	1	-	-	1
Responder (12 animals)	10	2	1	-	-	-	1
Nonresponder (5 animals)	5	-	2	-	-	-	-

among nonresponders is high and similar to the repeat frequency found with responders.

An important question relates to the frequency of repeats between responders and nonresponders. There are repeats between responders and nonresponders, but we do not have sufficient data to define the overlap in V-gene pools for these animals. More important, we have not defined which antibody clones account for specificity and whether there will be an overlap within this group. Specificity studies suggest that responders will have clones not present in nonresponders but these studies might miss small amounts of highly specific antibody in the nonresponders.

McDEVITT: By repeat, does Schlossman mean identical?

SCHLOSSMAN: In all probability they will be identical, but our data is still based only on identical isoelectric focusing migration.

In view of their pedigrees and lack of complete inbreeding in the animals we have studied, it is not surprising that responders and nonresponders share identical V-genes. It is, however, too soon to tell whether the B cell pools in these animals will be completely identical.[a]

[a]EDITORS' COMMENT: Since the editors were aware of the fact that the work referred to in Schlossman's discussion was completed shortly after the Brook Lodge meeting, they invited Schlossman to submit a brief addendum which would permit his results to be included in their final form. The results are as follows:

1. 36 guinea pigs immunized with α, DNP-Lys_{10} yielded 57 distinct clones by the method of isoelectric focusing.
2. 20 responder animals produced 39 separate clones; 13 nonresponder animals produced 18 separate clones.
3. 1 clone was seen 10 times in 10 individual responder animals and the same clone, by the criteria of isoelectric focusing, was seen 7 times in nonresponder animals.
4. Other clones were repeated 6,4,3,3,3,3 times, and all of these clones were equally divided between responder and nonresponder animals—i.e., each of these clones which was seen in more than one animal was seen in both the responder and nonresponder animals.

Thus, the antibody clones seen in responder and nonresponder animals appear to be identical and the variable region gene pool for antibodies to α,DNP-Lys_{10} appears to be identical in the responder and nonresponder animals. Schlossman informs us that he cannot account for what he considers to be real differences in nonresponder and responder antibody made to defined peptides that he referred to in the initial part of his discussion. He feels that this might be accounted for by a small fraction of antibody that is not readily detected on isoelectric focusing. However, Schlossman believes that these results resolve one major problem; that responders and nonresponders, by and large, have an identical B cell population, except perhaps for a small pool of antibody clones present in responders, either as a result of selection by the appropriate T cell, or existing in responders as an absolute difference between responders and nonresponders. This finding is to be contrasted with a totally different T cell response to the same antigen.

BODMER: I would like to follow up the discussion that McDevitt initiated earlier on the question of the genetic control of the various immune response phenomena, and whether Ir genes function both on B and T cells. The problem is that one is trying to establish the genetics of possible effects on B and T cells just by looking at the associations between different strains, or different animals, and various immunological parameters associated with T and B cell function. I think one has to be extremely cautious in this simplistic kind of approach. The first point that has been made, which applies especially in Schlossman's case, where he doesn't have congenic lines, is that he certainly does not know whether the genetic differences are the *same* differences that are linked to the major histocompatibility locus.

But even in the case where one has congenic lines, to talk about *the* Ir gene, as I am sure we will hear in the next session, may be a misnomer. One knows that there are a very large number of genes in that region. Because they are closely linked, one may be dealing with a *set* of genes affecting B cells and a *different set* of genes affecting T cells; both could be H-2 linked but not separated in the particular strain one is looking at. Until one has been able to do refined fine structure analyses that separate out different regions within H-2 and allow one to make strains that are congenic with respect to *everything*, it cannot be said that the *same* genes effect T cells and B cells.

I consider this to be an extremely important issue. During all our discussions we are looking at correlations between different sets of animals with a particular set of markers—the "H" antigens, for example, which are not the ones actually causing the effect being studied. The problem is similar to the one we were discussing earlier, regarding the association in the random-bred guinea pigs between the histocompatibility type and the response to certain antigens. Sometimes the association is good and sometimes it is not so good. The reason is that in some cases we may have started with an initial stock of genes which are very restricted in terms of the number of recombination products. There has not then been time to build up all combinations, so we find the association. In other cases the initial stock had lots of different combinations which are still there later, so we don't find the same strength of association. In other words, Schlossman is looking at associations due to linkage. Without going into some of the algebra of this problem, I simply want to emphasize that this is a complicated question, and that very closely linked genes eventually will be associated at random, in random populations, but the length of time it takes for this to occur is long. So my message is that this really has to be taken into account when one is looking at different genes that are closely linked. They may still be associated

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Schlossman interprets these results as supporting the hypothesis that the Ir genes are expressed exclusively in T cells.

quite persistently for a long time. Thus, I think we have to be careful in talking about the same genes affecting both B cells and T cells.

SCHLOSSMAN: I agree that this is still a problem in guinea pigs but may be resolved by development of better inbred lines as well as antibody studies in outbreds to determine linkage to histocompatibility antigens.

BODMER: That may be. But the logical point is extremely important; namely whether the same set or group of genes can be expressed at one and the same time in both B and T cells, or whether, in fact, we have one set of genes expressed in T cells and another set in B cells. This is, to my mind, an important logical distinction, because it asks the question of the control of expression of these genes. In one case, they are restricted to the different types of cells, even though they are closely linked, while in the other case the same set of genes can operate in both types of cells. I think that these two possibilities have not been distinguished by any of the data yet presented.

CHAIRMAN SIMONSEN: Would Bodmer like to tell us what experiments he suggests?

BODMER: I think we have to use the strains made by the investigators working on H-2 in which they have isolated different segments of the H-2 region and made mouse strains which are congenic with respect to these segments. Then we may answer this question. Of course, it follows that we must also identify the gene product.

CHAIRMAN SIMONSEN: But may these regions have the H-2 complex and not comprise many genes?

BENACERRAF: I would like to compliment Schlossman on a beautiful experiment which is in a sense a clear example of what I said in my introductory remarks. To observe B cell genetics, one must immunize with antigens capable of inducing antibody responses with limited heterogeneity, which is what Schlossman has done so expertly. The pattern of clonal responses to such an antigen by two inbred strains of animals such as strain 2 and 13 guinea pigs are expected to differ, since their genome differs, which is what Schlossman has observed. However, before making the conclusion that the PLL gene is responsible for the clonal pattern observed in responder animals immunized with DNP-oligolysines, the demonstration must be made that: 1. the strain 2 pattern is inherited together with the PLL gene in back-cross animals; or 2. that the pattern observed in strain 2 is also detected in PLL-responder random bred animals.

SCHLOSSMAN: We have not analyzed antibody clones made in Ir positive outbreds, and with back-crosses. Sera have been obtained in outbreds immunized to α, DNP-Lys_{10}, but they have not been analyzed.

I think one point might be stressed; whenever we see this extraordinary specificity of the B cell product (and presumably the receptor), we see a similar level of specificity at the T cell. When it is lacking at the T cell, as in Ir negative animals, it seems to be missing also at the B cell. One surprise has been that Ir negative animals (PLL nonresponders) produce such restricted antibody. I had thought that in the absence of T cells we would find a more heterogeneous antibody response. The restriction of heterogeneity in the nonresponder is equivalent to that of the responder.

NISONOFF: What are the approximate antibody levels in terms of micrograms per ml?

SCHLOSSMAN: I can't answer that precisely, but in earlier studies it ranged between 100-500 micrograms of antibody per ml, depending on the adjuvant used.

SELA: Working with DNP-protein antigens in mice, Williamson has shown a tremendous number of structurally unique proteins capable of binding dinitrophenyl haptens. Did Schlossman similarly check 15 or 20 different guinea pigs, by injecting them with a dinitrophenyl protein? Is it possible that in the guinea pigs the pool of different clones producing antidinitrophenyl antibodies is much more restricted and that he would find many more similar clones than in mice?

SCHLOSSMAN: Williamson and I looked at antibodies to both DNP-HSA and DNP-BGG in the guinea pig and found that these antigens induced a very large number of clones. In contrast, we have immunized with many defined DNP-peptides and found that the clonal pattern is *always* restricted. Still to be resolved is whether α, DNP-Lys_{10} induces the same clones as would be produced with α, DNP-Lys_{12}, for example. This would not be expected, inasmuch as we can distinguish differences in the specificity of these antibodies.

GREEN: I would like to call attention to another difference between responders and nonresponders in terms of their response to DNP poly-lysines, or DNP-PLL-BSA. I refer to the time of appearance of the antibody. In animals that are responders to DNP-PLL this occurs in about 5 days before the appearance of anti-DNP antibody in nonresponder animals that are responding to DNP-PLL-BSA. There is, thus, still another significant difference in the way individual animals look at these particular antigens.

I would like to query Schlossman in terms of what Benacerraf showed us earlier in this session, viz., that any hapten coupled to PLL can have antibody made against it in responder animals. How does this fit with the idea that the

polylysine gene may operate in B cells, since the many different haptens would have many different diverse antibodies produced in response to them?

SCHLOSSMAN: In specificity studies we have analyzed responder antibody to about 15 peptides. All animals produce antibody which, by quenching studies, can be shown to discriminate the homologous from heterologous antigens. This suggests that the total V-gene pool may be very large. However, the cloning studies comparing patterns with different peptides might help us resolve this problem.

BODMER: Yes, but at least we could get a positive answer. I would envision that it could be found that one does separate the B cell and T cell functions in some of them. One could still say that if he doesn't, he is not dissecting the region finely enough.

CEPPELLINI: I mentioned earlier a simple experiment which we should have done a long time ago and Green can easily do now. It involves differentiating H antigens from other possible structures expressed by Ir genes. The approach would be to absorb allogeneic antisera with nonlymphoid tissues, *viz.* fibroblasts. We should be mindful, however, that humoral antibodies developed after kidney transplantation are able to block MLC, and presumably, Ir expression in Green's model; at least that is what I would expect. If not elicited by passenger lymphocytes, the blocking activity of these antibodies would suggest either that blocking is through H antigens or that MLC (and by implication Ir) genes are also expressed on renal tissues.

I want to leave this conference with well-educated B (for brain) cells; therefore, let me pose another naive question. MLC in a "virgin" responder, i.e., in a subject who, as far as is known, has not been immunized to allogeneic H antigens by pregnancy, transplantation, etc., corresponds to an *in vitro* primary response. At any rate, such a subject is not producing humoral anti-H antibodies.

I am prepared to accept that MLC response is solely due to T cells. But *in vitro* activation by soluble antigens, such as tuberculin or tetanus toxoid requires previous *in vivo* sensitization, which is often accompanied by production of humoral antibodies. Can we be sure in the second situation that only T cells are involved? Can we *really* be sure that B cells do not play any role. We need to have an answer on that point.

GREEN: Yes, I believe I can respond to Ceppellini's query with cogent data. In collaboration with Elfenbein and Shevach at the NIH, we did a study of guinea pig lymph node cells which were undergoing blast transformation, in response to ovalbumin. Following three or four days of culture *in vitro* with the antigen, we could detect complement receptor lymphocytes (CRL), (a function of B lym-

phocytes), and simultaneously demonstrate incorporation of tritiated thymidine, a presumed T cell function. We then sedimented the cells on slides and looked for cells that had grains (T cells) and the cells which had rosettes (CRL positive lymphocytes or B cells). It is noteworthy that roughly 20% of all the grained cells *also* formed rosettes. The answer is, therefore, that when one does these lymph node cell cultures in guinea pigs, a significant proportion of the total ^{3}H thymidine incorporated is into *B* cells and, therefore, the amount of proliferation in these cultures should not be construed as an exact measure of T cell function as it may involve variable numbers of B cells. I am sure if this experiment were done in rabbits, one might get a possibly higher and thus even more different answer.

UHR: Since antigen-antibody complexes can stimulate lymphocytes, is it possible that in Green's work B cells were stimulated nonspecifically by ovalbumin-antiovalbumin complexes rather than specifically by ovalbumin?

GREEN: That is a possibility that cannot be ruled out entirely. This is however, inherent in any system in which one uses the mixed guinea pig lymph node cell population which contains the cells secreting antibody. I am, therefore, not saying precisely by which mechanism this B cell stimulation occurs. I am only putting on record the fact that the number one obtains with ^{3}H thymidine incorporation, also reflects *some* proliferation of B cells. I agree with Uhr that the mechanism he suggests is possible, but it is nevertheless inherent in any antigen system. In *in vitro* systems one has a small number of cells producing antibody. This antibody will complex with the antigen and the immune complexes are interacting to a greater or lesser degree.

CHAIRMAN SIMONSEN: As we approach the close of this session, it seems to me, in view of the information presented and our critical discussion, that no one would now question that Ir genes are in fact expressed in T cells. On the other hand, there is far from complete agreement as to whether they may also be expressed in B cells.

I would ask Benacerraf and Green, who gave us the basic information at this session whether they have been convinced by the evidence and arguments presented by others that Ir genes may also be expressed in B cells?

BENACERRAF: The "H"-linked Ir genes appear to affect in some cases the specificity of the antibody population produced. There are many examples of this phenomenon, which indicates that the gene has some influence on the specific recruitment of B cells. However, there are several possible explanations for this phenomenon besides the necessary expression of the Ir gene product on the

surface of B cells, as I stated in my opening remarks. Nevertheless this possibility must be seriously considered.

CHAIRMAN SIMONSEN: Would Green agree with Benacerraf's reply?

GREEN: I tend to favor the idea that the immune response genes are expressed in T cells either solely or predominantly, and that the influence one sees on antibody specificity has to do with the presentation of, or interaction with, antigen.

Thus, the antigen is complicated, having many parts as different as the carrier portions and the haptenic determinants. Accordingly, I feel that the immunological machinery represented by the Ir gene may recognize a specific site on the antigenic molecule as the site of the carrier function, but the specificity of antibody to this segment (the hapten) near the carrier site will be favored, and therefore, the precise specificity that the B cell recognizes is in a sense determined by the specificity of the immunological machinery in terms of which site the Ir gene carrier function recognizes on the antigen. In my view, this can explain variations in the fine specificity of the antibody to the determinant without necessarily invoking Ir genes on B cells. I freely admit, however, that this view reflects my personal prejudice.

CHAIRMAN SIMONSEN: Would Schlossman concur?

SCHLOSSMAN: I would agree with Green to the extent that he admits prejudice and bias. I personally believe that more data must be collected. I would recap for you that in our system the affinities of the antibodies are identical in responders and nonresponders; the frequency of repeats in the nonresponders suggests that the nonresponders have their own pool of V-genes and that the T cell has probably no role in B cell selection, but does magnify the intensity of the B cell response. The Ir gene, at least in the responders, exerts its effect both in T and B cells.

CHAIRMAN SIMONSEN: Two more conferees whose views I would particularly like to have on record are Sela and McDevitt.

SELA: I think, first of all, that Chairman Simonsen's question was imprecise, because it would be better to ask about Ir responses which are H-2 linked, or "H"-linked. I think this is what he assumes, namely whether "H"-linked responses are expressed in T cells or B cells, or in both of them.

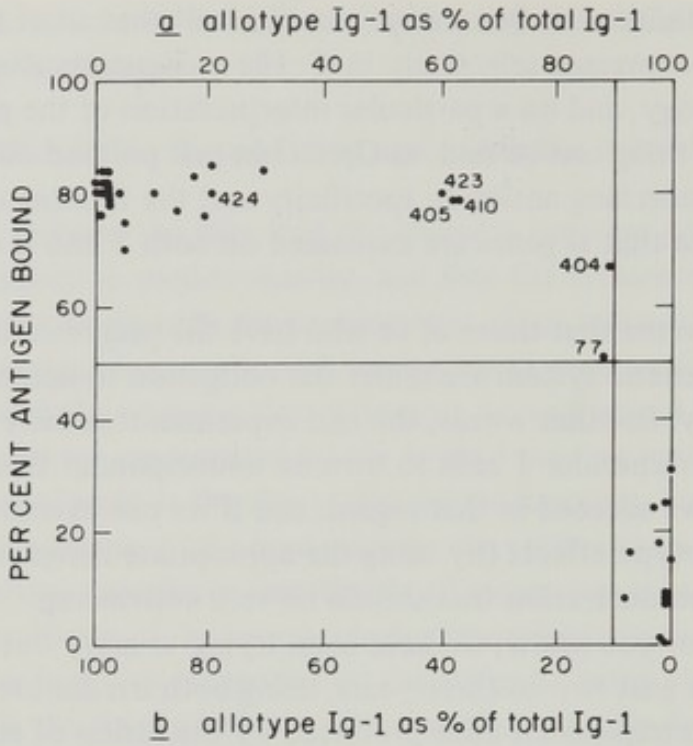
I would say, based on the pool of impressions at this first session, as well as on one's own load of—call it prejudice or opinion—that the T cell is definitely (at least in many cases) the main culprit. I would, however, also consider that cell-to-cell cooperation involving in some indirect way B cells could also play an important role. In other words, I am not yet giving up in the matter of B cells.

McDEVITT: I think even Benacerraf would admit that all of the evidence that the Ir genes are expressed selectively in T cells is circumstantial, indirect, based on phenomenology, and on a particular interpretation of the phenomenology.

The same thing can be said, as Green has just pointed out, about much of the evidence concerning antibody specificity and the limiting dilution assays, so that the evidence that Ir genes are expressed on both T and B cells is likewise circumstantial.

It seems to me that those of us who have the prejudice that Ir genes are solely a T cell genetic system are under the obligation to demonstrate that in an unequivocal way. In other words, the real experiment we have to do is to show that we *can* get responder T cells to turn on nonresponder B cells to make specific antibody. If we succeed in that regard, and if we can exclude allogeneic effects, and exclude allotype effects (by using the appropriate congenic strains), then we would have a demonstration that should be very convincing.

As many of you know, we have been trying to carry out this very experiment during the past two or three years, using both irradiation chimeras, and most recently, tetraparental mice produced by the fusion of eight-cell cleavage stage embryos from two different strains followed by reimplantation into the uterus of a pseudo-pregnant recipient. Until very recently, all of the evidence indicated that, in tetraparental mice created by the fusion of embryos derived from responder and nonresponder strains, all of the antibody that was produced was of the responder allotype, even in those situations in which there was a considerable amount of nonresponder immunoglobulin present in the nonantibody fraction of the serum immunoglobulins. I hesitate to discuss preliminary results in an area which I consider crucial to the resolution of this question, but some of our most recent evidence indicates that this may not always be the case. In the last group of 44 tetraparental mice, which were produced by fusion of one embryo from the C3H low responder strain, and one embryo from the congenic CWB high responder strain, the results are somewhat different. The majority of the animals turned out to be predominantly of either the responder immunoglobulin allotype and responders to (T,G)-A--L, or predominantly of the nonresponder immunoglobulin allotype and nonresponders to (T,G)-A--L (Fig. 18). However, there are seven or eight animals which have between 20 and 80% of their total serum immunoglobulin contributed by the nonresponder immunoglobulin allotype, and which are high responders to (T,G)-A--L. Of these, five have 50 to 80% nonresponder immunoglobulin, and in these five animals there are readily detectable amounts of specific anti-(T,G)-A--L antibody of the nonresponder immunoglobulin allotype. The amount of specific anti-(T,G)-A--L antibody of nonresponder allotype in these animals is considerably more than we ever find in the intact nonresponder, and this suggests, of course, that responder T cells *can*, in fact, interact with nonresponder B cells to result in



Tetraparental	total serum Ig-1 %a	%b	specific anti-(T,G)-A--L %a	%b	anti-(T,G)-A--L response level
404	89	11	68,60	32,40	67
405	62	38	35,30	65,70	79
410	62	38	13,10	87,90	79
423	60	40	47	53	80
424	20	80	31	69	79

Fig. 18. Analysis of the allotype of total serum immunoglobulin and specific anti-(T,G)-A--L antibody in C3H↔CWB tetraparental mice. *Top:* The percent antigen bound of ^{125}I-(T,G)-A--L following standard immunization of tetraparental mice is shown on the vertical axis. The lower horizontal axis plots the percent of *b* (responder) Ig-l (γG_2 a) allotype as percent of total Ig-l. The upper horizontal axis is a reciprocal of the lower horizontal axis and plots the percent *a* (nonresponder) Ig-l (γG_2 a) allotype as percent of total Ig-l. There is a distinct correlation between the presence of the responder allotype and responder status (responder status = > 50% antigen bound). All mice with a large amount of responder allotype are responders—no animal with less than ten % responder allotype gave a high response to (T,G)-A--L. *Bottom:* This table gives the *a* (nonresponder) and *b* (responder) composition of the specific anti-(T,G)-A--L antibody of five tetraparental mice shown in the plot above. All five animals are responders to (T,G)-A--L and have readily detectable anti-(T,G)-A--L antibody of nonresponder allotype. Where given, duplicate figures represent the results of separate runs through an anti-*a* allotype-Sepharose immunoabsorbent column.

the production of specific anti-(T,G)-A--L antibody. If we can exclude the allogeneic effect described by Benacerraf and Katz, this preliminary result would suggest that the Ir genes are expressed *exclusively* in T-cells. Before we can be

certain of this result, the allotype determinations have to be repeated several times and experiments have to be carried out to exclude the allogeneic effect. Even in these congenic tetraparental animals, there is a tendency to produce more antibody of the responder allotype than would be predicted on the basis of the allotype ratios in the total serum immunoglobulin. For example, an animal which has 80% of its total serum immunoglobulins of the nonresponder allotype, may have only 30% of its specific anti-(T,G)-A--L antibody of the nonresponder immunoglobulin allotype, while the remainder is of the responder type.

We suspect that there is a very real reason for this—namely that responder T cells can interact less efficiently with nonresponder B cells across a homozygous H-2 barrier, than with responder B cells. In other words, there is a preferential interaction between T and B cells of the same H-2 type. To test this hypothesis, we are now producing tetraparental mice which have a homozygous difference in immunoglobulin allotype markers, but have only a heterozygous difference at H-2. If our prediction is right, these animals should have readily detectable amounts of nonresponder anti-(T,G)-A--L antibody, reflecting our hypothesis that T-B cell interaction will occur more efficiently if the responder T cells share at least one H-2 allele with the nonresponder B cells.

CHAIRMAN SIMONSEN: Is Benacerraf in agreement on this matter?

BENACERRAF: In this opening session there is still doubt amongst us whether "H"-linked Ir genes *are* necessarily expressed in B cells. There is no doubt that they are necessarily expressed in T cells where they perform a specific function. In the third session of this conference we shall hear of allotype-linked Ir genes which control immunoglobulin specificity and are necessarily expressed in B cells; there is doubt that such allotype-linked Ir genes are expressed also in T cells. In summary, we have to deal with two specificity systems, each with its genetic control and its preferential expression in a class of immunocompetent cells. I repeat that I think we can leave it at that.

SELA: I would like to suggest that one way to approach the problem would be to find out whether it is possible to detect an H-2-linked genetic control using a genuinely thymus-independent antigen.

WARNER: In response to Sela, I would like to stress the point that I don't believe we are really sure that there is *any* absolutely thymus-independent antigen, at least not in terms of the IgG response, which is mostly concerned in these H-2-linked immune response studies.

COHN: I want to put the following proposition to Benacerraf: If somebody were to demonstrate that a B cell response was affected by the Ir gene, in what way would that change your position?

BENACERRAF: It has been demonstrated already, so it doesn't change a thing.

COHN: Therefore, you are saying it is of no use trying to demonstrate that B cells are affected by an *Ir-1* gene mutation, because in any case you would postulate that there is a receptor even on B cells which is not immunoglobulin-related and which is coded for by the *Ir-1* gene. Is that correct?

BENACERRAF: I have repeated several times that there is now evidence from several laboratories that the specificity of B cells may in some systems be affected by an "H"-linked Ir gene which is predominantly expressed in T cells. The problem we have to explain is: by what mechanism is the result achieved? I believe that the various possibilities have been clearly described in the course of the discussion at this first session.

RAFF: I think this discussion has brought out why it would be very useful to study antigen-binding cells in responder and nonresponder animals. For example, it would be interesting to know whether antihistocompatibility sera could inhibit the binding of GT or GL to B cells in $(2 \times 13)F_1$ guinea pigs and whether nonresponder thymocytes bind the "nonresponse" antigens and what the effect of anti-"H" sera might be on such binding if it occurs. Has Green done such experiments?

GREEN: No, not as yet.

CEPPELLINI: In collaboration with Kunkel, we have done an experiment that may be relevant to Raff's question. Anti-HL-A sera prevent formation of the so-called "cold rosettes" a typical T cell reaction in man.

RAFF: Would Ceppellini please identify the experiment?

CEPPELLINI: As you well know, 60 to 70% of human peripheral blood lymphocytes bind SRBC even in absence of any demonstrable antisheep antibody. These rosette-forming cells do not show any Ig receptors by immunofluorescence, and thus, are classified as T lymphocytes. In our experiment, when we treat the lymphocytes with the four anti-HL-A sera for which they are positive (full house), then wash and set them up for rosette formation, binding of SRBC is reduced from 60 to 70% all the way down to 2 to 5%. This finding seems to be another piece of evidence suggesting that H antigens (or related structures) play a special role in the surface functions of T cells. In our test situation, results obtained with SRBC sensitized by antisheep antibody and B cells (chronic lymphocytic leukemia cells) do not seem to be at all inhibited by anti-HL-A sera.

RAFF: It may be, for example, that T cells recognize histocompatibility (and perhaps some other related or nonrelated) antigens by some mechanism other than Ig receptors. In contrast, T cell recognition of haptens and various protein antigens involves 7S IgM receptors. Wouldn't it be interesting if the binding and responses of T cells to "H" antigens and those antigens which give rise to responses controlled by "H"-linked Ir genes could be inhibited only by anti-"H" sera, while T cell binding and responses to other antigens could be inhibited only by anti-Ig?

BENACERRAF: You can bet your life that this proposition is being explored.

RAFF: Yes.

McDEVITT: Günter Hämmerling, working in our laboratory, has studied the binding of (T,G)-A--L to normal and immunized spleen cells from responder and nonresponder mice. It is clear that the majority of antigen-binding cells are B cells, and it is equally clear that anti-"H" antisera have no effect in inhibiting this binding of antigen to B cells. The picture with respect to T cells is much less clear and is currently being studied.

SESSION II

GENETIC ORGANIZATION OF H-2 AND ITS RELATIONSHIP TO THE Ir AND MLC GENES

The two locus model of H-2 – Mapping of Ir genes – Mapping of MLC genes – Genetic distinction between MLC and HL-A – Interrelationships amongst H-2, Ir, and MLC – Implications for the nature of the Ir gene product.

Editors' Foreword

The material presented in this session deals primarily with the genetic fine structure of the major (H-2) histocompatibility complex in the mouse. Much of the discussion requires an understanding of H-2 nomenclature and of the derivation of the recombinant H-2 chromosomes which were used to obtain most of the critical experimental data in this session.

We are dealing with an extraordinarily complex genetic region, and one that is as yet imperfectly understood. Even after considerable revision by the individual discussants and by the editors, the information presented in this session makes for slow and difficult reading. This difficulty is increased by the lack of a formal notation system for many of the genes described in this session, because our information on these genes is new and rapidly evolving. The editors, therefore, felt it advisable to include a note of caution to the reader and to attempt to resolve some of the inconsistencies in nomenclature that might confuse the reader who is not familiar with the immunogenetics of the H-2 complex.

The H-2 complex in the mouse is not a single gene, but a large chromosomal region which includes not only genes coding for serologically detected transplantation antigens, but also genes controlling a variety of other functions. In the current notation system there are two separate genes coding for the serologic transplantation antigens: *H-2K* and *H-2D,* shown in Fig. 17 in the discussion by Shreffler. The symbols *H-2K* and *H-2D* denote genes which are present in any H-2 chromosome in one of several different allelic forms. The allelic forms of each of these genes is denoted by a number, which represents a particular antigenic specificity detected by serological techniques. A given combination of numbered antigenic specificities for *H-2K* and *H-2D* are said to constitute a particular H-2 type. The H-2 type is then designated by H-2 followed by a small letter superscript, e.g., $H\text{-}2^a$ or $H\text{-}2^b$ or $H\text{-}2^k$.

This notation system then permits the designation of the H-2 type of a particular inbred strain by a relatively simple notation form. Unfortunately, many individuals refer to different H-2 alleles of different inbred strains. Since H-2 is actually a combination of at least two genes these are not alleles in the true sense, but a combination of alleles at at least two different loci. This is the origin of the designation of H-2 alleles as pseudoalleles. To avoid a hopelessly complex series of numbers designating particular alleles of the *H-2K* or *H-2D* loci, the aforementioned notation has been adopted, so that the particular allele of the *H-2K* or *H-2D* locus is designated by a small letter superscript identical to the superscript used to classify the H-2 type of the particular H-2 chromosome carrying the *H-2K* or *H-2D* locus to be specified. Thus, $H\text{-}2^d$ is said to be made

up of the *H-2K*^{d} and *H-2D*^{d} alleles. Confusion will inevitably arise when the reader considers *H-2*^{a}. Since this strain is thought to represent a natural recombinant between *H-2*^{k} and *H-2*^{d}, it is considered to carry the *H-2K*^{k} in combination with the *H-2D*^{d} allele. Additional confusion will arise from the fact that some of the recombinant H-2 chromosomes derived from known crossovers between *H-2K* and *H-2D* are denoted by a double superscript letter, e.g., *H-2*^{oh} and *H-2*^{ol}. In this case, the h and l denote the genotype at the *Ss* locus in the center of the H-2 complex and have nothing to do with transplantation antigen specificities. On the other hand, *H-2*$^{y\text{-}Sg}$ is an *H-2*^{y} recombinant chromosome derived by Stimpfling (Sg) and is so denoted to distinguish it from the *H-2*^{y} chromosome derived by Klein. The distinction is important because the crossovers resulting in the production of the two *H-2*^{y} recombinant chromosomes occurred at different positions in the H-2 complex. The confusion is further compounded by a series of rather arbitrary strain designations which sometimes reflect the H-2 type of the strain, and sometimes reflect other aspects of the genetic derivation of a particular inbred strain.

The reader would thus be hopelessly confused without a score card, and for this purpose the editors have taken the liberty of rearranging the information in Shreffler's Table 28 so that it presents the inbred strains carrying recombinant H-2 chromosomes, along with the chromosome designation and the designation of the derivation of each of the genes in the H-2 complex, more or less in the order and in the manner that they are taken up in the text of this session. There is no easy way through this table, and the reader must familiarize himself at least with the strain designation, the designation of the H-2 type of the recombinant H-2 chromosome, and the position of the crossover, which is based on the map given in Fig. 17.

Because no formal notation has yet been developed for assigning the numbered serologic antigenic specificities of H-2 to the *H-2K* or *H-2D* loci, we

Probable Composition of Some H-2 Recombinant Chromosomes

Type strain(s)	Recombinant chromosome	Parental combination	Region of H-2*			
			K	*Ir*	*Ss*	*D*
B10.A(1R); B10.A(2R)	h	a/b	k	k	d |	b
B10.A(4R)	h	a/b	k	k |	b	b
B10.A(3R); B10.A(5R)	i	a/b	b	b |	d	d
C3H.OH	oh	d/k	d	d	d |	k
C3H.OL	ol	d/k	d	d |	k	k
A.AL	al	d/k	k	k	k |	d
A; B10.A	a†	d/k	k	k |	d	d
A.TL	tl	al/s	s |	k	k	d
A.TH; B10.S(7R)	th	a/s	s	s	s |	d
AQR	y	a/q	q |	k	d	d
B10.T(6R)	y-Sg	a/q	q	q	q |	d

*Vertical lines indicate probable crossover position.

†Postulated recombinant.

have used the older system of designating a particular H-2 antigenic specificity as H-2.3 or H-2.33, etc. Since these specificities are clearly presented in diagrams as well as mentioned in the text, this should not be a source of great confusion.

The reader is also forced to deal with considerable complexity in the nomenclature for the immune response (Ir) genes. Initially, when it was discovered that the immune response to (T,G)-A--L was linked to the H-2 locus and associated with the $H\text{-}2^b$ allele, this gene was designated as immune response-1, following the accepted convention that genes are given official designations as soon as they are located in a particular linkage group. Immune response-1, or *Ir-1*, in the strictest sense could be designated as $Ir\text{-}1^{(T,G)\text{-}A\text{--}L^{lo}}$, or $Ir\text{-}1^{(T,G)\text{-}A\text{--}L^{hi}}$. In this notation, the *lo* and *hi* denote low and high immune responses to (T,G)-A--L. Since it is not yet known whether the Ir genes responsible for immune response to (H,G)-A--L and (Phe,G)-A--L are simply different allelic forms of $Ir\text{-}1^{(T,G)\text{-}A\text{--}L}$ or represent different loci, no formal nomenclature has been adopted for this particular set of genes. Throughout the discussion, *Ir-1* is occasionally used to refer to the immune response genes as a group and at other times used to refer to only that gene or those genes controlling the immune response to the three synthetic polypeptides, the structure of which was defined in "Glossary of Terms and Definitions for Session I". As additional immune response genes have been discovered, they have been given informal notations such as *Ir-IgA* and *Ir-IgG*, which represent genes controlling high or low response to the *a* allotype of IgA and γG_{2a} myeloma proteins. As Lieberman makes clear in her discussion, there is considerable evidence that the genes at *Ir-1* are separate from those at *Ir-IgA* and *Ir-IgG*. It does not seem justified at this point to construct a notation system clearly reflecting this separation since the matter has not been completely resolved by direct experiment.

CHAIRMAN McDEVITT: In any discussion involving the transplantation antigens of the mouse and particularly the major H-2 transplantation antigens of the species, we must acknowledge an enormous debt to Gorer, Snell, and the others who first began to study the genetics of the murine histocompatibility antigens, and who, over a period of many years developed the principles, the insights, the reagents, and the inbred strains which have contributed so much to our understanding of the number, range, and diversity of transplantation antigens. These early workers and the followers that they trained and inspired have continued to advance our understanding of transplantation antigens and cell-surface antigens. Their studies have established the existence of a large number of separate transplantation antigens, most of them now designated as minor or weak histocompatibility antigens. The continuing analysis of the major transplantation antigens has revealed an extraordinarily complex genetic region within the ninth mouse linkage group which appears to comprise a large number of closely linked genes controlling a variety of functional effects that seem related and may well reflect an underlying structural relationship and a remote common genetic origin.

Our purpose in this session is to review a series of recent findings which have clarified our understanding of the H-2 region and which have considerable bearing on the relationship between histocompatibility-linked genetic controls of the immune response and the genes controlling histocompatibility antigens themselves. We will depart from the usual format for these sessions by having three (instead of one) discussion introducers, each describing recent findings related to the genetic organization and functional expression of the H-2 region. Klein and Shreffler have recently developed a new model for the genetic organization of those parts of the H-2 region which affect the serologically defined H-2 antigens, and the antigens responsible for graft rejection. This model and the hypothesis and the assumptions underlying it will be presented by Shreffler.

The existence of a large number of histocompatibility-linked specific immune response genes suggests some type of causal or functional relationship for this remarkable association between genes controlling cell-surface antigens and genes controlling specific immune responses. A precise understanding of the genetic relationship between these two types of genes is essential to our understanding of this problem, and I will present studies carried out in collaboration with Klein, Shreffler, Stimpfling, and Snell, which have given us a fairly clear picture of the position of at least one immune response gene, *Ir-1*, within the H-2 region.

It has been known for some time that in addition to graft rejection antigens, the serologically defined H-2 antigens, and the specific immune response genes, the H-2 region also controls the expression of antigens responsible for the mixed lymphocyte culture (MLC) reaction. Bach and Klein have recently obtained new and exciting information on the relationship between the loci responsible for the

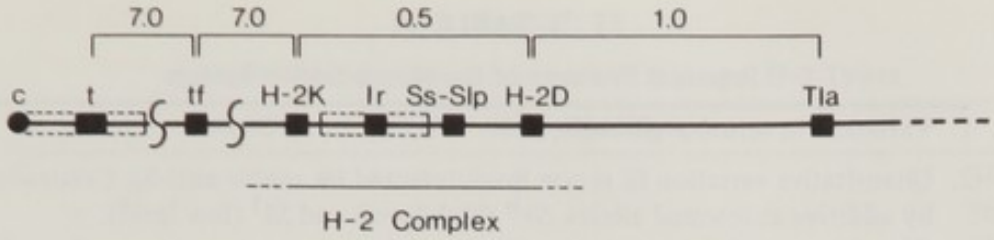


Fig. 19. Chromosome 17 (linkage group IX) of the mouse.

MLC reaction and the other genes which comprise the H-2 complex, which will be presented by Bach.

Taken together, the evidence from these separate but closely related studies suggests that the H-2 complex is a fairly extensive chromosome region including a large number of genes responsible for the serologically defined H-2 antigens; graft rejection antigens; the specific immune response genes; and genes for the antigens eliciting the MLC reaction *in vitro*. There are compelling reasons for believing that many of these phenomena may be functionally related, and this of course raises the speculation of a structural relationship among them as well. These possibilities will be taken up in our general discussion. We will now proceed with Shreffler's consideration of the two-gene model for the H-2 locus.

SHREFFLER: My primary function as I see it, is not to go over the detailed data on mapping of the H-2 region, but rather to assess in a general way our current view of H-2 genetic fine structure as a background for the understanding of the data on mapping of immune response genes and genes responsible for the MLC reaction.

Figure 19 shows our current fine structure map of the H-2 complex on chromosome 17 (linkage group IX) of the mouse. For those who have been reading the literature on H-2 over the years, note the centromere position at the left; the entire map must be reversed from the previous versions because recent data have shown that the centromere is at the K end of H-2. Linked to the H-2 complex are the equally complex t region, close to the centromere, and on the other side of H-2, the *Tla* locus, which controls the thymus-leukemia antigens and several other genes which we need not discuss.

Our current picture of the H-2 complex is rather different from that which has been published over the last 10 to 15 years. We now believe that all of the serologically detected specificities controlled by the H-2 complex are determined by only two genetic regions, *H-2K* and *H-2D*. Further, we think that the *H-2K* and *H-2D* regions are probably single genes, each controlling a single polypeptide which is the serologically detected H-2 antigen. The latter point is based primarily on the chemical evidence of Nathenson and others. I will return later to a consideration of our reasons for now thinking in terms of only two loci.

TABLE 21

Principal Features of the Mouse Ss-Slp System

1. Variation in serum β-globulin, M.W. 150,000 to 1,000,000.
2. Quantitative variation in serum level detected by rabbit anti-Ss. Controlled by additive autosomal alleles, Ss^h (high level) and Ss^l (low level).
3. Structural (allotypic) variation in Ss protein, detected by mouse anti-Slp. Controlled by autosomal dominant allele Slp^a (positive for antigen) and recessive Slp^o (negative for antigen).
4. Expression of Slp antigen normally confined to males, under control of testosterone. Induced in Slp^a/-females by testosterone.
5. *Ss* and *Slp* map within H-2 region. *Ss* and *Slp* have not been separated by recombination.

Allele(s)	Relative Ss level	Slp antigen (in males)
Ss^h Slp^a	20	+
Ss^h Slp^o	20	—
Ss^l Slp^o	1	—

Ss = serologically detected serum protein.
Slp = sex-limited protein.

Between the *H-2K* and *H-2D* loci, there are two additional genetic regions. The one which is most familiar to all of us is the Ir region, which controls at least some of the immune responses we have been talking about, and is also, as will be shown later, involved in the MLC reaction. I have indicated some uncertainty about the length of this Ir region. As will be evident later on, the Ir region is very probably composed of a number of distinct loci mapping between *H-2K* and *Ss-Slp*. In this regard, I might point out that the recombination frequency between the *H-2K* and *H-2D* loci is of the order of 0.5%. One can calculate the number of DNA base pairs which that recombination frequency represents, assuming that the recombination frequency in this region is directly proportional to the physical length of DNA in this segment of chromosome. This amounts to approximately 500,000 base pairs, or enough for about 500 genes of 1,000 base pairs each.

Also located between the *H-2K* and *H-2D* loci is the *Ss-Slp* region (cf. Table 21).

The *Ss-Slp* system involves two traits, the Ss serologically detected trait, and the Slp sex-limited protein trait. Both of these traits involve variations in a specific serum β-globulin. The Ss variation is a quantitative variation, detected with a rabbit antiserum by immunodiffusion. The variation is controlled by alleles Ss^h, which determines a high serum level and Ss^l, which determines a low serum level of Ss protein. The Slp variation is allotypic, detected by alloimmune mouse anti-Slp, which is produced by immunizing mice of one Slp type with Ss

TABLE 22

Some Antigeneic Specificities of the Principal H-2 Types

Chromosome	H-2 Specificities														
a	1	–	3	4	5	6	8	–	23	–	–	–	–	35	36
b	–	2	–	–	5	6	–	–	–	–	–	–	33	35	36
d	–	–	3	4	–	6	8	–	–	–	31	–	–	35	36
k	1	–	3	–	5	–	8	–	23	–	–	32	–	–	–
q	1	–	3	–	5	6	–	17	–	30	–	–	–	–	–

protein from a genetically different type. This allotypic variation occurs in the Ss protein. The variation is controlled by an autosomal dominant allele which we call Slp^a, which determines the presence of the allotype, and a recessive allele, Slp^o, which determines its absence. An interesting point about the Slp allotype is that it is sex-limited in expression. It is normally restricted to males. Its appearance is under control of testosterone and the antigen can be induced in females with the Slp^a allele by administration of testosterone. Ss and Slp map between the *H-2K* and *H-2D* regions. This is based upon data from 18 intra-H-2 recombinants which are entirely consistent in locating *Ss-Slp* in this position. So far it has not been possible to separate the *Ss* and *Slp* traits by recombination, i.e., they behave as though controlled by a single genetic locus. However, they could be controlled by two closely linked loci, and our present guess would be that we are dealing with two linked, related loci for these two different traits. *Ss-Slp* differences do not play any discernible role in histocompatibility, as we have tested by skin grafting a number of strain combinations which differ only in the *Ss-Slp* region and find no graft rejection.

I might say that the listing of these four regions on the map is not meant to exclude the possibility of more regions, i.e., more genes, between *H-2K* and *H-2D*. For example, there could be additional loci controlling antigens that might be detected by skin grafting, but which are not serologically detectable, or there could be genes for other unrelated traits. What has been called the two-gene model for the H-2 complex is concerned only with the positioning of the genes controlling serologically detected specificities.

Now I would like to consider the genetic control of the serological specificities. Table 22 shows a very simple H-2 chart, which depicts a few selected H-2 types and their associated antigenic specificities. The point is simply that associated with each different H-2 chromosome or haplotype there is a series of different alloantigenic specificities, for the most part detected with relatively monospecific antisera by hemagglutination tests or cytotoxic tests. These sets of specificities, as you know, can be separated by genetic recombination. This was shown first by Gorer and his associates more than 15 years ago.

TABLE 23

Arrangement of the H-2 Specificities by Region

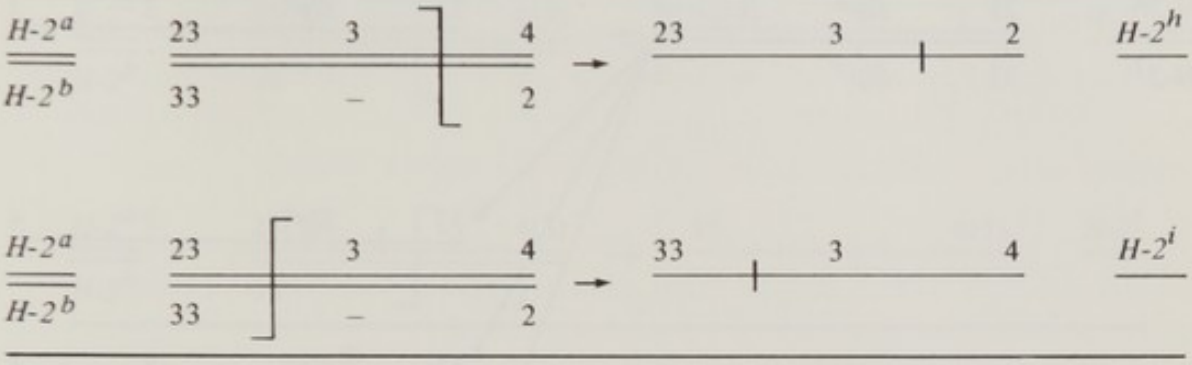
	H-2 K Region							H-2 D Region						
Chromosome	Private	Public						Public						Private
a	23	1	3	5	8	–	–	–	3	–	6	35	36	4
b	33	–	–	5	–	35	36	–	–	–	6	–	–	2
d	31	–	–	–	8	–	–	–	3	–	6	35	36	4
k	23	1	3	5	8	–	–	1*	3*	5*	–	–	–	32
q	17	1*	?	5	–	–	–	?	3	?	6	–	–	30

Under our two-locus model, we have divided these specificities into two groups, an *H-2K* set and an *H-2D* set, on the basis of recombination. Table 23 shows an example of the type of H-2 chart we are now using. There are two things on this chart I wish to emphasize. The first is that each different *H-2K* or *H-2D* region, on a different chromosome, is characterized by a different *private* specificity. By private specificity I mean a specificity unique, or almost unique to that particular chromosome. In addition, a number of so-called *public* specificities, which are shared by many different H-2 types, are associated with each *H-2K* or *H-2D* region. For example, H-2.1, 3, 5, 6, etc., are shared by a number of different *H-2K* region antigens.

The $H\text{-}2^a$ chromosome is almost certainly an example of a recombinant chromosome; that is, it appears to be made up in part of chromosomal material derived from the $H\text{-}2^k$ chromosome and in part from the $H\text{-}2^d$ chromosome. The recombinational event must have occured in a mouse which was an $H\text{-}2^d/H\text{-}2^k$ heterozygote, with the actual recombinational event taking place between the K and the D ends of H-2. Note that the *H-2K* region of $H\text{-}2^k$ is identical to the *H-2K* region of $H\text{-}2^a$. Note that the *H-2D* region of $H\text{-}2^d$ is identical to the *H-2D* region of $H\text{-}2^a$. In fact, this is the origin of the K and D region terminology. $H\text{-}2^a$ was originally envisioned as a recombination between chromosomes $H\text{-}2^k$ and $H\text{-}2^d$, having two chromosome segments, one from $H\text{-}2^k$, the other from $H\text{-}2^d$. The second and key point about this chart with respect to the new model for the H-2 complex is the fact that some specificities seem to occur in *both* regions, namely, H-2.1, 3, 5, 35, and 36. This was the fundamental assumption which led to the clarification of what was previously a very complex picture. We now believe that certain public specificities may be determined by either the *H-2K* or the *H-2D* loci of different chromosomes (e.g., H-2.35 of $H\text{-}2^b$ and $H\text{-}2^d$), or in some cases, by both genes on the same chromosome (e.g., H-2.3 of $H\text{-}2^a$). To state the principle in another way, we think that the antigens determined by certain alleles at the *H-2K* and *H-2D* loci may crossreact serologically, and these crossreactions are reflected in certain public specificities.

TABLE 24

Original Positioning of Specificity 3 Based on Gorer's Recombinants, $H\text{-}2^h$ and $H\text{-}2^i$



Over the years there were not only K and D regions, but C and V and E and A regions postulated within the H-2 complex. The single assumption of K-D cross-reactivity has permitted us to eliminate all of these regions, because the C and V and E and A regions involved the specificities H-2.1, 3, and 5, which are represented in the chart as crossreactive (by asterisks).

Now I want briefly to focus on the evidence that led to this new concept. Table 24 shows two of the original recombinants detected by Gorer *et al.* in 1955, $H\text{-}2^h$ and $H\text{-}2^i$, both derived from the heterozygous $H\text{-}2^a/H\text{-}2^b$ combinations. I have represented on this table only the private K specificities and the private D specificities of these two chromosomes. Gorer and his associates found that the $H\text{-}2^h$ type recombinant had the private specificities H-2.23 of $H\text{-}2^a$, the private specificity of H-2.2 of $H\text{-}2^b$, and a public specificity H-2.3.

At that point in time it had not been recognized that some specificities were private and some were public. It was found that $H\text{-}2^i$ had just the opposite specificities with respect to $H\text{-}2^a$ and $H\text{-}2^b$, i.e., H-2.33 from $H\text{-}2^b$ and H-2.4 from $H\text{-}2^a$, and once again the specificity H-2.3 was present.

Under the standard approach that one would use in mapping a three-point genetic cross, it was concluded that, assuming a single crossover as the origin for each of these two recombinant types, one must position specificity three in the middle of the H-2 map, in order to obtain the two observed recombinants. This was reasonable enough at the time. Later Stimpfling confirmed the Gorer observation with respect to the $H\text{-}2^h$ and $H\text{-}2^i$ recombinants, finding a total of seven recombinants of either the $H\text{-}2^h$ or $H\text{-}2^i$ type, and showed again the same separations of the K and D specificities. He also noted at that point that all of the recombinants which had been found from this $H\text{-}2^a/H\text{-}2^b$ heterozygous combination were positive for H-2.3, even though one would expect, if this map is correct, that equal numbers of recombinants with 3 and without 3 should occur. The type without 3 was not detected.

TABLE 25

Inconsistency in the Position of Specificity 3 Revealed by Typing Recombinants for Slp

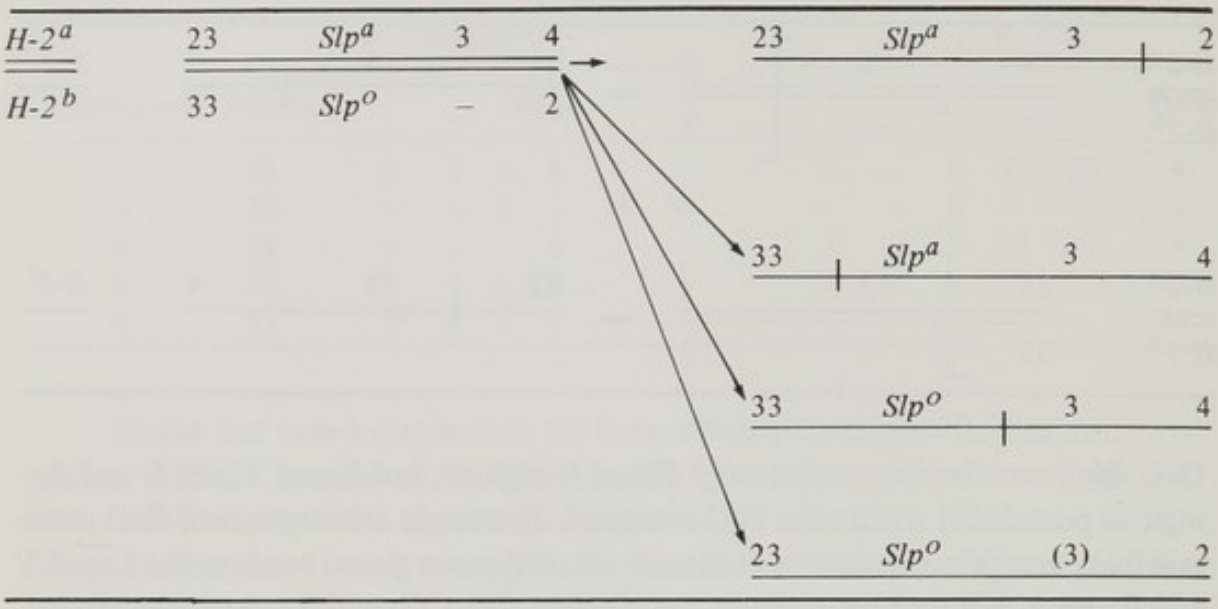


Table 25 discloses a further difficulty with the map which arose when the $H\text{-}2^h$ and $H\text{-}2^i$ recombinants were classified for the Slp trait. It was found that the $H\text{-}2^h$ and $H\text{-}2^i$ recombinant types could be classified into two sets, one Slp positive and one Slp negative. As already indicated, the evidence positioning Slp between K and D is quite conclusive, but when we began to try to order Slp with respect to 3, we encountered difficulties.

According to the old map with the order 23, Slp^a 3, 4, for $H\text{-}2^a$, one can account for the first three of these four recombinant types by single crossovers, but for the fourth, one must postulate a triple crossover. If one tries to position 3 on the other side of Slp^a, then there is the same problem with the third recombinant. In fact, wherever one positions 3, one must assume either a triple crossover or that something is wrong with the map.

Now, one could certainly accept that one triple crossover might have occurred, and that this was the best explanation. However, there were other difficulties with specificity 3 in some other recombinant combinations which have been described elsewhere. We also encountered the same types of difficulties with respect to specificity 1, specificity 5, and in later data from Snell *et al.*, specificities 35 and 36. Again, it was impossible to position these specificities in any consistent way, assuming the origin of the recombinants by single crossovers. This led us to the concept that perhaps certain public specificities like 3, were somehow reflections of some crossreactivity between very similar *H-2D* and *H-2K* antigenic products. Table 26 shows the explanation with respect to the $H\text{-}2^a/H\text{-}2^b$ crossovers. If one assumes that specificity H-2.3 can be determined by either the K end or the D end of the complex, then clearly a recombination anywhere between K and D and in either direction will give rise to a 3-positive recombinant.

TABLE 26

Resolution of the Inconsistency in the Position of Specificity 3

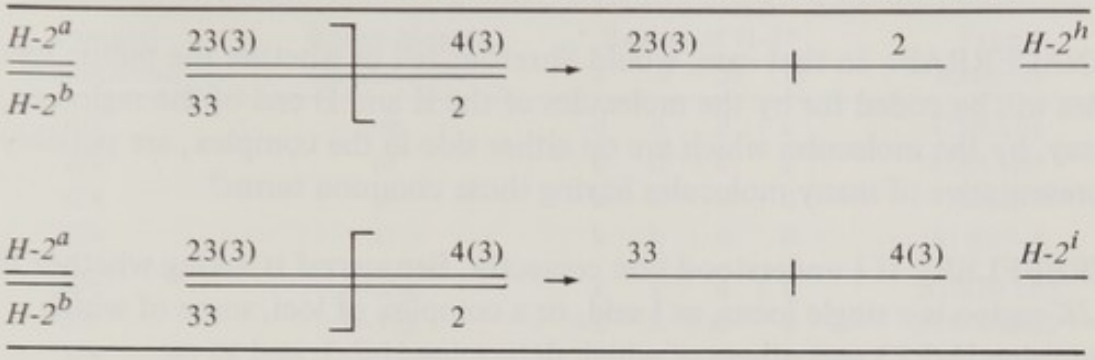
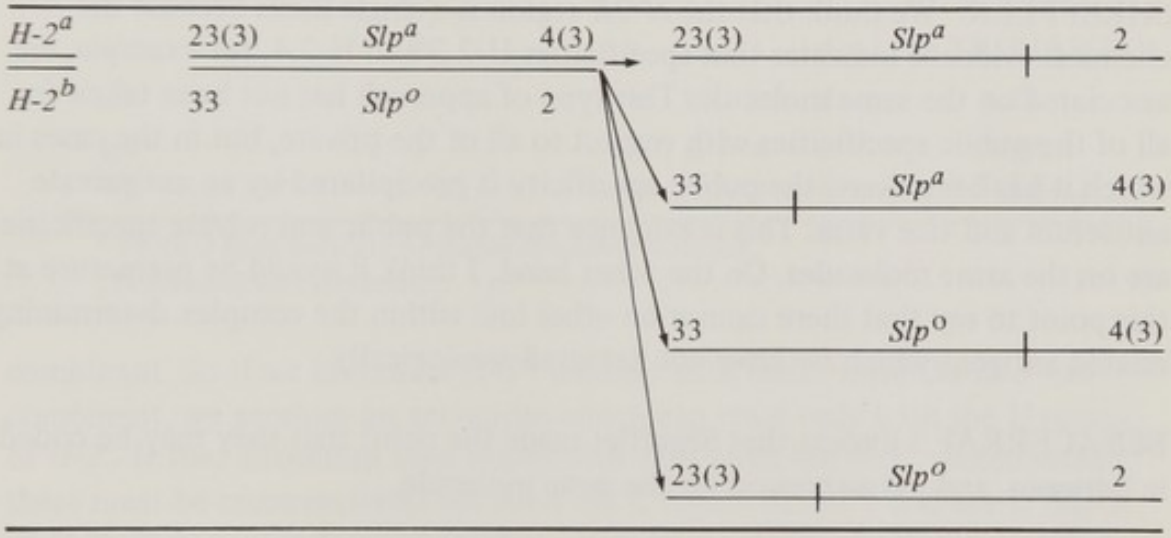


TABLE 27

Resolution of the Position(s) of Specificity 3 with Respect to Slp Type



If H-2.3 is very intimately associated with H-2.23, on the one end and H-2.4 on the other, no recombination between these factors would be expected. One might assume, of course, that H-2.23 and H-2.3 are determined by different mutational sites in the same gene and that one could, on occasion, observe intragenic recombination, but this would presumably occur at a much lower frequency.

This then explains the failure to find 3-negative recombinants; Table 27 shows the way out of the difficulty in positioning H-2.3 with respect to the Slp marker in the middle of the complex. The four different recombinant types are now very simply obtained by the two reciprocal types of crossovers in the two regions between K and Slp and between Slp and D. Now, to avoid taking time to go into a great deal of additional data, let me simply say that this kind of argument when applied to the other difficulties involving specificity 3, and also with respect to specificity 1 and 5, removes all of the inconsistencies.

So, at this point the two-gene model is not proven, but it is consistent with all of the data and circumvents many previous problems.

BENACERRAF: In that case, would Shreffler tell us whether the public specificities will be coded for by the molecules of the K and D end of the region or, let us say, by the molecules which are on either side in the complex, are possibly representative of many molecules having those common terms?

SHREFFLER: If I understood him correctly, Benacerraf is asking whether the *H-2K* region is a single locus, as I said, or a complex of loci, some of which determine H-2.23 and others of which determine H-2.3, and so on.

BENACERRAF: Yes, that is correct.

SHREFFLER: We think that the *H-2K* region is a single locus because the chemical evidence indicates that specificities H-2.3 and H-2.4, for example, are associated on the same molecule. This type of approach has not been taken for all of the public specificities with respect to all of the private, but in the cases in which it has been done, the public specificity is precipitated by an antiprivate antiserum and vice versa. This is evidence that the public and private specificities are on the same molecules. On the other hand, I think it would be premature at this point to say that there cannot be other loci within the complex determining related antigens which we have not detected serologically.

BENACERRAF: Except that Shreffler made the point that they may be coded in between, and yet expressed on the same molecule.

SHREFFLER: Yes, that is so. In fact this was Stimpfling's original explanation for the failure to find three negative recombinants, that the three specificities or three molecules, if you like, are somhow necessary to the expression of the private specificity, H-2.4 or H-2.23. That is not ruled out.

Now the key underlying assumption of the two-locus model, again, is the one of crossreactivity between *H-2K* and *H-2D* products, i.e., that a certain antiserum, which we call in this case anti-3, may react with both the K and D products of $H\text{-}2^a$, for example. The sort of approach which we have been taking to test that assumption of crossreactivity between the two regions is illustrated in the preceding table. In this representation of the $H\text{-}2^h$ and $H\text{-}2^i$ recombinants, you can see that H-2.3 should be associated with the K region of $H\text{-}2^h$ and associated with the D region of $H\text{-}2^i$. The data of Snell, Demant, and Cherry were consistent with some three-family specificity in both regions, but their antisera could have contained other antibodies than anti-3. We avoided this difficulty by immunizing the parental animals with tissue from one recombinant, then testing the antiserum against the other re-

TABLE 28

Probable Composition of Some H-2-Recombinant Chromosomes

Parental combination	Recombinant chromosome	Region of H-2* *K*	*Ir*	*Ss*	*D*	Type strain(s)
d/k	oh	d	d	d |	k	C3H.OH
d/k	ol	d	d |	k	k	C3H.OL
d/k	al	k	k	k |	d	A.AL
d/k	a†	k	k |	d	d	A; B10.A
a/b	h-2Sg	k	k	d |	b	B10.A(2R)
a/b	h-3Sg	k	k |	b	b	B10.A(4R)
a/b	i-2Sg	b	b |	d	d	B10.A(5R)
a/b	i-Go	b	b	b |	d	HTI
d/b	g	d	d	d |	b	HTG
k/q	m†	k	k	k |	q	B10.AKM
a/s	th	s	s	s |	d	A.TH; B10.S(7R)
al/s	tl	s |	k	k	d	A.TL
a/q	y	q |	k	d	d	AQR
a/q	y-Sg	q	q	q |	d	B10.T(6R)

*Vertical lines indicate probable crossover position.
†Postulated recombinant.

combinant. So if we immunize *H-2*^{b} animals with tissue from the *H-2*^{i} recombinant, we produce an antiserum which can react only with the D region of *H-2*^{i}. If that antiserum then reacts with cells from the *H-2*^{h} recombinant there must be crossreactivity between the K region of *H-2*^{h} and the D region of *H-2*^{i}. One cannot account for this reactivity in terms of any intermediate overlap region on the basis of controls which indicate that these are reciprocal recombinants. The preliminary evidence from this approach is that crossreactions do occur between the D and K region.

Another approach which is too complex to be covered in detail is one that Jan Klein has taken with my collaboration, using skin grafts as a means for detection of possible internal regions controlling H-2 antigens. These data have just been published (*J. Exp. Med.*, **135**, 924, 1972). Accordingly I do not propose to take up time for the details. They give no evidence for central regions between K and D coding for antigens detectable by skin graft.

So at the moment all of the data are consistent with the idea that there are only two loci or regions, *H-2K* and *H-2D,* which control serologically, or skin graft detectable, H-2 antigens. I think, however, that we should not exclude the possibility that new loci may be revealed by new recombinants or by different means for detection of antigenic differences.

Table 28 is intended to provide a sort of road map for consideration of the immune response and MLC mapping data which will come later. On it I have listed all of the recombinants that will be discussed. A few points should be made about these recombinants. First, in the left-hand column are listed the parental heterozygous combinations from which the recombinants were derived. In the next column are chromosome or haplotype symbols. Next I have listed under the K, Ir, Ss and D regions the genetic information which is carried in each of these regions according to the new model. Thus, for example, in the case at the top, *H-2*^{oh} has the *H-2*^{d} allele (or sets of alleles), in the K, Ir and Ss regions, but has in the *H-2D* region, the allele or alleles from the *H-2*^{k} chromosome. In the last column are the various type strains for each of these recombinants.

Now I would like to point out some of the types of comparisons one can make using these recombinant strains. For example, I indicated earlier that there is no skin graft rejection associated with the Ss region. If one exchanges skin grafts between strains C3H.OH and C3H.OL, since they are identical in the K, Ir and D regions, and differ only with respect to Ss, one is then testing for histocompatibility differences associated with the Ss region. Similarly, if we compare the *H-2*^{oh} type with the *H-2*^{d} type, then we are dealing with a difference restricted to the D region.

Finally, I would like to point out the two very interesting strain combinations, ATH-ATL and AQR-B10.T(6R), which will be discussed later, with respect to mapping of Ir and MLC. These two pairs of strains are identical serologically, by all present data—identical in the K region and identical in the D region, but they differ in the Ir and Ss regions, and as we will hear later, they have been critical in localizing the genes controlling the Ir and MLC traits. In any event, by making comparisons of this sort one can evaluate the roles of each of the individual regions in a variety of tests: skin grafting; serological tests; MLC; GVH; and immune response. I think these recombinants will prove more and more useful in the future for unravelling the genetic control of immune responses.

CHAIRMAN McDEVITT: From this point on let us restrict discussion to specific questions on this new interpretation of H-2.

BODMER: My question to Shreffler is on the nature of the public antigen, which is based, to some extent, on a consideration of the comparison between H-2 and HL-A. What proportion of the public antigens crossreact between the D and the K end, as opposed to crossreactions only amongst K or amongst D antigens?

SHREFFLER: At the moment we would be required, in order to sustain the two-gene model, to assign specificities 1, 3, 5, 35, and 36 to one or the other region in various combinations. This leaves about 10 to 12 public specificities thus far assigned only to one or the other region.

ALLISON: I am wondering whether a simple consideration which would explain the public antigens could be that the *H-2D* and *H-2K* genes arose from duplication of a precursor gene some time back in the evolutionary process and that the public antigens were sequences which had been retained while the others were sequences which had mutated subsequently.

SHREFFLER: I think that Allison's is an excellent explanation. This is exactly the way I would interpret it.

LILLY: This problem of reduplication brings up what to me is the greatest mystery of the H-2 locus and of the presumably equivalent complex loci in other species. If one assumes that *H-2K* and *H-2D* originated as gene duplications, then the question becomes: why have they remained together through so many thousands of years, thousands of generations in evolution; I wonder if Shreffler has any comment on that? The alpha and beta loci of the hemoglobin system presumably originated by the same means and were separated onto different chromosomes in the further course of evolution. In the H-2 system in the mouse, these two genes originated by reduplication, according to hypothesis; they managed to become separated by a significant amount of nucleic acid and yet have remained together. There are some indications that this is true in other species as well.

SHREFFLER: I agree with Lilly that it implies there may be some force keeping them together. One might postulate some sort of selection operating over a long (super-gene) chromosome region.

SELA: A question of clarification. When Shreffler uses the expression "antigen" is he referring to antigenic molecules or to antigenic determinants, possibly on the same molecule? Is there any difference between two antigenic determinants when they are on the same molecule or when they are on different molecules?

SHREFFLER: Sela is entirely correct. I must admit that at least sometimes I erroneously use "antigen" when I mean "specificity" or "determinant." But the definitions are quite clear, I think. I view an H-2 antigen as a single polypeptide product which probably has a number of specificities. We don't know, however, whether these multiple specificities reflect a single determinant group reactive in different ways with different populations of antibodies, or multiple determinant

groups. I think we lack good evidence at the moment to distinguish between those two possibilities.

GRUMET: Would Shreffler tell us whether the crossover frequency varies much based on factors such as different background or different H-2 alleles? I was under the impression the crossover frequency covered a wide range with some strain combinations where crossing over was very infrequent and others where it was frequent.

SHREFFLER: Yes, this seems to be the case. The $H\text{-}2^a/H\text{-}2^b$ combination gives a recombination frequency in the order of 0.5% which is about the average frequency for all combinations. In the $H\text{-}2^d/H\text{-}2^k$ combination the data indicate a recombination frequency more like 0.2%. In the $H\text{-}2^d/H\text{-}2^b$ combination, on the other hand, the frequency is about 1.5%. But the problem here is that if we put standard errors on these frequencies they are as large as the frequencies themselves, so none of these are significantly different from the others. We can only say there is some suspicion that there might be a difference.

GRUMET: Would Shreffler tell us whether there are any combinations for which there is an extraordinarily rare crossover?

SHREFFLER: Yes, the $H\text{-}2^b/H\text{-}2^k$ combination was screened rather extensively by both Stimpfling and myself but no recombinant was found. However, Amos recently found one in a rather small series. So it is a matter of chance.

BENACERRAF: It is known that H-2 is expressed to a different extent in different types of host cells. Are private specificities and public specificities expressed in parallel in all cells to that extent? Does Shreffler have any data with respect to the expression of private specificities and public specificities on lymphocytes?

SHREFFLER: A number of different studies have been done on tissue distributions of various individual H-2 specificities. These were done before the public-private distinction was recognized, but in some cases with rather good antisera. The general conclusion from these studies was that the different specificities are represented concordantly in various tissues. In other words, the *relative* levels of public and private specificities are comparable in different tissues even though the *total* level of H-2 antigen in different tissues may vary widely.

BODMER: One question to Shreffler on the recombination fraction. Is there a sex difference?

SHREFFLER: In Stimpfling's data there appeared to be a higher frequency in females. In some of my own data there was little difference. There may be some difference, but I think in the overall average frequency, it would not be very significant.

HERZENBERG: Is there evidence of crossreaction between the isolated proteins of the D and K region, which I gather are separated now?

SHREFFLER: That has not yet been tested, but hopefully will soon be.

HERZENBERG: There is no crossreaction between the isolated H-2 and Ss. Does Shreffler agree that there is no reason to think that Ss has anything to do with H-2 except that it is nearby?

SHREFFLER: That is correct.

HERZENBERG: The same thing may be true for Ir; that it has nothing to do with H-2 except for the fact that the gene happens to be located there?

SHREFFLER: I know of nothing that directly relates Ir to the serologically detected H-2 antigen.

CHAIRMAN McDEVITT: Would it help if I were to go through the evidence against it?

HERZENBERG: If it contributes anything new, yes.

CHAIRMAN McDEVITT: Based on surveys of many different strains and many H-2 recombinants by Grumet, the *Ir-1* gene is separable from the genes determining the H-2 serologic specificities; i.e., *Ir-1* responses do not appear to be uniquely associated with any particular H-2 specificities.

ALLISON: Since there are recombinants in which H-2 and Ir genes are separated, one of the original arguments in support of the view that H-2 proteins and Ir gene products are the same has been weakened. This should be taken into consideration when evaluating the triad put up by Simonsen in the first session.

SHREFFLER: Yes, I might say that one rather obvious experiment which should be done is to exchange grafts between the A.TH and A.TL strains, which differ only in the Ir and Ss regions, to see whether Ir has any effect on graft survival. We don't have the answer to that yet, but hopefully we soon will.

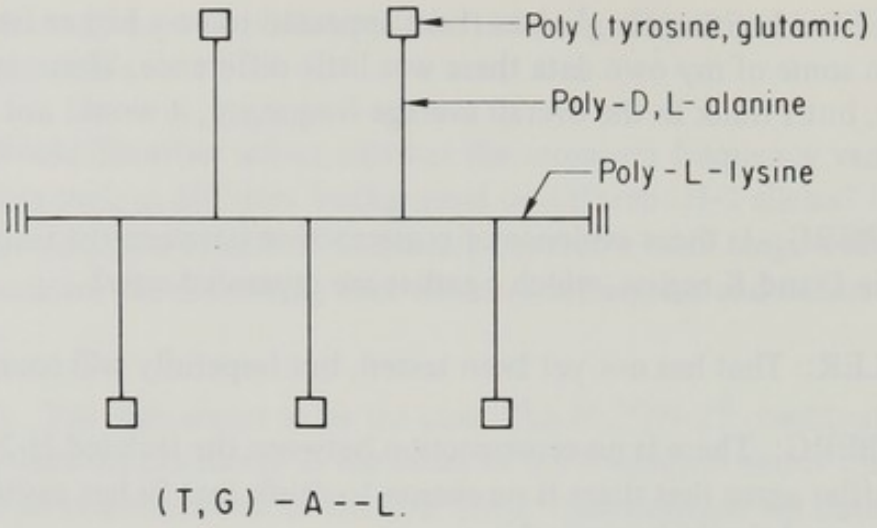


Fig. 20. A schematic structural diagram of (T,G)-A--L.

SIMONSEN: I fail to see the point Allison was trying to make. The serologically identified antigens might be on the same molecules as the not yet identified Ir products, and what we now identify as Ir products could well prove to carry histocompatibility antigens as well.

CHAIRMAN McDEVITT: We can now proceed to the second part of this session. The groundwork we are establishing here deals with the genetic relationship between specific immune response genes linked to histocompatibility loci, and the genes controlling histocompatibility antigens themselves. It is now clear that immune response genes exist for a large number of specific antigens and specific antigenic determinants in at least three species, the mouse, the rat, and the guinea pig. As Benacerraf showed in Session I, the major characteristics of this type of genetic control of a specific immune response, where it has been analyzed, appear to be similar for all the H-linked specific immune response genes identified in these three species.

Genetic mapping studies are possible only in the mouse and I will describe our studies which have succeeded in mapping the *Ir-1* immune response gene within the H-2 region. *Ir-1* is a gene (or genes) controlling the immune response to a related series of branched synthetic polypeptide antigens; poly-L(Tyr,Glu) -poly-D,L-Ala--poly-L-Lys [(T,G)-A--L]; poly-L (His,Glu)-poly-D,L-Ala- -poly-L-Lys [(H,G)-A--L]; poly-L (Phe,Glu)-poly-D,L-Ala--poly-L-Lys [(Phe,G)-A--L]. Figure 20 is a schematic diagram of the structure of (T,G)-A--L, showing the poly-L-lysine backbone, the D,L-alanine sidechains, and the amino terminal tips of tyrosine and glutamic acid in a random mixed sequence. The related members of this series have either histidine or phenylalanine replacing tyrosine, and are listed on Table 29. All of these synthetic polypeptides are antigenic in some strains of mice. They will not cross-*immunize*, although antibody to them will cross-*react* extensively.

TABLE 29

Structural Homologs of (T,G)-A--L	
Antigenic determinant (side chain tips)	Abbreviation
Tyrosine, glutamic acid	(T,G)-A--L
Histidine, glutamic acid	(H,G)-A--L
Phenylalanine, glutamic acid	(Phe,G)-A--L

Using these three antigens, we have studied a large number of inbred mouse strains bearing several different H-2 alleles. The results, summarized in Table 30, have shown that the response of a particular inbred mouse strain can be predicted by its H-2 type and that, within the inbred mouse populations, any particular H-2 type has a distinctive pattern of immune response to these three related and crossreacting antigens. For example, all inbred mouse strains bearing the $H\text{-}2^a$ allele are low responders to (T,G)-A--L, high responders to (H,G)-A--L, and high responders to (Phe,G)-A--L. On the other hand, all mouse strains bearing the $H\text{-}2^b$ allele are high responders to (T,G)-A--L, *low* responders to (H,G)-A--L, and high responders to (Phe,G)-A--L. This characterization of a particular H-2 allele with respect to its immune response to these three antigens can be termed an immune response pattern and is a way of phenotyping an unknown strain or a strain bearing a recombinant H-2 chromosome for its immune response phenotype. These results are summarized in Table 30, which is simply a listing of the immune response patterns of all the inbred mouse strains bearing the most common H-2 alleles, namely $H\text{-}2^{a,b,d,k,q,}$ and ^{s}. As is evident in this table, $H\text{-}2^a$ and $H\text{-}2^k$ have the same immune response pattern to these three antigens, while all the other H-2 alleles tested have distinctive patterns. It is also worth noting that there are many other antigens under the control of H-2 linked immune response genes and that, for the majority of these antigens, the pattern of responding and nonresponding H-2 alleles in both different from that shown in Table 30 and, for the majority, unique to each antigen under this type of genetic control. While it is possible that such a picture could be the result of a single gene with an extremely large number of different alleles, it is much more likely that it is the result of a large number of closely linked genes, each controlling the response to a particular antigenic determinant. There is more specific evidence on this point which I will return to later.

The major point of Table 30 is to show the method by which a particular inbred strain can be genotyped for its *Ir-1* gene, the locus or loci controlling the response to these three branched synthetic polypeptide antigens. This method can, of course, be extended to genotype any strain or group of animals which are sufficiently inbred to be homozygous for the ninth mouse linkage group, in

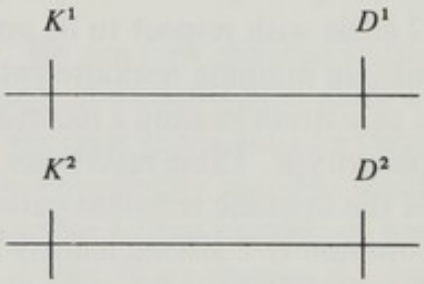
TABLE 30

Response of Inbred Mouse Strains, Grouped by H-2 Type, to a Series of Synthetic Polypeptide Antigens

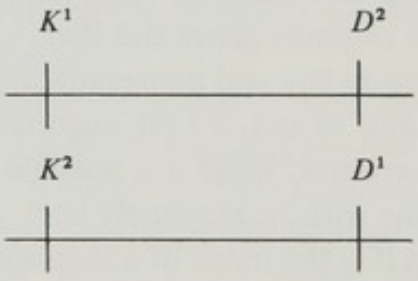
H-2 Type	Antibody response to:		
	(T,G)-A--L	(H,G)-A--L	(Phe,G)-A--L
a	Lo	Hi	Hi
b	Hi	Lo	Hi
d	Med-Var	Med-Var	Hi
k	Lo	Hi	Hi
q	Lo	Lo	Hi
s	Lo	Lo	Lo

order to determine the *Ir-1* genotype of that strain. This is the method that we have used to determine the *Ir-1* genotype of many of the recombinant H-2 chromosomes which were described in detail by Shreffler earlier in this session.

The approach is quite simple. For example, let us take two strains, both homozygous, one bearing H-2 type one, and the other bearing H-2 type two. These can then be shown schematically as follows:



A search for crossovers between the *K* and *D* loci of H-2 will give rise to two types of recombinants which can be diagrammed as follows:



Once these recombinants have been detected and verified by progeny testing, two backcross offspring, each of which bear the recombinant H-2 chromosome, can then be crossed to produce a line of animals which are homozygous for the new recombinant H-2 chromosome. These animals are then given a new strain designation, as Shreffler described earlier (cf. Table 28), and it is these H-2 recombinant strains which can then be typed for their immune response pattern to the three related synthetic polypeptide antigens, and from this their genotype at the *Ir-1* locus or loci can be deduced. Since the crossover giving rise to the

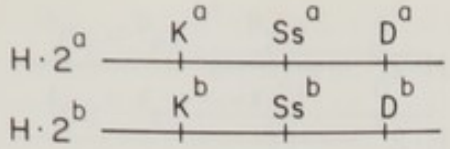


Fig. 21. A simplified schematic diagram of the H-2 complex for the *H-2*^{a} and *H-2*^{b} alleles.

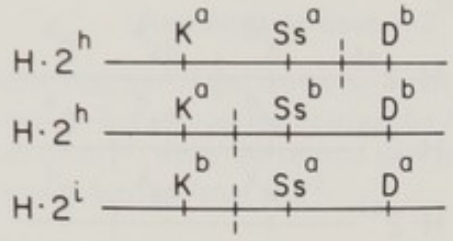


Fig. 22. Schematic diagram of the recombinant H-2 chromosomes derived from crossovers between *H-2*^{a} and *H-2*^{b}.

recombinant H-2 chromosome is known to have occurred between the *K* and *D* loci of H-2, we can then determine whether the *Ir-1* genes are associated with the *K* locus of H-2 or the *D* locus of H-2. In addition, because of the fact that the *Ss* locus is in the central part of the H-2 complex, it is possible to localize the initial crossover event with respect to the *Ss* locus as well as the *K* and *D* loci.

We have done immune response pattern typing, i.e., *Ir-1* genotyping, on eleven inbred strains bearing H-2 recombinant chromosomes. Assuming that Klein and Shreffler are correct and that the K and D regions of H-2 are in fact two genes, I would now like to present the evidence on the *Ir-1* genotypes of these H-2 recombinant chromosomes. If *Ir-1* is closely associated with *H-2K* or lies anywhere on the ninth mouse linkage to the left of *H-2K*, the H-2 recombinant strains should give an immune response pattern similar to that of the donor of the *H-2K* part of the new H-2 chromosome. On the other hand, if *Ir-1* is closely associated with *H-2D* or lies anywhere to the right of the *H-2D* locus, then the H-2 recombinant strains should give an immune response pattern identical to that of the donor of the *H-2D* locus in the new H-2 chromosome. I have taken the liberty of simplifying Shreffler's diagram of the H-2 region even further and Fig. 21 shows a schematic diagram of the *H-2*^{a} and *H-2*^{b} alleles. I have designated the *K*, *Ss* and *D* loci of *H-2*^{a} as K^a, Ss^a, and D^a. This was done for clarity, but is *not* the accepted nomenclature, which is given in Table 28. Stimpfling has detected and isolated five separate recombinant H-2 chromosomes derived from crossovers between *H-2*^{a} and *H-2*^{b}. These recombinant chromosomes, of three different types, are shown in Fig. 22. There are two *H-2*^{h} chromosomes, each bearing the *K* locus of *H-2*^{a} and the *D* of *H-2*^{b}. They differ in that in one the crossover is to the right of Ss and in the other it is to the left of

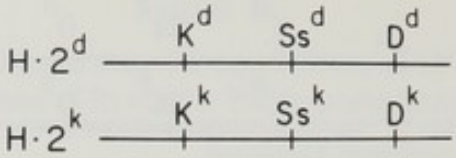


Fig. 23. A simplified schematic diagram of the H-2 complex of the *H-2*^{d} and *H-2*^{k} alleles.

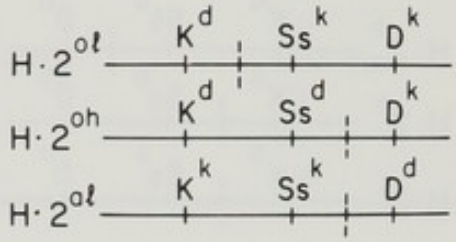


Fig. 24. A diagram of the recombinant H-2 chromosomes derived from crossovers between the *H-2*^{d} and *H-2*^{k} alleles.

Ss. The third type of recombinant, *H-2*^{i}, is of the reciprocal type and bears the *K* locus of *H-2*^{b} and *D* locus of *H-2*^{a}.

All five of these H-2 recombinant inbred strains, when immunized with (T,G)-A--L and (H,G)-A--L respond with the same immune response pattern as the donor of the *K* locus of the recombinant H-2 chromosome. This result implies that *Ir-1* is associated with either the *K* locus or is very close to it on the right hand side or may lie at any point to the left of the *K* locus.

With this information, we went on to test a second set of H-2 recombinant inbred strains which were derived from crossovers between *H-2*^{d} and *H-2*^{k} by Shreffler. The parental H-2 alleles are shown in Fig. 23, again in my oversimplified terminology; and the recombinant H-2 chromosomes derived from them and the probable locus of the crossover event are shown in Fig. 24. *H-2*^{ol} and *H-2*^{oh} bear the *K* locus of *H-2*^{d} and the *D* locus of *H-2*^{k}, and differ only in the position of the crossover, one carrying *Ss*^{l}, and the other *Ss*^{h}. *H-2*^{al}, on the other hand, carries the *K* locus of *H-2*^{k} and the *D* locus of *H-2*^{d}. The immune response pattern of these three recombinant H-2 chromosomes were in every case identical with the immune response pattern of the donors of the *K* locus of the recombinant H-2 chromosome. This again says that *Ir-1* is either closely associated with the *K* locus, identical with it, or lies somewhere to the left of it in the ninth mouse linkage group.

On the basis of the evidence presented so far, there is no way to obtain a more precise localization of *Ir-1* with respect to the *K* and *D* loci of the H-2 complex. However, a more precise localization was strongly implied when we determined the immune response pattern of yet another H-2 recombinant detected by Shreffler. The crossover event which gave rise to this new H-2 chromosome is diagrammed in Fig. 25, which shows that this was a crossover between *H-2*^{al} (shown in the preceding diagram) and *H-2*^{s}. The recombinant H-2

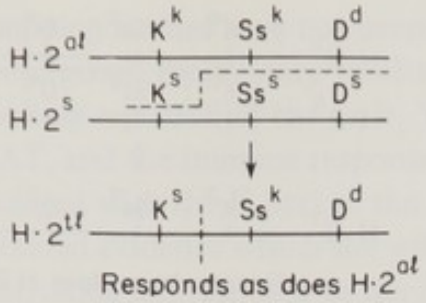


Fig. 25. A simplified schematic diagram of the crossover event occurring between *H-2*^{al} and *H-2*^{s} to give rise to the new H-2 chromosome bearing the *H-2*^{tl} allele. The *H-2*^{tl} chromosome bears the *H-2K* locus of *H-2*^{s}, while the central part of its H-2 complex is derived from H-2^{k}, and the *H-2D* locus is derived from *H-2*^{d}.

chromosome, designated *H-2*^{tl}, carries the *K* locus of *H-2*^{s}, the *Ss* locus of *H-2*^{k}, and the *D* locus of *H-2*^{d}. Mice homozygous for this recombinant H-2 chromosome responded to (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L as do mice bearing the *H-2*^{al} and *H-2*^{k} alleles, and *not* as do mice bearing the *H-2*^{s} allele. Taken at face value, this result indicated that *Ir-1* is located just to the right of the *H-2K* locus and lies between the *H-2K* and *Ss* loci in the left center part of the H-2 complex. This is so because the recombinant *H-2*^{tl} chromosome behaves as does the *H-2*^{al} parental allele, which indicates that *Ir-1* is to the right of the crossover giving rise to *H-2*^{tl}. Since all of the other H-2 recombinants would place *Ir-1* to the left of the crossover event, it must be assumed that this crossover is farther to the left towards *H-2K* than any of the other crossovers previously studied, and sufficiently far to the left that *Ir-1* is placed to the right of this particular crossover event.

There is regrettably an alternative possibility—that *Ir-1* actually lies to the left of the *H-2K* locus in the ninth mouse linkage group and that the crossover event giving rise to the *H-2*^{tl} allele was actually a double crossover, with one crossover occurring between the *K* and *Ss* loci and the other occurring somewhere to the left of the *K* locus and between the *K* locus and the *Ir-1* genes. Such a double crossover event cannot be ruled out because the original cross between *H-2*^{al} and *H-2*^{s} did not involve outside markers to the right of the *D* locus and to the left of the *K* locus which would permit an unequivocal statement as to whether the crossover event was a single or double crossover.

Fortunately, Klein has produced an H-2 recombinant strain derived from a known (by the use of outside markers) single crossover event. The derivation of this new H-2 chromosome is shown in Fig. 26, again using my simplified designation for genes on the *H-2*^{a} chromesome.

The parental input chromosomes involved in this cross were *H-2*^{a} and *H-2*^{q}. However, the *H-2*^{q} allele was part of a ninth mouse linkage group which carried readily detectable genetic markers on either side of the H-2 complex—namely, the T (brachyury) gene to the left of the *K* locus of H-2, and the

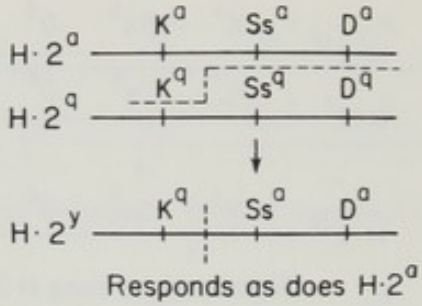


Fig. 26. A schematic diagram of the crossover event between *H-2*^{a} and *H-2*^{q} (derived from T-138 line and bearing outside markers on either side of *H-2*^{q}) to give rise to the *H-2*^{y} recombinant H-2 chromosome.

translocation, T138, to the right of the *D* locus of H-2. The crossover event, shown in dotted lines in the figure, was known to be a single crossover event by the concomitant recombination between the outside markers T (brachyury) and translocation-138 originally carried on the parental ninth mouse linkage group carrying the *H-2*^{q} allele. This crossover gave rise to the *H-2*^{y} chromosome shown at the bottom of the figure, which bears the *K* locus of *H-2*^{q} and the *Ss* and *D* locus of *H-2*^{a}. Mice homozygous for this recombinant H-2 chromosome respond to (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L as do mice bearing the *H-2*^{a} allele. This result is the same as that given by the *H-2*^{tl} allele, and again indicates that *Ir-1* lies just to the right of the crossover and therefore maps between the *K* locus of H-2 and the *Ss* locus in the center of the H-2 complex. Since the *H-2*^{y} chromosome is known to have been derived from a single crossover event, this assignment of position for *Ir-1* can be considered as established.

At the same time that we were carrying out these studies on the recombinant H-2 chromosomes, we carried out a standard four-point mapping cross. In this cross, one parental strain carried the T or brachyury gene, the tf or tufted hair pattern gene, and the *H-2*^{q} allele. The other parental strain was wild type at the *T* and *tf* loci and carried the *H-2*^{s} allele. The F_1 animals from this cross were than backcrossed to the strain carrying the *H-2*^{s} allele, and the progeny of this cross were scored for T, tf, and H-2 type. All those animals which showed a crossover were then tested for their immune response genotype. Of 484 potential recombinants, only two animals were detected that appeared to be recombinants between *Ir-1* and the H-2 complex. Subsequent testing of these recombinants and their progeny established that both of these recombinants were in fact crossovers between the *K* and *D* loci of the H-2 complex. Thus, the only animals which appeared to show recombination between *Ir-1* and H-2 on preliminary screening were shown on subsequent testing to be recombinants within the H-2 complex itself. These results are entirely compatible with those given earlier and support the conclusion that *Ir-1* maps in the left center part of the H-2 complex, between the *K* locus of H-2 and the *Ss* locus.

To date, definitive mapping studies have not been carried out on a number of the other immune response genes. Preliminary results indicate that the immune response genes controlling rejection of the male, Y, transplantation antigen, the immune response to GAT, and the immune response to ovalbumin, indicate that these Ir genes are associated with the K part of the H-2 complex. However, Lieberman has recently obtained evidence which she will present later that the immune response genes controlling the response to the Ig^a alleles of γ_A and γ_{2a} immunoglobulin allotypes appear to be localized in precisely the same region of the H-2 complex, between the *K* locus and *Ss* locus of the H-2 complex. In addition we have recently obtained preliminary evidence that the immune response genes controlling immune response to two other antigens map in precisely the same region. Taken together all these results indicate that the immune response genes are arranged in a linear array in the chromosome region lying between the *K* locus of H-2 and the *Ss* locus, which is apparently somewhere in the middle of the H-2 complex.

It is difficult to make any estimate as to how many specific immune response genes comprise this linear set, or whether some of them might not lie to the left of the *K* locus or to the right of the *Ss* locus. Assuming for the reasons mentioned earlier that immune response genes for different antigens represent the effects of different gene loci, we can only make the crudest preliminary estimate that there are a large number of such genes. Almost 20 of them have already been identified in the mouse. These genes control the response to synthetic polypeptide antigens, transplantation antigens, immunoglobulin isoantigens, and complex protein antigens, and are being detected with increasing frequency. This would lead us to speculate that there are certainly more than ten of these genes and that there may be several hundred, if we could detect all of them.

We thus have quite firm evidence for the map position of the *Ir-1* locus, strong suggestive evidence that other immune response genes map in the same area, and a crude preliminary estimate of the number of these specific immune response genes. We are in much greater difficulty when we consider the possible nature and significance of this close association between Ir genes and histocompatibility antigens, and of the nature of the Ir gene products themselves. We have heard the evidence at the preceding session indicating that these genes are almost certainly expressed in thymus-derived, antigen-reactive lymphocytes, or T cells. There is still controversy as to whether they are also expressed in antibody-producing B cells. On balance, I would interpret that evidence as indicating that the Ir genes are expressed exclusively and only in T cells, and that they are intimately bound up with the mechanism of antigen recognition by T cells.

At this point, it might be most useful to cite several lines of evidence which indicate the Ir genes are not in any way related with immunoglobulin structural genes for either the light or heavy chains of the known immunoglob-

ulin classes. First, we know that the *Ir-1* gene is not linked to the mouse immunoglobulin allotype loci associated with the constant regions of the mouse heavy chain linkage group. If we accept the allotype linkage data obtained in the rabbit, which indicates that the heavy chain variable region and constant region genes are closely linked, this would rule out any association of the Ir genes with heavy chain structural genes.

The principle light chain in the mouse is the κ chain. There is no allotype marker available for the κ chain in the mouse but we know that in man the HL-A antigens which are formally analogous to the H-2 genes in the mouse are not linked to the Inv locus, which is a human κ chain constant region genetic marker. Assuming that the constant and variable regions of the light chains are linked as they have been shown to be in the rabbit for the heavy chain this would indicate that the Ir genes are not associated with light chain structural genes. In addition our very earliest studies showed that there is a genetic control of the ability to respond to (T,G)-A--L in the rabbit. This could not, of course, be linked to histocompatibility genes in the rabbit, but if we can assume that this is, in fact, an example of a rabbit histocompatibility-linked specific immune response gene, our earlier unpublished findings (in collaboration with Gell and Kelus) indicate that there is no association between the ability to respond well to (T,G)-A--L and the *a* or *b* immunoglobulin allotype loci associated with the heavy chain linkage group and the κ chain of the rabbit, respectively. There is thus considerable evidence, based on both direct observation and several assumptions concerning the genetics of immunoglobulin synthesis, all of which indicates that the Ir genes are not associated with structural genes for the variable regions of either light or heavy chains of the known immunoglobulin classes.

There are I think three major possibilities which have to be entertained concerning the nature of the Ir gene product. The first is that the Ir genes represent a new heavy chain class, analogous to the IgX or IgT which has been postulated by others and which functions as the specific antigen receptor on thymus-derived cells. Aside from being a unique heavy chain class this new type of immunoglobulin heavy chain could be in other respects similar to the other heavy chains, but only bound to cell surfaces and never free in the circulation. If this were the case, the mechanism of generation of diversity of this class of specific antigen receptors could be assumed to be similar to that for the other immunoglobulin polypeptide chains, whatever the nature of that mechanism may be. While there are some suggestions that the specificity of antigen recognition by T cells is of a different nature than antigen recognition at the B cell level, all of this evidence is indirect and I do not think it sufficient to exclude the possibility of a new heavy chain immunoglobulin class restricted to T cells.

The second possibility is that we are dealing with a completely new type of antigen recognition which has a molecular basis distinct from that of the clas-

sical immunoglobulins. Whether this is antigen recognition mediated by a complex array of histocompatibility antigens on the surface of T cells or some completely new molecular mechanism, is entirely a matter of speculation. We simply have no evidence for or against these possibilities at the present time.

The hypothesis that is most appealing to me is that primordial cell surface receptors which arose early in the course of the evolution of multicellular organisms gradually developed the ability to undergo some type of somatic variation. This might very well have originated in a series of reduplicated genes coding for a variety of cell surface receptors subserving several different types of recognition function. At some point in evolution some of these cell surface antigens may have taken on the functions of a constant portion of the final cell surface product, while others took on the ability to develop somatic variation. As evolution proceeded this primitive system of developing cell surface recognition units could have reduplicated sufficiently to give rise to cell-bound recognition units on the surface of T cells on the one hand, and to gene complexes ultimately located on other chromosomes and coding for the heavy chain and light chain linkage groups on the other hand. Such an hypothesis would postulate a common genetic origin and some type of structural analogy between histocompatibility antigens and immunoglobulin molecules. This hypothesis is of course not new and has been stated in other forms by Burnet and others. I find it difficult to choose between these two major possibilities—that the Ir genes represent a new heavy chain class, or that they represent a unique antigen recognition system distinct from the classical immunoglobulins, but perhaps the genetic ancestor of the present day immunoglobulin light and heavy chains.

A third major possibility for the mechanism of Ir gene action is that the Ir gene-products are in fact the effects of a series of cell surface antigens, perhaps closely related to the histocompatibility antigens, which are in close association with classical immunoglobulin receptors on the surface of T lymphocytes. During the early course of these studies when there was only one association between a specific immune response gene and histocompatibility antigens, I found this a much more attractive hypothesis. However, it now appears that there are a large number of specific immune response genes with a great deal of immunologic specificity. There is also considerable evidence that most of the readily identifiable molecules known to be present on the cell surface and in the cell membrane are free to move around and rearrange themselves in the rather viscous lipid sea that comprises the cell membrane. To me these two findings make it unlikely that the Ir genes are the result of a modulating effect of classical histocompatibility antigens on the interaction of immunoglobulins with their specific antigens. There are of course other possible explanations of the Ir genes, but given the evidence which we have reviewed in the preceding session and in the present session, I find these three to be the most appealing at the present time.

BENACERRAF: I don't understand the differences in McDevitt's view between hypothesis 1 and 2. Basically, it is the same thing. The only thing that he is saying is that one is analogous to the V region but not the V regions themselves.

CHAIRMAN McDEVITT: There is a big difference between hypothesis 1 and hypothesis 2. Hypothesis 1 postulates that the Ir genes are, in fact, a new and distinct heavy chain class. Hypothesis 2 is in reality two variations on the same theme: 1. that Ir genes are a new and completely distinct type of antigen-recognition system with a unique molecular basis, of a type that can only be guessed at; or 2. that the Ir genes are closely related to the histocompatibility antigens in structure, and may be the primordial genes which, through the course of evolution and reduplication gave rise to the structural genes for the immunoglobulins. Other possibilities which come to mind and which I have not mentioned earlier are that Ir genes are short nucleotide sequences which are inserted into the hypervariable regions of the immunoglobulin variable region genes. I think this is a remote hypothesis but one that has to be considered.

HERZENBERG: I think a strong argument against McDevitt's possibility 1, is that all the known heavy chain genes of each species that have been looked at are linked. What he now proposes would be a new heavy chain gene *not* linked to the other known heavy chain genes. I think this is very unlikely.

CHAIRMAN McDEVITT: Are the genes for the kappa and lambda light chains linked?

HERZENBERG: No, these two are known not to be linked, but all the heavy chains are linked. As in the hemoglobins, all the nonalpha chain genes are linked, but the alpha and the beta group are not linked.

CHAIRMAN McDEVITT: That is just the point. We know that there are separate genes on separate chromosomes for two different types of immunoglobulin light chains. If this is possible it seems to me equally possible that there could be two distinct and genetically separate chromosome regions for two different heavy chain linkage groups, one including the known circulating immunoglobulins, and a second coding for the IgX or IgT immunoglobulins restricted to the surface of T cells.

HERZENBERG: Naturally—as McDevitt knows—everything in this world is possible, but I think it helps to limit possibilities to those which are testable; perhaps even beyond that, to those which seem to have some precedent.

CHAIRMAN McDEVITT: The precedent is the one I have already cited: the existence of independent linkage groups for two different types of immunoglob-

ulin light chains. The amino acid sequences of the kappa and lambda variable regions show that these are distinct, so that whatever the mechanism of generation of diversity may be, there is one for the kappa light chains, one for the lambda light chains, and one for the heavy chain linkage group. All I am postulating is one additional heavy chain linkage group with its associated generator of diversity, whatever its mechanism.

HERZENBERG: Maybe we should call what you are proposing a fourth type of immunoglobulin chain, a further type. I find that I agree with Benacerraf; just what is the difference between hypothesis 1 and 2?

CHAIRMAN McDEVITT: I am talking now about a class of molecules that may have no structural homology of any sort with the immunoglobulins, but are entirely different.

HERZENBERG: I want to remind McDevitt—that is Benacerraf's argument. In fact I would like to suggest that McDevitt take out the word new; if he insists on postulating, I would suggest he say old, evolutionarily speaking.

CHAIRMAN McDEVITT: I agree with Herzenberg. I should say new to *us*.

BODMER: I think the subject that McDevitt mentioned before, namely the evolutionary relationship, is one that must be borne in mind. The difference between his first two hypotheses ends up as being a semantic argument, unless one says that what he is arguing about is whether the recognition system is or is not evolutionarily related to the V and C chains of the immunoglobulin system.

The question of whether one calls it a new set of variable H chains is irrelevant. The question is, what is the evolutionary relationship, or origin of the T cell receptors. If, as I and others have suggested elsewhere, the genes in the HL-A region and the immunoglobulin gene have a common evolutionary origin, then one reconciles those two hypotheses, and can allow for any level of difference he wants between them, according to the difference that has evolved in the eons since these two sets of genes split.

So the question really is: Is the recognition system evolutionarily derived from the same sets of genes as the immunoglobulin genes or not?

SELA: In molecular terms, wouldn't hypothesis 1 and 2 be completely distinguishable? In proposition 1 it is assumed that there is a difference in the combining site due to a new heavy chain class. In contrast proposition 2 assumes a completely new recognition mechanism, a new molecule which may have a different specificity.

BENACERRAF: I feel that Sela is taking us to the Talmud!

SELA: Perhaps, but this is a molecular Talmud.

BENACERRAF: It's a molecular Talmud, but a Talmud anyhow. Nonetheless it is good that we are talking about it, as it is an important point. Since H-linked Ir genes are not associated with heavy chain structural genes of known immunoglobulin, in all likelihood they do not code for the V regions common to all heavy chains. Each of us will make his choice of these alternatives—but I will vote for hypothesis number 2.

HERZENBERG: Biozzi urges me to make this point, but he is not to blame. Later on he will be telling us why he believes that there are at least ten different genes which affect the immune response to a variety of antigens; one is allotype-linked, one is H-2-linked, etc. Accordingly, is there any reason for choosing *Ir-1* as the gene which controls a receptor—any more than the numerous others?

CHAIRMAN McDEVITT: All the evidence we heard during the first session implies that H-linked Ir genes affect antigen recognition at the level of T cell function.

HERZENBERG: That may be—but none of us have studied the other eight. By McDevitt's reasoning we will have eight more immunoglobulin classes controlled by these eight genes.

CHAIRMAN McDEVITT: Only if Herzenberg or others show that they affect antigen recognition and are *not* linked to the immunoglobulin allotypes.

BENACERRAF: Since I worked with Biozzi on this problem, permit me to contribute to this discussion. The major genetic differences of the Biozzi high and low antibody lines concern the genes which regulate differentiation of B cells and their proliferation, and also the amounts of immunoglobulin, regardless of specificity. Nevertheless, the lines were selected on the basis of their ability to respond with the production of antibody to sheep erythrocytes; it is, therefore, likely that Ir genes concerned with the recognition of sheep arythrocyte antigens have been selected against in the low line, which may explain why one of the genes concerned with the selection has been shown to be linked to the heavy chain allotypes.

CHAIRMAN McDEVITT: At this point I think we should proceed to a consideration of the relationship between genes controlling those cell surface antigens that stimulate the MLC reaction, and the other genes which are known to comprise the H-2 complex. This part of the discussion will be developed by Bach.

BACH: I would begin by stating that I am painfully aware of the good fortune we have had to be dealing with a system associated with H-2 and the consequent opportunity to use the strain recombinants derived over the past years. The great majority of the mouse data I will present has been obtained in collaboration with Klein. We have, moreover, received mice from Shreffler, Stimpfling, and Bailey. Of great aid has been Michael Widmer, a student in our laboratory who has worked with my wife and myself.

First, I would identify the MLC test as we have used it, and its relationship to the major histocompatibility complex as it has been defined here. For the studies on human cells we use peripheral blood—in the murine studies cells are obtained from spleen. To identify the cell interactions I will designate one donor, human or mouse, as A, and the second donor as B; in all cases we examine the response *in vitro* of the lymphocytes of A to "foreigness" associated with the major histocompatibility complex on the cells of B. In order to assure a one-way reaction, we treat the cells of B with mitomycin C; the treated cells are designated with a subscript "m." To determine whether we have stimulation, we compare counts per minute (cpm) of radioactive thymidine incorporated into such an allogeneic mixture with an isogeneic (or syngeneic) control in which we mix the cells of A with that individual's own mitomycin C-treated cells.

The observations in both man and mouse suggest that the major genetic control of stimulation in the MLC (measured by the incorporation of radioactive thymidine in allogeneic mixtures and compared with control mixtures) is associated with the HL-A or H-2 complex.

Although there is some evidence obtained by Festenstein and others that suggests that loci in the mouse, the alleles of which segregate independently of H-2, can cause MLC activation. This is the exception, rather than the general rule. For the most part, then, the MLC reaction can be equated with differences for the major histocompatibility complexes.

In man as in the mouse (and the two systems are very similar), we are dealing with a chromosomal region in which there are two loci, the products of which are defined with antisera, i.e., serologically defined loci. In man these are designated *Four* and *LA*; in the mouse *H-2D* and *H-2K*. It has been thought for a long time that the MLC reaction was consequent to the recognition by A cells with a foreign *Four* or *LA* locus, or *H-2K* and *H-2D* locus antigens-serologically defined antigens associated with this region.

The data I would like to present deals with the genetic control of the MLC and suggests that a major part of the stimulation of MLC is due to genetic loci *other* than the serologically defined ones, in both mouse and man. This work evolved from studies on human cells, and I therefore would like briefly to review the present situation in man. I would point out that in man, since we are not dealing with homozygous strains, we have two antigens of the *LA* locus and two

TABLE 31

HL-A Typing on a Family with Two MLC-Reactive HL-A Identical Siblings

Family	H^a
Father	2,R^a/3,7
Mother	9,12/L1-FE71.3, FJH-AJ
J.H.	2,R^a/L1-FE71.3, FJH-AJ
V.H.	2,R^a/L1-FE71.3, FJH-AJ

[a]Typing performed by Dr. Flemming Kissmeyer-Nielsen.

of the *Four* locus, so we frequently deal with four antigens which are defined in any one individual.

Table 31 shows HL-A typing results on two siblings, JH and VH, included in a study by Amos and myself in which we established the initial correlation between the major histocompatibility complex and MLC reactivity in man. The general rule, of course, was that if two siblings had inherited the same HL-A chromosomes or haplotypes from their parents, that is the same four antigens from the HL-A system, the cells did not stimulate in the MLC.

However, in that initial series we found these two particular siblings who clearly inherited the same four antigens from their parents, yet their cells did stimulate in the MLC. The hypothesis which Amos and I advanced at that time is shown in a much more elegant form in Fig. 27, in harmony with recent suggestions by Yunis and Amos. What we brought forward, in fact, was that MLC reactivity may not be controlled entirely by the serologically defined antigens, but also by a locus separate from, but linked to the serologically defined or definable loci. Alleles of this other locus are designated W, X, Y, and Z, whereas the numbers refer to antigens of HL-A.

We speak of a mating between the two parents shown. The basic observation made was that these two siblings inherited the same two HL-A haplotypes from their parents, and yet their cells *did* stimulate in mixed culture. Accordingly we hypothesized another locus linked to the HL-A system which was difficult to define serologically, and yet, because of a recombinational event in the mother which resulted in a 1, 8, X haplotype in one sibling, the two siblings differed for this "third locus;" consequently their cells stimulated because of the difference at this locus which was serologically difficult to define.

This phenomenon will be discussed further by van Rood and other conferees. I would only point out that at that time it seemed possible that such loci—which could cause lymphocyte activation, and yet were difficult to define serologically—existed in man associated with the major histocompatibility complex (MHC). A map of the human major complex would include, as shown in

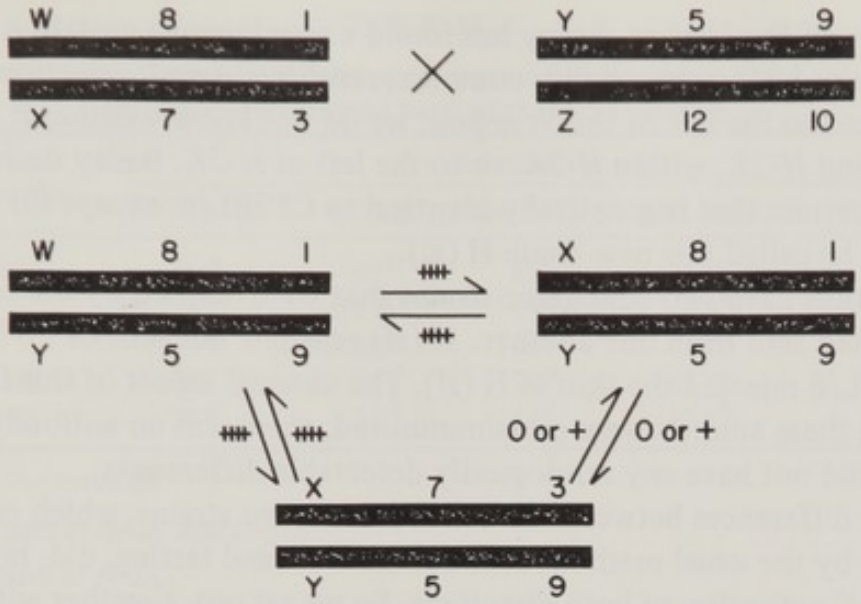


Fig. 27. Schema of the HL-A system with a linked LD locus. Presumed major histocompatibility complex (MHC) chromosomes in a human family. The numbers refer to the HL-A antigens of the *LA* and *FOUR* loci; the letters to presumed alleles of an LD locus. The one parent carries a 1,8 W and a 3,7 X haplotype; the other parent a 9,5 Y and 10,12 Z haplotype. Two siblings who inherit the same HL-A loci alleles–and thus the same HL-A antigens, can differ for the LD locus secondary to a recombinational event in one of the parents. In this particular case, a recombinational event in the first parent resulted in a 1,8 X haplotype. These two siblings thus stimulate in MLC. The + signs refer to MLC stimulation in a quantitative sense.

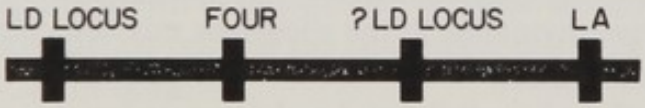


Fig. 28. Genetic map of the human MHC. In the manner that the HL-A loci are written in this map, an *LD* locus is placed to the left of FOUR to explain the data described. The possible *LD* locus between FOUR and LA would explain stimulation between siblings that are identical for the *LD* locus to the left of FOUR, but whose cells still stimulate. In siblings that have the same *LD* locus alleles but differ for the HL-A antigens there is either no stimulation or weak stimulation; the weak stimulation could be caused by difference at an *LD* locus between FOUR and LA.

Fig. 28, the *LA* locus, the *Four* locus, and another locus to the left of the *Four* locus which could be defined by lymphocyte reactivity. Without wanting further to complicate the nomenclature, I will refer to such a locus as a lymphocyte defined or *LD* locus. It seems possible that *LD* loci exist between Four and LA.

We have studied whether there are genetic differences in this region in the mouse which can result in lymphocyte activation in the MLC, and which are nonetheless difficult to define by the present technology, which utilizes cytotoxic or agglutinating antibody. Our initial studies were done in collaboration

with Bailey of Bar Harbor. Bailey has found a spontaneous mutation of the murine major histocompatibility complex, resulting in graft rejection, which he has localized to the left of the *Ss* region; we do not know whether it was between Ss and *H-2K*, within *H-2K*, or to the left of *H-2K*. Bailey derived a coisogenic strain that is genetically identical to C57BL/6, except for this single mutation; he called this new strain H (zl).

We thus have two coisogenic strains that were defined by the fact that they rejected skin from one another. H (zl) rejected the skin of C57BL/6 and C57BL/6 rejected the skin of H (zl). The unusual aspect of this finding was that when these animals were crossimmunized, there was no antibody response, and they did not have any serologically detectable differences.

The differences between the cells of these two strains, which could not be picked up by the usual methods of immunization and testing, did, however, result in MLC activation in both directions. So we set out, together with Klein, to see whether we could dissect this region further and make use of the mutants and the recombinants that had already been identified, to find out where in the H-2 complex the loci were located which caused activation of the MLC.

I want to point out that we tested over 300 combinations, all of them reciprocally. What I propose to show is the MLC test results for mice that differ only for the *H-2K* region, only for the *Ir-Ss-Slp* region, and only for the *H-2D* region. Some comment is required to provide assurance on how the data is interpreted. The critical points are listed in Table 32. I have already indicated that what we looked for was a significant difference between counts per minute (cpm) of radioactive thymidine in the allogeneic mixture culture and the isogeneic or syngeneic control.

This can be done by a "t" test; i.e., by expressing the result as a ratio of cpm incorporated in the allogeneic MLC to those in the isogeneic control mixture, or by comparing the cpm in any given allogeneic mixture with the cpm in other allogeneic mixtures in the same experiment, using the same responding cell (Table 33). We have used all these different methods to try to illustrate the point we would like to make.

TABLE 32

Evaluation of Significance of Stimulation in MLC

1. "t" test on log transformed data.
2. Ratio method. Expression of ratio of cpm incorporated into the allogeneic MLC to cpm in the control isogeneic or syngeneic mixture.
3. Comparison of cpm incorporated into a given allogeneic mixture with the cpm in other allogeneic mixtures using the same responding cell.

TABLE 33

MLC Reactions between Congenic Strains of Mice Differing in Restricted Parts of the H-2 Complex

Responding cells	Stimulating cells				
	C57BL/10	B10.Br	B10.A	B10.A(1R)	B10.A(5R)
C57BL/10	(7)[a]	57[b]	88[b]	54[c]	20[d]
B10.Br	78[b]	(17)	19[d]	18[d]	46[b]
B10.A	129[b]	26[d]	(10)	14[d]	59[c]
B10.A(1R)	72[c]	36[d]	26[d]	(12)	44[b]
B10.A(5R)	28[d]	73[b]	59[c]	56[b]	(9)

[a]cpm in thousands.

[b]Differences at *H-2K* and *H-2D*.

[c]Differences at *H-2K*.

[d]Differences at *H-2D*.

Table 33 shows a combinatorial experiment in which we are testing the response of cells of the five different strains that are listed. The responding cells are listed on the left; the stimulating (or mitomycin C-treated cells) are listed across the top. The control of C57BL/10 incorporates 7,000 cpm, and the stimulation in the allogeneic mixtures varies from 20,000 to 88,000. All these values of stimulation with C57BL/10 responding cells are highly significant with p values of less than .001.

One of the factors shown in this tabulation is the designation of the difference between the stimulating cell and the responding cell with respect to differing by *H-2K, H-2D,* or both.

The first point to note is that we could get stimulation, in contrast with the finding of Rychlikova *et al.*, even with differences only at the *H-2D* end.

By means of Tables 34 through 39, I want to lead you through a dissection of this region. Table 34 shows results for mice that differed only at the *H-2D* end. The two strains used were the same for *H-2K*, Ir and *Ss-Slp*, but they differed for the *H-2D* end. In the response of B10.T (6R) to B10.G stimulating cells in three different experiments, the average ratio of cpm of the allogeneic cells to control cells was 1.4, with the range 0.82-2.14; further, if we tested these responses by a "t" test, they were generally insignificant, or only weakly significant. In the opposite direction, it can be seen that in the three experiments we had varying responses in terms of significance, with the stimulation ratio generally low. If one wants to make quantitative comparisons (although we hesitate to do this) generally the strength of stimulation when there are *H-2D* differences alone is relatively weak. The data in Table 35 represent a summary of all the experiments we have done with *H-2D* differences alone. Twelve strains were

TABLE 34

Results of MLC Studies Using Lymphocytes with *H-2D* Differences Only

	H-2K	*Ir-1*	*Ss-Slp*	*H-2D*
B10.T(6R)	q	q	qq	d
B10.G	q	q	qq	q

MLC	No. of expts.	Ratio (exp/control)		Significance of stim.			
		Average	Range	N.S.	<.05	<.01	<.001
$6R(B10.G)_m$	3	1.39	0.82-2.14	2	1		
$B10.G(6R)_m$	3	3.44	1.99-5.36		1	1	1

TABLE 35

Summary of MLC Results in All Combinations with *H-2D* Differences

No. of strains	No. of combinations	Ratio	
		Average	Range
12	26	1.56	0.24-2.34

tested in 26 different combinations, each at least twice, several many more times, and the average stimulation ratio was 1.56. The p values ranged from insignificant to highly significant. Thus, some *H-2D* differences stimulate and some do not, by the criteria I have given, but on the average they certainly stimulate much less than when differences for all of the various regions are present.

Now, let us turn to the other end of the major histocompatibility complex where there are *H-2K* differences. One combination studied (Table 36) AQR and B10.A, is shown. These strains also differ for minor H loci. They differ for *H-2K*, they are the same for Ir, *Ss-Slp*, and *H-2D*. The stimulation is again relatively weak. In Table 36, it is seen that the stimulation ratio is on average 1.4 to 1.5, and all of the p values are either not significant or just less than .05. Similarly, if one tests A.AL with A.TL (Table 36), it is again seen that stimulation ratios are very low, and are either insignificant or just barely significant. So differences for *H-2K* alone result in very weak stimulation.

Table 37 shows results for strains that are identical for the serologically defined loci, e.g., B10.A(2R) versus B10.A(4R), in which *H-2K* and *H-2D* are identical. The only differences between these two congenic strains must lie between *H-2K* and *H-2D*. 2R and 4R differ for *Ir-Ig*, as described by Lieberman; the fact that they are both listed as K under *Ir-1* does not include recent information that they are *Ir-Ig* different in the study by Lieberman; 2R and 4R also differ in the *Slp* trait. Table 37 shows the response of 2R to $4R_m$. In four dif-

TABLE 36

Results of MLC Studies Involving *H-2K* Difference Only

	H-2K	*Ir-1*	*Ss-Slp*	*H-2D*
AQR	q	k	dd	d
B10.A	k	k	dd	d
A.AL	k	k	kk	d
A.TL	s	k	kk	d

MLC	No. of expts.	Ratio (exp/control)		Significance of stim.			
		Average	Range	N.S.	<.05	<.01	<.001
AQR(B10.A)$_m$	4	1.49	0.82-2.21	2	2		
B10.A(AQR)$_m$	4	1.35	0.79-1.99	3	1		
A.AL(A.TL)$_m$	1	0.63			1		
A.TL(A.AL)$_m$	1	1.97		1			

TABLE 37

MLC Tests between Lymphocytes
with Identical Serologically Defined Antigens

	H-2K	*Ir-1*	*Ss-Slp*	*H-2D*
B10.A(2R)	k	k	dd	b
B10.A(4R)	k	k	bb	b

MLC	No. of expts.	Ratio (exp/control)		Significance of stim.			
		Average	Range	N.S.	<.05	<.01	<.001
2R(4R)$_m$	4	1.40	0.92-1.75	3		1	
4R(2R)$_m$	4	2.58	1.67-3.75		1		3

ferent experiments there is a low stimulation ratio, with three of those values insignificant, and one significant with a p value of less than .01. However, in the opposite direction, 4R responding to 2R$_m$, despite the identity of *H-2K* and *H-2D*, there was a ratio of 2.6 with three of the experiments highly significant and one of borderline significance.

This is the first example of stimulation in one direction between cells with identical serologically defined loci.

Table 38 gives the data on the last example of this particular phenomenon which I would like to show. It is, again, a case where the serologically defined loci, *H-2K* and *H-2D* are identical, but there are differences at *Ir-1* and *Ss-Slp*. Note first the results with AQR and B10.T(6R). Clearly these two strains stimulate each other. The average stimulation ratio here is 5 and 6.5; the tests are all highly significant. Yet these strains differ only *between* D and K with respect to

TABLE 38

MLC Tests between Lymphocytes
with Identical Serologically Defined Antigens

	H-2K	*Ir-1*	*Ss-Slp*	*H-2D*
AQR	q	k	dd	d
B10.T(6R)	q	q	qq	d
B10.A	k	k	dd	d

MLC	No. of expts.	Ratio (exp/control)		Significance of stim.			
		Average	Range	N.S.	<.05	<.01	<.001
AQR(6R)$_m$	3	4.98	3.20- 9.00				3
6R(AQR)$_m$	3	6.55	2.75-12.84			1	2
AQR(B10.A)$_m$	4	1.49	0.82- 2.21	2	2		
B10.A(AQR)$_m$	4	1.35	0.79- 1.99	3	1		

TABLE 39

MLC Response of B10.A(2R) Lymphocytes
to Stimulation by Cells of Various Congenic Strains

Stimulating cells	*K*	*Ir-1*	*Ss*	*Slp*	*D*	Ratio
B10.G	q	q	q	q	q	5.93
B10	b	b	b	b		5.81
B10.A(3R)	b	b			d	9.71
B10.A(4R)			b	b		1.75
B10.Br			k	k	k	1.89
B10.A					d	1.74
HTG	d	d				8.59
AQR	q				d	3.14

the major histocompatibility complex. Sadly, these strains also differ at minor loci. However, it is possible to show that it is not the minor loci by themselves which cause this very pronounced stimulation. This is shown by comparing the combination of AQR with B10.A and with B10.T(6R). The latter two strains have the same minor loci. Thus, the response of AQR to B10.A, if the stimulation with B10.T(6R) were due to minor loci, should also be a strong response. Table 38 shows that the AQR response to B10.A, even with an *H-2K* difference plus the minor loci differences, is a very weak response. Therefore, we have to ascribe the strong reactivity between AQR and B10.T(6R) to the genetic differences mapping *between* the serologically defined loci, i.e., the lymphocyte-defined differences.

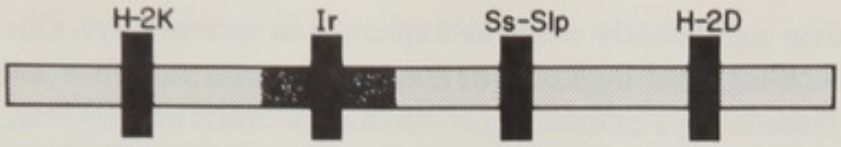


Fig. 29. Map of the mouse MHC. The four regions of the mouse MHC are designated. The region around Ir is heavily stippled since in this region there are known *LD* loci. The rest of the MHC chromosome is stippled since it appears that throughout this region there may be genetic differences which can lead to MLC activation.

Table 39 gives a summary of further data supporting the general conclusion which we would like to draw from these studies. This shows the response of B10.A(2R) cells to a variety of different stimulating cells. The table indicates the genetic regions by which the responding cells differ from the stimulating cells. As one glances down this chart it can be seen that wherever there is an *Ir-1* difference, the ratios are 6, or higher. Wherever there is not an *Ir-1* difference, the ratios are below 2 in three cases, and 3.14 in one case.

The general impression from all of these experiments is that strong stimulation is associated with differences which map with the *Ir-1* locus. Therefore, we wonder whether there are not a series of differences between *H-2K* and *H-2D* which we can define with lymphocytes in the MLC but which are very difficult to define by serology; this is shown schematically in Fig. 29. I hope that Klein will comment on some of the data which he has accumulated in this regard. We have shown, I believe, that insofar as the recombinants allow us to map these lymphocyte-defined, LD differences, they map with *Ir-1*.

It seems conceivable that the Ir product is, in fact, the recognition molecule on the responding cell in the MLC, but in addition, the Ir product can act as a stimulating molecule on the mitomycin C-treated cell—the molecule recognized as foreign by a lymphocyte and in response to which the lymphocyte proliferates. It is understood that something which maps with the *Ir* locus is not necessarily the same product. Obviously the *LD* loci, as I have referred to them, could be separate genes. We have obtained evidence that some of these loci are genetically separable from the serologically detectable loci. I would stress that although LD differences may be difficult to detect serologically by the usual cytotoxicity methods, it may be possible to show that antisera which are raised by AQR against B10.T(6R), for instance, will specifically block the MLC test. In addition, we have not ruled out the possibility that in the mouse there are *LD* loci to the left of *H-2K*, as we would have to hypothesize is true in the human. Similarly we do not yet know whether in man there may be *LD* loci between *Four* and *LA*.

At this point, we also do not know whether the serologically defined antigens play any role in MLC activation. The *H-2K* and *H-2D* end differences

which stimulate significantly could be explained in several ways. One is that the *H-2D* and *H-2K* locus serologically-defined antigens do stimulate, or, that in addition to there being loci between *H-2K* and *Ss* which are *LD* loci, there are also *LD* loci between Ss and *H-2D*. It may be those *H-2D* differences which also differ by *LD* loci stimulate similarly for *H-2K*. We do not have critical evidence one way or the other. I would like to mention very briefly a point which I hope will be discussed later. In man, there seems to be some correlation between the serologically defined antigens and lymphocyte responsiveness in MLC. So we must hypothesize either that the serologically-defined antigens, Four and LA, (and perhaps *H-2K* and *H-2D*) play some role in causing direct activation of MLC, or that linkage disequilibrium allows some prediction of MLC reactions by typing for the serologically-defined antigens.

BENACERRAF: I would ask Bach whether mice which are MLC positive, but identical at the *K* and *D* loci, accept skin grafts indefinitely?

BACH: In some cases mice which are MLC positive but identical at *H-2K* and *H-2D do* reject skin—in fact in a quite rapid fashion. However, in one case, B10.A(2R) versus B10.A(4R) there is *one-way* stimulation in the MLC, serological identity for *H-2K* and *H-2D,* and *no* skin graft rejection.

CHAIRMAN McDEVITT: Let us now come back to Shreffler, who has some further evidence on this very point.

SHREFFLER: I would like to report further data which also demonstrates MLC stimulation in another case involving only Ir and Ss region differences. This work has been done in collaboration with Meo, Vives, Miggiano, and Ceppellini, at the Basel Institute for Immunology. Table 40 shows our results with the congenic strains A.TH and A.TL. These are results with lymph-node cell cultures. The data are presented with values for level of stimulation in cpm, the indices of stimulation, and their statistical significance, based on the t test. The regions which are different between responding cells and stimulating cells in each of the combinations tested are indicated above the cpm values. First, it will be noted that with only an Ss difference (the A-A.AL combination), there is a rather low level of stimulation, which is nevertheless significant. In the opposite direction, there is essentially no stimulation. With a K plus Ss difference (A vs A.TL), a weak nonsignificant stimulation was found, but when there is a difference also involving Ir (A vs A.TH), quite a high and significant stimulation was found. With a K difference alone, the stimulation was weak or essentially absent (A.AL vs A.TL).

With a D difference (A.TH vs B10.S), there is no stimulation. In the K + D incompatible case (B10.AKM vs A.TL) stimulation is perhaps a bit higher, but

TABLE 40

MLC Reactions between Lymphocytes of Congenic Strains Differing at Restricted Parts of the H-2 Complex

Responding cells	Stimulating cells					
	A	A.AL	A.TH	A.TL	B10.S	B10.AKM
		Ss	*K,Ir,Ss*	*K,Ss*		
A	1,500	3,300	11,900	3,000		
	-	2.2[b]	7.9[c]	2.0		
	Ss		*K,Ir,Ss*	*K*		
A.AL	4,200	4,400	13,400	5,200		
	0.9	-	3.0[c]	1.2		
	K,Ir,Ss	*K,Ir,Ss*		*Ir,Ss*	*D*	
A.TH	44,900	58,400	16,800	42,100	22,500	
	2.6[b]	3.4[c]	-	2.5[b]	1.3	
	K,Ss	*K*	*Ir,Ss*			
A.TL	1,100	1,100	4,700	700		
	1.5	1.5	6.7[c]	-		
	K,Ir,Ss,D		*D*	*Ir,Ss,D*		
B10.S	63,700		12,900	45,800	17,800	
	3.5		0.7	2.5[a]	-	
				K,D		
B10.AKM				1,100		500
				2.2		-

[a] $P < .05$

[b] $P < .01$

[c] $P < .001$

the difference is not significant. In all of the cases involving an Ir difference, on the other hand, significant and relatively high levels of stimulation were observed. The key point in the data is that in the A.TH-A.TL comparison, where the differences involve Ir and Ss without either a K or a D difference in the reciprocal combinations, the stimulations are quite significant. Thus it seems quite clear both in our data and those of Bach, that the Ir region plays a very important role in the MLC reaction independent of any differences in the serologically detected antigens, which give only weak stimulation.

CHAIRMAN McDEVITT: I believe I would summarize the foregoing by saying that the evidence presented in these three principal discussions attests to the H-2 complex including the *K* locus, a series of Ir genes, the *Ss* locus, and the *D*

locus, and that the genes controlling antigens which stimulate the MLC reaction appear to be localized in the same region of the H-2 complex as the Ir genes.

BACH: I think we really have to go further than that. In fact, I think we have to extend it to include the region to the right of Ss, and perhaps the *D* locus as well.

CHAIRMAN McDEVITT: What Bach has shown us is K, D, Ss, a certain number of Ir genes in the left center region, and evidence that the sites controlling the MLC are located in the same place as the Ir genes. But what can Bach tell us with respect to K and D? Can they also stimulate in the MLC reactions?

BACH: Most of the *H-2D* differences do, in fact, stimulate weakly. I believe I showed some selective examples; some of these do stimulate in repeated testing. Isolated *H-2K* differences have been weak stimulators, but we have only a couple of these to test, and one of them we have tested only once. Let me stress again, we do not know whether it is the *H-2K* or *H-2D* products or LD products closely linked to these loci which stimulate.

CHAIRMAN McDEVITT: In addition to this we have Green's evidence that antisera to histocompatibility antigens in the guinea pig will selectively block the function of histocompatibility-linked specific immune response genes in an F_1 heterozygote. This raises a number of questions concerning the relationship between serological histocompatibility antigens, genes for the MLC antigens, and for the Ir genes, and for graft rejection antigens. In particular, if strain combinations exist which differ only at Ir and MLC but are identical with respect to the serologically-defined histocompatibility antigens and histocompatible on skin graft testing, then we must explore the relationship between Ir genes and MLC genes and determine whether these are closely related but separate functions, or in fact represent the effects of the same gene products. We do not have very much information on this point. This region codes for the Ir genes which appear to control recognition of specific antigenic determinants. It also appears to be responsible for cell surface antigens which are capable of the strongest stimulation in the MLC test. It is not clear to me whether this type of stimulation is due to Ir gene products or whether the two functions are served by different gene loci within the same chromosome region.

It might be worthwhile at this point to bring out specific data on strain combinations which differ only at Ir but are histocompatible in skin graft testing. Most pertinent in this regard is the B10.A(2R) and B10.A(4R) recombinants which differ for Ir genes controlling immune response to mouse immunoglobulin allotypes, stimulate in one direction in the MLC test, but are histocompatible.

BACH: I think the combination which is of interest insofar as we have data, is first, the Bailey mutant, which is a single mutation to the left of Ss in which

there is reciprocal graft rejection and reciprocal MLC stimulation; second, the combination of 2R-4R, which shows evidence of good stimulation in one direction (a very unusual finding), and in which there is no skin graft rejection; and third, the combination of B10.T(6R) and AQR in which (despite minor H loci differences) Klein has shown that skin grafts are rejected due to the differences in the major histocompatibility complex and there is reciprocal MLC stimulation. I hope he will speak of these studies.

CHAIRMAN McDEVITT: At this point, it might be appropriate for Lieberman to present her evidence on the immune response gene differences between B10.A(2R) and B10.A(4R). This strain combination appears to be identical for the *K*, *Ir-1*, *Ss* and *D* loci, but different for the *Ir-Ig* loci, and some MLC antigens.

LIEBERMAN: I would like to report on a collaborative investigation which included Paul, Stimpfling, William Humphrey, Jr., and myself. One of the questions we posed was whether *Ir-1* was a single locus with multiple alleles. I think that our findings with two Ir genes, each controlling the immune response to a different immunoglobulin allotype, indicate that we have at least two separate *Ir* loci, and if we look at our findings in terms of the *Ir-1* data of McDevitt and Chinitz, it seems that there may be three separate Ir genes at separate loci.

In our past work we reported that the immune response to the *a* allotype allele of IgA in the mouse was under the control of a gene linked to H-2, designated *Ir-IgA*. Partial chromosome mapping of the *Ir-IgA* gene showed it was associated with the K region, similar to *Ir-1*. We were not able to localize it precisely between Ss and the K region as McDevitt had done, but rather associated it with the K region specificities. The *Ir-IgA* gene could have been to the left of the K region or somewhere in the K region. Since then we have investigated another immune response gene controlling the immune response to another class of immunoglobulin of *a* allotype; i.e., the IgG (γ_{2a}) immunoglobulin on *a* allotype myeloma proteins of the BALB/c mouse. It is noteworthy that the *Ir-IgG* gene is associated with different H-2 types than the *Ir-IgA* gene. The *Ir-IgA* high response allele is associated with $H\text{-}2^a$, whereas the *Ir-IgG* high response allele is associated with $H\text{-}2^b$. Thus, linkage of the two different high response alleles with two different H-2 types permitted us to examine the recombinants of $H\text{-}2^a/H\text{-}2^b$ that were available in order to determine the relationship of the *Ir-IgA* gene to the *Ir-IgG* gene in the H-2 locus. Table 41 shows the congenic strains that have been immunized with γ_{2a} myeloma proteins derived from BALB/c. The immunoglobulins of the strains shown here have none of the *a* allotypic determinants that are present on the BALB/c myeloma protein that was used for immunization. The only difference, therefore, was in the H-2 type of each strain.

TABLE 41

The Immune Response of C57BL/10 (B10) H-2 Congenic Strains to BALB/c IgG (γ_{2a}) Myeloma Protein (MOPC 173)

H-2	Strain	No. of mice	Log 2 HA titer	
			Idio	Allo
a	B10.A	9	2.88	2.77
b	B10	15	7.28	6.83
bc	B10.129(6M)	10	9.12	6.75
d	B10.D2	10	1.20	1.20
k	B10.BR	8	2.00	2.00
p	B10.P	6	11.33	8.66

Antisera reacted with the specific myeloma protein used for immunization and may identify both the idiotypic myeloma specific determinant present on the Fab segment and the allotypic determinants present on the heavy constant region (CH).

None of the strains shown have any of the known IgG (γ_{2a}) allotypic determinants present on the BALB/c IgG myeloma protein immunogen.

The HA method of Gold and Fudenberg, *J. Immunol.* **99**, 859, 1967, was used.

As is evident, there is a clear association with specific H-2 types and the ability to produce an immune response to BALB/c γ_{2a} myeloma proteins. The *H-2*^{b}, *H-2*^{bc}, and *H-2*^{p} types are all associated with high immune responses to both the idiotype and to the *a* allotype present on the immunizing myeloma protein. It is well known that the allotypic determinants are present on the constant region of the heavy chain. The idiotypic determinants are myeloma specific and are present on the Fab segment. Table 42 identifies the H-2 types associated with the *Ir-IgG* gene controlling the immune response to γ_{2a} (IgG) *a* allotype and the association with the *Ir-IgA* gene controlling the immune response to γA *a* allotype myeloma proteins. It is apparent that *H-2*^{a} is associated with a low immune response to γG_{2a} *a* allotype and a high response to γA *a* allotype. *H-2*^{b} is associated with a high response to γG *a* allotype and a low response to γA *a* allotype. A few other differences are also emphasized because later on we will be discussing the *Ir-1* genes, that is, *Ir-(H,G)-A--L* and *Ir-(T,G)-A--L* in terms of the H-2 types associated with *Ir-IgG* and *Ir-IgA* genes. You will recall that McDevitt showed that *H-2*^{s} is associated with a *low* response to (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L. However, in Table 42, it can be seen that *H-2*^{s} is associated with a *high* response to both γG and γA myeloma proteins of BALB/c origin. Similarly, *H-2*^{p} also differed in its immune response from that obtained by McDevitt with (H,G)-A--L. It was high for both γG and γA BALB/c myeloma proteins, and low for (H,G)-A--L. Thus, this analysis shows three chromosome types (*H-2*^{p},^{r}, and ^{s}) in which a discordance was noted be-

TABLE 42

The H-2 Alleles Associated with Ir-IgG and Ir-IgA Genes Controlling the Immune Responsiveness to IgG(γ_{2a}) and IgA Myeloma Proteins Respectively, of BALB/c Origin

H-2	IgG		IgA	
	Idio	Allo	Idio	Allo
a	L	L	H	H
b	H	H	L	L
bc	H	H	L	L
d	L	L	L	L
k	L	L	H	H
m	N.T	N.T.	M	L
p	H	H	H	M
q	L	L	L	L
r	H	H	H	H
s	H	H	H	M
v	H	H	L	L

L = low; H = High; M = medium; NT = not tested.

Antisera reacted to the immunogens (M173 or M467) and may identify both (CH) allotypic and (Fab) idiotypic determinants.

TABLE 43

Immune Response of *H-2^a/H-2^b* Recombinant Strains to BALB/c IgG (γ_{2a}) (M173) and IgA (M467) Myeloma Proteins

Strain	H-2	γG (γ_{2a})			γ A		
		No. of mice	HA (log 2)		No. of mice	HA (log 2)	
			Idio	Allo		Idio	Allo
B10	b	15	7.3	6.8	10	0.4	0.1
B10.A	a	9	2.9	2.7	8	10.8	2.0
Recombinants							
(1R) B10.A(2R)	h	20	2.3	0.4	10	9.5	2.0
(3R) B10.A(5R)	i	20	9.8	5.2	10	0.1	0.1
B10.A(4R)	h	6	8.3	1.6	6	7.8	3.2

Antisera reacted with specific myeloma proteins used for immunization and may identify the myeloma specific (Fab) idiotypic determinants and also the (CH) allotypic determinants.

tween the immune response to *a* allotype myeloma proteins and the multichain synthetic polypeptides.

Figure 30 and Table 43 show the immune response of the *H-2^a/H-2^b* crossovers that we have discussed during much of this session. The recombinants

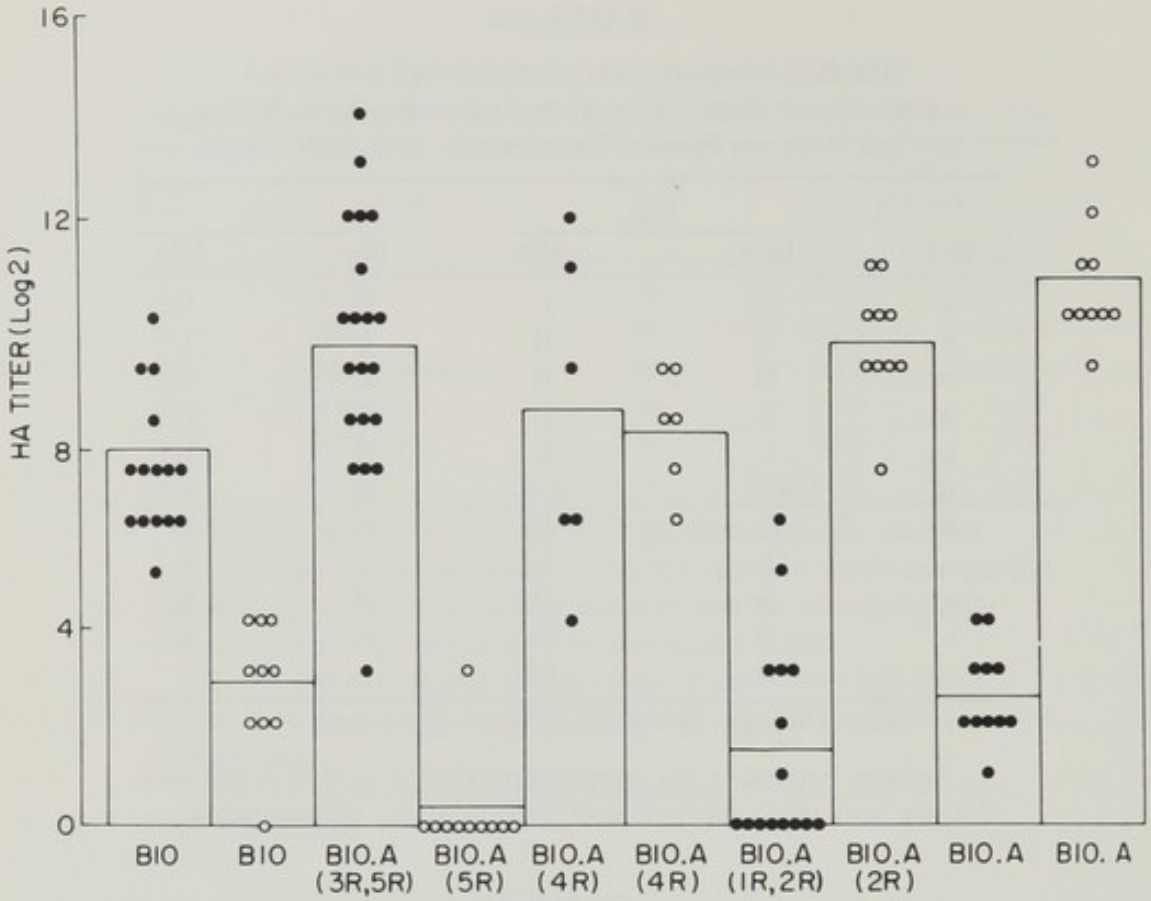


Fig. 30. Immune Response of $H\text{-}2^a$, $H\text{-}2^b$, and $H\text{-}2^{a/b}$ recombinants. The immune response of B10 ($H\text{-}2^b$), B10.A ($H\text{-}2^a$) and $H\text{-}2^a/H\text{-}2^b$ crossover mice B10.A (1R), B10.A(2R), B10.A(3R), B10.A(4R) and B10.A(5R) to IgG (γ_{2a}) and IgA myeloma proteins of BALB/c origin. The 1R and 2R are combined as the H-2 products have not yet been distinguished. This also holds for 3R and 5R. Solid circles indicate HA titer to IgG myeloma proteins; open circles to IgA myeloma proteins. Geometric mean titers are shown for each strain.

B10.A(1R), B10.A(2R), and B10.A(4R) are $H\text{-}2^h$ recombinants. The K region of their chromosomes are derived from $H\text{-}2^a$ and the D regions are derived from $H\text{-}2^b$.

The difference between B10.A(4R), and B10.A(1R) and B10.A(2R) is in *Ss-Slp*; B10.A(4R) is $Ss^h\ Slp^o$, whereas 1R and 2R are $Ss^h\ Slp^a$. The crossover occurred in different regions of the H-2 chromosome of these recombinants. The immune response to γG *a* allotype was *high* for B10.A(4R) and *low* in the B10.A(1R) and B10.A(2R) strains. In all these strains the K regions of the chromosomes are derived from $H\text{-}2^a$. In Figure 30, it is seen that B10.A(2R) and B10.A(4R) are high responders to γA *a* allotype and this is indicated by the open circles. Thus, *only* the B10.A(4R) recombinant responded well to *both* the γG (solid circles) and γA (open circles) *a* allotype myeloma proteins.

Figure 31 is a schematic design of the chromosomes of the recombinants classified in Fig. 30. The $H\text{-}2^a$ and $H\text{-}2^b$ chromosomes are shown as broken and

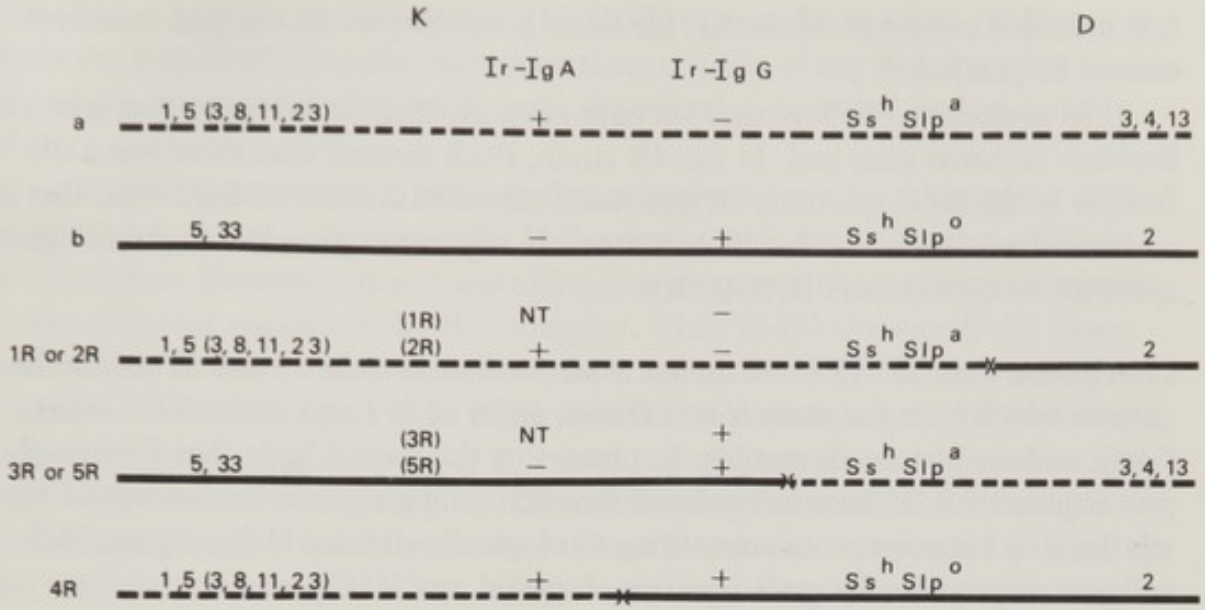


Fig. 31. Schematic representation of the chromosomes of B10.A ($H\text{-}2^a$), B10 ($H\text{-}2^b$) and the $H\text{-}2^a/H\text{-}2^b$ crossover chromosomes of B10.A. The region of crossover in the generation of each recombinant chromosome is indicated by an X. It has previously been established that the B10.A(4R) chromosome resulted from a crossover event between the *K region of H-2* and the *Ss Slp* genes. The data presented in Table 41 indicate that the crossover separated the *Ir-IgA* and *Ir-IgG* genes which occur in this region. This indicated that *Ir-IgA* and *Ir-IgG* are at separate loci. The 1R and 2R are combined as the H-2 products have not yet been distinguished. This also holds for 3R and 5R.

EDITORS' COMMENT: The reader is advised to compare the map of the $H\text{-}2^a$ and $H\text{-}2^b$ chromosomes given in this figure with the simplified version given in Table 28 and in the editors' foreword to this session. In essence, Lieberman has used the numbered antigenic specificities to represent the alleles of the *H-2K* and *H-2D* loci which are present in these chromosomes and has added postulated loci for *Ir-IgG* and *Ir-IgA*.

solid lines, respectively. Inspection of the chromosome maps show the linkage between the *H-2K* region and the *Ir-IgA* and *Ir-IgG* genes in these recombinant strains.

The B10.A(1R) and B10.A(2R) strains possess the *H-2K* region of $H\text{-}2^a$ and have the same responsiveness as B10.A ($H\text{-}2^a$). The B10.A(3R) and 5R strains share the same *H-2K* region gene of $H\text{-}2^b$ and have the same immune responses to IgG and IgA *a* allotype myeloma proteins as B10 ($H\text{-}2^b$).

The immune response of B10.A(4R) is exceptional. Since it shares the *H-2K* region gene of the $H\text{-}2^a$ type, it would have been expected to be a high responder to IgA *a* allotype and a low responder to IgG *a* allotype. However, the response to both IgA and IgG *a* allotypes was high. On this basis, the crossover event resulting in the H-2 complex of the B10.A(4R) strain appears to have occurred between the *Ir-IgA* and *Ir-IgG* genes. We place the order of the genes on the chromosomes as *Ir-IgA, Ir-IgG* to the left of the *Ss-Slp* for the simple reason that this would entail a single crossover. Any other order would necessi-

tate a double crossover. However, the latter possibility or an unequal crossover cannot be precluded.

In summary, I believe that we have good evidence attesting to two separate immune response gene loci. In the 4R strain, Bach showed that there was a difference in the MLC reactivity of this strain versus B10.A(2R). I think that this is additional evidence that the 4R chromosome region associated with the K region specificities differs from B10.A(2R).

CHAIRMAN McDEVITT: Klein has evidence that there are other strain combinations which have the same *K* and *D* loci, differ at *Ir-1* and at the *MLC* locus (loci), and are histoincompatible. In presenting this data, I hope that Klein will pull together for us these interrelated functions and give us some concept of the relationship between genes controlling serologically-defined H-2 antigens, H-2 antigens responsible for graft rejection, Ir genes, and MLC genes.

KLEIN: It is clear that we are dealing with an extremely complex region, the products of which can be detected by at least four different approaches: serological, transplantation (mostly skin grafting), methods for studying the genetics of immune responses, and finally methods of activating lymphocytes *in vitro* (MLC).

As far as the serological evidence goes, it seems clear that the complex contains at least two genes, *H-2K* and *H-2D*, which control two series of antigenic specificities. Although additional genes for serologically detectable products in the H-2 region cannot be completely ruled out, so far everything seems to indicate that there are only two. Skin grafting data also seem to indicate that there are only two loci, *H-2K* and *H-2D*, identical with the loci controlling serologically detectable antigens. Either of these two loci can cause skin graft rejection. Again, although it cannot be completely ruled out that there are other histocompatibility loci inside the H-2 complex, all the skin grafting results are at least compatible with the two-gene hypothesis. However, they do not rule out the possibility that there are H genes outside of but closely linked to the H-2 complex as we know it today.

What is the relationship between serologically detectable H-2 antigens and H-2 antigens causing skin graft rejection? In the overwhelming majority of cases, serological identity is associated with permanent skin graft survival and serological difference with skin graft rejection. However, there are exceptions to this rule. One example is Bailey's mutant which is serologically indistinguishable from the original strain, and yet skin grafts exchanged between the two strains are rejected. Another example are the B10.T(6R) and AQR recombinant lines. We have tested them with a large panel of H-2 antisera and do not find any serological difference. We have also crossimmunized them and found no detectable antibodies. Yet the two strains are not histocompatible, as witnessed by the fact

that (B10 x AQR)F_1 hybrids reject B10.T(6R) skin grafts at 14 to 16 days. Since the B10.T(6R) line also carries markers outside of the H-2 complex (T and tf), it is possible that in this case the rejection is caused by loci outside of H-2.

The MLC data seem to indicate that stimulation can be achieved by differences at almost any point across the H-2 complex with the exception of the *Ss-Slp* region. However, there are striking quantitative differences in stimulation by the different regions of the H-2 complex. While *H-2D* alone or *H-2K* alone lead usually to only weak stimulation, the Ir region alone gives a very strong response. There also seems to be some additive effect between the different regions in the sense that differences at several regions provide a stronger stimulus than a difference at a single region. The relationship between MLC stimulation and skin graft rejection is at present not clear. However, once there is at least one well-documented case where MLC stimulation occurs despite permanent skin graft survival, namely the B10.A(2R) and B10.A(4R) case, it seems possible to conclude that the two phenomena operate independently.

The picture emerging from the Ir studies is that of multiple genes located primarily between the *Ss-Slp* and *H-2K* regions. Of these, two are now firmly established, namely the *Ir-1* genes of McDevitt and those of Lieberman. The evidence for additional Ir-genes in the *H-2K* region is incomplete or circumstantial. The possibility of Ir genes between *Ss* and *H-2D* regions or outside the H-2 complex should at least be kept in mind. In general, however, it seems that the Ir genes are concentrated in the chromosomal segment which gives the strongest stimulation in the MLC test.

On the basis of these findings, the H-2 complex can be interpreted in one of the following ways: First, the molecules detected by the different methods mentioned are all different and have very little in common. This possibility is hard to accept in view of the intimate relationship between the phenotypic effects of the different products. Second, at least some of the approaches detect the same products. It can be speculated that the serological and transplantation methods detect one type of product, and the MLC and Ir methods detect another type of product. This possibility is supported by the finding that strains which are serologically identical and accept each other's skin grafts, but differ in their Ir genes, also activate each other's lymphocytes in the MLC.

The third possibility is that all the genes in the H-2 complex determine the same type of products and the different manifestations of these products are due merely to the differences in the methodology. This hypothesis would, of course, require the *H-2D* and *H-2K* genes to be expressed differently from genes in the middle portion of the H-2 complex. Although this last possibility is certainly the most attractive one, it will be a matter for future research to decide which interpretation is the correct one. I have no doubt that in the near future,

indirect evidence will be forthcoming from both genetic and immunological studies which will support one or the other interpretation. I am equally convinced that the final decisions regarding the various hypotheses will be possible only after the individual products of the genes in the H-2 complex are isolated.

CHAIRMAN McDEVITT: Klein's contribution opens the way for some comments that Bodmer would like to make concerning the population genetics of closely linked loci.

BODMER: This is a topic that comes up frequently in these discussions. Why do loci with related functions tend to be near each other, and why do we have associations between phenotypes determined by linked loci? The population genetics of linked loci is a subject that has evolved since 1930, when Fisher first speculated on this question.

Let me take a completely general model unrelated to any of the genetic symbols we have seen here. Consider two linked loci, each with two alleles, A and a, and B and b. These are loci on the same chromosomes and give rise to four genetic combinations: AB, Ab, aB, and ab. One can look separately at the A-a locus and the B-b locus and ask what is the population frequency of A as compared to a, and similarly for B and b.

Now we can ask the question—if one looks at the combination of the two loci—is there an association? Let the frequencies of A = P and a = Q, then Q = 1 − P; and of B = p and b = q, similarly q = 1 − p.

If there is independence between the two loci, then the frequencies of the genetic combinations should be just the products of the relevant frequencies. That is Pp for AB, Pq for Ab, QP for aB, and Qq for ab. In other words, there should be no particular association between the two loci.

It is a standard result of population genetics that in the absence of selection, whatever the recombination fraction between these two loci, the population eventually arrives at this complete independence between the two loci. In other words, *at equilibrium,* there is a fundamental difference between association of loci due to linkage *in families* (which if there is close linkage may be almost complete if the recombination fraction is small) and association at the *population level,* where there may be no tendency at all for the two loci to show any sort of association.

It can be shown that the rate at which this tendency to an independent pattern is approached, if we don't start off with independence, is a function of $(1\text{-}r)^t$ where t is the time and r the recombination fraction. In other words, in the absence of selection, and given enough time, however closely two loci are linked, they will not show their linkage at the population level.

When we are looking at pairs of loci which are closely linked, and *do* find a tendency for association at the population level, what is the explanation? The

explanation in formal terms is that certain gametic combinations are not obeying the rule that they should occur with a frequency that is the product of the gene frequencies, and this phenomenon is called linkage disequilibrium.

In the extreme situation, if we started off with a population with only the combinations AB and ab, we would start off with an absolute association between A and B, and between a and b, before the recombinant types had been produced. Eventually, by recombination, we would produce Ab and aB and these would increase in frequency, until in the absence of selection the frequencies which give rise to independence had been achieved.

So one explanation for the lack of independence is that there has not been enough time, since a given combination arose, for alleles of the two loci to reassociate until they are, in effect, independent. In other words, if a particular combination arose comparatively recently there simply may not be enough time to reassociate them at random with other combinations already present in the population.

You may recall that during the preceding session, after Benacerraf's discussion on the association of histocompatibility genes with immune response genes in random-bred guinea pigs, I asked whether the random guinea pigs were derived from a large or a small founding stock. If they were derived from a relatively small stock, then, initially, there may have been a very limited number of genetic combinations of a given H antigen with a particular immune response gene. Suppose, for example, the high response gene was on the chromosome which had a type 2 antigen and the low response gene on the chromosome which had type 13. Then there might not have been enough time to produce the low:2 and high:13 recombinants in reasonable number, and so, because one started with a small number of genetic combinations, there remains in the random-bred stock a tendency for an association between the two phenotypes, immune response and H antigens. If, however, one started with a much larger stock in which *all* possible combinations were present to start with, (perhaps with equilibrium frequencies as expected for a population which has been interbred for a long time) then one would not see this association. In other words, one can get a population association due to close linkage, because he started with a limited number of genetic combinations which were not in the situation of being independent one from the other in the founding population.

The difference between the behavior of linked loci in *populations* as opposed to *families* is the main reason why the genetics of HL-A in man progressed in a completely different way from the genetics of H-2 in the mouse. So much of the data in man has been based on studies of the population at large, where one has to interpret associations in terms of population genetic criteria. In the mouse on the other hand, we are working with a limited number of genotypes represented by the available inbred strains, and we do not have data on all the possible combinations which can occur.

I think this is an extremely important point because it means that when there are a lot of closely linked genes, as in the H-2 and HL-A regions, the more genes there are the less likely it is that any finite set of genotypes is a proper random sample of all possible combinations. And so, the more likely it is that among that particular set, there will be found associations due to the particular combinations which were there to begin with. It is for these reasons that one has to be so careful in extrapolating from associations between given phenotypes and genotypes in inbred strains and the presence of one or more specific genes controlling the phenotypes. Lieberman's contribution is an excellent example of this, and illustrates how one can begin to separate functions which may be very closely linked only if we look at carefully chosen recombinant genotypes.

What about the more general question of why there is a region with so many linked genes with apparently related functions? What holds it together? This relates to the question of how we can have a situation in the population genetics of linked loci where in fact we do not end up with the genes associated at random even after a long period of time. What Fisher suggested in 1930 is that, if there has been selection which favors particular combinations of genes, say AB and ab, relative to the other combinations (AB and aB), we may arrive at an equilibrium state where we no longer have the loci associated at random.

Since 1930 there have been a great many papers on theoretical population genetics which have examined the conditions under which equilibrium does not result in random recombinations of a pair of linked loci. In general terms, the conditions are that the selective interaction, namely the tendency to favor particular combinations over others, must be of the order of the recombination fraction or less. This means that the closer the linkage between two genes, the smaller the selective interaction which is needed to tend to hold them together.

In population genetic studies there are many examples known in higher organisms of sets of genes which tend to be held together, presumably by the mechanism of selection. We all heard earlier the term "supergenes" coined by E. B. Ford. This is a term I do not particularly like because it has no clear-cut meaning. What it intends to refer to is a cluster of genes held together because of their interactive selective advantages.

I think it is fairly clear that what we have in the HL-A and H-2 regions is a super example of a supergene! Here we have a set of genes with entirely interrelated functions, that are presumably held together in evolution by this mechanism of selection which tends to favor combinations which interact with each other.

I think this is the answer in very simple terms, to the question Lilly raised earlier of why we have this particular and unique genetic structure with two loci with very similar genes: D and K in the mouse, LA and Four in man, separated by hundreds or even thousands of other genes. I expect there is probably a simi-

lar region in all mammalian species which has been held together throughout the course of evolution of the mammalian species.

It is a natural speculation, of course, as Shreffler and others pointed out some time ago, that the homology between LA and Four, and between D and K, and more generally between this whole set of genes, is produced by successive steps of duplication. This is a mechanism of expanding a genetic region which is well known and described in the classical genetics of Drosophila.

CHAIRMAN McDEVITT: Would Bodmer tell us whether he thinks the Ir genes are basically the same as the K or D molecule?

BODMER: McDevitt's proposition is certainly quite possible. One has to ask the question—what was the initial unit that started to get duplicated? It could have been a single gene or it might have been a small cluster which was then reduplicated. The question then arises of what might have been the relationship among the genes in that initial cluster. It is worth pointing out that there are two basic ways of duplicating genetic information in evolution.

One is, as I have stated, tandem duplication of a restricted region by unequal crossing over. The other well known mechanism is to reproduce whole chromosomes. Thus, a likely speculation in the case of the hemoglobins (and probably also the immunoglobulins) where one has α chains on the one hand, and $\beta,\gamma,\delta,\epsilon$ chains, on the other, which are all related, is that we had a primordial hemoglobin chain gene and the chromosome in which the gene occurred duplicated. One duplicate became the chromosome which carried the α chain gene and the other became the chromosome that carried the β chain genes, and these further differentiated in a way that was different from the α chain gene.

Going back to the earlier discussion on the possible relationship of H genes to immunoglobulin genes, the evolutionary speculation would be that these two are derived from a common ancestor in an essentially similar way. Namely, that we have immunoglobulin genes on the one hand, and H genes on the other, and that both sets may originally be derived from a common set of ancestors that were duplicated by duplicating the chromosomes. Subsequently they have evolved to have different functions.

The speculation which I have made elsewhere, following Jerne and Burnet, is that the common origin of these functions, and particularly the origin of those genes linked to the H genes, is connected more generally with cell-cell recognition during differentiation, which certainly predates the evolution of the immune response.

CEPPELLINI: I would like to add a few comments to what has been so clearly explained by Bodmer. For man, another case of linkage disequilibrium is known, again related to immunology because it is between the alleles coding for the con-

stant part of γ chains. The region is "γ_1 (Gm^a, Gm^f); γ_3 (Gm^b, Gm^g); γ_2; γ_4." The sequence in writing corresponds to the most likely chromosomal map according to Kunkel. The Gm types in parentheses indicate the alleles for γ_1 and γ_3 which are more common among Caucasians. Nonetheless, only two chromosomal combinations (or haplotypes) are found: "$\gamma_1 Gm^a$; $\gamma_3 Gm^g$" and "$\gamma_1 Gm^f$; $\gamma_3 Gm^b$." Haplotypes "Gm^a; Gm^b" and "Gm^f; Gm^g" are extremely rare in Europe. It is true that the linkage between γ_1 and γ_3 is very close, ($<10^{-4}$), but this polymorphism is very ancient, because chimpanzees have the same Gm markers as man. Thus, there has been enough time in generations to reach equilibrium among all possible haplotypes. We, therefore, are justified in assuming that in the European environment some selective advantage favors the observed haplotypic combinations.

Very likely selective advantage is also instrumental in maintaining the close linkage between the genes coding for heavy chains. In this case we may think of a good justification; in fact, closeness on the chromosome may be one prerequisite for joining different V genes with different C genes through intrachromosomal translocation. If the joining of the variable and constant part takes place at the DNA level, the Ig regions must act as integrated functional units, almost as chromosomal organelles.

The compartmentalization of the chromosomes into functional units composed of many genes is probably a common phenomenon in higher organisms and it may also apply to the many genes included in the main histocompatibility systems.

HERZENBERG: I would like to present an idea which can be shot down rapidly, but it may perhaps serve to provoke some discussion. I perceive a way which might bring together this very interesting observation—that MLC and Ir map in the same genetic region or are the same genes, and the question of whether it is the T or B cells on which Ir is expressed.

In fact, when (T,G)-A--L binds to certain B cells, which we know it does, it may not be recognized as free (T,G)-A--L, but as a new cell-type determinant. The T cell, working on this determinant in the same way in which Ir functions in its other manifestation as a cell recognition unit, recognizes a lymphocyte which has a genetically determined different surface configuration. So put the two together: T cells expressing Ir (equals MLC or LD, as Bach labels it), recognize other cells which have a different cell surface configuration, either genetically determined or determined by combination with antigens like (T,G)-A--L, although they could not recognize (T,G)-A--L in the first place.

I have one final point to make. There is another experiment which might be reinterpreted in this way, the Katz-Benacerraf type of observation (the "allogeneic effect") and Grumet's observation that the GVH reaction turns nonresponders into responders. This is really but another way of getting T cells stimu-

lated so as to get B cell differentiation. After T cell recognition of the complex, we then get the production of factors which are able to permit differentiation of B cells to antibody-producing cells.[a]

BACH: I would like to return to the question Benacerraf raised earlier regarding graft rejection. I think this is a very interesting point, and whereas I agree with almost everything Klein has said, I have a couple of points I would like to raise. The first is that in the AQR-B10.T(6R) combination there is a difference *between H-2K* and *H-2D*. There is stimulation in the MLC. There is also graft rejection, and whereas I readily agree that the locus which leads to graft rejection may be different from the one which leads to MLC activation, I would think that it is also possible that the locus between *H-2K* and *H-2D* which leads to MLC activation also leads to graft rejection.

The Simonsen finding of a very high frequency of responding cells in the GVH system and the same observation in MLC has intrigued all of us. I think that perhaps we can, at this stage, say that this may not be such a surprising finding because now we really have no idea of how many different structures are being recognized on one allogeneic cell, and we have very little idea how many *LD* loci there are.

It would seem to me the critical test which we should be aiming for in answer to Benacerraf's question is whether in these situations, where there is an *in vitro* lymphocyte response, there is also a corresponding *in vivo* lymphocyte response—such as for example the GVH reaction. This is a thesis which we are now testing.

[a]AFTERTHOUGHT BY BRAUN: Much of the discussion on the role of Ir genes has focused on their control of specific receptors for antigens. However, I would like to reiterate that these genes, in addition to controlling recognition at the T and B cell level, may also regulate the critical interaction between helper T cells and antigen-reactive B cells.

We have good evidence now that the detectable performance of B cells, which is the criterion in all of the experiments discussed here, requires two signals to the relevant B cell: 1. a specific antigenic signal and 2. a signal for the activation of the cAMP-mediated amplification system. Elsewhere (*Ann. N.Y. Acad. Sci.*, 1972), I discussed the evidence that the second signal may be provided by the interaction of antigen-reactive B cells with antigen-activated T cells. Earlier, I referred to the evidence that such interactions, if they lead to an excessive stimulation of the cAMP system in B cells, may inhibit B cell functions. Insufficient stimulation of the amplification system in B cells also may result in a failure to obtain detectable B cell functions.

The question then is: To what extent may the influence of Ir genes on the obligatory amplification system account for low responses versus high responses? The fact that different antigens may trigger either high or low responses in a given genotype might, in fact, be a reflection of the extent to which a given antigen interacting with the appropriate receptor, may in itself affect the regulatory cAMP system. Thus it appears entirely possible that genetic control of Ir may operate not only at the level of specific recognition of antigen, but also at the less specific level of regulation of the response by B cells.

BODMER: I would like to make two further comments. First, I think the case of the guinea pig is very nice, because it shows the transition from mouse to man. The mouse is at the top with complete association between immune response and H antigens, and man at the bottom, differing very little from random association in a large population.

The other point concerns what might seem to be an apparent contradiction between the data in the mouse and man, on the position of the MLC locus. As Bach explained, the fact that one gets HL-A identical sibs which respond in MLC, suggests that the *MLC* locus is outside the FOUR-LA region, whereas the elegant mapping studies in H-2 suggest a series of genes between K and D which control the MLC response.

I would like to suggest that this difference is more apparent than real. It is a function of the techniques used to pick up the different types of recombinants. In man we are forced to look at matings involving four different chromosomes and then to look at those offspring which are HL-A identical but which differ with respect to MLC responses. In the mouse we have a careful control of the genetic situation, so that one can do backcross studies with a given heterozygote and pick out the recombinants for the serological differences in the first place, and then ask whether these differ for MLC.

In man I don't think one would expect to have picked up the MLC differences that could be controlled by genes between LA and FOUR because of the focus on the HL-A identical sibs. To do the same study in the mouse, it seems to me, one should take a cross between two F_1's and look at the offspring that are identical serologically and seek to determine whether there are any that are identical serologically but different for MLC. Correspondingly in man, one should look more intensively at the recombinants between the loci detected serologically to see how they perform with respect to MLC (van Rood has some data on this, which we should hear). In this way I suspect we will once again see the two systems, H-2 and HL-A, converging as far as their genetic organization is concerned.

CHAIRMAN McDEVITT: I would like now to call on van Rood to present his evidence at this juncture.

VAN ROOD: I would like to comment on four topics which have been discussed during this session:

1. The localization of the *MLC* locus.
2. The effectiveness of serotyping for LA and FOUR to predict MLC reactivity.
3. The relevance of the *MLC* locus for cellular immune response *in vivo*, that is to say, skin graft rejection, and
4. The serological recognition of the *MLC* locus.

TABLE 44

HL-A Typing and Haplotypes of Family KA

P	2- 8/11-W5	ab
M	3- 7/ 1- 8	cd
Child		
1.	11-W5/ 3- 7	bc
2.	11-W5/ 3- 7	bc
3.	11-W5/ 1- 8	bd
4.	11-W5/ 1- 8	bd
5.	11-W5/ 1- 8	bd
6.	2- 8/ 1- 8	ad
7.	2- 8/ 1- 8	ad
8.	2- 8/ 3- 7	ac
9.	2- 8/ 3- 7	ac
10.	2-W5/ 3- 7	a/b c

W5 = R*

TABLE 45

Bilateral MLC's Involving Recombinants between LA and 4 Segregant Series of HL-A in Family KA

H-L-A difference	Sib//	Cpm
lst series only:		
a/b c + bc	10 + 1	62
	10 + 2	63
2nd series only:		
a/b c + ac	10 + 8	3.035
	10 + 9	11.069

The results of the one way reactions, the controls as well as the standard error can be found in the original publication. No one way reaction was negative when the bilateral reaction was positive.

I will lead into these issues by presenting data on a family we studied in Amsterdam, together with Eijsvogel, that is family KA. This family was unusual in that there were two different crossovers in two siblings; this family thus had six different chromosomes. Table 44 shows the relevant HL-A data. The last child had a crossover between FOUR and LA; the 2 specificity of the a chromosome combined with W5 of the b chromosome of the father. Table 45 shows some of the MLC test results: testing this child who carries a crossover between the *FOUR* and *LA* loci against siblings carrying bc haplotypes and ac haplotypes. There are differences for the LA series but there is virtually no thymidine in-

TABLE 46

Bilateral MLC's Involving the Siblings 1, 2, 3, 4, 5, 6, 7, 8, 9 of Family KA

HL-A genotype combination	Sib	Cpm
bc + bc	1 + 2	33
bd + bd	4 + 5	37
	4 + 3	2,124
	5 + 3	3,982
bc + bd	1 + 4	1,969
	2 + 4	1,655
	1 + 5	2,108
	2 + 5	3,078
	1 + 3	122
	2 + 3	105
a/b c + bd	10 + 3	353
	10 + 4	2,452
	10 + 5	2,394
ad + ad	6 + 7	117
ac + ac	8 + 9	167

The results of the one way reactions, the controls as well as the standard error can be found in the original publication. No one way reaction was negative when the bilateral reaction was positive.

corporation. When there were differences for the FOUR series, there was significant stimulation. So this confirms the data of a number of workers who also found that stimulation in the MLC goes together with the antigen of the FOUR series and not (or only minimally) with the LA series.

Table 46 shows the MLC data with the other siblings, which had no crossovers between LA and FOUR. Two HL-A identical siblings carried the haplotypes b and c and as expected they did not stimulate. There were three siblings which carried the haplotypes b and d (siblings 3, 4, and 5), and as expected 4 and 5 did not stimulate. This might be due to a crossover between the *MLC* locus and the *FOUR* locus.

Eijsvogel had the opportunity to test sibling 3 with the presumed crossover between MLC and FOUR against the sibling which had the crossover between FOUR and LA. There was virtually no stimulation, while there was a difference for three HL-A antigens. This, then, I believe gives us a possibility to map the *MLC* locus to the left of the FOUR locus. Were it to the right, we would have to assume that there were two crossovers which is possible but most unlikely. That is one argument. The same conclusion was reached by Bach and Eijsvogel be-

TABLE 47

MLC between Unrelated HL-A Identical Individuals

Phenotype		No. of unrelated individuals	No. of combinations tested	MLC-negative/ MLC-total
1,3	7,8	7	14	3/14
1,2	7,8	4	6	0/6
1,2	8,12	3	3	0/3
2,3	7,W15	2	1	0/1
2,3	7,12	2	1	0/1
1,9	8,12	2	1	0/1
		20	26	3/26

cause MLC stimulation between HL-A identical siblings occurs as frequently as crossover between LA and FOUR.

Assuming that such a stimulation is due to a crossover between MLC and FOUR this implies that if the *MLC* locus would be at the right side of the *FOUR* locus, it would lie near the *LA* locus; the other data do not support this. So for the time being, based on two different sets of data, i.e., the family I have shown and the combined world data, we believe tha the prime *MLC* locus in man is to the left of FOUR. The question that follows is, how well can we predict reactivity of MLC if we serotype only for LA and FOUR? This is a point that Bodmer also raised and Table 47 provides an answer. There were only three out of this series of 26 combinations of HL-A identical, unrelated individuals which did not stimulate in the MLC test. This is roughly 10% and we have now over 100 of these combinations and the percentage remains between 10 and 20%. The negative ones are as low as HL-A identical siblings; when there is stimulation between HL-A identical unrelated individuals, there are generally speaking lower than the average between mismatched controls, as Sørensen, Bach, and others have pointed out.

So the answer to my question is, if one serotypes only for LA and FOUR, he will predict correctly only in 10-20% of the situations for the *MLC* locus. I think this is a very relevant question when one comes to the correlation of serotyping for HL-A with disease. For that reason, to study the problem of skin graft survival and MLC reactivity we not only serotyped for LA and FOUR, but also determined MLC reactivity. In Fig. 32, MLC reactivity is expressed as a stimulation index in the same way that Bach showed us. We have previously presented the data of one large experiment in which there was no correlation between MLC reactivity and skin graft survival. This, then is a follow-up of the study, and now it can be seen that there *is* a correlation, although this correlation is perhaps not notably impressive. There are several symbols in this figure. The circles with an outer circle or two outer circles refer to recipients who received

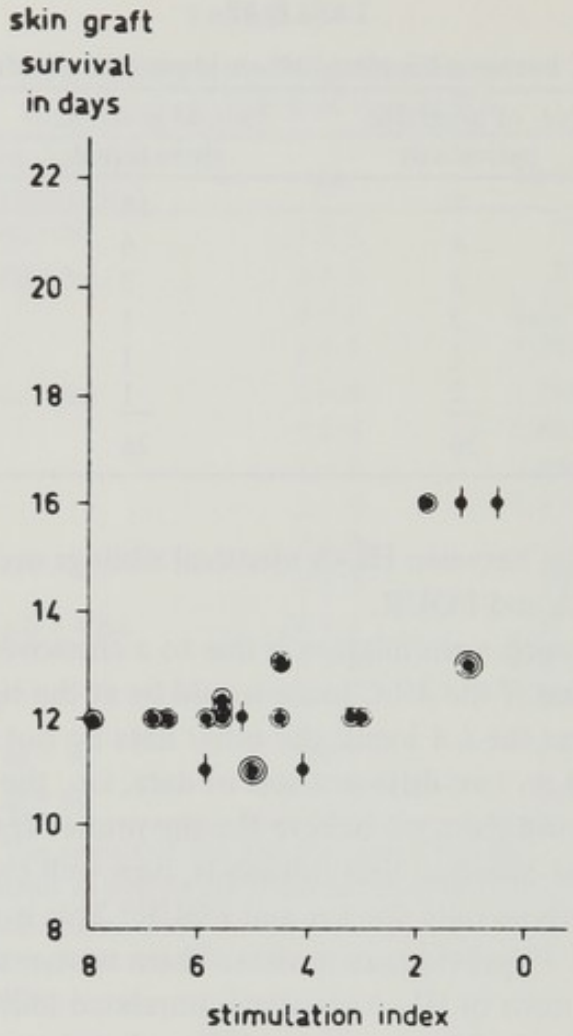


Fig. 32. Relationships between MLC stimulation and skin graft survival.

either two or three skin grafts. It will be seen that between these recipients the correlation between reactivity and skin graft survival is not very impressive. On the other hand, ♦ symbols refer to the recipients who got only one skin graft and here it appears that in these preliminary data there is a much better correlation between MLC and skin graft survival.

Finally, a point that was raised several times in our discussion during this session was whether it will be possible to recognize the product of the *MLC* locus serologically. We are currently investigating a protocol, which was based on the observations of Ceppellini. The problem is that this requires unrelated individuals who are identical for HL-A and different for MLC. If one immunizes such individuals by skin grafting it is then necessary to exclude the possibility that there exist unrecognized heterogeneity for FOUR, LA or both. In other words, that the serum obtained after skin grafting is not against MLC but against a portion of a FOUR or LA antigen. Serological tests have so far been negative. We have further used the most sensitive test we know of, which is antigen elimination *in vivo*, i.e., survival of ^{51}Cr labeled platelets of the donor. Platelets do

not carry the product of *MLC* locus, but do carry the product of the *FOUR* and *LA* loci, and we know that platelet survival is always shortened after an incompatible skin graft. In over 90% of our tests where donor and recipient were unrelated but HL-A identical, platelet survival after skin graft rejection was normal, indicating that there are no antibodies against FOUR or LA. However, these sera are in some instances able to suppress MLC reactivity between HL-A identical unrelated individuals and might thus recognize an MLC determinant.

SIMONSEN: I have found it delightful to be at this conference and to be exposed to so many new facts, but it can also be a little tiring. By the end of an intensive session such as this, one feels the urge to try and make out what the essential message is, and what is more or less random background noise. What seems to me the essential message comes very close to the last point Klein was making, viz, in all likelihood, we are dealing with a huge complex of genes and corresponding gene products with essentially similar physiological functions. This function relates to receptors, at least T cell receptors. As a side effect, some of these receptors, or most of them, perhaps all of them, are also transplantation antigens—if you pick your test system right—and this is where all the background noise comes in. For example, when a point is made of the fact that the 2R with 4R combination gives an MLC reaction but no skin graft rejection, this to my way of thinking is a trivial point.

Obviously transplantation reactions depend on many things, such as, for example, the relative concentration of the relevant antigens on various cell types. Some antigens may be better expressed on skin than on other cells, and other antigens may be best expressed on lymphocytes, or indeed on erythrocytes. Also, the observed end point of the transplantation reaction may vary tremendiously in sensitivity from one test system to another. For example, if we take the vast majority of the known histocompatibility loci in the mouse and the rat, which are identified by skin graft rejection, these appear not to be antigens at all if one looks at them in kidney transplantation or in heart transplantation—nor do they cause MLC reactions with the techniques usually employed. So this is all rather uninteresting, unless one happens to be particularly concerned with transplanting this or that organ or tissue.

To my way of thinking some antigens are transplantation antigens in one system and not in another and some are stronger and some are weaker. But I would think it likely that all of these Ir products (= T cell receptors) have some genetic polymorphism which is demonstrable by some transplantation reaction or other, provided one looks at it in the right way. It is a question of much more general interest whether the antigenic determinants responsible for the various transplantation reactions are themselves part of the recognition site. But I can see no way of telling that just yet.

There is one point we have left out altogether, and that is the existence of all the weaker loci of histocompatibility. If it is true as some of us feel, that as far as the H-2 complex goes, the gene products are both receptors and transplantation antigens—if one looks at them in the right way—then one must also expect to find one day that the products of the weaker loci possess a receptor function. It is of interest in this context that Festenstein and coworkers have found an *MLC* locus which is unlinked to the H-2 complex. Also, if transplantation antigens are determinants sitting on receptor molecules, it actually follows that B cells have their receptors too, but they may be much less important here, relative to Ig receptors. These, then, are the main points that for me emerged from our extraordinary discussions at this session.

CHAIRMAN McDEVITT: Simonsen's view that all of the complex genetic systems which are included in the mouse major histocompatibility complex and which we have discussed during this session are essentially similar, and that the differences that we detect are a result of different test systems may be an oversimplification. It seems clear that all of these genes—*H-2K*, Ir, MLC, and *H-2D*, as well as genes which are serologically silent but responsible for graft rejection and perhaps for GVH reactions—are related in two important ways: first, they seem to be involved in some type of recognition phenomenon; and second, they are all closely linked and may well have arisen by a repeated series of intrachromosomal duplications. There is thus reason to believe that there is some functional relationship, as well as a common structural and genetic origin. However, during the course of evolution it is quite possible that these closely linked genes evolved, in the process of selection, to serve rather different functions. The possibility, therefore, must be kept in mind that some of these genes, such as *H-2K* and *H-2D* and closely associated, serologically silent antigens responsible for graft rejection, may be involved in cell-cell recognition during the course of differentiation and organogenesis. At the same time, other genes in this complex may have evolved towards different functions. Thus it is possible that the Ir genes may represent a set of genes which are selectively expressed only on certain classes of immunocompetent lymphocytes, and it may even be that certain Ir genes are selectively expressed on subsets of a particular immunocompetent cell population, i.e., there may be clonal expression of Ir gene products, a state of affairs decidedly different from that found for most transplantation antigens. A great deal more experimental work must be done before the interrelationships between these different genes can be sorted out. Even if we accept Klein's hypothesis that all of these genes are involved in some type of recognition, Bodmer's hypothesis that many or all of these genes are involved in cell-cell interaction during the course of differentiation, and Simonsen's view that all of these gene products are essentially transplantation antigens which have a variety of physiologic effects, there is still room (both theoretically and in the ninth mouse

linkage group) for the possibility that all of these functions exist and that superimposed on them is a series of highly specialized functions such as those represented by the immune response genes and perhaps others controlling very specific types of cell-cell interactions which have not yet been detected. Fortunately, the availability of a large number of precisely characterized recombinant H-2 chromosomes on standard inbred backgrounds should permit rapid progress in obtaining direct experimental answers to many of the questions which our discussions have raised.

SESSION III

IMMUNE RESPONSE GENES LINKED TO IMMUNOGLOBULIN ALLOTYPES AND OTHER TYPES OF IMMUNE RESPONSE GENES

Genetic control of monoclonal antibody production – Relationship between idiotypy and allotypy – Linkage of idiotype to Ig allotype – Idiotype suppression – B cell determination of idiotype (carrier independence) – Selective breeding for high and low antibody production – Ir genes linked neither to H-2 nor Ig allotype – Strain variation in specificity of spontaneous antihapten antibodies.

Definition of Terms for Session III

This session deals extensively with the association between the production of homogeneous antibody, or of idiotypic antibody, and immunoglobulin allotypes. Since different speakers (notably Krause and Ceppellini) use slightly different notations for the rabbit immunoglobulin allotypes, and since the notation system for the mouse immunoglobulin allotypes is not familiar to many immunologists, the editors felt that it would be helpful to the reader to include a brief definition of allotype nomenclature.

The rabbit immunoglobulin allotype markers (with a few exceptions) are the serologic expression of allelic differences in amino acid sequence in several different regions of the immunoglobulin heavy chains, or allelic differences in amino acid sequence in undefined positions in both kappa (κ) and lambda (λ) light chains. A particular allotype locus is designated by a small letter in italic type, (e.g., *a, b, c*) and the different alleles which occur at that locus are represented by a series of numbered specificities (e.g., 1, 2, 3). The major allotypic markers in the rabbit are:

a 1,2,3 — A set of three alleles representing allotypic differences in amino acid sequence in the *variable* region of IgG, IgA, and IgM heavy chains.

b 4,5,6,9 — A series of allotypic markers found on kappa light chains.

c 7, 8 — Allelic allotype markers found on lambda light chains.

In addition, there are several other allelic allotype differences (*a*11, 12 and *a*15, 16) which are localized to the constant region of IgG heavy chains. These are not referred to specifically in the text, although several speakers in Sessions II and III refer to them indirectly in discussing the rabbit C (constant) region allotype genes.

The major source of confusion arises from the fact that Krause uses the more recent convention of designating particular allotypes as *a*1, *b*4, etc., while Ceppellini uses the older convention that dictates that all the immunoglobulin allotypes will be preceded by a capital *A* (e.g., *Aa*1, *Ab*9, or *Ab*5). The reader should be aware that *b*5 or *b*9 are the same as *Ab*5 or *Ab*9.

Even greater confusion has been introduced into the text by those speakers referring to the mouse immunoglobulin allotypes. This arises because some speakers designate the Ig (immunoglobulin) type as "strain C57BL allotype" or "BALB/c allotype", while others use the more generally accepted and technically correct notation, and still others use *both* methods of designating the immunoglobulin allotype of a strain in their discussion and in their tables. Fortunately

for the reader, all of the mouse immunoglobulin allotype genes are included in a single linkage group which includes four different allotype loci, representing allelic differences in amino acid sequence in the constant region of γG_1, γG_{2a}, γG_{2b}, and γA heavy chains. Among the *inbred* mouse strains, all strains having a particular allotypic specificity on their γG_{2a} heavy chains have the same set of allotypic specificities on the other three heavy chains of this linkage group. Thus, for the inbred mouse strains, it is possible to use a single letter designation (e.g., *a, b, c, d, e*) to designate the particular allelic group of allotypic specificities on all four heavy chains possessed by a particular inbred strain. Since the designation of this set of allotype markers in the mouse is *Ig*, a strain can be designated as Ig^a or Ig^b, etc. An alternative way is to state that the C_H allotype of a particular strain is *a,* or *b,* or *c.* A third alternative way of expressing this is to state that a strain carries the allotype linkage group identical to C57BL, or to BALB/c, or to some other prototype strain. All three methods are used by different speakers and are given in different tables. In addition, in Table 63, Biozzi uses the notation developed by Lieberman and Potter which designates the *Ig* allotype loci as a linked set of loci – *G, H, A,* and *F.* Since Biozzi also includes the heavy chain class on which these loci are found and the numerical designation of allotypic specificities used by Lieberman and Potter, this notation should not cause any further confusion.

CHAIRMAN WARNER: I think it might be fair to say that we concluded from Session II that there are immune response genes demonstrable in several species, which are linked to major histocompatibility loci, and are expressed in T cells. There is still some controversy as to whether those same genes are also expressed in B cells.

This session intends to focus on whether there are other types of immune response genes, which are not H-2 linked, but which may be either linked to immunoglobulin (Ig) allotypes, to minor histocompatibility loci, or perhaps are not associated with any presently recognized genetic marker.

The second issue to which we should address ourselves is whether these other genes are expressed in T cells, B cells, macrophages, or still other cell types which we have not yet recognized, but which may be involved in the immune response. Accordingly, this session will deal with three general topics. The first will be to consider specific immune response genes which are linked to Ig allotypes, and secondly, those allotype-linked controls of the immune response which are associated with responses to more than one single antigenic determinant. A third matter will be to discuss whether there are other genetic controls of immune responses which are neither H-2 linked nor allotype-linked.

Krause will introduce the discussion with an assessment of the state of this field and the major issues as he sees them.

KRAUSE: My discussion is concerned with the association between genetic control of the immune response and the structural genes which code for the antibody molecule. Among the issues considered will be: whether there is a relationship between a specific immune response and the structural genes which code for a specific antibody; and if a specific allotype or a specific allotypic combination is more compatible with certain specific antibody-binding sites than with others. Thus, in serologic terms, we seek to determine if there is a relationship between allotypy and idiotypy. Other questions could range further and consider the relationship between other structural genes of the IgG molecule (e.g., constant region structural genes) and the immune response. For example, would a wider search in man of the immune response to the large number of carbohydrates of microbial origin, bear out a preferential selection of the IgG_2 sub-group, a point first emphasized by Yount, Kunkel, and Kabat?

I propose to concentrate on three particular aspects: the association between the genetic control of the immune response and allotype; the inheritance of idiotypy; and the association between idiotypy and allotypy. I am confident that others will expand these comments with their own bias and point of view.

First, a few brief remarks are in order regarding the influence of genetic factors on the immune response to streptococcal carbohydrate, especially as in the discussion thus far, the genetic control of the immune response to bacterial antigens has not been examined in any detail. Furthermore, the immunization

procedure which is now employed in a number of laboratories to raise antibodies to microbial carbohydrates is quite different from that which is employed for the immunization with synthetic antigens; I believe it is worth keeping these differences in mind.

Whole heat-killed streptococci are injected intravenously. A rather monotonous antigenic paint coats their surface. Pepsin treatment removes all surface antigens except the group specific carbohydrate. It is a remarkable fact that although the whole bacterium is injected intravenously, 90% of the antibody response is directed against the carbohydrate. Indeed, almost all the IgG made by the rabbit is antibody to the carbohydrate. The immunization schedule involves giving the vaccine intravenously three times a week for four weeks. This is followed by a rest period of three to four months before a second series of injections. If the rest period is omitted, exhaustion of some sort occurs, and a secondary response is not seen. In general the amount of antibody after a third or a fourth series of injections is no greater than that following the second series.

These streptococcal group A and C carbohydrates are relatively simple branched polymers of rhamnose, on which there are terminal amino sugars. In the case of the group A polysaccharide, the terminal sugar is β-*N*-acetyl-glucosamine, and in the case of group C polysaccharide, the terminal amino sugar is α-*N*-acetyl-galactosamine. These two different terminal amino sugars account for the antigenic specificity of the group A and C carbohydrates.

Results on antibody responses for a large number of rabbits immunized with streptococcal vaccines suggest that there is a genetic influence on the immune response to these surface carbohydrates. In Table 48 are given the immune response data to the group C carbohydrate for 31 random rabbits following a second immunization. The average antibody concentration is 14 mg/ml and ranged from 1 to 36, with a median of 12. High responders and low responders were selected from this random population for an F_1 generation; from this F_1

TABLE 48

Immune Response to Group C Carbohydrate

		Antibody concentration mg/ml		
Rabbit	Number	Average	Range	Median
Random	31	14	1-36	12
F_2 high responders	52	21	5-58	18
F_2 low responders	14	7	1-14	7

Random vs. high	Random vs. low	High vs. low
$t = 2.649$	$t = 4.191$	$t = 6.233$
$p < 0.01$	$p < 0.001$	$p < 0.001$

generation, the lowest of the low and the highest of the high were selected to produce the F_2 high responders and the F_2 low responders shown in Table 48. For the high responders, the average was 21 mg/ml, with a range from 5 to 58, and a median of 18. For the 14 low responders, the average was 7 mg/ml with a median of 7. The t test indicates statistical differences. The high responders have been selectively bred through the F_5 generation, but it became necessary to terminate the continuation of the low responder line; they were, moreover, of no special value for the procurement of large amounts of antibodies with molecular uniformity. I should stress that in both the low and high responders, almost all of the serum IgG was antibody to the carbohydrate. It would, therefore, appear that the two groups differ greatly in the proliferation of lymphoid tissue generally as opposed to plasma cells. This may well prove to be a fruitful subject for further study.

These studies have been extended by Eichmann to a number of inbred mouse strains. He has also immunized intravenously with streptococci. Later in this session Rajewsky will comment on these data. Antibody responses as high as those obtained in the rabbit are seen in the mouse, but not in every inbred strain; indications are that in both rabbits and mice there are genetic factors which influence the immune response to microbial carbohydrates. I should say parenthetically that some of our high responders have been sent to Pincus at the NIH, and indeed they also respond extremely well to the pneumococcal polysaccharide when given in the form of whole pneumococcal vaccine.

A separate facet of this immune response in rabbits, as well as in mice, concerns the influence of genetic factors on the heterogeneity of the immune response. Shown in Fig. 33 are the microzone electrophoretic patterns of immune sera for 12 random rabbits. Almost all of their γ globulin (seen on the left) is IgG antibody to the streptococcal carbohydrate. In some, such as in number 12, there is a striking instance of homogeneity of the antibody response, but in others, such as number 9, there is a high response with considerable polyclonal heterogeneity. In others, such as number 1, there is almost no antibody response, and there is no electrophoretic evidence of restriction in heterogeneity.

Studies were begun several years ago to determine if the restricted heterogeneity was genetically transmitted. This is a difficult issue to resolve, partly because restricted heterogeneity is an ambiguous trait to define in any precise way. There is no entirely satisfactory system to score for this trait. Nevertheless, what was done was to score as best we could the electrophoretic patterns shown. Antisera were classified as: heterogeneous, viz., numbers 1 and 9; restricted, viz., numbers 7 and 4; monoclonal, viz., number 12. In Fig. 34 are microzone electrophoretic patterns of antisera from examples of several breeding pairs from the inbred colony. The heterogeneous low responder parents gave rise to offspring which were low responders without evidence of restricted heterogeneity.

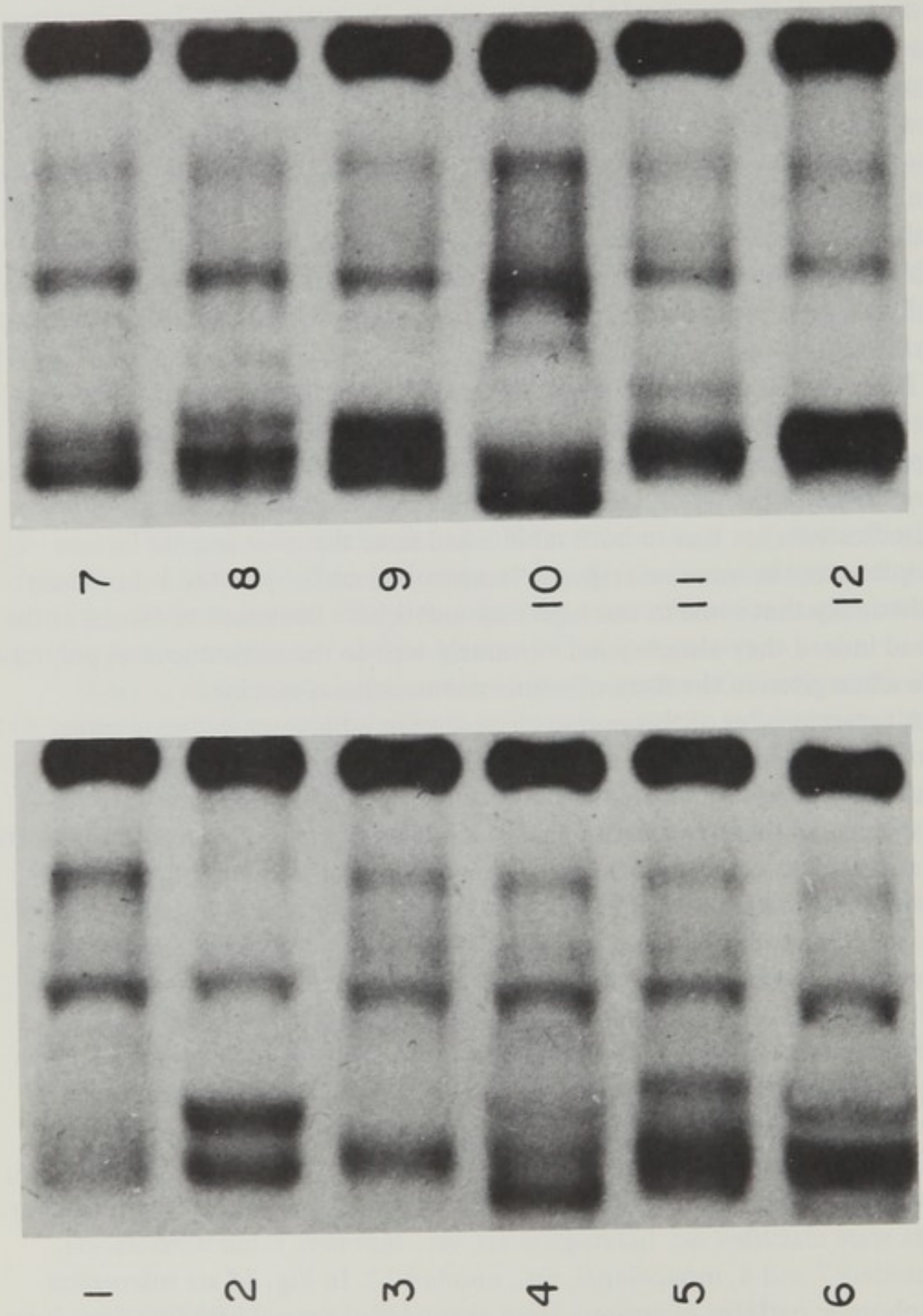


Fig. 33. Microzone electrophoretic patterns of antisera from 12 random rabbits immunized with group C streptococci. Antisera were collected after a second series of I.V. immunizations.

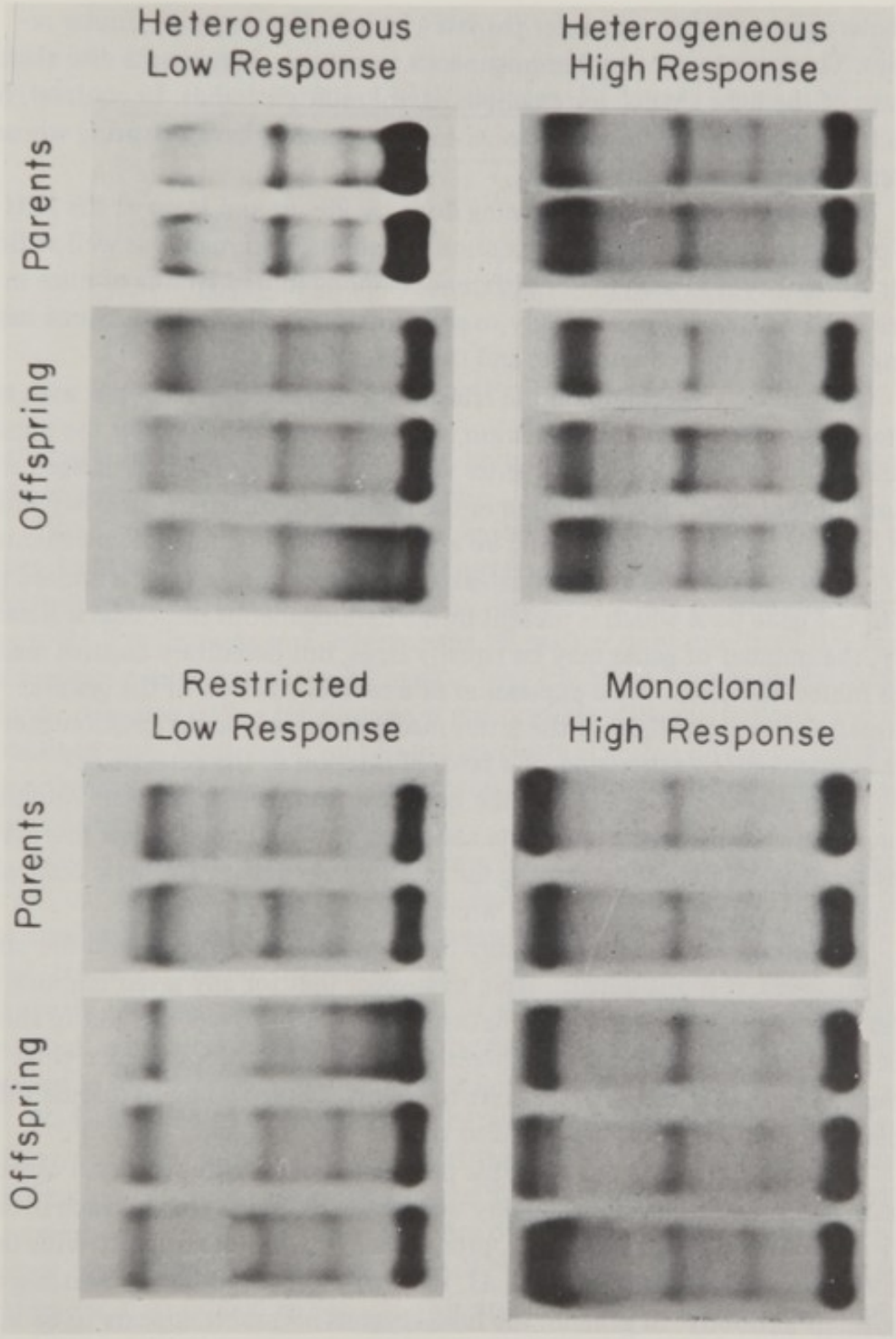


Fig. 34. Breeding studies demonstrating the influence of genetic factors on the heterogeneity of the immune response. Microzone electrophoretic patterns of the antisera are shown for parents and their offspring following immunization with group C streptococci. The gammaglobulin is to the left. The bulk of the IgG is antibody to the group C carbohydrate.

The heterogeneous high responder parents yielded offspring with similar responses. These are really very heterogeneous responses. Acrylamide disc electrophoresis of the light chains, for example, reveal multiple bands. In contrast, both restricted low response and monoclonal-high responders have offspring whose response mimics that of the parents.

Studies of a similar sort are being done by Pincus and Mage at the NIH with pneumococcal vaccine immunization of rabbits. Eichmann, as well as Briles in our laboratory, has seen major differences among inbred strains of mice in the degree of heterogeneity of antibody to streptococcal carbohydrate. Some inbred strains show this type of response, and others do not.

One can only speculate on the reasons for an antibody response with restricted heterogeneity; one explanation is that this is determined by the number of possible binding site structural genes which can be expressed on antigen stimulation. In that event, the limitation in the number of different antibody molecules, as seen in these studies, could be achieved by several possible mechanisms. It is conceivable that the monoclonal-like responders possess only a limited portion of the gene pool which is present in the heterogeneous responders. Alternatively, the number of genes may be equally large, but hereditary control mechanisms inhibit the phenotypic expression of a certain fraction of the genetic information. As a corollary of the latter mechanism, one could envision that there is an enhanced expression of a favored fraction of the gene pool.

From this brief comment on the genetic aspects of the immune response to microbial carbohydrates in rabbits and mice, it would appear that this system might be a useful one for examining the relationship between genetic control of the immune response and the genes which code for IgG structure.

This brings us to the relationship between Ig allotype genes and the immune response. It is not unreasonable to suspect that for any given immune response there may be a relationship between the immune response and Ig allotype genes, because either directly or indirectly the specific antibody is the final product of both. Inasmuch as speakers to follow are also going to discuss this problem, my remarks will be limited to studies from our laboratory.

A systematic search for allotypic preference of antistreptococcal carbohydrate antibody in our rabbit colony was begun by Kindt. In one such breeding study the mother, a high responder, produced 17 mg/ml of antibody with the a2, b4 allotype; the father, allotype a3, b9, was a low responder, producing only 3 mg/ml of antibody. In general, the homozygous b9 rabbit appears to be a low responder. There were ten offspring in two litters, all of which were a2, a3, b4, b9. IgG preparations from the preimmune sera of parents and offspring were tested for the presence and distribution of the allotypic markers. Similarly, the immune IgG was tested for the presence and distribution of the allotypic markers. The results, given in Table 49, show the average percent distributions of

TABLE 49

Concentration of H and L Chain Allotypes in Preimmune and Immune IgG in 10 Heterozygous F_1 Progeny

	Allotype; mg/ml serum			
	a2	a3	b4	b9
Preimmune	1.1	2.2	2.8	0.6
Immune	10.1	14.5	25.7	1.1
Increase	9.0	12.3	22.9	0.5
Total inc.	21.3		23.4	
% increase	38	52	97	2

the allotypic markers of preimmune and immune IgG for the ten siblings; the numbers given are actual mg/ml of serum.

There was no major shift in the proportion of IgG with the a2 or the a3 marker, but there was a major shift with preferential selection of the b4 marker in the immune sera. The b4 concentration rose from 2.8 in preimmune specimens to 25.7 in immune sera, whereas the b9 marker only rose from 0.6 to 1.0. This suggests a selection of the b4 marker over the b9. It may be that a major antibody response with a b9 allotype is not possible with this antigen, and would be obtained only when these rabbits are immunized with a different antigen. Such a finding would indicate a preferential association between antibody specificity and particular allotypic alleles.

Why is b4 preferentially selected over b9 in this specific immune response? One can only speculate. It is conceivable that certain structural genes may be more compatible than others with the genes which control the antibody response. It is also possible that antibodies of one allotype have a higher affinity for a specific antigen than do antibodies of a different allotype. Further quantitative studies on genetic markers of antibodies to limited antigenic determinants produced in heterozygous animals should help resolve this issue.

We now come to the relationship between response genes and genes coding for idiotypic determinants. The view that the idiotypic determinants include the unique amino acid sequence related to the binding site is supported by studies too numerous to recount. For the purpose of discussion, we will consider two different types of idiotypy. These are really operational definitions of idiotypy and they are diagrammed in Fig. 35. This nomenclature, which was discussed at the First International Congress of Immunology, was proposed by Kunkel. I will consider this nomenclature briefly within the framework of our own system; heterologous idiotypy is detected by an anti-antibody raised in a different species; homologous idiotypy is detected by antibodies raised in an allotypically matched animal of the same species; isologous idiotypy is detected by antibodies raised in inbred animals.

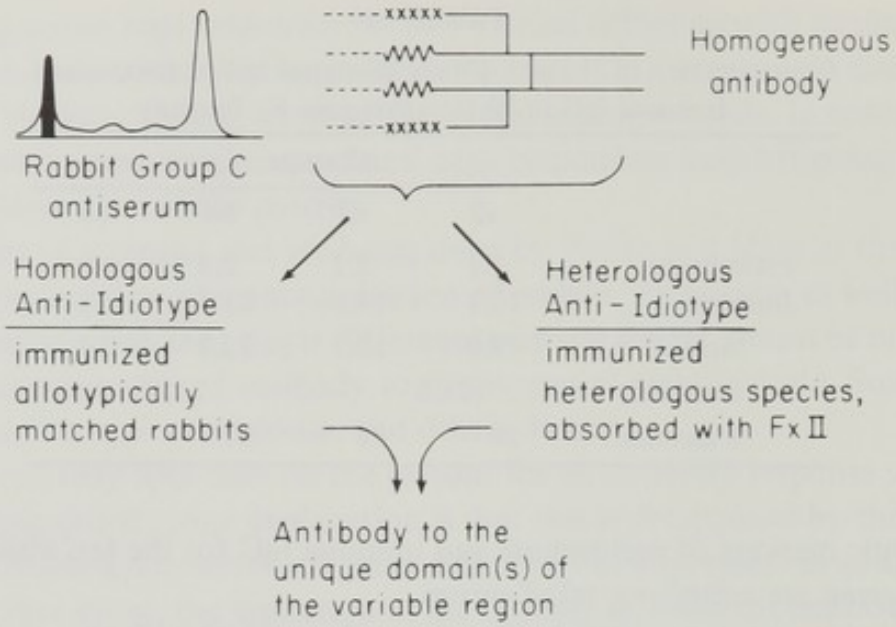


Fig. 35. Diagram demonstrating the method for the preparation of homologous and heterologous antiidiotypic sera to a group C antibody with molecular uniformity.

Heterologous idiotypy of myeloma proteins was first called individual antigenic specificity by Kunkel. In our own laboratory, antiidiotype antibodies are produced by immunization with antibodies which possess uniform properties, as shown in Fig. 35. Insofar as possible, it is our goal to use only those antibodies for idiotypic studies which have been isolated with care. Such an antibody preparation is predominantly a single molecular species, and clearly the idiotypic antisera directed against it will have specificity for the predominant idiotypic determinant(s). We have concentrated primarily on preparing heterologous idiotypic antisera by immunization of guinea pigs with isolated monoclonal-like antibodies. The guinea pigs were previously made tolerant to rabbit IgG by an intravenous injection of five mg of IgG.

Figure 36 shows an example of one family in which there were a large number of restricted responders born to parents which gave highly restricted responses. It was an examination of pedigree patterns such as this which stimulated Eichmann and Kindt to speculate on genetic factors which might place limits on antibody heterogeneity. Such a high incidence of monoclonal responses in an inbred family might be the result of limited idiotypic variability—which would suggest that antibody heterogeneity is determined by a relatively small number of different genes which are activated by antigenic stimulation. It was speculations such as these which led these investigators to search for the inheritance of idiotypic markers in an inbred family such as the one shown here. Figure 37 shows duplicate pedigrees of a family bred through the F_2 generation. Each member of this family was immunized with the same streptococcal vaccine. Antibodies with molecular uniformity, isolated from the two rabbits indicated

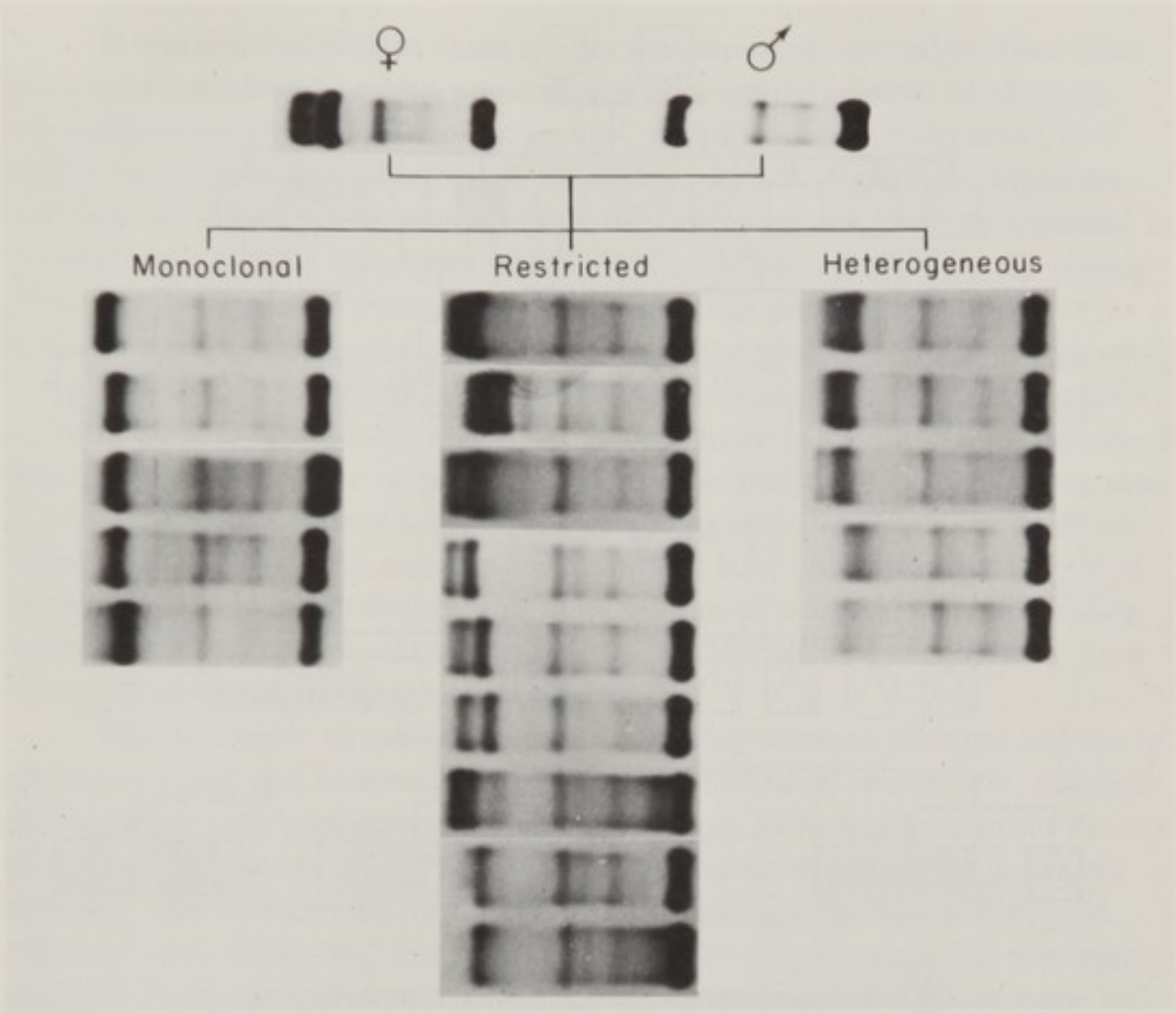


Fig. 36. Microzone electrophoretic patterns of antisera from offspring of parents with antibodies of restricted electrophoretic heterogeneity. The antibody was obtained by immunization with group C streptococci.

by the solid symbols, were termed proband antibodies; they were used as the immunogens for preparing antiidiotypic sera. Preimmune and immune sera of all the rabbits in these families were tested with the two antiidiotypic sera for the occurrence of antibodies with these idiotypic determinants. No preimmune sera reacted with these antiidiotypic sera, nor did the sera of 48 unrelated rabbits.

In the upper frame, the family members which reacted with the x-marked idiotypic antiserum are indicated by the open x's. In the bottom frame, the family members which reacted with the triangle-marked antiidiotype antiserum are indicated by open triangles. The streptococcal antiserum from one rabbit indicated by a dotted line extending from the upper frame to the lower frame showed reactivity with both idiotypic antisera. This rabbit produced one antibody which has one idiotypic determinant, and another antibody which has another idiotypic determinant.

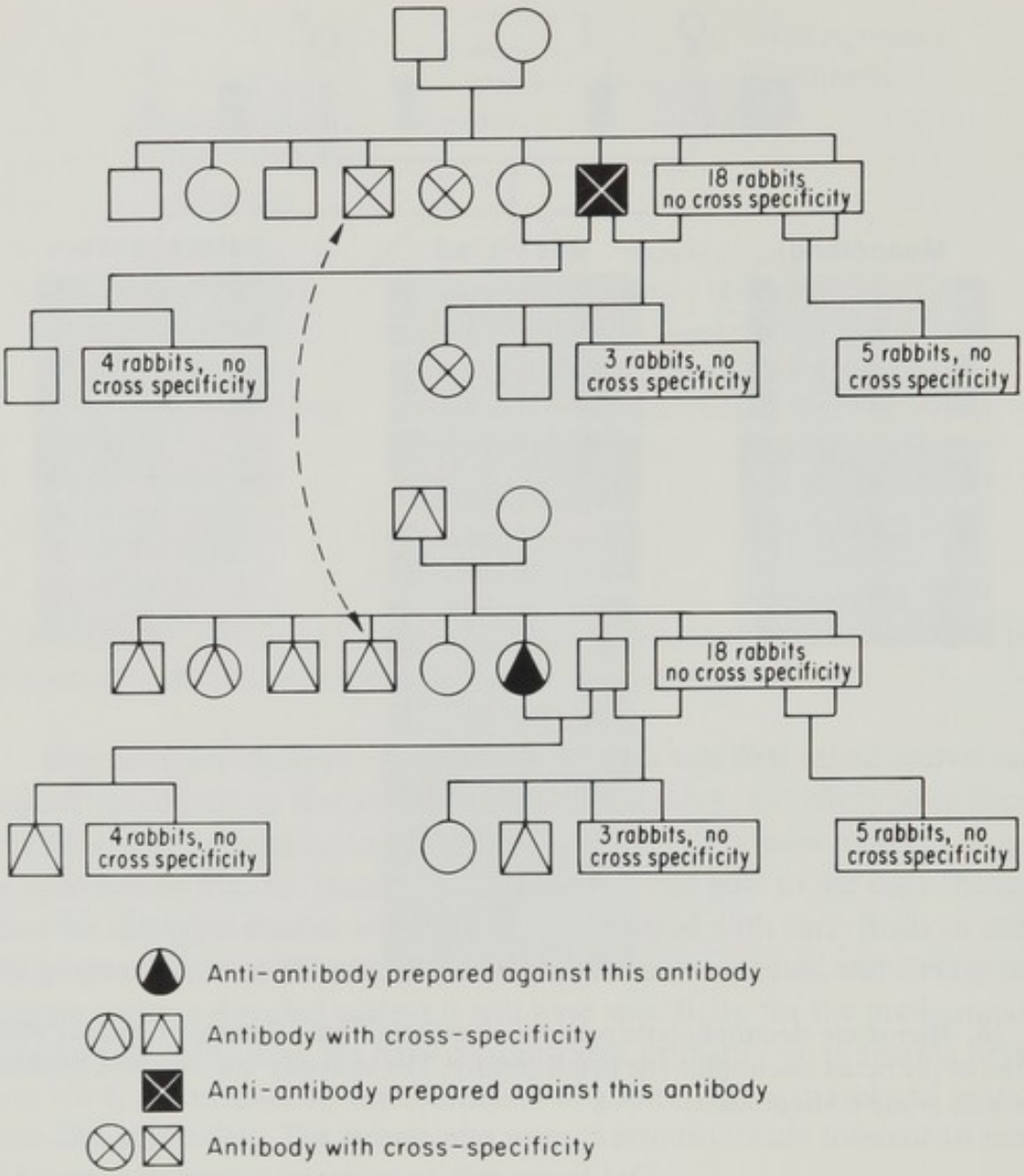


Fig. 37. Duplicate pedigrees of one inbred rabbit family, all members of which were immunized with group C streptococci. Antiidiotype sera were prepared against isolated antibody components from the rabbits indicated by the closed symbols.

This occurrence in a single rabbit of two antibodies, each with a different idiotype, requires emphasis. If all the antibodies in this antiserum had been used to prepare the antiidiotype serum, the number of crossreactions observed in this family would have been ten instead of the seven with the x-marked idiotype and four with the triangle-marked idiotype. Obviously an antiidiotype serum prepared with antibodies from this rabbit would have detected at least two different idiotypes, rather than a single idiotype. This possibility must be kept in mind in all discussions concerned with experimental observations on idiotype crossreactions. Clearly a careful dissection of the genetics of idiotypic determinants requires the use of monospecific antisera insofar as it is possible to obtain them.

It was concluded from these studies that repeated selected brother-sister mating had produced a rabbit population with a limited number of idiotypic alternatives for the response to the group C streptococcal carbohydrate. In a rabbit family with limited idiotypic variability, antigen stimulation frequently selected antibodies with a similar or identical idiotype, and idiotypy appeared to be preserved over the three generations. This could be explained by the assumption that the pertinent genes are present as multiple pseudoalleles and that inbreeding had led to idiotypic selection through the generation of homozygosity.

The absence of highly inbred rabbits has hampered any thorough genetic investigation of this variable region antigenic marker. Idiotypic studies on inbred mice are obviously more suitable for such genetic analysis. These have been conducted in several different laboratories, including our own. I will not dwell on these studies as they will come up for discussion later on. Suffice it to say that antibodies of the same idiotype are found in mice of one strain and antibodies with this idiotype are absent in another strain. Clearly this opens the way for a thorough genetic investigation of idiotypy.

The next point to consider is the relationship between idiotype and the Ig allotypes; Kindt and Eichmann have already published on this subject, and their data requires only brief comment. The principle of these studies involves the use of normal preimmune IgG to inhibit the heterologous antiidiotypic precipitin reaction. This is illustrated by the double diffusion analysis shown in Fig. 38. The top frame shows that this streptococcal antibody gives a specific idiotypic reaction. In the lower frame is shown the inhibition by pooled nonimmune IgG (fraction II) of the precipitin line. This is a phenomenon which Kunkel has observed in the heterologous idiotypic reaction with myeloma proteins. Rather than using pooled nonimmune IgG as an inhibitor, Eichmann and Kindt used preimmune IgG from single rabbits with various known allotypic combinations. As shown in Table 50, preimmune IgG of the same allotype as the idiotypic antibody was the most effective inhibitor of the antiidiotypic precipitin reactions. Shown also are the I_{50} values of various preimmune IgG's used as inhibitors of the antiidiotypic precipitin reaction. Quantitative radio-precipitin inhibition tests were performed here with five μg of radio-labeled proband antibody and 25 microliters of absorbed antiidiotypic serum. The test was performed over a range of inhibitor concentrations to determine the 50% inhibition value. The amount of proband antibody used as the inhibitor in the test was set as equal to one. Sixty-four times as much preimmune IgG of the proband rabbit was needed than of the proband antibody to give 50% inhibition when compared to proband antibody. In the last column is given the excess of inhibitor over the proband preimmune IgG to achieve the I_{50} value. The values are shown for several different preimmune IgG preparations. Two siblings of the proband which had preimmune IgG with the same allotypes as the proband were just as effective as

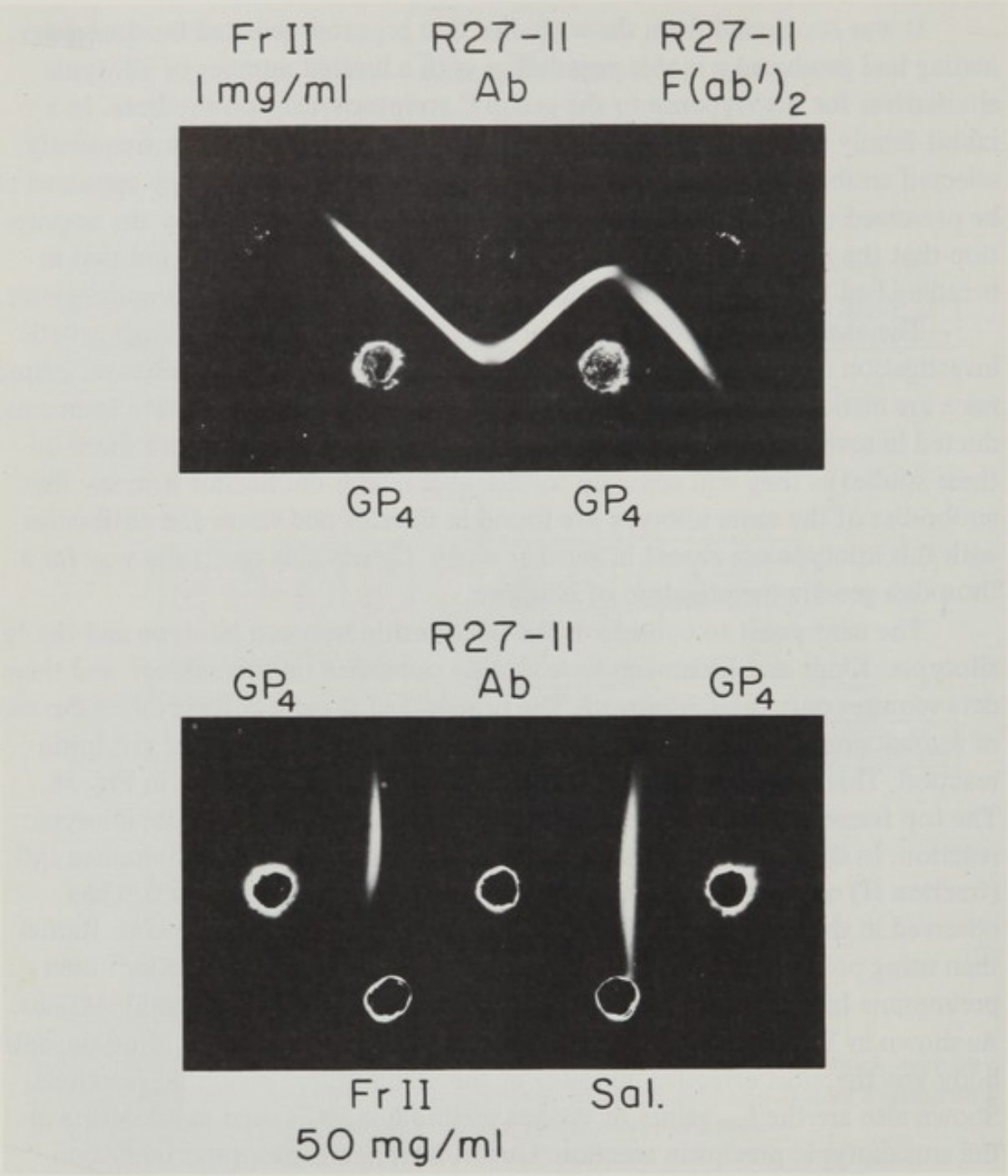


Fig. 38. Double diffusion analysis of antiidiotypic antisera. *Upper frame*: Double diffusion analysis shows the idiotypic reaction of an isolated antibody component R27-11. GP_4 indicates an antiidiotype serum prepared in a guinea pig. *Lower frame*: Inhibition of the precipitin reaction between the antibody R27-11 and the antiidiotype serum by a gammaglobulin fraction II preparation at a concentration of 50 mg/ml.

the proband's own preimmune IgG. It is noteworthy that the random rabbit R11, sharing the same allotypes a2/b4 as the proband, has preimmune IgG which is an effective inhibitor. On the other hand, random rabbits which had entirely different allotypic combinations are progressively less effective inhibitors of the

TABLE 50

Inhibition of an Idiotypic Precipitin Reaction by Various Preimmune IgG; I_{50} Values (Relative Concentration Required for 50% Inhibition)

Inhibitor	Allotype	I_{50}	Excess over proband preimmune IgG
Proband antibody	a2, b4	[1]	--
Preimmune IgG	a2,3, b4	64	[1]
Preimmune IgG			
Sibling 1	a2,3, b4	64	1
Sibling 2	a2, b4	70	1
Sibling 4	a2, b4	122	2
Random R11	a2, b4	113	2
Random R12	a3, b4	250	4
Random R13	a2, b9	280	4
Random R15	a1, b5	800	12

idiotypic reactions. This would suggest that the idiotypic specificity of the proband antibody is preferentially associated with the a2,b4 allotypic combination.

These inhibition studies suggest some participation of the allotypic determinants in the idiotypic determinant, but they may also reflect a linkage between genes responsible for idiotype and genes coding for the a and b allotypes. These introductory remarks will, I trust, make it clear that there are provocative relationships between the immune response, allotypy, and idiotypy, and that the way is now open for a genetic study.

CHAIRMAN WARNER: In these studies with the rabbit model, Krause has brought out strongly almost all of the major areas for discussion in this session; namely, that there is genetic control of the amount of antibody produced, whether it be of restricted or of heterogeneous type, that in certain instances this may be allotype-linked, and that there may be an association of idiotype with Ig allotype in these antibody responses.

I would suggest that we deal with these areas more or less separately, and begin by having Rajewsky comment first on the production of antiidiotype antibodies in mice.

RAJEWSKY: It may be appropriate to discuss at this point the work of my colleague, Eichmann, on the antistreptococcal polysaccharide response in mice. Figure 39 demonstrates the results of a first series of experiments in which a variety of inbred strains of mice were immunized with group A and group C carbohydrates.

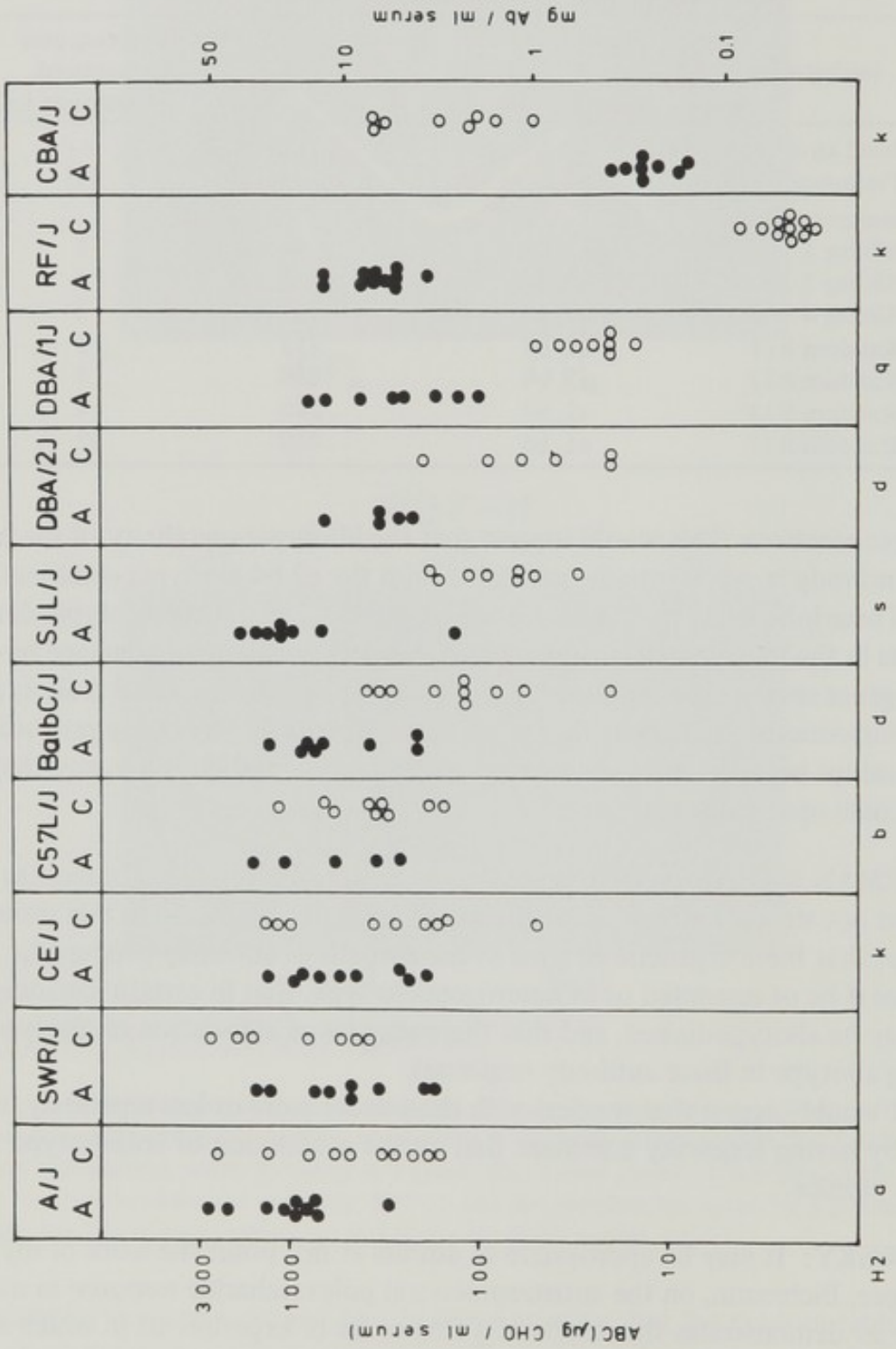


Fig. 39. Immune responses of various inbred strains of mice to group A and C streptococcal carbohydrates.

BENACERRAF: Were the mice immunized with pure carbohydrates or with intact streptococci?

RAJEWSKY: They were given bacterial vaccines.

It may be noticed first of all that the amounts of antibody formed in the various strains differ dramatically. In addition, some strains produce more anti-A than anti-C antibody, whereas in others the situation is reversed. It should be noted that strains carrying identical H-2 alleles differ considerably in their anticarbohydrate response. However, it is not only the amount of antibody formed upon immunization in which the various strains differ from each other, but also the heterogeneity of the immune response. This became apparent upon analysis of the antisera by isoelectric focusing. According to this criterion, the anticarbohydrate response was quite heterogeneous in some strains, whereas others (the A/J strain in particular) developed a rather homogeneous response.

Eichmann was able to passage the major antibody clone of A/J mice in the Askonas-Williamson fashion several times through irradiated syngeneic recipients. Enough homogeneous antibody could thus be obtained to raise antiidiotypic antibody in both A/J mice and guinea pigs. In the A/J strain, upon immunization with group A streptococci, four out of five individual mice produced essentially homogeneous anticarbohydrate antibodies, which were indistinguishable in isoelectric focusing and which carried the same idiotype. In one mouse, a homogeneous response was obtained, but the antibodies had a different isoelectric focusing pattern and also shared only part of the idiotypic determinants with the four others. In two other mouse strains, CE/J and SWR/J, the A/J idiotype of antigroup A carbohydrate antibodies could not be detected. Genetic studies are now under way to determine the pattern of inheritance of the idiotypic marker.

CHAIRMAN WARNER: From the strains reported on by Rajewsky, it would appear that there was no obvious association of response with allotype.

We will now proceed to hear from Nisonoff who will tell us about studies on idiotypic specificities in congenic mice.

NISONOFF: We have been working in collaboration with Laura Pawlak, David Hart, Ai-Lan Wang, as well as Michael Potter of the NIH, on idiotypic specificities of antihapten antibodies in inbred mice. The two haptens that we have studied are the para-azo-benzoate, and the para-azo-phenylarsonate groups. Ordinarily, we immunize mice with KLH coupled to the hapten and eventually obtain hyperimmune antiserum of high titer against the hapten. The antihapten antibody is then precipitated with another protein having the same hapten coupled to it to selectively precipitate the antihapten antibody. This precipitate is then washed and dissolved at pH 3.5, which may or may not liberate all of the

antigen in the complex, but which dissolves the precipitate. The dissolved precipitate is injected into a rabbit or a mouse of a different strain.

Nearly all of this work has been done with antiidiotypic antibody made in rabbits injected with a milligram of dissolved precipitate two or three times; almost invariably large amounts of antibody against mouse IgG are produced. One then absorbs these antisera with immunoglobulin from a mouse of the donor strain, and ordinarily with mouse serum as well. There are various types of evidence that the absorbed antiserum is specific for idiotypes. For example, if one absorbs the antihapten antibody from the immune serum of the donor mouse, it will no longer react with the antiidiotypic antibody. Also, the preimmune serum of the donor mouse does not react with the antiidiotypic antibody. Antibodies to other haptens or other proteins obtained in the same strain of mouse also fail to react. Accordingly, after absorption, we are dealing with an antiidiotypic antibody directed to the antihapten antibody of the mouse that provided the immunogen.

I want to comment briefly on three separate topics. The first is the suppression of idiotype; one can suppress the appearance of a specific idiotype by injecting into a mouse idiotype-specific rabbit antibody. This suppression has continued for as long as five months. The second topic is the linkage of idiotype to the heavy chain allotype of the mouse (localized in its Fc region). The third topic is the effect of changing the carrier on the idiotype of antihapten antibodies. If one inoculates A/J mice with KLH-p-azophenylarsonate, one produces antibodies with a certain group of idiotypic specificities. It becomes of interest to determine what happens if one immunizes a mouse of the same strain with the same hapten but on a different carrier. Does one elicit molecules with related idiotypic specificities? Our earliest investigations seemed to indicate that there are generally strong intrastrain idiotypic crossreactions. With the data we now have, we would have to say that the existence of intrastrain crossreactions is rather unpredictable, depending on the antigen, and on the strain of mouse.

Table 51 shows the method used for quantitation of specific idiotypic antibody. We allow rabbit antiidiotypic antiserum to react with purified mouse antibody labeled with ^{125}I; the complexes are then precipitated with goat anti-rabbit Fc. Controls include the use of rabbit antiovalbumin in place of the anti-idiotypic antiserum, or labeled normal mouse IgG in place of the labeled anti-hapten antibody. Unlabeled preimmune or hyperimmune sera are tested as inhibitors of binding.

Table 52 summarizes the types of crossreactions we have seen so far; for example, with the A/J strain of mouse, there are very strong intrastrain idiotypic crossreactions. Virtually every serum from an A/J mouse immunized with KLH-azo-phenylarsonate will react strongly with an antiidiotypic serum prepared against such antibodies from an individual A/J mouse. This generalization

TABLE 51

Quantitation of Antiidiotypic Antibody
by Inhibition of Indirect Precipitation

^{125}I-labeled specifically purified mouse antibody (0.1 to 0.15 μg)
+ test antiserum
+ excess absorbed rabbit antiserum
+ excess goat antirabbit Fc(absorbed with mouse serum)
Controls – A) Rabbit antiovalbumin in place of rabbit antimouse antibody
B) ^{125}I-normal mouse IgG in place of labeled mouse antibody

Unlabeled test sera containing the same or crossreacting idiotypes will inhibit the precipitation.

TABLE 52

Cross-Reactions of Rabbit Antiidiotypic Antisera
Directed against Antihapten Antibody of an Individual Mouse

Strain	Hapten	*Intra*strain cross-reactions	No. of antisera tested
A/J	Azophenylarsonate	Strong	3
A/J	Azobenzoate	Moderate	4
BALB/c	Azophenylarsonate	Very weak	4
BALB/c	Azobenzoate	Moderate	2
C57/BL	Azophenylarsonate	Very Weak	2

has held up with the three antisera tested so far. With A/J antibodies directed to the p-azo-benzoate group, the intrastrain crossreactions are moderately strong. Immune serum from almost every mouse crossreacts, but it takes considerably more antibody in general to cause the same degree of inhibition of indirect precipitation as compared with the antibodies of the donor mouse.

SELA: Is there any idiotypic crossreaction between antiphenylarsonate and antibenzoate antibodies?

NISONOFF: No. When we change antigens, there is no crossreaction at all. Furthermore, if one immunizes a mouse with the KLH-azo-phenylarsonate, and absorbs out the antihapten antibody, the antiserum loses its capacity to react with the antiidiotypic antiserum. In other words, the remaining anticarrier antibody does not crossreact idiotypically with the antihapten antibody.

Let us now get back to the A/J azo-phenylarsonate system. This is the best one we have studied so far, because the intrastrain idiotypic crossreactions are very strong. In this system, antiidiotypic antiserum is a reagent that can be used to study the antibodies of any mouse of the A/J strain that is immunized with the azo-phenylarsonate hapten.

TABLE 53

Inhibition of Binding of 0.01 μg of ^{125}I-Labeled Antiphenylarsonate Antibody from Mouse 413 to Its Antiidiotypic Antibody[a]

	Data expressed as % of control value					
	Preimmune	Week 5 bleeding			Week 11 bleeding	
	serum (10) μl)	0.3	3	10 μl Serum	0.3	3	10 μl Serum
Controls	95(2)	26(0)	23(3)				
	100(2)	76(7)	37(0)		56(0)	23(2)	
	95(2)	45(3)	22(0)		25(2)	7(3)	
	95(2)	41(0)	28(0)		41(0)	10(3)	
	102(1)	79(2)	28(1)		34(1)	17(1)	
	95(1)	59(0)	25(1)		36(4)	12(1)	
	102(2)	52(1)	28(1)		22(1)	8(1)	
	98(1)	50(5)	30(3)		22(3)	11(3)	
	98(1)	48(0)	29(3)		49(4)	12(1)	
Suppressed	91(6)		95(0)	87(5)		82(2)	70(4)
	97(1)		95(1)	88(1)		100(1)	90(2)
	102(3)		90(0)	80(3)		90(1)	84(1)
	96(2)		101(2)	N.D.		91(2)	92(1)
	100(0)		92(2)	81(3)		77(1)	75(2)
	100(0)		93(1)	82(3)		93(3)	94(6)
	100(0)		90(1)	88(2)		85(0)	84(1)
	98(1)		96(0)	86(2)		84(6)	78(1)
	109(3)		87(0)	77(3)		93(2)	82(1)

[a]All mice, including the donor (no. 413) are of the A/J strain. Indirect precipitations were carried out with rabbit antiidiotypic antiserum and excess goat antirabbit Fc.

To demonstrate suppression of idiotype, one prepares an IgG fraction of rabbit antiidiotypic antibody specific for A/J anti-azo-phenylarsonate antibodies.

BENACERRAF: What percentage of that antibody crossreacts in that strain?

NISONOFF: One can inhibit indirect precipitation to the extent of at least 75 to 80% with the anti-azo-phenylarsonate antibody of almost any A/J mouse. If one injects a mouse with rabbit anitiḍiotypic IgG intraperitoneally, twice with one mg, the suppression lasts for months. The idiotype does not appear on subsequent immunization with azo-phenylarsonate-KLH even when significant amounts of anti-KLH and antiphenylarsonate antibodies are found.

Table 53 shows some of the data on suppression. In the case of the control mice, which were injected with normal rabbit IgG, the idiotype appears after a

couple of weeks, and persists during subsequent immunization. The antisera of the suppressed mice, that received the antiidiotypic antibody, are very poor inhibitors of the idiotypic reactions.

HERZENBERG: Would Nisonoff identify the values in parentheses?

NISONOFF: They are the average deviations of duplicate experiments.

BENACERRAF: Is the amount of antibody also decreased in absolute terms?

NISONOFF: We don't know for sure. We have, however, carried out Ouchterlony tests, and found that the suppressed mice all produce substantial amounts of antihapten antibody.

COHN: I see lots of zeros in Table 53. How does Nisonoff get zero standard deviation?

NISONOFF: The zeros refer to duplicate determinations that yielded the same results to within 1%. As can be seen the suppression lasted for at least eleven weeks; with a few mice, it has persisted for five months.

HERZENBERG: Did Nisonoff have idiotype antibodies still present in the mice at five or eleven weeks?

NISONOFF: I can't answer that question, but in control mice the amount of idiotypic antibody produced exceeds by a very large factor the capacity of anti-idiotypic antibody to absorb these antibodies.

HERZENBERG: But wouldn't it be an easy matter to check whether there is idiotypic antibody in the suppressed animal by assay via direct precipitation of the labeled antihapten antibody?

NISONOFF: That would not be possible since the amount of antihapten antibodies in the serum of these mice is much too high.

UHR: Does rabbit gamma globulin persist in the sera of these mice?

NISONOFF: We have not determined that. However, we have looked for mouse antirabbit globulin and have not been able to detect it.

Figure 40 shows results obtained with congenic mice raised by Potter. What is shown here is linkage between allotype and idiotype. First, it is evident that antiphenylarsonate antibodies raised in A/J mice don't crossreact idiotypically with antiphenylarsonate antibodies of BALB/c mice. Each point on Fig. 40

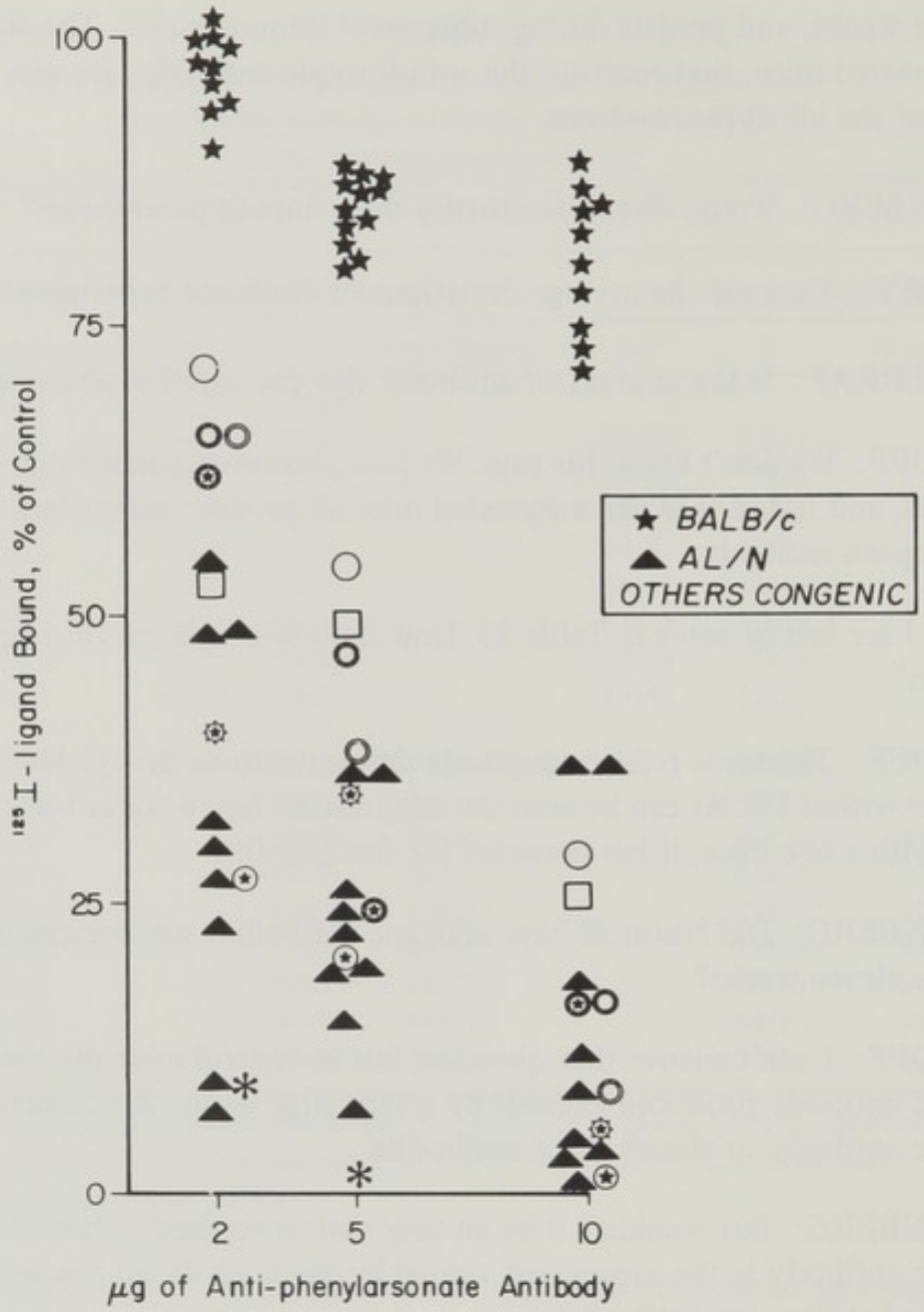


Fig. 40. Inhibition of antiidiotypic antibodies directed against anti-Ar antibodies of A/J mouse 413. The labeled ligand is specifically purified anti-Ar antibody of the donor mouse. The data show that congenic mice bearing the allotype of AL/N strain on a BALB/c background are as effective as anti-Ar antibodies of AL/N mice in inhibiting the binding reaction, whereas BALB/c antibodies are ineffective.

represents a different mouse. AL/N antiphenylarsonate antibodies do crossreact with antiidiotypic antibody within the strain, and these are shown by the filled triangles. Antiidiotypic antibody prepared against A/J anti-phenylarsonate antibody does crossreact with AL/N antiphenylarsonate. It is seen that with ten μg of antibody each antiserum inhibits to the extent of at least 70%. The other symbols that are not filled represent congenic mice which have the AL/N allo-

TABLE 54

Inhibition of A/J Antiazophenylarsonate to Its Binding of Antiidiotypic Antibody by Various Antisera

	Unlabeled inhibitors – Whole antisera					
Antibody to →	AR	AR	BENZ[a]	KLH	AR	AR
Carrier →	KLH	BGG	KLH		KLH	KLH
Strain →	A/J	A/J	A/J	A/J	BALB/c	C57/BL
	^{125}I-Labeled ligand bound (% of control)					
6(1)	8(1)	87(3)	86(0)	86(2)	80(3)	
0(2)	6(2)	94(2)	88(1)	89(1)	85(2)	
14(4)	18(2)	87(1)	83(3)	92(3)	89(4)	
3(1)	8(1)	88(2)	88(2)	97(1)	75(6)	
8(2)	2(1)	80(3)	90(1)	78(2)	85(3)	
2(1)	44(5)	90(1)	84(4)	90(2)	90(2)	
11(2)	18(1)	85(1)	89(2)	88(3)	88(1)	
18(2)	24(3)	90(2)	82(1)	82(2)	82(4)	
15(3)	59(4)	90(3)	85(3)	86(2)	82(2)	
10(1)	10(3)	83(1)	86(4)	94(3)	85(3)	
Additional A/J mice	(3-20)	(9-25)				
	(12 mice)	(5 mice)				

Inhibition of binding of 0.01 μg of purified ^{125}I-antiazophenylarsonate (Ar) antibody from A/J mouse 413 to its antiidiotypic antibody by various antisera. Inhibitors were unlabeled whole antisera, each containing 20 μg of precipitable antihapten antibody. The anti-KLH antisera (column 4) all gave heavy precipitates with KLH, but their antibody content was not determined. Each value in the table was obtained with the antiserum of an individual mouse. In the absence of inhibitor 55 percent of the labeled ligand was bound. Experiments were carried out in duplicate. Average deviations are given in parentheses. Indirect precipitations were carried out using the labeled anti-Ar antibody of mouse 413, its rabbit antiidiotypic antibody and excess goat antirabbit Fc.

[a]azobenzoate.

type on the BALB/c background, after they were backcrossed nine times, then rendered homozygous for the AL/N allotype. It is apparent that all of the congenic mice that we tested produced the crossreacting idiotype. This shows that the idiotypic marker is linked very closely to the allotypic marker, which is of course, on the heavy chain. Thus, the V_H gene must be closely linked to the C_H gene. We do not yet know the role of the light chain.

Table 54 indicates that one can change the protein carrier for the hapten and still get idiotypic crossreactions. The original donor was immunized with KLH-p-azo-phenylarsonate. Antisera prepared against BGG-p-azo-phenylarsonate show good idiotypic crossreactions although larger amounts are needed to produce the same degree of inhibition (data not shown). This suggests that one can change the carrier and still stimulate the same clones of cells. These data

are not conclusive because it is possible that the antibodies raised against BGG-azo-phenylarsonate are crossreacting idiotypically, but are not structurally identical. However, I believe the simpler interpretation is that one can stimulate some of the same clones of cells regardless of the carrier, either KLH or BGG. In my view, this indicates that idiotype is determined at the level of the B cell rather than the T cell, since it appears to be independent of the carrier.

CHAIRMAN WARNER: I think at this stage we have several clearly documented examples of allotype-linked immune response genes. Could we get on the record any other specific data on this point, as we will then proceed to the question of whether there are other levels of control of antibody production associated with allotype.

COHN: The studies I propose to summarize are detailed in a series of current publications: Blomberg, Geckeler, and Weigert, *Science*, **177**, 178, 1972; Sher and Cohn, *J. Immunol.*, **109**, 176, 1972; Carson and Weigert, *Proc. Nat. Acad. Sci.*, in press; Sher and Cohn, *Eur. J. Immunol.*, in press.

I want to consider two cases of a very restricted response in mice to complex antigens, dextran B-1355S which contains α-1,3 α-1,6, and α-1,4 like linkages, and pneumococcal C-carbohydrate which has dideoxyglucose, N-acetyl galactosamine phosphate, phosphorylcholine, etc.[a] I propose to show that mouse strains recognizing the same immunodominant group of these antigens, α-1,3 glucoside on the one hand and phosphorylcholine on the other, differ in the structure of their antibody. This structural difference is due to a locus linked to the allotype marker on the constant region of the heavy chain (C_H), presumably a V_H gene. In addition, the level of the response is associated with the structural polymorphism, implying that germ-line V-genes can control respon-

[a]Editors' Comment: In reading Cohn's discussion of the association of the murine immune response to dextran and pneumococcal polysaccharide with immunoglobulin allotype, the reader should be aware of the origin of the recombinant inbred strains referred to in Table 57, and of the fact that in the mouse 95% of the light chains are of the kappa class, and lambda light chains comprise a very small part of the normal immune response. The recombinant inbred strains referred to in Table 57 were derived by Bailey, by crossing BALB/c and C57BL/6. Random mating pairs were chosen from F_2 generation and used to establish seven new inbred lines, each of which contains a new random combination of the input genes donated by the C57 and BALB/c parental lines. By typing each of these lines for the various genes from the two parental lines, each new recombinant inbred strain can be designated to be of a particular H-2 type and a particular immunoglobulin allotype. Immunization of these recombinant inbred lines with dextran then permits a determination of association of dextran immune response with H-2 type, immunoglobulin allotype, or with neither linkage group.

TABLE 55

Specificity of the Immune Response to Dextran

Mouse strain	Plaques/10^6 spleen cells assayed on		
	B-1355S-SRBC	B-512S-SRBC	SRBC
BALB/c	1580	5	3
C57BL/6	465	32	33

Dextran B-1355S is a branched polymer (~ $3X10^7$ M.W.) containing 57% α-1,6, 35% α-1,3, and 8% α-1,4 like linkages. Dextran B-512S contains 95% α-1,6 and 5% α-1,4 like linkages; no α-1,3 being detectable.

Mice were immunized with B-1355S dextran in complete Freund's adjuvant, boosted at day 7 with 10 μg dextran in incomplete Freund's adjuvant, and assayed at day 12 by the plaque technique. Red cells for assay were sensitized with either dextran B-512S (without α-1,3 linkages) or dextran B-1355S (with α-1,3 linkages). Red cells sensitized with B-512S were agglutinable by γAκ myeloma proteins with antidextran activity (not anti-α-1,3 glucosyl linkage) showing that determinants on B-512S are accessible. Unimmunized mice gave less than 13 plaques/10^6 with B-1355S sensitized cells, therefore, background plaques were not subtracted from values reported in this and the following tables. *Science,* **177,** 178, 1972.

siveness, a point of considerable importance for the theories of diversity. Further, the responsiveness difference is due to a gene-locus expressed in B cells.

The key reagent is an anti-idiotypic serum directed against a reference myeloma protein. In the case of the dextran response, the reference myeloma protein is an IgA, J558, with specificity for nigerose, an α-1,3 glucoside, one of the repeating units in dextran B-1355S. In the case of the C-carbohydrate response, the reference myeloma is an IgA, S107, with specificity for phosphorylcholine. The suitably absorbed antiidiotypic sera show an interaction with the myeloma protein which is abolished upon addition of the specific ligand, a phenomenon described by Nisonoff.

About 1% of the myelomas induced in BALB/c mice produce immunoglobulin which show specificity for α-1,3 dextran and an additional 1% show specificity for phosphorylcholine. The anti-α-1,3 dextran myeloma proteins are always associated with a λ chain of unique sequence and similar idiotype. However, the antiphosphorylcholine myeloma proteins are all in the κ class but only 80% have κ chains in the same subgroup and similar idiotype. These are the highest binding proteins.

The immunization of BALB/c mice results in antibody restricted to the α-1,3 linkage in the case of dextran, or to phosphorylcholine in the case of the C-carbohydrate. The response is uniquely in the IgM class, even after many weeks of repeated immunization and remains restricted.

First, let us consider the dextran system. As is evident in Table 55 BALB/c is a high, and C57BL/6 a low, responder. The difference between them is initially quite large (at five days as shown in Table 56), but gradually disappears as the

TABLE 56

Response of Various Mouse Strains to α-1, 3 Glucosyl Linkages in Dextran'

Mouse Strain	Plaques/10^6 cells at 5 days (mean)*	H-2**	Allotype†	L-chain class	Idiotype
BALB/c	1,710	d	BALB/c	λ	J558
129	1,635	b	BALB/c	λ	J558
C58	685	k	BALB/c	λ	J558
F_1 (BALB/c × C57BL/6)	1,140	d/b	BALB/c C57BL/6	λ	J558
(C57BL/6)	110	b	C57BL/6	κ	not J558
SJL	40	s	C57BL/6	κ	n.t.‡
CBA	41	k	BALB/c	κ	n.t.
AKR	40	k	AL	κ	n.t.
A/He	10	a	AL	κ	n.t.
NZB	33	d	AL	κ	n.t.

*Range for BALB/c (20 mice) was 1000-2800 PFC/10^6, for C57BL/6 (15 mice) 50-160 PFC/10^6.

Transplantation, **2, 777, 1964.

†_Cold Spring Harbor Symp. Quant. Biol._ **32**, 187, 1967.

‡Not tested.

immunization is continued (Table 55). This is to be expected as I will later show. The point of the data in Table 55 is that BALB/c and C57BL/6 both manifest an anti-α-1,3 dextran response *only*, although they were immunized with B-1355S which has α-1,6 and α-1,4 linkages. There is no reaction with B-512S (which lacks α-1,3 linkages) over that seen with SRBC alone.

The high responding strains (e.g., BALB/c listed in Table 56) all produce antibody in the λ class and carrying the idiotypic determinant of the reference myeloma J558. The low responding strains, e.g., C57BL/6, all produce antibody in the κ class lacking the reference idiotype. The F_1 (BALB/c × C57BL/6) hybrid is a high responder like BALB/c and produced antibody in the λ class carrying the reference idiotype. Therefore, responsiveness is dominant and as expected the antibody produced is BALB/c-like. I show Table 56 primarily to illustrate how difficult is is to argue linkage from strain surveys. BALB/c and NZB have the same H-2 locus, but BALB/c is a high and NZB a low responder. This argues that responsiveness is not H-2 linked, which happens to be correct. Likewise BALB/c and CBA have the same heavy chain allotype locus yet they differ in responsiveness. This argues that responsiveness is not heavy chain linked. This conclusion happens to be wrong as will be seen, and probably means that during the inbreeding to make a CBA or BALB/c mouse the responsiveness V_H-gene and the C_H-allotype gene were separated by recombination.

TABLE 57

The Linkage of Antidextran Immune Response to the Heavy Chain Allotype

	Recombinant inbred strains*						
Locus	C × BD	C × BE	C × BG	C × BH	C × BI	C × BJ	C × BK
H-2**	C	B	B	C	B	B	B
C_H†	B	B	C	B	B	C	B
dextran‡ response	B	B	C	B	B	C	B

*C = BALB/c allele, B = C57BL/6 allele. *Science*, **177**, 178, 1972.

***Transplantation*, **11**, 325, 1971.

†The heavy chain allotype.

‡Mice were assayed for their response to dextran 5 days after immunization by a hemagglutination assay or by the plaque assay. The response of strains C × BG and C × BJ can be inhibited (80-100%) with antidiotypic antisera directed against myeloma antibody J558.

The demonstration that both the idiotype and responsiveness are linked to the heavy chain allotype is shown in Table 57. The trick here is the use of recombinant inbred lines between BALB/c and C57BL/6. Essentially one inbreeds randomly chosen pairs from the F_2 generation so that in a sense one establishes clones of F_2 mice. The lines are then assayed for the various genes of BALB/c or (C) or C57BL/6 (B) they carry. The use of recombinant inbred lines minimizes the problem of inadvertent recombination so that the linkage groups of BALB/c (C) or C57BL/6 (B) are preserved. The linkage of dextran responsiveness to the heavy chain allotype and not to the H-2 locus can be seen because the phenotype of the response, B or C, follows the allotype, B or C, not the H-2 type. For example, the C × BD strain has the C H-2 type and the B dextran response. Therefore, the dextran response and H-2 loci are unlinked. The linkage of responsiveness to the heavy chain allotype has been confirmed by backcross and the two loci are linked to within 1% recombination.

The interpretation of these data is that a germ-line V_H gene closely linked to C_H determines responsiveness to α-1,3 dextran. The product of this gene complements with a germ-line V_λ subunit in the high responding BALB/c to produce an immunoglobulin which is of α-1,3 dextran specificity. In other words, this is the antibody specificity of a germ-line encoded immunoglobulin. The product of the germ-line V_H allele in C57BL/6 complemented to a germ-line V_λ subunit is postulated to require one or more mutational steps to express high α-1,3 dextran binding. Hence the relatively slow initial response compared to BALB/c. Eventually C57BL/6 catches up.

Now let us consider the response to the C-carbohydrate. I am not going to deal with the level of the response in this case, only the idiotype of the antibody

TABLE 58

Linkage of Antiphosphorylcholine Immune Response to C_H Allotype*

Mouse strain†	H-2 type	C_H allotype	Phenotype response‡
BALB/c	d	a	+
C57BL/10	b	b	+
A/WY, A/J	a	e	-
AL/N	a	d	-
C.AL	d	d	i
A. BY	b	e	i
B10.A	a	b	+

**Eur. J. Immunol.* in press 1972.

†C.AL is congenic with BALB/c but carries the C_P allotype locus of AL/N. A.BY is congenic with A/WY but carries the H-2 locus of C57BL/10. B10.A is congenic with C57BL/10 but carries the H-2 locus of A/WY. These are congenic lines references for which are found in Sher & Cohn, 1972.

‡The idiotype of the response is determined as percent inhibition of expression of plaque forming centers by a reference anti-idiotype serum prepared against an antiphosphorylcholine myeloma protein (S107) and used at a limiting dilution to give 90% inhibition of BALB/c plaques.

+ = idiotype indistinguishable from that of BALB/c,e.g. C57BL/10.

- = idiotype distinct from that of BALB/c,e.g. A/WY.

i = idiotype intermediate between BALB/c and A/WY.

produced. The antibodies to phosphorylcholine produced in the A and BALB/c mice are indinstinguishable in their affinity for phosphorylcholine. Yet the A mouse antibody is distinguishable in its idiotype from BALB/c.

Table 58 provides a summary of studies on selected strains congenic at the H-2 and C_H loci; they show the following characteristics:

1. BALBC/c and C57BL/10 are indistinguishable in the idiotype of the antibody to phosphorylcholine which they produce. They are described as "+".

2. A/WY, A/J and AL/N mice do not carry the reference idiotype on their antiphosphorylcholine antibody. They are described as "–".

3. The C.AL mouse which is congenic with BALB/c except for the heavy-chain locus which is that of AL/N, is of an intermediate behavior, the structural basis for which has not yet been analyzed.

Strains which are + never show less than 85% inhibition of plaque formers by the standard dilution of antiidiotype serum and strains which are – never show more than 20% inhibition. The intermediate inhibitions fall between 40 and 70%. There is no overlap in the scatter of values into the intermediate category in studying individuals of a + or – strain. A result similar to that of C.AL is found for the A.BY mouse.

4. The B10.A congenic strain carrying the H-2 locus of a negative strain (A/WY) and the C_H-locus of a positive strain (C57BL/10) is a positive strain. This means that it is indistinguishable from C57BL/10 or BALB/c in the idiotype of its antiphosphorylcholine antibody. It appears that an antibody carrying the correct heavy chain alone behaves as an idiotype + whereas one carrying the correct κ-chain alone is intermediate in its idiotype.

The conclusions are:

1. Two genetic factors may be involved in determining the idiotype, one of which is heavy chain linked, whereas the linkage of the other is unknown.

2. Since this is a κ response and since idiotype is being measured, the two factors would be guessed to be a V_H and a V_κ gene

3. The assignment of the V_H gene as one factor is a strong guess; the assignment of V_κ (linked to H-2) is a weak guess, based solely on the intermediate phenotype of the A.BY mouse. Other explanations are possible.

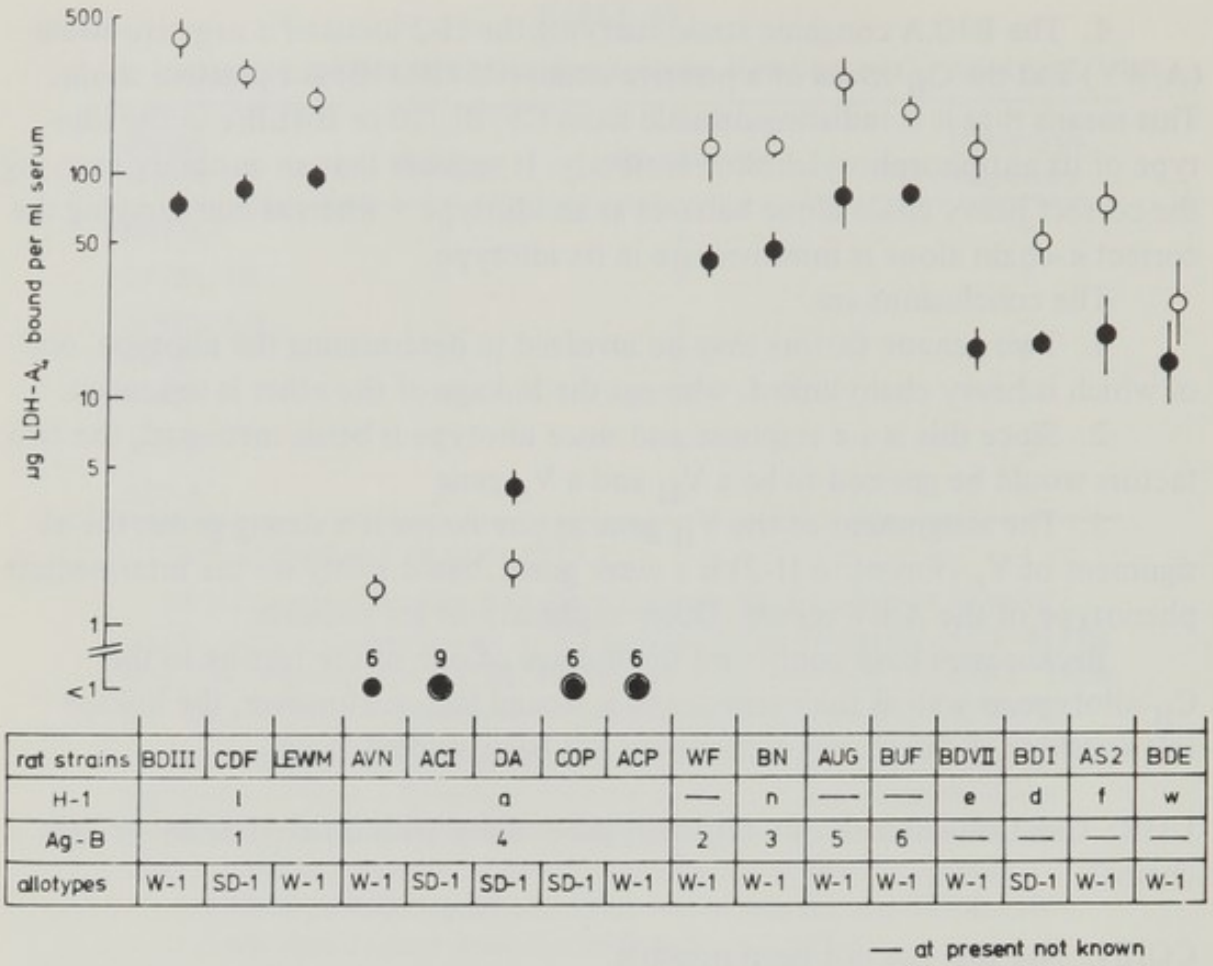
Backcrosses have confirmed the linkage of one of the factors to the C_H-allotype as well as the existence of a second factor. However, the linkage relationships of the second factor have not been studied as yet.

UHR: Can Cohn absorb out antiidiotypic activity with isolated heavy or light chains?

COHN: No, that has not been possible.

RAJEWSKY: I would like to contribute a salient fact or two and a comment to this discussion. The facts concern a possible relationship of κ light chains with the Ir locus which has been alluded to. Wurzberg in my laboratory has established an immune response system in rats, in which responsiveness to porcine lactic dehydrogenase is linked to the major histocompatibility locus (Fig. 41). There is no linkage, however, to a light chain allotypic marker which in all probability is located on κ light chains (Table 59).

The comment I would contribute is that it is not the linkage of idiotype with allotype which is the new message from Cohn's data, since we already know that in rabbits the allotypes on the Fc portion and the Fd portion of the heavy chain are linked. What I believe is more important here is the fact that the idiotypes which Cohn is describing, and which Nisonoff has reported, seem to be expressed on *any* strain background. This is of crucial importance for theories of the generation of somatic diversity, such as the one proposed by Jerne. One must, however, be cautious and look at more idiotypes in different congenic strain combinations to ascertain whether the expression of the idiotype is consistently independent of the histocompatibility background.



rat strains	BDIII	CDF	LEWM	AVN	ACI	DA	COP	ACP	WF	BN	AUG	BUF	BDVII	BDI	AS2	BDE
H-1	l			a					—	n	—	—	e	d	f	w
Ag-B	1			4					2	3	5	6	—	—	—	—
allotypes	W-1	SD-1	W-1	W-1	SD-1	SD-1	SD-1	W-1	W-1	W-1	W-1	W-1	W-1	SD-1	W-1	W-1

— at present not known

Fig. 41. Immune response to porcine lactodehydrogenase A_4 in various rat strains. Filled circles represent primary, open circles secondary antibody titers in a standard immunization procedure.

TABLE 59

Association of Anti-LDH-A_4 Response with Major Histocompatibility (H-1) Type and Immunoglobulin Allotype in F_2 Hybrids

	H-1			Allotype		
	aa	al	ll	SD/SD	SD/W	W/W
High responder	0	9	8	4	10	3
Low responder	7	0	0	2	4	1

F_2 = LEW (H-1^1, W-1) × DA (H-1^a, SD-1).

COHN: At this moment I do not intend to argue that question. I think it is an open question as to whether *Ir-1* is the κ locus, although as has been pointed out, all of the available evidence is against it. There are two likely competing hypotheses. In the mouse, either *Ir-1* codes for κ chains, or for a new heavy chain locus, the product of which complements with the product of the κ locus. Since it is essential to rule out *Ir-1* as the κ locus, I prefer, at this moment, to play devil's advocate.

CHAIRMAN WARNER: There is one more specific example worthy of discussion on allotype linkage to immune response genes, and involving congenic mice, and this will be dealt with by Herzenberg.

HERZENBERG: The work I will summarize was done in collaboration with Benjamini, Cherry, Leung, and Noble, and it was based on an accidental finding, one of those serendipitous things. Using congenic strains is a good idea for almost any comparison, because if a difference is found, one can then ascribe it to the gene which differentiates the congenic strains. In one of the many types of experiments Mitchell was doing in our laboratory last year, he used tobacco mosaic virus (TMV) protein as an antigen, and found paradoxical results. The variable turned out to be that he sometimes used one congenic strain and sometimes another for his experiment. We have now pursued this lead and found that if one immunizes the two congenic strains of mice, CSW and CWB, with whole TMV protein, and assays the antibody obtained against the immunodominant decapeptide of this protein, (which Benjamini has worked with for a number of years) we then find in each strain a very different antibody response in the antibody to the decapeptide.

Figure 42 shows the difference obtained in groups of mice. What we really wanted to see was whether this difference was due to gene(s) expressed on T cells or B cells by doing various kinds of T and B recombinations. However, we found the results were not at all as clear as we had hoped. Accordingly, we proceeded to examine individual mice. Table 60 shows the results of titrating the sera of a number of individual congenic mice of either the homozygous Ig^a allotype or homozygous Ig^b allotype, and the *a*/*b* heterozygotes. In most animals, we found either a clear high or low response and a few intermediates. It is apparent that there is not an absolute correlation; the correlation in each direction is about 90%. The heterozygotes then come out to be about half responders and half nonresponders. This is not the clear-cut dominant or recessive relationship one would like to see.

We also looked at a number of different inbred and congenic strains. Table 61 shows that the immune response to even a fairly simple decapeptide such as this, does not give an absolute correlation with either Ig allotype or H-2. However, the *a* allotype mice are in general among the high responders, except for BALB/c. The *b* allotypes are all down at the bottom, except for two interesting strains, LP and SJL. It is evident that the correlation with H-2 is by no means absolute.

CHAIRMAN WARNER: I wonder if we can say without argument that at this stage, some of the data with the allotype congenic strains indicates that in different inbred strains there is a different library of V-region genes, in that some strains lack certain combining sites. It is implicit that these V-region genes are

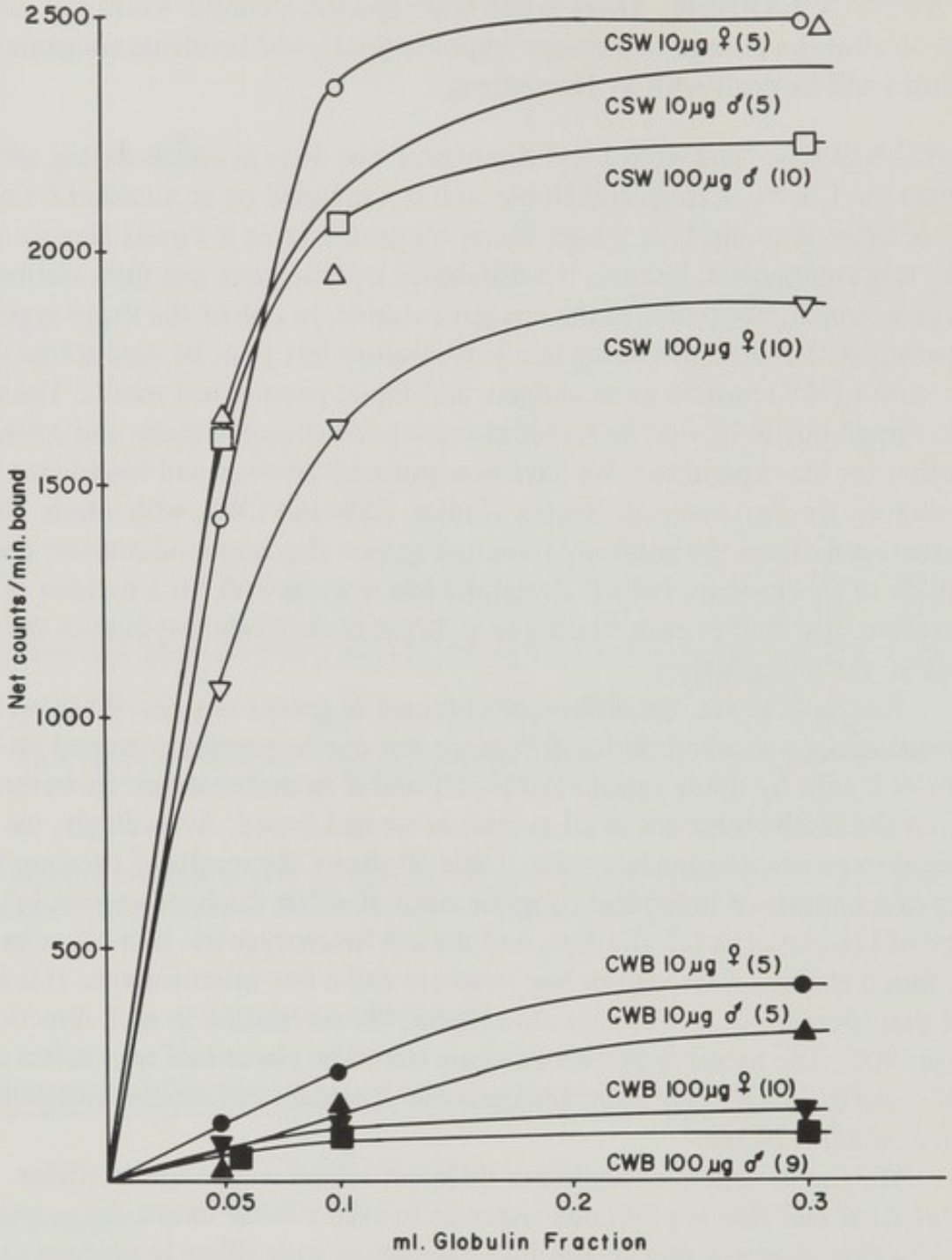


Fig. 42. Decapeptide binding activity of globulins from congenic mice differing only for Ig (C_H) chromosome region. Immunization was with Tobacco Mosaic Virus Protein (TMVP) by footpad injection in complete Freund's adjuvant followed three weeks later with aqueous TMVP in the footpads. Serum was obtained 14 days after this booster injection, the globulin fraction obtained by ammonium sulfate precipitation and ^{125}I-Tyr-decapeptide binding measured by a Farr technique. Number of animals per group is given in parenthesis. The sera tested were pooled specimens.

TABLE 60

Response to Decapeptide after Immunization with TMVP. Correlation with Allotype in Congenic Mice[a]

Ig allotype	No. of mice			Total	% High
	High[b]	Low	Intermediate		
a/a	17	1	1	19	90
b/b	7	59	2	68	10[c]
a/b	34	41	5	80	43

[a]All mice were C3H.SW, its congenic partner, CWB/13, or hybrids of these congenic mice.

[b]Most mice were clearly high or low responders (> 20% or < 8% decapeptide bound respectively). Intermediates had values between these two groups.

[c]If intermediates included as high, value becomes 13%.

TABLE 61

Response of Various Mouse Strains to ^{125}I-Tyr Decapeptide after Immunization with 10 μg TMVP

Strain	Decapeptide bound*	Alleles	
	%	Ig	H-2
CSW	47	a	b
NZB	36	e	d
A/J	34	e	a
LP/J	22	b	bc
SJL/J	16	b	s
CE/J	15	f	k
AKR/J	15	d	k
C3H/HeJ	11	a	k
DBA/2J	9	c	d
B10/Sn	8	b	b
CWB/13	6	b	b
B10.D2	5	b	d
BALB/cN†	0	a	d
BAB/14	0	b	d
RIII/2J	0	g	r
B10 (brown)	0	b	b
B10.Br	0	b	k
B10.A	0	b	a

*Percent of 0.01 μg ^{125}I-Tyr decapeptide bound by 0.3 ml globulin from pools of five mice per group on day 14 of secondary response.

†In an earlier experiment BALB/c gave about 14% binding of decapeptide after immunization with 100 μg TMVP plus. *B. pertussis* in complete Freund's adjuvant.

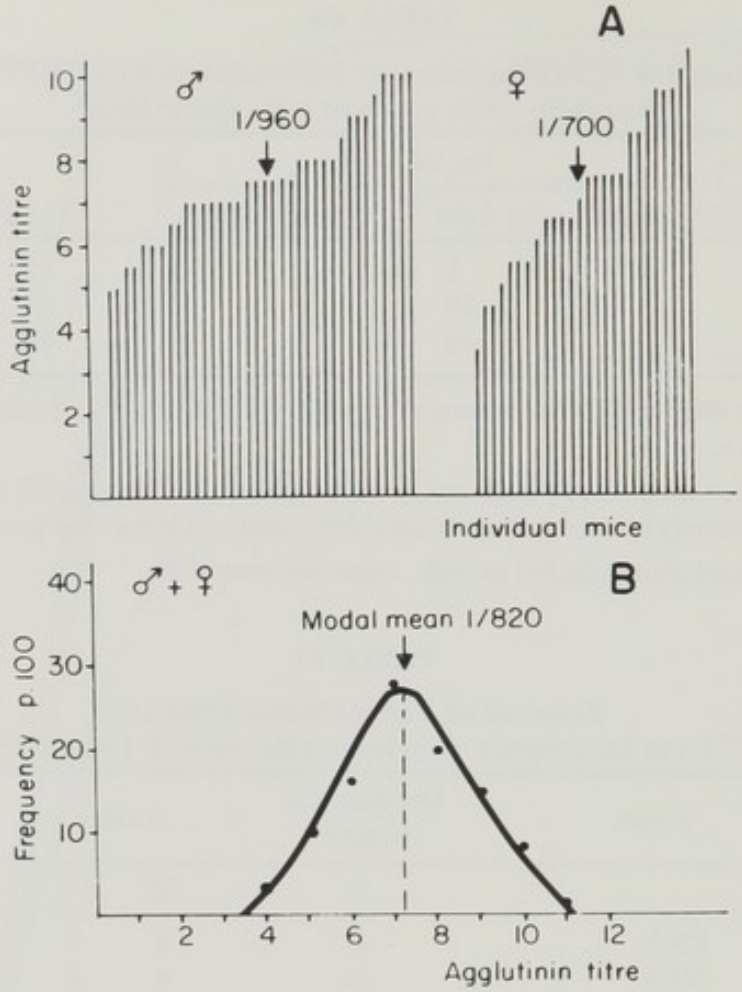


Fig. 43. Distribution of anti-SRBC agglutinins in the original population of random bred albino mice.

linked to the C region as shown by the heavy chain allotype linkage data, and this is consistent with the *a*, *d*, and *e* locus linkage data in rabbits. This is clearly one of the major issues, but unless anybody has any strong wish to discuss this further, I would suggest that we accept that there is a B cell expressed V-region gene control of certain immune responses.

A question that might also be raised is to ask how many different immune responses are involved in this type of genetic control as compared to the H-2 linked immune response genes. What we would now like to consider is whether there are any other levels of genetic control, such as Krause's work has indicated, concerning heterogeneous antibody responses and the amounts of antibody produced.

Let us first consider the example provided by Biozzi who will introduce the concept that there may be allotype linkage associated with more than one specific antibody response.

BIOZZI: I would like to acquaint you with a model of genetic regulation of antibody synthesis which is different from that involving specific immune re-

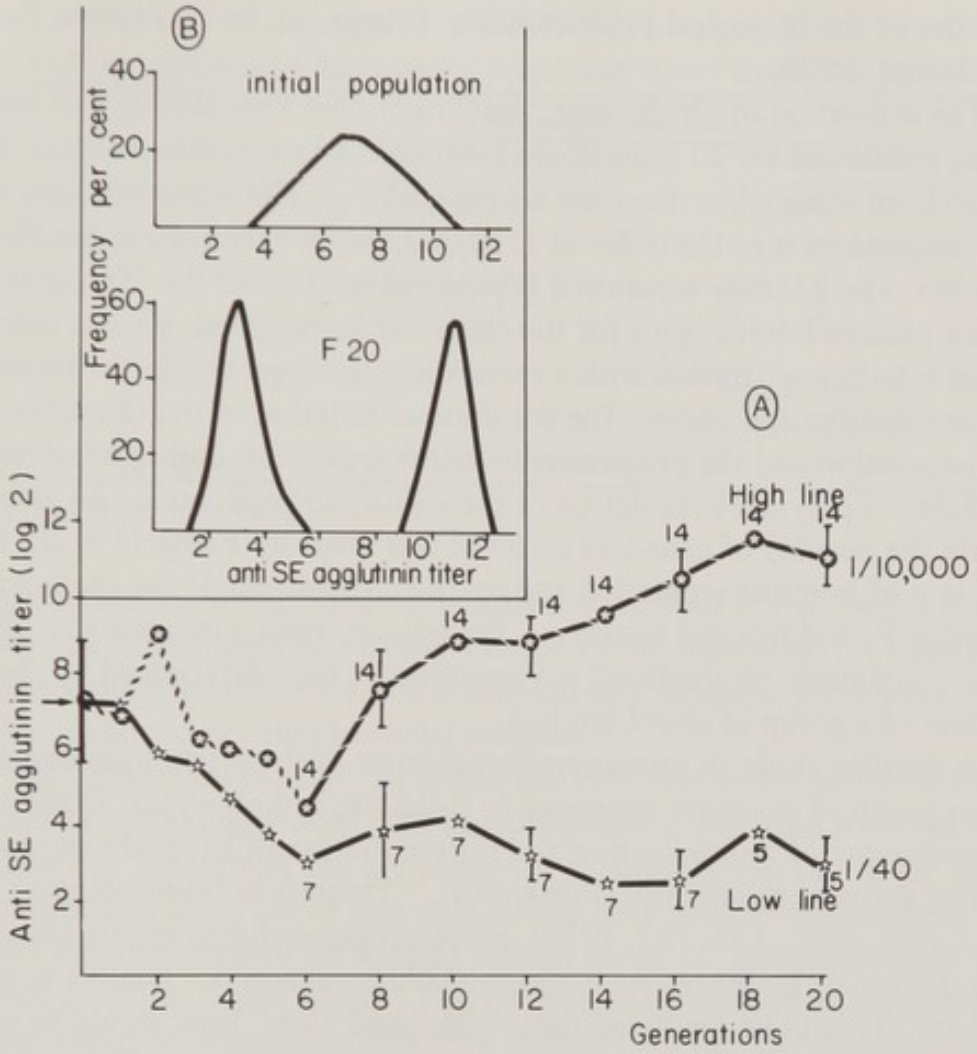


Fig. 44. Separation of "high" and "low" responder mice in the course of selective breeding.

sponse genes. In fact, in our model the genetic regulation operates at the level of immunoglobulin synthesis irrespective of their specificity. Random-bred albino mice have been immunized i.v. with an optimal dose of SRBC. The peak level of agglutinin production has been established in each animal. This peak level, expressed as $\log_2$ serum dilution, has been taken as the phenotypic character with respect to agglutinin production.

In Fig. 43 is represented the individual anti-SRBC agglutinin titers established for a group of female and male mice; there is a large individual variation with no evident sex-linked difference. The frequency distribution curve of this character in the total population is harmonic; the modal mean is close to the arithmetic mean. Therefore, the measure of the phenotypic character "agglutinin production" is correct. From this starting population "high" and "low" responder lines have been separated by selective breeding via appropriate matings. The details of the selective breeding program have been described (Biozzi *et al.*,

in Protides of the Biological Fluids, Colloq. Brugge, ed. by H. Peeters, Pergamon Press, Oxford, 1970).

The separation of "high" and "low" responder lines throughout selective breeding continued for 20 generations (Fig. 44). A remarkable interline difference had been attained by the time we reached F_{20}. The agglutinin titer of "high" responders is of the order of 1:10,000, but is only 1:40 in the "low" responders. The interline separation is progressive. Around the 20th generation, each line became homozygous for the character investigated, since it reached the maximal interline separation with a remarkable homogeneity in the respective frequency distribution curves. The continuous variation of the character in the initial population and the progressive interline separation during selective breeding indicates a polygenic regulation of the agglutinin production. An attempt to calculate the number of gene-loci involved was made by Feingold *et al.*, based on the rate of interline separation and on the distribution of the character in segregating F_2 hybrids and backcross. Preliminary results indicate that the character of "agglutinin production" is a quantitative trait determined by the cumulative effect of a group of about ten loci.

A detailed study of immunoresponsiveness of these two lines demonstrates that this group of genes are expressed in B-derived lymphocytes. These genes regulate the rate of multiplication and differentiation of antibody-producing cells after antigen stimulation. (Biozzi *et al.*, "Progress in Immunology," **1**, 529, 1971; *J. Exp. Med.*, **132**, 752, 1970). Since antigen-induced multiplication and differentiation of lymphocytes is a general phenomenon not relative to antigen specificity, it could be predicted that "high" and "low" lines would be separated in their immune responsiveness to any of a variety of antigens. This has, in fact, been verified for many non crossreacting antigens as is shown in Table 62. The

TABLE 62

Responsiveness of "High" and "Low" Responder Mouse Lines to Different Antigens

Antigens	Peak of serum antibody[a]		
	High line	Low line	Assay
SRBC	1/15,000	1/80	Agg
Pigeon erythrocytes	1/7,000	1/100	Agg
S. typhi O somatic	1/500	1/30	Agg
S. typhi H flagellar	1/1,000	1/60	Agg
Hemocyanin	1/400	1/10	Pass. hemaggl.
Bovine serum albumin	1/20,000	1/30	Pass. hemaggl.
DNP-BGG	1/2,000	1/80	Pass. hemaggl. DNP-SRBC
Egg albumin	1/64	1/3	PCA

[a]Primary or secondary response.

TABLE 63

Distribution of Immunoglobulin Allotypes in "High" and "Low" Responder Lines of Mice

	Immunoglobulin allotypes	
	"High" line	"Low" line
Phenotypes	1) 8% BALB/c 2) 43% C_{57}BL 3) 49% (BALB/c × C_{57}BL)F_1	G(γ_2a) 3, 5, 7; 8 H(γ_2b) 9, 11 A(IgA) - F(γ_1) f Nonexistant in inbred strains (wild mice)

J. *Exp. Med.* **132.** 752, 1970.

interline separation for antibody synthesis as presented in Fig. 44 has been extended to all the antigens and haptens tested so far, including, for example, pneumococcus polysaccharide, histocompatibility antigens, picric acid, T_4 bacteriophage, and many other bacterial antigens.

The differential capacity to synthesize antibody in the two lines involves all classes of immunoglobulins. The immunoglobulin allotypes involved has been ascertained by Lieberman and are presented in Table 63. In the "high"line, the immunoglobulin allotypes of two pure strains have been found, whereas "low" line mice are homozygous for a single linkage group of immunoglobulin allotypes not found in inbred strains. This restriction of allotype distribution in the two lines could be determined either by linkage between the allotype locus and a quantitative locus segregated in each line by our selective breeding, or by intraline consanguinity. Though close inbreeding has been excluded during genetic selection, a considerable degree of consanguinity results from the limited number of pairs selected in each generation (closed colony). The inbreeding coefficient calculated by Feingold *et al.* in F_{21} mice is of the order of 0.55 and 0.71 in "high" and "low" lines respectively.

The demonstration of a positive correlation between the distribution of the immunoglobulin allotypes and anti-SRBC responses in segregating interline F_2 hybrids has been found, as shown in Table 64. Such a correlation has been confirmed in F_1 hybrids backcrossed with the two parental lines as demonstrated in Table 65.

The quantitative difference in agglutinin production found in segregating F_2 hybrids and backcross (Tables 64 and 65) represents only a fraction of that separating homozygous "high" and "low" lines (Fig. 44). This indicates that only one of the group of quantitative loci segregated in each line by the selective breeding is linked with the immunoglobulin allotypes. This locus may be closely linked to the one coding for allotype specificity without necessarily having a

TABLE 64

Correlation between Immunoglobulin Allotype and Anti-SRBC Agglutinin Titer in Interline F_2 Hybrids

Allotype	Anti-SRBC agglutinin (5 days)	
Homozygous "low" 3,5/3,5	1/500	$p < 0.01$
Homozygous "high" 2/2	1/1,000	
Heterozygous ("high" × "low")	1/750	

TABLE 65

Correlation between Immunoglobulin Allotype and Anti-SRBC Agglutinin Titer in Back Cross

	Allotype	Titer of anti-SRBC agglutinins five days after immunization
F_1 × "high line"	Homozygous "high" 2/2	1/1,100
	Heterozygous 2/3,5	1/750
F_1 × "low line"	Homozygous "low" 3,5/3,5	1/130
	Heterozygous 2/3,5	1/250

functional relationship with the structural genes for immunoglobulin heavy chains.

BODMER: I have a point to make in relation to the statement by Biozzi concerning the possible chance of linkage to the allotypes that was not functionally significant. I think that this is extremely unlikely, because even though one has 20 chromosomes and there is a one in two chance that one of ten markers will be on the chromosome with the allotype marker, that is not enough to detect significant linkage. A significant association will not be found unless the linkage is quite close. Consequently, I would have thought that if there really is an association, it is very unlikely to be just a chance association with the allotype marker.

BENACERRAF: Possibly because of lack of time, Biozzi did not present all the data obtained on these mice which is relevant to this problem. The low line has substantially lower amounts of immunoglobulin before and after immunization

than the high line, with respect to just about every class of Ig. These measurements were made by Asofsky. No relationship whatsoever could be demonstrated in the F_2 and backcross generations between the allotype linkage groups and the level of immunoglobulin in the individual animals.

CHAIRMAN WARNER: I think the point that we need to discuss at this moment is whether there are allotype associations of the genetic control of antibody production which have nothing to do with the V-region gene library. I wonder if Ceppellini would like to discuss the situation with regard to b4 and b9 in rabbits; as to whether there are certain allotype patterns which are associated with many different deficiences of antibody production.

CEPPELLINI: I would like to have the opinion of the experts on another aspect of the genetic control of Ig and hence antibody synthesis. My colleague Carbonara some years ago identified a new allele of the Ab locus of rabbit allotypes that later was confirmed to be qualitatively identical to Dubiski's Ab9. The mutant found in the Torino rabbit colony, however, was also characterized by a very low level of Ab9 molecules in the serum. Homozygous 9/9 rabbits had an abnormally low level of total Ig with a high level of an unusual kind of light chain (now known to be λ chains). In 4/9, 5/9, and 6/9 heterozygotes, the ratio between the two allelic products was always unbalanced, *against* the new allele. In some combinations, b9 represented only ten percent of the total κ chains. Specific humoral responses were mainly of the other allelic type, although occasionally, even in heterozygotes, a prominent antibody response with b9 chains was observed. All in all, the situation was highly reminiscent of some hemoglobinopathies where some abnormal hemoglobins are also characterized by a deficient synthesis of one chain.

This is in agreement with the known fact that each structural mutant shows a specific rate of synthesis, which seems to be related to the primary structure of the polypeptide. In the case of immunoglobulins, however, the levels of the different molecular populations observed in the serum are a rather faithful reflection of the types of immunocytes that are found in the lymphoid organs (taking into account Ig catabolism and other factors). In the case of b4/9 heterozygous rabbits studied by Carbonara, the b4 cells observed by immunofluorescence in stimulated lymph nodes greatly outnumbered (up to 10:1) cells showing the b9 allotype. This observation suggests that "allelic exclusion," and more generally, the differentiation of the immunocytes towards one or another of the alternative immunoglobulin patterns, may be, at least in part, a function of the primary structure and consequently of the relative rate of synthesis of the various H and L chains. A similar imbalance is well known for the λ subclasses (pseudoalleles) of man.

As we all appreciate, allotypes, by definition, are "constant" parts; but the same reasoning may be valid for the antibody combining site which is characterized by amino acid substitutions. Should this be the case, it then follows that some (possible) antibodies are only rarely expressed because of their primary structure in the *variable* part, which makes improbable the differentiation of the clone. This, then makes for one more element of restriction to nucleotide changes in either the germinal or the somatic theories.

CHAIRMAN WARNER: I think Ceppelini's point deserves further discussion. One could cite further examples such as the IgG class in man. There are at least three laboratories that have documented that Gm5 homozygotes have in general twice as much serum IgG_3 as Gm21 homozygotes. If we accept that there can apparently be deficient amounts of immunoglobulin associated with certain allelic constant region genes, Ceppellini then raises the point that we must discriminate between an actual difference in rate of synthesis of that allelic product or whether one can demonstrate, for example, that b4 is preferentially present over b9 on the cell surface. As this may occur in the absence of antigenic stimulation, the issue of such a preferential association of variable region genes with b4 rather than with b9 needs to be answered.

HERZENBERG: I think we may be getting a bit confused by this b9 story. There is no question that there are different rates of production of immunoglobulins belonging to different loci, and that one can get what is called immune deviation, i.e., a change in the ratio of production, say of λ 1 and λ 2, according to the method of immunization. The *b* locus so-called allotypes have been thought to be alleles, but there may be some question as to whether they really are true alleles, or in fact, manifestations of genes at different loci. The ratio of b4 to b9 in 4/9 heterozygotes can, for example, be altered by treating cells *in vitro* with anti-b9 serum for 24 hours, and then putting them back into allogenic rabbits (Frenzdorf, *et al., Science,* **171**, 391, 1971). In this way, one gets a great increase in production of b9. On the other hand, there are ways of suppressing the production of one of the allotypes; such suppression is a well documented phenomenon. I don't think that it is quite like the thalassemia situation, because with allotypes it is likely to be a problem of what stimulates these cells to divide.

COHN: I think that we now believe that the V-genes and the C-genes coding for each subunit are closely linked and that the V and C regions are expressed *cis* on the chromosome. Therefore, one of the two factors in the expression of a C gene is which V locus it is linked to. Mage has shown very clearly that there is a quantitative relationship between the concentration or level of a heavy chain when assayed by a constant region genetic marker which is *cis* to it. The V-locus

determining the specificities which can be recognized is one factor. The other factor determining the level is the switch. It is not known what factors, hormonal or others, determine to which class the IgM producing cell switches. When we say that a given heavy chain class, like IgG_3, is associated with V regions which are odd, or that it is present in a high or low concentration in serum, we have still to consider these two points.

CHAIRMAN WARNER: In another way, Cohn is asking whether all the other allotype associated effects are not also a result of V region immune response gene controls. The question, therefore, in terms of the IgG_3 levels in man, is whether the higher IgG_3 level in Gm5 homozygotes represents certain specific antibodies which are under this type of genetic control.

COHN: The level of IgG_3 in man could also be due to some hormonal effect or something else essentially nonimmunologic. The apparent association of IgG_3 level with a specific V region depends upon what chromosome one starts with. Since we have no V markers in man, we can't study this association. If a given specificity and a given function are related, e.g., resistance to certain helminths and IgE, then V-C selection due to the interaction of the two factors occurs.

CHAIRMAN WARNER: It should be stated, however, that it is not known whether the high responder individuals in man to a given antigen, such as flagellin, may not also be high responders to other antigenic determinants.

MORRIS: It should I believe, be pointed out that this so-called linkage between Gm allotype and response to flagellin, just referred to, is in fact, at best a very weak association. In the study by Wells, Fudenberg, and Mackay, a population of high and low responders to primary immunization with flagellin were selected from a very large group of people so immunized. The low responders had a low natural antibody titer (<100) while the high responders had a high natural antibody titer (>600) and gave a predominantly IgM good primary response ($>10{,}000$). Gm allotypes a, x, b, g, f were determined in these people and an association emerged between the Gm a, g genotype (combined homozygotes and heterozygotes) and the high responders, which was just significant at the 0.05 level. However, the application of a corrective factor for the number of comparisons made would render this value nonsignificant. Thus, this one possible example of an allotype-linked immune response in man must really be considered doubtful. Incidentally, we are looking at the HL-A profiles in this same group of people and, with approximately half of the group already studied, no association with responsiveness has thus far appeared.

CEPPELLINI: I think that Herzenberg's objection is not pertinent. The problem is that in those heterozygous 4/9 rabbits, the number of free molecules in the

serum and of cells in the lymphoid organs is not equal for the two allelic κ chains. The same is true for λ_3 chains in heterozygous Gm^b/Gm^g humans.

The question is whether this imbalance is due to the different primary structure of the two allelic polypeptides in the constant or in the variable part. How is this reflected in the differentiation of the immunocyte?

Cohn suggest another, not very dissimilar, explanation, i.e., that the probability of choice between the two allotypes depends on the variable genes which are linked in coupling with the constant part carrying the allotype. However, Mage has evidence for a low but significant recombination frequency between the Aa (in the variable part) and the Ad (in the constant part) markers of rabbit heavy chains. Bodmer will surely tell us that in the context of evolutionary time span, because of crossing over, there should emerge all possible combinations between alleles of the linked V and C genes. Thus, we cannot explain a stable characteristic of a C gene at the population level on the basis of properties of linked V genes. Still less can we explain the different levels of λ_1, λ_2, λ_3, and λ_4 subclasses which use the same V regions.

ALLISON: One of the ways to sort out that particular problem is to obtain rabbits from other sources and see how widely this particular phenomenon is found.

I should like to return to Herzenberg's point of allotype suppression. It may be that this type of active suppression is a good deal more common than we suppose. We have the example of the suppression of idiotype formation by foreign antibodies earlier in this session. There are other experiments on immunization of sheep with BGG where Spoener, in Edinburgh, has found that after a short course of immunization, good antibody responses are obtained. However, as one presses on with further immunization these antibodies disappear, again suggesting some kind of active control. This phenomenon deserves to be looked at more carefully, as it might affect differentially certain allotypes.

HERZENBERG: I would like to go back to the data Cohn presented earlier in this session, which I thought extremely interesting–I suppose all of us think so. But we must recognize again that associations are not proof, for example, of V and C linkage. If we consider Cohn's data, as presented, we might conclude that λ structural genes are very closely linked to the heavy chain constant region genes in mice. But it may well be that this is an association which is not due to genetic linkage, but rather to a determination of the kind of light λ chain which gets produced, and by what kinds of heavy chains V regions are favored in the immune response of these animals.

COHN: It should be noted that I didn't present *that* hypothesis, but it is valid given the data I presented. Herzenberg has keen vision and in spite of orders to

my secretary to make the protocols unreadable, he sees the details on them that other people miss.

I want to come back to this linkage of V to C in selecting heavy chains. It is possible that the Bodmer argument applies and that one would expect eventual mixing of the allelic V loci of the total population by recombination. However, many of these recombinants could be lethal. It might require a certain number of different functional V genes in the germ line, functional as determined by their ability to recognize specific antigens on pathogens of the kind that Krause discussed. If we lose these germ-line encoded activities, then we are dealing with an animal which is a nonresponder that is selected against. I simply don't understand the mouse lines Biozzi described for us. If we have a low responder to everything, and all of the responses are linked to allotype, we would be tempted to postulate that the V region is largely deleted, or that some other kind of scrambled V structure has appeared.

BENACERRAF: In reply to Cohn's comment on the Biozzi experiments, all that was shown to be linked to heavy chain allotypes in the Biozzi lines is the capacity to respond to SRBC, which happens to be the major antigen used in the selection.

I would like to address a question to Nisonoff. In his experiments where he managed to suppress the idiotype with antiidiotype antibody in the line immunized with the hapten conjugate, did he have any measurement of the affinity of the antibody produced by that particular suppressed line? One would predict that suppression of the preferential antibody which that line would normally make would result in production of antibody of lower affinity than normal.

There is a further comment that should be made; in the genetic system discussed in this session, the differences have been more qualitative than absolute and related to specificity of the antibody produced in one form or another, which is what would be expected in these allotype-linked Ir gene systems. However, I question whether the term responders and nonresponders are adequate to describe these differences—maybe "selective" responders and nonresponders would be more appropriate terminology.

NISONOFF: It would have taken too much time to go into all the details or data, but I would now say that if one makes an anti-idiotype antibody against mouse antihapten antibody, it does not react with 100% of the mouse antibody. A typical value for the antiphenylarsonate system in the A/J mouse would be 60 to 80%. I would then assume that in a suppressed mouse the other 20 or 40% rises and replaces the suppressed population. We don't have quantitative data as to whether the replacement is complete; if there is some suppression of antihapten antibody synthesis is not certain. In this regard we examined Ouchterlony plates prepared by using a heterologous carrier with the same hapten, to

ascertain whether in the suppressed mice the line was closer to the antigen well or the antibody well. We examined many such plates prepared with suppressed or control sera and could not discern much difference among the two groups. I therefore suspect that the overall suppression of antihapten antibody synthesis is small. If that unsuppressed population is merely a random population of molecules that happens not to have induced anti-idiotypic antibody, it might very well have an affinity roughly equivalent to the suppressed population.

COHN: Benacerraf awakens in me a desire to answer him, and I want to return now to the α 1,3 dextran response. There are high and low responders—if I used the term nonresponder, that was a mistake which I didn't intend. I really meant to refer to high and low. What is being said here is that one has high and low responders to dextran; BALB/c versus C57BL, and that in the F_1 cross the response is dominant. In addition, I can tell you that there is a structural difference between these two responses. Their heavy chains are different, they have different idiotypes.

If I hadn't told you anything about this system other than it was linked to H-2, you wouldn't be able to tell from the phenotype of the response what the linkage was like. It is not correct to say that in H-2 linked responses we are looking at high and low responders while in allotype-linked responders we are looking at structural differences or carbohydrates or special classes of antigen. In other words, given a particular antigen and knowledge of a high or a low response to it, we cannot *a priori* tell what that response will be linked to!

CHAIRMAN WARNER: At this stage we might proceed to the final general area of discussion. As this is the last session that will consider different types of immune response genes, the main point of this segment of our discussion might be to ask whether there are other immune response genes which may be expressed in other cell types, or at least if there is any evidence of non-H-2, nonallotype associated genetic control of the immune response. I think that Sela might lead off on this issue.

SELA: I would like first of all to orient us in a general way to the problem of genetic controls at the molecular level. I think that the first precise data obtained on genetic differences were with pauci-determinant immunogens, that is, immunogens which contain few determinants, and this was the reason the genetic differences were rather clear. At that time our working hypothesis was that genetic differences are not seen clearly with proteins, as these contain a mosaic of different determinants. Indeed, recent work showing genetic differences in proteins using low doses is nothing more than reducing a complicated multideterminant protein to one in which only one or a few determinants are immunopotent, and therefore brings us back to a pauci-determinant immunogen.

Evidence in this direction was really first obtained by Mozes *et al.*, (*J. Exp. Med.*, **130**, 1263, 1969), using the polymer (Phe,G)-Pro--L, in which they showed equal amounts of antibodies in different strains. However, the specificity of such antibodies was different, in one case being mainly directed against the peptide composed of phenylalanine and glutamic acid, while in the other it was mainly against polyproline.

I think that direct evidence for this phenomenon comes from our recent work on a unique region in a native protein. I would like to stress that it is a conformation-dependent determinant in hen egg white lysozyme, a molecule about which we happen to know a lot. Both the amino acid sequence and the three-dimensional structure are known. The region of interest to us is the "loop" which is still linked with a disulphide bridge and it is one of the most immunopotent regions of the molecule (Arnon *et al.*, *Proc. Natl. Acad. Sci.*, **68**, 1450, 1971). It is characteristic of these proteins for which the crystallographic structure has been solved, that it is usually the corner which has the most immunopotent determinants. When we inject lysozymes in a dozen different strains of mice, all of them produce antilysozyme antibodies. But if we take this loop region, either isolated from lysozyme or synthesized in the laboratory, and attach it to a synthetic polymer such as polyalanine or polyproline, some mouse strains make antibodies and some do not. (Mozes *et al.*, *J. Immunol.*, **106**, 862, 1971).

The question that arose was: In a strain making antilysozyme in response to lysozyme, but not making antiloop in response to synthetic loop-polymer conjugate, would this strain make antiloop when injected with lysozyme. As seen in Table 66, the answer obtained was in the negative. Thus, for the SJL strain, when we injected the loop attached to polyalanine, or the loop attached to polyproline, there was essentially no response in terms of reaction with lysozyme. In contrast, when we injected lysozyme into the SJL strain, there was a high response to it, but a very low response to the loop. SJL thus makes antibodies to other determinants of lysozyme but not to the loop determinant. In Table 66 we have a comparison between two strains of mice, one of them is a good responder to the loop determinant, independent of whether it is part of the native protein or the synthetic polymer. One can conclude that in this situation

TABLE 66

Immune Response of Intact SJL and DBA/1 Mice to the "Loop" Peptide of Lysozyme

Immunized with:	Lysozyme		Loop-A--L		Loop-Pro--L	
Assayed with:	Lysozyme	Loop	Lysozyme	(T,G)-A--L	Lysozyme	(T,G)-Pro--L
SJL strain	High	Low	Low	Low	Low	High
DBA/1 strain	High	High	High	Low	High	Low

we have genetic control at the level of unique determinant regions. In this particular case it is a conformation-dependent determinant, because if the disulfide bridge of the loop is opened, there is no longer any crossreaction.

This brings me to the original family of branched polymers with which McDevitt and I started working on the determinant-specific genetic control of immune response. The first three are so universally abbreviated that I believe most people don't even know how they look; I refer to (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L. In these polymers we kept the carrier constant, so it was worthwhile to change to another carrier, and we chose polyprolyine, and worked with two additional multichain polypeptides (T,G)-Pro--L and (Phe,G)-Pro--L, but there was still one member of the new family missing, and I refer to the one we could call (H,G)-Pro--L. This is the polymer in which peptides of L-histidine and L-glutamic acid are attached to multichain poly-L-proline. There were good chemical reasons why we couldn't make it earlier, but we have now solved that problem and we have succeeded in preparing it (Shaltiel *et al.*, *Israel J. Chem.*, in press).

It proved to be the most immunogenic of all the six polymers; on primary immunization it gives good agglutinin titers, in contrast to the others (Table 67). In Table 68 is shown the immune response in different strains; what I want to emphasize is that specificity to the (H,G) is definitely linked to the $H\text{-}2^k$ allele with a poor response in $H\text{-}2^b$ and $H\text{-}2^s$ mice (*Ir-1*). On the other hand, as is already known from studies with (Phe,G)-Pro--L, antipolyproline response is not linked to H-2 (this response is controlled by *Ir-3*). Moreover, in this case, we have some mouse strains, such as BALB/c or DBA/l and SWR, which yield good titers to the original molecule and develop very poor titers to either (H,G)-A--L or (T,G)-Pro--L, indicative of some additional complexity. This then is a good example of one molecule, one immunogen, with several determinants, the response to some of these determinants being linked to H-2 and the response to other determinants not linked to H-2.

I think we should distinguish between two general cases. On the one hand are polylysine conjugates in strain 13 guinea pigs, and polyalanine conjugates in SJL mice. These seem to be poor responders to any determinant attached to polylysine in the case of strain 13 guinea pigs, or to polyalanine in the case of SJL mice. So in this situation the genetic control is carrier-specific and not determinant-specific. On the other hand, in many of the studies discussed in the course of these three sessions, we considered determinant-specific genetic control. I think it is worth remembering that we face situations in which even within the same molecule there may be determinants with different linkages leading to different responses.

CHAIRMAN WARNER: May we have a point of clarification; is it specifically clear that the *Ir-3* gene is neither H-2 nor allotype-linked?

TABLE 67

Primary Response of Inbred Mouse Strains to Synthetic Antigens Built on Multichain Polyalanine and on Multichain Polyproline

Mouse strain	Serum titers after immunization with			
	(T,G)-A--L	(H,G)-A--L	(T,G)-Pro--L	(H,G)-Pro--L
C3H.SW	1:4	< 1:4	1:8	1:32
C3H/HeJ	1:4	1:16	1:4-1:8	> 1:128
AKR/Cu	< 1:4	1:32	1:8-1:16	> 1:128
DBA/1	1:4	< 1:4	1:4-1:8	1:32

TABLE 68

Immune Response of Inbred Mouse Strains to (H,G)-Pro--L After Secondary Immunization

Strain	H-2 type	Serum titer assayed with the indicated antigen		
		(H,G)-Pro--L	(H,G)-A--L	(T,G)-Pro--L
A/J	a	> 1/128	1/32	1/64-1/128
C57BL/6	b	1/16-1/32	0 -1/4	1/8 -1/16
C3H·SW		1/32-1/64	1/4-1/8	1/32
DBA/2	d	1/32-1/64	1/8-1/16	1/16
Balb/c		1/64-1/128	1/8	1/16
C3H/HeJ	k	> 1/128	1/64-1/128	1/8-1/16
AKR/Cu		> 1/128	1/128	1/16
DBA/1	q	1/64	1/8-1/16	1/4-1/8
SWR		1/128	1/8-1/16	1/32
SJL	s	1/64	0-1/8	1/32-1/64

SELA: I think it is clear that it is not H-2-linked. For (T,G)-Pro--L, it was also shown that it is not linked to allotype (Mozes *et al.*, *J. Exp. Med.*, **130**, 493, 1969), but I think the possibility of its being linked to allotype should be re-examined.

COHN: Upon scanning the complex genetic data that have been presented on responsiveness to H-2.2, erythrocyte antigens, and (Phe,G)-Pro--L, it appears to me that a two-factor cross would be the preferred means to fit the data. The manner in which the data have been analyzed and the way they have been presented make it more difficult to ascertain when we *don't* have linkage to allotype, because it is being obscured by the distribution of the other factor. In the case of (T,G)-Pro--L, very probably the response to the T,G portion is H-2 linked, whereas the response to the Pro--L could be linked to C_H allotype. I am

not convinced that it is other than linked to allotype. In any case, it would be helpful to have identified for us the strains used by Mozes, Jaton, Sela, and McDevitt.

McDEVITT: The experiment was done with DBA/l and SJL, using (T,G)-Pro--L and (Phe,G)-Pro--L, looking for an association between H-2 type and immunoglobulin allotype and the ability to produce anti-(Phe,G) or anti-(Pro--L) antibodies. Cohn has a point in that there may be a hidden partial association with allotype, but the data as they stand show that with (Phe,G)-Pro--L the immune response to (Phe,G) is H-2 linked, but the ability to make anti-Pro--Lys antibody is not H-2 linked and is not allotype-linked. I think Shearer has some data on how to circumvent the *Ir-3* (antipolyproline) defect that makes it unlikely that it is either an allotype-linked or an H-2 linked response.

SHEARER: I would like, first of all, to make a statement about the *Ir-3* gene concerning the genetic data that was published a few years ago by Mozes, McDevitt, Jaton, and Seal. At that time there appeared to be some complicating factors in the genetic analysis of the (DBA/l × SJL) F_1 × SJL back-crosses, in that they found that the antibody responses of these animals segregated as intermediate and low responders. If one were to assume that a simple Mendelian dominant genetic phenomenon operated here, one would have expected to find the back-crosses to have been high and intermediate.

From those earlier data it was postulated that there were complicating factors involved in the expression of these genes. After reviewing the experiments that had been done, Mozes realized that the back-cross mice that were used in those studies were *old* animals. She has now repeated those genetic experiments using *young* mice, and as shown in Fig. 45, she finds that the (DBA/l × SJL) F_1 × SJL back-cross responses segregate as high and intermediate responders. Although these data are not yet published there is now evidence to indicate that complicating genetic factors do not exist for this particular *Ir-3* gene and that it appears to be a single gene.

The other point that I want to make is that we have been interested in the cellular analysis of the *Ir-3* response, and the limiting dilution data I gave in Session I was compatible with one of two hypotheses: Either the immune response gene is expressed in B cells or is expressed at the level of cell-to-cell interaction. In studies that we carried out in collaboration with Braun, poly A:U treatment of DBA/l low responder animals restored their immune response to (T,G)-Pro--L all the way to the level of the high responder strain. Similar treatment of the SJL high responder had no effect. The same sort of phenomenon, i.e., transformation of low responders to high responders, can also be achieved by injecting syngeneic DBA/l low responder peritoneal exudate cells or macrophages. In my opinion, the macrophage and the poly A:U effects are likely to be

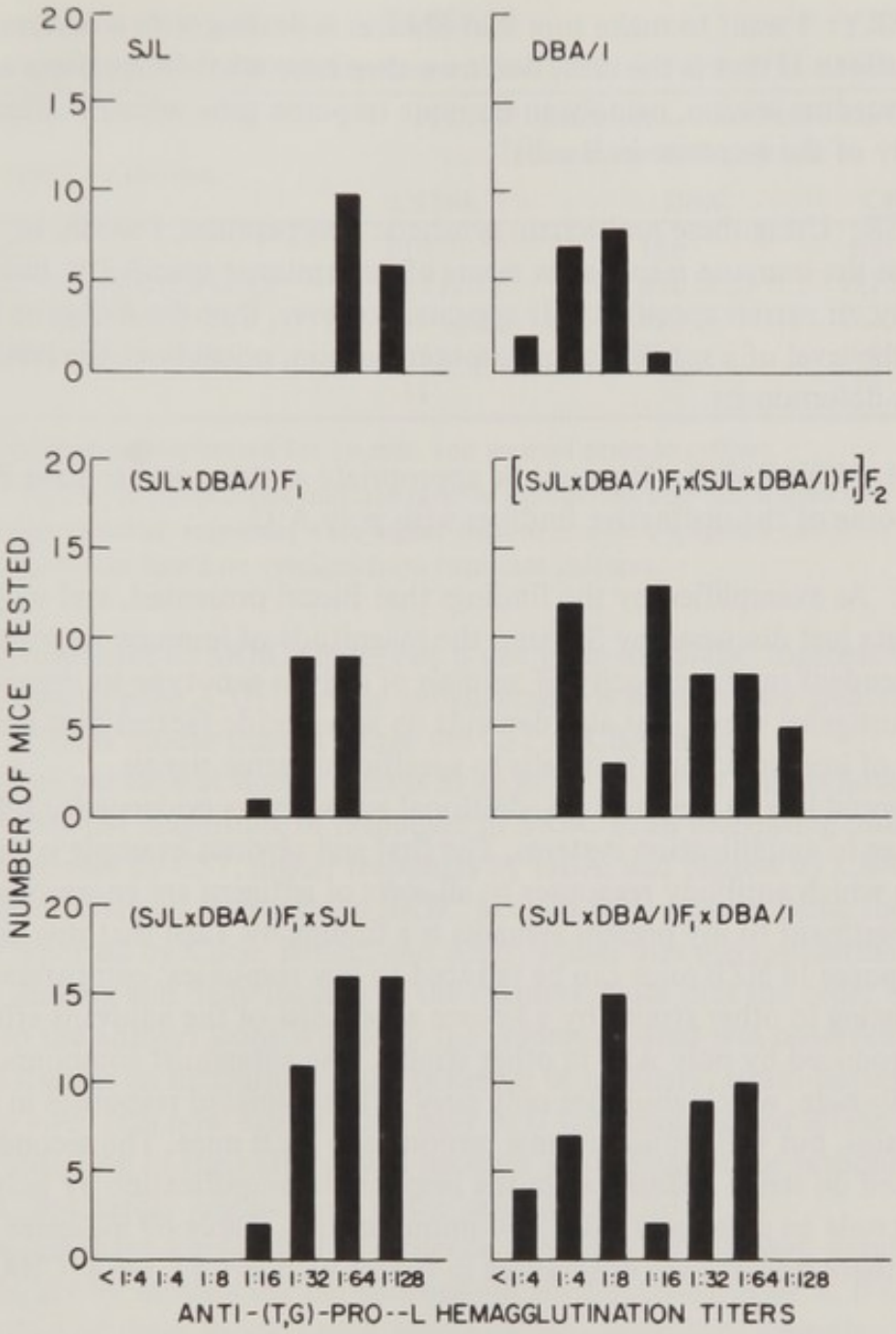


Fig. 45. Anti-(T,G)-Pro--L hemagglutination titers as a function of the number of mice tested for the parental, F_1 F_2, and backcross responses.

nonspecific. Therefore, our present interpretation of the cellular analysis of this genetic defect is that *Ir-3* is an example of a defect in cell-to-cell interaction. There may very well be sufficient B cells present in the low responder DBA/1 mice, but for some unknown reason these B cells are not triggered by the normal method of immunization. Thus, stimulatory factors such as macrophages, poly A:U, or other nonspecific agents may be needed to activate the low-responder B cells.

RAJEWSKY: I want to make sure that Shearer is dealing with a determinant-specific effect. If that is the case, don't we then have what Herzenberg asked for in the preceding session, namely an immune response gene which is affecting the specificity of the response in B cells?

SHEARER: Using these multichain synthetic polypeptides, I would be reluctant to express the immune response in terms of determinant specificity, hapten specificity, or carrier specificity. It appears, however, that the *Ir-3* gene is operating at the level of a specific immunopotent region, possibly at the level of an antigenic determinant.

CHAIRMAN WARNER: It would be appropriate at this point to have Braun present some of the distinctive findings with poly A:U.

BRAUN: As exemplified by the findings that Biozzi presented, and suggested by the data just discussed by Shearer, the magnitude of immune responses is not only dependent on the capacity of animals of a given genotype to respond to a specific antigenic signal, but also depends on nonspecific factors that amplify the response of immunocompetent cells to specific antigenic signals.

I would like to present two additional examples as evidence for inherent differences in amplification systems. The first and obvious example is the NZB mouse in which antibody responses to all sorts of antigens are enormously high. What is pertinent to my present remarks is a finding by Talal that this high antibody response in NZB mice can be reduced to low responses, comparable to those existing in other strains by a known antagonist of the adjuvant effects that can be produced by poly A:U in other strains. The antagonist compound is kinetin riboside, which abolishes only poly A:U stimulated responses in most other strains, but will reduce normal responses in NZB mice. The second example is based on strain differences in the response to amplification by poly A:U, using as crude an antigen as SRBC for immunization. Table 69 indicates that not only are there well-known differences in strains such as C57, DBA, CBA, and in

TABLE 69

Effects of Poly A:U on Antibody Responses to SRBC *in Vivo*

Treatment of spleen donors	PFC/10^6 spleen cells, 48 hours after immunization of:		
	C57BL	DBA	CBA
SRBC(10^8)	188 ± 27	368 ± 80	793 ± 50
SRBC + poly A:U (300 γ)	569 ± 74 (3.0)[a]	3745 ± 179 (10.2)[a]	3223 ± 435 (4.1)[a]
None	5 ± 2	10 ± 0.1	138 ± 4

[a] Ratio of stimulated/nonstimulated.

5 animals per group.

TABLE 70

Effects of Poly A:U on Antibody Responses to SRBC *in Vitro* (EHAA Medium)

Added to cultures	PFC/10^6 spleen cells, 4 days after start of cultures with cells of:		
	C57BL	DBA	CBA
SRBC	319	166	63
SRBC + poly A:U (10γ)[a]	342 (*1.1*)[b]	370 (*2.2*)	117 (*1.9*)
SRBC + poly A:U (1γ)	375 (*1.2*)	167	122 (*2.0*)
SRBC + poly A:U (0.1γ)	350 (*1.1*)	162	64
–	11	7	12

[a]Cells were pretreated for 10 min. and washed prior to culture.

[b]Figures in parentheses indicate ratio of stimulated/non-stimulated in cultures in which responses were higher than in non-A:U-exposed cultures. All figures are based on averages from triplicate cultures.

regard to responses to SRBC alone, but if one gives the SRBC together with immunoenhancing poly A:U, then the enhancement is significantly greater in the case of the DBA mouse than in either the C57 or CBA mouse.

Before we look at similar effects in an *in vitro* system, please note that Table 69 lists the magnitude of responses to SRBC in an interesting order: lowest responses by C57, higher responses by DBA, and highest by CBA.

Table 70 shows responses to SRBC in an *in vitro* system, using the new medium reported by Click, Benck, and Alter, which uses mercaptoethanol and obviates shaking and daily feeding of the cultures. Note that the order of responses to the antigen alone is exactly the reverse of what was observed *in vivo*, which in itself is an indication of the presence of auxiliary, strain-dependent factors *in vivo*. It is now evident that poly A:U treatment of the spleen cells prior to initiation of the cultures will produce a doubling of the response in DBA and CBA, but hardly any increase in the response in C57.

We now have a great deal of evidence that poly A:U, as well as many other adjuvants, act by influencing the endogenous level of cyclic AMP in B cells. At the very first of these Brook Lodge immunology conferences a number of years ago, Cohn predicted that in order to trigger a detectable activation of B cells, separate signals to relevant B cells would be required. As indicated in Fig. 46, experimental data now indicate that B cell activation does indeed require two signals: a specific antigenic signal, and a second signal to the receptor site for the activation of a cyclic AMP-dependent amplification system. Either of these signals *alone* gives no detectable activation, but *both* signals give full activation. Recent studies, such as those by Katz and Benacerraf, Kreth and Williamson, and our own studies with Matsumoto, have indicated that T-B cell cooperation in antibody formation may be a natural process for the activation of the cyclic AMP-dependent amplification system. This conclusion is supported by the find-

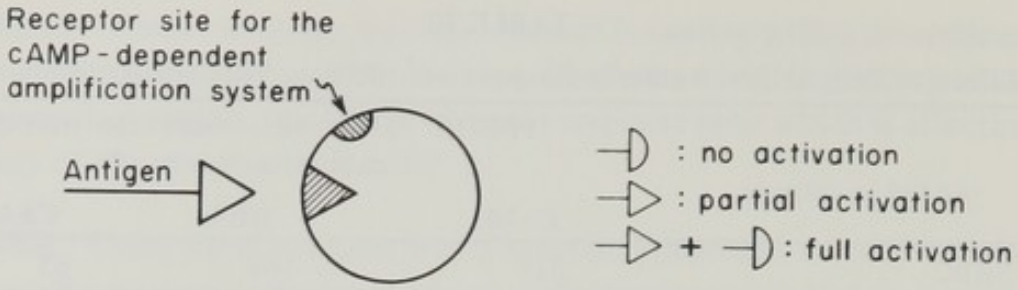


Fig. 46. Presumed steps in the triggering of detectable B cell functions.

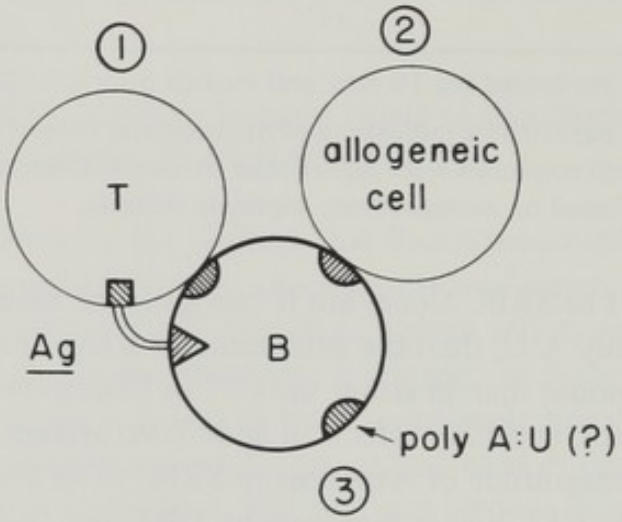


Fig. 47. Three alternative mechanisms for activating the cAMP-dependent amplification system of antigen-exposed B cells.

ings summarized in Fig. 47; both *in vivo* and *in vitro*, in the absence of a sufficient number of T cells (i.e., after thymectomy or anti-θ + C′ treatment), one can substitute for the usual T cell requirement to obtain responses to T cell-dependent antigens, either allogeneic normal spleen cells or poly A:U. Either substitute will lead to an effective "turning on" of B cells. We know now that these two substitutes operate via an elevation of endogenous cyclic AMP levels; this has been measured directly. This apparent dependence of amplification on the cAMP system raises the question of whether strain differences in general immunoresponsiveness might be associated with strain differences in the dynamics of the cyclic AMP system. As Fig. 48 indicates, the available data suggest that this may be so (Shiozawa and Braun, unpublished data). We compared the activity of adenyl cyclase, the enzyme responsible for the formation of cyclic AMP from ATP, in spleen cell populations of C57 and CBA following exposure to PHA which is known to lead to enhanced adenyl cyclase activity (Winchurch and Braun, *J. Immun.*, **106**, 1399, 1971). The pattern of response is very different in CBA and C57. In CBA cells, 30 minutes after PHA exposure, the level of adenyl cyclase activity is still increasing, and subsequent tests showed that it does reach

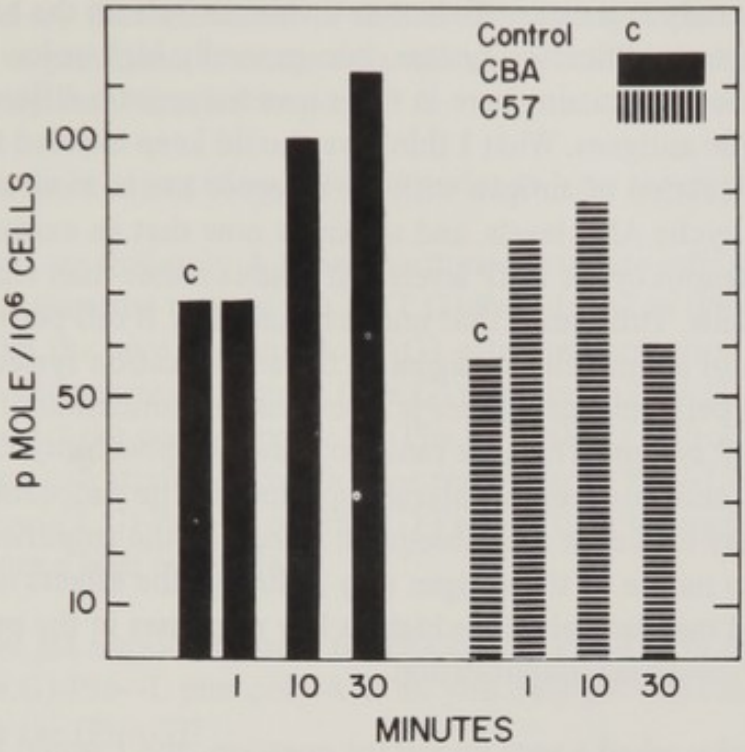


Fig. 48. Changes in endogenous cAMP levels of PHA-exposed normal spleen cells from C57BL and CBA mice. Cyclic AMP levels were determined in spleen cell suspensions (2×10^7 per culture) by the procedures of Gilman (*Proc. Nat. Acad. Sci.*, 67, 305, 1970).

its peak in 60 minutes. In contrast, in C57 there is a rapid early increase, detectable one minute after PHA exposure, and adenyl cyclase activity reaches its maximum at ten minutes and then declines. We must note that what is occurring here is the function of a membrane-associated enzyme, and therefore, what we have in terms of strain differences in the dynamics of the cAMP system may reflect differences in the composition of the membranes of the two strains. The results shown in Fig. 48 have been obtained in repeated experiments and suggest that any consideration of the genetic control of immune responsiveness, also should include appropriate consideration of genetic differences in host responses to nonspecific modifiers of the immune responses, which in the examples that I have cited has focused on the regulatory effects of the cyclic AMP system.

OLDSTONE: Did I understand Braun correctly, that he could, in effect divide all mouse strains specifically into either high or low responders assuming that there are different amounts of cyclic AMP receptors or genetically controlled release of cyclic AMP? I certainly disagree with Braun that qualitatively New Zealand mice are hyperresponders. The point is that there are certain antigens to which they can respond well and other antigens to which they respond poorly.

BRAUN: Obviously it is not sufficient to divide animals on the basis of inherent differences in the amplification system, into generally high or low responders; we have, after all, been discussing here in three sessions genetic differences in response to specific antigens. What I think we should keep in mind is the probability that the interaction of antigen with the receptor site in a cell may *also* modify endogenous cyclic AMP levels, and we know now that an excessive stimulation of endogenous cyclic AMP levels will inhibit rather than stimulate the function of B cells. This means that one may have low B cell performance either in the presence of an insufficient signal to the amplification system, or one can have low B cell performance if there is an excessive stimulation of the endogenous cyclic AMP system. Thus, we must consider the possibility that whatever the first signal (antigen-receptor interaction) does to the endogenous cyclic AMP system may influence the subsequent effects of the amplification system; accordingly, the nature of the antigen may influence the effects of the amplification system and may lead either to high or low responses in the presence of a uniform cAMP-mediated amplification.

COHN: Rajewsky asked a very important question, and I would like to come back to it since I believe that we are dealing now with nonspecific effects exerted by poly A:U. We were discussing the response to (Phe,G)-Pro--L, a response believed to have two controlling genes, an *Ir-1* gene and an *Ir-3* gene. Poly A:U has been said to substitute for or enhance this response. Now we would like to know if it is determinant-specific? Does it enhance the (Phe,G) response, and in the SJL mouse, do Shearer or Sela have the reciprocal control?

SHEARER: The question put by Cohn is indeed important. The original experiments and the ones that have been most completely analyzed were done using (T,G)-Pro--L. We have tried to enhance the low response of SJL mice to the (Phe,G)-specific immunopotent region of (Phe,G)-Pro--L by poly A:U, but this proved unsuccessful as were attempts to enhance the (Phe,G) response which is *Ir-1* and H-2 linked.

BRAUN: There are several ways in which one may account for a lack of response to poly A:U. First of all it could mean that the relevant cells are absent, in which case a stimulator could not accomplish anything. However, another possibility is suggested by the fact I referred to earlier; one can have low B cell performance in the presence of an excess of stimulation of the amplification system. In such a situation, of course, any further stimulation would not alter anything. If the latter is true, it would be interesting to see whether the antagonist kinetin riboside would restore a high response in the low responders that fail to respond to poly A:U.

SELA: I would say that whichever of these various antigens one uses, it is clear that poly A:U or peritoneal exudate cells will not touch the *Ir-1* response, but will correct completely the *Ir-3* response.

COHN: That is an interpretation. I would prefer to have the data.

SELA: The data are that poly A:U and peritoneal exudate cells correct completely the poor response to poly Pro in (T,G)-Pro--L and in (Phe,G)-Pro--L, but do not affect the poor response to (T,G), after immunization with (T,G)-Pro--L, nor do they correct the poor (Phe,G) response in (Phe,G)-Pro--L. If we try to correct the poor response to (Phe,G) in (Phe,G)-Pro--L, we can not, but if we go to a strain which is a poor responder to Pro--L irrespective of whether we inject (T,G)-Pro--L or (Phe,G)-Pro--L, then we correct completely and it becomes a high responder.

COHN: Do you get a good response to (Phe,G) and Pro--L? In the strain where you inject (Phe,G)-Pro--L plus poly A:U do you only get response to poly Pro--L and not the (Phe,G)?

SELA: That is correct.

SHEARER: We obtain no further enhancement to (Phe,G) because the strain which responds poorly to Pro--L is a genetic high responder to (Phe,G).

COHN: That is what I thought.

SHEARER: The poly A:U effect is seen only for the antibody specificity controlled by the *Ir-3* gene. If we switch to immunogenic macromolecules such as (Phe,G)-A--L or (T,G)-A--L, which involve the *Ir-1*, H-2 linked system, we are unable to correct the low response situation.

CHAIRMAN WARNER: Merchant will now present data which are distinctive in that they deal with the genetic implications of antibody responses in unprimed animals, a background situation.

MERCHANT: I would like to share with you some unusual B cell information which represents cooperative efforts with Inman and Claflin. It is in large part, the product of new technical developments that involve the enlargement and extension of haptenic determinants employed in hemolytic plaque assays.

We have used a series of 14 different haptenic determinants each coupled at the distal end of either one or two different tripeptides, *N*-acetyl-tyrosyl-glycylglycine or β-alanylglyclglycine, both of which couple almost exclusively to lysine residues on indicator red cells, giving us in effect a series of 14 different

hapten tetrapeptide determinants. The haptenic groups on the distal end of such enlarged determinants are mostly azo-phenyl hapten analogues, for example, azobenzene-arsonate, azobenzene-sulfonate, and azobenzoate. Also included in our series are derivatives of the DNP, TNP, and dansyl groups coupled through β-alanylglycylglycyllysine. We employed large haptens of dimensions that approximated the estimated size of the average antibody combining site.

A major by-product of utilizing such large haptens is that we attained profound increases in the sensitivity of hemolytic plaque techniques. As compared with immunizing with a small hapten-azoprotein and plaquing with the same small hapten, we found that assay of the same immune cell suspension with the corresponding large hapten usually results in a five- to ten-fold increase in PFC, a marked increase in the sensitivity of our assay system. This increased sensitivity is also manifested, for example, by the fact that use of the DNP tetrapeptide hapten of our series regularly allows us to detect 90% of MOPC 315 tumor cells as plaque formers.

We immunized inbred animals with one of these large haptens, and then assayed the cells with each of the 14 haptens, the data enabled us to construct a 14-component profile of hapten-specific PFC for each individual mouse. Initially we found notable similarities in the plaque profiles from one immunized inbred mouse to the next. However, animals of the same strain, sex, and age given identical immunization but kept in different quarters showed marked profile differences which were eventually attributed to environmental differences. Accordingly, our studies shifted to germ-free mice; and since we enjoy a much increased level of sensitivity, the studies so far have not required immunization of germ-free mice, but have simply involved the screening of natural or innate "background" IgM plaque activity in these mice on an individual mouse basis.

We also established in specific pathogen-free nude (athymic) mice versus their normal heterozygous litter mates, that the natural background plaque activities of these nude mice equaled or exceeded the activities in their litter mates for each of the 14 test hapten specificities. Thus the hapten-specific background IgM secretion under study is considered a thymus-independent phenomenon. Figure 49 shows the background level of hapten-specific PFC for each of our 14 different haptens in each of three individual BALB/c male mice, and in a pool of three additional BALB/c mice. It can be seen that the similarities in these hapten-specific IgM background profiles are rather remarkable. Arbitrary code letters are used to identify individual haptens. These are identified in Table 71. Since it was possible that such profile similarities might well stem from stimulation by common antigens in the diet or bedding to which all these germ-free mice were exposed, we determined the background profiles in germ-free *outbred* Swiss mice, given the same sterile diet and kept on the same bedding. Figure 50 shows the profound profile differences which existed between indi-

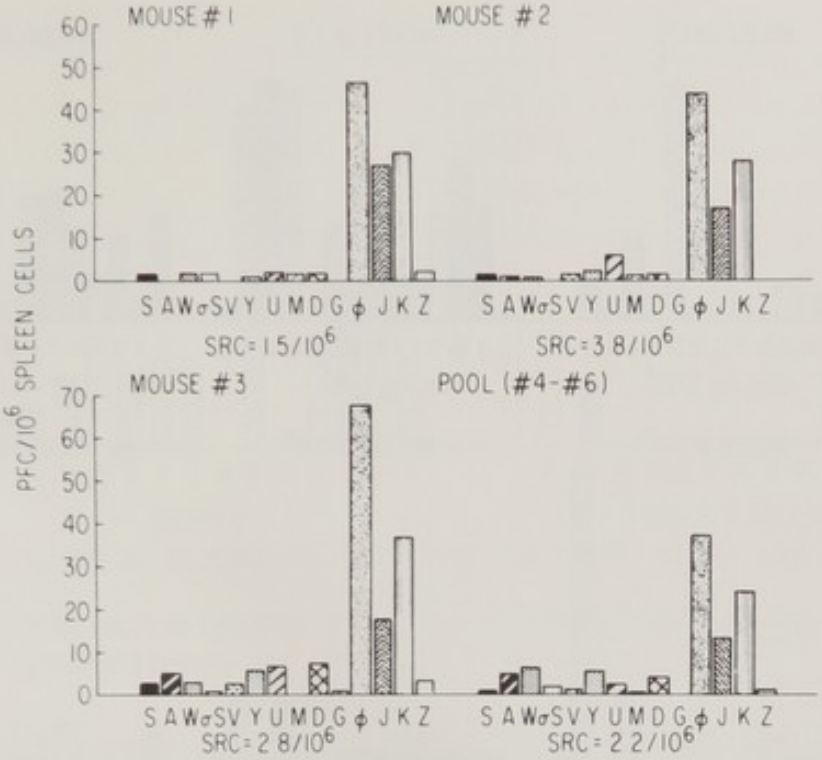


Fig. 49. IgM Hx-TGG profiles in untreated germfree ♂ BALB/c mice. Experiments were performed on animals seven weeks of age.

TABLE 71

Progressive Appearance of Hapten Reactive Direct PFC in Pooled Lymphoid Tissues of Normal Unimmunized Fetal Mice

Indicator Erythrocytes		PFC/10^7 Nucleated Fetal Cells							
		NZB fetuses			BALB/c fetuses				NIH fetuses
Days of Gestation →		19	20	21	19	19	20	21	20
SRBC controls	(P)	8	3	26	7	11	13	16	6
SRBC derivitized with									
p-Azobenzensulfonate TGG	(S)						7		6
p-Azobenzenearsonate TGG	(A)							6	2
m-Azobenzenesulfonate TGG	(W)				1	1	1	1	2
o-Azobenzenesulfonate TGG	(O)							10	
p-Azobenzoate TGG	(V)								
p-Benzoylphenylazo TGG	(Y)			9					8
p-Bromophenylazo TGG	(U)							26	2
p-Azophenyltrimethyl ammonium TGG	(M)			6					
Dansyl-AGG	(D)	2	2	15			8	63	2
TGG	(G)								
phenylazo TGG	(Ø)		3	40					2
Dinitrophenyl AGG	(J)						48	7	3
Trinitrophenyl AGG	(K)		2	2					
Benzyloxycarbonyl AGG	(Z)			13					

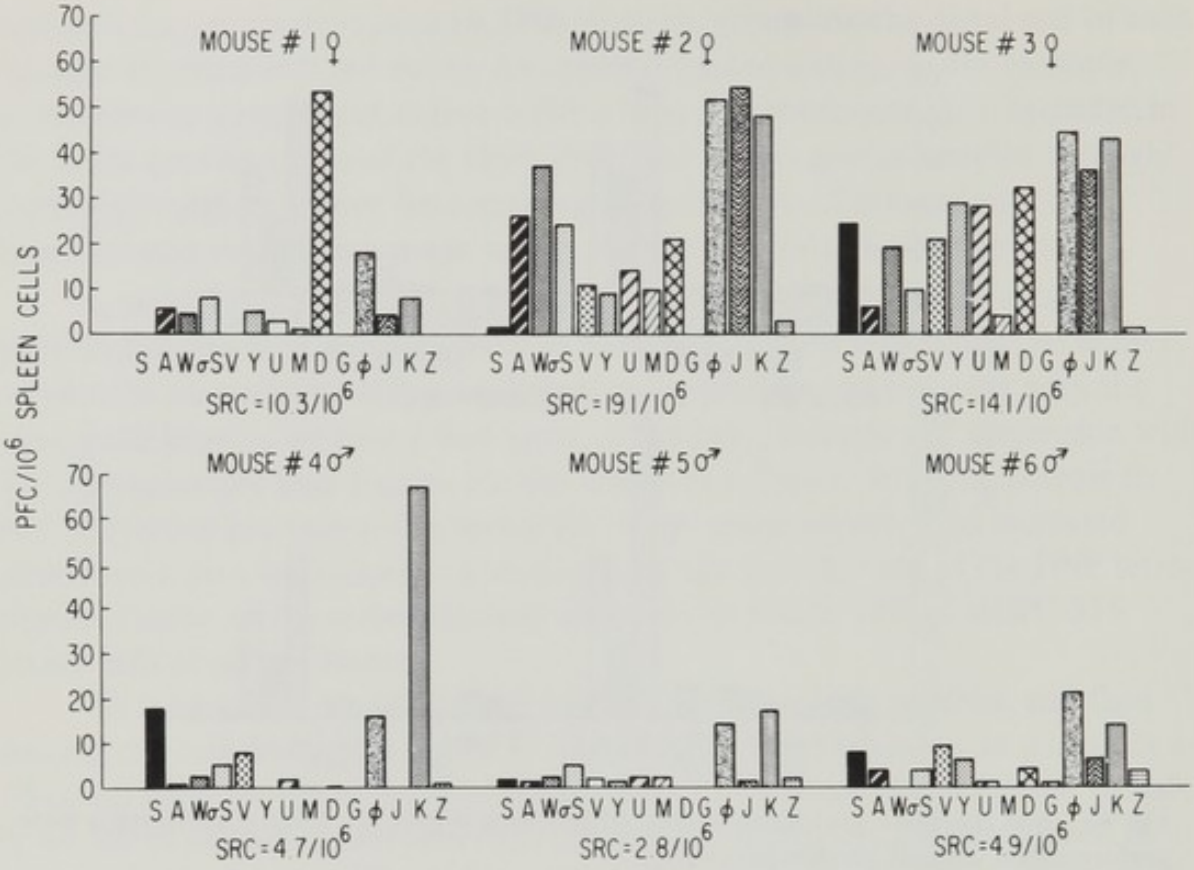


Fig. 50. IgM Hx-TGG profiles in untreated germfree random bred ♂ and ♀ (NIH Swiss) mice. Experiments were performed on animals seven weeks of age.

vidual *outbred* mice. Moreover the absolute numbers of hapten-specific background PFC in germ-free mice were always substantially higher, usually on the order of two- to five-fold higher, than for conventional mice of the same age, sex, and strain. Accordingly, this raised the possibility that some of these activities might actually be spontaneous.

It is well nigh impossible to approach a truly antigen-free environment short of a "return to the womb." Therefore, we sought spontaneous IgM secreting cells in unimmunized fetal mice. In working with fetal mice, we have taken a great many precautions to exclude the possibility that the effects we are measuring were due to transfer across the placenta of antigens or maternal antibody or maternal cells.

Figure 51 shows the levels of PFC in a gravid BALB/c female and in the pooled lymphoid tissues of her litter of fetuses. One typical finding to be stressed is that the most important IgM specificity in the female, K (which represents activity against TNP-AGG) was not detectable in the pool of fetal lymphoid tissues derived from that gravid female. Thus, TNP-AGG reactive maternal IgM antibody and/or cells appeared not to affect TNP-AGG activity in her fetuses.

Table 72 summarizes the data from seven separate fetal experiments. First, it is emphasized that we have never observed *any* background PFC in fetal tissues

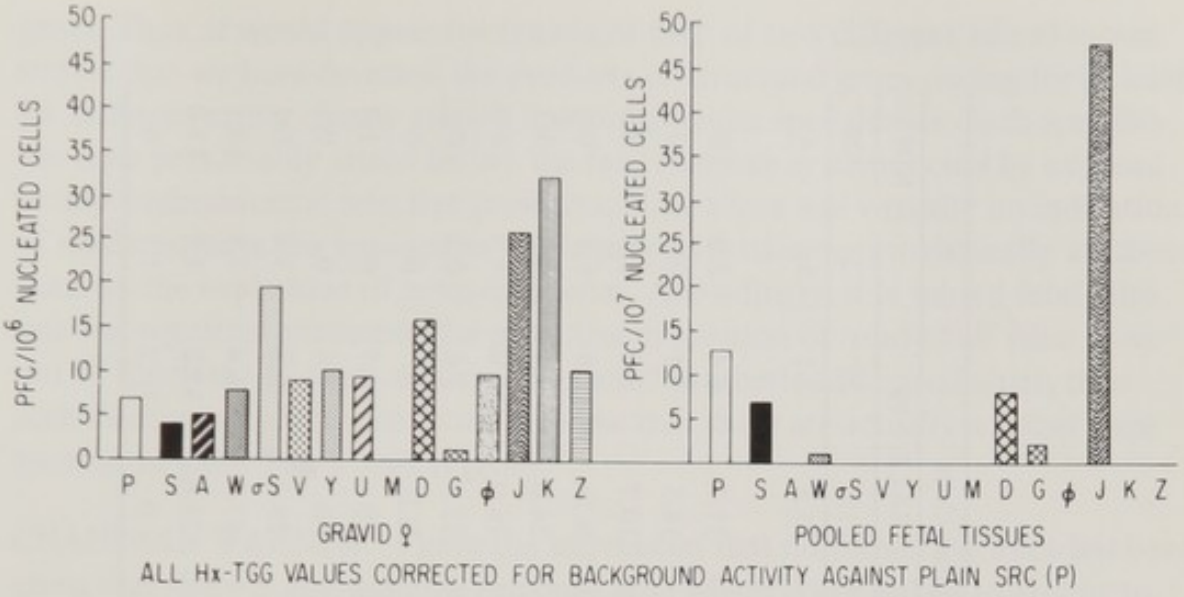


Fig. 51. Hx-TGG reactive IgM PFC in normal gravid BALB/c at 20 days gestation and in pooled fetal lymphoid tissues.

at 17 or 18 days gestation. However, when we observed a certain specificity at 19 days, we again regularly observed the same specificity in separate experiments with fetuses of the same strain on subsequent days. Thus, when a specificity arose either in NZB or in BALB/c mice, it would then regularly be observed in successive experiments with more mature fetuses of the same strain. Another major point is that of the six hapten specificities detected in NZB fetuses on day 21 (the final day of gestation) there was only one overlap (which was the dansyl AGG specificity) with six specificities represented in BALB/c fetal mice on their final day of gestation. Finally, there were a larger number (eight) of specificities detected on the penultimate day of gestation in a pool of outbred mice, and these specificities broadly overlapped those observed in each of the two inbred strains.

Table 72 gives control data for Fig. 51, providing simultaneously determined profile values for maternal spleens in the same seven experiments. Comparison of Table 72 with the data in Fig. 51 reveals a lack of correlation both in magnitude and in specificity of most maternal-fetal PFC values. Maternal IgM-PFC status then, was not reflected simultaneously in fetal IgM-PFC.

The major point I would make is that the regular correlation in separate repeated experiments of certain IgM specificities with certain inbred strains of adult and fetal mice suggests genetic control. Although the observed specificity differences between BALB/c and NZB mice may in part reflect differences in the function of strain-specific regulatory genes, it is emphasized that we have been following a thymic-independent process and that the observations which have been made were made directly on Ig gene products coded for by structural

TABLE 72

Direct Hx TGG PFC Activities in Gravid ♀ Mice

Indicator Erythrocytes		PFC/10^6 Spleen Cells							
		NZB ♀♀			BALB/c ♀♀				NIH ♀♀
Days of Gestation →		19	20	21	19	19	20	21	20
SRBC controls	(P)	8.2	15.9	18.5	3.4	35.5	7.1	5.5	4
SRBC derivitized with									
p-Azobenzensulfonate TGG	(S)	0.4	22.2	0	0	31.7	4.4	2.8	0
p-Azobenzenearsonate TGG	(A)	0	14.0	0	0	14.5	5.1	0	0
m-Azobenzenesulfonate TGG	(W)	5.6	29.4	0	2.1	10.7	8.0	2.8	2.5
o-Azobenzenesulfonate TGG	(O)	10	23.6	0	2.4	N.D.	19.6	6.4	0
p-Azobenzoate TGG	(V)	4.5	11.6	8.8	3.8	24.0	9.1	3.6	3.1
p-Benzoylphenylazo TGG	(Y)	9.3	11.3	0	2.4	26.0	10.0	4.0	4.0
p-Bromophenylazo TGG	(U)	3.3	21.7	0	1.8	35.5	9.6	0.2	0
p-Azophenyltrimethyl ammonium TGG	(M)	0	9.2	0	0	N.D.	0	0	0
Dansyl-AGG	(D)	16.5	36.6	5.9	1.1	0	15.7	0	1.8
TGG	(G)	0	16.0	0	0	0	1.4	0	0
Phenylazo TGG	(Ø)	18.7	47.2	7.3	5.5	29.3	9.5	3.4	0.4
Dinitrophenyl AGG	(J)	44.9	113.2	33.7	9.8	47.4	26.4	7.9	18.1
Trinitrophenyl AGG	(K)	35.6	118.1	53.7	12.9	23.6	32.2	4.5	17.0
Benzyloxycarbonyl AGG	(Z)	0	18.0	0	0	0	10.1	1.1	0

genes. Thus, it would appear for fetuses of each of two different inbred mouse strains that we have detected the products of structural genes coding for at least six different rather closely related immunoglobulin specificities. Such specificities have presumably arisen *before* the fetal immune systems could be exposed to any environmental selective pressures. Since there was virtually no indication of randomness in the appearance of these specificities, we provisionally attribute them to the expression of germ-line genes. Accordingly, if in inbred fetal mice one can regularly encounter the germ-line expression of nearly half (that is six out of fourteen) of one's arbitrarily selected synthetic test specificities, then such findings are consonant with the view that there are actually a rather large number of germ-line V region genes.

CHAIRMAN WARNER: I have the impression that no formal evidence has been given that there is any immune response gene which is not linked to H-2 or to immunoglobulin allotype. Is this correct? Nor have we, in fact, seen any formal evidence that there is an immune response gene operating other than in the T or B cells. Are there comments on these points?

LEVINE: Vaz and I have studied a system of genetic control of immune response which is not correlated with H-2 and furthermore appears not to be correlated with allotype. This is the immune response to reagin in mice.

We have one mouse strain which is very well worked up with many antigens which makes IgG antibody in good amounts, but makes little or no reagin. Our experiments have compared IgG_1 and reagin. The differences in reagin titers are 1:640 in the good responding strain as compared to 1:10 or less in the low responding strain, so the differential is considerable. There are no consistant differences in IgG_1 antibody titers.

We have tested another *H-2*^{s} strain, A.SW, which is a good reagin producer. In addition, in breeding experiments we have done back-crosses to the SJL recipient, where one can then segregate out good reagin producers, poor reagin producers (similar to the two parents) and a new intermediate reagin-producing group. These are not correlated with the *H-2*^{s} allele by typing of the red cells for "s" antigen.

This system is quite different from other systems described previously in that it is not antigen-specific. It applies to a variety of antigens (we tested about eight or nine) and it seems to be unique for a single immunoglobulin class—reagin, an IgE immunoglobulin.

CHAIRMAN WARNER: Does Levine have any data on allotype?

LEVINE: Thus far we have thoroughly worked up only the SJL strain. We have several other strains that appear also to be poor reagin producers, but we have

only studied them with three or four antigens; they are BUB, SWR, and the ST/b. I believe these have different heavy chain allotypes. In contrast the very good reagin responding strains are DBA/l, C57BL, A/He. I believe these strains represent different allotypes.

CHAIRMAN WARNER: Different allotypes are indeed represented *within* Levine's reagin responding and nonresponding groups of strains, which would indicate a *lack* of association of this reagin type of immune response with heavy chain allotype.

RAJEWSKY: I make reference to Chairman Warner's earlier statement that to date date only the H-2 linked and allotype-linked immune response genes are clearly defined. I thought that *Ir-3* was an immune response gene which is *neither* linked to H-2 nor to allotype. *Ir-3* would therefore represent a gene which affects the specificity of the immune response of B cells without itself being the receptor for antigens.

CHAIRMAN WARNER: I had thought *Ir-3* might be our closest example of this, but there seems some doubt raised about the allotype linkage.

McDEVITT: Regarding the objections that Cohn raises—the data are clear in the experiments that were done—there was no linkage to allotype. If he wants to raise the question as to whether there are multiple factors; experiments have to be done in congenic strains. That would be a fair objection, but the present data, as far as they go, indicate that there is no linkage of the immune response to (T,G)-Pro--L to either allotype or to H-2.

HERZENBERG: For the record, there is another example published by Gasser which should be identified.

SHREFFLER: The *Ir-2* locus was originally defined by Gasser in a cross between YBR and BALB/c. In that cross, response to Ea-l antigen was due to a single genetic factor which is linked to Agouti as well as H-3 and H-6 in the fifth linkage group. In a second cross between YBR, which is a responder, and C57BL, which is a nonresponder, however, the data indicated two or more genetic loci. Subsequent analyses indicated that one of the two genes was associated with H-2. The second gene has not yet been identified. It is not clear whether that second gene is *Ir-2*, in the fifth linkage group, or some new gene.

McDEVITT: The way Gasser originally did the study, it appeared to be linked to H-3 and H-6. He then went back and did a different cross, and showed that there are two genes that are controlling the response to Ea-l, the erythrocyte antigen on mouse red cells analogous to the ABO system in man. One of them is

linked to H-2 and the other to H-3 and H-6. So it is fair to say that *Ir-2* should be assigned to one or the other of these loci. To the best of my knowledge Gasser has not yet done that.

CHAIRMAN WARNER: One would hesitate to attempt a comprehensive summary of this intensive session. However, I would say that at this stage of our discussions we have at least two clearly documented types of immune response genes. One type, H-2-linked is operating in T cells, and it is still not certain whether it is also operating in B cells. At this session we had several examples that formally documented the second distinct type of immune response gene which is operating in B cells and is linked to Ig allotype. It is assumed to be associated with V-region genes. The contrasts in the nature of the antigens used to detect each type of gene, their distinctive characteristics, and the striking symmetry of the expression of one type of gene in T cells and the other in B cells, suggests that we are dealing with two completely separate but complementary genetic systems.

The question which I think is still unanswered, is whether the other documented examples of allotype association, such as with the rate and amount of antibody production, are in fact, reflections of this same immune system operating through V genes. Alternatively, there may be many other levels of control over total antibody synthesis. Finally, it seems clear that other types of immune response genes, perhaps expressed in macrophages, exist, although their mechanism of action remains unknown.

SESSION IV

RELATIONSHIP BETWEEN HISTOCOMPATIBILITY-LINKED AND ALLOTYPE-LINKED IMMUNE RESPONSE GENES AND ANTIGEN-SPECIFIC RECEPTORS ON T AND B CELLS

Ig identity of B cell receptor – Failure to detect Ig on T cells – Evidence for presence of Ig on T cells – Assessment of the conflicting data – Specificity of the receptors on T and B cells – Blocking of T cell functions by anti-H and anti-Ig – Distinguishing characteristics of thymus-independent antigens.

UNANUE: I propose to consider some of the information at hand on the nature of antigen receptors on both T and B lymphocytes, recognizing that in the past five years there has been extensive work in this field. Consequently, I intend to be selective in considering some recent and most important developments, noting particularly those in which there is disagreement and controversy, such as the issue of immunoglobulin receptors on T cells.

There is general agreement that the receptor for antigen on the B cell is an antibody molecule, that it is localized on the surface membrane of the cell, and that it can interact with and bind antigen. For many years the evidence for this kind of receptor on lymphocytes was indirect and stemmed mainly from the work of Gell and Sell, who had shown that rabbit peripheral lymphocytes, in culture, developed into blasts upon exposure to antiimmunoglobulin antibodies. They concluded that this reaction was the result of the formation of immune complexes involving surface immunoglobulin molecules. It was only recently that Coombs and his associates were able to demonstrate such Ig molecules directly with the use of the immunocytoadherence test. This was followed by the exciting work of Raff, Taylor, and collaborators in which the immunoglobulin was directly visualized by immunofluorescence. Indeed, viable lymphocytes in suspension, upon incubation with fluorescent antiimmunoglobulin antibody, showed small discrete spots on the entire cell surface. In this technique there is no background fluorescence and the cell exhibits many small discrete spots which can readily be identified. Figure 52 is a representation of a B lymphocyte from a spleen of a mouse, stained with a fluorescent polyvalent antimmunoglobulin antibody. Such a positive reaction is abolished completely if the antiimmunoglobulin antibodies of the fluorescent conjugate are specifically absorbed. We have extended these studies to the ultrastructural level in collaboration with Perkins and Karnovsky. Figure 53 shows a B lymphocyte after reaction with ^{125}I antiimmunoglobulin. It is noteworthy that the grains representing the surface immunoglobulin molecules are distributed all around the plasma membrane of the cell. The cell carrying the immunoglobulin is a typical splenic small lymphocyte.

A consensus has been reached that the molecules of immunoglobulin observed on B cells are actually synthesized by these cells and do not represent cytophilic antibody. Free monomeric γG will not bind to the lymphocyte surface. More importantly, one can strip off the receptors of the cell by treatment with trypsin, for example, then place the cell in culture media devoid of mouse proteins and follow the reappearance of the surface immunoglobulin molecules. The predominant class of immunoglobulin in the lymphocyte varies from species to species, and within species varies with age and environmental conditions of the experimental animal. In general, the evidence is that at a given time individual lymphocytes contain on the surface membrane a single class of immunoglob-

Fig. 52. Fluorescence micrograph of a viable spleen lymphocyte after exposure to fluorescent antiimmunoglobulin antiserum. Note the positive ring reaction. About 50% of spleen cells representing the B cell population show detectable immunoglobulin.

ulin. Pernis found that in rabbits the predominant immunoglobulin was of the IgM type. We found by immunofluorescence that about 50% of the lymphocytes in mice carry IgM and the remainder carry IgG and IgA. In humans, about 30-50% of Ig bearing peripheral lymphocytes have IgM. In guinea pigs the predominant lymphocyte surface immunoglobulin is IgG.

Most of the lymphocytes that have easily detectable surface immunoglobulin molecules are the B type. Table 73 summarizes a series of experiments that we and others have done attesting to this conclusion. For example, mice that are thymectomized as adults, then lethally irradiated and reconstituted with

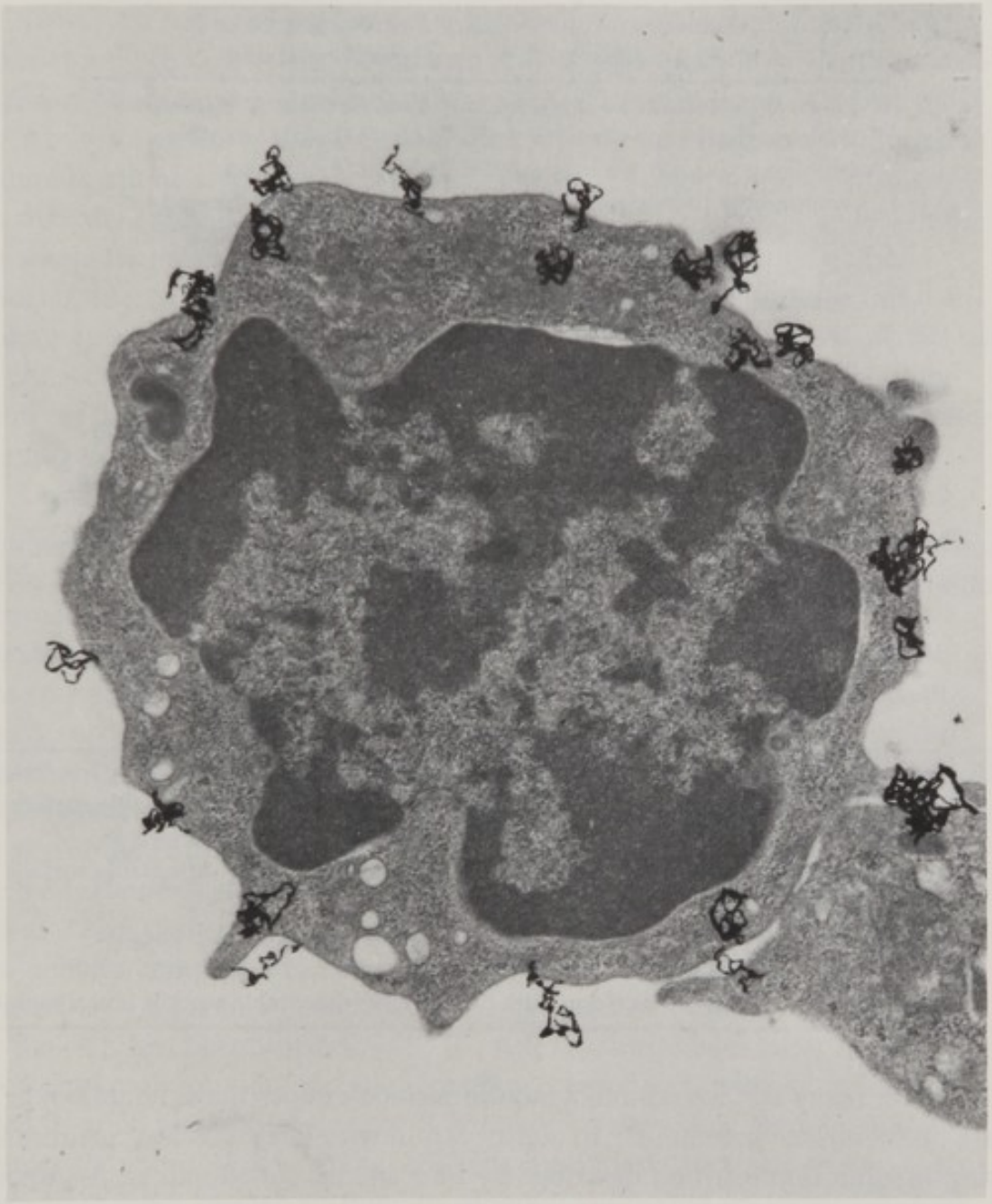


Fig. 53. Electron microscopy autoradiograph of a mouse spleen lymphocyte after reaction with ^{125}I-labeled antiimmunoglobulin antibody at 4°C. At this temperature the immunoglobulin molecules are distributed over the entire cell surface. *J. Exp. Med.*, **135**, 267, 1972.

bone-marrow cells contain the same number of lymphocytes with surface immunoglobulin as control mice that were not thymectomized. Similarly, neonatally thymectomized mice as well as mice with congenital thymic aplasia (nude mice) show a normal number of lymphocytes with surface immunoglobulin. The thymic-deprived mice, on the other hand, lack the population of lymphocytes with no detectable surface immunoglobulin molecules. Furthermore, it has been

TABLE 73

Evidence for the Origin of Lymphocytes
with Easily Detectable Surface Immunoglobulin

1. Adult thymectomized mice, lethally irradiated and transplanted with syngeneic bone-marrow cells, 8-12 weeks later exhibit lymphocytes *with* surface immunoglobulin in numbers comparable to control mice not thymectomized but treated otherwise in the same way. The thymectomized mice lack the populations of lymphocytes *without* detectable surface immunoglobulin.
2. Nude (athymic) mice have lymphocytes *with* surface immunoglobulin.
3. In birds, bursal cells have detectable surface immunoglobulin; thymectomy does not deplete this population of immunoglobulin-positive lymphocytes.

shown by Raff that cells carrying the isoantigen θ do not have surface immunoglobulin demonstrable by immunofluorescence (Table 74).

TABLE 74

Evidence for the Origin of Lymphocytes
without Readily Detectable Surface Immunoglobulin

1. F_1 thymocytes transplanted to adult parental thymectomized mice (lethally irradiated and reconstituted with syngeneic bone-marrow cells) do *not* show detectable surface immunoglobulin when identified in peripheral lymphoid organs 6 weeks later.
2. Most thymocytes primed or "educated" to an antigen do *not* have detectable surface immunoglobulin.
3. In birds, bursectomy *depletes* the population of the immunoglobulin-positive lymphocytes.
4. Lymphocytes having the alloantigen theta do *not* have detectable surface immunoglobulin.

How many surface immunoglobulin molecules does the B lymphocyte have on its surface membrane? In collaboration with Grey this was quantitated using an inhibition test first described by Farr and his collaborators. A suspension of live lymphocytes is incubated with a small amount of antiimmunoglobulin antibodies; subsequently, these antiimmunoglobulin antibodies are incubated with nanogram amounts of ^{125}I-labeled immunoglobulin. The extent to which the prior incubation with lymphocytes inhibits the standard antiimmunoglobulin ^{125}I-immunoglobulin reaction is used as the basis to estimate the number of immunoglobulin molecules available. With this test we came up with figures of about 50,000 to 150,000 molecules of immunoglobulin per B lymphocyte. In most experiments the mean number is about 10^5 molecules per cell. Such figures have been quite consistent for the five different strains of mice that have been analyzed.

How are the immunoglobulin molecules distributed on the surface of the B cell? Karnovsky has been studying the distribution of these immunoglobulin molecules with the use of the freeze etching technique, employing antiimmunoglobulin antibodies labeled with ferritin or hemocyanin. Figure 54 shows the surface of a B lymphocyte after reaction with ferritin conjugated antiimmunoglobulin. It can be seen that there is a random distribution of the ferritin molecules over the entire surface of the cell. This study has been done under conditions where there is very little displacement of the surface immunoglobulin molecules, i.e., by incubating the cells with antibody at 2°-4°C. With this technique, temperature control throughout the procedure is most important. We can actually account for most of the immunoglobulin previously calculated to be present on the surface membrane. Absorption of the ferritin antiimmunoglobulin conjugate with immunoglobulin completely suppresses the reaction.

Immunoglobulin molecules are not fixed rigidly on the membrane, but they can be displaced very easily subsequent to a reaction with antigen or with an antiimmunoglobulin antibody. The first report of this very interesting phenomenon was made by Taylor, Raff, Duffus, and de Petris, who showed that there was a rapid movement of the surface immunoglobulin molecules subsequent to reaction with a fluorescent antiimmunoglobulin antibody. We have recently carried out a series of morphological and biochemical studies on this reaction with Karnovsky and his associates. Lymphocytes incubated at 4°C with a suitably labeled antiimmunoglobulin antibody show immunoglobulin molecules distributed at random over their entire cell surface, as was shown in Figs. 53 and 54. However, if subsequent to these reactions the lymphocytes are incubated at 37°C, there is a very rapid displacement of the antiimmunoglobulin-immunoglobulin complex into one pole of the cell, into what is termed a "cap". Figure 55 is an electron micrograph of this phenomenon exactly one minute after the cells had been warmed to 37°C. A few minutes later most of the immunoglobulin-antiimmunoglobulin complex present on the cap is rapidly internalized in small vesicles, leaving the B lymphocyte devoid of immunoglobulin receptors for about 24 hours (Fig. 56). The formation of a single large complex in the cap requires enough antiimmunoglobulin antibodies for crosslinking all the surface molecules and the presence of a viable, metabolically active cell. We think that crosslinking stimulates the cell to move, a process that propels the complex to one area of the membrane.

In other electron microscopic studies using hemocyanin-labeled antiimmunoglobulin molecules, it has been possible to observe that in the region of the cap the immunoglobulin molecules are stacked in several layers, one on top of the other. This raises the possibility that the actual complex has been pulled out of the membrane prior to the internalization. In fact, other experiments do suggest that the immunoglobulin molecules are rather loosely bound to the plasma

Fig. 54. A freeze etch preparation of a murine spleen B lymphocyte after incubation with ferritin labeled antiimmunoglobulin. Immunoglobulin is present in a close random network. *J. Exp. Med.*, **135**, 267, 1972.

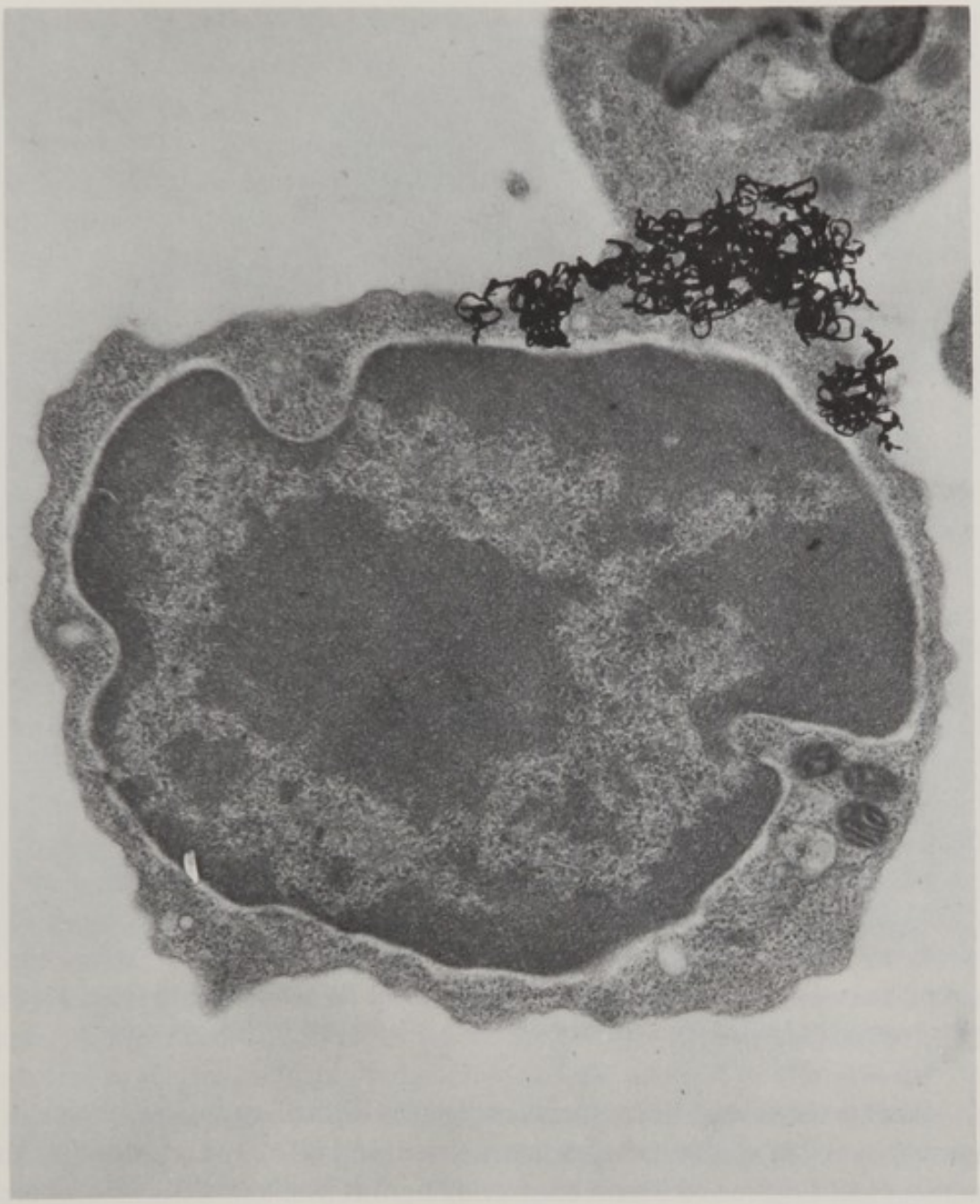


Fig. 55. Electron microscopy autoradiograph of a murine spleen lymphocyte after exposure to ^{125}I antiimmunoglobulin at 4°C and subsequent incubation at 37°C for one to two minutes. Note aggregation of the labeled antibody at one pole of the cell. This preparation is from the same study shown in Fig. 52.

membrane: Lymphoid cells are incubated at 4°C with fluorescent antiimmunoglobulin, then washed and incubated for one to five minutes at 37°C in order to allow the formation of a single agglutinate at one pole of the cell; the cell is returned to 4°C and subjected to heavy agitation; under these conditions, it can be noted that many of the cells rapidly lose the antiimmunoglobulin complex into the culture supernatant.

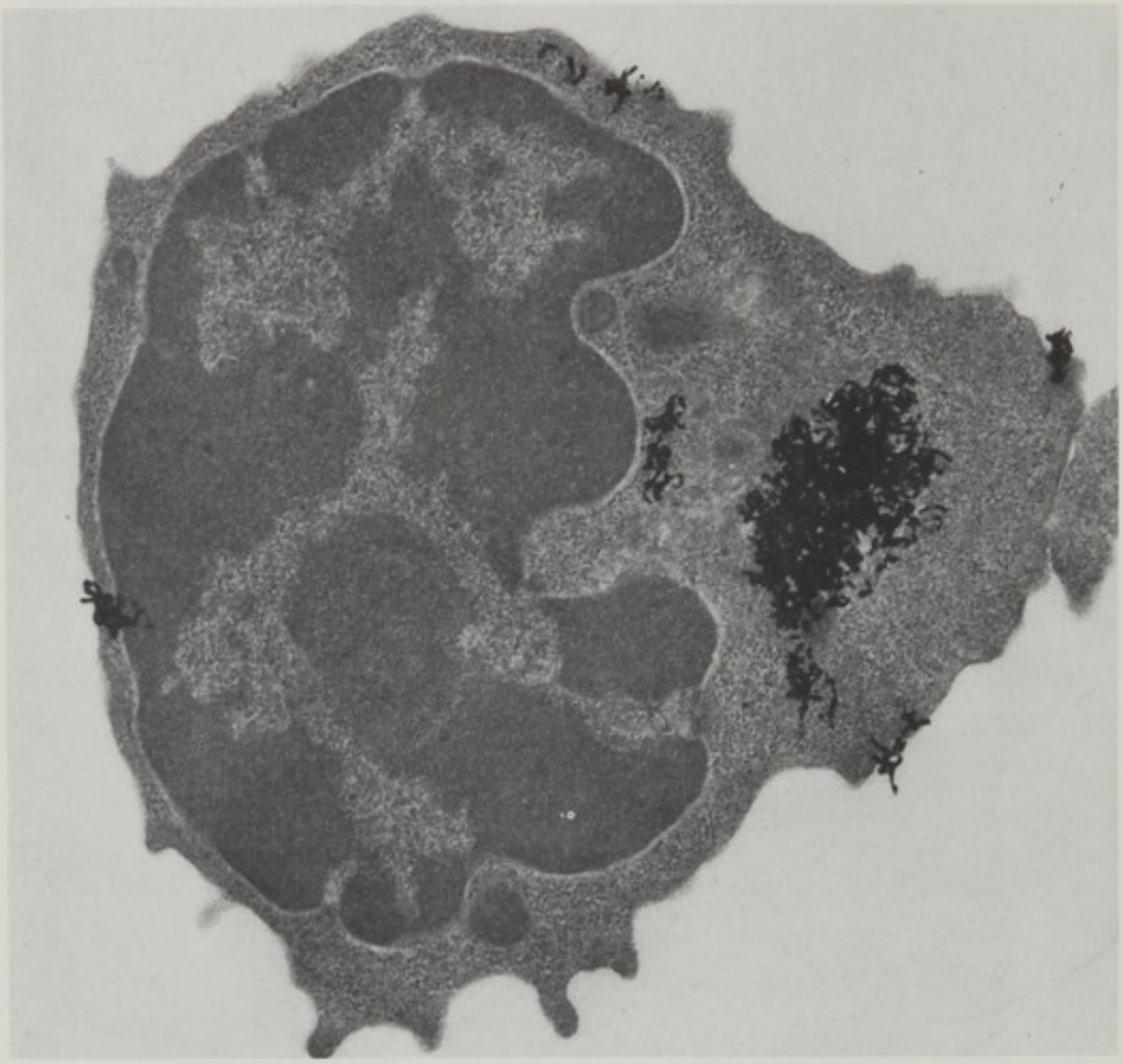


Fig. 56. The same study as Figs. 52 and 55 except that the cell has been kept at 37°C for five minutes. The labeled antibody has now been interiorized by the lymphocyte.

What is the fate of the immune complexes formed on the membrane of the B lymphocytes? Is all the complex internalized, and if so, is it degraded by the lymphocytes? Engers and I have been studying this problem using ^{125}I-labeled antiimmunoglobulin antibody. The experiments consisted of following biochemically the fate of the ^{125}I-labeled rabbit IgG molecules (with antimouse immunoglobulin activity) subsequent to immune complex formation on the B lymphocyte membrane. Indeed, it appears that the complex can follow two pathways: In one, about 10-20% of the labeled molecules are released from the cell as large aggregates, presumably as immune complexes of antiimmunoglobulin with immunoglobulin; in the other pathway, the molecules are partially digested. Thus, 24 hours after culture, 40-60% of the radioactivity is present in the culture supernatant mostly in the form of small peptides or low molecular weight protein fragments; the remaining 40% is still present in the cell, now in

the form of low molecular weight proteins. Thus, the B lymphocyte does have the capacity to effectively degrade the internalized immune complexes. These two observations may explain why several years ago Nossal and Humphrey and their collaborators were unable to detect molecules of antigen in plasma cells, since it is to be expected that the B lymphocyte would handle antigen molecules in a manner similar to rabbit immunoglobulin. However, it is most likely that we will see variations in the degree of shedding, internalization, and digestion of the complex depending upon the physicochemical state of antigen molecules.

What is the fate of the B lymphocyte subsequent to the internalization of the complex? We have done a small number of experiments along these lines. If the lymphocytes that have eliminated the immunoglobulin from their membranes as a result of complexing with antiimmunoglobulin are placed in culture in media containing ten % fetal calf serum, they will show reappearance of the surface immunoglobulin in a period of about 24 hours. In essence, it appears that the B lymphocyte has the capacity to resynthesize the new receptor subsequent to the internalization of the immune complex.

There is strong indirect evidence that the surface immunoglobulin molecules of the B lymphocyte serve to bind antigen. This information has been obtained in several ways. We have examined antigen-binding cells by autoradiography after exposure of lymphocytes to radioactively labeled antigen. Using this technique, it can be estimated that in a spleen a very small number of lymphocytes, on the order of about one per 10,000, can bind molecules of antigen. If prior to exposure to the antigen, lymphocytes are incubated with antiimmunoglobulin antibody, the binding is markedly suppressed. The immunoglobulin receptors for antigen on the B lymphocytes appear to be restricted in their specificity to antigen, as would be predicted from clonal selection theories. Some of the best evidence to this effect comes from "suicide" type experiments, in which treatment *in vitro* with highly radioactive antigen is found to abrogate specifically the immune response to the labeled, but not to other unrelated antigens. In summary, insofar as the B cell is concerned, there is not very much controversy about the nature of the receptors. The antigen receptors on B lymphocytes are immunoglobulin molecules present on the membrane in a random fashion, showing specificity restrictions and capable of binding antigen. These immunoglobulin molecules are not tightly bound to the membrane, and can be readily displaced upon complexing with a ligand, a process that leads to their shedding or interiorization. Obviously, we still have much to learn of the relationship between the movement of the surface immunoglobulin molecules subsequent to the binding of antigen and the triggering of the B cell. At present there is little information along these lines.

In the field of cellular immunology the problem of the antigen receptor on the T lymphocyte has been a subject of great controversy. Many have sought a

type of recognition unit similar to that on the B cell for antigen molecules, in preference to facing the complicating features of another type of receptor molecule. However, present information on the issue of immunoglobulin on T cells is confusing, to say the least. There is a marked disagreement by functional analysis, as to whether T lymphocytes show surface immunoglobulin molecules. By direct methodologies, such as the ones presented with the B lymphocyte, there has been no clear, reproducible demonstration of such an immunoglobulin-type receptor. This is the situation for most of the antiimmunoglobulins that have been employed by the majority of immunologists. It should be noted that with techniques such as immunofluorescence and autoradiography, about 10^3 molecules of immunoglobulin can be easily detected on a lymphocyte membrane.

What are the experiments that have shown immunoglobulin on lymphocytes from the thymus? These experiments consist of inhibiting a series of immunological reactions by incubation of T lymphocytes with antiimmunoglobulin antibodies. For example, GVH reactions, MLC, T cell rosettes, and "suicide" (with hot antigen) have been blocked by such procedures. Most of these experiments show reasonable controls, and in many of them the presence of natural anticell membrane antibody appears to be eliminated. There are two puzzling problems with these results: First, in many of these experiments there is a need to use highly selected antiimmunoglobulin antibodies. However, the particular specificity that differentiates these antisera from those that do not block T cell reactions is far from clear. The second problem is that many other investigators have not been able to reproduce such results, despite the utilization of a rather large range of antiimmunoglobulin antibodies. These contradictory findings have given rise to uncertainty as to whether immunoglobulins are in fact the antigen receptors on T lymphocytes.

The kinds of experiments we have done along these lines can be summarized as follows: We have tested a variety of antibodies made against murine Fab fragments of immunoglobulins (in chickens, rabbits, and rats) by immunofluorescence or by autoradiography (using short or long exposure times). Thus far, we have been unable with these reagents to demonstrate immunoglobulin molecules on T cells. A very convincing demonstration to this effect has been performed by Karnovsky with the technique of freeze etching in which a great part of the surface membrane of the cell is readily visualized. This method fails to demonstrate *any* immunoglobulin on thymic or peripheral T cells exposed to ferritin conjugated antiimmunoglobulin antibodies. With this technique most of the B lymphocyte immunoglobulin molecules could be detected. An interesting observation for which we have no clear explanation, is the presence in the thymus of a very small but consistent population of cells which *do* bear surface immunoglobulin molecules. The number is usually about one per 300 thymocytes. The lymphocytes which bear surface immunoglobulin molecules have a peculiar cytology which sets them apart from the rest of the thymocytes. Their

Fig. 57. A thymic cell labeled with ^{125}I antiimmunoglobulin. Most of the few labeled cells show a peculiar cytology that distinguishes them from most thymocytes (and peripheral lymphocytes). Note the large amount of cytoplasm with lesser density of ribosomes. *J. Exp. Med.*, **135**, 267, 1972.

cytoplasm is less dense and they contain fewer numbers of polyribosomes in their cytoplasm (Fig. 57). Our suspicion is that a great many of these lymphocytes which have surface immunoglobulin may actually be B lymphocytes that have settled in the thymus. The literature provides indications that lymphocytes from peripheral blood can actually penetrate and settle in the thymus. Thus, McCluskey and his associates have studied the distribution of lymph-node lymphocytes labeled *in situ* with tritiated thymidine and found that a small number of these lymphocytes actually home to the medulla of the thymus. Histologists have long noted the presence of plasma cells in the thymus. Since the plasma cell is derived from the B lymphocyte, one must then postulate that there must have been a small number of B cells settling in the thymus medulla. We have investi-

gated whether the immunoglobulin-positive thymocytes are sensitive to anti-θ treatment. Thymocytes were incubated with anti-θ serum and complement, and then placed on a Ficoll-Hypaque gradient in order to separate the live from the dead cells. The anti-θ treatment killed 98% or more of the thymocytes. There were about 50% macrophages and 50% typical lymphocytes with surface immunoglobulin among the one to two % of viable cells recovered. This experiment tells us that the lymphocytes with surface immunoglobulin that are in the thymus are either B cells, or T cells with a low density of θ receptors. In summary, it would appear that with methods that are capable of detecting a few hundred molecules, the majority of peripheral T cells are found to lack antigenic determinants recognized by most of our antibodies.

The search for antigen-binding cells among thymocytes or peripheral T cells has also been a problem. Engers, in my laboratory has carried out a series of experiments searching for cells capable of binding an antigen of high molecular weight, hemocyanin, among thymic cells. He has done this by: 1. an autoradiographic method in which the cells are first incubated with the antigen and then with a ^{125}I-labeled antibody; the cells binding the antigen are studied by autoradiography and 2. by quantitating the amount of antigen binding to the lymphocytes by a Farr inhibition test (Table 75). In the spleen about 0.1 to 0.2% of the cells bind antigen; in the thymus we find about 1/10 the number of antigen-binding cells found in the spleen. The binding of antigen to spleen cells is inhibited to the extent of 85% or more, if the cells are incubated with antiimmunoglobulin antibody. However, the binding of antigen to T cells has been inhibited, at most, by 35-50%. The very low number of antigen-binding cells in the thymus and the error in counting, make this last result of borderline significance. The quantitation of antigen bound to spleen and thymus cells yielded interesting results (Fig. 58). For example, we have arrived at the figure of about 8,000

TABLE 75

Procedure for Assaying Cell Bound Hemocyanin

Test:	a) 6 ng of ^{125}I-hemocyanin (from KLH) is reacted with a dilution of mouse anti-KLH sufficient to bind 30-50% of this specimen.
	b) Bound KLH is measured by coprecipitation of complex with antimouse immunoglobulin.
Establish Inhibition Curve:	Unlabeled KLH is reacted with anti-KLH prior to addition of ^{125}I-KLH.
Quantitation of Cell Bound KLH:	Lymphoid cells are incubated, generally, at a concentration of 3-5 × 10^7 cells, with 100-500 μg of KLH at 4°C; washed; the cells are then used in a standard test to determine the degree of reduction of binding of ^{125}I-KLH by prior incubation of mouse anti-KLH with the cells. The degree of inhibition is converted to amounts of KLH bound to cells by use of a standard inhibition curve.

Fed. Proc., **31**, 735, 1972.

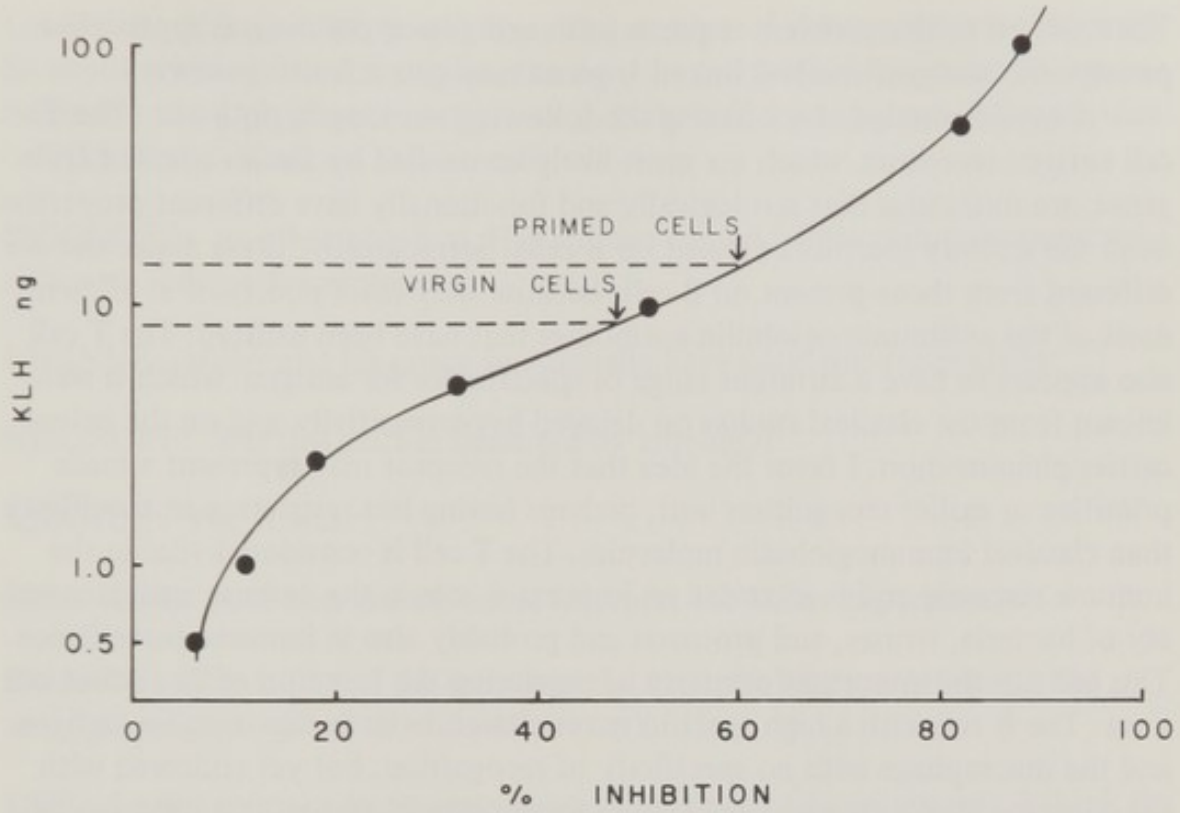


Fig. 58. Results of quantitation of cell bound hemocyanin on immune and unimmunized (virgin) spleen cells. The vertical axis gives the amount of free hemocyanin used in the standard reaction; degree of inhibition is shown on the horizontal axis. The curve is the standard one obtained with hemocyanin.

molecules of hemocyanin bound per spleen cell, calculated on the basis of the degree of inhibition of binding of ^{125}I-KLH, the antihemocyanin-hemocyanin and the assumption that all the antigen is bound to the lymphocytes detected by autoradiography. The thymic cell population also binds antigen, although this binding is about 1/3 to 1/2 of that bound to the spleen cell population. When we calculate the number of antigen molecules bound per T cell we find that the range is 10,000-30,000 molecules. Once again, this assumes that all the antigen is bound exclusively to the cells that we detect by autoradiography, which in the case of the thymus is perhaps not a valid assumption. In these quantitative experiments we can inhibit most of the binding to the spleen lymphocytes by incubation with antiimmunoglobulin antibody, but no such inhibition occurs with the T cell population. These are preliminary results that need further evaluation.

In summary, I confess to complete failure in actually detecting the presence of immunoglobulin molecules on T cells by a series of functional and direct morphological observations. How are we to reconcile these negative observations which many have made, with the few positive results that have appeared in the literature? This is rather difficult, but perhaps exchanging reagents and comparing methodologies might provide a clearer picture of this fundamental problem.

The solution to this problem requires fresh and new experimental approaches; perhaps the study of the H-2 linked Ir genes may give a fruitful answer.

I would conclude by offering the following working hypothesis: The T cell antigen receptors, which are most likely controlled by the H-2-linked Ir genes, are molecules that serologically and functionally have different properties from the antibdy receptors present on B cells. Serologically, these molecules are different from those present on B cells because they react poorly, if at all, with most of the antiimmunoglobulin antibodies that have been utilized. The T cell also appears to have a different range of specificities for antigen, which is well known from the classical studies on delayed hypersensitivity and on the helper carrier phenomenon. I favor the idea that the receptor may represent a more primitive or earlier recognition unit, perhaps having less restriction in specificity than classical immunoglobulin molecules. The T cell is considered vital in the immune response and is accorded an important role in the defense against a variety of bacteria, viruses, and protozoa and probably also in immune surveillance. This cell has the important property of regulating the function of two other cell lines: The B cell with a high specificity restriction in its recognition for antigen, and the macrophage with no specificity of recognition, but yet endowed with the capacity to effectively degrade microorganisms. However, it is conceivable that this important biological role could be carried out by a T cell with a recognition system of less restricted specificity.

CHAIRMAN RAJEWSKY: Although we will have two additional contributions on this general subject, we could nonetheless, have a few questions directed to Unanue's presentation.

SELA: Would Unanue tell us what happens when he does antigen-binding tests with completely irrelevant cells, such as erythrocytes or liver cells? Doesn't he find a few that bind KLH, and isn't such binding not inhibited by antiimmunoglobulin?

UNANUE: Perhaps. The antigen-binding cells in the thymus look like typical small lymphocytes. However, I have not done binding tests with red cells or with liver cells.

NISONOFF: In Fig. 57 you showed us that a thymus cell population had B cells in it. Is that correct?

UNANUE: I believe those are indeed B cells, yes.

NISONOFF: Wouldn't it be reasonable to expect that there might be some B cells binding KLH in the thymus?

UNANUE: Yes, that is a good point. The number of contaminant B cells must be about ten % in order to explain the data on antigen-binding cells. This number seems too high, consequently we conclude that the antigen-binding thymus cells are likely to be true thymic cells.

NISONOFF: Another question. Has anyone caused helper cells to commit suicide with radioactive protein?

UNANUE: Miller and his group in Australia have done this successfully.

NISONOFF: Was this suicide inhibited by anti-IgG?

UNANUE: Yes, it was.

NISONOFF: And how does Unanue interpret the Miller experiments?

UNANUE: This is one of the controversial matters. Why are some selected anti-IgG reagents effective while others are not?

UHR: I want to make two points concerning Unanue's experiments. I think the experiments on antigen binding are extremely exciting. By taking advantage of the polyvalency of antigen he has developed another approach to the issue of the antigen-specific receptor of T cells. Since in his hands the receptor is not immunoglobulin, proof of the specificity of the binding is critical. Therefore, two types of controls are needed: Other cell populations to study, which presumably do not have receptors and, in addition, other proteins which resemble the specific one biochemically, but are not crossreactive. Secondly, it would be interesting to see the effect of pretreatment with congenic anti-θ serum and C$'$ on antigen-binding by thymus cell suspensions, to evaluate quantitatively the role of non-θ bearing cells on binding.

UNANUE: I agree with Uhr. These are experiments that we began recently and they need to be expanded. I intend to try several other antigens. One of the things we have thought about in terms of specificity, is to inject antigens in situations where we know we can tolerize the thymic cell population; we would then look for the presence of specific antigen-binding T cells.

WILSON: Unanue suggested that some of the cells in the thymus might be plasma cells, hence presumably derived from B cells. These bind antigen and cannot be blocked with anti-Ig.

UNANUE: I believe they are B cells.

WILSON: Isn't it possible that these cells might after all be binding, and nonetheless fail to be blocked with anti-IgG? Is it known that plasma cells can be blocked with anti-IgG?

UNANUE: I have not said the antigen-binding cells in the thymus are B cells. What I *am* saying is that some of the cells in the thymus that bear surface immunoglobulin might be B cells, or T cells with a low density of θ receptors, since they are not killed by anti-θ sera. In support of the hypothesis that they might be B cells are the observations of a few plasma cells in the thymus.

WILSON: Agreed. The statement was that the thymic antigen-binding cells could not be blocked with antiimmunoglobulin.

UNANUE: Antigen-binding cells in the thymus were not blocked to a significant degree by our antiimmunoglobulin antibodies.

McDEVITT: What happens if Unanue tries to inhibit with anti-H-2?

CEPPELLINI: Yes, that experiment is especially important.

UNANUE: In a single experiment, we failed to inhibit binding.

BENACERRAF: Could Unanue show us a picture of the H-2 distribution on T cells, as that would have a bearing on this discussion? We should see for ourselves where these receptors are located.

UNANUE: Fig. 59 referred to by Benacerraf is from a freeze etch study with Karnovsky, using anti-H-2 antibodies on thymic cells. It is evident that H-2 is present in patches all over the cell surface. The patches are about 1,000-3,000 Å apart from each other. These distances are too far apart to be linked by antibody (i.e., span of 140 Å) or by a sandwich reaction (i.e., about 420 Å). These H-2 sites do not aggregate or cap, probably because of the wide spacing of these domains of antigen.

RAFF: It hurts me to hear people still talking about a patchy distribution of surface antigens or receptors, which have been demonstrated by multivalent antibody or ligands as if it were the real distribution. Stephanello de Petris and I have shown that when one uses monovalent Fab antiimmunoglobulin labeled with ferritin, one does not see a patchy distribution. On the other hand, whole antiimmunoglobulin even at 4°C induces striking patch formation. Surely the obligation now lies with those who maintain that a patchy distribution is real and not artifactually induced, to demonstrate the patchiness with monovalent reagents.

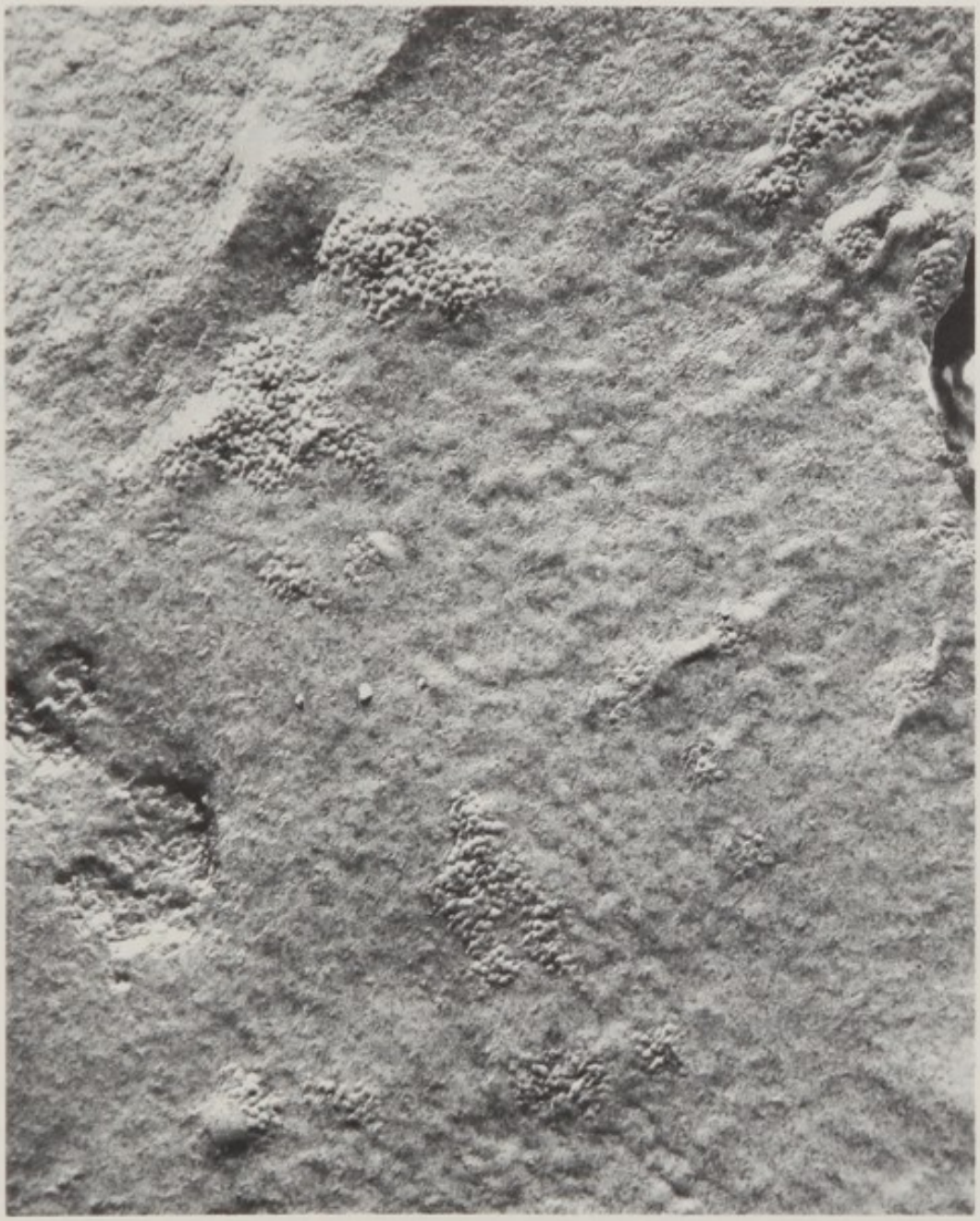


Fig. 59. A freeze etch analysis of a murine thymic cell following exposure to mouse anti-H-2 and ferritin-conjugated antimouse immunoglobulin. H-2 is found in patches. Similar results have been obtained directly using anti-H-2 iabeled with ferritin. *J. Exp. Med.*, **135**, 267, 1972.

As regards the very few cells in the thymus that have readily demonstrable Ig on their surface and that Unanue has suggested may be B cells, their unusual morphology makes them different from the B cells in the peripheral lymphoid tissues. Thus, either they represent a small subpopulation of B cells or are another cell type, such as stem cells with passively absorbed immunoglobulin.

I have a question for Unanue concerning the KLH-binding cells in the thymus, which appear to bind more molecules of KLH than spleen B cells. If these are really thymus lymphocytes binding KLH, then one might expect to detect KLH-binding T cells in the spleen. Has he been able to inhibit any of the KLH-binding cells in the spleen with anti-θ and complement?

UNANUE: No. I have not done that experiment as yet. The distribution of immunoglobulin or other receptors can be studied with bivalent antibodies at 2-4°C under conditions where there is minimal aggregation. Strict control of temperature during the whole procedure is most important; as soon as the cells are warmed, aggregation takes place.

RAFF: But I recall distinctly that the second picture Unanue showed (Fig. 53) was an autoradiograph, where spleen cells were labeled at 4°C (with iodinated antiimmunoglobulin) and the distribution was clearly patchy. This is precisely what we see with ferritin-labeled antiimmunoglobulin when labeling is done at 4°C.

UNANUE: At 2-4°C ferritin-labeled antiimmunoglobulin is distributed homogeneously without aggregation.

RAFF: In that event, your experience is very different than what we have observed. To my way of thinking, it is very clear that patching can be an *induced redistribution*. On the other hand, I find it hard to imagine how a diffuse distribution could be an artifact.

CHAIRMAN RAJEWSKY: Patching is certainly an interesting phenomenon but for now I don't think we should pursue that line further.

LANDY: Artifact or not, I would like to come back to the type of distribution of the H-2 sites on T cells. I ask Unanue whether he has directly compared his experimental model with the cell slice reconstruction model that Stackpole, Boyse, and Old have reported. They found a characteristic patchy distribution; has Unanue lined up his cells side by side by the Stackpole diagram—and if so—how do they compare?

UNANUE: The mapping of H-2 on T cells done by Stackpole *et al.* matches our results. In their mapping of peripheral lymphocytes there was a heavier distribution of markers but this could be accounted for by the presence of mouse immunoglobulin on the membranes apart from H-2 sites.

COHN: A few years ago we left the question of the distribution of *H-2K* and *H-2D* in the belief that there was a K-island and a D-island on the surface of

the membrane, these two islands being independent. I am referring to the studies of Boyse *et al.*, who carried out cross-saturation studies with anti-K and anti-D. Today I would like to have the experts tell us whether they still believe that K and D are independently associated on the membrane?

CEPPELLINI: I believe I can answer Cohn's question, if we can assume it is appropriate to extrapolate from LA and Four (the two recognized loci of the HL-A region) to D and K of the mouse. We have induced cap and spot formation with a monospecific anti-HL-A serum followed, after washing, by anti-human Ig rabbit serum (anti-HL-A sera even if bivalent do not easily induce cap formation) on lymphocytes of a doubly heterozygous donor. After this treatment only the HL-A factor corresponding to the anti-HL-A serum used, but *not* the other three HL-A factors, becomes inaccessible to further addition by antiserum and C′ (and the cells are not lysed).

Our interpretation is that the four HL-A genes produce four different populations of molecules which have independent mobility at the cell surface. By contrast, determinants controlled by the same gene (and hence likely carried by the same polypeptide as with the Gm^a and Gm^z allotypic factors in human γ_1 chains) do "co-cap". These results have been confirmed visually with the use of two different fluorochromes (Bernoco, Cullen, Scudeller, Trinchieri, Ceppellini, "HL-A Molecules at the Cell Surface" in *Histocompatibility Testing*, Munksgaard, Copenhagen, 1972).

LILLY: I too can report on the very point raised by Cohn. This involves the results of preliminary experiments conducted in collaboration with Néauport and Kourilsky. These experiments are based on conjugation of red and green fluorescent molecules to antibodies monospecific for K or D end determinants of H-2. The experiments have been done in several combinations and I refer only to one example. The first step consists of incubation of a cell with the proper red-conjugated D end antiserum, followed by warm incubation so that cap formation takes place. The next step is incubation in the cold with green-conjugated anti-K end specificity. When this is done, the green fluorescence is seen to be evenly distributed over the surface of the cell at the same time that there is a red cap. These experiments so far have been done only in homozygous cells; we have not yet gotten the results from heterozygous cells.

RAFF: The experiment that Lilly has described is a very exciting result. We have spent six months trying to do this type of experiment and have failed for technical reasons. It should be made clear that mobility studies of this kind are not necessarily analagous to the mapping experiments that have involved blocking effects. In the blocking experiments of Boyse and his colleagues, thymus cells are coated with one alloantibody and the subsequent absorption of other

alloantibodies is studied. If two alloantibodies reciprocally block each other's uptake, then the corresponding alloantigens are considered to be side by side on the membrane. In such experiments it was found that the gene products of the Tla and *H-2D* loci mapped side by side. On the other hand, Boyse and colleagues showed many years ago that if one treats thymocytes with complement and antibody to Tl, Tl is removed from the membrane, but *H-2D* remains in increased amounts.

So it would seem that associations demonstrated in this way may be labile and may be broken by antibody-antigen interactions, and thus not be demonstrable by mobility studies. If two antigens are found to segregate together (either in patches or in a cap), it is likely that they will be found to be part of a single molecule, although at the moment this is only my prejudice. Thus far it has been shown that surface immunoglobulin moves independently of histocompatibility antigens and of the various mitogen (PHA, Con-A, Pokeweed) receptors.

CHAIRMAN RAJEWSKY: Clearly the studies of Boyse and his associates would show that there is some sort of association which can be broken up but which still exists.

RAFF: It is very hard to get around Boyse's observation that alloantigens on mouse thymocytes are not randomly distributed. But it may be that their arrangement is a statistical association, where mobile protein molecules come together and form loose, noncovalent associations.

UNANUE: Whether or not several receptors move independently or are associated with each other is critical. Thus far we have found that Con A receptors, H-2, immunoglobulin, and antigens reactive with ALS move independently of each other. These are the four that we have checked against each other.

CHAIRMAN RAJEWSKY: For the record I wish to say that Hämmerling, in collaboration with us, has done studies of very much the same type as Unanue (*Eur. J. Immunol.*, 1, 447, 1971). In his hands there is labeling of practically *all* peripheral lymphocytes with a number of different antiimmunoglobulin sera including anti-κ, anti-F(ab)$'$ and anti-μ. These studies are thus not restricted to one special antiserum but rather have been carried out with a battery of antisera yielding identical results. It would now be very helpful if Unanue were to exchange the appropriate reagents with Hämmerling to help clarify this situation.

UNANUE: Warner and his group find immunoglobulin on T cells with selected antibodies using a highly sensitive approach. No work I know of except

that of Hämmerling, has detected immunoglobulin with ordinary methods and using all antisera. I have no explanation for this discrepancy. We have used the same methods including hybrid antibody with completely negative results. I have already referred to the negative freeze etch analysis made by Karnovsky.

McDEVITT: How high a concentration of immunoglobulin molecules were found on T cells by Hämmerling?

CHAIRMAN RAJEWSKY: To give a precise answer to McDevitt is difficult. Looking at the total lymphocyte population, the distribution of surface labeling was heterogeneous, ranging from a few marker particles to almost complete coverage of the cell perimeter. However, at this stage it was not possible to correlate labeling density with lymphocyte subpopulations.

NISONOFF: Hämmerling reported this work at the Midwinter Conference of Immunologists earlier this year. He showed a rather high density of Ig molecules on T cells. It was an extremely large number.

SIMONSEN: A brief question to Unanue. Were these antigen-binding experiments with T cells carried out at different temperatures or did he try only one temperature?

UNANUE: I have done it only in the cold, at 4°C.

SIMONSEN: Would it not be possible to do them at a higher temperature also?

UNANUE: I have deliberately avoided doing these tests at 37°C because we know that with spleen cell populations, tests at this temperature lead to rapid capping.

SIMONSEN: I think this may possibly be an important point. If one tries to demonstrate heterogeneity in the T cell population by absorption on monolayers which Brondz in Moscow was the first to do (Brondz and Golberg, *Folia Biol.*, **16**, 20, 1970) this works only at 37°C or at room temperature, but not at 4°C, according to Wigzell, in Stockholm, who has successfully repeated such absorptions. It might, in fact, be that Unanue's antigen-binding T cells would be found to be much more numerous if he were to examine them at 37°C.

CHAIRMAN RAJEWSKY: This discussion has very logically taken us to the kind of rigorous experiments required to resolve the issue of immunoglobulin on T cells. We will next hear from Uhr about his work in this controversial area.

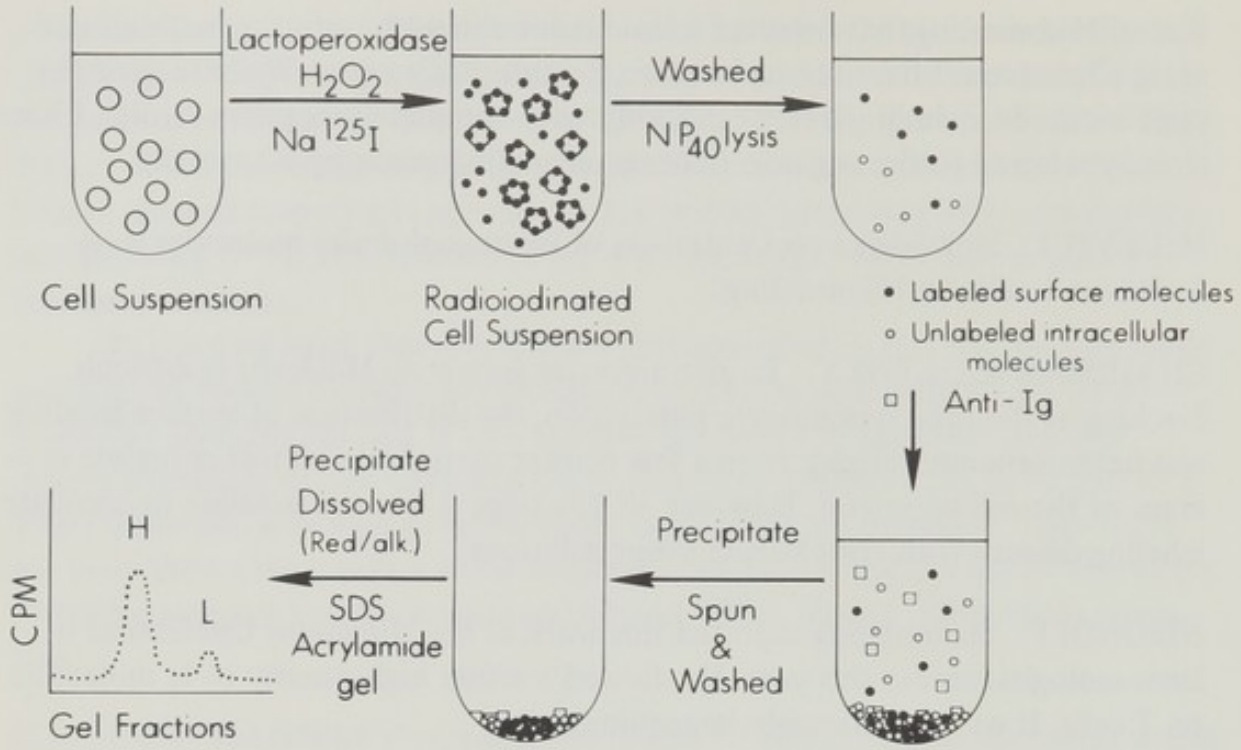


Fig. 60. Technique for radioiodination, isolation, and characterization of cell surface Ig of lymphocytes.

UHR: The first question I want to discuss is the presence of immunoglobulin on the surface of thymocytes and T cells. Capitalizing on the observation of Phillips and Morrison, *Biochem. Biophys. Res. Comm.*, **40**, 284, 1970, that the surface of intact erythrocytes can be enzymatically radioiodinated, we developed an approach for isolating labeled Ig from the surface of lymphocytes (Baur *et al.*, *J. Immunol.*, **106**, 1133, 1971). Figure 60 summarizes the method. A suspension of lymphocytes is radiolabeled with lactoperoxidase, minute quantities of hydrogen peroxide and ^{125}I. The washed radioiodinated cells are lysed in a nonionic detergent giving a solution of unlabeled intracellular molecules and labeled surface molecules. These are then precipitated with antibody, in this case anti-Ig. The washed precipitate is dissolved in sodium dodecyl sulfate (SDS), frequently is reduced and alkylated, and electrophoresed on acrylamide gel in SDS.

I would like to recapitulate our results on the distribution of cell surface Ig among thymocytes, bone-marrow cells, and their derived populations. These experiments were done by Vitetta in my laboratory in collaboration with Nussenzweig and Bianco. Table 76 summarizes the results. Thymocytes and cortisone resistant thymocytes gave negative results for Ig if parathymic lymph nodes were removed from the thymus gland. Other subpopulations that were negative included: θ-positive lymph node cells which were radioiodinated, killed with anti-θ and complement and the ghosts captured on an albumin cushion; a line of θ-positive lymphoma cells given to us by Horibata; CRL^- splenocytes and activated, θ-positive splenocytes.

TABLE 76

Distribution of Ig on Subpopulations of Lymphocytes

Cell type examined	Presence of Ig
Thymocytes	0
Cortisone-Resistant Thymocytes	0
θ^+ Lymphocytes	0
θ^+ Lymphoma Cells	0
CRL^- Splenocytes	0
T-activated, θ^+ splenocytes	0
Bone-marrow cells	+
CRL^+ Splenocytes	+
θ^- Lymphocytes	+

In contrast, immunoglobulin was easily demonstrated on bone-marrow cells or B cells. These were CRL^+ splenocytes, captured by virtue of rosette formation on an albumin density gradient, and θ-positive lymphocytes. By negative, I mean that precipitation with antiimmunoglobulin and a control precipitate pelleted equivalent amounts of radioactivity, and that analysis on acrylamide gel electrophoresis of the reduced and alkylated specific precipate did not reveal H and L chain peaks.

Incidentally, our precipitations are performed by "sandwich" technique. Thus, the control is a hyperimmune rabbit antiserum to an unrelated antigen.

Figure 61 shows the results with activated T cells; 10^8 activated T cells from a GVH reaction (90% θ-positive) gave negative results. In contrast, normal spleen cells gave prominent μ and L chain peaks.

Since there are a variety of reports by others suggesting that Ig *is* the T cell receptor, we considered various possibilities by which our approach might have missed immunoglobulin on T cells. Thus, cell surface immunoglobulin might be loosely bound and removed by thorough washing. Washing more gently or allowing the hypothetical receptor to be regenerated in serum-free medium before iodination did not change the negative results. Perhaps the receptor is hidden in the plasma membrane as suggested by others. This possibility is unattractive because in our laboratory, Schenkein, who has studied the enzymology of the lactoperoxidase reaction has extended older observations and determined that molecular iodine is the major or sole mediator of the labeling. In fact, he can use lactoperoxidase in a dialysis bag and label proteins on the outside. Presumably, molecular iodine can iodinate any available tyrosine residues on the cell surface. Nevertheless, we iodinated *lysates* of thymus cells and labeled thymocytes with ^{3}H-tyrosine. Again, no Ig was detected. We then considered the possibility that the cell concentration used for iodination might be suboptimal. We iodinated at concentrations from 10^5 to 10^9 cells/ml. The results were negative. The possibility was considered that the antibody molecule might be insolu-

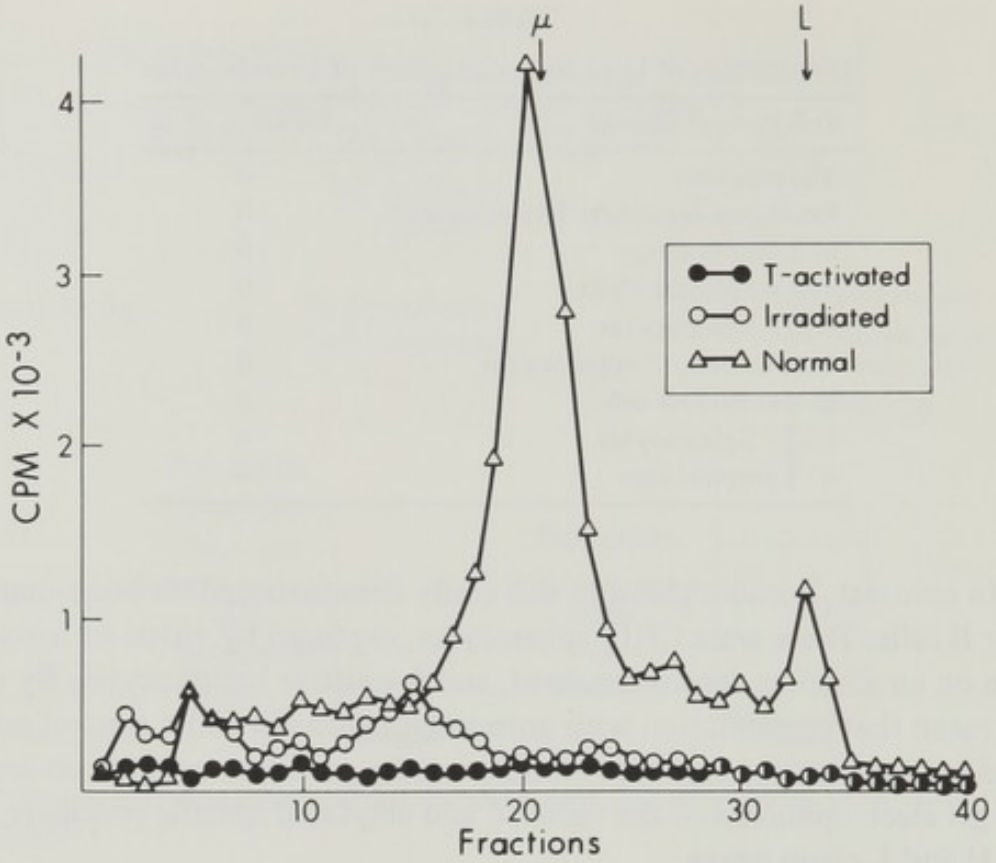


Fig. 61. Electrophoresis of anti-Ig precipitates obtained from lysates of surface radiolabeled activated T-spleen cells and control cells (from normal and irradiated mice). Precipitates were reduced and alkylated prior to electrophoresis. The positions of μ and L chains electrophoresed on companion gels are noted.

ble in detergent. Marchalonis *et al.*, *J. Exp. Med.*, **135**, 956, 1972, has described an extraction procedure with 9M urea and mercaptoethanol. We found that such an extraction results in an 80% loss of acid precipitable radioactivity in the nuclear pellet. Our extraction with five % NP40 results in a loss of only ten %.

However, a precipitate forms when thymus lysates are allowed to stand. Tryptic digestion of this precipitate and cofingerprinting with mouse IgM revealed no evidence of structural similarity.

We considered the possibility that the specificity of the antibody might not be "appropriate," which is unlikely because precipitation is carried out in solution, not between antibody and cells. Our conventional anti-Ig antiserum contained specificities to μ, γ, κ, and λ. In addition, however, we obtained from Dutton an antiserum which blocks T cell function *in vitro*. This antiserum gave negative results with thymocytes.

Finally, in a critical way we asked whether our method is a sufficiently sensitive one. Vitetta therefore iodinated a mixture containing a small number of B cells (5×10^6) with a large number of thymocytes (2×10^9) as well as the same number of thymocytes by themselves.

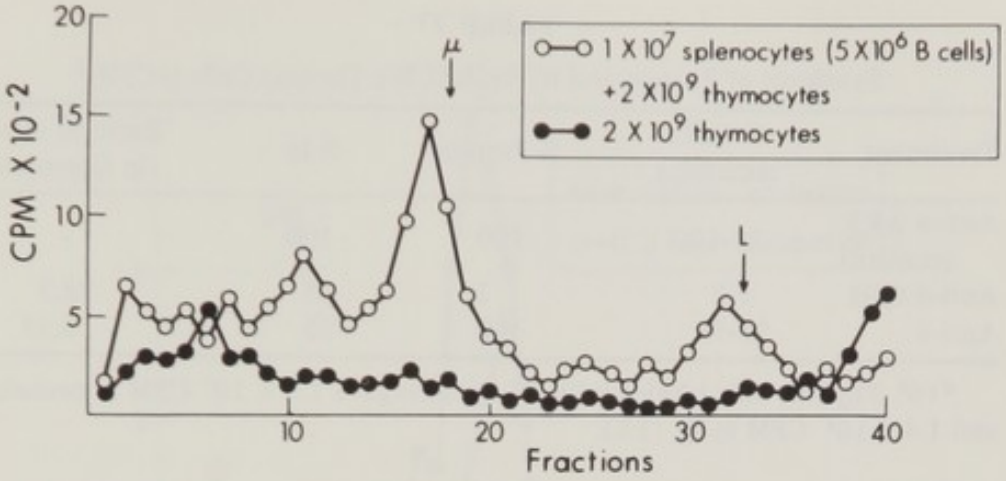


Fig. 62. Electrophoresis of anti-Ig precipitates obtained from lysates of surface radiolabeled thymocytes and a mixture of thymocytes and splenocytes. See Fig. 61.

As seen in Fig. 62, the thymocytes themselves gave negative results whereas the mixture containing one B cell per 400 thymocytes gave *easily* detectable μ and L chains. If there are approximately 100,000 Ig molecules per B cell, if all the thymocytes have Ig, and if the iodination reaction is not dependent upon the density of Ig per cell, we should have been able to detect 250 Ig molecules per thymocyte.

Shortly after these studies were completed Vitetta made an unexpected observation. She labeled thymocytes with ^{3}H-tyrosine to study secretion of cell surface proteins. She examined the incubation medium for Ig and found evidence that trace amounts were present. We had not previously examined thymic secretions, and since passage across the plasma membrane represents a very effective biological purification it was possible that we had missed detecting the immunoglobulin by examining only lysates.

To pursue this observation further, we asked whether θ-bearing or non-θ bearing cells secreted this Ig. Table 77 summarizes the results of a representative experiment. BALB/c thymus cells [θ-C3H were pretreated with anti-θ-AKR (control) or anti-θ-C3H and complement] . Anti-θ-AKR (control) resulted in no killing of the spleen cells; the amount of incorporation of ^{3}H-tyrosine into protein and into specifically precipitable Ig (specific precipitate minus control precipitate) is considered as 100%. Anti-θ-C3H killed virtually all the cells, inhibited virtually all protein synthesis, but suppressed the synthesis of Ig only to a trivial degree. In contrast, anti-κ killed only one % of the cells, did not affect protein synthesis and markedly depressed Ig synthesis. By pooling secretions from several experiments, there was sufficient radioactivity in specific precipitates to analyze on acrylamide gels. The results revealed γ, μ, and L chains analogous to secretions of labeled B cells from the spleen. I consider this experiment very strong evidence that the trace amounts of immunoglobulin secreted by a thymus cell preparation is due to contamination with B cells.

TABLE 77

Synthesis of Protein and IG by BALB/c Thymus Cells (θC3H)[a]

Treatment	% Cells remaining	% Protein	% Ig	Ratio of change in Ig protein
Anti-θ AKR (control)	99-100	100	100	-
Anti-θ C3H	1-2	3	85	28.3
Anti-κ	98-99	100	35	0.35

[a] 10^8 Thymus cells labeled with ^{3}H tyrosine gave 1.5×10^7 CPM in protein and 1.4×10^5 CPM in Ig (1%).

My interpretation of both sets of data is that there is little or no immunoglobulin on the surface of thymocytes and T cells, and that there is little or no immunoglobulin synthesized by thymocytes. It should be appreciated that I have come to this conclusion reluctantly. In summarizing the second Brook Lodge conference on "Mediators of Cellular Immunity," I recall saying that I considered it "unthinkable" that there would be a molecule *other* than immunoglobulin responsible for the specificity of T cells. However, I believe we must now consider the nature of the receptor as unanswered. Should we now consider the possibility that it is other than immunoglobulin?

I want to make a final point that bears on the findings by others associating 8S IgM with T cells. Figures 63 and 64 show that radioiodinated spleen cells release about one-third of their surface immunoglobulin during three hours of incubation. It is of interest that H-2 alloantigens are *not* released from the same aliquot of cells during this period of incubation. Thus, even if 8S IgM *is* found in secretions of T cells, it could have arisen from contaminating B cells.

WARNER: Following hard on the summation of Uhr's work, I would say at the outset that *every* experimental approach that has been carried out in our laboratory leads to the conclusion that the T cell *has* on its surface something which antigenically reacts with certain antiimmunoglobulin sera. Our thesis is, therefore, that the T cell is synthesizing some type of immunoglobulin gene product. While much of the data is published, I would like to recast it for the conferees once again so as to facilitate the comparisons many of you may want to make. The studies I will describe have been performed in collaboration with Harris, Bankhurst, Rouse, and Dwyer.

First of all, I would say that we totally accept the view that there are typical B cells in the thymus. I would disagree with Raff's view that it is a stem cell, because Basten has shown, using a marker of receptors for antigen-antibody complexes (which are not on T cells), that the thymus of certain mouse strains contains 0.2 to 2% of cells that react in this test; the values vary, depending on the mouse strain.

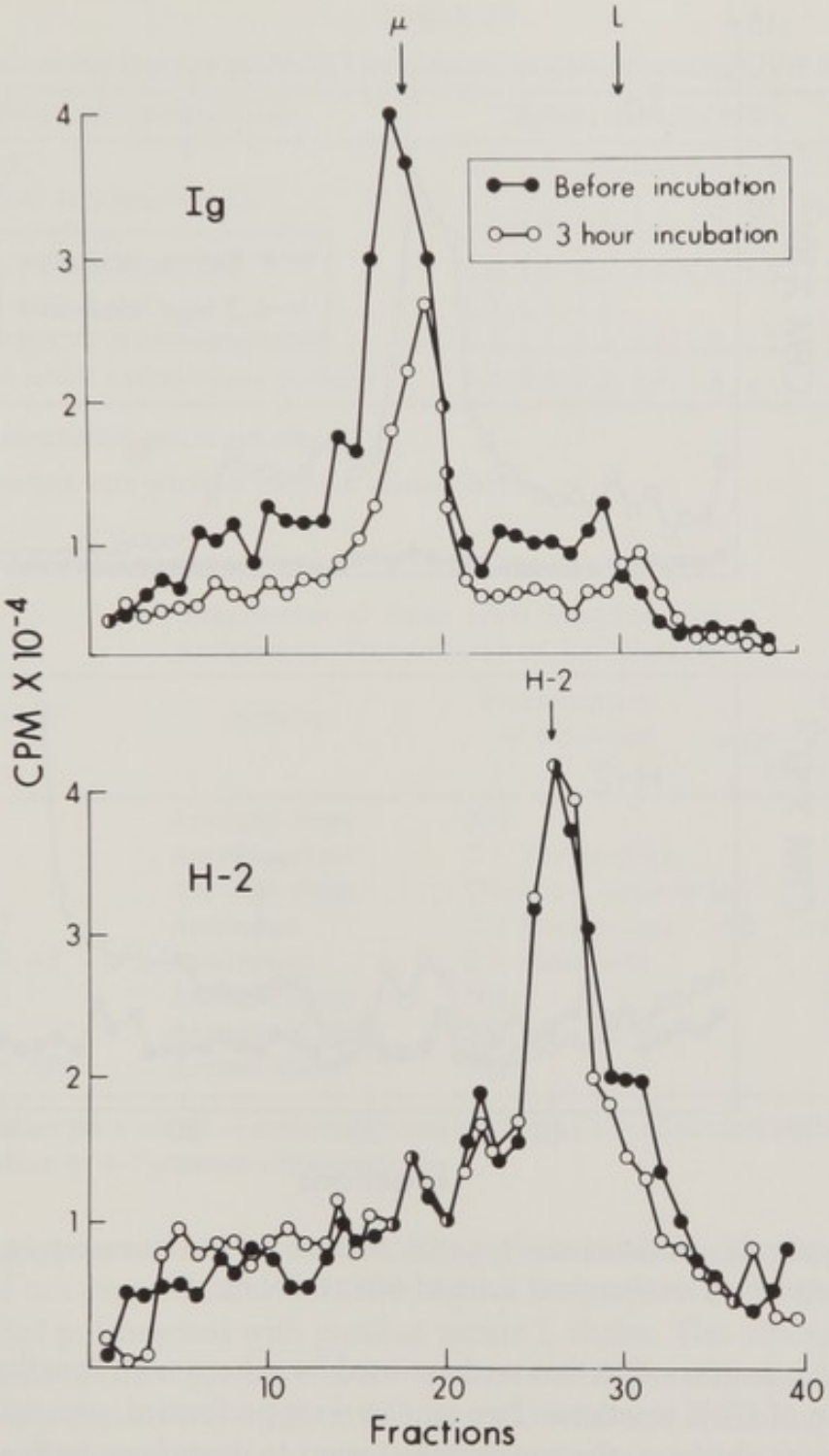


Fig. 63. Radioiodinated Ig and *H-2*^{a} from A/J murine splenic lymphocytes incubated *in vitro* for three hours. Samples of lysates and secretions were prepared before and after incubation and the Ig precipitated by a "sandwich" technique (using rabbit antimouse Ig and goat antirabbit Ig). Supernatants of the precipitates were then treated with anti *H-2*^{a} and the complexes precipitated with goat antimouse Ig. Both anti-Ig and anti-*H-2*^{a} precipitates were dissolved, reduced and alkylated and electrophoresed on SDS-acrylamide gels.

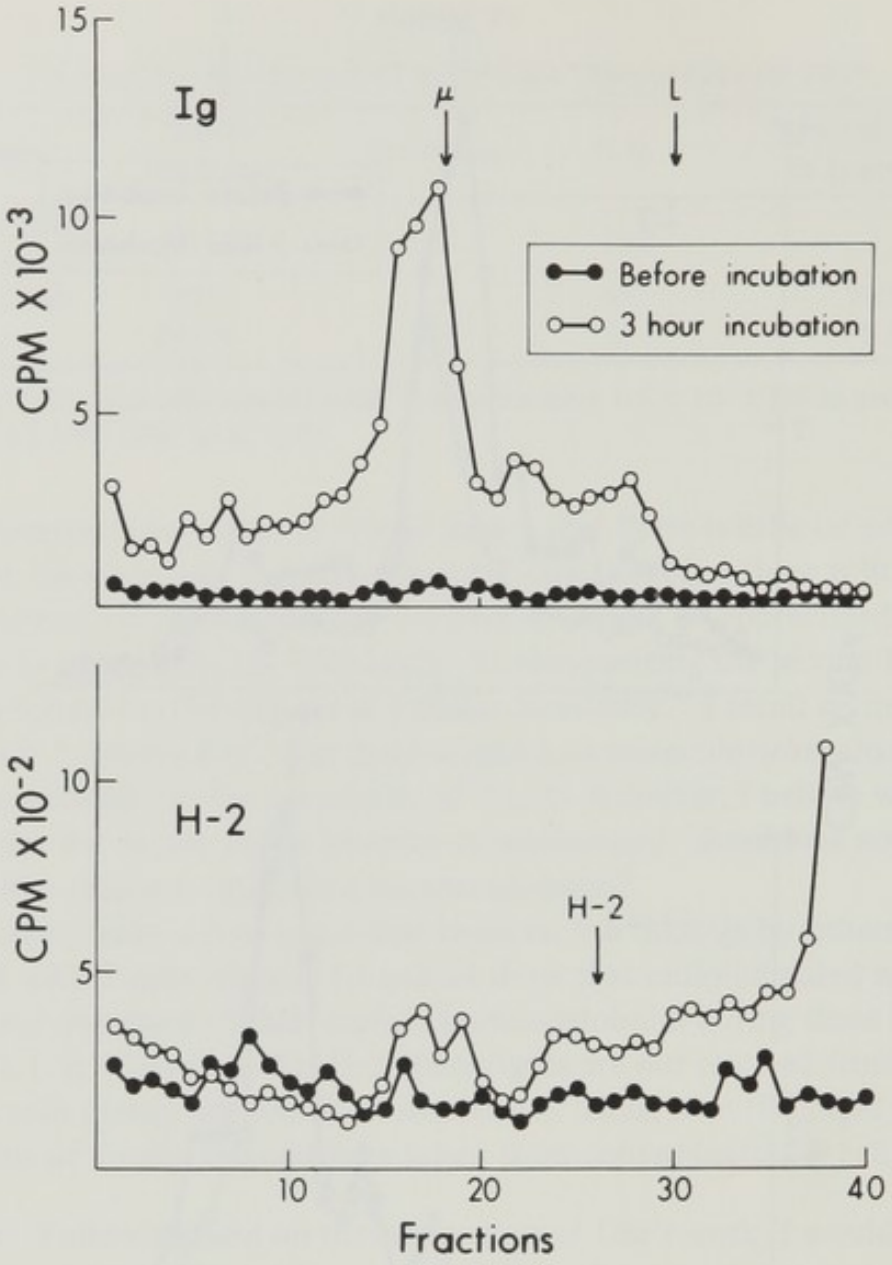


Fig. 64. Radioiodinated cell surface Ig and *H-2*^{a} recovered from the incubation medium of A/J splenic lymphocytes after three hours of culture. See Fig. 63.

The first approaches that we have used have been with functional tests on suppression of GVH reactions. Two studies were performed, one with mice and the other with chickens; the conclusions I want to draw from both are exactly the same.

Table 78 stresses the point that only certain antiglobulin sera will give the suppressive effect on T cells. The results show the chimeric spleen indices in which each value is the mean of a single experimental group, each value in turn being a mean for six to eight mice. There are three antiimmunoglobulin sera which have markedly suppressed, if not abolished, the reactivity of the T cells in inducing GVH. There was one experiment where this was not achieved, but in

TABLE 78

Effect of Antiimmunoglobulin Pretreatment of Cells Mediating GVH Reactions

Donor cell pretreatment	Spleen chimeric index	Mean
Eisen's B.S.S.	1.7, 2.1, 2.3, 2.3, 2.4, 2.6, 2.7	2.3
Specific rabbit anti-heavy chain sera (μ, γ, γ^2, α)	2.5, 3.4, 3.5, 5.3, 1.9, 2.3, 2.7	3.1
Rabbit anti-L chain (HPC-2)	1.0, 1.1, 1.1, 1.1, 1.3, 1.8,(2.7)[a]	1.2
Rabbit anti-HPC-3 (IgG_{2a})	1.3, 1.1, 1.2	1.2
Rabbit anti-HPC-1 microsomes (IgA)	1.2, 1.1, 1.0, 0.9, 1.2, 1.2,(2.5, 3.4)[b]	1.1
Unabsorbed rabbit antimyeloma proteins	3.4, 2.5, 2.3, 2.4, 1.8	2.5

[a]Preimmunization serum sample.

[b]Preabsorbed with purified mouse L chains (BJP).

TABLE 79

Suppression of Avian GVH Reaction with Antiglobulin Pretreatment of Lymphocytes

Antiserum	Activity	Preabsorption of antiserum	GVH[a] (% NRS treated control)
R71	Antilight chain	NIL	22.7
R71	Antilight chain	2 × Thymocytes	30.0
R71	Antilight chain	Chicken L chain or IgG	85.2
R76, 77	Antibursal	2 × Thymocytes	120.0
R78, 79, 43	Antithymus	2 × Bursa cells	14.0
R70, 45	Antilight chain	NIL	105.0
R75, 24	Antigamma chain	NIL	89.0
R69, 68, 6P1	Antimu chain	NIL	80.0

[a]Median pock count of antiserum treated group as a % of control NRS treated cells. (Mean of 4-7 separate experiments.)

the main, suppression was repeatable. The preimmunization bleeding of these rabbits did not suppress, and the main control is a preparation of the antiserum that was first preabsorbed with purified mouse L chains. This absorption completely removed the suppressive action of the serum. The second point that this table shows is that several other antiimmunoglobulin sera, including three different antisera having antilight chain activity in similar concentration (as measured by radioimmune precipitation assays) did not inhibit the GVH reaction, nor did any of the antiheavy chain sera that were used.

I would now like to indicate that we also have one antiserum against chicken immunoglobulin which blocks focus formation on chick chorioallantoic membranes. The reaction involves inoculating peripheral blood samples of adult chickens onto the chorioallantoic membrane of chick embryos. The results in Table 79 show the percent of the median focus count in the test group as com-

pared to the control, in which the blood was pretreated with normal rabbit serum. With rabbit antiserum to chicken thymus, which has been absorbed with normal bursal cells, suppression of the reaction was observed. Antiserum against bursal cells absorbed with thymus cells gives no inhibition. We are therefore dealing with a T cell-mediated immune reaction. One rabbit antichicken light chain serum also reduced the number of foci to about 25% of the control. This involved six to seven individual experiments and in all cases suppression was observed with this serum. Again, I would stress that the essential control which must be used in this type of study is the same preparation of antiserum that was preabsorbed with purified immunoglobulin. This was done and it resulted in a considerable removal of the suppressive activity of the serum. Absorption with thymus cells did not remove this suppressive effect. Again, however, we have two other antichicken light chain sera which have similar titers as determined by radioimmunoassays, but which show no suppression of GVH.

Also different antiheavy chain sera showed no effect. I would generally conclude that the sera that do work, do so because of an antiimmunoglobulin activity and not because of any nonspecific cytotoxicity for T cells. Suppressive activity is not related solely to the amount of antilight chain antibody in the serum; rather we would conclude that there may be a difference in the quality of the antisera.

Table 80 summarizes our results with B cells, and indeed is very consistent with the work Unanue described. Our purified B cell population is derived from athymic nude mice and contains about 97% B cells. With labeled anti-κ globulin we find about 94% of cells labeled. This is a specific reaction of the antiserum as pretreatment of the serum with purified light chains removes its binding activity. The results with B cells also indicate that there is a population of cells which have more than one heavy chain class of immunoglobulin on the cell surface.

TABLE 80

Labeling[a] of Lymphocytes from Nude and Normal Mice by ^{125}I-Antiimmunoglobulin Protein

Reagent	Nude TDL		Normal TDL	
	% cells[b]	Average %	% cells[b]	Average %
Antipolyvalent heavy chain	86.0, 87.3, 89.0	87.4	26.0, 33.0, 18.0	25.7
Antipolyvalent heavy chain blocked	0	0	0	0
Anti-K	90.0, 91.6, 100	93.9	27.0, 27.0, 18.0	24.0
Anti-K blocked	0	0	0	0
Anti-γ_2[c]	29.0, 43.0, 28.0	33.3	12.0, 18.0	15.0
Anti-μ[c]	78.0, 76.0, 76.3	76.3	25.0, 28.0, 18.0	23.6
Anti-α[c]	45.0, 40.0, 51.0	45.3	17.0, 20.0	18.5
Normal rabbit globulin	6.0, 2.1, 2.0	3.0	0 2.0	1.0

[a] 5×10^6 viable cells were reacted at 0°C for one hour with 6500-8000 nanograms of ^{125}I-antiimmunoglobulin IgG. These conditions provide near-optimal percentage labeling of cells.

[b] Each value was obtained from a cell suspension pool collected from 2-5 nude mice. Normal TDL labeling was performed with the same reagent simultaneously.

[c] These reagents have been shown to be specifically blocked by purified immunoglobulins in previous experiments.

TABLE 81

Labeling of Mouse "T" Lymphoid Cells with Antiglobulins

^{125}I IgG preparation[a]	% of labeled cells[b]	
	Thymus	T-TDL
Anti-K	17.0	49.0
Anti-K (blocked K)	0	0
Pool antiheavy chain	1.3	1.3
Normal rabbit	1.0	-

[a] 6-8 μgm per 10^7 cells.

[b] 54-60 day exposure of autoradiographs.

The main thing to stress about this approach with T cells is that we must use a different set of conditions for autoradiography. When autoradiographs are exposed for periods of around six to eight days, only a few percent of labeled cells are found in the thymus. Table 81 shows exactly the same method, but with autoradiographs exposed for about *two months*. Under *these* conditions, the thymus now shows about 17% of labeled cells, which is beyond any estimate of B cell contamination. Again, this finding is specific in that the blocked reagent does not label cells. In this instance, polyvalent antiheavy chain sera also do not show any labeling of cells.

With a population of T cells derived from the thoracic duct lymph of irradiated F_1 mice previously injected with adult thymus cells, and which are about 99% θ-positive, we found that at least half of them labeled with the antilight chain sera, but not with the antiheavy chain serum (Table 81). From these two approaches, it is suggested that T cells may have some sort of immunoglobulin on their surface. The percentages of labeled cells in these results cannot be explained by contaminating B cells.

These two methods do not, however, discriminate between synthesis of Ig and the possiblility that the T cell has cytophilically bound some serum immunoglobulin. We have, therefore, considered that the only specific way to demonstrate what the T cell expresses must involve synthesis studies. In Fig. 65 I would like to suggest that we have shown, in fact, an *Ir-1* gene product. The figure depicts a radioimmunoelectrophoresis of short-term cultures of mouse tumors. A labeled line, albeit faintly, is present in the lower two gels, and the obvious line in the top gel is from a plasma cell tumor. The approach used was the technique described by Thorbecke of short-term culture (24 hours) of fragments of tissue in roller tubes together with ^{14}C-labeled amino acids. What has been involved in this interaction is a mixture of secretion and intracellular contents, inasmuch as the cells had been disrupted, repeatedly extracted, dialyzed free of amino acids, and concentrated. This, then, is the complex mixture that was run in immunoelectrophoresis using carrier serum to develop the precipitin lines with

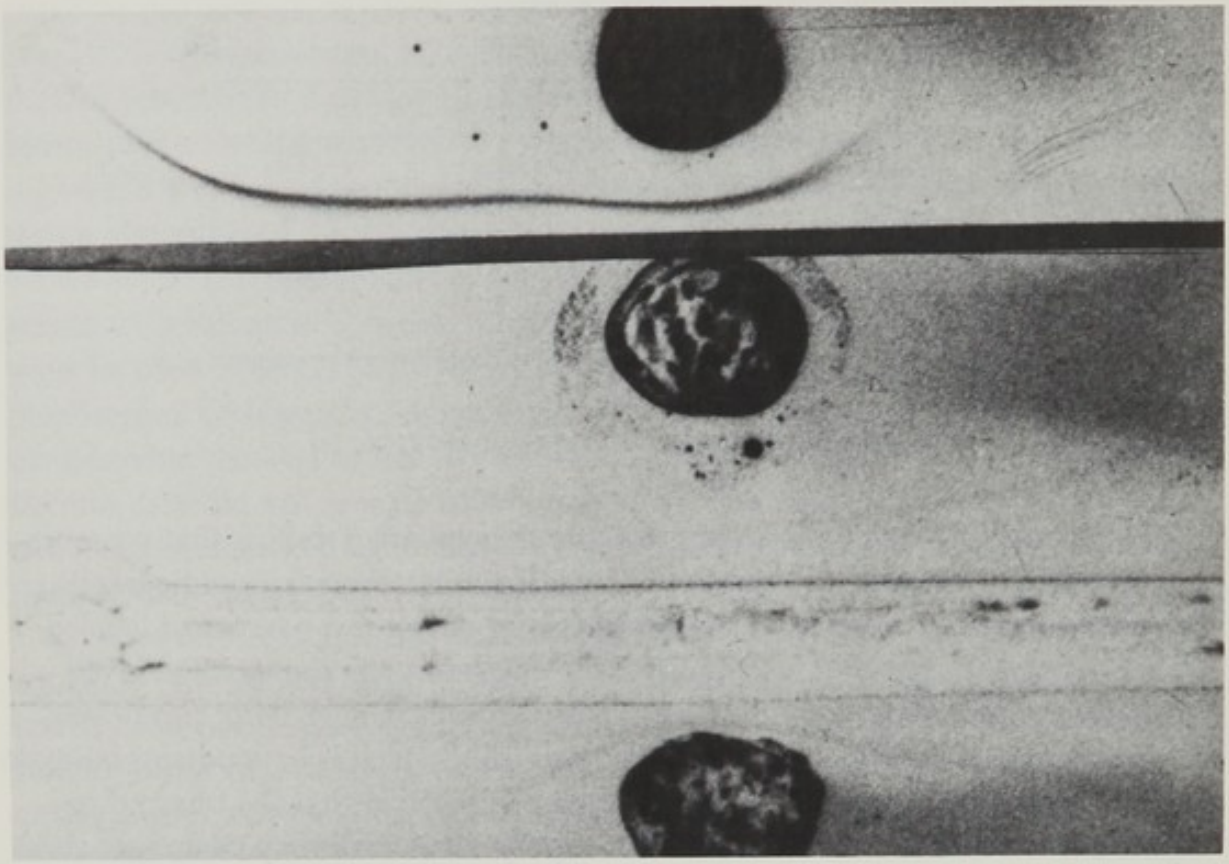


Fig. 65. Radioimmunoelectrophoresis of mouse thymoma.

polyvalent antiglobulin sera, and then prepared for radioautography. Figure 65 shows a culture from a plasma cell tumor in the upper gel; in this instance we have exposed the film for only a few days. An intensely labeled IgG_1 line is evident. In contrast, the lower two gels are cultures of radiation-induced thymomas; in this case the x-ray film has been exposed for two months. Again only a faint labeled line is evident, but the point to note is that there is labeling of only a *certain part* of the IgG line.

Although we have not yet performed ultracentrifugal gradient studies on this type of material, because of the low degree of labeling, we can say that with other types of cultures, such as those from plasma cell tumors secreting only light chains, this pattern of labeling is indicative of synthesis of only free light chains. The reason for the indicated distribution of label is that the migration of free light chains is different from that of intact immunoglobulin, but precipitation occurs as the antiserum *does* have antilight chain activity.

BENACERRAF: Was this particular experiment carried out with Warner's special type of antiserum, or could it be done with any other similar reagent?

WARNER: Development of the radioimmunoelectrophoresis involved antisera which had been used in the *in vitro* blocking studies of T cell immunity. We have

not yet used for precipitation a specific antilight chain serum that was shown to inhibit T cell functions *in vitro*.

Table 82 summarizes 19 radiation-induced thymomas studied for immunoglobulin synthesis. There are six lines which show this pattern of minimal light chain synthesis as detected only after long periods of exposure of the autoradiographs. With 12 lines, no immunoglobulin synthesis was detected in that period of time. One line (WEHI-22) shows specific IgM synthesis. In Table 83 the results of surface immunoglobulin labeling on WEHI-22 are given. This shows the percent of cells labeled with both antilight chain globulin and polyvalent antiheavy chain globulin, together with control specifically blocked antisera. The

TABLE 82

Immunoglobulin Synthesis by Murine Lymphoid Tumors

Tumor line	Type	Strain	Immunoglobulin synthesis
WEHI - 7	Thymoma[b]	BALB/c	None detected
- 14	Thymoma[b]	BALB/c	None detected
- 16	Thymoma[b]	BALB/c	None detected
- 20	Thymoma[b]	BALB/c	None detected
- 22	Thymoma[b]	BALB/c	IgM
- 24	Thymoma[b]	BALB/c	None detected
- 26	Thymoma[b]	BALB/c	Trace L chain
- 27	Thymoma[b]	BALB/c	None detected
- 31	Thymoma[b]	BALB/c	Trace L chain
- 56	Thymoma[b]	BALB/c	L chain
- 105	Thymoma[b]	NZB	L chain
- 106	Thymoma[b]	NZW	None detected
- 112	Thymoma[b]	NZB	None detected
- 113	Thymoma[b]	NZB	None detected
- 114	Thymoma[b]	NZB	None detected
- 124	Thymoma[b]	NZW	L chain
- 126	Thymoma[b]	NZB	L chain
- 128	Thymoma[b]	NZB	None detected
- 129	Thymoma[b]	NZB	None detected
- 2	Lymphoma[c]	(NZB × BALB/c)F_1	L chain $^{+++}$
- 5	Lymphoma[c]	(NZB × BALB/c)F_1	L chain, trace IgM
- 6	Lymphoma[c]	(NZB × 57 BL)F_1	IgM
- 37	Lymphoma[c]	(NZB × 57 BL)F_1^1	L chain $^{+++}$

[a] Detected by radioimmunoelectrophoresis of cell content after short term culture with ^{14}C amino acids.

[b] Radiation induced lymphoid thymomas.

[c] Spontaneous lymphomas arising in spleen or lymph nodes.
Thymomas: 12/19 No Ig synthesis detected.
6/19 Minimal L chain synthesis.
1/19 Minimal IgM synthesis.

TABLE 83

Surface Immunoglobulins on a Murine Thymoma Line

Cell source	% Labeled cells			
	Anti-K	K blocked	Anti-H	H blocked
(CBA × C57)F_1 spleen	48	2	46	0
(CBA × C57)F_1 thymus	1	0	1	0
WEHI-105-7	0	0	0	0
WEHI-22	89	4	90	0

first lines of the table show that this experiment was performed with B cell labeling conditions, i.e., short-term exposure of the autoradiographs, in that thymus does not label but the spleen shows about 46% of labeled cells.

The tumor cells used in this study are not from *in vivo* thymic tumor tissue, but an *in vitro* cloned line, derived by Harris, cultured from a single cell and grown continuously in tissue culture. Both of these thymoma lines are completely killed by anti-θ sera. Line WEHI-22 also shows most cells labeling with anti-light and anti-heavy chain sera, which is not found in the case of WEHI-105. This other line (WEHI-105) is the line that showed light chain synthesis. It would seem that the WEHI-22 line that synthesizes IgM, has B cell-type concentrations of IgM on its cell surface. The problem that we have at the moment with this line, is that we have now also shown that this line carries the receptor for antigen-antibody complexes, and in inhibition studies with myeloma proteins that the specificity of this receptor on this line is exactly that of B cells. In this particular instance we are faced with two alternative explanations of the results. First, that we have a line of cells which are typical B cells, synthesizing IgM and holding it on the cell membrane, but because the clone was present in the thymus, at least at the time of tumor induction, had also expressed the θ gene, and hence the cells are positive for both θ and IgM.

The alternative explanation is that the clone is a typical T cell, but the IgM that is carried on the cell surface has an antigammaglobulin specificity. This would explain why the line gives rosettes with immunoglobulin coated SRBC. At the moment this WEHI-22 line has to be evaluated further so the best data that we can cite on T cell immunoglobulin is the line (WEHI-105) which is θ-positive, is cloned in culture and synthesizes a protein which behaves like a light chain or a light chain component in radioimmunoelectrophoresis.

Figure 66 shows a similar result from a normal cell population as it may not be justifiable to extrapolate results on immunoglobulin gene expression in malignant cells to normal cells. There is a faint but definite labeled line in the lower gels which are from cultures of spleens from bursectomized agammaglobulinemic chickens. These animals have no B cells as shown by absence of binding of labeled antiglobulins. If one presumed that there may be a small number of B

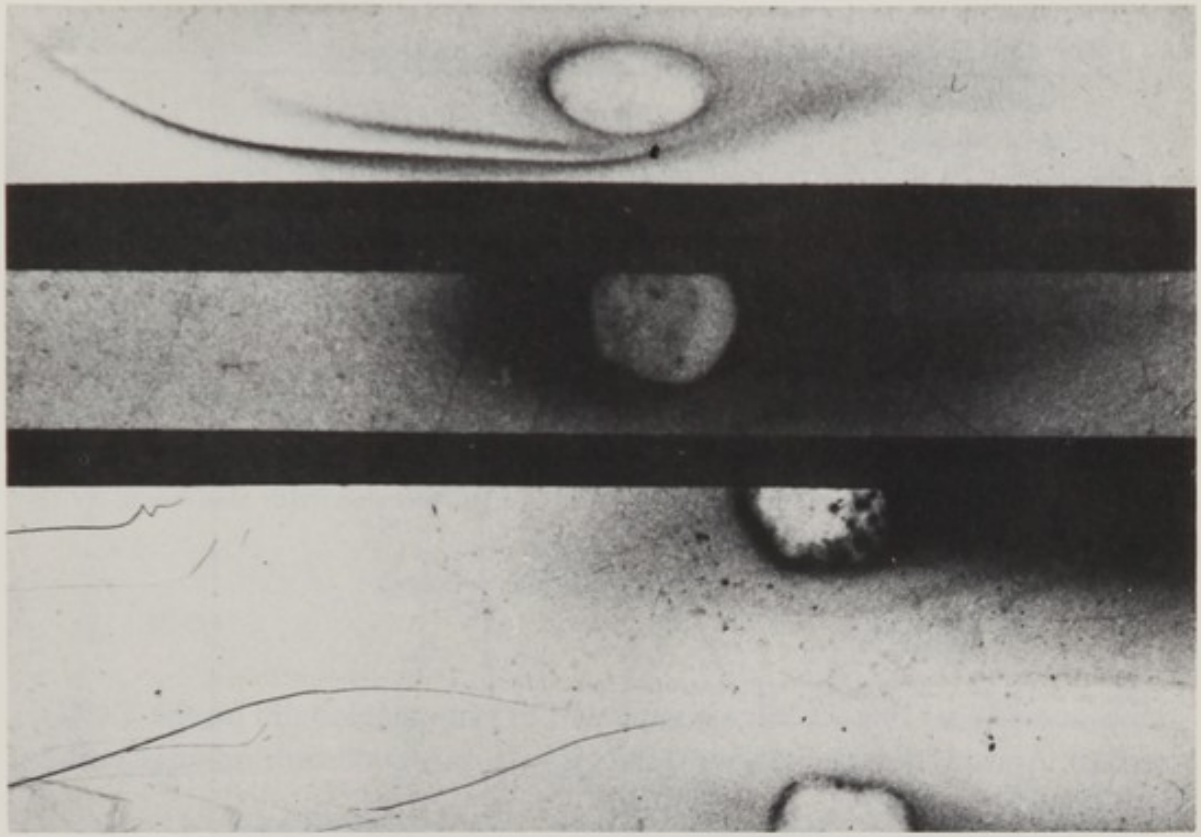


Fig. 66. Radioimmunoelectrophoresis of spleen cultures of agammaglobulinemic bursaless chickens.

cells still left, these chickens should have shown a small amount of intact IgM synthesis, but instead we find the differential labeling of the anodal end of the IgG arc, indicative of free light chain synthesis. The upper gel shows normal IgM and IgG synthesis in a control age-matched chicken spleen.

Table 84 gives results on antigen-binding cells in the thymus. These studies, performed with Dwyer, have used fetal and neonatal thymus of mouse and man; in both cases we do find antigen-binding cells. The detection of these antigen-binding cells can be totally inhibited by either antilight chain or anti-μ chain pretreatment of cells in both man and mouse. Therefore we conclude that there are immunoglobulin bearing antigen-binding cells in the thymus, but in this instance we do not have any direct proof that these are T cells and they may possibly represent a small population of B cells which have cytophilically-bound IgM.

BENACERRAF: Does Warner imply that *any* garden variety antiimmunoglobulin serum is effective?

WARNER: All antiimmunoglobulin sera to light chains studied will show this inhibition of antigen-binding. On the basis of these extensive studies on T cells

TABLE 84

Inhibition of Thymic Antigen-Binding Cells (ABC) with Antiimmunoglobulin Sera

Antiserum pretreatment	% Inhibition of ABC formation[a]			
	Mouse		Human	
Anti-k	73, 100, 100	(91%)	41, 46, 53, 61, 66	(53%)
Anti-λ	nt		40	(40%)
Anti-μ	86, 89, 100	(92%)	80, 88, 88, 99, 100	(91%)
Anti-α	0, 0, 0	(0%)	0, 11	(5%)
Anti-γ_1[b]	0	(0%)	0, 0, 0, 0, 8, 10	(3%)
Anti-γ_2	0, 0, 0	(0%)		

[a] % inhibition relative to control cell with no antiserum pretreatment. Each value is for an individual thymus cell suspension with the mean values given in brackets.

[b] For human, anti-γ reacting with all γ-classes.

nt: not tested.

we conclude that there is some component of immunoglobulin on the T cell surface. A major question of our results is why only some antiimmunoglobulin sera show this result.

A hypothesis to explain this is that there is a difference in the quality of the sera; it may be that only antisera with antivariable region activity will show these results. It could also be suggested that there is a separate variable region gene which is expressed only in T cells, but reacts with some other anti-V region antisera.[a]

[a]For completeness of the record, the editors invited Warner to summarize the work of his colleagues, Marchalonis, Cone, Atwell, and Sprent, which also strongly supports the concept that T cells possess surface immunoglobulin. Their direct isolation and partial characterization of the surface immunoglobulin, moreover, has provided evidence that the molecule is composed of light chains and μ-type heavy chains. T cells of CBA mice were activated to BALB/c or C57BL histocompatibility antigens by injection of CBA thymus cells into lethally irradiated F_1 hybrids (CBA × BALB/c) or (CBA × C57BL). Thoracic duct lymphocytes taken from the recipient animals at day four after reconstitution (CBA-T-TDL's) were 95-100% θ-positive CBA cells. The activated T cells were surface radioiodinated with ^{125}I iodide using the lactoperoxidase catalyzed technique (Marchalonis, Cone, and Santer, *Biochem. J.*, **124**, 921, 1971). Soluble cell surface protein was obtained by metabolic turnover (Cone, Marchalonis, and Rolley, *J. Exp. Med.*, **134**,1373, 1971), and the amount of high molecular weight material assessed by gel filtration on Sephadex G-25. Eight % of this high molecular weight protein was specifically coprecipitated by rabbit antiserum possessing activity towards the light chains and μ chains of mouse γM immunoglobulin (Marchalonis, Cone, and Atwell, *J. Exp. Med.*, **135**, 956, 1972). Counts in ^{125}I-labeled surface proteins precipitated by the antiimmunoglobulin were six to ten fold higher than those brought down by normal rabbit serum, and four to seven fold higher than counts precipitated by hemocyanin- antihemocyanin or BSA- anti-BSA. The coprecipitated cell surface protein was solubilized in 9M urea-ten % acetic acid and resolved by polyacrylamide gel electro-

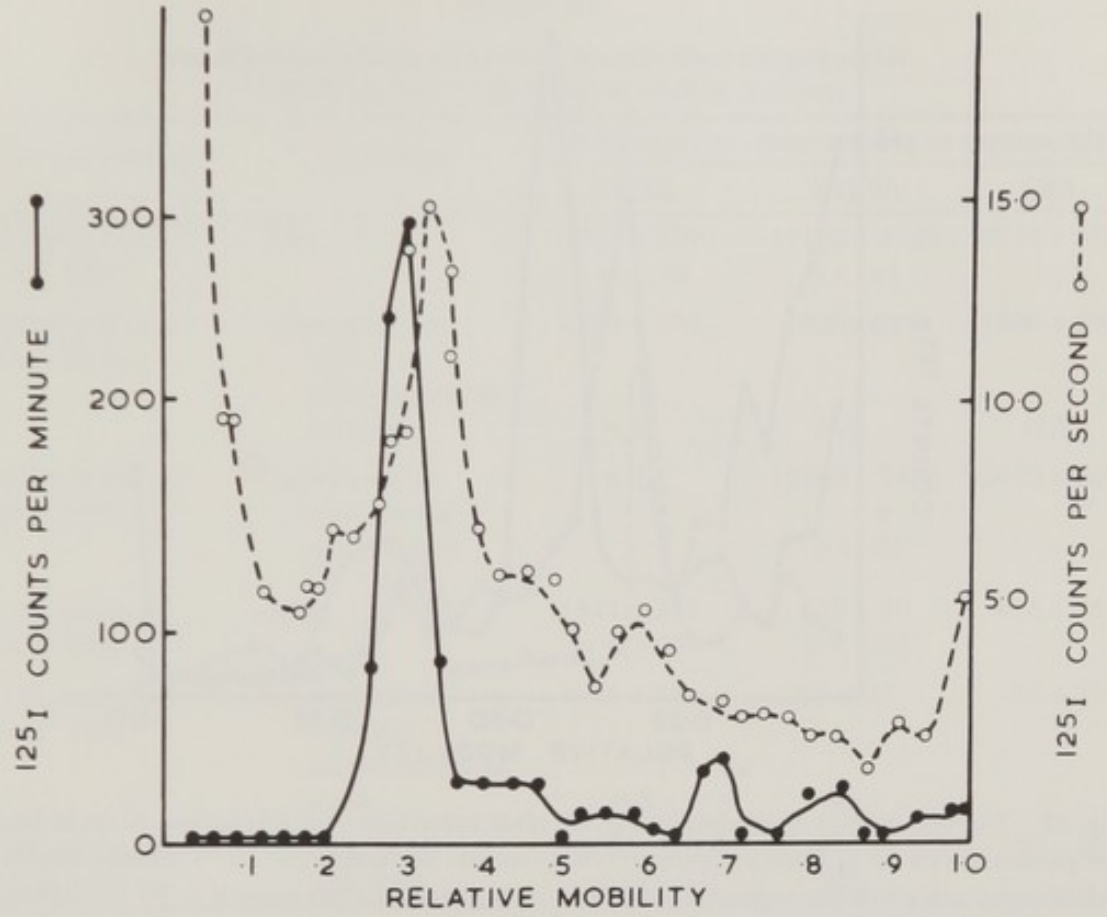


Fig. 67. Acrylamide gel electrophoresis in acid urea of cell-surface Ig from radioiodinated activated T cells. Closed circles indicate the pattern of specifically coprecipitated T cell Ig (counts per minute). Open circles (dotted line) indicates the pattern shown by ^{125}I-labeled mouse IgG coprecipitated under the same conditions (counts per second).

Since division of > 20% of CBA T-TDL's could be initiated specifically by contact with the histocompatibility antigens used in the activation process (Cheers and Sprent, Fourth Int. Conf. Lymph. Germinal Centers in the Immune Response, 1972), it was feasible to ascertain the binding specificity of cell surface immunoglobulin. Soluble ^{125}I-labeled cell surface protein was obtained

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phoresis in acid urea. As shown in Fig. 67, only one component (heavy line) was observed. It migrated slightly more slowly than the peak of purified mouse γG immunoglobulin coprecipitated in the same fashion (dotted line). Gel filtration analysis of this component indicated that it possessed a molecular weight of approximately 180,000 daltons. The specifically coprecipitated cell surface immunoglobulin was reduced and alkylated and subjected to disc electrophoresis under conditions which resolve polypeptide chains (Fig. 68). Coprecipitated, reduced alkylated γG (•—•) and γM (○--○) served as controls to indicate the position of light chains (RF .55-.75), γ chains (RF .40-.48) and μ chains (RF .27-.33). The surface immunoglobulin from activated T cells (*—*) possessed light chains and heavy chains similar to μ chains in gel penetration.

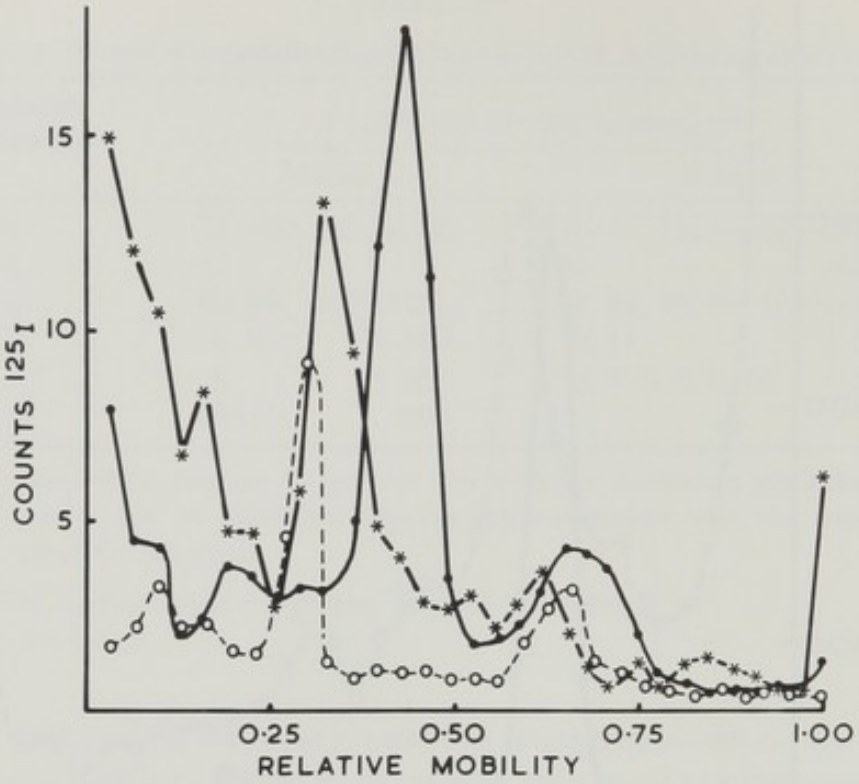

Fig. 68. The cell surface Ig shown in Fig. 67 after reduction and alkylation. Stars indicate the pattern given by Ig from activated T cells (counts per second $\times 10^{-2}$). Closed circles indicate pattern given by coprecipitated IgG control (counts per second $\times 10^{-1}$). Open circles (dotted line) give data points for coprecipitated IgM standard (counts per second $\times 10^{-1}$). Relative mobilities of standard chains: light, 0.55-0.75; γ, 0.40-0.48; μ, 0.27-0.33.

and incubated at 4°C with 10^7 allogeneic or syngeneic thymus cells with the results shown in Table 85. Binding specificity was present in labeled protein from CBA T-TDL's activated to C57BL or BALB/c antigens. This binding specificity was totally abrogated by coprecipitation with antiimmunoglobulin, but treatment with normal rabbit serum had no effect. Crossreaction between BALB/c and C57BL was observed, this being consistent with the fact that these strains share certain H-2 antigens. The possibility that the immunoglobulin detected here resulted from contamination with trace amounts of B cells was considered. However, this complication was thought to be unlikely because the methods used allow the isolation of comparable amounts of immunoglobulin from T cells and B cells. In addition, even if T cells contained many fewer surface immunoglobulin molecules than do B cells, the possibility of these results arising from B cell contamination was improbable because at this low level of B cell contamination (<0.3 percent, Basten, Miller, Sprent, and Pye, *J. Exp. Med.*, **135**, 610, 1972) all of the B cells would have to react to the particular antigens used, and furthermore these cells would have to possess about 100 times more surface immunoglobulin than that of typical B cells. Similar results were obtained with T cells activated to protein and erythrocyte antigens.

TABLE 85

Specificity of Thymus-Derived Thoracic Duct Lymphocytes
^{125}I-Cell Surface Proteins for Activating Antigens

Cell surface proteins	Treatment	Radioactivity (cpm) binding to thymus cells		
		C57BL	BALB/c	CBA
BALB/c-activated CBA T. TDL	none	7900 ± 2500 $p < .05$	17180 ± 4700 $p < .01$	3730 ± 550
BALB/c-activated CBA T. TDL	coprecipitated with anti-immunoglobulin antiserum	1300 ± 300 N.S.	1800 ± 400 N.S.	1400 ± 200 N.S.
BALB/c activated CBA T. TDL	coprecipitated with normal rabbit serum	n.d.	12200 ± 2400 $p < .01$	2400 ± 800
C57BL-activated CBA T. TDL	none	1544 ± 252 $p < .001$	630 ± 30 $p < .001$	416 ± 35
C57BL-activated CBA T. TDL	coprecipitated with anti-immunoglobulin serum	46 ± 30 N.S.	107 ± 97 N.S.	33 ± 20
C57BL-activated CBA T. TDL	coprecipitated with NRS	900 ± 100 < .001	240 ± 10 N.S.	180 ± 40

p-values given here represent comparisons of counts bound to the cells indicated with those bound to the CBA control. The Student's t-test modified for small sample size was used in the calculations. For BALB/c activated T. TDL, the *p*-value for the comparison BALB/c vs. C57BL was < .05. For C57BL activated T. TDL, the *p*-value for the comparison C57BL vs. BALB/c was < .001.

Populations of T. TDL activated to H-2 antigens were obtained by the method of Sprent and Miller *Nature, New Biol.*, **234**: 195, 1971. Briefly, three month old (CBA × C57BL)F_1 or (CBA × BALB/c)F_1 mice were injected i.v. with 2×10^8 CBA thymus cells within 2 hours of receiving 750r total body irradiation. Four days later the thoracic ducts of these mice were cannulated and lymph collected over the first 12-16 hours of drainage.

95-100% of thoracic duct cells obtained in this way are cells of donor thymus origin. Such preparations contain less than 0.3% B lymphocytes. CBA thymus cells activated to BALB/c antigens are termed BALB/c-activated CBA T.TDL; CBA thymus cells activated to C57BL H-2 antigens are termed C57BL-activated CBA T.TDL.

BENACERRAF: If I understood Warner correctly, he has two sets of observations, one of them obtained with just about any antiimmunoglobulin serum. These are concerned primarily with the cells synthesizing immunoglobulin. He has another set of observations which require the use of peculiar antiimmunoglobulin sera, and not others, to obtain inhibition of T cell function.

WARNER: To be specific, we used one standard serum for the development of all the radioimmunoelectrophoreses, and that antiserum has not been used in

any of the blocking studies. We clearly need to do more tests with all sera in all situations before we can be sure of the answer to Benacerraf's question.

BENACERRAF: Sela has the greatest experience concerning specificity and crossreactivity. I would like to ask him to what extent can Warner's results which are obtained with selective antisera, be explained by a weak crossreactivity between the anti-L chain sera and some unknown protein on the surface of the thymocyte?

SELA: The general answer to Benacerraf's query is that one can obviously have two proteins which crossreact to a small extent and there can be crossreactions of the kind he suggests. Since Warner mentioned fibroblasts, I would like to add that we can kill them with both anticollagen sera and with antibodies against a synthetic antigen resembling collagen, which is a case in point of such a crossreaction (Maoz, Fuder, and Sela, unpublished data). As to the question of whether there can be something which is not immunoglobulin and may nevertheless crossreact with an immunoglobulin system, the answer is, yes of course, until proved to the contrary.

UHR: I would like to integrate these *seemingly* disparate investigations and make several points. First of all, the results of three independent studies that have been presented at this conference regarding T cell receptors are not that far apart: Unanue's data and our own, obtained from two entirely different approaches fit together like hand and glove. Warner's data suggests to him that there is a small amount of immunoglobulin, possibly a fragment, on thymocytes or T cells. These three studies stand in sharp contrast with the results of Hämmerling and Rajewsky (*Eur. J. of Immunol.*, **1**, 447, 1971), suggesting that there is no major difference between the density of Ig on T and B cells as observed by immunoelectron microscopy[a] and the results of Marchalonis *et al.* (*J. Exp. Med.*, **135**, 959, 1972), using a technique remarkably similar to our own, that there are equivalent amounts of immunoglobulin on thymocytes, T cells and B cells. Incidentally, the last two studies disagree with each other because Hämmerling and Rajewsky find *no* Ig on thymocytes.

Another point is an amplification of Benacerraf's comment It is an uncomfortable and probably untenable position to have a half dozen or so antisera which are not distinguishable in terms of their reactivity with κ and μ chain, but

[a]AFTERTHOUGHT BY RAJEWSKY: Since this point came up here again, it appears appropriate to cite from the discussion of our paper: "Note in this connection that the hybrid antibody technique as used in these experiments does not enable one to easily distinguish between classes of cells which differ in the density of a given surface antigen. Our data do, therefore, not argue against the possibility that T cells have a lower receptor density than B cells."

only one of which will block a T cell function. Clearly there is a possibility that *other* specificities are responsible for the blocking. What evidence is there to exclude the possibility that the rabbit antisera that do the blocking may have specificities for other surface antigens on mouse thymocytes. Certainly some rabbit sera are cytotoxic for thymocytes. The immediate question that arises is how can one explain absorption with Ig or Bence-Jones protein. I want to emphasize that both the B lymphocyte and the thymocyte are very actively releasing many of their surface proteins. I mentioned that release of H-2 alloantigens has *not* been detected, but many other nonimmunoglobulin proteins *are* rapidly released. In fact, with murine B lymphocytes, the proportion of nonimmunoglobulin proteins in the incubation medium is similar to that present on the cell surface. It is possible, therefore, that surface proteins might be present in the serum and urine, and might not be readily separated from myeloma proteins.

WARNER: One could not formally distinguish between the immunoglobulin and the possibility that there is a nonimmunoglobulin product released by cells which could pass into the urine and be of similar molecular size to light chains; and that an antibody to this component is also present in the antilight chain sera. However, on acrylamide gel electrophoresis of our light chain preparations there appears to be only a single band, and furthermore ^{125}I-labeled light chains are specifically precipitable with virtually all of the antilight chain sera. If there were another protein admixed in the preparation, it would have to be very similar in charge and size as to Bence-Jones protein and be in relatively low amount. That possibility could not be excluded.

My own feeling as to why there is a difference in antilight chain is based on our observations with certain antilight chain sera made against human purified Bence-Jones protein, in that some, but not all, can be shown to have low levels of anti-V region activity, by their ability to distinguish κ subgroups. I would estimate that the amount of the anti-V region antibody would only be of the order of one to ten % of the total antilight chain activity of the antisera. What we need to do, and have not yet done, is to attempt to demonstrate that these sera in mice that act on T cells may be ones that show anti-V region activity.

CHAIRMAN RAJEWSKY: One should cite Greaves' results in this context; he found that T cell rosettes were inhibited only by those anti-μ sera which contained antibody to the Fd-piece and the hinge region of the μ chain.

SELA: I would like to ask the various contributors at this session whether any effort has been made to unveil possible cryptic immunoglobulin receptors by such approaches as cleavage with trypsin or neuraminidase?

UNANUE: We tried, with negative results, i.e., treated cells did not reveal Ig.

RAFF: Ashman, Greaves, and I have independently done similar experiments without exposing immunoglobulin on T cells.

McDEVITT: What did Raff do?

RAFF: Basically we have found that trypsin and neuraminidase treatment of spleen or thymus cells does not seem to expose Ig on thymus or T lymphocytes.

McDEVITT: Were you finding immunoglobulin on the lymphocytes?

RAFF: No.

BENACERRAF: I would ask Warner whether his interpretation, therefore, is that the antiimmunoglobulin antisera which are active in the inhibition test owe their activity to the fact that they are specific for some "V" region sequences of the κ chain. The activity of these sera may be absorbed by κ, so therefore, they must be specific for some "V" region of the κ chain.

WARNER: The antisera are made against κ chains, but I am suggesting that the "V" region presented on T cells may not be κ, but rather the product of this gene antigenically crossreacts with the κ chain.

BENACERRAF: That may be—but the "V" region genes of the heavy chain and the variable region genes of the κ chain are, by sequences, believed not to be related.

WARNER: I realize that but there is a paucity of data on antigenic crossreactions.

BENACERRAF: Regarding the crossreactivity hypothesis, I believe that Warner must consider two possibilities: 1. he is dealing with selected κ V region on the surface of thymocytes without κ constant regions; 2. he is dealing with a new set of V regions unrelated to κ, but crossreacting with it.

WARNER: Yes. I agree with Benacerraf's reasoning. The principal motivation for my preferring to say that it may be a different V region gene is on the basis of the immune response gene study, which demonstrates antigen-binding B cells *in vitro* with antigens to which the T cells are nonresponsive. We would say that this B cell must have a V-region gene associated with this particular antigen, but if the T cell is different (nonresponsive) that is why we would suggest it might be a different V-region gene in the T cell, rather than being κ.

BODMER: As a complete outsider in this technically involved area, I would like to ask the following question: Is it completely ruled out that any of these sera contain specificities not directed at proteins at all, but rather at the polysaccharide components? The reason I ask this is that it is fairly clear that the natural antibody present in the sera of all normal rabbits, which is reactive with human cells, is directed against polysaccharide components. Some preliminary work we have done suggests that it can be inhibited with considerable specificity by the polysaccharide side chains of human IgM.

WARNER: If there is polysaccharide on the purified light chains used then the possibility is not ruled out.

SIMONSEN: I am of course very interested, but a little disturbed by the fact that Warner has been able to neutralize the GVH reaction, particularly in the chicken, by means of antilight chain antibodies, albeit that only one out of many sera worked. Now, as Warner knows, in our work (*Transplant. Rev.*, **10**, 36, 1972), it was possible to get neutralization of the GVH reaction with sera of antimembrane specificity in high dilutions. The contamination of an anti-L chain serum with say one part in 10,000, would be fully adequate to explain this. It is very difficult to rule out that such degree of contamination could have occurred. Therefore, the only part of Warner's experiment which really disturbed me was his failure to absorb the blocking effect with thymus cells. Consequently, I ask him whether the inhibition he got with that exceptional serum was done with undiluted serum. Finally, did Warner test a preimmunization bleeding from the same rabbit?

WARNER: The cell incubations are done with approximately 20-30% serum concentrations. The antiserum was made against purified chicken immunoglobulin light chains, prepared by salt precipitation and column chromatography, from the serum of normal chickens. The same light chain preparation was used for the blocking of antiserum. Finally, we did *not* test a prebleed serum of this rabbit. I fully recognize that many rabbit sera can have cytotoxic effects but this activity may change in the rabbit serum with time, which is why we do not consider a prebleed as the best control. To my way of thinking the only control is to take the same preparation of antiserum which we are using and to block it by absorption with purified immunoglobulin.

SIMONSEN: Yes, that is so; however, the blocking is of course in doubt if Warner assumes that the light chain preparation he started with might have been contaminated with membranes.

WARNER: Again, one cannot strictly rule out the possibility. If there is a membrane component which migrates electrophoretically in the same way as light chain and is the same size, then one cannot rule that out.

UHR: I had previously intended to mention that immunoglobulin released from the B cell is attached to a fragment of membrane. This is deduced from the facts that labeled lipid can be shown to be bound noncovalently to the released immunoglobulin, and that density gradient analysis of secretions shows that 70% of the released immunoglobulin molecules have a density corresponding to high density lipoprotein. If secretions are pretreated with detergent, all of the released immunoglobulin has the density of protein. These observations may bear on the absorption studies with Bence-Jones or other myeloma proteins.

VAN ROOD: It is significant and relevant to this discussion that HL-A antigens have been shown to be present in normal serum, albeit in minute amounts. I cannot visualize all the experiments that have been presented to us but it is not at all inconceivable that Warner's rabbits formed anti-H antibodies against this small amount of H antigen present in normal serum.

WARNER: I would make the same answer as my last reply to Simonsen. van Rood has reminded us of an important, cogent possibility—I accept it as a possibility—we will have to do the appropriate experiments and see.

CHAIRMAN RAJEWSKY: We should now proceed to the problem of T cell receptors in a more general way. In particular, I think that we should make an attempt to see the problem of the presence or absence of Ig on T cells which we have been discussing so far, in the context of *other* studies which have revealed highly relevant features of T cell recognition.

Besides the considerable difficulties encountered in attempts to demonstrate antigen-binding T cells, I should like to mention Wigzell's finding, which we have confirmed, namely that helper T cells in contrast to B cells, pass through antigen-coated columns. This tells us that there must be *something* peculiar about the receptors on T cells which distinguishes them from B cell receptors in some way. They may, for example, have a much lower density than B cell receptors or they may be more easily shed. This could be relevant to the fact that many investigators have had difficulty in detecting Ig. Raff will assess this entire issue for us.

RAFF: I would hate to see the "immunoglobulin receptor on T cell" notion die here without at least some attempt at a last stand. There are four types of studies that are pertinent:

1. *The lacto peroxidase iodination studies* have just been reviewed in detail by Uhr and it is clear that there is no obvious way at the moment of reconciling his results with those of Marchalonis. Certainly the Vitetta and Uhr experiments are the most compelling negative experiments to date and suggest that burial of Ig determinants, or falling off of Ig molecules from the membrane cannot be the only reasons for failing to detect Ig on T cells.

2. *The direct methods,* which involve transformation of lymphocytes with anti-Ig serum, or labeling of lymphocytes with various types of labeled anti-Ig antibodies—by and large have failed to detect Ig on T cells as Unanue has just noted—with but few exceptions, perhaps the most compelling being the study of Hämmerling and Rajewsky. It is difficult to explain their startling results since in their work, virtually all T cells had IgM, and *not* just a few hundred molecules.

3. The studies involving *inhibition of T cell function* are particularly interesting in that some type of pattern seems to be emerging. Attempts to inhibit T cell helper function with antilight chain, anti-μ, sera have for the most part been successful. This now includes both *in vitro* and *in vivo* assays of helper function, and I am not aware of any published failures. On the other hand, attempts to inhibit T cell responses to histocompatibility antigens with anti-Ig sera have mostly failed. In the case of the rare anti-Ig serum that inhibits GVH responses, I am sure Warner would agree that the inhibition is unlikely to be due to a steric interference with "recognition" of antigen—as the receptors are almost certainly turning over rapidly, and would presumably regenerate in time to respond to the alloantigens of the adoptive host. The inhibition is more likely to be related to opsinization or altered migration of injected cells, and says little about the nature of the receptor interacting with the alloantigens. All attempts to inhibit T cell-mediated killing of allogeneic target cells *in vitro* have failed and most attempts to inhibit MLC also have been unsuccessful. Although Greaves and Roitt were able to inhibit MLC of human lymphocytes with anti-L sera, recently Roitt has not been able to repeat these observations with a variety of different anti-L sera. And so it may be time to give up the idea that T cell recognition of histocompatibility antigens involves Ig receptors.

4. On the other hand, if one looks at studies involving *antigen-binding by T cells*, two important points emerge. Firstly, as Rajewsky pointed out, it has been exceedingly difficult to demonstrate T cells binding antigen. Thus, no matter what the chemical nature of the T cell receptor, there is an important difference between T and B cells in this respect, which has not been explained. Secondly, it looks as if T cells may "prefer" seeing antigen on the surface of *living* cells; thus, T cells have been shown to bind specifically to the surface of allogeneic target cells and it *is* easier to demonstrate antigen-binding T cells by the rosette techniques than by those other methods that involve labeled soluble antigen. It may be that *in vivo* T cells "recognize" antigens preferentially, or

solely, on the surface of macrophages and/or B cells. Even in the rosette-forming systems, it is clear that T cell rosettes are extremely fragile and that unless special precautions are taken, they will break up and not be detected. This is almost certainly the reason for the prolonged controversy as to whether T-RFC's really exist. Recently Ashman and I have been able to definitively demonstrate T-RFC's using fluorescein-labeled anti-θ serum, which could be shown to be entirely specific for the θ alloantigen and for T cells in immunofluorescence assays. Thus, the existence of T-RFC's should no longer be a point of debate. I personally find that many experiments showing that T-RFC can be inhibited by anti-Ig sera very compelling, in view of the rigorous controls concerning the specificity of the anti-Ig sera in the case of the studies by Greaves and Hogg. Although Unanue could not inhibit the KLH-binding cells in the thymus with anti-Ig sera, others have been able to inhibit antigen-binding thymus cells, but here the specificity controls of the anti-Ig sera leave something to be desired. And so perhaps we should not abandon T cell Ig entirely.

For example, it may be that T cells have two recognition systems—one for recognizing histocompatibility antigens and another for recognizing other types of antigens. This should not be a distressing proposition, inasmuch as invertebrates seem to be able to recognize foreigness (with apparently some degree of short-term memory and specificity) and yet do not make Ig. Why should such a recognition system have been lost in evolution? The possibility that this system may involve receptors coded for by genes of the major histocompatibility complex is an exciting one and is consistent with the findings that MLC reactions can be inhibited by anti-H sera. On the other hand, the failure to inhibit T cell cytotoxicity against allogeneic target cells with such sera presents obvious problems for this hypothesis. The elegant studies of Shevach, Green, and Paul, that Green described for us earlier, suggests that perhaps this "primitive" recognition system recognizes antigens other than histocompatibility antigens. The second T cell recognition system would be Ig—and would account for the findings of Schlossman and Rajewsky that T cells seem to be able to discriminate between different antigens to the same extent as B cells and soluble antibody, and would also account for the ability to inhibit T cell helper function and antigen-binding by anti-Ig sera.

SIMONSEN: I do not object in principle to Raff's contention that there might be two recognition systems working in T cells, one for rosette formation and helper function, and another for recognition of transplantation antigens; however, I don't think it is very likely.

If the studies we heard from Uhr and from Unanue are taken as fact, then it will be difficult indeed to see Ig receptors accounting for helper function or rosette formation. Consequently, I would like to offer for our consideration an alternative explanation of these T cell functions, which is that the T cells have

acquired IgM receptors by some specific, passive mechanism. In other words, I believe they have an indigenous T cell receptor which is not Ig but is nevertheless good enough to bind some antigen and having done that, there will be neighboring B cells producing IgM which provide this as a second layer of the sandwich. Thus, we could have a T cell with specific IgM, though *not* produced by that cell. I particularly favor this speculation when it comes to rosette formation because in our own experience and also that of other people, bursectomized chickens form no rosettes, which is an experiment saying that in chickens, all rosette formation is B cell dependent.

McDEVITT: Raff's comments about the fragility of rosettes rang a bell in my subconscious. In human peripheral blood there is found a large number of rosette-forming cells for SRBC only; these are temperature-dependent and dependent on incubation conditions, the values range from 4% up to 40% of the total peripheral blood lymphocytes. Imagine—40% of the total peripheral blood lymphocytes! I don't think anyone would argue that all of these are specific for SRBC.

Again I remind you that they form *only* with SRBC and in Coombs' experience, they are not inhibitable by antiimmunoglobulin antisera, particularly when the antisera have first been absorbed with human cells before they are used in tests on rosette formation. I wonder if a significant portion of the type of rosettes that are very fragile and difficult to reproduce in other laboratories is not something similar to what occurs in the human, which is certainly rosette formation by a nonimmunologic mechanism.

RAFF: No, I think one can exclude that as a possibility, for as McDevitt said, one can't inhibit the rosettes in man with antiimmunoglobulin, and the studies in mouse suggest that for the most part the RFC are specific. Thus, Ashman has found that if he immunizes mice with sheep and pigeon erythrocytes, less than 1:200 T-RFC bind *both* types of red cells. This does not get around Simonsen's objection which I think is a very good one. For if, as he suggests, antigen binds first and then specific antibody, you wouldn't expect double binding cells.

BIOZZI: I would like to mention some recent results obtained by Jean-Francois Bach in Paris which attest to the thymus origin of rosette-forming cells in the mouse. Thymectomized and irradiated CBA/H mice were restored with CBA/H bone-marrow and CBA/H T_6-T_6 thymus cells. Three days after immunization with pigeon erythrocytes the totality of rosettes in mitosis were of thymus origin as proved by the presence of the translocation chromosomes T_6-T_6. On the other hand, by using an appropriate technique we demonstrated that a large majority of rosette-forming cells can be inhibited by heterologous anti-

mouse immunoglobulin sera (anti-Fab). These results are consistent with the view that the antigen receptors on T lymphocytes may be immunoglobulins.

WARNER: Raff suggests that the simplest hypothesis is to say that there are two totally different mechanisms, but I would think that a simpler mechanism would be that there may be two kinds of T cells, one predominantly involved in cytotoxic-type functions as in delayed sensitivity and transplantation rejection, and the other involved in T cell helper functions for B cells. It then may be that the divergence between them is based on a differential expression of immunoglobulin genes which are, nevertheless, evolutionarily related genes.

The question I pose for Raff is that although there is much data on the existence of T cell rosettes to xenogeneic erythrocytes, there are also studies which demonstrate similar types of T cell rosettes against allogeneic erythrocytes. Where would these be placed in a scheme involving an immunoglobulin and a nonimmunoglobulin T cell receptor?

RAFF: I think it is an error to think of this problem solely in terms of rosettes and SRBC, because surely the fragility of T cell rosettes simply reflects the fragility of T cells binding antigen in general.

CHAIRMAN RAJEWSKY: In the rather limited time remaining for this session, I would propose that we take up the question of whether there are differences in specificity in T and B cell receptors.

BACH: I would begin this phase of our discussion by referring to the observation of Simonsen in which he noted that in the GVH up to 3% of the cells could respond to allogeneic differences on a chorioallantoic membrane. Subsequently Wilson, and our group, showed that in the MLC the responding cell also showed this very high frequency of initially responding units, despite what we consider the very great number of molecules or antigens which had to be recognized.

I would like to demonstrate that we must consider not only the response in allogeneic MLC, but also the response in xenogeneic MLC. In Table 86, it is clearly evident that mouse cells not only respond to allogeneic mouse cells (a control of 1,549 cpm vs experimental 33,902 cpm), but also to xenogeneic cells, the control being the 1,549 cpm again and xenogeneic stimulation varying between 5,000 and 67,000 cpm with stimulating cells of different species.

A similar response pattern of human cells is shown in Table 87, again attesting to the fact that there can be marked xenogeneic responses. A major problem we have to concern ourselves with is the issue of whether responding cells in the MLC are totipotent, as Simonsen has suggested.

TABLE 86

Xenogeneic MLC Responses

Responding cells	Stimulating cells	Reactivity (expressed as mean cpm)
Mouse-1	Mouse-1	1,549
Mouse-1	Mouse-2	33,902
Mouse-1	Human-1	67,429
Mouse-1	Human-2	19,048
Mouse-1	Rabbit-1	27,348
Mouse-1	Rabbit-2	41,945
Mouse-1	Dog	5,274

TABLE 87

Xenogeneic MLC Responses

Responding cells	Stimulating cells	Reactivity (expressed as mean cpm)
Human-1	Human-1	1,966
Human-1	Human-2	66,985
Human-1	Mouse-1	2,076
Human-1	Mouse-2	19,592
Human-1	Rabbit-1	65,841
Human-1	Rabbit-2	41,992
Human-1	Dog	18,196

I would like to remind conferees of the experiments by Zoschke in my laboratory, similar to those of Salmon, in which he showed that there is specificity as far as the MLC response goes. This was demonstrated, as given in Table 88, by killing the responding, dividing cells in an activated MLC with 5-bromodeoxyuridine and light. A mixed culture, ABm, without the BUdR, incorporates 4,277 counts; when this MLC is treated with BUdR and light, the responding cell can be completely eliminated, and essentially no thymidine is incorporated. Similar results were obtained with ACm.

Table 89 shows that where A cells have initially been stimulated with B allogeneic cells and then the responding cells have been wiped out with BUdR and light, there is no subsequent response to B cells added at a later time. There is a good response to a different allogeneic cell, Cm; on the other hand, if A cells are first stimulated with Cm, then treated with BUdR-light, there is no secondary response to Cm but a good one to Bm. In view of these findings, I would say that we must worry about this at two levels of complexity. The first of them is what is being recognized.

TABLE 88

Effect of BUdR-Light Treatment on Mixed Leukocyte Cultures

Mixture	BUdR	Reactivity (expressed as mean cpm)
AB_m	No	4,277
AB_m	Yes	45
AC_m	No	7,329
AC_m	Yes	108

TABLE 89

Specificity of the Response in the MLC

Initial Mixture	Secondary stimulant	Reactivity (expressed as mean cpm)
AB_m	A_m	214
AB_m	B_m	511
AB_m	C_m	2,715
AC_m	A_m	414
AC_m	B_m	5,313
AC_m	C_m	360

I think that what we showed in an earlier session is that we no longer think that it is simply the serologically defined antigen of the histocompatibility complex which is recognized. It is the region between *H-2K* and *H-2D* in the mouse, as well as the serologically defined antigens, and therefore, we don't know how many antigens have to be recognized.

The other point I want to make most strongly is that this kind of critical evidence, says only that the cells are not functionally totipotent. It clearly leaves open the possibility of even extensive pluripotentiality.

CEPPELLINI: I would like to present some results on cell-mediated immune cytolysis in man, since they are especially pertinent to the discussion about the respective role played by "serologically defined" and "lymphocyte defined" allogeneic differences, or SD and LD antigens, according to Bach's terminology.

It is well known that *responder* lymphocytes activated *in vitro* through unidirectional MLC with allogeneic (mitomycin-C treated) *stimulator* lymphocytes, acquire the ability to destroy appropriate *target* cells. At variance with other systems such as the activation of lymphocytes through PHA, this effector phase of cell-mediated immunity is endowed with a good degree of specificity

towards selected targets, as first shown by Solliday and Bach, (*Science*, **70**, 1406, 1970). In this respect, the phenomenon approaches the classical model of Cerottini and Brünner, where however activation is obtained *in vivo*. Experimentation in man has until now been limited by the fact that normal lymphocytes of the peripheral blood are quite resistant to cell-mediated lysis. We knew, however, that lymphoblasts obtained via PHA stimulation become much more sensitive to the lytic action of alloantibody + complement, without losing their original antigenic profile (at least for HL-A). This greater sensitivity is due to the increased presence of some lysosomal enzymes in premitotic cells which could conceivably contribute to self destruction after an initial, externally induced, damage of membranes. The same mechanism probably contributes to the acquisition of lytic ability by activated lymphocytes.

It is in this context that we worked out the simple but effective technique depicted in Fig. 69. It will be seen that while the responder lymphocytes are being activated through an unidirectional MLC, a number of targets are prepared from the peripheral blood of normal donors, by separating lymphocytes as for the MLC, keeping them in nutrient medium at 37°C for three days, and then adding PHA. On the sixth day the PHA-transformed lymphoblasts are washed and labeled with ^{51}Cr (actually we now prefer to mark them by incorporating ^{14}C thymidine during the terminal hours of the culture). The responder cells, which by now are MLC-blasts, are mixed in a 50:1 ratio with the labeled targets

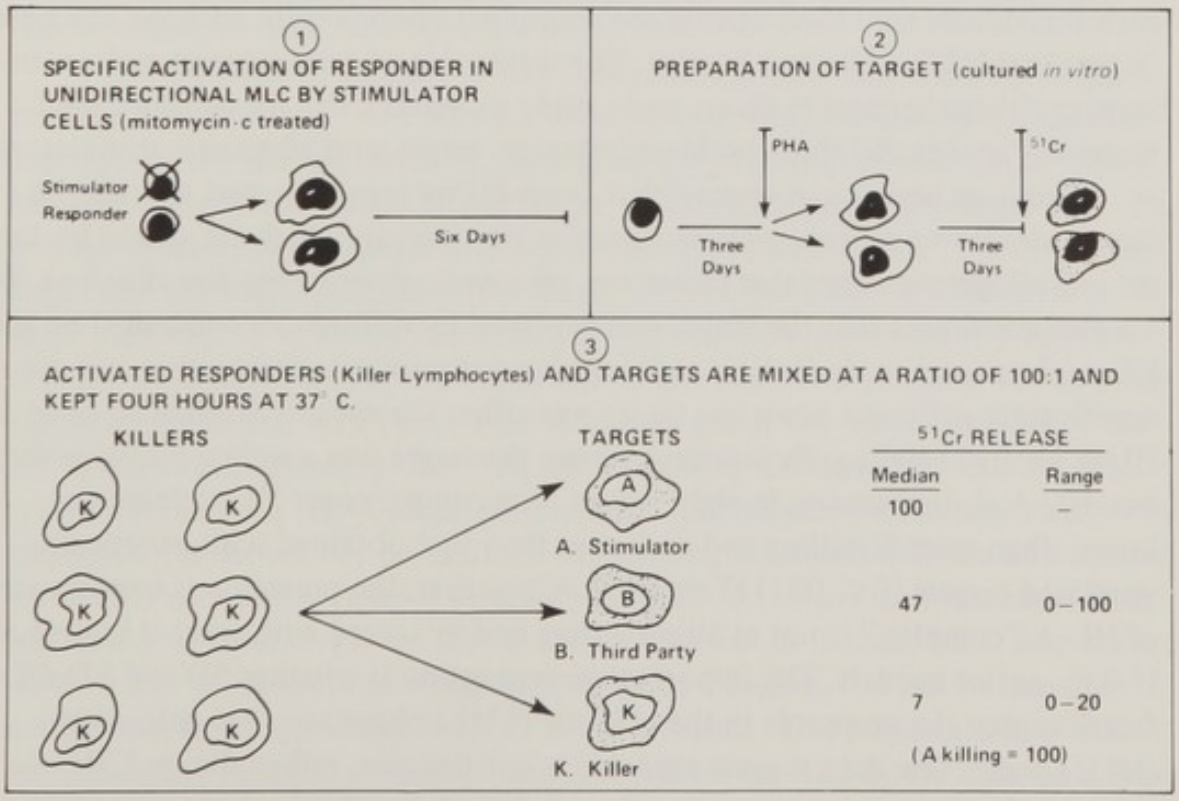

Fig. 69. Cell-mediated lymphotoxicity in man.

for four hours at 37°C. The amount of cell lysis is expressed, as usual, in percent of ^{51}Cr release (Lightbody *et al.*, *J. Batt. Virol. Immunol.*, **64**, 243, 1971; Miggiano *et al.*, *Transplantation Proc.*, **4**, 231, 1972). Under these experimental conditions, activation by PHA, even when PHA is added again in the final effector phase of the reaction, does not of itself induce any significant destruction of target.

By contrast, subsequent activation through allogeneic MLC induces the results summarized in Fig. 69: 1. the destruction shows a good degree of specificity because, with virtually no exceptions maximal lysis is obtained when target and stimulator are isogeneic (specific killing). For facility of comparison this effect has been equated to 100. In absolute terms *specific* ^{51}Cr release may vary from to 20 t0 90% of total release; 2. there is usually insignificant ^{51}Cr release above spontaneous release when the target is isogeneic to the killer cells. About two to five % of normal individuals show a significant degree of *autologous killing*. This has been observed in the same indivuduals on different occasions in separate experiments; it is, therefore, not a technical error and the phenomenon deserves further study; 3. targets allogeneic to both effector and stimulator are usually destroyed to a significant degree (cross-killing) although at a level that is on the average 50% lower than specific killing. The range does, however, vary widely, from 0 to 100.

The test has some interesting clinical applications such as, for example, on the lymphocytes of transplanted patients undergoing rejection crisis. The cells of such individuals are killers against the transplant donor's cells, without any need of previous MLC activation *in vitro*. The test is also of great interest and use in basic problems because it allows us to study crossreactivity for cell-mediated immunity among the three partners (effector, target, and allogeneic stimulator).

We have become convinced that *cross-killing* is in large part specific, i.e., due to structures necessary for recognition by effector, which are shared by target and allogeneic stimulator (these are, of course, absent from the effector). We are also convinced that the major role is played by differences controlled by the HL-A chromosome. In fact, in eight of nine comparisons, destruction was not significantly different when the target was either the specific stimulator or an HL-A identical sibling. By contrast, where the target was a sibling differing for two HL-A chromosomes, in eight out of nine comparisons, ^{51}Cr release was lower than specific killing and no better than that obtained with genetically unrelated targets ($P < .001$) (Table 90). Altogether, the preeminent importance of HL-A "complex" is not at all surprising and in accord with what is known for H-2 in murine models. The important new question is whether SD and LD differences play the same role in the effector (CML) phase as in the activation (MLC) phase. Our data suggest that this is *not* the case, rather that in CML the serologically detected antigens (products of the LA and Four loci) are *more im-*

TABLE 90

MLC and CML between Related and Unrelated Individuals Chosen on the Basis of HL-A Genotype

Stimulators	Effector: Ch-like Target: Child		Effector: Ch-unlike Target: Child	
	^{3}H-Thymidine cpm	% ^{51}Cr Release	^{3}H-Thymidine cpm	% ^{51}Cr Release
Child (Ch)	111,000	13.6	111,000	71.6
Father	120,900	9.3	70,100	33.8
Mother	55,400	3.5	82,100	62.0
Ch-unlike	101,200	4.1	3,300	4.1
Ch-like	1,200	−1.7	73,900	71.2

HL-A genotypes

Family members		Unrelated donors	
Father:	2, 5, 4a/ 3, W18, 4b	Ch-like:	1, 8, 4b/ 2, W15, 4b
Mother:	1, 8, 4b/11, W15, 4b	Ch-unlike:	2, 5, 4a/Da22, 12, 4a
Child:	1, 8, 4b/ 3, W18, 4b		

For minimizing nonspecific individual variability the target is always the child (Ch). The effectors are an unrelated donor. HL-A similar to the target (Ch-like on the left) and an unrelated donor, HL-A dissimilar (Ch-unlike on the right). *Transplant. Proc,* **4**, 231, 1972.

portant than the differences controlled by the third (MLC) locus of the HL-A region, while for MLC, the opposite holds true.

The evidence in support of this conclusion is four-fold and may be summarized as follows: 1. not only between siblings, but also between unrelated individuals, the sharing of HL-A serological differences (either at LA or Four) between allogeneic stimulator and target, significantly increases target lysis. The average cross-killing (expressed as percent of specific killing) is 72% where the target and the stimulator share two HL-A factors, but only 30% when they share no factor ($P < .001$). Moreover, in this situation, instances of a high percentage of killing can often be explained on the basis of serological HL-A cross-reactions (as 4a and 4b). From what is known about the polymorphism of the HL-A region, the crossing-over frequencies among the three loci and the MLC activation between HL-A identical unrelated individuals, (as discussed earlier by van Rood), it should *not* be assumed that among unrelated individuals a similarity for SD necessarily reflects similarity for LD; 2. earlier in this session Bach has shown us most elegantly that in the mouse, just as in man, MLC activation *in vitro*, is due to differences which map in the H-2 region, but not in the same areas known to control the *H-2D* or *H-2K* antigens. This important new information makes possible a reevaluation of much of the data in the literature, showing that once activation is achieved, the effector is able to destroy targets sharing

TABLE 91

Effect of Anti-HL-A on Unidirectional MLC and CML

Unidirectional mixed lymphocyte culture (MLC)				Lymphocytolysis (CML)			
Identity of interacting lymphocytes		Sera added to cell culture	Immune response incorporation of ^3H	% ^{51}Cr release from 2 different target cells			
				A		B	
Resp.	Stim.		CPM	Ns	Ab	Ns	Ab[a]
B	A	Ns	39,900	29	25(3)	0	20
B	A	Ab(1)	8,800	7	–	3	–
A	B	Ns	39,600	4	3	41	79(4)
A	B	Ab(2)	600	0	–	0	–
C	–	–	–	4	3	2	55(5)

A is a recipient of multiple blood transfusions who has developed a strong cytotoxic antibody (Ab) for which B (an unrelated individual) is positive. C is a third donor, Ab-negative. Ns is normal human serum. Ab added during MLC (in absence of complement) impairs activation when directed either against responder (1) or mitomycin-C treated stimulator (2). In contrast Ab added during the effector CML phase does not alter cytolysis against effector(3), whereas against the target(4), Ab greatly increases lysis. The latter effect is also obtained when the target is interacted with fresh nonactivated lymphocytes of individual C, in presence of Ab(5) and absence of complement, i.e., lymphocyte antibody lymphocytolytic interaction.

[a] ^{51}Cr release is reduced to < 5% when intact Ab is replaced by $F(ab')_2$.

Trinchieri *et al.*, Histocompatibility Testing, 1972, Munksgaard, Copenhagen.

only SD differences with the stimulator and having the same Ir allele (and presumably MLC) as the effector (e.g., Manuel *et al.*, *Immunology*, **18**, 517, 1970), C3H (K^k, Ir^k, Ss^k, D^k) lymphocytes activated by DBA/2 (d,d,d,d) give 59% ^{51}Cr release against the specific target, DBA/2, and 38% against target A (k,k,d,d); 3. as shown by Cerottini and Brünner, humoral antibodies against SD factors completely inhibit cell-mediated cytolysis. This is true also in the present system if we use $F(ab')_2$ instead of intact anti-HL-A sera. In fact, when intact Ig molecules are used, the block of CML in our system is masked by lysis due to a "Lymphocyte-Antibody-Lytic-Interaction" i.e., the acquisition of cytolytic properties by B lymphocytes when interacting with targets covered by antibodies, as described by Möller and by Perlmann. For the latter system too, PHA-blasts are excellent targets (Table 91). Blocking of CML by anti-HL-A $F(ab')_2$ against target suggests that HL-A antigens are the sensitive sites to both cytotoxic antibodies and activated lymphocytes. Other explanations, however, cannot be ruled out: a. sera containing cytotoxic antibodies against SD antigens may also contain blocking antibodies against LD antigens that are not recognized by current cytotoxic tests. It still remains for us to carry out appropriate absorption experiments, as I pointed out in Session I; b. alternatively steric hindrance by anti-SD antibodies could affect the nearby LD sites: 4. there is, however, another difference between MLC and CML: Allogeneic sera, notably effective in blocking MLC when directed against either responder or stimulator,

do *not* impair cytotoxicity when directed against the effector (Table 91). If the block of MLC is not a trivial and nonspecific phenomenon (Fig. 7), this contrast would in effect suggest that structures of the responder which are involved in MLC activation, are not the same structures of the effector cells which are responsible for target recognition.

Although in my view there can be no doubt about the biologic implications of MLC, we continue to ask what its real relevance may be *in vivo.* From what I can understand from the murine studies, a good activation *in vivo* with a consequent very efficient *in vitro* CML, can also be obtained when responder and stimulator differ for SD only, but not for Ir (e.g., Brondz and Snegiröva, *Immunology*, **20**, 456, 1972); responder k,k,k,k, stimulator and target k,k,d,d, yielded 80% lysis. The data cited suggest that LD differences are not as essential *in vivo* for lymphocyte activation as they appear to be in the *in vitro* system.

Is is tempting, as I have discussed repeatedly with Bach, to equate SD structures with the haptenic moiety of a complex antigen which easily elicits humoral antibodies. In contrast, LD is visualized as representing the carrier whose importance is preeminent for activation of cell-mediated immunity. It is, however, often dominated by the nearby haptenic groups as regards production of humoral antibody. I can foresee, however, a possible objection to this model; the reasoning is along the following line. Carrier and hapten should be topographically in *very close* proximity, i.e., located on the same molecule or supramolecular unit. We know now that the products of the four HL-A genes are located on independent units at the cell surface (Bernoco *et al.*, Histocompatibility Testing 1972, Munksgaard, Copenhagen). This is difficult to understand if the SD antigens must be inserted on the product of the MLC locus (on the assumption that this is the only LD locus of the HL-A complex). In order to explain the independent mobility of SD determinants (and especially of the products of the two LA alleles, which map at more than one % recombination frequency from the MLC locus, on the other side of Four), we must assume that HL-A molecules are linked *only* to the products controlled by the MLC allele located on the same, but not on the homologous, chromosome. Otherwise the different HL-A specificities would move together at the cell surface because they would be physically linked to the same carrier.

In any case it seems to me important to be aware of the fact that MLC activation although controlled (solely or preeminently) by the third locus, nonetheless induces, in the effector, immunological specificity for determinants controlled by other loci. One could suggest that MLC differences are only necessary for "nonspecific" derepression of the quiescent lymphocyte; recognition then involves other allogeneic structures of the cell surface. However, I prefer to end these remarks by quoting Bach once again: "Anyone who isn't confused about MLC isn't thinking clearly."

AFTERTHOUGHT BY CEPPELLINI: Eijsvoogel *et al.*, (Histocompatibility Testing 1972, Munksgaard, Copenhagen) found that two siblings, different for the serological HL-A factors, do not stimulate each others' cells in MLC, presumably because they are recombinants (e.g., 1st sibling: "LA^a, $Four^a$, MLC^a" and recombinant sibling "b,b,a"). If, however, "a,a,a" lymphocytes were activated in the MLC by "b,b,b," lymphocytes of a third sibling, they were then able to kill in CML equally well with "b,b,b" and "b,b,a" lymphocytes. This constitutes strong evidence in favor of the proposed hypothesis that it is the HL-A SD antigens rather than LD that are recognized in the effector phase of cell-mediated cytolysis, just as they are the targets of Ab + C′-dependent lysis.

BACH: The questions so provocatively raised by Ceppellini are it seems to me very difficult to answer at this time. He has formulated them very clearly; however, it may be worthwhile to speculate a little further about the problems being raised in this area.

I think it important that one observation is clarified. The LD loci, as we have called them, for the most part map with the presently defined Ir loci. This does not of course say they are the same. On the other hand, as I have already discussed, MLC stimulation is associated with some *H-2D* region differences, a finding which has alternative explanations. It is possible that either the *H-2D* locus SD antigens themselves are stimulatory in MLC or that there are LD loci closely linked to the *H-2D* locus which are stimulatory but which have not been separated by recombination from the *H-2D* locus. Likewise it seems possible that Ir loci will be found to the right of Ss-Slp. This is important since it complicates the interpretation of cytotoxicity which is found with differences of only the *H-2D* end of the major histocompatibility complex.

Let me for a moment come back to a question which has been raised several times at this conference: that of the relationship between the LD loci (as reflected by stimulation in the MLC test) and graft survival. I do this since for the moment I equate the *in vitro* cytotoxicity or CML test with the effector phase of the homograft reaction. Actually I rather doubt that the CML test is an adequate representation of the entire, probably varied, efferent arm of this reaction. There are three mouse strain combinations which we must consider, which differ for the major histocompatibility complex but appear to be serologically indistinguishable. First, C57BL/6 and H(zl) differ by a spontaneous mutation in the left part of the MHC. They appear to be serologically identical by crossimmunization. Their cells stimulate in both directions in MLC and they reject skin grafts from one another. Barbara Alter in our laboratory has done some very preliminary experiments to see whether CML activity is demonstrated after MLC sensitization. Although I must again stress that these results are very preliminary, she finds that the cells *do* kill in CML with only this difference. This combination then, it seems to me, warns that we may not need SD antigenic differences in *all* cases to obtain CML, although the target specificities for cytotoxicity may well be different from the molecules which are stimulatory in MLC.

Second, the combination of AQR and B10.T(6R). Here there is reciprocal skin graft rejection (in all probability due to the MHC differences) as there is reciprocal MLC activation. Skin graft rejection does not necessarily say to me that there will be CML activity after MLC activation. There are probably many different mechanisms which can lead to graft rejection; even if the CML test is a meaningful measure of at least some of these, skin graft rejection might occur without that phase of the homograft reaction represented by CML. No CML results have been obtained in this combination.

Third, the combination of B10.A(2R) and B10.A(4R) which is most informative. Here there are LD differences in only one direction: 4R responds to 2R but there is no response in the other direction in MLC. This combination is of such very great interest since there is no skin graft rejection in either direction. One explanation of these findings would be that the two strains differ by LD loci but do not differ by those loci which are necessary for skin graft rejection. Consequently I would thus hypothesize as Ceppellini has just done, that the effector arm of the homograft response, at least in some cases and as reflected in CML, is directed at differences other than the LD loci products. Ceppellini's own studies showing the very strong correlation between the antigens 4a and 4b and the CML test, provide the strongest evidence to date that the target may be the serologically defined (SD) antigens. We must remember, however, that it may be again as Ceppellini and I have so often discussed, that the CML target is determined by genes closely linked to the SD loci, with linkage disequilibrium leading to the correlation. The exciting possibility that the targets for the CML effector cells are different from the molecules which are the stimulatory ones in MLC, does not depend on the targets being the SD antigens.

I would find it most curious and would in fact suggest it most unlikely that the T cell response in MLC will not reflect some phase of the various reactions best grouped in the concept of the homograft reaction. The *in vivo* reaction which I have always associated with the MLC is that phase of the GVH reaction which involves donor cell proliferation, and I think GVH studies in these combinations will be of the greatest interest.

When a situation becomes sufficiently complicated and provocative, I find that a model which would explain the results is frequently useful. Let me propose one such model. We could think of one T cell population, one which may have the Ir product as its receptor molecule, which responds to LD differences, and leads to a proliferative response. Mechanisms could be proposed by which this population could by itself result in a rejection response. However, I would think of it primarily as a facilitating or cooperating T cell population. In this regard, this first T cell population would be analogous to the T helper cell which facilitates the response of B cells. In analogy with that model I would expect that this first response would not be necessary in *every* case. However, response

of this population would be, at least in most cases, a prerequisite for the response of a second population of lymphocytes. Given a response of the LD responsive population, a second population of lymphocytes, perhaps with a molecule similar to Ig for its receptor, can respond. This second population might well respond to the SD antigens or some other such structure which is determined by genes closely linked to the SD loci genes. This second population alone or with the LD responsive population would lead to CML. I believe that this same model could be built using only one cell population which responds first to the LD antigens and then to the SD or their equivalent, but I find such a model much less satisfying. In proposing this model I am obviously influenced by the findings of Cantor and Asofsky showing that T cell collaboration does exist in some cases. Also, by the contradictory findings regarding the presence of Ig on the membrane of T lymphocytes. Whereas I am convinced by the findings which Uhr has presented here, I wonder whether under certain circumstances another population of T cells can express a receptor which is similar to Ig. If this were true, there would be a certain elegance if this second population recognized the SD antigens—which are themselves defined with antibodies.

Finally, I want to point out that Ceppellini has argued largely from findings in man, whereas my comments are based on experiments in mice. It might, however, be best if we all considered the possibility that the LD loci in the mouse, which seem to map between the two SD loci, may in fact be different from the presumed locus in man outside the two SD loci.

CHAIRMAN RAJEWSKY: Ceppellini's contribution and Bach's as well, on the issue of specificity is most welcome, but we still need to discuss the basic question, i.e., whether one can distinguish two sets of specificity in the T and B cells. I would ask Schlossman to extend our discussion in that direction.

SCHLOSSMAN: The real question of whether the repertoire of specificity of T cells is much different than the specificity of B cells is indeed important. In our studies with defined DNP-oligolysines, T cell specificity, as defined by antigen induced thymidine incorporation, delayed hypersensitivity or MIF production, is capable of discriminating a series of very closely related DNP oligolysines differing only in hapten position and lysine chain length. Sensitized cells from animals sensitized to 9, ϵ DNP lys_9 are not readily triggered to undergo proliferation of biosynthesis by closely related crossreactive antigens. The T cell thus has extraordinary discriminatory power.

Similarly, if one looks at antibodies to these peptides, one again sees the capacity of antibody to the peptides to discriminate one from another. In our view, the important observation is that in terms of T and B cell specificity, the range of specificities in these cells is not different. When a responder reacts to a defined DNP-oligolysine, both its T cells and its B cells respond and discriminate

closely related antigens. Nonresponders, on the other hand, injected with the same peptide in the same adjuvant mixtures do not elaborate specific T cells, and their B cells are also not as discriminatory.[a] Studies such as these suggest that both T and B cells have similar ranges of specificity and perhaps identical recognition systems.

The differences in behavior in T and B cells seen in terms of specificity may not be attributable to different recognition, but rather to differences in requirement for triggering and initiating a signal. Thus, T cell specificity for antigens may be identical to B cell specificity, utilize the same immunoglobulin or immunoglobulin-like receptor, but differ in the quantity or distribution of receptor which leads to different requirements to initiate a signal. This does not say the Ir gene product may not be involved in antigen recognition at a different level which is separate from the other very specific "recognition system." This very specific T cell recognition system is best accounted for by an antibody molecule, despite the present difficulties in finding antibody on T cell surfaces.

CHAIRMAN RAJEWSKY: Our own, by now rather extensive data on the specificity of helper function, are entirely in line with Schlossman's findings. The issue of differences in specificity in T and B cells has been raised repeatedly in this session. Is there any further defense of this issue?

WARNER: I think it is worth mentioning that Ada's group has presented work which demonstrates, using two flagellins which are *non* crossreactive in terms of humoral antibody, complete cross-reactivity for induction and elicitation of delayed sensitivity. This could indicate that *in vivo* there is a different T versus B cell recognition of these flagellins.

The other comment I would like to make is that if the T cell is expressing only part of the total immunoglobulin genes that are involved in complete immunoglobulins, for example, only the variable region gene product, then one might expect a different degree of specificity of binding as compared to binding mediated by a heavy and light chain in combination as a complete molecule.

SELA: Similar to Ada's results, I may mention two other cases of efficient crossreaction in cellular immunity in the *absence* of antibody crossreaction. One is from the work of Maron and Arnon, concerning crossreaction for delayed sensitivity and also cross stimulation, using thymidine uptake, between native hen egg white lysozyme and native bovine alpha lactalbumin; these two proteins do not crossreact at all at the antibody level. Similar results were obtained in our

[a]Editor's Comment: The reader is referred to the invited contribution by Schlossman in Session I. His conclusions there are presumably valid for his remarks here.

recent work on the delayed sensitivity crossreaction between collagen and a synthetic polymer resembling collagen (Maoz *et al.*, *Israel J. Med. Sci.*, in press).

GRUMET: I too would like to cite an experiment, but it is one in which an effect is shown which is just the opposite of that given by Sela, i.e., *non* cross-reactivity at the T cell level and yet marked crossreactivity at the antibody level. If aqueous (T,G)-A--L is given to an $H\text{-}2^b$ mouse, a pure IgM primary response occurs and the IgM persists at a stable level for at least a month. If a second antigen challenge is given seven days after the primary antigen challenge, the mouse shows a classical secondary response, producing high titered IgG antibody. If (H,G)-A--L is used instead, a primary IgM antibody [that is highly crossreactive with (T,G)-A--L] develops, and persists unaffected by subsequent challenge with (H,G)-A--L, and IgG antibody does not appear.

If one gives (T,G)-A--L as the first antigen challenge and gives (H,G)-A--L as the second antigen challenge, there is no secondary response of any kind. There is, thus, no cross-stimulation (as McDevitt showed previously using antigen in adjuvant). If on the other hand, one were to give (H,G)-A--L, one day preceding the (T,G)-A--L primary challenge, followed by a second (T,G)-A--L challenge seven days later, the primary IgM antibody to (T,G)-A--L appears normally, but the secondary (i.e., IgG antibody) response is ablated. The (H,G)-A--L pretreatment thus effectively blocked the switchover from IgM to IgG. This finding is interpreted as indicating that the (H,G)-A--L may be behaving as a carrierless hapten which can interact with B cell receptor for the crossreacting hapten on (T,G)-A--L and (H,G)-A--L and induce B cells to do whatever they are supposed to. One then follows up with the (T,G)-A--L which will stimulate both the B cell receptor and the T cell receptor. Prevention of the switchover from IgM to IgG may have been prevented either by asynchronous stimulation of the T cells and B cells, or alternatively by paralysis of T cells by (H,G)-A--L. Although I favor the former explanation there is currently no data to select between these two mechanisms.

SELA: My reply to Grumet is to remind him that the specificity of (H,G) and (T,G) is different, but they do share the carrier moiety (polyalanine), a portion of the molecule which may be important in tolerance and delayed sensitivity.

CHAIRMAN RAJEWSKY: Could it be that there are two different specificities in the two cell lines?

BENACERRAF: In all deference to our chairman, I think that he has opened a Pandora's box with his question. All the information which is available on the comparative specificities of cellular immune reactions and of humoral antibodies is largely indirect and operational, although I agree quite significant.

1. Many investigators have observed a broader crossreactivity in cellular immune reactions than in antibodies specific for the same antigen. Many years ago, with Gell, we made such an observation with respect to the crossreactivity between BSA and HSA among themselves, and with denatured BSA and HSA.

2. On the other hand, there have been many valid observations, particularly in hapten-protein systems, showing that the specificity of delayed hypersensitivity reactions and of their *in vitro* correlates is much more precise than the specificity of the antibodies produced against the same antigen.

3. Then, too, there is another important difference observed with DNP-guinea pig albumin in guinea pigs. Anti-DNP PFC's can be easily suppressed by relatively low concentrations of DNP-lysine whereas even near toxic concentrations of this ligand inhibit very little the *in vitro* increased DNA synthesis stimulated by the immunizing DNP-GPA in sensitized guinea pig lymph node cells.

All these operational differences are well substantiated and they establish differences in specificity of both forms of reactivity, but it is difficult to ascertain what they mean at the level of the individual cellular receptors. Therefore, I feel that the final answer to explain these differences must await the isolation of the T cell receptor.

CHAIRMAN RAJEWSKY: I thank Benacerraf, as the points he has made are actually what I hoped would come out of this discussion; namely that while the specificity studies permit one to demonstrate the tremendous heterogeneity in T cell receptors, they so far fail to provide data on the basis of which one could definitely establish that the range of receptor specificities is different in the two cell compartments.

BENACERRAF: There is one further safe generality; that *certain types* of molecules do not stimulate thymocytes. These are the so-called thymus-independent antigens such as most complex polysaccharides.

RAFF: It seems to me that Benacerraf is falling into the trap that he just warned against. For when he tells us there are antigens that T cells don't recognize, what is he really saying? He means that there are antigens that don't seem to elicit a T cell response, but that is not to say that T cells lack receptors for these antigens. For example, B cells appear to have as many receptors for Con-A as T cells, and yet only T cells respond, which serves to point out that we cannot say anything about the number, specificity or affinity of receptors simply by measuring responses!

CHAIRMAN RAJEWSKY: To continue this debate—one must consider in this context that in T and B cell populations, regulatory mechanisms are operative which may differentially affect the apparent specificity of recognition. For ex-

ample, we, as well as Mitchison, have established that low-zone tolerance to serum albumin is exclusively localized in T cells. Such a mechanism effectively deletes certain specificities in the T cell compartment. Thus, differences in receptor specificity become apparent in the two cell compartments, although the physical nature of the receptor molecules may well be identical.

BENACERRAF: In reply to Rajewsky, I would say that there are distinctions that can be made between measuring and detecting differences in specificity—and in detecting nothing at all. In the sense that when one wants to measure whether something is more specific than something else, he has to have two reactions which he can compare—either at different concentrations—or under different conditions. This is what I was referring to previously. When one wants to compare the specificity of delayed sensitivity reaction with that of antibody, operational comparisons have been made on the basis of the existence of these phenomena.

I am simply saying that the comparisons that are made can sometimes be misleading because we do not know the *level of sensitivity* of the phenomena themselves. But this does not alter the fact that some molecules do not trigger or elicit thymus-derived cell reactions. Whether they fail to elicit because they do not bind or for other reasons, we do not know at present, but it is nonetheless clear that certain molecules do *not* elicit the function of cellular immunity. Many of these molecules have been classified as so-called thymus-independent antigens.

UHR: The problems regarding specificity cannot be separated from those of affinity. The binding of divalent antibody to a polyvalent ligand is quite different from its binding to a cell which may have thousands of recognition units. Does Warner know if Ada has examined the capacity of the two flagellins (which crossreact at the level of delayed hypersensitivity but not serum antibody) to crossreact in terms of binding to B lymphocytes? This might be another approach to the question of whether specificity is different between T and B cells.

WARNER: I believe there is data on those same crossreacting flagellins with human cells in antigen-binding tests; they show that there is an additive number when the two are used together, which suggests that they are binding to different cells at the B cell level.

COHN: Benacerraf said that there are antigens which cannot be recognized by thymocytes, namely the T independent antigens. How does he explain the fact that induction and tolerance are, nonetheless attained with T-independent antigens?

BENACERRAF: Cohn is, in effect, asking me whether I belong to one church or another. This is a matter of faith. In this matter, I plead the fifth amendment. I don't have to answer that question.

COHN: Does Benacerraf believe that an animal can be rendered tolerant to lipopolysaccharides, to polyvinyl pyrrolidone, to pneumococcal polysaccharides, etc.?

BENACERRAF: Cohn is a great logician whom I admire, but I would point out that in order to make a cell tolerant to an antigen, and to verify the degree of tolerance, one has to measure the state of tolerance of that cell. If that cell is never able to respond in the native state, or to be in any way affected by that antigen, how can one tell whether he had, in fact, made it tolerant? Cohn can postulate it was tolerant before he saw it, but that is an act of faith; it is not an experimental datum.

SELA: I would like to make a comment here on thymus-independent antigens and their nature, which is I believe germane to the orientation of this session. If there *are* thymus-independent antigens, should we see genetic differences in immune response in various strains of animals, and if there are genetic differences, is it possible that there will also be H-2 linkage, or not?

I do not have the answer to the last of these questions, but I want to mention that it has been rather generally accepted recently, at least for the series of immunogens Cohn referred to (polyvinyl pyrrolidone, *E. coli* lipopolysaccharide, pneumococcal polysaccharides, and polymerized flagellin) that the common denominator is their makeup of repeating antigenic determinants. We are in disagreement here because most of the antigens we have been talking about during the preceding three sessions are synthetic polymers which possess repeating antigenic determinants; but they are nevertheless, thymus-dependent. It seems that there is one other common denominator to all of the thymus-independent antigens, namely, that they either do not undergo degradation at all or are very difficult for the host to metabolize.

Using the synthetic approach, we were able to build a series of polymers which have very similar size, shape, molecular weight, amino acid composition, and most probably sequence, but which differ only in their optical configuration, having L-amino acids throughout, or D-amino acids throughout, or L-amino acids inside and D outside or *vice versa,* and we have checked all of these antigens for their thymus-dependence.

We have compared the immune response of DBA/l and SJL mice to four versions of the *same* multichain (tyrosyl, glutamyl) poly-lysine polymer which differ only on the optical configuration of their component amino acids. When it is composed only of L-amino acids, it is a good producer of antibodies in SJL

TABLE 92

Cell Cooperation as a Function of the Optical Configuration of the Amino Acids Composing the Immunogen Multi-copoly(Tyr, Glu)-polyPro--poly Lys[a]

Immunogen	10^8 thymocytes	2×10^7 marrow cells	10^8 thymocytes and 2×10^7 marrow cells
L(Tyr, Glu)-LPro-LLys	0	8	100
L(Tyr, Glu)-DPro–DLys	ND[b]	73	56
D(Tyr, Glu)-LPro-LLys	0	70	64
D(Tyr, Glu)-DPro-DLys	0	63	59

[a]Percentage of syngeneic irradiated SJL/J mice producing antibodies (passive hemagglutination) after cell transfer.

[b]Not done.

and poor in the DBA/l mice, whereas the other three polymers are good antibody producers in both strains. It would thus seem that in this case a genetic difference was abolished by a change in the optical configuration of amino acids in the immunogen.

Table 92 shows that of the four polymers examined only the all L polymer is really thymus-dependent, and one cannot transfer reactivity to it with bone-marrow alone, whereas the polymers which are all D, as well as the polymer which is L inside and D on the outside are thymus-independent. We know that there is a good chemical reason why the latter are not metabolizable. There are no endopeptidases capable of splitting a bond between two L-proline residues, consequently a long chain of poly (L) proline locked between a peptide of D amino acids and the ϵ amino group of lysine in the polylysine backbone is not digestable. Table 93 shows that the same results hold true for thymectomized animals.

As seen in Table 94 in the DBA/l strain of mice, a polymer in which the inside is poly-D-proline and the outside a polymer of L-phenylalanine and L-glutamic acid is immunogenic, and both of these regions are immunopotent, so that a good response is obtained to both of them. We were, therefore, able to check the thymus-dependence or independence, not at the level of an antigenic macromolecule, but at the level of single determinants. Table 95 shows clearly that the antibody formation to the metabolizable moiety, the peptide of L-phenylalanine and L-glutamic acid, is thymus-dependent, and one must transfer thymocytes as well as bone-marrow for an efficient antibody response, whereas the antibody production to the non-metabolizable poly-D-proline moiety is thymus-independent.

The question remains whether some such thymus-independent antigens will relate to genetic differences between various animal strains; if the answer should be in the affirmative, it will be of interest to find out to what genes such responses are linked.

TABLE 93

Cell to Cell Interaction for Immune Responses to D(Tyr, Glu)-DPro--DLys in Thymectomized SJL Mice

Thymocytes injected	Marrow cells injected	Fraction of positive sera[a]	% of positive sera[a]
1×10^8	0	0/8	0
0	2×10^7	7/11	63
1×10^8	2×10^7	8/11	72

[a] Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.

TABLE 94

Immune Response of DBA/1 and SJL Mice to Multi-copoly (LPhe, LGlu)-polyDPro--polyDLys Assayed by Passive Hemagglutination

Mouse strain	Serum titers assayed with		
	L(Phe, Glu)-DPro--DLys	L(Phe, Glu)--LPro--LLys	D(Phe, Glu)-DPro--DLys
DBA/1	1:1024	1:512	1:256
SJL	1:128	1:8	1:128

TABLE 95

Cell Cooperation as a Function of the Optical Configuration of the Antigenic Determinants within the Immunogen Multi-copoly(LPhe, LGlu)-polyDPro--polyDLys[a]

Antigen used for assay	10^8 thymocytes	2×10^7 marrow cells	10^8 thymocytes and 2×10^7 marrow cells
L(Phe, Glu)-DLAla--LLys [for (Phe,Glu) specificity]	20	20	82
D(Tyr, Glu)-DPro-DLys [for Pro--Lys specificity]	10	60	67

[a] Percentage of syngeneic irradiated DBA/1 mice producing antibodies (passive hemagglutination) after cell transfer.

LEVINE: We obtained some data which I suspect has relevance with regard to the nature of the T cell receptor. Basically, my experiment was an attempt to view the nature of the T cell receptor by varying the poly-lysine carrier. What I did was synthesize polymers of lysine, argine, homarginine, and ornithine and prepare lightly coupled DNP conjugates. There are two assumptions; one is that the same PLL-gene permits recognition of the four carriers. This appears to be true. The same animals are responders to all four conjugates in outbred and also in inbred strains of guinea pigs. The other assumption is that the recognition of the carrier is rate-limiting in terms of the ability of these materials to induce sensitization. The third point is that once these animals are sensitized, they will recognize the immunogen better than the other three conjugates. Thus, though

poly-homarginine is a relatively poor sensitizer, once guinea pigs are sensitized, the animals will recognize poly-homarginine better than they will recognize poly-lysine. Thus, immunological recognition exists for all four conjugates, and there is no rank order in terms of immunological specificity of classical type. But in terms of induction of sensitivity, there was a clear-cut rank order for induction of both serum antibody and delayed hypersensitivity. The order was lysine > arginine > homarginine > ornithine.

Now, having identified the system, my point is, could this rank order reflect the nature of this hypothetical T cell receptor? Specifically this rank order is reminiscent of the specificity of certain proteases specific for basic amino acids. We found that the specificity of trypsin is somewhat different, i.e., arginine > lysine > homarginine > ornithine. However, there are some mammalian proteases, such as those involved in metamorphosis of sea urchins (as studied by Troll), which give the same rank order, of specificity as found for the PLL gene. Thus, the point I make is that the hypothetical T cell receptor may be akin to a degenerate enzyme, not an immunoglobulin, which recognizes these basic polyaminoacid carriers with specificity resembling that of protease-substrate binding. The specificity could be different from the classical immunological specificity seen in response to immunization with these antigens.

CHAIRMAN RAJEWSKY: In concluding Session IV, I would like briefly to try to summarize my impressions on what has emerged from our lively discussions. There is an obvious controversy with respect to T cell recognition. Some have strongly expressed the view that T cell receptors may be not immunoglobulin. Their main arguments are the nonlinkage of the Ir genes to any known immunoglobulin gene, and the failure of a number of workers to directly or indirectly demonstrate immunoglobulin on the surface of T cells. The latter point, although strongly made by two speakers, cannot in my opinion (at this stage), be considered as entirely convincing, partly because of opposing data from other laboratories.

Others, myself included, still consider it likely that T cells as well as B cells carry immunoglobulin receptors. In particular in the case of helper T cells, evidence for immunoglobulin receptors is available. In the discussion of the specificity of T and B cell recognition, the tremendous heterogeneity of both T and B cell receptors became apparent. There was no cogent reason to postulate, on the basis of studies on the specificity of recognition, a different nature for T and B cell receptors.

The possibility has been raised and must certainly be considered that T cells may carry two different recognition systems, one mainly responsible for cell-cell recognition, the other resembling that of B cells.

As with most good discussions, ours has certainly raised more issues than it resolved.

SESSION V

ANIMAL MODELS OF MULTIGENIC CONTROL OF SUSCEPTIBILITY TO DISEASE

Multiple gene control of viral leukemogenesis in the mouse – Natural history of murine leukemia viruses – H-2 linked effect on susceptibility to LCM infection – Immune complex mediated injury in chronic LCM infection – Influence of virus infection on autoimmunity in NZB mice – Multigenic control of autoantibody production – Genetic control of immune response to self antigens – Differential susceptibility of high and low antibody-producing mouse lines to infection and tumor transplantation – H-2 linked control of marrow graft rejection in irradiated mice.

CHAIRMAN ALLISON: I should like to outline the territory to be covered, which might be useful for orienting our discussions in this session. First of all we should distinguish between single-gene and polygenic effects as illustrated in Fig. 70, and should then try to define the areas in which these are operating. We know that some effects are manifested in all cells of the body, *viz.* the capacity to support multiplication of certain viruses; some interesting single-gene effects operate at this level. There are also a number of effects which are expressed at the level of macrophages. Macrophages obviously play an important role as barriers to the spread of infection. Many viruses, whether introduced into the lung, skin, or elsewhere, are known to be taken up quickly in macrophages. If they can multiply in the macrophages, they then have the potential to spread to the rest of the body and thus disseminate the infection, whereas if they cannot multiply in macrophages the infection is contained.

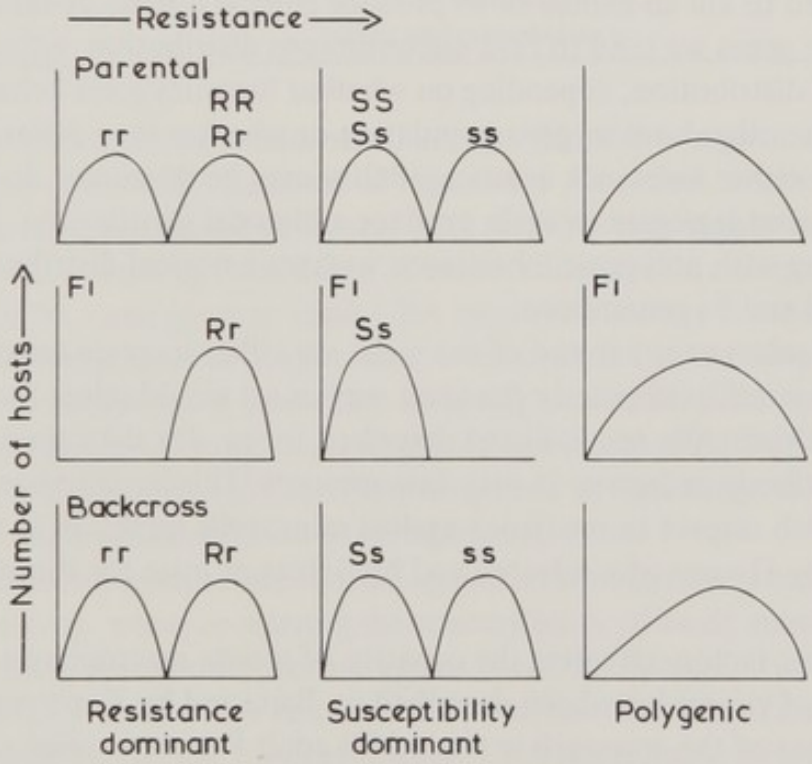

Fig. 70. Diagrammatic representation of the distribution of susceptibility and resistance in populations when single major genes segregate and when there are polygenic effects. Expected modes of inheritance of resistance against an infection based on three hypotheses: A (left) that a single pair of alleles with resistance dominant makes an important contribution; B (middle) that a single pair of alleles with susceptibility dominant makes an important contribution; C that many genes affect susceptibility. Upper curves show parental generations with resistance plotted on the abscissa in arbitrary units (e.g. proportion of animals surviving a given virus dose or dose of virus required to produce a particular response). Middle curves show progeny of matings of highly susceptible and highly resistant animals. Lower curves show backcrosses of F_1 with suitable parental type.

Some genetic differences in the susceptibility of mice, based on single genes, are manifested in the capacity of virus to replicate in macrophages. Then too, there are effects on leukocytes themselves, the discussion of which we shall have to forgo. The inherited defects in mink making them susceptible to Aleutian mink disease and chronic granulomatous disease in man are examples. For the purpose of this conference we are especially interested in genetic factors which are manifested in immunocompetent cells, namely the capacity of immunocompetent cells to react against infections and prevent their spread, or to prevent development of tumors. The relative contributions of T and B lymphocytes vary in different situations, and we should give this issue its full and proper attention.

Considering first the distinction between single-gene and multiple-gene effects, there are different distributions of resistance in populations. The resistance can be manifested in different ways; for example, in the number of organisms required to kill an animal or to produce clinical disease or tumors. With single major genes we tend to find discontinuous distribution, either a bimodal or trimodal distribution, depending on whether heterozygotes behave similarly to one or the other homozygous population or whether they differ. In the former case either resistance or susceptibility may be dominant. In backcrosses the major genes segregate to again produce a bimodal distribution. In contrast, when dealing with polygenic inheritance we have a normal distribution curve in the parental and F_1 generations.

If we select at either end of the scale, we either increase resistance or increase susceptibility in exactly the same way as we would select for bristle number in Drosophila or—as Biozzi described for us—for the capacity of mice to produce antibody in high or in very low amounts. This has also been done, for example, with respect to resistance against salmonella infection in the now classical work by Gowen who selected and bred lines of mice for this distinctive attribute.

Genetic factors affecting the capacity of mouse macrophages to support replication of viruses have been described by Bang and by Koprowski. Mouse hepatitis virus of the appropriate strain kills adult Princeton mice which are susceptible, whereas mice from the resistant C3H strain are not killed. Peritoneal macrophages from Princeton mice are able to support multiplication of virus, whereas those from resistant mice do not, and as the resistance genes are followed through F_2 and later generations, the susceptibility of animals is paralleled by the capacity of their macrophages to support virus replication. The same thing is seen in the case of the arthropod-borne viruses of the B group, i.e., yellow fever and West Nile viruses.

Moving on to consider genes manifested at the level of immunocompetent cells, it is known that polyoma virus produces tumors when inoculated into newborn mice of susceptible strains, while other inbred strains are resistant. C57

TABLE 96

Incidence of Tumors in Normal and Thymectomized CBA Mice Inoculated as Adults with Polyoma Virus

Preliminary treatment	Restoration at 7 weeks	No. of animals	% with tumors
Normal rabbit globulin	None	24	0
Thymectomy + antilymphocytic globulin	None	14	100
Thymectomy + antilymphocytic globulin	Nomral lymphoid cells	10	90
Thymectomy + antilymphocytic globulin	Sensitized lymphoid cells	11	0
Thymectomy + antilymphocytic globulin	Sensitized lymphoid cells	10	0
Thymectomy + antilymphocytic globulin	Sensitized lymphoid cells treated wthi anti-θ serum and complement	6	83

is one of the resistant strains; under standard conditions if one gives virus to newborn animals, very small numbers of tumors are produced, whereas animals that have been thymectomized or treated with ALS become highly susceptible. Furthermore, the normal age limitation of tumor induction is abrogated. As is shown in Table 96, virus given to adult CBA mice produces no tumors, whereas animals that have been immunosuppressed, even as adults, all develop tumors. Development of tumors is not prevented by giving antibodies after virus, but it is prevented by transfer of syngeneic, sensitized lymphoid cells. This constitutes an example of a T cell effect of major importance in determining the inherited resistance of certain strains of mice to an oncogenic virus.

It is possible to analyze the relative importance of antibody and cell-mediated immunity, when comparing the susceptibility of adult animals to virus infection, by using immunosuppressive agents (cyclophosphamide is a convenient example), followed by selective restoration of immune function. Zisman and I have worked with Coxsackie B3 virus in adult CBA mice. Table 97 shows that the normal untreated control adult animals are entirely resistant, but if given a single dose of cyclophosphamide, they all die. This can be prevented by administration of antibody. So it looks as though in this situation antibody itself suffices to bring about control of infection.

There are, however, certain virus infections as shown in Table 98, where this situation does not obtain and where one can show the critical role of cell-mediated immunity. A classic example among nononcogenic viruses is herpes simplex virus. Procedures such as thymectomy or administration of ALS greatly

TABLE 97

Mortality of Adult CBA Mice after Intraperitoneal Infection with Coxsackie B-3 Virus, Showing the Increase by Cyclophosphamide and Protection by Passively Administered Antidoby

Treatment	No. of mice	Mortality %
Untreated control	16	0
Cyclophosphamide (72 h. after virus)	16	100
Cyclophosphamide + antibody (4 and 15 days after virus)	14	0

TABLE 98

Effect of Suppression of Cell-Mediated Immunity on Outcome of Virus Infection in Mice

Co Virus	Age (weeks)	No. of mice	Treatment	% Mortality
Herpes simplex	4	25	NRS	0
		14	Thymectomy	71
		13	ALS	100
Yellow fever	4	58	None	16.4
		24	ALS	16.6
Coxsackie B-3	6	25	None	0
		22	Thymectomy	0
Ectromelia (Blanden)	Young Adults	26	NRS	20
		26	ALS	100

increase the mortality of infections of this sort and of poxviruses as well. Antibody administered after the infection is established does not protect.

The same sort of thing probably also occurs in man. Table 99 shows the types of virus infection potentiated by immune defects. We now know that patients with uncomplicated agammaglobulinemia are unusually susceptible to paralytic poliomyelitis but not to most other viruses. Defects in cell-mediated immunity, whether congenital or acquired, lead to greatly increased susceptibility to poxvirus, herpesvirus, and measles virus infections.

There may be other situations where the segregation of single genes in man is important in relation to the persistence of virus infections, which probably results from inadequate host immune responses. The work of Blumberg and his colleagues suggests that individuals homozygous for a susceptibility gene show persistence of Australia antigen in South-East Asian populations. Ceppellini has developed comparable data from Sardinian populations which I believe he will

TABLE 99

Virus Infections Potentiated in Different Types of Immunodeficiency

Immunodeficiency	Virus infection potentiated	Reference
Hypogammaglobulinaemia with intact cell-mediated immunity	Paralytic poliomyelitis	Schur, Borel, Gelfand, Heper, and Rosen (1970)
Deficient cell-mediated immunity (± normal immunoglobulins)	Vaccinia	Fulginiti *et al.* (1968) O'Connell, Karzon, and Barron (1964)
	Herpes simplex	Cooper *et al.* (1968) Kretschmer, August, Rosen, and Janeway (1969)
	Varicella zoster	Hayes, Beam, and Valentine (1965) Lux *et al.* (1970)
	Cytomegalovirus	Miller and Schieken (1967) Haworth, Hoogstraten, and Taylor (1967) Cooper *et al.* (1968)
	Measles	Nahmias, Griffith, Salsbery and Yoshida (1967) Cooper *et al.* (1968)

For references: Allison, A. C. (1972) Immunity against viruses. In: *The Scientific Basis of Medicine Annual Reviews.* London; Athlone Press, pp. 49-73. 1972.

describe for us in Session VI. So I hope that by now I will have made my point; that we have no lack of examples to illustrate each of the situations which I outlined.

Lilly will now develop for us, in some detail, one particularly well studied situation; the genetics of susceptibility of mice to leukemogenic viruses.

LILLY: Several lines of evidence in the literature indicate that susceptibility to leukemia in man is controlled to a considerable extent by genetic factors. However, it seems equally obvious that it is not a single-gene disease, but is controlled by a number of genes. Multiple gene characters have been studied since the relatively early days of genetics, notably the period from 1910 until well into the 1920's, centering partly around East and his co-workers. In the course of these studies and subsequent refinements of them, many ways have been found to describe in mathematical terms characteristics which are controlled by multiple genes and their effects within populations. This is the subject of quantitative genetics. The difficulty is that in trying to understand the basic mechanisms involved in polygenic control there is no substitute for reaching into the black box

TABLE 100

Genes Controlling Susceptibility to Leukemogenic Viruses

Gene	Effect on	Systems studied
Fv-2	Spleen focus-forming virus (SFFV) component of Friend virus complex	Friend virus
W, Sl, f	Hemopoietic cells (hereditary anemias)	Friend virus
Fv-1	Helper virus component of Friend virus complex	Friend virus, naturally occurring MuLV's
Rgv-1 (H-2)	Immune response to virus-associated antigens	Gross virus, B/T-L virus, Friend virus, RadLV, mammary tumor virus

and picking out the genes one at a time, identifying them, mapping them, and studying their individual mechanisms of action.

I shall describe the disease induced by Friend virus as a model of multiple-gene diseases. It is perhaps not the ideal model for leukemia in man, since spontaneous leukemia even in the mouse is due not to exogenously acquired virus but to endogenous virus. Nevertheless, as will become apparent, I hope the genes that have been identified as being important in the genetic control of the Friend virus disease act similarly in the case of spontaneous leukemia in the mouse.

This problem can be considered at three levels of complexity. We are dealing with multiple gene systems, so our first level of complexity is that of the genetic control. Table 100 lists the four categories of genes which I shall discuss. It should be emphasized that each of these categories appears to exert its influence on the development of the disease by a completely different mechanism. The first level of complexity is that of the genetic control exerted by the host genome on the disease process. A second level of complexity is the complex character of the disease itself. Classically, the disease induced by Friend virus in a highly susceptible host is described as erythroleukemia; that is, the target cell of virus infection and subsequent multiplication is a relatively early cell in the erythroid series. This fact distinguishes Friend virus disease from that induced by most murine leukemia viruses, which tend to induce lymphoid neoplasms.

The predominant symptom of Friend virus disease is illustrated in Fig. 71, as rapid, almost explosive proliferation of cells in the spleen. Very soon after inoculation of a high virus dose into a susceptible host, the spleen starts enlarging, continues to enlarge at a logarithmic rate during a period of about seven or eight days, with a doubling time of approximately 1.6 days. These kinetics will be even more impressive when I tell you that my calculations show that, were this rate of growth to continue for only five more days the entire mouse would be spleen. Fortunately for the mouse, that does not happen. The rate of

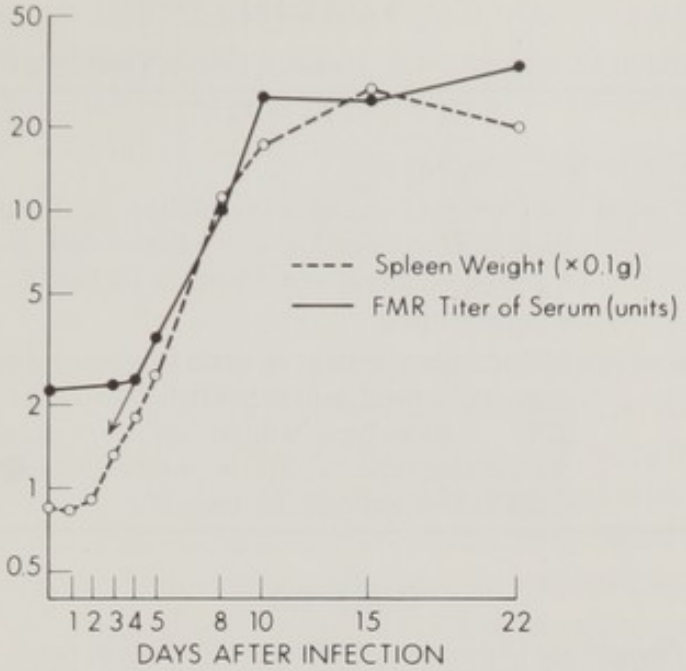

Fig. 71. Logarithmic increase in spleen weight and FMR titer in the course of Friend virus disease.

growth levels off; the spleen ceases to enlarge. Most of the animals do not die; not yet. The proliferation of the spleen ceases at about the end of the second week; the disease tends to be fatal at about four weeks.

This in itself indicates two levels of complexity of the disease: What I have referred to as the virological phase of the disease (that portion of the disease in which the spleen is growing quite rapidly) and the immunological stage of the disease, in which the rate of proliferation of the cells decreases. The disease is also complex in that the specific erythroleukemia-inducing property, i.e., the ability to induce the erythroid malignancy can be eliminated from the virus population by repeated passage of the virus through certain resistant animals. This maneuver yields a virus preparation which induces classical lymphatic leukemia, and no longer the rapidly developing erythroid leukemia. Such a lymphatic leukemia of mice has a very much longer latent period and is essentially monoclonal in origin.

We have thus dealt with the complexity of the disease induced by Friend virus, but there remains a third level of complexity to be considered, and that is the complexity of the virus itself as reflected in Table 101. Steeves and Axelrad discovered that Friend virus induces in susceptible hosts spleen foci readily detectable approximately nine days following intravenous virus inoculation. This test has been widely used in defining the virological aspects of the Friend virus disease.

According to Steeves' idea, also arrived at independently by Fieldsteel and his collaborators, the virus involves at least two different virus populations, simultaneous infection with *both* being necessary to produce erythroid disease.

TABLE 101

Postulated Composition of Standard Friend Virus Preparations

FV = SFFV + LLV

FV = Friend virus complex

SFFV = spleen focus-forming virus (a defective virus requiring simultaneous infection with a helper virus for virion maturation; shows a high level of specificity for erythroid cells)

LLV = lymphatic leukemia virus (a strain of virus, probably complex in itself, which has helper activity for SFFV, induces lymphatic leukemia in animals of certain genotypes, and shows a much broader range of tissue specificity than SFFV)

TABLE 102

Phenotypic Expression of Different *Fv-2* Genotypes

Fv-2 Genotype	Phenotype
$Fv\text{-}2^s/Fv\text{-}2^s$ $Fv\text{-}2^s/Fv\text{-}2^r$	Highly susceptible to focus formation by SFFV
$Fv\text{-}2^r/Fv\text{-}2^r$	Highly resistant to focus formation by SFFV

However, upon passage of the virus through animals resistant to the erythroid disease, a lymphatic leukemia virus is recovered; the interpretation is that one component of the complex Friend virus has been lost—the component which induces spleen foci and has been designated spleen focus-forming virus, or SFFV. What is left, which now induces classical lymphatic leukemia in susceptible host strains has been referred to as lymphatic leukemia virus, or LLV. Thus, as shown in Table 101, the Friend virus complex consists of a mixture of SFFV and LLV. LLV is what is left from a Friend virus preparation when SFFV is removed. It will, I hope, become clear that there is considerable reason for believing that LLV itself is complex.

The first gene of major importance governing the virological phase of the Friend virus disease is the *Fv-2* locus (Table 102). There are two alleles at the *Fv-2* locus, and all inbred mice are homozygous for one or the other of them. There is an allele for susceptibility ($Fv\text{-}2^s$) and an allele for resistance ($Fv\text{-}2^r$). The gene has been mapped and is linked to the *dilute* locus in linkage group II of the mouse. It is therefore unrelated to H-2 in linkage group IX. Susceptibility at the *Fv-2* locus is dominant; that is, animals homozygous or heterozygous for the susceptibility allele are indistinguishable.

Resistance due to the $Fv\text{-}2^r$ gene is essentially absolute; that is, animals homozygous for the resistant allele at *Fv-2* do not make spleen foci at all. *Fv-2*

apparently governs the SFFV component of the Friend virus and has no direct influence on the LLV component.

There exists at least one strain of Friend virus which contains an apparent mutant form of SFFV, very much less sensitive to the resistance conferred by homozygosity for the *Fv-2*^{r} allele. This brings up a still further level of complexity which I will not detail; i.e., single-gene differences among virus populations. It should be fairly obvious that those properties which define SFFV as opposed to LLV, would be very hard to explain by a single-gene difference between the two viruses. Since susceptibility is completely dominant in the *Fv-2* system, there is some reason for believing that the control exerted by this gene acts very early in the process of infection. One thinks naturally of receptor sites, or of uncoating of the virus, or of some phenomenon associated with viral penetration of the susceptible cells. There is a problem, however, since one can force passage of the Friend virus through animals that are resistant at *Fv-2* (although these animals do not make spleen foci), but it takes several virus passages, rather than a single passage, to eliminate the SFFV component. This is evidence against the receptor site hypothesis, altough not totally incompatible with it. The phenotypic expression of the different *Fv-2* genotypes are given in Table 102. The gradual loss of the SFFV component of Friend virus complex during passage in genetically resistant hosts represents, then, a selective pressure in this environment against the SFFV component, but as I have indicated there is no apparent effect of the *Fv-2* gene on susceptibility to LLV.

The second class of genes which strongly influence the host response to Friend virus is that of the hereditary anemias. Three examples of these have been found to influence Friend virus disease: *W, Sl* and *f*. Since, as I noted earlier, the classical Friend virus disease is highly specific for erythroid tissue, one might expect that genes which influence the maturation of erythroid cells would influence the disease process, and this has proved to be the case in all three of the situations that have been investigated.

This might, parenthetically, bring up one further question. Given the fact that the Friend virus is highly tissue-specific for erythroid precursor cells, one wonders whether the *Fv-2* gene, which is not known to be linked to a gene with an effect on the hemopoietic system, might not be in itself a developmental gene of sorts, which is expressed exclusively in erythroid cells. This restricted expression of the *Fv-2* gene might then explain the high degree of tissue specificity of Friend virus. This is, however, purely conjectural and is a subject for future investigation.

The third gene I would like to discuss at some length is a gene designated *Fv-1*, which governs the host response to the helper virus component of Friend virus. Helper virus activity is a property of LLV and also of other leukemia viruses. Recall that in order to induce the classical Friend virus disease, simulta-

neous infection with both SFFV and LLV is necessary. SFFV alone is not infectious in any cell; that is, it is a defective virus. It will grow only in the presence of simultaneous infection with LLV, which apparently allows the maturation of the SFFV virus particle, a function which it cannot carry on for itself. This missing property is supplied by the LLV component of the virus, or by some population within the LLV component of the virus, which thus serves as a helper for SFFV. It is this helper activity that is governed by the *Fv-1* gene. LLV is not defective in this sense and will grow in the absence of SFFV.

The *Fv-1* gene has two alleles, which were originally designated the $Fv\text{-}1^s$ (susceptible) and $Fv\text{-}1^r$ (resistant) alleles; these names have since proved inappropriate and have been changed to $Fv\text{-}1^n$ and $Fv\text{-}1^b$, respectively. The distinction between the two alleles resides in their ability to foster the proliferation of the infectiousness of different types of helper virus. The helper component in Friend virus can be one of three possible types. These are designated as N-tropic, B-tropic, or NB-tropic. These tropisms of the virus are defined in terms of their interaction with the host genotype at *Fv-1* (Table 103). That is, animals of the homozygous $Fv\text{-}1^n$ genotype are permissive for the growth of N-tropic viruses. The reverse is true of animals homozygous for the $Fv\text{-}1^b$ allele; that is, they are permissive for the growth of B-tropic viruses, but nonpermissive for the growth of N-tropic viruses.

A third type of helper component is said to be NB-tropic, and this is an artificially acquired property of the virus, following forced passage of an N- or B-tropic virus through cells of a $Fv\text{-}1^{b/b}$ or $Fv\text{-}1^{n/n}$ nonpermissive host. This acquired NB-tropism presents itself as an insensitivity to the potential barrier represented by the *Fv-1* gene. That is, NB-tropic viruses grow equally well in hosts of any *Fv-1* genotype. All inbred strains of mice tested fall into one of two patterns with respect to their N or B properties. That is, N-tropic viruses grow much better in animals and cells of the $Fv\text{-}1^n$ genotype than B-tropic virus,

TABLE 103

The Tropisms of the Friend Virus, Defined with Regard to Their Interaction with the Host Genotype at *Fv-1*

Fv-1 Genotype	Gene: *Fv-1* Alleles: $Fv\text{-}1^n$ and $Fv\text{-}1^b$ Phenotypes		
	Hitness of dose-response curve with FV's of different topisms		
	N-tropic (F-S)	NB-tropic (F-B)	B-tropic (F-T)
$Fv\text{-}1^n/FV\text{-}1^n$	One-hit	One-hit	Multiple-hit
$Fv\text{-}1^n/Fv\text{-}1^b$	Multiple-hit	One-hit	Multiple-hit
$Fv\text{-}1^b/Fv\text{-}1^b$	Multiple-hit	One-hit	One-hit

whereas animals of the *Fv-1*^{b} genotype allow the proliferation of B-tropic viruses to a very much greater extent than N-tropic viruses.

Fv-1 has not been mapped in the mouse genome, but it segregates independently of *Fv-2* and also of H-2; we also know a fair number of other places in the mouse genome where the gene is *not* located. It should be emphasized that in this *Fv-1* system, in contrast with the *Fv-2* system, resistance is dominant in heterozygous mice. It should be remembered that susceptibility was dominant at the *Fv-2* locus, but at the *Fv-1* locus resistance is dominant. Further, this resistance is not complete, as is the resistance conferred by the *Fv-2* gene. Resistance in the *Fv-1* system is relative. It is reflected in the kinetics of the titration curve in a given host. Using the spleen focus assay, N-tropic virus titrated in mice homozygous for *Fv-1*^{n} shows a one-hit titration curve, whereas the same virus titrated in animals heterozygous or homozygous for the *Fv-1*^{b} allele shows multiple-hit titration patterns. B-tropic virus shows a reciprocal pattern, that is, it shows single-hit titration curves in *Fv-1*^{b} homozygous mice, but multiple-hit kinetics in mice homozygous or heterozygous for *Fv-1*^{n}. Again, animals homozygous for *Fv-2*^{r} show no spleen foci regardless of their *Fv-1* type. Let me reemphasize that mice heterozygous at the *Fv-1* locus are relatively resistant to *both* N- and B-tropic viruses.

In addition to its being manifested in the spleen focus assay system, the *Fv-1* gene is also the basis of N- or B-tropism of the same virus product as detected *in vitro* in the XC assay, a modified plaque assay. Since there are many strains of virus which are active in the XC assay but which show very little or no leukemogenicity *in vivo*, the *Fv-1* gene thus appears to influence a large variety of C-type RNA tumor viruses.

The phenomenon of multiple-hit kinetics has been demonstrated both *in vitro* and *in vivo* in *Fv-1* nonpermissive hosts and has several implications. One is that LLV might itself be complex, since we think that the basis for two-or-more-hit kinetics is a requirement for simultaneous infection with two or more virus particles. There is no intrinsic necessity that the two virus particles required for an infectious event be different from each other, but since this is likely, there is a strong possibility that LLV consists of two different types of particles, both of which are necessary for a successful infectious event in animals of a nonpermissive *Fv-1* genotype.

Since relative resistance is dominant, the possession of a single nonpermissive allele at the *Fv-1* locus can convert what would otherwise be a one-hit titration curve to a multiple-hit titration curve. This implies that the gene product of the *Fv-1* locus is, itself, a regulatory factor in the initiation of viral infection, although that is certainly not proved. One tends to think of genetically dominant phenomena, in this case resistance, as being active phenomena.

I might conclude the discussion of *Fv-1* with the statement that there is considerable evidence that in addition to influencing the Friend virus disease, *Fv-1* rather strongly influences the occurrence of spontaneous leukemia in mice.

As the last example of a gene to be analyzed in some detail, I would like to discuss what has been named the *Rgv-1* locus (Resistance to Gross Virus), which is the name for that portion of the complex H-2 region of linkage group IX which influences leukemia virus susceptibility. This effect of *Rgv-1* has been demonstrated for a variety of viruses: not only Friend virus, but also Gross virus, B/T-L virus, radiation leukemia virus, and the mammary tumor virus. There are also suggestive data in the literature for an effect on murine sarcoma virus and the Abelson myelogenous leukemia virus.

All of these viruses, I might say, are influenced by a given H-2 allele in the same direction. That is, the $H\text{-}2^b$ allele confers relative resistance, whereas the $H\text{-}2^k$ allele confers relative susceptibility to all of these viruses. This suggests, although it does not prove, that the effects of *Rgv-1* on the various leukemia viruses are all governed by a single gene rather than by several closely linked genes.

Attempts to map the *Rgv-1* locus within the H-2 region have shown that the *H-2D* subregion is irrelevant in the *Rgv-1* phenomenon. Examination of the strain distributions of *Ss-Slp* and *Tla* fail to show any correlation with the *Rgv-1* influence, and one can assume these two loci are not responsible for the activity. This leads to the conclusion that *Rgv-1* maps somewhere in close association with either the *H-2K* subregion or the *Ir-1* subregion of the H-2 system. For the moment, it is not possible to be any more precise than that.

The effect of *Rgv-1* on leukemia virus is relative, that is, quantitative; it is an effect on the dose-response curve to the virus. Animals that are relatively susceptible because of their *Rgv-1* genotype show a lower dose-response curve with the virus than animals with a resistant *Rgv-1* type.

When the *Rgv-1* effect of the H-2 region was first introduced it was a great temptation to think that since H-2 antigens were on cell surfaces, the mechanism of action of H-2 in the control of virus susceptibility might be that H-2 antigens served as receptors for the virus. It is very clear now that, whatever the mechanism of *Rgv-1*, it does not operate as a receptor site. That is first of all apparent from the fact that the *Rgv-1* effect is relative and quantitative, rather than an absolute, all or none phenomenon. Furthermore, the effect of *Rgv-1* operates relatively late in the disease. This is shown by the fact that the spleen focus assay, which is performed within nine days following virus inoculation, reveals only a very minor influence of the H-2-type of animal. Animals differing at *Rgv-1* only, being relatively susceptible and relatively resistant, show only a two-fold difference in virus susceptibility by the spleen focus assay. If one looks at criteria associated with late stages of the disease, however, one can see a differ-

ence between these same host strains of much greater magnitude, in the range of 10- to 20-fold difference in their response to the virus.

Is *Rgv-1* identical with *Ir-1* or with some portion of it? It could be. If we look at the production of anti-FMR (Friend, Moloney, Rauscher common antigen) antibodies in animals of the two strains, we do find that animals susceptible to the virus by virtue of their *Rgv-1* type make somewhat less antibody than resistant animals. However, here again the difference is small, being of the order of magnitude of two-fold, which is not terribly impressive. One further fact which might be in favor of *Ir-1* is the observation that in favorable systems where one can make a sensible assessment of the question, animals heterozygous for the resistant and susceptible alleles at *Rgv-1* show an intermediate phenotype by comparison with animals homozygous for the two different alleles.

The last finding I would like to discuss is perhaps not greatly in favor of the idea that *Ir-1* and *Rgv-1* might be identical. I have mentioned the FMR antigen, the tumor-specific transplantation antigen induced on the surfaces of cells infected with Friend virus. I would now like to consider the appearance of this antigen in two strains of mice: BALB/c, which has the $H\text{-}2^d$ allele, and a congenic strain which we have made, the BALB/c-H-2^b. We call it the BALB.B strain.

Following inoculation of a high dose of virus in animals of these two congenic strains, the FMR antigen on spleen cells, starting from a zero level of expression appears at approximately the same time in both strains of animals and attains at around day ten the same maximum level of expression in both strains of animals. During the course of the first two weeks of the disease there does not seem to be any significant difference in the development of the FMR antigen in spleen cells of these two strains of mice. However, if we continue following the level of expression of the antigen it appears that, in animals susceptible at the *Rgv-1* locus, the FMR antigen disappears almost completely from the spleen cells, whereas in animals that are relatively resistant because of their *Rgv-1* genotype, the level of expression of the FMR antigen remains high.

On the basis of this observation, one could then hypothesize that the mechanisms whereby *Rgv-1* controls susceptibility or resistance to the virus is as follows: The animals of both *Rgv-1* types are equally able to make anti-FMR antibodies. However, in the one case, the $H\text{-}2^d$ animals lose the antigen, and therefore the antibodies are irrelevant and do not protect the animals. On the other hand, in the relatively resistant $H\text{-}2^b$ animals, where the antigen remains at a high level of expression, the antibodies are efficacious in killing the tumor cells. Susceptibility to the disease, then, would appear to be due to a loss of the FMR antigen specificity in the spleen cells and one can ask many questions about this disappearance. Is it equivalent to modulation? This question becomes even more

intriguing when we examine one of our controls for this experiment. Being rather surprised at this loss of the antigen, we thought that as a control, we might look at a normal cell antigen found in the same cell population. We decided to follow, in a developmental fashion the expression of H-2 antigens on the spleen cell surfaces of these two mouse strains, at the same time that we were monitoring the FMR antigen. The antigens determined by the *D* end of the H-2 region were seen not to vary significantly throughout the course of the disease in mice of either strain; that is, they remained at or near their initial level of expression.

This was not true, at least not in both kinds of mice, of antigens determined by the *K* locus of the H-2 region. In this case we found that the spleen cell antigens of the *K* locus of H-2 followed the same pattern of expression in the late phase of the disease as the FMR antigen; that is, in the highly susceptible $H\text{-}2^d$ animals, as the FMR antigen was disappearing the *H-2K* antigen, H-2.31 in this case, also disappeared at about the same rate.

In the $H\text{-}2^b$ animals, in which the FMR antigen remained at a relatively high level of expression to the end of the disease syndrome, the *H-2K* antigen, H-2.33 in this case, also remained at a high level of expression. This is mysterious to say the least. I do not have a really good hypothesis to explain it. One could speculate that in $H\text{-}2^d$ mice, anti-FMR antibodies cause the formation of polar caps of FMR antigen-antibody complexes, thus sweeping the FMR from the cell surface. Why this would not happen in $H\text{-}2^b$ mice is unclear. If one postulates that this clearance of the FMR antigen also carries along with it the H-2.31 antigen for some reason, then the problem is still only partially solved.

I have discussed four types of genes which govern Friend virus leukemogenesis. It seems likely that if this work is carried on for a few more years, there will be several additional genes to add to the list, although my suspicion is that we have already identified the major ones. There are certainly other genes, currently under study, of importance in spontaneous leukemogenesis and separate from all those I have considered so far. These include the genes which control the expression of endogenous C-type RNA tumor viruses and the genes governing the expression in untreated mice of virus-related antigens (gs-1, GSA and G_{IX}, for example). Whether these genes influence susceptibility to Friend virus complex is still unknown, but it seems clear that they are highly relevant to spontaneously occurring leukemia in mice.

I hope that my discussion has helped focus the problem of how the genetic control of a disease, governed in part by a gene which may be an immune response gene, can be very complex. Knowledge of the immune response genes in various hosts is but one segment of the information we need in order to predict susceptibility or resistance to a given disease process.

CHAIRMAN ALLISON: Lilly has shown us what may well prove to be a classical example of the way in which genetic analysis makes it possible to unravel the

interactions of several susceptibility factors in the genesis of a disease. This theme will now be pursued further by Steeves.

STEEVES: All of the genes described by Lilly as controlling susceptibility to Friend leukemia virus represent challenging problems for the geneticist. To the virologist, however, the *Fv-1* gene has special significance.

You will recall that the $Fv\text{-}1^b$ allele by B-type mice confers resistance only to N-tropic strains of murine leukemia virus. Lilly also pointed out that the resistance which is directed against the spleen focus-forming response to Friend leukemia virus infection is most pronounced near the dilution end-point for SFFV. That is, titer estimates of SFFV made at increasing dilutions diminish drastically instead of remaining constant, hence the multiple-hit dose-response relationship. It has been suggested that the host range of SFFV may be determined by the helper virus, which naturally accompanies it, called LLV-F. The $Fv\text{-}1^b$ gene is thought to reduce the infectivity of N-tropic LLV-F (usually present in considerable excess) so that the multiple-hit requirement for successful infection and focus formation is revealed.

This interpretation is supported by several lines of evidence. First, multiple-hit curves are not primarily due to diminishing degrees of virus-induced host immunodepression at increasing dilutions of virus, because the same sort of dose-response curves are observed in mice previously immunodepressed by cortisol treatment. Second, addition to the Friend virus diluent of LLV-F (itself devoid of any spleen focus-forming activity) will convert the multiple-hit curve to a one-hit form. The difference between the titer estimates of SFFV (at a given dilution) with or without the addition of helper virus is said to represent the helper activity of the helper virus preparation. Analysis by sucrose-gradient density centrifugation shows a close association between helper activity and particles with the density and morphology of RNA tumor viruses. Also, helper activity is proportional to the dose of helper virus used and is eliminated by incubating the helper virus with specific murine antiserum directed against it.

We have, therefore, a nine-day helper assay in mice and it shows a remarkable pattern of specificity. It can detect not only leukemia viruses of mice but also viruses which are leukemogenic in cats, chickens, and turkeys. However, it does *not* detect DNA-containing viruses or RNA-containing nononcogenic viruses. This leads us to examine thoroughly our recent finding of helper activity in 65 % of extracts prepared from leukemic human spleens, but *not* detected in normal human spleens. Such helper activity of human leukemic origin can be sedimented readily, or can be passed through 0.2 micron filters, but it does not tend to form a discrete band in centrifuged sucrose gradients. Even if human activity is not associated with C-type particles characteristic of leukemogenic viruses in other mammals, we plan to study its possible role as an etiologic factor in human leukemogenesis.

Let us now pursue another avenue of research made possible by the *Fv-1* gene-induced inhibition of LLV-F. We have recently found that we can make pseudotypes of SFFV by coinfecting B-type mice with N-tropic Friend virus complex, and with NB-tropic murine leukemia virus of a different origin. The latter, of course, will compete successfully with N-tropic LLV-F in B-type mice, so that two passages of SFFV in this manner will usually result in complete antigenic alteration of SFFV (i.e., acquisition of new antigens and complete loss of the Friend-specific antigens). This change is permanent on subsequent passage of the pseudotype unless LLV-F is added back into the virus stock, whereupon it reverts readily to its original form. My former graduate student, Echner, carried out a detailed antigenic analysis of nine pseudotypes of SFFV prepared from different murine leukemia virus isolates. He followed their neutralization kinetics in the presence of murine antisera directed against the virus-induced leukemias, and he was able to classify ten commonly used murine leukemia viruses into five antigenically distinct categories. It remains to be seen if this classification matches with a similar analysis utilizing the new XC plaque test for titrating murine leukemia viruses *in vitro*. If so, then the relationships among the murine leukemia viruses may be considerably more complex then the rather simplified version suggested by Ludwik Gross.

I hope I have indicated in these remarks how *Fv-1*, one of the genes that controls the susceptibility of mice to murine leukemia viruses has been utilized as a tool for studying the relationships among leukemogenic viruses. These studies have as yet revealed very little concerning the mechanism of action of the *Fv-1* gene, about which we must learn a great deal more before we will understand how leukemic tissue extracts substitute for some function that the *Fv-1* gene can inhibit.

CHAIRMAN ALLISON: The delineation of these models for genetic control of disease susceptibility by Lilly and by Steeves has opened up a whole series of interesting possibilities. Now we shall hear from Pincus about the XC system.

PINCUS: My own concern has been primarily with the genetic control of murine leukemia viruses in their natural hosts, based on studies conducted under the tutelage of and in collaboration with Rowe, Hartley, and Lilly.

A major problem in the study of murine leukemia viruses has been methodological. For the first 15 years of their recognition these viruses could be identified only by measurement of leukemogenesis as an end point, a parameter which proved both time-consuming and costly; experiments utilizing new data could be initiated only at lengthy (yearly) intervals. In the course of the last seven years, various *in vitro* assays have been developed which allow identification of the presence of murine leukemia viruses in a period of days or weeks, leading to study of these viruses in greater depth. These assays include those

utilizing immunologic viral markers in complement fixation, immunofluorescence, cytotoxic and other methods, spleen weight and focus formation by erythroleukemic viruses, presence of DNA reverse transcriptase, and plaque formation on XC cells. It is, of course, recognized that the various assays may detect different entities associated with virus or with different stages in viral replication. The tissue culture studies I will describe have been performed with the XC plaque assay; the *in vivo* studies have been based on the Friend virus spleen focus assay.

The natural history of murine leukemia viruses in high and low leukemia mouse strains and the relation of these viruses to leukemogenesis is remarkably complex. Viruses are certainly found in nonleukemic cells, both *in vivo* and *in vitro*, and virus is detectable at birth in animals which will develop leukemia in later life. In high leukemia AKR mice, virus is found starting at about 17 days of gestation with a dramatic increase from several days after birth to 16 days of life. Over the lifetime of the AKR mouse, there is a fall in titer with a later divergence; mice which develop leukemia show a significant rise in mean virus titer at about the time of disease onset. A possible interpretation of this phenomenon, for which Oldstone has more direct data, is that immune complexes of this virus develop, and the virus may in fact circulate as an immune complex. There appears to be a coincident rise in virus titer associated with leukemogenesis; however any inference at this point of a causal relationship is speculative.

Some years ago Hartley, Rowe, and Huebner began to devise methods for isolation of leukemia viruses from their natural hosts. These isolations were carried out on embryo fibroblasts of either NIH Swiss or BALB/c mice. The important observation was made that all naturally occurring murine leukemia viruses showed significant preferential replication on either NIH Swiss or BALB/c cells, but not on both (Table 104). The naturally occurring viruses could thus be classified into two groups: N-tropic, with a 100- 1,000-fold greater efficiency of growth on NIH than BALB/c cells; and B-tropic, with the reciprocal pattern. It is worth emphasizing that both N- and B-tropic viruses have been isolated from the BALB/c strain; thus far all viruses isolated from the AKR and C58 high leukemia strains have been found to be N-tropic. One can

TABLE 104

The Interaction of Viruses of N, B and NB Tropism in Cells of N Type (NIH) and B Type (BALB/c)

Host	Virus-titer-$\log_{10}$		
	N-tropic (AKR)	B-tropic (RAD-LV)	NB-tropic (Rauscher)
NIH	5.0	2.0	6.0
BALB/c	2.0	5.0	6.0

sometimes adapt N- or B-tropic viruses to growth on both types of cells through forced passage on resistant cells; such viruses are referred to as NB-tropic. Certain laboratory-derived murine leukemia viruses, such as Moloney and Rauscher virus, show the NB property. There has never been a direct conversion of virus from N-tropic to B-tropic except in complex transcapsidation experiments.

Influenced by the work of McDevitt and Sela showing genetic control of the immune response with evidence of linkage to the H-2 locus, and also the work of Lilly who described the H-2 linked *Rgv-1* locus with regard to susceptibility to Gross leukemia virus, we were led to the study of a possible genetic basis for N- and B-tropism.

A number of titrations utilizing many N- and B-tropic viruses derived from various strains of mice and murine tumors indicated that all mouse strains tested resembled either NIH or BALB/c (Table 105). Those strains showing a 100- 1,000-fold greater susceptibility to N-tropic viruses are referred to as "N-type"; all other strains showed the reciprocal pattern and are referred to as "B-type." It must be emphasized that significant differences are present within these broad categories. A notable example involves the NZB strain which is relatively insensitive to both types, but significantly more sensitive to N-tropic viruses, rendering it N-type. As you know, autoimmune phenomena are seen in this strain and the murine leukemia virology of this strain is unique. The NZB is the only strain in which viral antigen is found, but no *in vitro* activity is demonstrable in the XC plaque assay; furthermore, this is the only strain in which both viral antigen and antibody are found in the same host within its lifetime. Other differences, such as between NIH and DBA, and BALB/c and A and C57BL are also noteworthy. Nonetheless, the overall patterns of N- and B-type strains is apparent.

The superscripts^{d} in Table 105 refer to the presence of spontaneous virus in secondary tissue culture preparations. These strains include the high leukemia AKR and C58, as well as the RF and B10.BR strains. B10.BR ($H\text{-}2^{k/k}$) mice are congenic with the C57BL ($H\text{-}2^{b/b}$) strain in which virus can be "activated" by radiation; it is of interest that when the $H\text{-}2^{k}$ allele is put on a C57BL background to produce B10.BR a certain percentage of these mice show spontaneous leukemia virus presence. It is noteworthy that all strains which show significant spontaneous leukemia virus in secondary fibroblast cultures are of the $H\text{-}2^{k}$ type. However, as can be seen there is no linkage between H-2 and the N- or B-type of mouse strains; the b, d, and k alleles at H-2 are represented in both categories.

The evidence that determination of N- and B-type has a genetic basis will be briefly reviewed. F_1 hybrids of N-type and B-type mice are resistant to both N-tropic and B-tropic viruses. Resistance appears to involve a two-hit kinetic mechanism in both F_1 hybrids and resistant strains; thus one has only a titer estimate at a particular point in testing plaquing efficiency in nonsusceptible

TABLE 105

Plaquing Efficiency of Various N-, B-,
and NB-Tropic Viruses on Embryo Cells of 23 Mouse Strains

Cells	H-2 type	No. of tests	Viruses		
			N-tropic[a]	B-tropic[b]	NB-tropic[a]
N-type					
NIH/N	–	10	(100)[c]	<0.1	(100)[c]
AKR/N[d]	k	2	200	<0.1	250
C58/J[d]	k	1	120	<0.1	120
C57BR/cdJ	k	2	100	<0.1	100
C57L/J	b	5	100	0.2	100
Ha/ICR	–	1	80	0.5	140
ST/bJ	k	2	60	0.2	80
CBA/J	k	2	50	<0.1	100
CE/J	k	2	50	<0.1	100
C3H/HeN	k	3	50	<0.1	60
DBA/2N	d	7	40	0.3	100
NZW/N	?	2	15	0.3	100
129/J	b	4	15	<0.1	120
RE/J[d]	k	2	10	<0.1	100
NZB/N	d	7	2	<0.01	30
B-type					
BALB/cN	d	10	0.8	(100)[c]	80
A/HeJ	a	1	0.3	80	40
AL/N	a	2	0.3	60	40
B10.BR/J[d]	k	2	0.3	60	50
I	l	1	0.1	50	NT
C57BL/10J	b	3	0.2	30	40
A/J	a	6	0.4	25	80
C57BL/6N	b	7	0.3	25	50

[a]Plaquing efficiency relative to NIH ME (%).

[b]Plaquing efficiency relative to BALB/c ME (%)

[c](By definition).

[d]Primary ME cells used as secondary ME cells showed spontaneous plaques.

NT: not tested.

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embryo monolayers. In backcross studies (Table 106), two types of mice were observed: (a) those with titers of an N- or B-tropic virus similar to the parental strain with a much reduced titer of the reciprocal virus; (b) those with similar but 30- 100-fold reduced titers of both N- and B-tropic viruses when compared to the appropriate susceptible strain. These two types of susceptibility patterns are denoted homozygous and heterozygous respectively. Results from 12 different individual mating pairs are summarized in Table 106; 48 homozygous and

TABLE 106

Classification of Backcross Hybrid Progeny of 12 Mothers by Plaque Titrations on Cell Cultures from Individual Embryos

N-B type	Mating	Number of embryos in litter	Genotype (homozygous or heterozygous)	
			NN	NB
N × (B × N)F_1	DBA/2N × CDF_1	9	3	6
	C57L/J × CDF_1	9	7	2
	C57L/J × CLF_1	10	8	2
	C57BR/cdJ × CLF_1	6	3	3
	Total	34	21	13
(B × N)F_1 × N	CDF_1 × DBA/2N	8	2	6
	CDF_1 × C57L/J	9	3	6
	CLF_1 × C57L/J	7	2	5
	CLF_1 × C57BR/cdJ	11	7	4
	Total	35	14	21
			BB	NN
B × (B × N)F_1	BALB/cN × CDF_1	9	1	8
	BALB/cN × CLF_1	8	4	4
	Total	17	5	12
(B × N)F_1 × B	CDF_1 × BALB/cN	9	6	3
	CLF_1 × BALB/cN	5	2	3
	Total	14	8	6
Total of all embryos		100	48	52

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52 heterozygous embryos were found, compatible with the expected 1:1 ratio if a single major gene is involved. It should be noted that the figures involving the backcross mice were not as clear-cut as in the parental suceptible and resistant strains, indicating other factors than the locus we are describing are involved in susceptibility patterns. However, strict criteria were used to define homozygotes and heterozygotes, and all embryos showed a clear resemblance to either the parental (susceptible) or F_1 (resistant) type.

In reviewing the literature it was apparent that the patterns noted in regard to the "NB" locus were quite similar to those observed by Lilly with regard to the locus described as *Fv-1* in relation to Friend virus susceptibility. The "NB" studies had been performed utilizing an *in vitro* tissue culture technique, while the *Fv-1* locus was described in studies with the *in vivo* spleen focus assay. In collaboration with Rowe and Lilly, it appeared reasonable to exchange reagents and mice to determine if the "NB" and *Fv-1* genetic loci were in fact identical. Accordingly, the two variants of Friend virus utilized to establish the *Fv-1* locus were tested using tissue culture techniques; as predicted, the F-S Friend virus

TABLE 107

Titer (LOG_{10}) of Friend Murine Leukemia Virus Variants on Mouse Embryo Cells

N-B Type	Cells	F-S (N-tropic)	F-B (NB-tropic)
N	NIH	5.5	5.6
($Fv\text{-}1^s$)	DBA/2N	5.8	5.4
	C3H/HeN	5.6	5.3
	C57L/J	5.7	5.5
	NZB/N	5.2	5.0
B	BALB/cN	3.1	5.5
($Fv\text{-}1^r$)	A/J	2.8	5.1
	C57BL/6	2.2	5.3
F_1	C57L × BALB	3.0	5.6
	BALB × DBA	2.5	5.2

variant was found to be an N-tropic virus with inefficient propagation on B-type strains while the F-B variant (known to grow in all mice after adaptation) is an NB-tropic virus (Table 107). Further studies utilizing strains established by Lilly which are congenic at the *Fv-1* locus (as well as at the *Fv-2* locus, which is irrelevant to tissue culture susceptibility patterns and not included in this discussion), indicated that the D-2 RB strain, which is *Fv-1* sensitive, behaved as an N-type strain, and the D-2 RS strain, resistant at the *Fv-1* locus, behaved as a B-type strain. Finally, knowledge of the *Fv-1* type which has been obtained through tissue culture studies in the XC plaque assay was used to predict spleen focus response in previously-untyped strains; all predictions as to response to F-S and F-B were verified. These experiments established that the "NB" locus and *Fv-1* locus were indeed identical or very closely-linked. It appeared reasonable that a new nomenclature be developed to designate the alleles at *Fv-1;* rather than the use of s (sensitive) and r (resistant), the designations n (N-type) and b (B-type) seemed preferable (Table 108). In denoting the alleles, the lower case is utilized to indicate dominance of resistance.

TABLE 108

Correlation between *Fv-1* Genotype and N- and B-Tropism

$Fv\text{-}1^s = Fv\text{-}1^n$
$Fv\text{-}1^r = Fv\text{-}1^b$

We recognize that these studies have something to do with virology and genetics, but what does all this have to do with leukemia? The answers from a prospective study are not yet available; Rowe has a large experiment directed toward studying the relation of the *Fv-1* locus to natural viral expression and

TABLE 109

Leukemia Virus Susceptibility Correlated with *Fv-1* Type

Fv-1	H-2	Mouse	Leukemia %		
			Passage A	RAD-LV	B/T-L
N	b	C57L			0
	d	DBA/2			8
	k	C3H/B1	98	27	
	k	C57BR	94		9
	q	DBA/1			18
B	a	A	11		100
	b	C57BL/6		91	68
	b	C57BL/10		89	80
	d	BALB/c		71	100
	I	I	22		

leukemogenesis in mice. However, available host-range data in the literature suggests a possible role for *Fv-1* in spontaneous leukemogenesis. The data illustrated in Table 109 is compiled from host-range studies of Gross with the passage A virus, Kaplan with the Rad-LV virus, and Tennant with the B/TL-L virus; the mouse strains studied are grouped according to "NB", or *Fv-1* type, rather than as presented by these workers long before knowledge of the *Fv-1* locus. It would appear quite plausible to conclude that the passage A virus is an N-tropic virus (derived from the N-type AKR mouse) and the Rad-LV and B/T-L viruses are B-tropic (derived from the B-type C57BL and BALB/c strains respectively). Significant variations are seen among strains in the two groups, reinforcing our concepts concerning the multifactorial nature of host range susceptibility to leukemogenesis. Indeed, Lilly and Tennant have demonstrated the importance of H-2 linked determinants in susceptibility to leukemia, as discussed earlier. It does appear, however, that the *Fv-1* locus is a primary determinant of leukemogenesis as denoted by the data in Table 109, though this remains to be confirmed by prospective studies in progress.

WARNER: Would Lilly tell us whether *Rgv-1* is operating in an immune response gene manner? He commented that there is very little difference in humoral antibody production of *Rgv-1* susceptible and resistant strains. We have data in our own laboratory with plasma cell tumors probably involving a Gross virus antigen, which indicate that *in vivo* tumor immunity is mediated by a θ-bearing cell, i.e., a T cell. Is there any evidence for a difference in T cell-mediated immunity in *Rgv-1* susceptible and resistant strains?

LILLY: We have no data on this point. There are data from the laboratories of Jean-Paul Levy and also Bianchi in Italy which suggest that there is some T cell

factor involved. Whether this is a reflection of the *Rgv-1* genotype or not is an entirely open question.

BACH: Did Lilly mean to suggest that the reason *Rgv-1* could not be a receptor site is because the effect is a partial or quantitative effect rather than a qualitative effect?

LILLY: Yes. That was my point.

BACH: I don't think that is a critical argument—far from it. It seems to me that a mutation at a receptor site could lead to less affinity; all kinds of changes which would, in fact, result in a quantitative difference.

LILLY: The quantitative effect that I spoke of in connection with *Rgv-1* is one that is manifested at the level of an approximately 10- or 20-fold difference, late in the disease process. However, in systems where one can observe early in the disease process, the quantitative difference between the two hosts is essentially negligible, certainly not more than a two-fold difference.

For example, animals otherwise susceptible to Friend virus, differing only at *Rgv-1*, show extreme susceptibility to focus formation by the virus, and there are cogent reasons for believing that focus formation is a major factor in the development of the disease syndrome. In such animals the results of the spleen focus assay, which is conducted nine days following virus inoculation, show only a two-fold difference. I really don't think that this is enough to indicate a receptor difference, particularly since the difference only a few days later by several other criteria is now amplified enormously (*Rgv-1* susceptible hosts are susceptible in the spleen focus assay to a level of an approximate virus titer of 10^5, whereas the resistant hosts show a titer of 0.5×10^5). I think this argues against the receptor hypothesis, but Bach's objection has merit. In the long run, the affinity of the receptor molecule might amplify the effects late in the course of the disease.

PINCUS: In this regard it is worth noting that *Rgv-1* is a recessive gene. I think that the dominance for resistance at this locus also is against a receptor being involved.

BODMER: I would like to ask Lilly about his very interesting observation on the change in the H-2 antigens during the course of the disease associated with the *Rgv-1* gene. This seems to raise a number of questions, which as he has indicated may provide a clue to the events involved.

The main question is—what are the cell types which Lilly is studying with respect to the H-2 antigens? Further, is he using a cytotoxic assay and has he checked that there are changes as judged by absorption as well as by direct assay? Is it conceivable that the cells being studied are actually the tumor cells

and that the apparent change is connected with overall changes in these cells during tumor progression, and is therefore not directly related to the H-2 antigens. HL-A typing, for example, does sometimes give different results in people with diseases, and changes in the HL A types on lymphoid cells have been reported. Also, HL-A antigens cannot be demonstrated on HeLa cells by direct typing, as is also the case with H-2 on tumor cells such as Ehrlich ascites. Similarly, HL-A antigens cannot be detected on granulocytes by direct cytotoxicity even though absorption tests provide us with evidence that in fact they are there. Accordingly, it seems to me important to study further the H-2 change associated with *Rgv-1*, and try to find out what is the nature of the change in the H-2 antigens and what clues that might provide to the function of the *Rgv-1* gene.

LILLY: We have tested the disappearance of the FMR and H-2.31 antigens by several techniques, absorption included. None of these methods rule out the possibility, of course, that what we are looking at is not antigen disappearance in the strict sense, but antigen blocking by some mechanism.

CHAIRMAN ALLISON: The interesting differential effect on one H-2 antigen but not another is certainly significant.

LILLY: Exactly, it is genotype specific; the analogous antigen in resistant animals does not disappear.

CHAIRMAN ALLISON: Does anything like this happen in the human situation?

LILLY: It may, but we don't know.

VAN ROOD: To my knowledge, there are only two documented cases that Bodmer refers to. One study was by Amos, and another one by Bertrams in Essen. The case of Amos was, I believe, checked by absorption; the case of Bertrams was not. The interesting aspect of the case of Bertrams was that the 4a specificity did remain while the other specificities disappeared.

LILLY: Was there an associated disease syndrome?

VAN ROOD: These were patients with late stages of Hodgkin's disease.

BODMER: I would like to have answers to my other questions, Mr. Chairman.

LILLY: As regards the cell type involved—we have looked almost exclusively at cells in the spleen. In the very early stage of the development of the disease this, of course, includes the entire mixed population of spleen cells. However, soon after the rapid splenomegaly begins the spleen contains a largely monoclonal population of virus-infected tumor cells, so that we are looking at essen-

tially a pretty homogeneous population of cells—cells of the early erythroid series, often referred to as erythroblasts.

BODMER: These are the cells of the tumor?

LILLY: Yes, they are indeed the cells of the tumor.

BODMER: So if I correctly interpret Lilly's statement, the tumor cells are the ones that in one case lose a particular antigen and in another case don't.

LILLY: Yes. I should say that as far as I know the tumor cells are the only cells which express the tumor-specific antigen, the FMR antigen. Lymphocytes from these same hosts do not in our experience detectably express the FMR antigen, so that for the purpose of that aspect of the study we are restricted to looking at the tumor cells. I might also say that preliminary and perhaps incomplete studies suggest that whereas the H-2.31 antigen disappears from these tumor cells, as a late phenomenon in the disease process, they do not at the same time disappear from lymphocytes.

RAFF: Has Lilly tried modulating the FMR antigens *in vitro*?

LILLY: No.

CHAIRMAN ALLISON: In lymphocytic choriomeningitis it looks as if the disappearance of virus-specific antigen from the surface of infected cells may be interesting and important, according to recent work by Lehmann-Grubbe. Does anybody want to speak on this point?

BODMER: I would like to make one further comment on this question of typing different kinds of cells, because I think it is important. Some observations we have made suggest that there may be situations in which one tends to lose the LA antigens, and not the Four antigens of the HL-A system, as detected by direct typing. The observations are on hybrid cells we and others have made between HeLa cells, on which one cannot detect HL-A antigens, and peripheral blood lymphocytes or fibroblasts, on which one can. On the hybrids one generally finds the HL-A types only of the lymphocytes or fibroblasts. We have found, however, that the LA antigens may be expressed much more weakly than the Four antigens.

So I think that an analogous alteration on tumor cells may be a relatively nonspecific change that is just reflecting some aspect of tumor progression, since the tumor is presumably growing in a different and more virulent fashion in the susceptible animal.

BENACERRAF: The discussion has centered on *Rgv-1* and the mode of action of the H-2 linked histocompatibility, but my query is whether immunity has

ever been demonstrated to the antigen of the Friend leukemia virus? If that is the case, it should be possible to demonstrate with pretreatment with irradiated isogenic tumor cells some form of immunity in that system. One would then have a means of demonstrating whether there is weaker immunity in strains which are susceptible to the leukemia than in those which are not. Alternatively, does thymectomy or ALS abolish the strain difference?

LILLY: Thymectomy *certainly* increases susceptibility to the Friend virus disease. I do not think that the studies have progressed to the point where we can say that it abolishes the *Rgv-1*-determined difference, since *both* strains are more susceptible following neonatal thymectomy. There is considerable evidence that the production of anti-FMR antibodies, the humoral response to the tumor-specific antigen, is in fact, a factor in resistance to Friend and to all of the other leukemia viruses. As I mentioned in response to Warner's question a while ago, the evidence for cell-mediated immunity, as a factor in resistance to the disease is considerably less firm, but is really very suggestive. The experiments Benacerraf suggested would be worth carrying out.

BENACERRAF: Has disease induction by transplantation of existing transformed cells in these animals which bear the antigen, either irradiated or in numbers so low as to reduce immunity in other systems, ever been followed?

LILLY: The answer is I think, not at all extensively.

BENACERRAF: Too bad.

CHAIRMAN ALLISON: Oldstone will now tell us about two models in which the production of disease is dependent on the host immune response itself.

OLDSTONE: The disease that I will discuss first is lymphocytic choriomeningitis (LCM) virus infection in mice, and second, the autoimmune disease of New Zealand mice.

Let us begin by directing our attention to LCM virus infection—limiting our discussion to the acute disease in adult mice. When LCM virus is injected into mice, the pattern that occurs is similar to most acute viral infection; that is, depending on the virulence and dose of the virus and susceptibility of the host, either the animal dies acutely from infection with manifestations predominantly in the choroid plexus, brain, liver, and heart, or clears his tissues of virus and becomes immune. Death is dependent on the host immune response to the infecting virus. The evidence is as follows:

1. LCM *per se,* is noncytopathic. When this virus is grown *in vitro* in tissue culture, no cytopathic effect occurs.
2. When an animal is challenged with an ordinarily lethal dose of virus, if the host is immunosuppressed in any way, (ALS, x-irradiation, metho-

trexate, neonatal thymectomy), these animals are protected from the ordinarily fatal outcome of acute viral disease.

3. If either immune sera and complement, or sensitized cells are added to virus infected cells, they are injured.

Thus, the evidence is overwhelming that this acute virus disease is *not* due to the virus *per se*, but rather to the interaction between the host immune response and the virus.

In discussing susceptibility and resistance in this model, I shall not dwell on nonspecific factors such as interferon, body temperature, hormones, etc., which obviously play a role in *any* infection. Instead, I would like to focus attention on the immune response in LCM infection.

When we first looked at this model, it was soon clear that certain strains were highly susceptible, while others were resistant to LCM virus infection. As disease is caused by the immune response against virus, the question was asked: "Is this response linked to H-2?" To answer this question we looked at a number of strains, but I shall refer only to two, the SWR/J, which is ($H\text{-}2^{q/q}$), and the C3H/J, which is ($H\text{-}2^{k/k}$). The SWR/J was the most susceptible, while the C3H/J was most resistant. The question posed was what would be the susceptibility in the F_1 (SWR/J and C3H) and backcrosses to parental strains. Obviously, if the antiviral immune response(s) which killed the animal were H-2-linked, one would predict that any animal bearing the $H\text{-}2^{q}$ alleles would be susceptible while those homozygous for $H\text{-}2^{k}$ would be resistant.

To carry this point further, backcrosses of the $H\text{-}2^{q/k}$ F_1 mice to the susceptible strain would be expected to be susceptible, whereas backcrosses to the resistant strain would be expected to segregate to susceptible and resistant. These experiments, shown in Tables 110 and 111, were carried out in collaboration with McDevitt and Mitchell. The experimental design as described involved animals that were all H-2 typed, coded, and then injected intracerebrally with

TABLE 110

Relationship of the k and q Alleles of H-2 to Susceptibility in Acute LCM Virus Infection

Strain	H-2	Acute LCM virus infection	
		Susceptible	Resistant
SWR/J	qq	+	
C3H	kk		+
(SWR/J × C3H)F_1	qk	+	
F_1 × SWR/J	qq	+	
	qk	+	
F_1 × C3H	qk	+	
	kk		+

TABLE 111

Relationship of the k and q Alleles of H-2 to Susceptibility in Acute LCM Virus Infection

Congenic strain	H-2	LD_{50}[a]
C3H	kk	4.3
C3H Q/SF	qq	5.7

[a]Reciprocal of the log dilution needed to kill 50% of the animals. 1 log or more difference is significant. Titration done with stock LCM virus passaged through mouse L cells.

LCM virus. The endpoint was death due to leptomeningitis. The essential points that emerged were as follows: If one injected such animals at day zero with a lethal amount of virus, it could be expected that about a third to a half of the susceptible animals would start to die by day six or seven and generally all would be dead by two weeks.

As is evident in Table 110, backcross or hybrid mice bearing at least one $H\text{-}2^q$ allele died; in contrast, the parental C3H mice and backcross mice homozygous for $H\text{-}2^k$ survived and are still alive. On the other hand, the $F_1 \times$ C3H mice bearing one $H\text{-}2^q$ allele all died.

The next experiment studied these differences in congenic strains obtained from Shreffler. These were C3H ($H\text{-}2^k$) and C3H.Q ($H\text{-}2^q$) mice. Again, $H\text{-}2^q$ mice were significantly more susceptible following challenge with virus, than were congenic C3H ($H\text{-}2^k$) mice (Table 111). The point to be made here is that there is a positive correlation between H-2 type and susceptibility to LCM virus infection.

TABLE 112

Postulated Relationship between Histocompatibility Antigens and Susceptibility to Viral Infection

1. Provide receptor sites for viral attachment.
2. Share antigenic determinants.
3. Genetic control of specific immune response (Ir gene).

The next logical question was "what is this H-2-linked susceptibility due to?" I have listed three main possibilities in Table 112. One consideration is that the H-2 allele provides receptor sites for viral attachment. If this were the case we would predict that the most susceptible animal ($H\text{-}2^{q/q}$) would adsorb more virus onto the cell surface than the resistant animal ($H\text{-}2^{k/k}$). This can be tested experimentally. The experiment involved using cultured fibroblasts from either C3H.Q or C3H mice, adding virus, allowing adsorption for one hour, and then measuring the amount of virus adsorbed on or in the various H-2 cell types.

TABLE 113

Incidence of Chronic Disease in Mice with Persistent LCM Virus Infection[a]

3 months	H-2	% Disease
SWR/J	qq	100
B10.D2 old	dd	0
C3H	kk	0
12 months		
SWR/J	qq	100
B10.D2 old	dd	90
C3H	kk	0

[a] Chronic disease consists of immune complex mediated glomerulonephritis, arteritis, and focal necrosis in various tissues. Mice were inoculated with virus shortly after birth.

Results of such experiments show that there is *no* difference in the amount of virus adsorbed onto cells of either C3H or C3H.Q mice. Hence, we can exclude the possibility of H-2 receptor site or viral attachment as an explanation of this H-2-linked susceptibility.

The second hypothesis is that there is a sharing of antigenic determinants between H-2 and a viral antigen. This hypothesis requires that the host is unable to react immunologically against the virus. This could not be the case since the F_1 hybrid host mounts both an antibody and a cellular response against the virus. Thus, the second hypothesis seems untenable.

The third main hypothesis is that death is related to the *Ir-1* immune response gene. At this point, although there is no direct experimental evidence to support or reject such a contention, indirect evidence supports this theory. Other considerations are that the amount of virus carried on the plasma membrane in $H\text{-}2^q$ mice is greater than in $H\text{-}2^k$ mice. The virus or virus antigen is then in a position to be better hit by immune cells or antibodies. We simply have no evidence on this point at all.

A further interesting point involves the finding that one can cause a chronic progressive "autoimmune" disease with LCM virus in animals injected with virus at birth or infected naturally *in utero* by its parents.

As Table 113 shows, once again SWR/J is susceptible, developing the manifestation of disease associated with chronic viral infection while the C3H is quite resistant. The manifestations of chronic disease are immune complex-mediated glomerulonephritis and vasculitis and focal liver, brain, and other tissue injury. Again tissue injury is mediated by the immune response of the host against the virus. The principal evidence for this is:

TABLE 114

One Month Survival of C3H.Q Mice
Inoculated at Birth with LCM Virus

	% Mortality after birth at age			
	1 wk	2 wk	3 wk	4 wk
C3H.Q	18	44	74	83

1. The virus is noncytopathic in cultured cells.
2. Despite the fact that the virus grows in all or most all of the tissues of the chronically infected animals, only selective or focal areas show injury.
3. An immune response, both cellular and humoral, can be detected in chronically infected animals.
4. Parabiosis of chronically infected animals with immune animals, or transfer of immune cells, or of immune sera to chronically infected mice, results in initiation or enhancement of lesions of the chronic disease. The accumulated evidence makes it quite clear that these injuries are indeed due to the host immune response.

One can detect antiviral antibodies in the glomeruli of such mice. Such antibody presumably travels complexed to LCM virus in the circulation. It is quite clear that in the glomeruli of SWR/J mice there is a heavy IgG deposit; 10 to 15 times greater than the amount of IgG deposited in the glomeruli of C3H mice.

Table 114 shows the fate of C3H.Q mice infected at birth with LCM virus. These mice are highly susceptible and over 85% die by one month of age. The actual cause of their death is still unknown. It is interesting, of course, that they do start to die at a time when the mouse begins to mount his own immune response. Moreover, although nursed by the same mother, littermate C3H.Q mice infected at birth are much smaller than noninfected controls. At autopsy we have not yet found any pathological reasons why the infected animals do so poorly.

UNANUE: The group at Hopkins have shown recently that cell-mediated immunity is an important, if not the most important, component in acute LCM disease; they have shown in transfer studies that θ-positive cells mediate this disease. Could Oldstone conclude that this H-2-linked susceptibility to LCM is simply an expression of a T cell response on the part of his infected animals?

OLDSTONE: Yes, I do think it would be tempting to come to such a conclusion. However, the evidence from the Hopkins group (Gilden, Cole, and Nathanson), is not as clear as I would like. The data Unanue referred to is based on using heter-

ologous anti-θ, and is therefore open to objections. However, I do agree that it would be nice if the H-2-linked response proves to be due to a T cell-mediated immune response.

UNANUE: The evidence seems to me clear that it is a cell-mediated immune reaction transferred by cells, not by antibodies; moreover, the controls, too, indicate directly that this is a θ-positive cell.

OLDSTONE: If Unanue wants to debate this point, I would be glad to accommodate him. First of all the heterologous anti-θ sera used were not well characterized, or absorbed. With antiallotypic antibodies present, how can one be certain that one is not killing lymphocytes that have Ig receptors on their surface? How does one rule out the operation here of a Perlmann-like model in which antibody-coated target cells are killed by nonsensitized lymphocytes?

The second point about their model is that the animal is immunosuppressed with cyclophosphamide, the effect of which on this specific antiviral immune response has not been measured. Third, the model is quite different from that which ordinarily takes place in an acute infection. In the cyclophosphamide treated animal antigen is concentrated almost entirely in the central nervous system, while in acute LCM infection, virus is in all tissues.

The most interesting aspect of the Gilden-Cole-Nathanson model is the fact that they can show manifestations of acute CNS disease following transfer with sensitized cells. I like their model but I am not completely convinced of either their or Unanue's conclusions.

SELA: I want to be sure I understand the immunological system we are discussing. When one is dealing with susceptible animals and resistant animals, is the idea that the susceptible animals are those that have a powerful immune response—regardless, for the sake of the argument—whether it is antibody or cellular? Is the disease related to the immune response or is the immune response, like antibody formation, protective? I am not at all clear, in terms of the correlations implied between the disease and the immune response. What is correlated with what?

First, I thought that Oldstone was telling us that the susceptible animals are producing antibody, the antibodies form complexes, and it is this that induced the disease. Then, the question would be: "In the resistant animal, is antibody being produced?" In short, what is the correlation between the disease and the genetic control of the immune response?

OLDSTONE: Is Sela asking whether an immune response to the virus kills these animals?

SELA: Yes, I am.

OLDSTONE: My answer would be yes. Some of the questions Sela asked are most relevant, but very hard to answer at present. Clearly the nephritis and arteritis in *chronic* LCM infections reflect immune complex-mediated injury. In my view there is no reason to doubt that.

C3H ($H\text{-}2^k$) and SWR/J ($H\text{-}2^q$) mice mount both an antibody and a cellular response against the virus. It should be appreciated that the animal is in virus (or antigen) excess all the time; quantitation of the antibody in excess antigen, is at present impossible.

The acute manifestations of tissue injury are mostly in the CNS, liver, and heart. There is evidence that these are caused in part, as Unanue has pointed out, by cell-mediated immune responses, presumably to the virus. However, the antiviral antibody response may also play a role since complement depletion of animals acutely infected with LCM virus protects these animals. The relative roles of T and B cells have not been sorted out, but I would suspect that both are involved.

COHN: As this disease progresses, has Oldstone measured whether the animal produces autoimmune antibodies to normal tissues?

OLDSTONE: Yes, we have and this will be discussed later. Suffice it to say that autoimmune manifestations such as antired blood cell antibodies and antinuclear antibodies are not produced in the C3H, and the SWR/J, B10.D2-old or B10.D2-new strains. Antinuclear antibodies are made in New Zealand mice chronically infected with LCM virus. Hotchin has reported antibrain antibodies but we have been unable to confirm his results.

LEVINE: As I understand Oldstone's thesis concerning the acute disease, he is saying that one can isolate one factor which he thinks is necessary, but not necessarily sufficient to cause death, namely immune responsiveness of the mouse to virus antigens; this is what is correlated with H-2. There is something about this situation that I don't quite understand, but which rather surprises me. For example, we have found that when we look at correlations of protein immunogens with H-2, that there is an important dose effect. If the dose of antigen is lowered to minimal levels which barely induce any response, then we see an H-2 correlation with responsiveness. On the other hand, if we use high doses of an immunogen, then this correlation becomes progressively more difficult to discern, and in some cases is obliterated. As to the virus, we are in all probability dealing with a complex immunogen.

Thus, high doses of antigen would be expected to wipe out these differences so that the H-2 correlated effect would disappear. I am surprised that the H-2 correlation should be so clear. Surely there should be some $H\text{-}2^k$ strains which *do* give the acute disease. Perhaps Oldstone is prepared to respond to these provocative remarks.

OLDSTONE: The only reply to Levine is based on the available facts. When we look at an $H\text{-}2^{q/k}$ combination in the hybrid and backcrosses, only those mice that are homozygous $H\text{-}2^{k/k}$ are protected. Again, the degree of protection is relative, because some animals are dying; about 30% or so of the $H\text{-}2^{k/k}$ animals. Obviously, the virus dose that we are using is one that is limiting, since it does not kill all the animals.

SELA: I think that what Levine was referring to is that if one uses a protein which carries *many* determinants, one can detect the H-2 correlation *only* at dose levels at which but one, or a few of the determinants are immunogenic. This does cause one to wonder why it should be that the first determinant detected by the host in this case should be one which is under H-2-linked genetic control since we know that whereas some antigenic determinants may be H-2-linked, others are not. I assume that next to nothing is known about the chemistry of the virus coat. Until more is known, I would answer Levine's question by taking the analogy of tobacco mosaic virus, which when it is in the form of the virus shows outside a single unique determinant, whereas when the virus is destroyed and one injects the protein of tobacco mosaic virus, as many as half a dozen different determinants are expressed. So one may be in a better position with the protein; one may have in LCM virus just one or two determinants, in which case one will soon have a clear-cut correlation.

OLDSTONE: No comment. I like Sela's suggestion.

GRUMET: I was wondering whether killed virus preparations have ever been used as an immunogen both for *in vitro* tests, such as lymphocyte transformation, and in the induction of antibodies. In that manner one might be able, perhaps, to assess the immune response to viral antigens independently of problems of viral replication, disease, or possible attachment sites.

OLDSTONE: When mice are infected either with inactivated or with live virus, mostly a complement-fixing antibody response is made. I find that there is no significant difference between a CF antibody response made by the SWR/J as compared to C3H/J mice. In general, SWR/J respond a little better, but not significantly more. CF values are low and it is a poor test. Tests for cellular immunity have been made in the study of this viral infection; it is clear that in both the C3H and the SWR/J strains sensitized lymphocytes can kill cells, release lymphotoxin and MIF. However, quantitation of these phenomena is very difficult and we have not yet been able to compare C3H/J and SWR/J responses.

McDEVITT: I think Levine's question was a good one. Isn't the answer really that if a lot of virus is inoculated all the C3H and C3H.Q mice are killed, so the effect of interest to us is only demonstrable at higher dilutions i.e., lower doses of virus.

OLDSTONE: Yes. That is correct.

BENACERRAF: We should not be left with the impression that H-linked Ir genes are only demonstrated with synthetic antigens; these genes also control the response to native antigens. Thus, Rajewsky and associates have shown that the response to one isozyme of porcine LDH is under H-linked genetic control in the rat. There are also many other examples of similar genetic control of immune responses to native antigens.

LEVINE: I am still not really convinced that in Oldstone's situation one is segregating for H-2 correlated immune responsiveness *per se.* With Julia and Franco Quagliata, we are finding that when we study delayed hypersensitivity in mice by the footpad method, some strains, though good responders to a given immunogen do not give footpad delayed hypersensitivity reactions. These mice may or may not produce sensitized T cells, but they don't give detectable delayed sensitivity reactions to antigen injected into the footpad. If the acute disease is a manifestation of delayed hypersensitivity, I am wondering whether Oldstone may be segregating a factor having to do with this rather than to a factor relating to general immune responsiveness. Thus, are other $H\text{-}2^q$ mice equally susceptible to the acute disease?

OLDSTONE: We have only looked at two strains, the C3H.Q and the SWR/J.

CHAIRMAN ALLISON: Perhaps we should now go on to the second part of Oldstone's findings which deal with the effect of virus infection on manifestations of autoimmunity in New Zealand mice.

OLDSTONE: In considering this second model of immune response disease, I would like to first point out that the New Zealand mice are made up of two strains and their hybrid, and the scheme I want to present is as follows:

Strain	Antinuclear antibody	Anti-RBC Ab	Ab response to DNA_mBSA
NZW	Low	Absent to low	High
NZB	Moderate	Severe	Moderate
(NZB × NZW)F_1	Severe	Moderate	High

These mice show several autoimmune manifestations, and perhaps the most important are anti-erythrocyte antibodies and anti-DNA antibody or antinuclear antibody responses. The anti-red blood cell response leads to autoimmune hemolytic anemia, while the anti-DNA and antinuclear antibody response leads to immune complex glomerulonephritis.

I want to take up the antinuclear antibody response, as I know that Warner will tell us more about the anti-RBC response. In our laboratory, with Tonietti and Dixon, we noted that the New Zealand white (NZW) mouse shows mild anti-

nuclear antibody (ANA) responses, and practically no manifestations of antired blood cell antibodies. The New Zealand blacks (NZB) show perhaps slightly greater manifestations of antinuclear antibody, but are a good model for the autoimmune hemolytic anemia because of their high antired blood cell antibody responses. The F_1 cross, NZB × NZW, has high ANA and moderate antired blood cell antibody.

Interestingly enough, and central to my point, is that if the immune response to DNA methylated BSA(DNA_mBSA) is measured in these mice (this was done by Lambert and Dixon), it is found that NZW is a *hyper*-responder to DNA, NZB is a hyper-responder compared to other strains, but a lower responder responder than NZW, and the (NZB × NZW) hybrid is also a hyper-responder to DNA immunization.

The hypothesis that best fits the experimental facts that I am going to present is that the central defect in the New Zealand mice is their genetic hyperreactivity to nuclear antigen(s), DNA, and similar antigens. This genetic abnormality can be triggered by several environmental factors which may turn on or turn off this immune response. Hence, no one agent or virus is responsible, but many different ones are capable of playing a role.

I will focus on the antinuclear and anti-DNA antibody manifestation. In the mouse, this leads to formation of nuclear antigen-ANA and DNA-anti-DNA antibody immune complexes. These complexes become trapped in the glomeruli and cause nephritis which eventually kills these animals. A few years ago we began to examine the incidence of antinuclear antibody in chronic LCM and polyoma virus infections. At this point I remind you that polyoma virus is a DNA virus, whereas the LCM virus is an RNA virus. The important point of Table 115 is that the incidence of antinuclear antibody, whether one infects NZ mice with RNA or DNA virus is enhancement of antinuclear antibody production in *both* strains and their *hybrids.* Thus, at three months of age it is evident that the NZW have a very low incidence of spontaneous antinuclear antibody. However, when they are infected chronically with LCM virus or with polyoma virus these mice show a significant elevation of ANA.

In contrast, in infection of the SWR/J or C3H strains, which manifest negligible amounts of spontaneous antinuclear antibody, there is no such enhancement of elaborated ANA. Moreover, strains which spontaneously develop ANA, such as AKR mice (with about a 15-20% incidence of ANA), or C57BL mice, had no *enhanced* antinuclear antibody formation when chronically infected with either of these two viruses. Experiments showed that the immune response was directed *primarily* against *single-stranded* DNA and the antibody that is made is from three to six times higher in LCM-infected NZ animals than in noninfected NZ animals.

Finally, it is possible to turn off this type of spontaneous antinuclear antibody response with a different RNA virus. Chronic infection of NZ mice with

TABLE 115

Cumulative Incidence of Autoimmune Responses in New Zealand Female Mice Chronically Infected with LCM Virus or Polyoma Virus

	Antibodies to nuclear antigen at		
	3 mon.	6 mon.	9 mon.
NZW			
Noninfected	7[a]	38	52
LCM virus	64	94	96
Polyoma virus	33	47	77
NZB			
Noninfected	8	44	53
LCM virus	87	86	100
Polyoma virus	48	65	95
(NZB × W)F_1			
Noninfected	13	50	93
LCM virus	63	100	100
Polyoma virus	40	96	96
SWR/J			
Noninfected	0	2	2
LCM virus	0	1	1
Polyoma virus	0	0	3

[a]Percent of mice having antibodies to nuclear antigens

lactic dehydrogenase virus (LDV) prevents the further spontaneous development of ANA, but does *not reverse* an ANA response which is *already present.* Similarly, anti-DNA antibody in LDV infected NZ mice is about one third or less than that in noninfected NZ mice. In summary, NZ mice appear to be genetic hyper-responders to DNA stimulation. Certain viruses either turn on or turn off this immune response.

CHAIRMAN ALLISON: Warner will now continue the development of this discussion and tell us more about the genetics of the New Zealand mice.

WARNER: In continuing this discussion of NZB immunopathology, I would like to consider the point that there may be an immune response gene controling antired cell autoantibody production. The incidence of antired cell antibody production in inbred NZB mice is virtually 100% by 10 months of age, but in NZB hybrid mice is usually much lower when we are examining the development of antibody by nine to ten months of age. It is true that at around one and a half to two years, many hybrids develop antired cell antibody, but I now want to consider a third New Zealand mouse in this discussion, namely the New Zealand chocolate, NZC. The incidence of hemolytic anemia as determined by positive Coombs' tests around nine to ten months of age in (NZB × NZC)F_1 mice

TABLE 116

Coombs' Positive Reactions in Hybrid Mice

Strain combination	Coombs' positive[a]		Expected % Coombs' positive[b]			
	No.	%	1 or 2 Dom.	1 or 2 Rec.	2 Rec. (1 in NZC)	1 Dom. 1 rec. (rec. in NZC)
NZB	68/74	92	100	100	100	100
NZC	0/38	0	0	0	0	0
(NZB × NZC)F_1	54/60	90	100	0	0	100
(NZB × NZC)F_1 × NZC	15/45	33	50	0	0	50
Other NZB hybrids						
e.g. (BALB/c × NZB)F_1	2/27	8	100	0	0	0
(BALB/c × NZB)F_1 × (NZB × NZC)F_1	61/140	43	75	25	< 25	50

[a] At 11 months of age.

[b] On the basis of NZB genetic type.

is close to 100%. The inbred NZC are *not* Coombs' positive, and at no stage in their lives do NZC mice develop this antibody. At this particular age, other NZB hybrids are no more than about eight % positive in Coombs tests.

Oldstone introduced the view that there is indeed genetic control of autoimmune responses. I should like to extend it and say we feel there are at least two genes involved in the inbred NZB mice responsible for their antired cell antibody production. The data on incidence of Coombs' positive mice at 11 months of age (Table 116) shows a similar and full expression in NZB (NZB × NZC)F_1, but reduced incidence in backcross and intercross mice. If we consider these data in terms of the possibilities indicated for gene control, they closely fit the idea that there are two genes in NZB associated with the development of antired cell antibody, one of which is recessive and is also carried in NZC mice.

Of interest to us, therefore, is the level at which these genes are acting. Our hypothesis is that the dominant gene is in some manner operating at the level of determining whether an immune response will be initiated (whereas in other mouse strains, tolerance would normally be induced). We would also suggest that the recessive gene may be operating as an immune response gene. We therefore predicted that if the NZC mouse is carrying this gene, one could perhaps induce its expression in the inbred. An attempt was made along these lines by immunization with Freund's adjuvant containing either syngeneic or allogeneic red cells. A Coombs positive state was in fact induced in many of these mice (Table 117). This only occurred with allogeneic red cells. To our surprise, however, our control strain, in this case BALB/c, also became Coombs' positive, but again, only when allogeneic red cells were used for immunization.

TABLE 117

Induction of Coombs' Positive Reactions by Red Cell Immunization

Recipient mice[a]	Immunizing red cells in C.F.A.		
	Saline	NZC	BALB/c
NZC	0/16[b]	0/14	10/12
BALB/c	0/15	9/14	0/16

[a]Immunized at 3.5 and 6 months. Tested 6.5 months

[b]Number Coombs' positive/number tested.

This study was then extended to several other mouse strains using NZC red cells, and evidence of a genetic control operating in this system was found (Table 118). We therefore suggest that NZC and BALB/c mice both carry a gene that may be operating in an immune response fashion which is specific for antired cell autoantibody production. We would also suggest that these experiments imply, that to trigger the gene into action, one must use allogeneic red cells which may be acting in a hapten-carrier sense, in that the allogeneic determinant is acting as the carrier for the autoerythrocyte determinant. In these instances, the BALB/c mice do produce a serum antibody which is directed against NZC allogeneic red cells, and in the reciprocal direction, NZC mice make an anti-BALB/c red cell agglutinin.

TABLE 118

Strain Susceptibility to Induced Autoimmune Hemolytic Anemia

Strain	% Coomb's Positive[a]
NZC	0
BALB/c	43
C57 BL	0
CBA	0
(CBA × C57)F_1	0
A/J	0
129/J	0
(C57 × A/J)F_1	0
(CBA × DBA/2)F_1	0
DBA/2	0

[a]NZC red cells in complete Freund Adjuvant day 0,24. Coombs' test day 38.

Another genetic control of tumor susceptibility was observed in NZB and BALB/c mice. This relates to the induction of plasma cell tumors (PCT). Table 119 shows that following mineral oil induction treatments, BALB/c and NZB mice show a high incidence of plasma cell tumors. Our figures are misleadingly low because many of our BALB/c colony die from viral pneumonia, and NZB

TABLE 119

Genetic Control of Susceptibility to Plasma Cell Tumor Development in Mice

Strains[a]	No. of mice	% Tumor Incidence[b]	
		Plasma cell tumors	Other Tumors
BALB/c, NZB	107	36	3
(BALB/c × NZB)F_1	96	60	20
Other Inbreds	241	0.5	14
BALB/c Hybrids	200	3	15
NZB Hybrids	105	6	28
Control NZB Hybrids (No Mineral Oil)	108	0	6

[a] All mice treated with mineral oil.

[b] % with tumors of original number of mice.

mice die from glomerulonephritis. If we look at the hybrid, we see a much greater incidence of PCT as well in inbred NZB. In this time period, other inbred mice and other BALB/c and NZB hybrids showed a very low incidence of PCT, and we are wondering whether this is not also a manifestation of one of the autoimmune response genes.

In summary, we would be inclined to suggest the possibility that study of the three New Zealand strains, NZB, NZW and NZC, suggests that there are three genes involved in them concerning autoimmunity. One operates to control the elicitation of an autoimmune response. This may well be affected by environmental factors, as in Oldstone's work, and this gene is present only in the NZB mouse. We may have also immune response-type genes, one of which is involved in red cell autoantibody production, and it should be present in NZB and NZC, but not in NZW. The other gene controls anti-DNA responses and is also present in NZB and NZW, but absent in NZC; (NZB × NZC)F_1 mice do not develop glomerulonephritis in high incidence.

CEPPELLINI: Warner's last point was not entirely clear to me. Could he tell us whether they are at least of the same locus, or different loci?

WARNER: This is a very interesting question. There is a recent study by Noel Rose involving antithyroglobulin antibody production and thyroiditis incidence in mice. They find this is under genetic control and is H-2-linked. Its effects are manifested in severity of disease as assessed by lymphocytic infiltration of the thyroid, and also in antibody production. In particular, BALB/c was a non-responder mouse to thyroid extract, whereas in this present situation we find that BALB/c is a responder in antired cell antibody production. We would now like to determine with hybrid and backcross studies whether these two autoimmune response controls are behaving in allelic fashion.

BRAUN: As far as I know, the LDH virus is an immunoenhancing agent in other strains of mice, and Oldstone has told us that the LDH virus in the NZB mouse produces a reduction in the antinuclear antibody response. I am wondering if we may not again be confronted with a situation in which an excessive stimulation of the cyclic AMP level of relevant immunocompetent cells interferes with antibody formation. In that case, the effect of LDH virus on antibody formation in the NZB mouse would not necessarily be an exception to the general enhanced responsiveness of the NZB mouse.

OLDSTONE: Several points must be kept in mind. First of all, New Zealand mice are not hyper-responders to *all* antigens. Certainly the data by Cerottini and others have clearly shown that with certain antigenic challenges, such as KLH, NZ mice are relatively hypo-responders. So it depends again on the antigen that we are using. In the case of NZ mice it appears that DNA is the antigen with which we should be concerned. Secondly, we should remember that LDH infection causes immune enhancement, as far as I know, only after immunization with HGG. This was first reported by Notkins and we have confirmed his results. However, the immune response to SRBC and KLH is not enhanced by LDV infection, at least not in strains SWR/J, C3H/J, B10.D2-old, and B10.D2-new.

BRAUN: In CFW and C57BL mice we observed elevated responses to SRBC in the presence of LDH virus.

OLDSTONE: Then we would agree that the antigen and mouse strain used are important; as well as dosage and route of administration. I think one of the intriguing possibilities of LDH infection in NZ mice is that this agent is elevating enzymes. One of the germane questions is: does it elevate DNAse? That might account for some of the other effects we see, but as yet we don't know the answer.

SELA: Another autoimmune disease under genetic control should be mentioned: experimental allergic encephalomyelitis. Stone and his colleagues at the NIH showed considerable differences in the susceptibility to disease among guinea pig strains (*Proc. Soc. Exp. Biol. Med.*, **132**, 341, 1969). We have also studied it recently and found that under identical experimental conditions, 63% (15/24) of strain 13 guinea pigs showed clinical symptoms, whereas none of 11 strain 2 guinea pigs became sick (Webb, Teitelbaum, Arnon, and Sela, unpublished data). It would thus seem possible that susceptibility to experimental allergic encephalomyelitis is linked to the major histocompatibility locus in the guinea pig.

TABLE 120

Comparison of the Immune Responses of Strain 2 and 13 Guinea Pigs to Limiting Doses of Various Hapten-Protein Conjugates

Hapten-protein conjugate	Immune responses
DNP_6GPA	$13 > 2^a$
$DNP_{33}GPA$	$13 > 2$
Pipsyl-GPA	$13 > 2$
DNP_5-APA-GPA[b]	$13 > 2$
DNP_6GO	$13 = 2$
DNP_6BSA	$2 > 13$

[a] Linked to 13 locus by backcross analysis.

[b] In this conjugate the dinitrophenyl group is attached to the free carboxyl groups of the guinea pig albumin.

GREEN: Thus far at this conference we have considered mainly the histocompatibility-linked immune response genes which deal with either synthetic antigens or foreign proteins. My point is relevant to the comment by Sela, because in the course of our studies of guinea pigs with DNP-6-GPA at limiting doses, we have made an observation which strongly suggests that the immune response to native self proteins may also be under genetic control. The way this emerged will be seen in the protocols of studies carried out in collaboration with Paul and Benacerraf; in fact these findings were presented in Session I by Benacerraf (Fig. 2). We found that strain 2 and 13 guinea pigs responded similarly to 100 μg of DNP-6-GPA, but when the dose became limiting (1 μg), strain 13 gave a far greater response. We wanted to ascertain whether this was a hapten function, so to speak, or whether this was a carrier function.

Table 120 shows some of the maneuvers we utilized, including first, the same hapten on different carriers, for example with limiting doses of DNP on glucose oxidase, or DNP-BSA. Strain 2 responded better than strain 13 animals. So it appears that the better response to DNP-GPA by the 13 animals is not an effect due to the hapten. On the other hand, when we put another, largely noncross reacting hapten (pipsyl) on GPA, we still saw the same effect (13 was better than 2), and when we put DNP on the carboxyl groups rather than amino groups of lysine we still obtained the effect (13 better than 2).

There are two points to be made: (1) up to this time, most of us have felt that the reason that GPA is immunogenic to guinea pigs immunized with hapten-GPA conjugates is because the GPA became altered by the DNP hapten, or any hapten for that matter, and therefore the GPA became foreign to the guinea pigs; but (2) what we see here is that no matter *how* we alter the GPA, and it is presumed that each hapten alters GPA in a different way, we obtained clear evi-

TABLE 121

Theoretical Outline of Functional Components Necessary for Inbred Guinea Pigs to Mount a Complete Immune Response to Selected Antigens

Strain	Antigen	Ir gene	T cell	B cell	Ab response
13	GPA	+	—	—	—
	DNP-GPA	+	+	+	+
	BSA	—	+	+	—
	DNP-PLL	—	—	+	—
2	GPA	—	—	—	—
	DNP-GPA	—	+	+	—
	BSA	+	+	+	+
	DNP-PLL	+	+	+	+

+ Indicates presence.
— Indicates absence.

dence of genetic control (13 gave a better response than 2). It may, therefore, be that there is an Ir gene for unmodified GPA which cannot be detected unless tolerance to GPA is broken by altering the GPA by conjugation with a hapten. Table 121 shows the overall estimate of what may be happening. In the strain 13 animals, an Ir gene to GPA exists; however, T cell function and B cell function is absent because of tolerance to GPA. When DNP-GPA is used in limiting doses to immunize the strain 13 guinea pigs, a response can be obtained because the GPA has become altered and strain 13 pigs have an Ir gene for GPA. In strain 2 animals, immunization with DNP-GPA at limiting doses fails to produce a response because strain 2 animals lack an Ir gene for GPA.

Accordingly, the concept I would now propose is that the self proteins may have immune response genes which can be detected only by this type of maneuver.

WARNER: Could Green clarify for us the evidence that this is an immune response to native GPA, as against GPA modified by the actual hapten coupling procedure? This might therefore be a different determinant to the autologous GPA.

GREEN: The argument I am making is indirect since no one has yet studied, by direct physicochemical means, the changes made on DPA by hapten alteration. Cellular immunity to GPA, for example, is highly specific, e.g., there is no cross-reactivity of delayed sensitivity between pipsyl GPA and DNP-GPA. Consequently we assume that the GPA is modified in quite a different way (at least from the viewpoint of the guinea pigs' immunological machinery). Nevertheless, immunological responsiveness (presence of an Ir gene) to limiting doses of both pipsyl-GPA and DNP-GPA appears linked to the presence of strain 13 histo-

TABLE 122

Responsiveness of "High" and "Low" Lines to an Optimal Dose of Pneumococcus Polysaccharide III Injected Intravenously

Strain	Number of plaque forming cells[a] per spleen		
	Nonimmunized mice	Peak response 4th day	High/low
Low line	5,000	63,000	24-fold
High line	12,000	1,500,000	

[a] SIII sensitized sheep erythrocytes.
Eur. J. Immunol., 1972 in press.

compatibility antigens. Therefore, one possibility is that it is not the hapten-induced alteration on the GPA which is being recognized by the Ir gene, but rather the unmodified GPA itself.

CEPPELLINI: My query is directed to conference members who are experts in NZB immunopathology. What I would like to know is whether an autoantibody against lymphocytes is found in these animals? The rationale for my query is that in a human disease, systemic lupus erythematosus, which in many aspects is reminiscent of the NZB disease, a humoral antibody (evidently of B cell origin) is often present. This antibody is absorbed on lymphocytes and blocks the *in vitro* reactivity of T cells in MLC and in PHA stimulation. Thus, in agreement with Burnet's suggestion, the viral disease may alter the normal reciprocal regulation between B and T cells, by the distinctive means of inducing the former to produce an antibody acting on the function of the latter.

CHAIRMAN ALLISON: Mellors and Ziff and coworkers, have described such an antibody against lymphocytes in NZB mice. It would be interesting to go on with this discussion, but we still want to hear from Biozzi about reactions to infectious challenge in the two mouse strains which he described in Session III.

BIOZZI: I wish to supplement my earlier remarks by presenting some data dealing with resistance to experimental tumors and infections induced in the "high" and "low" responder mouse lines. These two lines of mice have been separated by selective breeding for the production of SRBC agglutinins. These same lines are also found to be separated for their humoral response to all the other noncross reacting antigens tested so far.

Table 122 shows the response of "high" and "low" lines to the type III pneumococcus polysaccharide, in terms of PFC using SRBC coated with SIII. The background of PFC in nonimmunized mice is not very different in the two lines. After immunization, however, the number of PFC is much greater in "high" responders. In the mouse this antigen is completely thymus-independent.

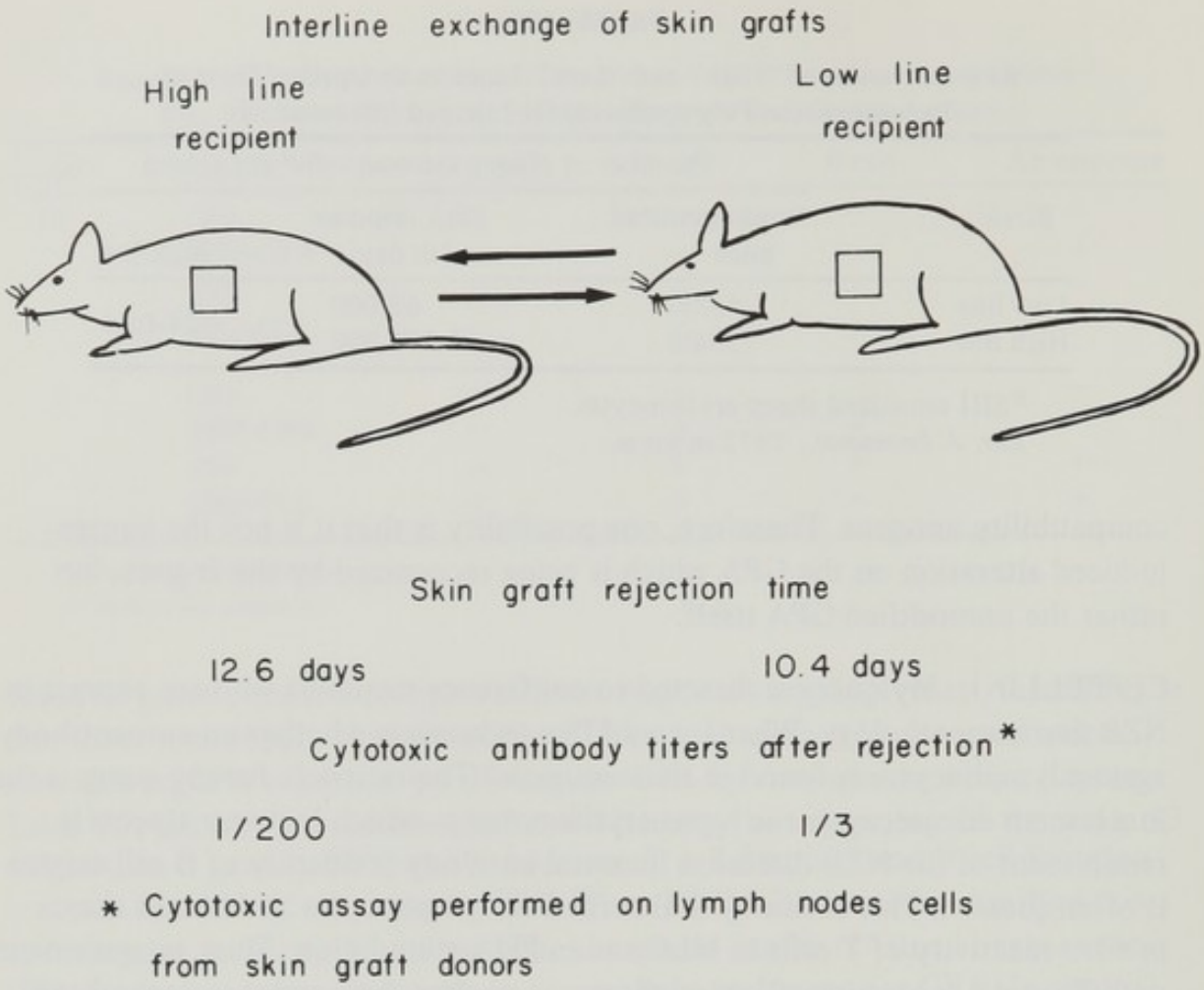
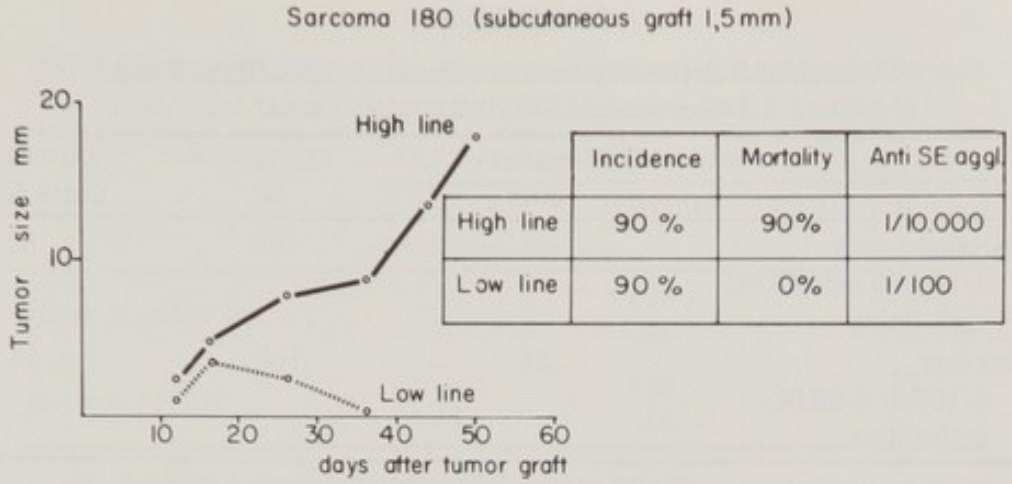


Fig. 72. Rejection of interline skin grafts and production of cytotoxic antibody in "high" and "low" recipients.

Therefore, the first conclusion which can be made from this experiment is that the two lines are certainly separated at the level of the potentiality of B-derived lymphocytes. Are they also separated at the level of the potentiality of T-derived lymphocytes? A very simple experiment represented in Fig. 72 shows that they are not. Skin grafts exchanged between "high" and "low" responder mice are rejected in about the same time. But the titer of cytotoxic antibodies against histocompatibility antigens is much higher in the "high" than in the "low" responders. Therefore, the two lines are also separated for antibody production in response to histocompatibility antigens but *not* for the cellular responsiveness to the same antigens. Cellular immunity mediated by T-derived lymphocytes is considered as the principal mechanism of skin graft rejection. This finding has been confirmed with all the models in which the cellular immune responsiveness of T-derived lymphocytes is involved, such as contact sensitization, PHA responsiveness, and GVH reactivity (Byfield and Howard, *Transplantation,* in press).



	Incidence	Mortality	Anti SE aggl.
High line	90 %	90%	1/10.000
Low line	90 %	0%	1/100

Fig. 73. Development of allogeneic sarcoma 180 grafted subcutaneously in "high" and "low" responder mice.

Our selective breeding has therefore produced a clear-cut dissociation between the potentiality of humoral antibody synthesis, which is very *different* in the two lines, and cell-mediated immunity which is *similar* in both lines. This is important since, when microorganisms or tumor cells are introduced in these two lines of mice the invading cells are faced by different humoral response but by the same cellular response. These animals therefore constitute a distinctive tool for the investigation of the respective roles of humoral and cellular defense against host invasion by bacterial or neoplastic cells.

As is illustrated in Fig. 73, Sarcoma 180 transmitted by subcutaneous implantation in "high" responder mice grows rapidly, inducing 90-100% mortality. In "low" responders, on the other hand, after some initial growth the tumors all regress and disappear. Sarcoma 180 is a tumor known to be particularly sensitive to the enhancing effect of facilitating antibodies. The regression of the tumor in "low" responders is probably due to lack of facilitating antibody synthesis. The cellular defenses may therefore play their full role. The Ehrlich ascites is another allogeneic tumor which is resistant to the enhancing effect of facilitating antibody. The data summarized in Table 123 show no difference in the resistance to this tumor in the two lines of mice. Only a small increase in the survival time in favor of "low" responders is observed.

We have studied the AKR and DBA/2 leukemias, but our "high" and "low" lines will not accept cell transmission of these leukemias, which is limited to the syngeneic strains. We have also observed that the character, "antibody synthesis," of "high" and "low" lines is inherited, to a great extent, in F_1 hybrids of each line with different strains of mice, as shown in Table 124. The DBA/2 leukemia is transmitted by intravenous (10^3 cells) and intraperitoneal route (10^4 cells) in (DBA/2 × High)F_1 and (DBA/2 × Low)F_1. A 100% mortality is produced in both hybrids without any significant difference of the mean survival times

TABLE 123

Lack of Relationship between Immunoresponsiveness and Resistance to Ehrlich Ascites Tumor

Host	Number of mice	Mortality [a] %	Mean time to death (days)
High line 1/10,000 anti-SRBC agglutinins	25	100	10
Low line 1/100 anti-SRBC agglutinins	25	100	13

[a] 10^6 Tumor cells i.p.

(about 10 days). Similar results are obtained in (AKR × High)F_1 and (AKR × Low)F_1 inoculated intraperitoneally with 100 leukemic AKR spleen cells (Table 125). We conclude that in spite of their different humoral immunoresponsiveness (Table 124), the susceptibility of these hybrids to syngeneic leukemia is not modified.

TABLE 124

Peak Level of anti-SRBC Agglutinins Attained in Different Mouse Lines[a]

Strain	Titer
"High" line F_{20}	1/10,000
"Low" line F_{20}	1/100
AKR	1/300
DBA/2	1/1,280
(AKR × high)F_1	1/2,000
(AKR × low)F_1	1/100
(DBA/2 × high)F_1	1/3,840
(DBA/2 × low)F_1	1/480

[a] Immunization with 5×10^8 SRBC i.v.

In previous experiments, we demonstrated a strong protection against syngeneically transmitted AKR leukemia produced by pretreatment with *Corynebacterium parvum*. The data presented in Table 125 indicate that such a protection is also produced in both "high" and "low" responder hybrids. The increased resistance produced by *C. parvum* treatment is therefore not related to the level of humoral responsiveness. The protective effect of *C. parvum* is stronger in "low" than in "high" responder hybrids. This may be due to the increased synthesis of facilitating antibody induced by the strong adjuvant effect of *C. parvum*.

TABLE 125

The Protective Effect of *Corynebacterium parvum* on the Susceptibility of "High" and "Low" Responder AKR Hybrids to AKR Leukemia

Mice	AKR leukemia (100 leukemia spleen cells i.p.)		
	C. parvum[a] treatment	Mortality %	Mean survival time (days)
(High × AKR)F_1	—	100	29
	+	31	—
(Low × AKR)F_1	—	100	27
	+	16	—

[a] C. parvum 1 mg intraperitoneally 6 days before.

TABLE 126

Effect of Vaccination of High and Low Lines on Their Resistance to *Salmonella typhimurium* Infection[a]

Immunization		Challenge	"High" line		"Low" line	
			Mortality	Mean survival time (days)	Mortality	Mean survival time (days)
	Controls	5,000 *S. typhimurium* i.d.	100%	10.4	45%	
		1,000 *S. typhimurium* i.p.	100%	5.4	100%	8.7
Vaccinated	Living nonvirulent *S. typhimurium*	1,000 *S. typhimurium* i.p.	100%	8.6	10%	
	Fraction extracted *from S. typhimurium*	1,000 *S. typhimurium* i.p.	100%	8	10%	

The resistance to *Salmonella typhimurium*, a natural pathogen of mice, was determined in the "high" and "low" responder lines as shown in Table 126. It is evident that "low" responders are markedly more resistant to this infection than "high" responders. For the extremely virulent infection produced by intraperitoneal challenge, this interline difference is only evident in terms of mean survival time. Intradermal challenge produces a less acute infection that is nevertheless fatal for 100% of "high" responders while 50% of the "low" responders survive. Specific vaccination with an avirulent strain of *S. typhimurium* or with an immunogenic extract is barely significant in "high" responders, yielding only a prolongation of survival time, whereas in the "low" responder mice 90% are definitely protected. This higher resistance of "low" responders is a broadly general phenomenon in that it has been demonstrated on each of the various other infections studied so far. Inoculation of virulent *Yersinia pestis* produces 100% mortality in both lines with a significantly more prolonged mean survival time in "low" responders. Specific vaccination with an immunogenic fraction of *Y. pestis* produces a total protection of "low" responders while it is without effect in "high" responders (Table 127).

TABLE 127

Effect of Vaccination of High and Low Lines on Their Resistance to *Yersinia pestis* Infection[a]

Treatment	High line		Low line	
	Mortality %	Mean survival time (days)	Mortality %	Mean survival time (days)
Control	100	4.5	100	7.7
Vaccinated[b]	100	5	0	

[a] 1,000 Microorganisms injected subcutaneously.

[b] 500 μg Extract from *Y. pestis* intradermally.

These findings attest to the greater susceptibility to infection of "high" responder mice. The implication is that resistance to infection is determined by the same group of genes involved in the regulation of humoral immunoresponsiveness. The interline difference in resistance to infection may be reasonably explained by the peripheral effect of facilitating antibodies. These antibodies are viewed as protecting the invading microorganisms against the destructive attack of cell-mediated immunity, which remarkably enough, has the very same potential in both lines.

BRAUN: I would like to comment on Biozzi's tumor data which clearly demonstrate that genetic differences in B cell performance do have an influence on the fate of tumor growth in syngeneic systems.

As I discussed previously, we can produce the phenotypic equivalent of genetically controlled high B cell performance, with the help of stimulators of endogenous cyclic AMP levels, and it has turned out that such stimulators, e.g., poly A:U and/or theophylline also have a pronounced antitumor effect, retarding the growth of a variety of chemically or virus-induced tumor cell lines in syngeneic hosts. But then we found that such antitumor effects of agents known to increase B cell performance occurred just as readily in irradiated animals as in nonirradiated animals (Well, Braun, and Plescia, *Cancer Res.*, in press).

Even pretreatment of the syngeneic tumors, prior to implantation, with the stimulators of endogenous cyclic AMP retarded tumor development. In other words, what I seek to convey is that in discussing any study dealing with tumor cells we have to keep in mind that in addition to the control by conventional immune responses, there is a direct susceptibility of tumor cells to agents that elevate endogenous cyclic AMP levels. Thus, for example, the striking effects that can be obtained with BCG and Corynebacteria may not all be due to the influence on either T or B cell populations, but rather to a mechanism which retards tumor growth in a more direct way.

HERZENBERG: Biozzi has shown that there are, perhaps, as many as ten genes which differentiate these high and low lines with respect to one particular response, i.e., SRBC. For the other responses he is studying, there may well be 10, 20, or even 50 genes which are involved. Further, in different responses it could be that different genes or different combinations of genes are playing a predominant role. I would therefore urge Biozzi, since he is working with a species that is amenable to genetic analysis, to focus on the isolation of these genes. This would be feasible by producing congenic lines following one or another or a half dozen of the effects he is studying in different lines. It might then be easier for the biochemist or the immunologist to ascertain which parameters are important in the different interesting effects he has described for us.

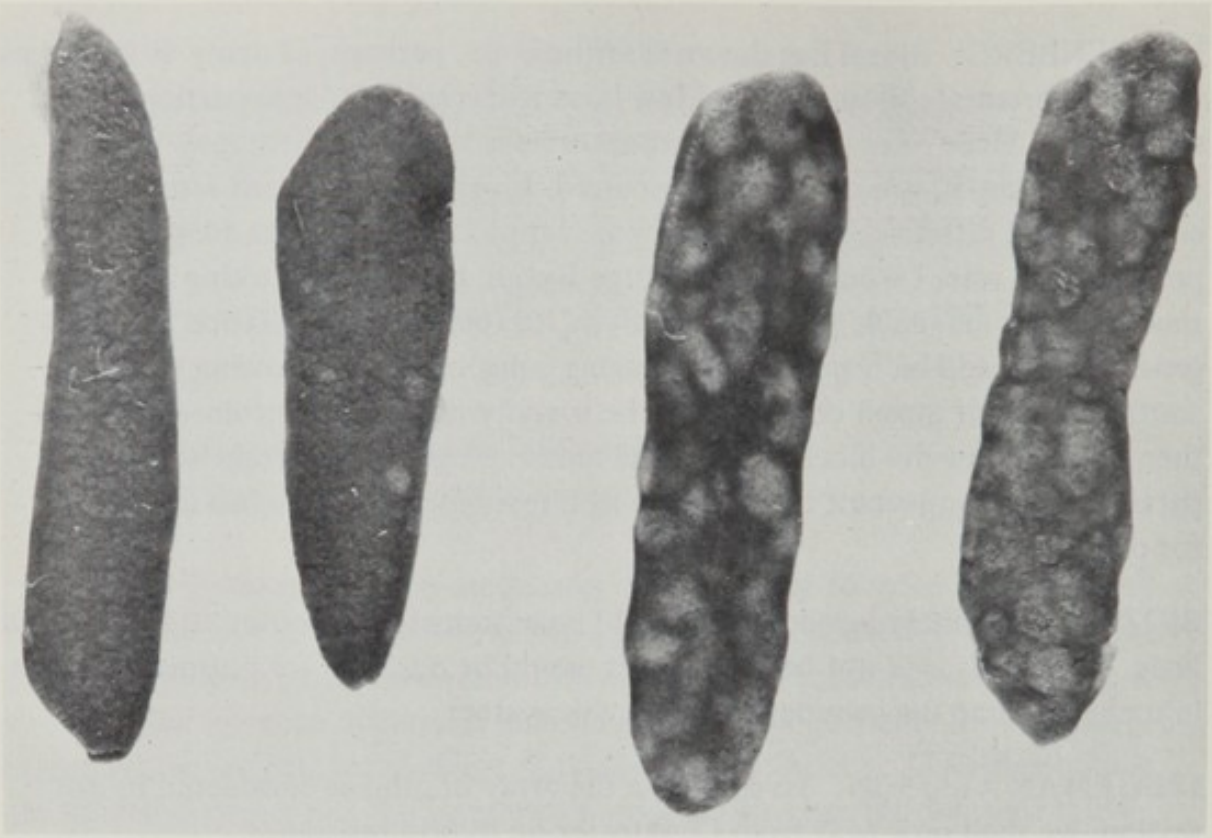
BIOZZI: I definitely agree that it would be extremely interesting to isolate such lines. It certainly will not be easy, but it would be essential for optimal progress in understanding the genetic control in this system.

CHAIRMAN ALLISON: To complete the array of models considered in this session, we shall now hear from Cudkowicz on hybrid resistance.

CUDKOWICZ: I intend to illustrate an animal model of genetic control over allograft reactivity which is highly unusual, for it involves the failure of hemopoietic cells in heavily irradiated mice. Such animals are expected to be unreactive after exposure to radiation, but apparently are not so for allogeneic and xenogeneic blood-forming cells. As the second unusual feature of the model, I wish to stress graft failures in parent-to-F_1 hybrids as well as in allogeneic cell transfers. Hybrid mice are regarded as universal acceptors of parental tissues by classical immunogenetic theory. The third unusual aspect of the model is the tissue specificity, i.e., the incompatibility restricted to hemopoietic cells.

Proliferation of grafted bone-marrow cells is evaluated qualitatively by gross inspection of recipient spleens fixed in Bouins fluid (Fig. 74), and quantitatively by labeling DNA of the cells entering mitosis with the DNA precursor 5-iodo-2′-deoxyuridine-^{125}I (Fig. 75). The spleens are nodular five days after transplantation of 10^6 nucleated cells in mice susceptible to marrow grafts, and the uptake of ^{125}IUdR ranges from 0.3 to 1%, but not so in resistant mice or in radiation controls.

One of the most dramatic examples of resistance to marrow grafts is shown in Fig. 75. The splenic uptake of ^{125}IUdR increases linearly in syngeneic hosts with the number of transplanted NZB cells, from 1.25 to 10×10^5 cells/mouse. However, 30-300 times as many cells fail to repopulate effectively (five days after transplantation), the spleens of irradiated (900-1000 rads of ^{137}Cs γ-rays) (NZW $\times$ NZB)F_1 mice.



A B C D

Fig. 74. Representative spleens of (C3H/He × C57BL/Ha)F_1 mice exposed to 900 r of X-rays and injected with saline (radiation controls) (A), 10^6 C57BL/Ha marrow cells (B), 10^6 C3H/He marrow cells (C), or 10^6 syngeneic cells (D). The mice were injected with ^{125}IUdR 5 days after marrow grafting, and the spleens were removed and fixed in Bouins fluid 17 hr later. The nodules grossly apparent represent foci of hemopoiesis. Note the similarity between A and B (0.01-0.05% uptake of ^{125}IUdR) and between C and D (0.3-1.0% uptake of ^{125}IUdR). *J. Exp. Med.*, **134,** 1513, 1971.

By studying an adequate number of F_1 hybrid strains, it becomes apparent that the genetic determinants of resistance are polymorphic. Hybrids of certain strain combinations are susceptible to cells of a given parent while others are not (Fig. 76). Similar results are obtained with several other strains; NZB is just given as a typical example. If one takes into account the H-2 type of donors and hosts, one can make the following generalizations:

1. H-2-homozygous hybrid animals are susceptible to parental marrow grafts.
2. Heterozygotes are either resistant or susceptible to parental grafts.
3. Substitution of one parental strain of an F_1 hybrid with a congenic strain differing at the H-2 region, often renders a resistant hybrid susceptible, or vice-versa(e.g., resistant B10 × NZB and susceptible B10.A × NZB mice of Fig. 76).

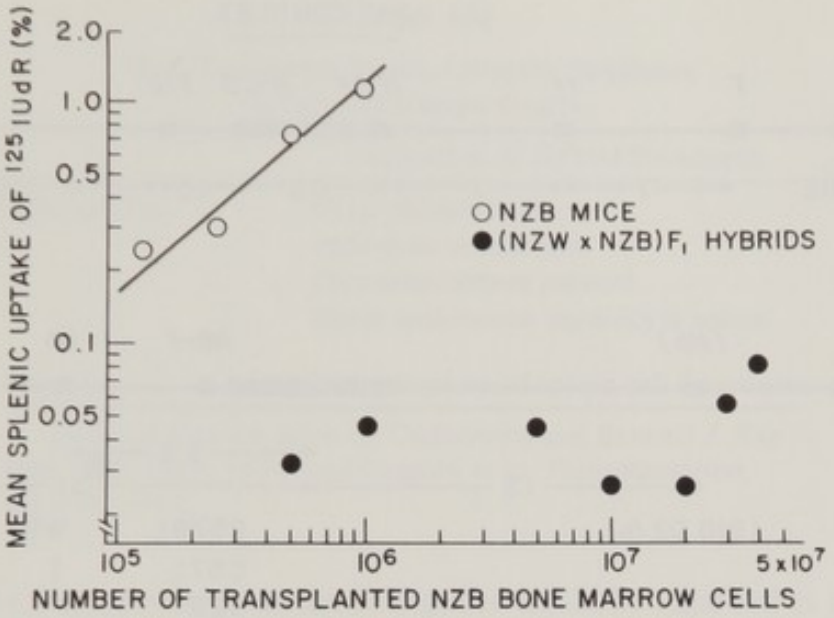


Fig. 75. Mean splenic uptake of ^{125}IUdR in relation to the number of bone-marrow cells transplanted 5 days earlier into heavily irradiated NZB and (NZW × NZB)F_1 mice 900-1000 rads of ^{137}Cs γ-rays, (5-10 animals of both sexes/point). Lotzová and Cudkowicz in *J. Immunol.* in press.

DONOR STRAIN: NZB (*H-2^d*)

SUSCEPTIBLE F_1 HYBRIDS	*H-2* TYPE	RESISTANT F_1 HYBRIDS	*H-2* TYPE
A x NZB	*a/d*	BIO x NZB	*b/d*
BIO.A x NZB	*a/d*	NZB x BIO.A(2R)	*d/h*
DBA/2 x NZB	*d/d*	BIO.A(5R) x NZB	*i/d*
BALB/c x NZB	*d/d*	BIO.BR x NZB	*k/d*
BIO.D2/n x NZB	*d/d*	C3H/He x NZB	*k/d*
HTG x NZB	*g/d*	BIO.*tf,T* x NZB	*q/d*
WB x NZB	*j/d*	NZW x NZB	*z/d*
DBA/1 x NZB	*q/d*		
A.SW x NZB	*s/d*		
SJL x NZB	*s/d*		

Fig. 76. Classification of F_1 hybrid mice as to susceptibility or resistance to grafts of 10^6 parental NZB marrow cells. The animals were classified by the ^{125}IUdR technique 5 days after irradiation and transplantation. Lotzová and Cudkowicz in *J. Immunol.* in press.

4. F_1 mice of the same heterozygous H-2 type may belong either to the susceptible or to the resistant classification group, as is the case for the *H-2^q/H-2^d* DBA/1 × NZB (susceptible) and B10.*tf,T* × NZB (resistant) hybrids of Fig. 76.

This type of result, verified in several strains other than NZB, illustrated here, leads to the conclusion that the primary genetic determinants of hybrid

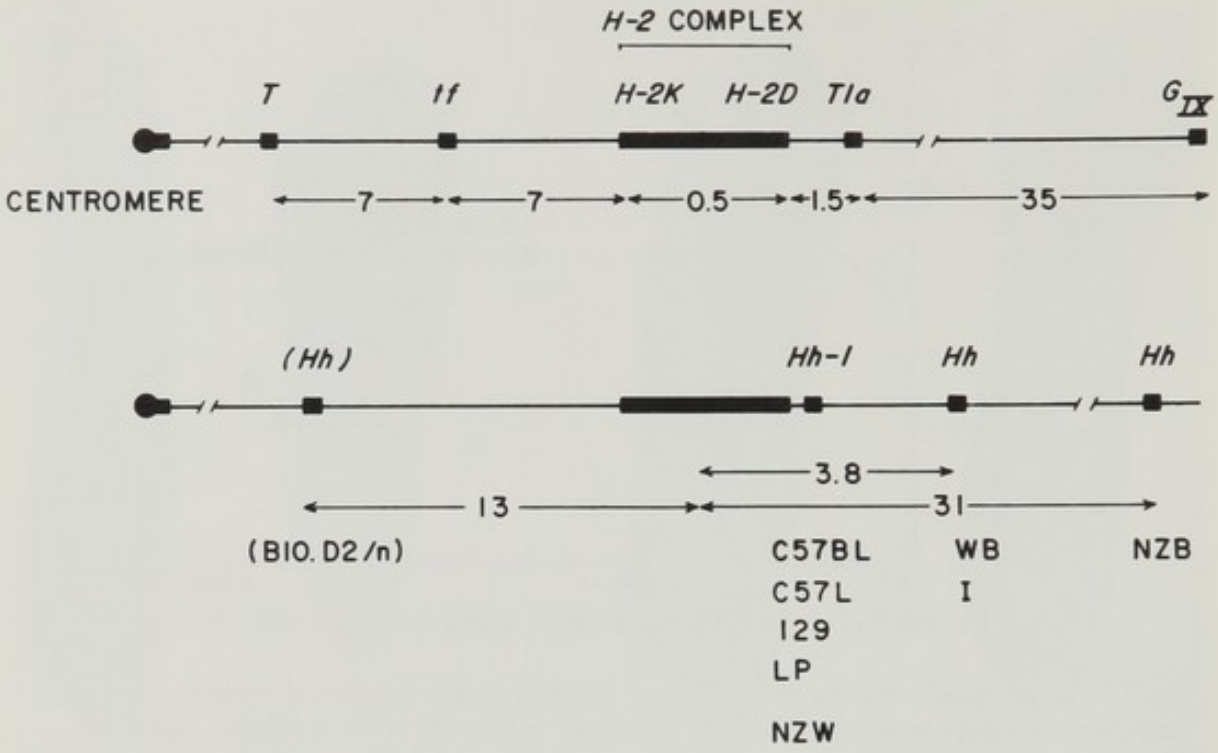


Fig. 77. Simplified maps of the H-2 region of linkage group IX. The upper map indicates the location and crossover frequency of marker genes and the lower map that of *Hh* genes of several strains of inbred mice. The *Hh* determinant of strain B10.D2/n is in brackets because it is not known on which side of H-2 it lies.

resistance are associated with the H-2 region (generalizations 1-3). However, it is also postulated that additional genetic factors (not linked to H-2) may regulate the manifestation of resistance (generalization 4), perhaps in a manner similar to Ir genes controlling manifestations of humoral and cellular immunity. These conclusions are borne out by backcross and intercross experiments and by analysis of H-2 recombinant lines. Consequently, the H-2 map is enriched by a few more genes designated as *Hh* for *Hybrid-* or *Hemopoietic-histocompatibility* (Fig. 77).

Hh-1 is closely linked to *H-2D* but separable from *Tla* and *H-2K* by crossing-over. Strains C57BL, C57L, 129, and LP share an allele of this locus; strain NZW possesses a different *Hh-1* allele. *Hh* determinants map 3.8, 13, or 31 crossover units from H-2 in several other strains. Three of the four *Hh* genes of linkage group IX lie on the D side of H-2. The exact location of the fourth *Hh* gene is still undetermined.

The product of *Hh* genes is expressed in homozygous cells of inbred mice, but is lacking in cells of heterozygotes. Thus far, *Hh*-gene products are recognized by the transplantation bioassay described, but not by serological reagents. *Hh* genes are likely to be recessive determinants of cell surface structures recognizable as nonself by some kind of radioresistant lympho-myeloid cell of F_1 hybrid or allogeneic host mice. Alternatively, one might assume that the expression of certain *Hh* alleles may be suppressed in the heterozygous con-

TABLE 128

Host Treatments Which Abrogate Resistance to Parental Marrow Grafts

	Abrogation of Hybrid Resistance
Non-specific	Cyclophosphamide
	Split-dose irradiation
	Corynebacterium parvum
	Horse anti-mouse thymocyte serum
Specific	Multiple parental spleen cell injections

Detailed data are given by Cudkowicz and Bennett *J. Exp. Med.* **134**, 1513, 1971, and Gregory *et al. Transplantation,* **13**. 138, 1972.

dition. Either way, resistance to marrow grafts would be regarded as a host-antigraft reaction endowed with specificity. In fact, abrogation of hybrid resistance is accomplished by treating prospective recipient animals with immunosuppressive and other biologically active agents (Table 128). More relevant to this argument is the immunogenetically specific abrogation of resistance which follows repeated injections of adult F_1 mice with incompatible parental cells. The tactics of this treatment are similar to those required for the induction of B cell unresponsiveness (for details see Cudkowicz and Bennett, *J. Exp. Med.*, **134**, 1513, 1971). Accordingly, the acquired susceptibility to parental marrow grafts is specific in an immunologic sense.

With regard to the question of whether Ir-like genes regulate hybrid resistance, one approach is to breed several F_1 hybrids of identical *Hh-1* type from different parental strains chosen among congenic lines of mice, and then, to measure resistance to parental grafts. In other words, the F_1 mice so produced are genetically resistant, and thus, are heterozygous at the H-2-*Hh-1* region, yet they possess the background genome of one given inbred (i.e., homozygous) strain. Fig. 78 illustrates the results obtained in a series of four such hybrids, each one presumed to possess exclusively the genome of strains DBA/1, A, C3H, and B10, respectively, except for a chromosome segment of linkage group IX including *Hh-1*. A fifth hybrid, C3H × C57BL, which does not meet these requirements, is also included. Since all hybrids are grafted with parental cells homozygous for the $Hh\text{-}1^a$ allele, the experiment is not assessing the effect of *Hh-1* itself, but the influence of genes other than *Hh-1* on hybrid resistance. It is clear that the strength of resistance, as judged from the displacement to the right of dose-response lines, varies greatly in hybrids of different congenic strains, decreasing in the order B10, C3H, A, and DBA/1. Resistance is not detectable in DBA/1 × D1.LP hybrids in spite of *Hh-1* heterozygosity, though B10.D2 ×

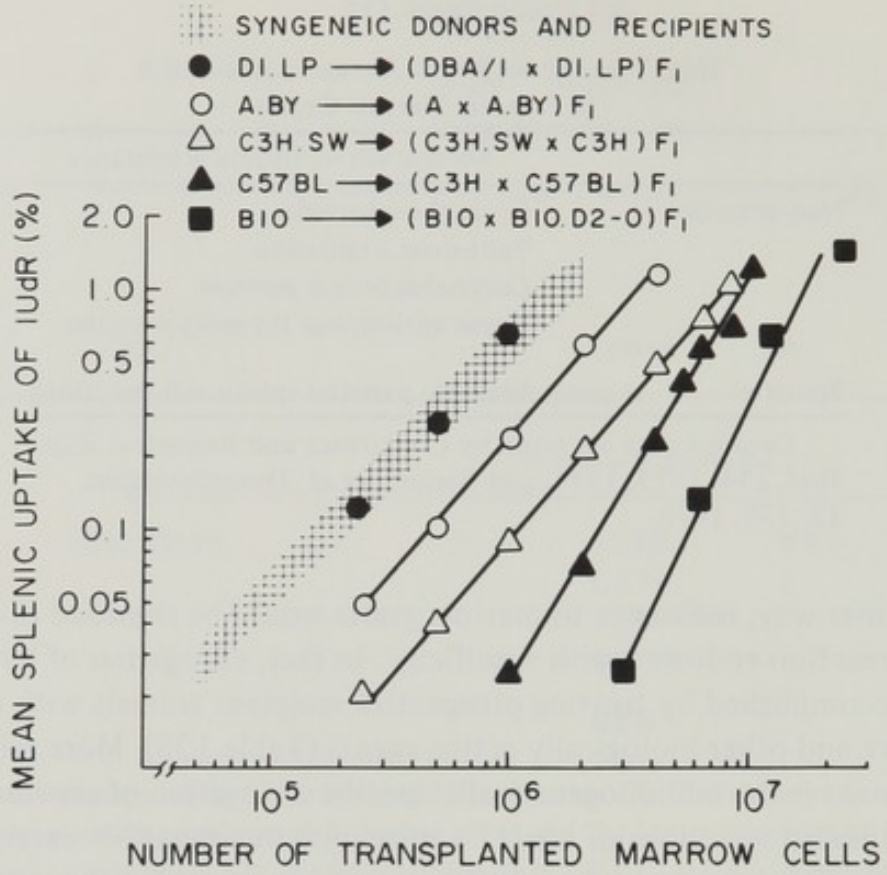


Fig. 78. Splenic uptake of ^{125}IUdR 5 days after transplantation of graded numbers of parental marrow cells into irradiated mice (900 r of X-rays). All donors shared the same *Hh-1* allele. The parents of all hybrids except C3H × C57BL were congenic pairs of mice. No resistance was observed in syngeneic and DBA/1 × D1.LP recipients. Resistance of differing strength was observed in the other hybrids in relation to the parental genetic background.

D1.LP and other hybrids are strongly resistant to D1.LP cells. These data show that genetic factors other than *Hh-1* regulate the strength of resistance to parental grafts.

The second approach is to set up crosses and backcrosses to estimate the number of genes involved in the regulation and to establish whether such regulator genes are dominant for reactivity.

The pair of (DBZ/1 × 129)F_1 and (DBZ/1 × D1.LP)F_1 mice is suitable for this study because both hybrids share H-2 ($H\text{-}2^q/H\text{-}2^b$) and *Hh-1* alleles, though the former hybrid is resistant and the latter susceptible to D1.LP cells (Fig. 79). (DBA/1 × 129)F_1 hybrids are mated to D1.LP mice to produce backcross offspring; DBA/1 and D1.LP are congenic-resistant lines and, furthermore, D1.LP and 129 are both $H\text{-}2^b$. Of the backcrosses, 90 mice are $H\text{-}2^b/H\text{-}2^b$ homozygotes and 80 $H\text{-}2^q/H\text{-}2^b$ heterozygotes. Only the latter segregants could include mice resistant to D1.LP marrow, since heterozygosity at the closely linked *Hh-1* locus is an absolute requirement for hybrid resistance. F_1 hybrids and $H\text{-}2^q/H\text{-}2^b$ backcross mice are irradiated; injected with 5×10^5 D1.LP marrow cells, and classi-

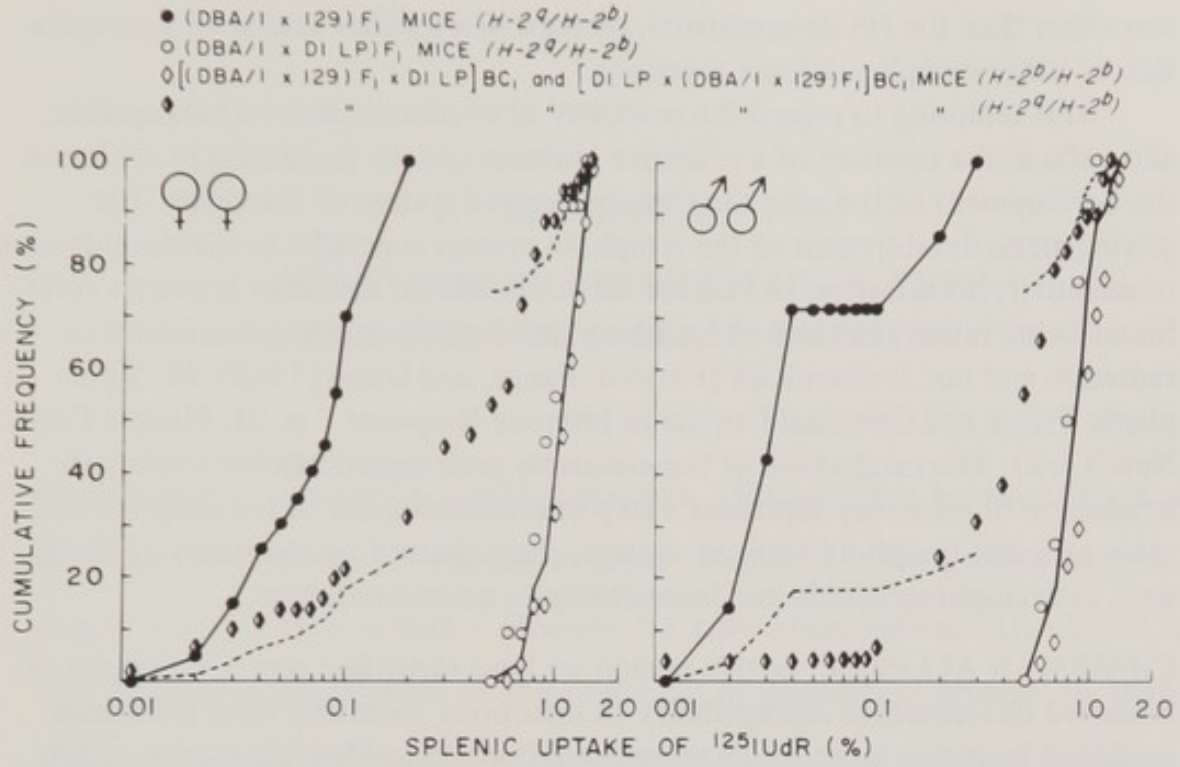


Fig. 79. Cumulative frequencies of splenic ^{125}IUdR uptake values in recipients of 5 X 10^5 DI.LP marrow cells. Strain and H-2 type of F_1 and segregating backcross mice are indicated in the upper portion of the figure. Solid lines represent observed frequencies and dashed lines calculated frequencies for backcross populations, assuming segregation ratios of the F_1 phenotypes as indicated in the text.

fied five days later for resistance or susceptibility. The cumulative frequency distributions of phenotypes (^{125}IUdR uptake values) are plotted in Fig. 79. The distributions of phenotypes in resistant and susceptible F_1 hybrids are regarded as reference distributions for model plots to be compared with observations in segregating backcross mice.

The results are unequivocal: The 80 *H-2*^{q}/*H-2*^{b} BC_1 mice segregate into classes of resistant, partially susceptible, and susceptible mice. The ratio of the three classes of segregants come very close to the model ratio of 1:2:1 which is expected on the assumption that two independent genes with additive effects regulate hybrid resistance and that the dominant alleles confer resistance. However, the data could also be interpreted as due to variable penetrance of a single regulator gene rather than to the effects of two genes. Progeny testing of segregants is the only way to discriminate between these alternatives. There is no association of resistance or susceptibility with the marker genes H-2 in linkage group IX, A^w (white-bellied Agouti) in linkage group V, *B* (brown) in linkage group VIII, and *d* (dilute) in linkage group II. The major conclusion is that hybrid resistance to parental grafts, as resistance to allogeneic grafts (Cudkowicz, *J. Exp. Med.*, **134**, 284, 1971), is regulated by a limited number of genetic fac-

tors other than the *Hh* determinants, perhaps in a manner similar to the regulation of other complex defense reactions.

It is tempting to regard the reactivity of irradiated mice to hemopoietic allografts as the remnant of a primitive immune system antedating in evolution the development of the adaptive thymus-derived system of mammals. The phylogenetic development of the lymphoid system is parallel to the development of sensitivity to radiation and certain toxic chemicals, the more primitive vertebrates being rather insensitive to both agents, and others being insensitive to radiation and not to chemicals [Finstad, Fange, and Good (1969), *In* "Lymphatic Tissue and Germinal Centers in Immune Response," p. 21, Plenum Press, New York]. The mechanism of bone-marrow graft rejection after total-body irradiation of mice may represent a step in evolution prior to the adaptive and more efficient lymphoid immune system, characterized by sensitivity to drugs such as cyclophosphamide and insensitivity to acute irradiation.

CHAIRMAN ALLISON: In this session we have identified several examples of inherited differences in susceptibility to infections, including virus infections, mediated by major genes which segregate according to Mendelian expectation. These have a powerful effect on host susceptibility or resistance, and are independent of major histocompatibility loci. In addition, there are associations among genes for major histocompatibility antigens and susceptibility to certain diseases, such as murine leukemia and lymphocytic choriomeningitis. These associations are, however, less strong than the ones to which I have alluded and their underlying mechanisms are still obscure. Systematic studies using immunosuppression and selective restoration of immune function should help to reveal how far inherited differences in susceptibility operate through functions of immunocompetent cells. While much work is required before any definite conclusions as to underlying mechanisms can be reached, the results which have already been obtained, illustrate the power of genetic analysis of factors involved in susceptibility and resistance to disease.

SESSION VI

ASSOCIATIONS BETWEEN HL-A TYPE AND SPECIFIC DISEASE ENTITIES

Homology between HL-A and H-2 systems – Relationship between HL-A and MLC genes or postulated human Ir gene – Problems in the genetic analysis of disease susceptibility in man – Reasons for association between HL-A and specific diseases – Application of the principles of population genetics to analysis of HL-A disease correlations – Complications raised by technical problems of HL-A serotyping – Assessment of the evidence for association of HL-A with Hodgkin's and with autoimmune diseases – Family studies of HL-A and Hodgkin's – Association between HL-A phenotype and production of anti-HL-A.

CHAIRMAN CEPPELLINI: I have been admonished by the organizers that in this session we should avoid as much as possible complicated tables with many HL-A numbers and long lists of supposedly associated diseases. We will of course do our best, but some compendia of data cannot be avoided if we are to establish some common *glossarium* between the human and nonhuman immunologists.

In Table 129 I have assembled an updated version of the results of the 5th Histocompatibility Workshop, held in June 1972, listing the HL-A specificities that are recognized by means of serological methods (mainly lymphocytotoxicity by humoral antibodies and complement), i.e., the SD antigens in the terminology of Bach. They are subdivided into two "segregant" series, *LA* (1st) and *FOUR* (2nd). The historical and operational reason for this separation is that members of each series are always carried by different chromosomes, therefore, when present together in a heterozygous parent they always "segregate" at meiosis in different gametes. In other words members of a series are alleles of the

TABLE 129

Segregant Factors at the 2 Loci of the HL-A System

1st or *LA* locus			2nd or *Four* locus		
	Frequencies			Frequencies	
Factors	Phenotypic	Genic	Factors	Phenotypic	Genic
HL-A1	0.243	0.125	HL-A13[a]	0.077	0.039
HL-A3	0.298	0.149	HL-A12[a]	0.135	0.076
HL-A11	0.096	0.048	W 18[b]	0.212	0.116
HL-A10[c]	0.192	0.101	HL-A5[a]	0.214	0.116
W 29	0.067	0.034	W 5[b]	0.365	0.204
W 30	0.106	0.053	W 15[a]	0.135	0.059
W 31	0.029	0.014	W 17[a]	0.058	0.020
W 32	0.163	0.082	W 21[a]	0.087	0.044
W 19.6	0.120	0.034	HL-A7[b]	0.097	0.047
HL-A2	0.388	0.216	W 10[b]	0.106	0.025
W 28	0.029	0.014	W 27[a]	0.048	0.024
HL-A9	0.240	0.130	W 22[b]	0.058	0.029
Blank			HL-A8[b]	0.135	0.069
			W 14[b]	0.087	0.048
			W 16[b]	0.144	0.074
			Blank	0.001	0.010

From Histocompatibility Testing, 1972., J. Dausset Ed., Munksgaard, Copenhagen.
Frequencies are representative for a Caucasian population.
HL-A "official" factors (W.H.O. Terminology report, Histocompatibility Testing, 1970).
W factors recognized at the 1970 and 1972 Workshops.
Brackets mean groups of crossreacting antigens sharing a common supertypic specificity.

[a]Factors sharing the supertypic specificity 4a.

[b]Factors sharing the supertypic specificity 4b.

[c]Split into W25 and W26. cf Ceppellini in "Progress in Immunology," 1971, D.B. Amos Ed., Academic Press, New York.

TABLE 130

Family Segregation in the "HL-A" System

Typing sera	ABO		LA series				Four series				MLC		Chrom	
	A	B	1	2	3	9	5	7	8	12			F	M
Father	+	+	+	+	—	—	+	—	+	—	X	Y	a	b
Mother	—	—	—	—	+	+	—	+	—	+	W	Z	c	d
Child 1	+	—	+	—	+	—	—	+	+	—	X	W	a	c
Child 2	+	—	+	—	—	+	—	—	+	+	X	Z	a	d
Child 3	+	—	—	+	+	—	+	+	—	—	Y	W	b	c
Child 4	—	+	—	+	—	+	+	—	—	+	Y	Z	b	d
Child 5	—	+	+	—	+	—	—	+	+	—	X	W	a	c
Child 6	—	+	+	—	+	—	+	+	—	—	Y	W	a/b	c
Child 7	+	—	+	—	+	—	—	+	+	—	Y	W	a/b	c

In this hypothetical family the parents are heterozygous at the 3 linked loci: LA (1st) and Four (2nd) recognized through serotyping, MLC (3rd) recognized through lymphocyte activation.

Paternal haplotypes (chromosomes) a: 1,8, x; b: 2,5, y. Maternal haplotypes c: 3,7, w; d: 9,12, z.

Child 6 is a recombinant between 1st and 2nd locus, Child 7 is a recombinant between 2nd and 3rd, for crossing-over at the paternal meiosis.

Activation of mixed lymphocyte cultures is obtained between individuals having (by definition) different MLC genotype. Thus in sibships, serological matching is usually a guarantee of MLC compatibility (nonactivation). Because of recombination, serological identity between unrelated individuals does not imply MLC identity. In families too, crossing-over may generate exceptions: sibs 6 and 7 are MLC compatible with sib 3, who has a different serological phenotype. Sib 7 is MLC incompatible with sibs 1 and 5 although they have the same serological phenotype. Blood groups ABO and HL-A segregate independently.

same locus and in the population their frequencies are expected to agree with the Hardy-Weinberg law.

In contrast, specificities belonging to different series are transmitted from a given parent either in coupling or in repulsion (but not independently) because they are controlled either by the same chromosome or by the two homologous chromosomes. In other words, the two series are controlled by two closely linked loci which together represent a complex "system." The concept of two loci has been validated by the occurrence of crossing-over with a frequency of 0.8% between *LA* and *FOUR* (e.g., sibling no. 6 in the family given in Table 130).

The analogy with the two loci, *D* and *K*, of the H-2 system is striking, especially now that, following the human model, mouse geneticists have fully understood the difference between *private* and *public* specificities. The numbers listed in Table 129 correspond to "private" or "subtypic" specificities, i.e., determinants which are unique and characteristic (as far as we know) for one allele. The brackets represent main families of crossreacting antigens, i.e., products of

HL-A alleles, which share common, or "public" determinants. I propose to call such determinants "supertypic" because their distribution in the population "includes" more than one subtypic specificity. (Ceppellini, *Progress in Immunology*, I, 973, 1971). Examples of supertypic determinants are the 4a and 4b specificities which characterize the two main groups of crossreacting alleles of the 2nd series. It is likely, although not yet fully proved, that each HL-A allele is a cistron controlling the synthesis of a polypeptide which has its own independent expression at the cell surface, viewed according to the fluid mosaic model of Singer. Therefore, on cells of heterozygotes at both loci (the commonest situation), four different molecular populations are recognized (Bernoco, *et al.*, Histocompatibility Testing, 1972). By definition, subtypic specificities represent the characteristic determinant of an allelic product, while supertypic determinants correspond to other areas of the same molecule, which are however common to more than one allele (Richiardi, *et al.* Histocompatibility Testing, 1972). Probably, an aminoacid substitution from point mutation may change one or maybe more determinants, but other parts of the molecule are unaffected. Therefore crossreaction is a consequence of relatedness in evolution.

The set of phenotypic traits which are transmitted together by one complex chromosomal region has been called a haplotype. In the case of HL-A, it is composed of two subtypic "serological" specificities, one at each locus (assuming that blanks correspond to unrecognized alleles). Because each antigen of one locus can be found associated in the same chromosome with each antigen of the other locus, according to Table 129, $16 \times 12 = 192$ different haplotypes can be generated. This corresponds to 19, 915 different genotypes and to a few thousand different phenotypes.

The average probability of finding two unrelated individuals who have the same serological HL-A phenotype is less than 0.001. In a sibship, however, if the rare occurrence of crossing-over inside the HL-A region is disregarded, only four different phenotypes can be found (in Table 130 children 1 and 5 are identical). Bodmer will discuss at some length the fact that some associations between alleles at the two loci, e.g., HL-A 1 and 8, HL-A 3 and 7, are more frequent than expected on the basis of their individual frequencies (linkage disequilibrium).

The close relationship of the HL-A system with *in vitro* activation of allogeneic mixtures of lymphocytes has already been discussed. On the basis of exceptions such as children 6 and 7 in the family shown in Table 130, MLC activation is thought to be controlled by a segment of the HL-A chromosome which maps outside the interval between *LA* and *FOUR*, on the side of *FOUR*. In this regard the perfect homology between H-2 and HL-A does not hold, because in the mouse, *MLC* maps between *K* and *D*, close to Ir. Thus, it is unjustified to assume by analogy with the mouse, that the centromere in the human chromosome is on the side of *Four*.

Recalling what has been said before, MLC is still a relative unknown; we still ignore how large a genetic segment controls it; whether it is composed of one or more cistrons; whether it is compact or is scattered along the entire region. We also ignore the identity of its product(s). As a working hypothesis, mainly based on the scanty evidence that allogeneic sera block the MLC, we assume that the MLC phenotype (the LD differences of Bach) is expressed as a component of the cell surface, possibly similar in structure to HL-A antigens, but for some reason unable to elicit cytotoxic antibodies. The other important but still indirect piece of information is the similarity between *MLC* and *Ir-1* in regard to chromosomal location and blocking by allogeneic sera.

My final message about HL-A is related to the extension of the region and the number of cistrons which compose this extraordinary "supergene." The recombination frequency between *Four* and *MLC* seems to be about the same as between *LA* and *FOUR*, hence the total length of the region is probably not less than 1.5%. On the basis of the average number of chiasmata observed at meiosis, 52, the human genome corresponds to $52 \times 1/2 \times 100 = 2{,}600$ centimorgans. Thus a recombination frequency of 1.5% represents 6×10^{-4} of the total map length. The DNA content of a human gamete corresponds to about 6×10^{9} nucleotides, thus to about 6×10^{6} genes (assuming an average of 500 pairs per structural gene). These calculations would support the contention that in the HL-A region there is room for $6 \times 10^{-4} \times 6 \times 10^{6} = 3{,}600$ genes. Even accepting the recent very conservative estimate of Crick, the region should include at least a few score of structural genes. Present evidence is rather in support of the view that each HL-A antigen may correspond to one polypeptide, thus to one cistron. Therefore, the great majority of the other genes of the region are either not expressed at the cell surface of lymphocytes (the only well studied cell lineage in man) or more likely are expressed as structures which cannot be recognized with current serological methods. (My colleague, Carbonara, has however, some evidence of an alloantibody which blocks MLC and can be seen with indirect immunofluorescence). As already mentioned, *MLC* and *Ir-1* are examples of such genes. A provocative hypothesis is that all (or the majority of) these hundreds or thousands of genes are related in origin because they are derived from some common ancestor through unequal crossing-over and then differential mutation. Because of this common origin, they are also probably related in function, insofar as they may control structures and specific activities of the cell surface. It is likely that they are variously expressed in different cell lineages. At this conference we are mainly interested in immunocytes (and therefore in *Ir* genes), but it is easy to visualize many other fundamental functions of the cell surface, e.g., contact inhibition, cell to cell communication, selective transport, etc.

Persistence of linkage disequilibrium between components of the region and the striking homology of chromosomal architecture in different species (two

linked series of H antigens with similar recombination frequencies, have been found in man, mouse, rhesus monkey, and dog), are two further reasons for thinking that the main histocompatibility system of mammals (vertebrates?) is "an epistatic block of genetic information" (R. Fisher) which is kept compact through evolution because it represents some sort of integrated functional unit, almost a chromosomal organelle. Other similar regions seem to be the systems of immunoglobulin chains. Because of the important and complex functions of HL-A, as here hypothesized, it is *a priori* likely that it may be related to a number of diseases, as Bodmer will shortly be telling us. I want, however, to emphasize the difficulty faced by the human geneticist in this case.

Even in an outbred species such as man, genetic analysis is easy when a product close to primary gene action is studied, since a polypeptide is easily recognized in its allelic variants (Hb S through sickling or electrophoresis or amino-acid sequencing; HL-A antigens through serotyping). But the more the phenotypic manifestation under study is removed from the gene, the more the picture becomes confused by the intrusion of other variables deriving from environment and the residual genome. A case in point is the situation of the Australia antigen, mentioned earlier by Allison. When we became aware of the relationship between H-2, viral leukemogenesis and the immune response, we decided to investigate the possible relationship between HL-A and the so-called healthy Au carrier, a condition which had been shown by Blumberg to have a high familial concentration. Through the study of 165 complete families from Sardinia, we could confirm that this is an individual mode of reaction to the viral infection (likely having an immunological basis) which is controlled to a significant extent by the genotype. The hypothesis of simple autosomal inheritance, however, could be maintained only by introducing some elaborate manipulations for taking into account incomplete penetrance of high variability (from 100 to 20%) according to age, sex, and of course, risk of infection. By family segregation we found no linkage between the Au carrier state and HL-A, but we have not analyzed the association at the population level (Ceppellini *et al.*, Alt Convegni Farinitalia, Minerva Medica, 1970). Should we have studied the population of Torino, we would probably have found a significant association with some HL-A, Rh, or MN phenotypes. In fact, Au carriers are very common in Torino among immigrants from Sardinia (up to 20% carriers in young males) but very rare among the aborigines ($< 1\%$). We can only speculate on how much of this difference is due to genetic factors and how much to the lower socio-economic status of immigrants. Because Sardinians differ greatly from North Italians for the frequencies of some blood antigens, the observed association would have been spurious and due to ethnic stratification. (Poverty, too, would have been found in association with some biological markers). This is one of the pitfalls of

human genetics, but for this reason I am most anxious to move on to the study of the immune response not only in inbred mouse strains, but also in the *wild* mice of Jan Klein.

These are some of the more urgent considerations in the human genetics that seem to me especially relevant for orienting this session. Now we look to Bodmer for an assessment of our present knowledge of the relationships between HL-A and disease.

BODMER: My contribution to this sixth session will be to discuss the problems that are faced by those of us who are especially concerned with studying some of the analogues of the murine models one might find in man. Specifically, I will consider some of the questions that arise when we look into human populations for associations between disease states and the HL-A system, using as models the situations in the mouse that were dealt with so exhaustively in Session V. The first such study to which I shall be referring again later, which came forth with a significant result, was published only five years ago by Amiel. I think it is fair to say that at the time neither I nor many others really appreciated the significance of his results; in more ways than one!

One might first of all ask the question: *Why* should we look for some evidence for genetic susceptibility in disease such as Hodgkin's disease, leukemia, or the autoimmune diseases, on which many of the HL-A studies thus far have been done, especially as there is no clear-cut evidence that these diseases are in any sense familial in the ordinary usage of this term? Generally, if one wants to look for a genetic basis, one looks to see whether there is significant clustering of the disease in families and if this can be interpreted in a simple way in terms of one or two Mendelian genes. It is certainly quite clear that for Hodgkin's disease the familial clustering is actually minimal. The incidence of Hodgkin's disease is of the order of five per 100,000, and published studies suggest at most a two- to three-fold increase in this incidence among first degree relatives of an affected individual (Razis, *et al.*, *Ann. Int. Med.*, **51**, 933, 1959).

There seem, nevertheless, to be good reasons *now* for looking for association of some of these diseases with genetic markers. I should like to say something about the rationale for doing this, despite the fact that one does not find a significant familial clustering.

One rather obvious explanation is that there are many genes involved in determining the disease susceptibility, as there are for the mouse models about which we heard from Lilly in Session V. In this situation, the familial clustering will be diluted by the fact that the genetic control of the disease is multifactorial.

A second reason why one might look for a genetic factor, even though there is no familial clustering, is that the effect of the gene(s) is by no means expressed in all or none fashion. Rather the influence of a gene may be to in-

crease the *tendency* to get the disease, or the susceptibility to infection. As Chairman Ceppellini has stated in another context, imperfect *penetrance* of a gene will tend to obscure a clear-cut familial incidence.

A third factor, which I personally consider will be the most significant in the diseases we are considering, is that there is, in fact, an environmental component which is major, and perhaps the most important in the emergence of disease. Thus, if we are talking about diseases which may be caused by a virus, then the virus itself is an overriding, important environmental factor. Exposure to the virus, for example, may itself be an infrequent event, or, even given exposure to the virus, the chance of getting the disease may be rare. The probability that the appropriate genotypes in a family will all have been exposed to this agent may be small, so one may not see the genetic clustering of the disease for purely *environmental* reasons.

Nevertheless, one could easily have a situation where a particular gene was an absolute requirement for infection by a virus, in order to give rise to the disease, but the *nature of exposure* to the virus was such that familial clustering was not seen, because effective exposure to the virus was a rare event. These then are some of the reasons why, even in the absence of any evidence of familial clustering one would nonetheless be justified in looking for associations between diseases and particular genetic factors.

There have been many studies on association of ABO and other blood groups, with stomach cancer and a variety of other diseases. The initial stimulus for doing these studies came from the suggestion that if one found such associations, they might be indicative of the evolutionary forces which are responsible for maintaining the blood group polymorphisms. The rationale was not, however, in terms of any possible explanation of the association with disease at the molecular level. I think it is fair to say that perhaps as a result of this, most of these studies have been disappointing; they were either equivocal, or proved to be not readily interpretable. Perhaps the only argument now, for looking for association of an arbitrary genetic marker with a disease (in the absence of any basis for expecting a functional relationship between the marker and the disease), is that there may be one or more genes which have *something to do* with the disease, that are closely linked to the marker under study. This then would be a form of *association due to genetic linkage*, which is one of my major theses, but one I would consider in a rather special context.

The case for looking for associations with HL-A was made rather clearly by the model studies in the mouse, which associate a variety of diseases and immune response phenomena with the H-2 system. Thus, one felt that perhaps there *was* a good rationale for HL-A disease associations, and we were not going to be faced with the frustrations of those who had looked for associations of other polymorphisms with diseases in man. Armed with this confidence, we all

started looking at the frequencies of HL-A antigens in a variety of diseases, which at least initially, were picked with the idea that they might be analogous to those for which an association with the H-2 system had been found in the mouse.

Before discussing the details of these studies and their interpretations I should, however, like to discuss some of the statistical and serological pitfalls that should be considered in the course of looking for such associations. First of all, the one that has bothered us more than *any other* is the possible bias in the interpretation of the data. As Chairman Ceppellini has already impressed on us, the HL-A system is indeed a complex system. There are now something like 30 antigens described that are distributed into the two series called Four and LA. Typically, one looks at the distribution of all of these antigens in patients with the disease and compares this, in some way, with a control. A chi-square value may be calculated to see whether the frequency of each antigen, in turn, in the diseased patients in significantly higher or lower than the frequency in the control series. If one comes up with a significant χ-square, well, here we have found an antigen which is associated with that disease.

The statistical tests one uses are such that, if one obtains a χ-square significant at the five % level, this means, on the average, one in 20 times he would expect to find as big a difference as the one under consideration—even if the distributions in the control and disease series were the same. Usually it is said that if such a difference is only found by chance once in 20 times, then there may be a real effect. But in situations where one is looking at *20* antigens, then each time a disease with 20 antigens is studied, one would expect one of these to differ by chance at the five % significance level between the disease population and the controls. This is not a truly significant result, because one is picking on the *extreme* difference, and therefore the standard type of statistical test is *not really* applicable. One has to take into account the fact that this extreme difference has been *selected* when assessing its significance.

There are, in fact, some well-known standard statistical corrections for doing this. Most simply, one multiplies the probability level found by the number of antigens that were studied. It is perhaps for this reason (with some justification), that Amiel's original observation on Hodgkin's disease was ignored, because by the factor I have just elaborated, it certainly was not truly significant. However, as is so often the case when clinicians initiate studies, they ignore some of the scientific considerations and end up by being correct, as we shall see later! There are two ways to overcome this statistical bias. One, as already mentioned, is to find a significance level which is *so* high that when one makes the appropriate corrections, there can be no question about its true significance.

The other, and perhaps more convincing way, is that if an initial study indicates a particular antigen is elevated in frequency, one must do a follow-up study, asking an *a priori* question about the behavior of that antigen in diseased

patients. In our follow-up study is that antigen associated with an increased frequency? In that event the statistical bias is removed and the statistical tests, when applied to the follow-up study, are quite valid.

There are two further statistical or epidemiological problems. One that is extremely important was referred to by Ceppellini and that is the question of the proper choice of controls. If the diseased population, as in the case of the Australia antigen in the Sardinians, comes from a sub-group which has a different distribution of the antigens than the "normal" control—say white European Caucasians—then the association with those antigens will be spurious. It will be due to a *stratification* in the population and have no particular connection with the disease under study. Accordingly, it is extremely important that the controls are chosen properly with respect to the comparison with the diseased population. Epidemiologists traditionally are most stringent in their choice of controls matching, at the very least, for age, sex, time, and racial group. It may be possible to be somewhat less stringent when comparing HL-A frequencies in disease and control populations, because there is available some information on the variability of the HL-A antigen frequencies in different groups of people. It is known that they are *not* sex linked, and that the distribution in old and young people is the same, at least so far, within experimental error.

The major factor which affects HL-A frequencies is the *racial* distribution, a problem to which those of us involved in the 5th Histocompatibility Workshop are addressing ourselves. Thus the overriding, important control must certainly be the one of proper matching with respect to racial origins of the people that are being studied. I feel that beyond that, an effect that is so small that it needs more careful adjustment of the control group is probably too small to be worth working with, and so of little real use for further study.

There is one further factor which I think must be emphasized in doing these studies; namely that the disease one is looking at may be a heterogeneous entity. There may be an association which is only with a *subcomponent* of the disease. In that event, the association may be diluted by not looking for an association with subcategories of the disease. In the case of Hodgkin's disease, for example, there is a great deal of uncertainty as to the significance of the different histological types of the disease and its different stages. There is some question as to whether people who get it late in life, in contrast to the young susceptibles, represent different *types* of Hodgkin's disease, or only different *expressions* of the same disease. Disease heterogeneity is, thus, an important factor to consider, though it is one which will tend to *dilute* associations rather than give *spurious* associations.

I should like next to mention some of the serological problems one must ponder in doing these disease studies. This is by way of anticipating what I know will be a topic giving rise to some later discussion. In the first instance, it is en-

tirely possible that there will be antigens expressed on the cells of patients with certain diseases which are, in fact, specific to those diseases. These are likely to have no relevance to the HL-A antigens, but there may well exist antibodies to them in our normal HL-A typing sera. Such antibodies will not ordinarily be picked up in the normal course of typing people.

There is, however, one rather simple and easy way in which such antibodies should be discovered. This involves using several sera, rather than a single one to type for each antigen; when one of these is out of line with the others in its reaction frequency with diseased patients, it clearly reflects an indication that it may be reacting with a specificity of the *disease* rather than the particular HL-A antigen it was intended to identify. So I think that, while disease-specific antigens may be a problem, it is one that should be rather readily discernible if the studies are done with the aforementioned proviso, i.e., by using several sera for each antigen being tested for.

The second problem, perhaps one which is not so easily resolved, arises if the diseased patient group has, in some way, an altered reactivity in their serological assay. In this situation, one may, for example, uncover expressions of crossreacting antigens, which are not normally detected in individuals without the disease. These may in turn lead to peculiar typing results that are inconsistent and give rise to an apparent association with a particular antigen.

Ceppellini has suggested other interesting possibilities; for example, there may be synergistic interactions with autoantibodies present in the sera of the patients under study. Again, I think it is extremely important to emphasize that if one uses several sera for each antigen, the *consistency* of the behavior of those sera is a major criterion of whether one is really looking at the HL-A antigens or at something else. Thus, if a set of sera which have, say, anti-HL-Al antibodies also consistently show interreaction with a particular autoantibody, but other sera do not, then that still may be of significance with respect to the association between the antigen and the disease.

Of course, the one sure way of removing doubts about the HL-A phenotypes of individuals is to establish the expected phenotypes on the basis of the genotypes, by doing family studies. The family members will, generally, be normal individuals as far as the disease is concerned. Now here one is really establishing what is needed, namely, the genotypes with respect to the HL-A antigens, and not merely the phenotypes. The phenotype is simply a handle, and often an easy handle for the genotype, which is what we are really interested in with respect to the disease association.

In the face of all these problems, one might well wonder how anyone has the audacity to claim that there might even be *some* significance to the association between HL-A antigens and disease.

I would, therefore, like to start by giving a little historical background for the case of Hodgkin's disease, which I am utilizing as my model, because more work has been done on it than on any other disease state. I have already mentioned that Amiel reported at the Histocompatibility Workshop in 1967 an apparent (approximately) two-fold increase (23 to 51%), in the frequency of an antigen, then called 4c, in patients with Hodgkin's disease. The rationale that he gave for looking for such an association was clearly based on the work of Lilly and others; nevertheless many of us simply believed there was no significance to Amiel's results. At first it was ignored—but nor for very long. Forbes and Morris (*Lancet* **II**, 849, 1970) and a group in Manchester (Zervas, *et al.*, *Lancet* **II**, 634, 1970) reported studies in which also appeared to indicate a significantly increased frequency of antigens related to the 4c antigens which Amiel studied. Since that time there have been at least seven further studies on the distribution of HL-A antigens in individuals with Hodgkin's disease. The total number of patients studied has now reached more than 680. If this series doesn't show anything significant, then I suppose nothing will; this is the data I intend to consider.

Before doing so, I would like to say a little bit more about the confusing story of the antigens associated with 4c, because in my view this, too, is extremely important for the interpretation of the data on Hodgkin's disease. The antigen 4c was originally described at Stanford by my wife Julia, myself, and Rose Payne. It is probable that as originally described, using agglutinating sera, it was equivalent to what is now called HL-A5 (Bodmer, *et al.*, *N.Y. Acad. Sci.*, **129**, 473, 1966). When we started to work with cytotoxic sera, the particular sera that worked best by agglutination to give the 4c antigen unfortunately did not work well in cytotoxic reactions. We therefore used another serum (Rafter) which we knew even then was not really the same. However, we had no alternative but to use Rafter; its reactions came subsequently to be called 4c. Studies which we reported very briefly in 1967 (Bodmer, *et al.*, Histocompatibility Testing, p. 347, 1967, Munksgaard, Copenhagen) showed that this serum could not be split by absorption. Thus, although it included reactions with all people who were HL-A5, there were people who were not HL-A5 who also reacted with this serum. It was therefore presumed there was present in the serum one or more antibodies that crossreacted with HL-A5 and other antigens. Subsequently, Thorsby and others showed clearly that there were, in fact, at least three specificities within what is detected by this serum; the original HL-A5, one that has come to be known as W5, and another one that is called W18.

In addition, there is an antigen, namely W15, which is of considerable interest as it is the one that was reported by Grumet, *et al.*, (*New Eng. J. Med.*, **285**, 193, 1971) and by Walford and coworkers (*Tissue Antigens*, **1**, 68, 1971), as increased in frequency in SLE patients. There is a variety of evidence associat-

TABLE 131

Comparison of χ-Square Values for Association between Hodgkin's Disease and 4c Cross-Reacting Antigens Versus All Other HL-A Antigens

	Number of χ-square values		
	Less than 3.8	Greater than 3.8	
4c antigens (HL-A5, W5, W18 and W15)	19	9	28
Other HL-A antigens	116	11	127
	135	20	155

The χ-squared for this 2×2 table is 9.25 ($0.5\% > P > 0.1\%$). The data are taken from the following nine studies: Zervas *et al.* 1970; Morris and Forbes, 1971; Coukell *et al.* 1971; Thorsby *et al.* 1971; van Rood and van Leuvwen, 1971; Kissmeyer-Nielsen *et al.* 1971; Bertrams *et al.* 1972; Falk and Osaba, 1971; Jeannet and Magnin, 1971.

ing W15 with these other antigens in a crossreacting set, though the crossreactions involving W15 may not be quite as strong. These four crossreacting antigens, HL-A5, W5, W18, and W15 are *all* determined by alleles at the *Four* locus. One of the confusions in the data on the association of 4c with Hodgkin's disease has been the question of which if any, of these antigens are really involved in the association.

Table 131 shows a summary of the data from nine of the ten studies on Hodgkin's disease taken from the literature. The one study that is omitted is Amiel's original study, because it is the one that suggested the association with the 4c set of antigens. All the later studies, therefore, can be considered as directed at the question of trying to establish whether or not this original, now *a priori* association is a *valid* one.

The problem here is statistical; with respect to each antigen in each study, one can calculate the χ-square for testing the significance of the difference in the frequency of the antigen in Hodgkin's patients, as compared with controls. The controls, in this case, were usually people from a European Caucasian panel; it should be noted that nearly all the studies have been done with Caucasians.

χ-square is a quantity which has a distribution, as given in the usual statistical tables. This is such that, were there no differences between the antigen frequencies in Hodgkin's patients and controls, on average one would then expect 1/20 of the χ-square testing these differences to have values greater than 3.8, which is the value corresponding to the five % significance level.

A priori I reason that one might expect an association involving the 4c antigens; consequently I have separated the data for these antigens from that for other HL-A antigens. The data are then tabulated in a simple way to show the distribution of the χ-square values in the nine studies for the two groups of anti-

gens. In other words, in these nine studies there were 28 comparisons between individuals with Hodgkin's disease and controls, with respect to the frequency of the 4c antigens. Out of the 28 corresponding χ-squared values, 9 were greater than 3.8, and 19 less than 3.8. If there were really no difference in frequency between these antigens in Hodgkin's patients and controls, then one would expect the proportion of chi-squares greater than 3.8 to be 1/20, i.e., one would have expected one or two out of 28, rather than the nine observed.

What about the χ-squares greater than 3.8 for the other antigens? If one looks at all the other HL-A antigens investigated in these nine studies, 127 comparisons were made among which there were 11 χ-squares which were greater than 3.8. This gives a frequency of a little less than ten % which is actually still significantly more than the five % expected. However, it is significantly far less than the nine out of 28 observed for comparisons involving the 4c antigens. In other words, if one tests for the significance of the difference between 9 out of 28 and 11 out of 127, a χ-square is obtained that has a value of 9.25 and is highly significant (0.5 percent $> P >$ 0.1%). Thus, if we look at the data in terms of the distribution of χ-square values there is a highly significant difference between the distribution for the 4c set as compared to that for all other antigens. In my view, this really *does* substantiate the idea that at least in the studies on Hodgkin's disease so far available, there is a definite, significant indication of a tendency to an increase in the frequency for one or another of the 4c antigens. I might say parenthetically, that this does not necessarily mean that increases involving other antigens are not also significant. There is a suggestion in some studies that HL-Al and/or HL-A8 are also increased in frequency; however these changes are not yet by any means as well established as those for the 4c set of antigens.

Thus, I believe that at least in this case, because of the large number of studies done, there is some consistency in the data indicating a significant association of changed HL-A frequencies with Hodgkin's disease.

This association appears not to be a simple one involving only one antigen; but before going on to discuss the possible interpretations, I should like to review briefly the overall current state of data on HL-A disease associations. Table 132 shows a simple minded attempt at summarizing the data on all diseases, that insofar as I am aware, have now been studied. I have sought to give some indication of the number of studies for each disease, which antigens are involved, and a subjective assessment of the consistency of the results. Some of the suggested associations, such as infectious mononucleosis, may already be contradicted. The diseases are arranged approximately in order of significance of the data.

Hodgkin's and lupus stand out as the diseases in which the data so far are most consistent. The relatively large number of studies on leukemia seem to give equivocal results. There are some studies on diseases of particular interest, such

TABLE 132

Reported Studies of Association between HL-A and Various Disease States

Disease	No. of studies	Antigens involved	Consistency or significance
Hodgkin's	10	HLA5,W5,W15,W18	++
SLE	4	HLA5,W5,W15	++
Malignant lymphomas	2	HLA12	+
Chronic glomerulonephritis	2	HLA2	+
Acute lymphatic leukemia	8	HLA2, HLA12	+−
Chronic lymphatic leukemia	3	(W5)	+−
Chronic myelogenous leukemia	2	HLA1,HLA3,HLA12(-)	+−
Multiple sclerosis	1	HLA3(HLA2,W10,W18)	+
Multiple myeloma	1	W18	+
Infectious mononucleosis	1	W5	+
Psoriasis	1	W17(HLA13, 10, 12)	+
Juvenile Diabetes	1		−
Rheumatoid arthritis	1		−
Asthma	1		−
Regional enteritis	1		−
Choriocarcinoma	3		−

as psoriasis, which hopefully will soon be confirmed. There are also at least a few diseases in which there is no claim for any association; this is something I find very reassuring! This summary should at least give some idea of the range of diseases studied and the amount of data which has been accumulated. When Cohn asks "why study man," I answer "because," as Alexander Pope said some 250 years ago, "the proper study of mankind is man." Here we have a demonstration of how quickly data for man are collected, given any suggestion that they might have some value.

I should like now to return to the question of why the association with Hodgkin's disease appears to involve a set of related antigens rather than just a single one. I believe the data can be interpreted best as indicating association not with the antigens themselves, but with the products of one of those hundreds (or thousands) of loci in the HL-A region which are linked to *LA* and *FOUR*, or in the H-2 region to the *D* and *K*. Let us consider again some of the problems of looking at associations which are due to linkage, a situation that we have already discussed extensively. It was pointed out that linkage may not be detectable by an association at the population level, even for quite closely linked genes which would, of course, give rise to readily detectable associations in family studies. More generally, association due to close linkage may be quite variable and com-

plex, depending as it does on the population frequencies of the relevant genetic combinations.

Suppose there is a particular locus in the HL-A region called say DS-A (DS for Disease Susceptibility) which is connected with susceptibility, and assume for the moment that the determination of susceptibility is by a dominant allele at this locus, *DS-A1*. Then the explanation for the association with a particular HL-A antigen, let us say HL-A5, would be that the genetic combination, or haplotype, involving the *Four* locus allele *HL-A5* with *DS-A1*, occurs substantially more frequently than the combination of *HL-A5* with other DS-A alleles, or the combination of other HL-A antigens with *DS-A1*. In other words, one would get an association between the effects of this allele, *DS-A1*, on the disease, and the presence of the HL-A5 antigen due to the fact that the genetic combination of *HL-A5* with *DS-A1* in the population, occurred with a higher frequency than other combinations including the allele *DS-A1*. The association is due to *linkage disequilibrium* between the allele causing susceptibility to the disease and the relevant allele of the *Four* locus. This could explain why an association may not be as significant as one might expect it to be. We are picking it up only by virtue of the linkage of the gene determining the antigen to the allele causing susceptibility. The same question can be asked, for example, with respect to the association between antigens of the *Four* and *LA* locus in human populations. Even though the genetic distance between *LA* and *Four* is only 0.8% recombination units, nevertheless most pairs of antigens taking one from each locus, are not significantly associated. Certain pairs, of course, are very significantly associated, such as the well-known combination of HL-Al and HL-A8 in Caucasian populations. Another pair known to be associated in Caucasians is HL-A7 and HL-A3. These are however, in general, the only pairs of antigens which are significantly associated in the Caucasian population. Other pairs are not, despite the close linkage between these loci, and that is the main reason why initially it was thought that the antigens of the Four and LA series might actually be determined by distant loci.

The same features could apply to associations between the *DS-A* and *Four* loci. Linkage disequilibrium may only be found for certain antigens of the *Four* locus and certain alleles of the *DS-A* locus. There may, moreover, not be any disequilibrium with antigens of the *LA* locus. Certainly, we know that in Caucasians HL-A5 does not associate significantly with any of the antigens of the *LA* locus.

It thus seems to me entirely possible that the explanation for the disease association with the 4c set of antigens could be that each of the corresponding alleles is associated with the product of an allele, *DS-A1*, of one of the many loci in the HL-A region, namely, that the particular allele involved, *DS-A1*, is the linkage disequilibrium with each of the four alleles corresponding to the 4c antigens.

Why should the disequilibrium involve more than one of the antigens? One plausible reason could be a common evolutionary origin of these combinations, which involve a crossreacting set of antigens. The fact that they are crossreacting suggests that they may have a common, relatively recent origin, either by recombination among each other or with other alleles, or by mutation (Bodmer, *Nature*, 237, 139, 1972). The association of the *DS-A1* allele with a precursor allele of one or another of these, has then been maintained throughout the production of the 4c antigens of the *Four* locus. The maintenance of the combinations could well involve a mild selective interaction between the product of the *DS-A1* allele and the four respective products of the *HL-A5*, *W5*, *W18*, and *W15* alleles. Incidentally, the fact that the disequilibrium is with the *Four* locus and not with the *LA* locus suggests that the *DS-A* locus may be closer to *Four* than it is to *LA*.

COHN: Is Bodmer assuming that *Four* is a single cistron?

BODMER: Not necessarily. The argument is the same whether *Four* is a single cistron or a cluster of cistrons.

McDEVITT: Are *HL-A5*, *W5*, *W15*, and *W18* alleles?

BODMER: Yes, they are all allelic in the sense that one does not find them together on the same haplotype.

There are standard ways of calculating the relative increase in the incidence of a disease associated with a particular phenotype, as compared to the incidence in a control group. These methods have been used especially in studies involving the ABO system (Cavalli-Sforza and Bodmer, "The Genetics of Human Populations," Freeman, San Francisco, 1971). If, for example, the frequency of one of the antigens increases from about 25% in controls to 50% in patients, as is approximately the case for Hodgkin's disease, the relative increase in incidence of Hodgkin's in people with one of the 4c antigens as compared to others would be about three-fold. In other words, the frequency of Hodgkin's among people with the relevant antigen phenotype would be about three times higher than among people with other phenotypes. It should be emphasized that this relative increase figure is associated *not* with the actual gene *DS-A1* that is causing the effect, but with the linked *Four* locus. It is possible, in fact, to calculate—on the assumption that having this particular allele was an absolute requirement for getting Hodgkin's disease—what magnitude of linkage disequilibrium with the relevant *Four* locus alleles would give only a three-fold increase in incidence. Preliminary calculations suggest that if linkage disequilibrium between the particular *DS-A1* allele and the *4c* alleles is comparable to that between *HL-A1* and *HL-A8*, then there could be an absolute requirement for all people

with Hodgkin's disease to have the *DS-A1* allele and nevertheless yield only a three-fold increase in the incidence of the disease among people with the 4c antigens. This is because genetic recombination is diluting out the effects of the association.

Were the disease association due to an effect of the *Four* locus itself, an increase in the frequency of an allele in patients with the disease would argue that the effect was dominant. If the effect was recessive, we would expect to find a decrease rather than an increase, i.e., there should be fewer, rather than more people with the relevant antigen among those individuals that have the disease. But if the effect is due to a linked locus, this distinction would no longer hold. This is because the *DS-A* allele that may be in disequilibrium with one or more *Four* locus alleles can either be the recessive allele or the dominant allele. In fact, one could get either a positive or a negative association with *Four* locus antigens due to linkage disequilibrium with the *Four* locus, whether or not the *DS-A* allele involved was dominant or recessive. Thus, if the explanation of the effect being due to a linked locus is correct, whether one gets an increase or a decrease in the antigen frequency in the disease he is looking at, does not tell whether the effect is dominant or recessive.

The question of dominance versus recessivity is, however, important when considering explanations for the disease associations. Explanations involving either the HL-A antigens themselves or the products of a closely-linked locus in molecular mimicry with pathogen (virus) antigens, or in interactions with viral receptors, lead to dominant effects on disease susceptibility. On the other hand, the simplest direct model of an immune response mechanism controlled by the *DS-A* locus would, by analogy with the mouse and guinea pig data, lead to recessive effects on disease susceptibility. This is because a positive immune response would be expected to be associated with disease *resistance*, and *this* should be dominantly controlled. More complicated hypotheses can, however, be envisaged which would give rise to a dominant immune response associated with susceptibility. For example, the disease could be associated with a positive autoantibody response or with the production of an excess of immune complexes. Another alternative is that the patients studied, especially if they come from an older age group, actually represent a class that has differentially *withstood* the disease and *so* is disease resistant rather than susceptible. On this hypothesis, the susceptibles either die or are otherwise prevented from being studied. As pointed out by Falk and Osaba (*Lancet*, **II**, 1118, 1971), such a mechanism might lead to different patterns of association for different age groups.

Thus, to summarize, I think that while the studies in man are extremely interesting, and support the idea that there may be substantial and important polymorphism with respect to genes affecting susceptibility to disease, these studies are difficult and may often be inconclusive because of the complexities

TABLE 133

Association between HL-A and Hodgkin's Disease in 2 Patient Series

HL-A	Normal Caucasians (n = 273)	Hodgkin's I (n = 127)	Hodgkin's II (n = 31)	Hodgkin's (total n = 158)
HL-A11	37 (13.6%)	36 (28.4%)[a]	1 (3.4%)	37 (22.8%)[c]
HL-A5	25 (9.1%)	16 (12.6%)	6 (19.4%)	22 (13.9%)
W5	25 (12.1%)	41 (32.3%)[b]	3 (9.7%)	44 (27.8%)[a]

[a] $p < 0.001$

[b] $p < 0.0005$

[a] $p < 0.01$

involved. The real goal must surely be to do family studies in which one can identify the activity of the particular loci involved, without the complications of environmental factors or of recombination at the population level.

MORRIS: I would like to consider some aspects of our own disease studies which will emphasize what Bodmer has mentioned, and possibly add one or two points that have not been mentioned. I will be identifying some of the diseases that we are interested in and their possible HL-A associations. I want primarily to confine my attention to Hodgkin's disease and some of the autoimmune diseases.

Table 133 provides a summary of our Hodgkin's disease data, giving the frequency of the HL-A antigens which we find to be associated with the disease, namely, HL-A11, W5, and possibly HL-A5. The data deal with a normal Caucasian Australian population. The Hodgkin's group is in fact made up of two prospective groups, previously published as such and, therefore, I have combined these into one group (Hodgkin's I) (Forbes and Morris, *Lancet*, **II**, 849, 22, 1970; Forbes and Morris, *J. Clin. Invest.*, **51**, 1156, 1972). This group showed a very significant increase in the frequency of HL-A11 and W5. A third prospective group (Hodgkin's II), being studied as part of the concurrent histocompatibility workshop, does not confirm, at least to date, the increased frequency of W5 in the previous two prospective studies. As Table 134 shows, taking all these together, viz, a total of 158 patients with Hodgkin's disease, we are left with a significantly increased frequency of W5 and HL-A11. From what has already been said, and from other published sources, I would suggest that we are dealing with something in the 4c region, that is increased in frequency in Hodgkin's disease.

Obviously one factor that has to be excluded in any of these various disease studies is that the increased frequency of a particular specificity or antigen is due to the disease itself, and that the expression of the antigen is a result of

TABLE 134

Results of Studies Searching for an Association between HL-A and Various Diseases

Disease	No. of cases	HL-A specificity associated
Hodgkin's	158	W5 (4c region), ?HL-A11
Malignant lymphoma	134	?HL-A12
ALL		None
Infectious mononucleosis	110	None
Autoimmune diseases	107	?HL-A8 –SLE, LH ?W15 – SLE ?W15 – RA ?HL-A1 – LH
Ca lung	56	None

the disease process. This can be established only by family studies. We have been able to obtain 40 families of patients with Hodgkin's disease in which we have determined the HL-A genotypes of the family members. This includes 8 families in which the patient had W5, and 12 families in which the patient had HL-A11, which were the two antigens we are suggesting as being increased in frequency in Hodgkin's disease. In all cases this antigen segregated in a normal Mendelian manner, so we can exclude the possibility that the antigen is *acquired* as a result of the disease (Forbes and Morris, *J. Clin. Invest.*, **51**, 1156, 1972). I should point out to those of you who are not clinicians that Hodgkin's is a very *heterogeneous* disease as regards its histological and clinical attributes, and in terms of the age of occurrence.

Bodmer felt this point was not very relevant in the overall picture of HL-A in Hodgkin's disease, and he may be right. However, I think we must bear in mind, in our study of Hodgkin's disease that it might be important and all the data should be accumulated with this in mind. For example, we find a highly significant increase of yet another antigen, HL-A7, in females with the nodular sclerosing type of Hodgkin's disease; and HL-A11, which is increased in all groups is predominantly increased in females, here again with the nodular sclerosing Hodgkin's disease.

This data is worth concentrating on, as the histology may reflect host immune responsiveness, first remembering that the histological definition of Hodgkin's disease undergoes changes during the course of the disease from its initiation to the terminal stages. Secondly, it is very difficult to get two histologists to agree about the grading of Hodgkin's; consequently, the ideal situation is to have

all histology studied by the same panel of histologists, which was in fact the case in our own data.

Furthermore, age is a major consideration, as Hodgkin's patients can be separated into two model groups on this basis. In the first, the disease occurs under fifty years of age, and in the second, over fifty. In our data, we can't make any comment on that, as 97% of the patients are in the first group; however, once again this would be information that the combined data from the international study in the Histocompatibility Workshop, going on at the moment, might show.

Now, I would like to devote a few moments to the serological definition of these antigens, because many seem to have the impression from HL-A specialists either in their publications, or discussions, that these specificities are clearly defined. I want now to stress that this is, in fact, not at all the case. This has become more apparent in some of our recent anthropological studies, something in which all the HL-A afficionados are involved, as part of the present Histocompatibility Workshop. Apart from providing an excuse to get us off to many

Fig. 80. A New Guinea highlander. Dramatically illustrative of the distinctive populations being explored globally as part of the anthropological orientation of the Fifth Histocompatibility Workshop. The point is that their lymphocytes react with standard anti-HL-A sera in patterns strikingly different from the cells of Caucasians, studied almost exclusively thus far.

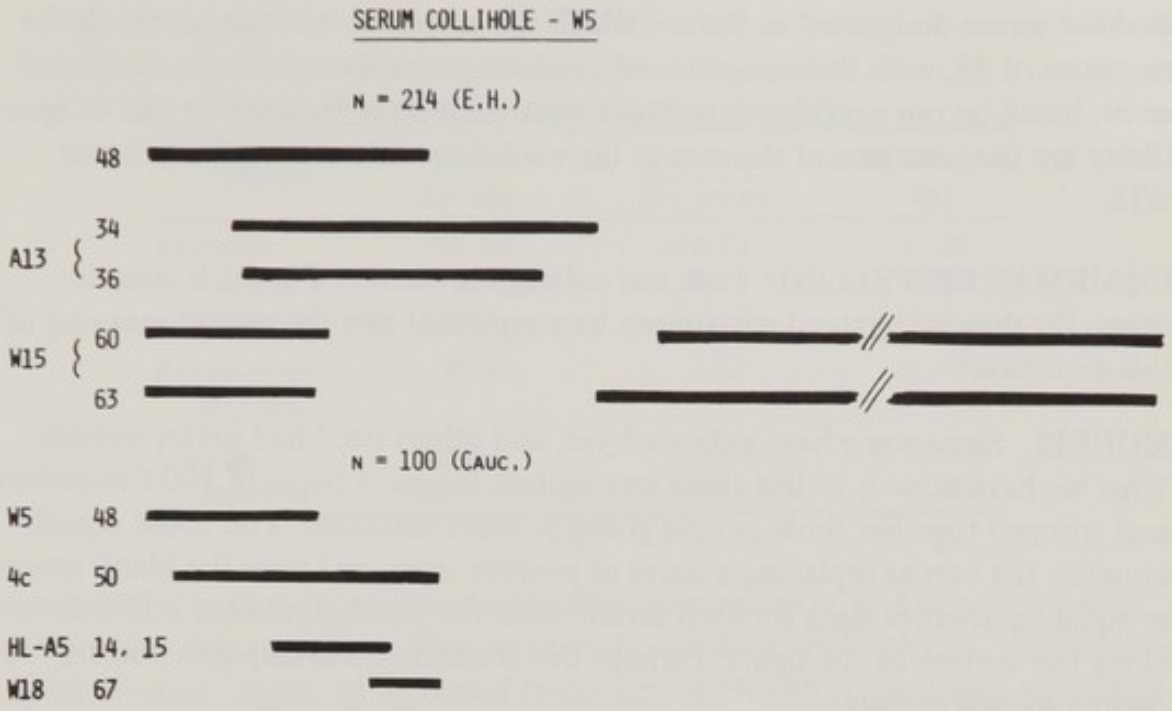


Fig. 81. Distribution of positive and negative cytotoxicity reactions. The solid bars reflect a positive reaction between the serum reagent and the lymphocytes of individuals under test. Any given solid bar represents the number of individuals whose cells are reacting positively. The length of the bar is proportional to the number of individuals involved.

romantic parts of the globe, it has already provided very useful information, and no doubt will provide a great deal more when all the data about some of the HL-A serological specificities are finally analyzed.

I would like to comment on one of the sera that we have used to define W5 in Hodgkin's disease, because this reacts quite differently with the cells of the New Guinea Highlanders such as the individual so strikingly depicted in Fig. 80. These people are quite different from us, and all conferees will agree, I think, that this person bears little resemblance to anyone in our group. Figure 81 is known as a serum map, in which the reactions of this W5 serum 48 (a Histocompatibility Workshop designation), are compared with a number of other sera, detecting other HL-A specificities. The length of the bars reflect the summation of the positive reactions of each serum against a panel of Caucasians in the bottom half of the figure and Highlanders in the upper half. Only the positive reactions with each serum are given. The negative reactions are not depicted. In the full elaborate charts our workshop colleagues are accustomed to using, the people tested would be lined up along the bottom of the figure, arranged so that those showing positive reactions are grouped together.

For the moment let us concentrate on the lower half of Fig. 81. In Caucasians, serum 48, which is a Melbourne serum and has been used for years by us, detects W5, and is positive in about ten % of the population. The next serum is a

Bodmer serum designated as Rafter, which we call 4c. It includes nearly all the reactions of 48, with the exception of 1, which probably represents a technical error, based on our previous experience with these sera. Included in this 4c specificity are the reactions of the sera in the workshop determining HL-A5 and W18.

CHAIRMAN CEPPELLINI: I ask our colleagues whether Fig. 81 is clear to them. Do they understand what those bars represent and the overall meaning of this depiction?

MORRIS: Since some have indicated yes, and others no, I had better explain. What we have done is to test these sera against the same panel of 100 Caucasians, and grouped together those people giving positive reactions. You could equally visualize the bars as replacing a series of positive signs, and view the blank spaces as equaling negative signs for each serum, with the panel numbered 1 to 100 along the bottom of the figure. Perhaps this statement will help give you the desired mental picture.

When we look at this same serum 48 in the New Guinea Highlanders, who *completely lack* eight of the *major* Caucasian HL-A antigens, we find that these sera defining 4c, HL-A5, and W18 are completely nonreactive, yielding not a *single* positive reaction. Yet we find this serum 48 reacts again with about ten % of the New Guinea population. But these reactions are completely included in sera detecting the specificities HL-A13 and W15.

My interpretation of this extraordinary type of serological data is that, in fact, this serum 48 is not only detecting the factors that make up W5, but is detecting some of the factors or determinants that make up HL-A13 and W15 in the New Guinea Highlanders. These *cannot* be the same factors that make up these same specificities in Caucasians, for in this race serum 48 is not positive with W15 or HL-A13. The reason for bringing up all of this esoteric information is that this raises the possibility that the additional factors that are being detected by 48 in the New Guineans, may be the ones that are associated with Hodgkin's disease.

So much for Hodgkin's, and now a brief word about the autoimmune diseases and HL-A. Table 135 summarizes the results of HL-A frequencies in the major groups of patients, SLE, rheumatoid arthritis, and lupoid hepatitis. We are not able to confirm the increased frequency of W15 mentioned by Bodmer, but W5 *is* increased and HL-A8 *is* increased. HL-A8 also was shown to be increased by Grumet and colleagues in their original study (*N. Eng. J. Med.*, **285**, 193, 1971). Again, in lupoid hepatitis we find an extraordinarily high frequency of HL-A 1 and 8 (Table 135). Admittedly, these groups are small, but in all our autoimmune disease patients studied HL-A8 *has been* increased. I think this is interesting, as we also find the frequency of HL-A8 to be increased, not always

TABLE 135

Correlation between HL-A Type and Rheumatic Disease and Lupoid Hepatitis

Disease	HL-A Specific	Positives No. tested	Significance (p)
Systemic	HL-A8	11/23	< .05
lupus	W5	8/23	< .01
erythematosus	W15	1/16	N.S.
Rheumatoid arthritis	W15	6/25	< .005
Lupoid	HL-A1	13/18	< .001
hepatitis	HL-A8	14/18	< .001

significantly, in high responders to *flagellin immunization*, and in patients who give positive skin tests to mumps, tuberculin, candida, trichophytin, and streptococcal varidase. Again, an increased frequency of HL-A8 is associated with childhood asthma which presumably has an immune background (Thorsby, *et al.*, *Tissue Antigens*, **1**, 147, 1971). In my view these admittedly preliminary observations are worthy of further study. Now, the last information I want to report involves a quite different area, namely the association between HL-A and capacity of the host to elaborate antibody against HL-A antigens. We have examined all the people in whom we have found anti-HL-A antibodies as the result of pregnancy, transfusion, transplantation, and immunization with lymphocytes or skin grafts. Altogether there are some 250-odd sera. In many of these we had genotype data both of donor and recipient. For the moment, let us focus on the phenotype data of the producers of the antibody.

About a year ago, van Rood demonstrated and directed our attention to a possible association with W15 and the capacity to produce anti-HL-A antibody. We could not confirm this in our total diverse group, but as is seen in Table 136, we did find a very significant influence of HL-A on the formation of *one* of these HL-A antibodies, viz, anti-HL-A2.

Table 136 summarizes the results in which we found that there is significant increase in frequency of HL-A3, and/or its crossreacting antigen HL-A11 in 45 people who made antibody directed against HL-A2. There is also a possible decrease in the frequency of HL-A9 in these same people.

The control group is a not fully acceptable group, in that it is not a group immunized with HL-A2 who do not make anti-HL-A2. It is a panel of 217 blood donors, from which have been deleted all HL-A2 positive people, leaving 97 in all.

What we have is a highly significant association between the ability to produce anti-HL-A2 and the presence of HL-A3 in the serum producer. This does

TABLE 136

Association between HL-A Type and the Capacity to Produce Anti-HL-A Antibody

		HL-A3		HL-A3/11		HL-A9	
		+	−	+	−	+	−
Anti-HL-A2	+	26	19	35	10	3	42
	−	27	70	37	60	25	72
		$\chi^2 = 11.78$		$\chi^2 = 19.318$		$\chi^2 = 7.088$	
		$p < .001$		$p < .001$		$p < .01$	

not mean an association between HL-A and an *Ir* gene, but this could be one possible explanation for this finding. We are presently seeking to define this finding more precisely, by immunization within HL-A genotyped families. The data we have obtained thus far would tend to confirm what I have stated, to the extent that it is certainly very easy to make an anti-HL-A2 antibody in individuals who are positive for HL-A3, and quite difficult in those who are negative for HL-A3.

CHAIRMAN CEPPELLINI: Do any other conferees have further information on the production of humoral antibodies and HL-A phenotype of the antibody producers? If not, I would like to quote the work of my colleagues in Torino and Parma (Curtoni, *et al.*, *Tissue Antigens*, in press 1972) who have followed antibody production in more than 50 volunteers immunized by planned transfusions of whole blood. We have found some interesting patterns of response, but the most significant observation ($P = <.01$) is that recipients who carry antigen HL-A3 respond poorly against antigens of the Four series and then only after many transfusions. This is of special interest, because the response is measured against antigens of the other series, and therefore is less likely to be influenced by crossreactivity between immunogens and tissues of the responder. But following Bodmer's admonition, we will repeat the observation in 50 more individuals before getting too excited. The second study will start with the *a priori* assumption that HL-A3 positive recipients are poor responders. We have recently obtained a new line of evidence in favor of a relationship between HL-A and the immune response. When comparing different genetic markers between populations (again from Sardinia), subjected until a generation ago to strong selective pressure by malaria (as also proved by a high incidence of congenital hemopathies), and control populations of identical ancestry, but living in nearby malaria-free highlands, the divergence between the "malarial" and "nonmalarial" populations is significantly higher than would be expected on the basis of random drift, only for Thalassemia, G-6-PD and locus *Four* (Piazza, *et al.*, Histocompatibility Testing, 1972), but not for 18 other markers (LA, Rh, MN, Gm,

etc.). It is very suggestive that locus *Four* tends to follow the trend of the two hemopathies which certainly are "selective" markers. Our working hypothesis was: (a) malaria is a strong selective agent (able to maintain polymorphism for lethal mutants such as Thalassemia); (b) under natural conditions, as pointed out repeatedly by Allison, immune mechanisms are powerful defenses against malaria, probably interacting with hemopathies for protection; (c) hence malaria should select for some special *Ir* alleles and thus, in evolution, bring with it the closely linked HL-A genes. If *Ir* genes in man map close to MLC, as shown by Bach in the mouse, that could explain why the effect seems to be significant only for the *Four*, but nor for the *LA* locus. There again, what we have to do is to repeat *ab initio* the research on other populations, possibly the African tribes studied by Allison, with high and low incidence of Hb S.

Observations in favor of an association between HL-A and the immune response, and HL-A and disease begin to accumulate. Nonetheless, as underlined by Bodmer, we should avoid the game of chasing statistically significant associations which may have little biological and clinical relevance. For instance, as Bodmer has pointed out, the risk of Hodgkin's disease is 5×10^{-5} for the general population, and only slightly increased, let us say five times, i.e., 25×10^{-5}, for the siblings of patients. The probability that siblings share the same HL-A genotype, however, is 25×10^{-2}. Thus, HL-A seems to contribute only one-thousandth of the total causative forces, assuming that all Hodgkin's concordant siblings have the same HL-A genotype. Many of the associations reported correspond to diseases that either directly affect the lymphocyte (e.g., acute lymphocytic leukemia) or induce autoantibodies (e.g., systemic lupus erythematosus). I wonder whether some correlations aren't due just to nonrandom mistyping, when for instance, the cells under test are coated with sublytic doses of autolymphocytotoxin. Of course, such an explanation does not hold when HL-A phenotyping has been confirmed through family genotyping, as in some of the studies presented by Morris.

VAN ROOD: At this juncture, I would like to fit together the diverse data and points of view presented to us. For those outside the international efforts on HL-A, I realize that our presentations are perhaps less fascinating and stimulating than other more basically oriented investigations; then, too, there is far less disagreement among this group as compared, for instance, to the T cell receptor workers. What can be the explanation for this contrast? Let us first consider the possibility that the field is simpler. Our field is the study of man and we are always told that it is *so* complex that we cannot hope to get anything meaningful out of it. Thus, solutions do not seem very likely.

The second possibility is that the field is really run by a dominant male, like the wild mouse. We have quite a few dominant males around, but I don't think their strength is sufficient to explain this homogeneity. I am, therefore,

really left with one possible explanation, and that is that we regularly exchange reagents and that this might be a major basis for our agreement.

To get at one of the factual points I would like to develop: Bodmer mentioned in passing something I regard as especially important, i.e., the data that are available indicate that gene frequency for HL-A does not differ between old and young people. This would be disappointing if it were really so, because it would imply that the influence of that region is only becoming apparent after the reproductive age. There are, I believe, data by Amos and Yunis which suggest just the opposite; i.e., that there *is* a difference between the gene frequency of young people as compared with the old. This, then, is one point which might be discussed, and perhaps others have data bearing on it. There is also general agreement that apart from the locus or cluster of the *Four* and *LA* loci, there are, of course, also loci **outside** *Four* and *LA*. I would like to recall for discussion the data we considered in Session II, from the single fortunate crossing-over family in which two cross-overs occurred. In that family, at least, it seems rather certain that the locus determining MLC activity is neither *Four* nor *LA*, but rather the one we now designate *MLC* and that we believe lies nearer to *Four* than to *LA*. In my view, this is one of the really new things in the human studies of which we can be rather certain.

There is another important point which emerged in this final session and which might fit in with the preceding observations. I am referring to the finding that most of the disease associations appear to relate to antigens of the *Four* series, not the *LA* series. If we agree on these facts, then at this stage of the game the really interesting question that surfaces is just how effective is our serum typing for the antigens of the *Four* and *LA* series in providing information on the *MLC* locus. Once again, there *is* factual evidence on this point, which indicates that serum typing is effective in about 10 to 20% of the situations. So if this *MLC* locus is adjacent to or identical with the "disease state" locus, we cannot hope for a better resolution that at 10 to 20%. This situation logically leads us to the question of the kind of sera that are being used in this global effort.

Some sera might crossreact with the product both of the *Four* and the *MLC* locus, and one's family study would not help him to discern whether this is, in fact, the case, because generally speaking these loci segregate together in families. Accordingly, one is left to think that he is only recognizing an antigen of the *Four* series. The excess of positive reactions which makes a set of data significant with a certain serum, could be due not to the specificity of the *Four* series, but rather the crossactivity of the *Four* and the *MLC* antigens. I am not saying that it is so. I only say that it is a *possibility* that we should keep in mind. This brings me to my last point; the only really satisfactory way to test this disease association is to test directly for the product of the *MLC* locus.

In private discussion with Benacerraf, he outlined the kind of ideal program he envisaged, among other things performing the MLC on patients with mammary carcinoma and also securing a large control series. I pointed out that to do this properly might take something like ten years. But I still think the idea is basically sound. What we need at the present time is to get exact information about polymorphism at the MLC locus. As I outlined in Session II, we are studying a protocol which was based largely on the work of Ceppellini and his group, using MLC inhibition; this *might* just do the trick. In some respects it may be unfortunate that we started with Hodgkin's disease because the familial incidence of Hodgkin's disease is very low, as Bodmer pointed out. I want to emphasize that it is never too late to correct our errors; it might be a good idea to change the direction of our efforts and study mammary carcinoma, for example, as it offers a higher familial incidence.

CHAIRMAN CEPPELLINI: Would Bach tell us why, in his HL-A schema he placed *Four* and *LA* in positions the reverse of what we understood as established?

BACH: Ceppellini may be the one who has been writing it in reverse. In difficult matters I read from right to left.

CHAIRMAN CEPPELLINI: Well, it is not simply a question of individual taste. I think we have no basis for pushing the homology so far. We don't even have reasons for equating *LA* to *D* and *Four* to *K*. Let us be a bit more patient and wait for amino acid sequences.

BACH: I want to make a few comments in reply to van Rood. First, with regard to the association between HL-A and what I referred to as the lymphocyte *LD* loci—the loci controlling activity in the MLC—the association is perhaps stronger than van Rood points out. Looking at the HL-A antigens leads one to find the case of unrelated individuals whose cells do not stimulate in the MLC. Moreover, if one seeks to correlate the number of antigens by which unrelated individuals differ, and the degree of reactivity in MLC, the studies of Sorenson (in Copenhagen) first, and now some studies by Miriam Segall in our laboratory, show that in fact, unrelated individuals, whose cells *do* stimulate despite HL-A serological identity, on the average stimulate somewhat less than cells of unrelated persons who type differently.

The second issue is localization of the *LD* loci. I would certainly agree with van Rood that the reasonable place for this locus is to the left of *Four* if one writes *LA* and *Four* in the way I have indicated. However, on the basis of the human studies by van Rood's own group with Eisjvoogel, as well as some of

the studies from elsewhere, I would not want to rule out the possibility that the loci controlling MLC could include *LD* loci between *Four* and *LA*.

On the basis of van Rood's findings where there is one complete haplotype difference with respect to the serological antigens, and where he has moreover presumed a recombinant between *Four* and the *LD* loci to the left of *Four*, he still gets a small but significant degree of reactivity. This stimulation could be caused by an *LD* locus between the two serologically defined loci, as we found in the mouse.

Finally, I also want to mention that I believe it may be possible to define the alleles of the *LD* loci. One way, following the lead of Ceppellini, would be to try to get blocking antisera which will block specifically the *LD* locus product.

This can certainly be tried directly in the mouse; in man perhaps with a little more difficulty. There may be a way of approaching this by using cells as a reagent, and with the combination of MLC and the specificity of killing, which Ceppellini has named CML, cell-mediated lympholysis. With Paul Sondel, I have wondered if we could get cellular reagents (killer cells) which would specifically define the phenotypic products of the *LD* locus.

Such cells can be used in a manner analogous to antisera, as groups of reagents defining antigens, or they can be absorbed following the lead of Brondz in Moscow, by absorbing them on monolayers.

CHAIRMAN CEPPELLINI: In reply to Bach, I would say that first of all, we do not know for sure whether *LA* or *Four* as antigens are important for the MLC. Are we perhaps looking at the equivalent of MLC as an antigen, and MLC as a receptor, i.e., a receptor for the histocompatibility reaction? In our experience, LA and Four differences, (SD antigens in Bach's terminology), have some bearing on the strength of MLC activation, but not as much as on the effector phase of cell-mediated cytotoxicity, where they seem to allow specific recognition of the target. What I want to know is how Bach visualizes the role played for initiating MLC activation by the products of MLC locus, his LD antigens: Are they the stimulating "antigens" in the stimulator lymphocytes, or are they related to immunoreceptors in the responder, or just pertinent surface differences between the two reacting partners?

BACH: It seems to me that on this issue there are two interpretations; one, the serologically defined antigens are part of the molecules stimulating in the MLC. Given an Ir difference, they may only stimulate or they may themselves serve as receptors. Or it is possible that the serologically defined antigens don't stimulate at all. I think that Ceppellini has brought up a very important point.

We would like to hypothesize that the products of the *LD* locus is the Ir product. Second, it is the series of *Ir* gene products which are important, not only as the recognition molecules, but also as the antigens which are recognized

in the MLC, and constitute at least a part of the antigenic spectrum which we can recognize.

PINCUS: As a clinician, I certainly agree with Bodmer that the proper study of mankind is man himself; however, perhaps some of the points of Session V regarding murine models should be borne in mind with respect to application of our concepts regarding histocompatibility antigens and disease.

As to the possible role of viruses in human leukemia, it should be recalled that naturally occurring murine leukemia viruses appear to be present from birth and may be latent in normal, and possibly all, mouse cells. There is no evidence for horizontal transmission of naturally occurring murine leukemia viruses. Thus, environmental circumstances emphasized by Bodmer may well influence the expression of virus and subsequent leukemogenesis; however, exposure to virus in the traditional sense may not be a significant determinant.

In the well known studies of Lilly demonstrating clearly that the H-2-linked *Rgv-1* locus plays a major role in determining whether hybrids of the C3H and C57BL strains develop leukemia induced by Gross passage A virus, Lilly was astute and recognized that a second locus, *Rgv-2* was involved in determination of leukemogenesis. The *Rgv-2* locus is not linked to H-2. Perhaps *Rgv-2* could be identical to the *Fv-1* locus discussed in Session V. Regardless, the *Fv-1* locus is a major genetic determinant of leukemogenesis or lymphoma in mice that is *not* linked to a major histocompatibility locus. Of course, we have no markers for this type of nonhistocompatibility locus in man, as a human leukemia or lymphoma virus has yet to be isolated. The identification of a major nonhistocompatibility locus affecting disease provides an approach to further understanding of polygenic control of leukemogenesis.

Finally, I would like to cite an example of a histocompatibility locus permissive of disease, which at first glance might appear to be of no particular significance in affecting disease susceptibility. In the case of the radiation leukemia virus, which is derived from a C57BL mouse ($H\text{-}2^b$, $Fv\text{-}1^b$), leukemia incidence in C57BL mice is approximately 90%. In C3H mice ($H\text{-}2^k$, $Fv\text{-}1^n$) the incidence of leukemia is approximately 30%. However, in C3H.SW mice ($H\text{-}2^b$, $Fv\text{-}1^n$) the incidence is further reduced to 10%. As C3H and C3H.SW mice differ only at the ninth linkage group containing H-2, it appears that the k allele at H-2 is more favorable to the development of leukemia than the b allele, despite the fact that leukemia incidence in C3H is only 30%. We would interpret the primary genetic determinant of leukemogenesis using the B-tropic radiation leukemia virus to be at the *Fv-1* locus, which as noted is not linked to a histocompatibility locus; both C3H and C3H.SW are resistant ($Fv\text{-}1^b$) at this locus. Nonetheless, the favorable histocompatibility-linked allele, the k allele at H-2, is in fact permissive for leukemogenesis in C3H, in contrast to C3H.SW, though only 30% of mice are affected. In this case, the influence of H-2 has biological as well as sta-

tistical significance, which is clearly understood, because other genetic determinants are recognized. This example involves a rather simple system with inbred mice. Both of the major determinants involving murine leukemia thus far recognized, *Fv-1* and H-2 (*Rgv-1*), show dominance for resistance. If one speculates by extrapolating from murine data, in which several resistant loci have been identified, to an outbred population, it would appear unlikely that any particular locus in a polygenetic system would involve the appearance of a favorable susceptibility allele in more than 30-40% of the affected population. This would in no way diminish the significance of that allele in promoting disease susceptibility.

MORRIS: At this point I am still a little confused concerning one concept which I think is relevant to this conference. Previously I understood Klein to say that in wild mice the MLC did *not* correlate with skin graft survival. We know that in humans where donor and recipient are related, both skin graft and kidney graft survival correlate *both* with MLC and HL-A serological matching. Where the donor and recipient are unrelated, there is no correlation between skin graft survival and either MLC or HL-A, unless as van Rood says, the donor and recipient are HL-A identical; whereas in kidney transplantation there is a modest association with HL-A, or so at least I find. I don't think there is adequate data available on MLC in this latter situation. One could further expand these facts from the tissue grafting data in rats.

Now, I would like to hear some speculations about the nature of the histocompatibility locus. It is the whole chromosomal region which includes *MLC*, *Ir*, the *D* and *K* loci in the mouse, and *MLC, Ir, LA* and *Four* in the human, as suggested by Simonsen. Or might the *MLC, D* and *K* loci in the mouse or the *MLC, LA* and *Four* loci in the human merely be linked to the histocompatibility locus, and histocompatibility in turn is determined by the product of the *Ir* gene? This might explain the discrepancies between MLC, serological typing, and tissue allograft survival between unrelated donors and recipients.

CHAIRMAN CEPPELLINI: Morris has brought up a very important issue. Indeed it is so fundamental that I feel it would warrant a completely separate meeting. Are there any other questions on basic issues?

BACH: I think Klein has been misquoted. He said that in the study we did in the inbred strains there is no obvious correlation, principally because in one combination, B10.A(2R)-B10.A(4R), with stimulation in one direction in MLC, there was no skin graft rejection. In the other serologically identical combinations where there is serological identity and stimulation of MLC, the H (zl) mutant and the AQR-6R combination, there is both MLC stimulation and skin graft rejection.

VAN ROOD: I would only reiterate what I said during Session II; that between HL-A identical, unrelated individuals, there appears to exist a significant correlation between skin-graft survival and MLC reactivity: The lower the MLC reactivity, the longer the skin graft, the higher the reactivity, the shorter the skin-graft survival.

BODMER: There are some points I would make in relation to further issues that have come up in our discussions.

The data quoted by Ceppellini on HL-A gene frequency differences, that may be associated with differences in malarial incidence are very interesting. Most studies on HL-A and disease association have so far involved diseases that probably have little evolutionary significance because they are rare or occur late in life. The suggested association of HL-A with malaria underlines the fact that associations may be found with much more important diseases from the point of view of natural selection, and that these may be of considerable significance in moulding the evolution of the HL-A system.

As far as the question of other loci is concerned, I did point out that we must *always* assume that we are looking at multigenic effects. If any of the conferees here could tell us what other markers we should use in order to identify the other loci that might be involved in the virus response, we would be happy indeed. HL-A is one we know to be a prime candidate, so we look for an association with it. The effects of other loci will be to dilute the association with HL-A, but I don't think they can give rise to spurious associations. It is clear that one should also be looking at the *Gm* and *Inv* loci. I suppose in family studies I would, in fact, do all the known polymorphisms, because one might by chance uncover still other linkages.

It seems worth pointing out that in the lupus studies which have been reported, the first two involved W15, which is a component of the same 4c complex of antigens involved in Hodgkin's disease. The two later studies, reported in abstracts, involve (possibly) HL-A5 and W5. It thus seems possible that in lupus the same set of antigens could be involved as in Hodgkin's. In our lupus study we tried to estimate the frequency of haplotypes in these patients to see whether the association involved any particular haplotype. We found no tendency for a particular W15 haplotype to be increased in frequency, suggesting that the association is strictly with the *Four* locus antigens. If the linked locus explanation is valid, then this emphasizes, as already mentioned, that the disequilibrium is with the *Four* and not with the *LA* locus. This is the reason for placing DS-A closer to *Four* than *LA*, though one would not have known on which side of the *Four* locus to place it.

On the question of whether we got induction of a disease connected with vertical transmission of a virus, it seems to me that we still have to have induction, and induction itself may be a rare phenomenon. Moreover, induction by

itself may not be enough to cause the disease. I don't think that such alternative mechanisms of disease causation are a serious challenge to the arguments about the possible low frequency of the environmental factors that lead to the disease.

On the matter of correlation with age, I should say that the familial studies which have been done so far show no disturbed segregations of HL-A antigens. I think this is evidence that if there is a change in the antigen distribution with age, in normal controls, it will be quite small and so, probably, much less than the possible disease effects.

I believe that one has to be very careful in interpreting the MLC data in unrelated HL-A identical individuals. It is very hard to distinguish an effect of the number of alleles that may be existing in the population at that locus, from effects of linkage disequilibrium, something Shreffler was pointing out when we were talking together previously. If there is linkage disequilibrium between the *MLC* locus and the *Four* locus, this will reduce the impression of the number of polymorphic alleles that may exist at the *MLC* locus. Without being able to identify the alleles separately, the two effects are, I think, completely confounded; i.e., the number of polymorphic alleles and the extent to which there is a linkage to disequilibrium.

VAN ROOD: I agree completely with Bodmer's last point. As to the correlation between HL-A, phenotype and age, I would like to point out that all of our present valuable information has been obtained in Caucasians. They, however, live in an extremely artificial environment. Thus, we find our drinking glass wrapped up in plastic each morning; this doesn't happen in the middle of Africa. If we want really meaningful information on this point, what we should do is study different age groups, and HL-A frequencies in tribes living in primitive conditions.

BODMER: There is, however, *some* information on primitive populations, such as the Pygmies. We have not yet analyzed age effects carefully, but there is no very obvious large difference. Even when one looks at a major example that Allison worked out (i.e., sickle cell anemia), though we find a significant difference in the frequency of the sickle cell gene as a function of age, the effect is quite small.

So I agree that one may find differences as a function of age. If, however, the differences with age which are due to natural selection are of the same order of magnitude as the disease associations, then I think that in practice one could not make good use of the disease association because the effect would be too small to be helpful.

CHAIRMAN CEPPELLINI: As a geneticist I agree with Voltaire's *Candide* that "this is the best of all possible worlds." I still believe in Darwinian selection and

I think that genetic systems in a wild population, under natural conditions, are the best of all possible collections of genes, taking into account also the past history of any group. Apart from obvious lethals (and even these may be of some advantage for the population as a whole, e.g., hemoglobinopathies in a malarial environment) it is impossible to say whether a gene is bad or good. In the case of immune response, an extreme selection for a set of *Ir* genes, specialized for one type of responsiveness, would probably be deleterious for the individual and for the population. Thus, either one is not able to respond to tissue antigens and he dies of cancer, or he has too much surveillance and develops autoimmune disease. Specialization of a population against prevalent pathogens, such as plasmodia, for example, may be paid for with a lower protection against some other agents, such as oncogenic viruses.

VII

CONFERENCE EVALUATION AND COMMENTARY

by

MELVIN COHN

Editors' Comment

In planning conferences in fields as fast-moving and productive as immunology, there is an overwhelming desire on the part of organizers to have them brought to a close with a tidy, concise statement summarizing their accomplishments. However, we submit that such attempts have had, on the whole, a rather poor record. Therefore, we sought instead a critique or evaluative synthesis of the ideas and concepts that emerged from these three days of free-ranging discussion. Dr. Melvin Cohn was approached for this assignment and after reviewing with us various possibilities, he accepted the task.

His impromptu commentary at the close of the sixth session, developed from his meeting notes, was excellent but did not achieve the synthesis he and the editors had sought. Accordingly, we mutually agreed to reassess this assignment. At a later date he was provided with the fully edited version of the conference proceedings, complete with tables and figures, so that he could begin anew this assignment in a more deliberate and thoughtful manner.

As a reading of this subsequent product attests, this proved to be an enormously complex and arduous task—but one that has, in our view, yielded an extraordinary and inspired critique. Not everyone will agree with all of Cohn's postulates, but he has nonetheless done us an enormous service by carefully separating all-too-frequent tacit assumptions from established fact, and by distinguishing theory based on a logical extension of the facts, from a simple restatement of observed phenomena. On behalf of our readers, we wish to express our thanks and deep appreciation to Dr. Cohn for his unprecedented effort and commitment, and for the immunologic *tour de force* that has emerged.

Introduction

As the excitement from an exposure to such a high density of facts subsides, it should not occasion surprise that I attempt the construction of a unifying theory. There are several reasons for doing this. First, we wish to have an insight into the immune system which goes beyond present experimental knowledge. Second, we must try to avoid being misled by the facts we already have. Third, we need a clearly defined conceptual framework in order to communicate to each other our different results and points of view, particularly now when it is clear that one individual's neology evokes a nonintended interpretation by others. I stress the importance of competing theories. It would be foolish to try to be the only wise man. As I hop around uncomfortably like a Yogi novice walking on hot coals, I insist that the reader, too, undertake the same trial by fire. Maybe, there is another route to nirvana.

I am aware that this analysis will not be easy to read. Nevertheless, I hope that most readers will persevere. Since I feel that we have reached a point where it is well to see how many of our widely accepted notions are founded on general principles, the effort to work through my arguments might not be wasted. I have pushed many of them to the limit deliberately, in order to sharpen the problems and their possible solutions. This outline should only be considered a guide to the reading, since the text does not have sharp demarcations. This reflects in part how intricately interwoven are the factors determining the expression of immune response genes, and in part, my limitations.

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The central problem of this conference is the role of the germ-line genes in determining immune responsiveness, the most difficult aspect of which is the regulation of the self-nonself discrimination. Many different recognition systems have been selected for during evolution. The unique feature of an immune system is that specific recognition is an inducible and paralyzable property depending upon the prior experience of the animal with respect to the given or related antigens. In other words, out of the total genetically determined potential, the repertoire of specificities actually expressed by an individual, i.e., the self-nonself discrimination, is not encoded in germ-line genes. It is acquired or learned. Thus we are faced with a new problem in somatic evolution and a classic problem in differentiation.

We have been dissecting specific and general responsiveness by the use of a molecular knife (mutation) with which we perform microsurgery on germ-line genes. Our experimental material is two animals differing by one or more products coded for by allelic genes. We try to segregate out the genes so that the animals differ by only one allelic pair and then determine from the phenotype what is the gene-product and its function. This is one example of a classical methodology for biologists, namely the removal of one part of an integrated system in order to observe the resultant perturbation. As a first step, we characterize the phenotype by challenging the animal with a battery of antigens and by analyzing the response with respect to the level (high or low) and class of serum antibody produced as well as "cell-mediated" behavior (GVH, MLC).

I. CATEGORIES OF IMMUNE RESPONSE GENES

The two immediate distinctions which we can make in considering the genetics is whether responsiveness is dominant or recessive and whether it involves one, or a few—specific responsiveness—or most, if not all, antigens—general responsiveness. Four possibilities exist; general recessive, general dominant, specific recessive, and specific dominant responsiveness. Only the last three are known experimentally, although general recessive responsiveness is predictable and would be very informative, because it would give us a key clue to the chromosomal control mechanisms for expression of responsiveness by implicating specific repressor proteins.

Since the paradoxical finding is specific dominant responsiveness, I will spend most of the discussion on this case. Let me begin, however, with some remarks concerning general dominant and specific recessive responsiveness. This will set the stage.

A. General Dominant Responsiveness

In the category of the dominant genetic determination of general responsiveness is:

- The example of the high and low responder mice analyzed by Biozzi (Sessions III and V).
- The cases not discussed by us here of the "nude" or "pituitary dwarf" mutation in mice, the various agammaglobulinemias in man and the X-chromosome-linked immune response in mice (Amsbaugh *et al., J. Exp. Med.* **136**, 931, 1972).
- The high *versus* low response of mice uniquely in the IgE class discussed by Levine (Session III).
- The response of mice to self-antigens, i.e., generalized autoimmunity (Session V; Section VI); a misleading example.

There are many steps in the differentiation of the immune system that could be blocked by a mutation in a structural gene coding for a hormone, a membrane protein, or the constant region of a heavy or light chain. Mutations blocking the ontogeny of the thymus could wipe out all responsiveness to any antigen. Mutations affecting the development of bone marrow-derived antigen-sensitive cells might eliminate all of the effector, but not the cooperating activities of the immune system. Mutations in the constant region of the light or heavy chain might eliminate only one immunoglobulin class and the effector activity it mediates. For example, a deletion of the gene coding for the constant region of the heavy chain of IgA or IgE would eliminate that class no matter what antigen was used to challenge the animal. In general, however, the mutations we study are not expressed as all or none (as would be expected from a deletion). They are more or less effective in inactivating the function of the gene product and we are forced to deal with high and low responders. Much can be learned about the pathway of ontogeny, the mechanism of induction and how antibodies effect functions from mutations affecting general dominant responsiveness.

B. Specific Recessive Responsiveness

In the category of specific recessive responsiveness is the response to antigens which are very similar to self-components and therefore tend to establish paralysis. Most often this is seen with antigens which are polymorphic in a species. For example, individuals of blood group A respond to immunization with the B substance and those of blood group B respond to immunization with A substance. The hybrid individual of blood group AB responds to neither A nor B substance. Responsiveness is specific and recessive. Known examples of this are legion. Responsiveness to any antigen which is allotypically variant in a species falls in this category. By extrapolation then, the agouti-linked *Ir-2* locus originally observed to control in a specific recessive fashion the responsiveness of mice to erythrocyte antigen ($Ea\text{-}1^a$), may be an as yet unresolved example of specific regulation of responsiveness by tolerance (Gasser, *J. Immunol.*, **103**, 66,

1969). The genetics of specific recessive responsiveness due to tolerance can be tricky. If one studies the antiidiotypic response to a test antigen which is an antibody to a polymorphic self-component, e.g., anti-H-2, then responsiveness can appear as dominant. Consider animal A expressing anti-B but not anti-A due to tolerance and animal B expressing anti-A but not anti-B due to tolerance. If one uses anti-B as an antigen, animal A would be a low and animal B, a high responder. Conversely, if anti-A were the antigen, animal A would be a high and animal B, a low responder. The F_1 hybrid AB would be a high responder making anti-idiotype to both anti-A and anti-B so that responsiveness would be dominant and controlled by the genes coding the self-component AB difference, e.g., H-2. I realize that this is an illustration likely to lead to difficulties if it were carried out experimentally as literally described. However, in essence this is the Ramseier-Lindenmann experiment (*Eur. J. Immunol.*, 2, 109, 1972). There is no new principle of genetic regulation of responsiveness involved here. Rather it is a warning that we can be faced with a "double bluff" situation requiring that the two steps be sorted out. I will return to this extremely important experiment in another context because it bears on the question of the use of "cell-mediated" assays to determine the nature of the t cell receptor. [I am using the symbol *t cell, not T cell,* for that population of thymus-derived antigen-sensitive cells the function of which is associative recognition (cooperation). The reason will be clear when I discuss Fig. 84; Section V A.]

C. Specific Dominant Responsiveness

Specific dominant responsiveness is the key phenomenon because the clarity with which we can interpret the findings is an accurate assay of our understanding of the origin and expression of antibody specificity. Individuals of inbred lines respond in a reproducible and different way, both quantitatively and qualitatively, to given antigenic stimuli. If the phenomenon were limited to only one or two antigens, it would not be quite so informative. Specific dominant responsiveness is seen with a variety of antigens in many species.

Let us start with an interpretation of the dominance and then consider the nature of the products of these Ir loci. I can think of only two "universal" ways by which genes could control specific immune responsiveness. (I use the designation universal to stress that there are *a priori* many ways that specific responsiveness could be regulated, were the phenomenon seen for only one antigen in one species.) These two universal mechanisms are either via tolerance or via structural genes coding for the receptors on antigen-sensitive cells.

As I pointed out, in general, a tolerance mechanism would lead to specific recessive responsiveness. The fact that the response is dominant is not sufficient grounds to rule out tolerance. There are several reasonable mechanisms via which dominant responsiveness could be due to tolerance. All that is required is that

the test antigen resemble a self-determinant which is absent in the responder parent and in the responding hybrid, but present in the nonresponder parent. We left this question at the 1970 Brook Lodge Conference on Immune Surveillance debating the likelihood of dominant tolerance and agreeing that if allophenic (tetraparental) mice made up of responder and nonresponder parents were responders, a strong argument could be made against "tolerance" models. We know now that allophenic mice do respond, but the argument appears to be somewhat weakened by the finding that they show autoimmunity or balanced enhancement (see Section VI F). However, it is unlikely that the many known responsiveness differences to randomly chosen antigens can be due to tolerance. The expectation would be that the responsiveness differences would map all over the genome not at the *Ir–1* locus as is found in most cases (Section II C). Consequently, I am prepared to extrapolate and say that for the cases we have discussed here, dominant responsiveness is not due to tolerance. However, if a paradoxical situation were to arise, we should be prepared to reexamine this question for that antigen simply because specific dominant responsiveness due to tolerance is not only predictable but it would tell us about the regulation of expression of surface antigens.

II. SPECIFIC DOMINANT Ir GENES ARE V GENES

This leaves us with the second proposal that specific dominant responsiveness is due to structural Ir genes coding for the receptors on antigen-sensitive cells. Since B cells express immunoglobulin receptors, the Ir genes determining their specificity must be the known variable (V) genes of the structural loci, kappa, lambda and the allotype marked heavy chain. It is only with respect to the Ir genes coding the receptors of t cells that four different interpretations have appeared. These are that the receptors on t cells are:

(1) Immunoglobulins coded by the same three loci as are expressed in B cells.

(2) Immunoglobulins coded by the two light chain loci, kappa and lambda, and a new heavy chain locus expressed uniquely in t cells.

(3) Proteins which are not immunoglobulins in that the known kappa, lambda, or allotype marked heavy chain loci do not code for any part of them.

(4) The same immunoglobulins expressed in B cells, but their specificities are modulated by the products of an Ir locus.

In their simplest form, these interpretations put the expected total number of Ir loci controlling specific dominant responsiveness at $\leqslant 4$, i.e., equal to the kappa, lambda, allotype marked heavy chain loci, and one new locus coding as above.

Although a major part of our deliberations dealt with these four interpretations, it is not with respect to the nature of the t cell receptor that the key differences among us lie. No theory of responsiveness is disproven by which proposal is correct because we would ascribe to any protein receptor the necessary properties to carry out physiological function. However, as I will demonstrate, we cannot discuss the phenotypic expression of Ir loci without stating our conceptual framework concerning the generation of diversity as well as induction and paralysis.

A good starting point would be to ask how reasonable does it appear on *a priori* grounds that Ir genes should code for germ-line V genes. The answer depends upon the model of diversification one has in mind.

A. Somatic Mutation Model Predicts That Ir Genes Are V Genes

What is the contribution of germ-line V genes and somatically derived V genes to the total recognition potential of the animal? I have analyzed the "germ-line" (Hood and Talmage, *Science,* **168**, 325, 1970) and "hyper-recombination" (Gally and Edelman, *Nature,* **227**, 341, 1970), models elsewhere (Cohn, *Ann. N. Y. Acad. Sci.,* **190**, 529, 1971; Cohn, "Biochemistry of Gene Expression in Higher Organisms," Lee and Pollak, eds., Australia and New Zealand Book Company, Sydney, 1972, in press). The major difficulty with these models as proposed, is that the problem of the selection pressures on germ-line V genes is not faced. Rather, the problem is seemingly resolved by the assumption that the total potential of an individual to respond effectively ($>10^6$ antibodies) is expressed independent of whether somatic antigenic selection is imposed.

If a large range of recognition specificities is generated in a random way by the animal so that the total potential of each animal is expressed whether or not antigenic selection is imposed, then one is not immediately led to the conclusion that structural germ-line V genes could control specific responsiveness to a wide variety of antigens. The reason is that, as a general rule, the induced antibody (associative and/or effector) must be reasonably restricted in its specificities in order for a response difference to be discerned. It is therefore no surprise that specific dominant responsiveness has never been considered by "germ-line" or "hyper-recombination" theorists in their discussions of the origin of diversity. A restricted response to a multideterminant antigen is not expected *unless one considers specific models of the selective pressures on germ-line V genes which would generate restriction.* Consider this rather striking example of restriction. The C carbohydrate of the pneumococcus because it is a complex structure, has many potential determinants. Yet, the pneumococcal C carbohydrate injected into rabbits results in a response restricted to the N-acetylgalactosamine phosphate determinant (Liu and Gotschlich, *J. Biol. Chem.* **212**, 478, 1967), whereas mice show a restricted response to the phosphorylcholine determinant (Sher and Cohn, *Eur. J. Immunol.,* **2**, 319, 1972).

I have analyzed elsewhere (Cohn, *Cell. Immunol.*, **1**, 461, 1970; Cohn, *In* "Biochemistry of Gene Expression in Higher Organisms," Lee and Pollak, eds., Australia and New Zealand Book Co., 1972, in press) the hypothesis that the germ-line V genes are selected upon during evolution so that certain $V_L V_H$ combinations code for specificities of immediate survival value to the animal, in its defense against the most commonly encountered pathogens. The optimal number of V_L and V_H germ-line genes is determined by a balance between two counter selective pressures. If there are too few germ-line V genes, then the initial rate of antigen-driven somatic diversification will be too slow and the animal will be selected against. If gene duplication overshoots the optimum number so that there are many redundant specificities, or if accumulation of mutants in the excess V gene pool dilutes the expression in cells of the specificities of initial survival value, or if not yet evolved special mechanisms become necessary to regulate the expression of a very large number of V genes, then the animal will also be selected against.

I estimate that there are of the order of 50 germ-line V_L and V_H genes respectively expressed as receptors in B cells. This number codes for roughly 2500 germ-line specificities ($50V_L \times 50V_H$). Assuming that selection for a given specificity can operate on only one $V_L V_H$ combination at a time there will be 50 germ-line antibodies with specificities of direct survival value and 2500 antibodies with fortuitious activities resulting from random combinations of the subunits of the fifty. These 2500 germ-line immunoglobulins are diversified by sequential and stepwise selection of somatic mutants by antigen. The total potential of the animal to respond effectively depends upon whether antigenic selection is imposed, and increases with time. How restricted the response would be depends upon how many mutational steps from each of the germ-line $V_L V_H$ combinations are required to generate a given specificity. This is why one is automatically led to the prediction that mutations in structural germ-line V genes will determine specific dominant responsiveness. However, it would be expected that somatic selection would correct germ-line controlled responsiveness so that eventually low and high responders should become indistinguishable as mutants affecting combining specificity are selected for in the low responder. I will return to the experimental test of this later (Section V A 3 b; V A 4 b).

B. Ir-V_H^B Locus Is Expressed In B Cells

I gave an example earlier in the symposium (Session III) of a germ-line $V_L V_H$ combination with a specificity which I would surmise is in the category of immediate survival value to the animal, and where a structural V gene controls specific dominant responsiveness. In mice the genetic control of the initial rate of response to the α-1,3 glucosyl determinants of dextran is linked to the heavy chain locus defined by an allotype marker. The high responders such as BALB/c,

produce IgM antibody to the α-1,3 glucosyl residue, of a restricted idiotype and in the λ light chain class. The low responders, such as C57BL/6 produce IgM antibody to the α-1,3 glucosyl residue of another idiotype and in the κ light chain class. Since the idiotypic marker depends upon the primary structure of the antibody, a V gene must code for it. The fact that the idiotype and the initial rate of responsiveness are closely associated (in fact have not been separated) indicates that the same V gene determines both properties. Since V_H genes are linked to C_H genes, it is a reasonable conclusion that the polymorphic structural V gene distinguishing a high responder, BALB/c, from a low responder, C57BL/6, is a V_H gene. In other words, one of the Ir loci codes for the known heavy chain itself. I will symbolize this Ir-V_H^B because this immune response locus is expressed in B cells. Nisonoff, Krause, and I (Session III) have discussed examples of the idiotype determined by the allotype-marked heavy chain locus. It is not surprising that the structure of the antibody would be determined in part by V_H^B. What is key about the example of the response to α-1,3 dextran is that *both* the structure and the level of responsiveness are determined by V_H^B. Other examples of V_H^B, V_κ, or V_λ gene control of responsiveness might appear when they are studied in more detail, e.g., SRBC in Biozzi mice (Session III and V), TMV (Herzenberg, Session III), the streptococcal A and C carbohydrates (Krause and Rajewsky, Session III).

The general point I wish to emphasize is that structural germ-line V genes can determine whether an animal will be a high or low responder. How far can we go with this hypothesis? Are all Ir genes controlling specific dominant responsiveness structural V genes? Is the precise way I posed the problem valid, i.e., are Ir genes V genes?

The known structural V gene loci code for the variable regions of the heavy chain (V_H^B) and of the two light chains, kappa (V_κ) and lambda (V_λ). Since lambda is such a minor component of the immunoglobulin of mice (the species we will be mostly concerned with), I would imagine that most of the cases discussed by us will not involve V_λ but rather either V_H^B, V_κ, or V_{other}.

The known Ir loci controlling specific dominant responsiveness are 1) Ir-V_H^B linked to the heavy chain allotype; 2) the *Ir-1* locus which maps between the K and D genes of the H-2 histocompatibility region; and 3) one (or more ?) Ir loci unlinked to either allotype or H-2. Thus there may well be only four V gene loci and only four Ir-loci controlling specific dominant responsiveness.

In order to remove any ambiguity as to what I mean by an Ir-V_L or Ir-V_H locus, I picture it as a series of contiguous V genes linked to C genes (Section II A). I have avoided the word cistron because the product, an immunoglobulin subunit, requires the translocation of one V to one C to produce a VC cistron. I am using the term Ir-V locus in the same sense as Gally and Edelman (*Adv.*

Genetics, **6**, 1, 1972) use the term "translocon." These loci are marked by allotype and idiotype determinants, sequence differences in their products (V and C) and responsiveness differences.

C. Ir-V_H^T Locus Is Expressed In t Cells

Consider the *Ir-1* locus. The first hypothesis is that *Ir-1* codes for the kappa chain. Since this is the most rational assumption and it appears to simplify the whole problem we should look closely at the evidence we have against it. There is one strong and one weak argument.

The strong argument is that, in man, InV, a kappa genetic marker, is unlinked to the HL-A locus. Since HL-A and H-2 are homologous, by extension to the mouse, the kappa and H-2 loci are unlinked. In the rat where the kappa locus and the major histocompatibility locus are unlinked (Rajewsky, Session III) the argument is less strong, because no evidence that this latter locus is homologous to HL-A or H-2 exists.

The weak argument is based on the existence of a genetic marker called the I_B peptide present in several V_κ subgroups (Edelman and Gottlieb, *Proc. Nat. Acad. Sci. U.S.*, **67**, 1192, 1970). I would imagine, after analyzing the data on the known sequences of mouse V_κ, that the I_B^+ allele is distinguished from I_B^- allele by a lysine to arginine interchange at position 24. The I_B peptide comprises positions 19-24.

This I_B peptide is absent from 18 strains of mice of many different H-2 alleles. It is present in three strains only, all of which are H-2^k.

H-2^k strain	I_B
AKR, C58, RFJ	+
CBA, CE/J, C3H, C57Br	–

The strength or weakness of the argument that the kappa and H-2 loci are unlinked depends upon the relative weight given to the two above facts:

- All I_B^+ strains are *H-2^k*.
- Not all *H-2^k* strains are I_B^+.

I weigh the second as slightly more meaningful because strains C57Br and C58 are derived from crosses between the same male No. 52 and females No. 57 and No. 58, all three from one litter (Potter and Lieberman, *Adv. Immunol.* **7**, 134, 1967). Since both strains C57Br and C58 are *H-2^k* but C57Br is I_B^- and C58 is

I_B^+ it is suggestive that the I_B locus and the H-2 locus are unlinked: However in the absence of genetic crosses this evidence is weak. This question is so important that we must have an answer based on formal genetics of the I_B and H-2 loci.

For our purposes, I will assume that the kappa and H-2 loci are unlinked. However, for didactic reasons, from time to time I will show how given results could be explained were *Ir-1* to code for kappa. This assumption that *Ir-1* is not the kappa locus will allow me to pursue what other product the *Ir-1* locus might code for and to deal with the profound differences we have concerning the expression of responsiveness.

If *Ir-1* does not code for the kappa subunit, the mapping of the kappa locus using structural and immune response markers is one next step. A search for linkage between the I_B marker and the Ir loci unlinked to either H-2 or the heavy chain allotype needs investigation. Clearly we expect responsiveness determined by V_κ genes to appear. I will symbolize this as yet putative immune response locus, Ir-V_κ and for completeness postulate an Ir-V_λ locus.

From now on I will be dealing largely with the *Ir-1* locus. We must try to understand why this locus is implicated in most of the cases of specific dominant responsiveness. In order to do this I must first analyze the mechanism of regulation of responsiveness by the *Ir-1* product.

The second proposal on my list (Section II) is that *Ir-1* codes for a new immunoglobulin subunit. The expectation is that this subunit would be a heavy chain expressed uniquely in t cells. This second heavy chain locus would be expected to have the same arrangement of genes as that found for the known allotype marked heavy chain locus, i.e., a number of contiguous V genes linked to a C gene. When referring specifically to this hypothesis, I will symbolize the *Ir-1* locus as the Ir-V_H^T in contrast to the known allotype marked Ir-V_H^B. Since *Ir-1* controls specific dominant responsiveness measured as serum antibody and since the serum antibody in mice is coded for by the known light and allotype marked heavy chain loci, the conclusion would be that *Ir-1* (coding neither kappa nor heavy chain) is *not* expressed as a B cell receptor. The only other way we know that specific dominant responsiveness could be genetically determined would be via cooperating t cells. This means that *Ir-1* codes for a subunit contributing uniquely to the specificity of the associative antibody receptor on the cooperating t cell. Hence the justification of my use of the symbols Ir-V_H^T to refer to the H-2 linked *Ir-1* locus expressed in t cells and Ir-V_H^B to refer to the allotype-linked locus expressed in B cells. The postulated Ir-V_κ and Ir-V_λ would be expressed in both t and B cells.

[I realize that, at this conference, we have arrived at the conclusion that B and t cells express receptors made up of different V regions or polypeptide chains because the antigen-induced specificity of serum antibody seems to be different from that of cooperating or cell-mediated activity (Benacerraf, Session

I; Katz and Benacerraf, *Adv. Immunol.,* **15**, 2, 1972). This conclusion unfortunately cannot be arrived at from the specificity studies thus far carried out. Since their interpretation depends upon a more general framework I will return to them (Sections V A 1 and V B 3) when I discuss the asymmetry in the t-B relationship and in what precise way cell-mediated immunity is a t or B cell function. In any case, there are no arguments other than the above cited genetic ones (Section II C) that *Ir-1* is not the kappa locus.]

III. ASSOCIATIVE RECOGNITION MODEL OF Ir GENE EXPRESSION

I stress that cooperating activity is the only known valid assay for the functioning of the thymus-derived antigen-sensitive cell (t cell). As I will point out, assays of induced cell-mediated reactivity are neither a more direct nor a more easily interpretable measure of the *Ir-1* coded t cell receptor than assays of induced serum antibody. What I will do now is to consider the physiology of the responsiveness to antigens in order to provide a background for our discussion of 1) the reasons why several hypotheses were introduced here concerning the chemical nature of the *Ir-1* coded receptor expressed in thymus-derived cells and 2) the expected and found behavior of the low and high responsiveness alleles of all Ir loci.

In order to do this let me state explicitly three assumptions which have been justified in several reviews (see below):

- Each antigen-sensitive cell (B or t, see Fig. 83 and 84) synthesizes and expresses a unique receptor identical in specificity to the antibody it would make upon induction. [In principle, the minimum requirement of allelic exclusion is that the cell become unipotential following induction (see footnote 8 in Cohn, *Ann. N.Y. Acad. Sci.,* **190**, 529, 1971; Cohn, *Cell. Immunol.,* **5**, 1, 1972)].
- Each antigen-sensitive cell (B or t) can be either paralyzed or induced.
- Antigen-sensitive cells of anti-self and anti-nonself specificities are generated continuously.

The logic of the system leads then to a model of the self-nonself discrimination the principles (not the detailed mechanism) of which go back to the first Brook Lodge symposium on immunological tolerance and which Bretscher and I have developed in detail in several papers (Bretscher and Cohn, *Nature,* **220**, 444, 1968; Cohn, "Control Processes in Multicellular Organisms," pg. 255, Churchill, London, 1970; Bretscher and Cohn, *Science,* **169**, 1042, 1970; Cohn, *Ann. N.Y. Acad. Sci.,* **190**, 529, 1971; Cohn, *Cell. Immunol.,* **5**, 1, 1972; Bretscher, *Transplantation Rev.,* **11**, 218, 1972; Bretscher, *Cell. Immunol.,* **6**, 1, 1973).

The key points for the minimum associative recognition model (Fig. 82) are:

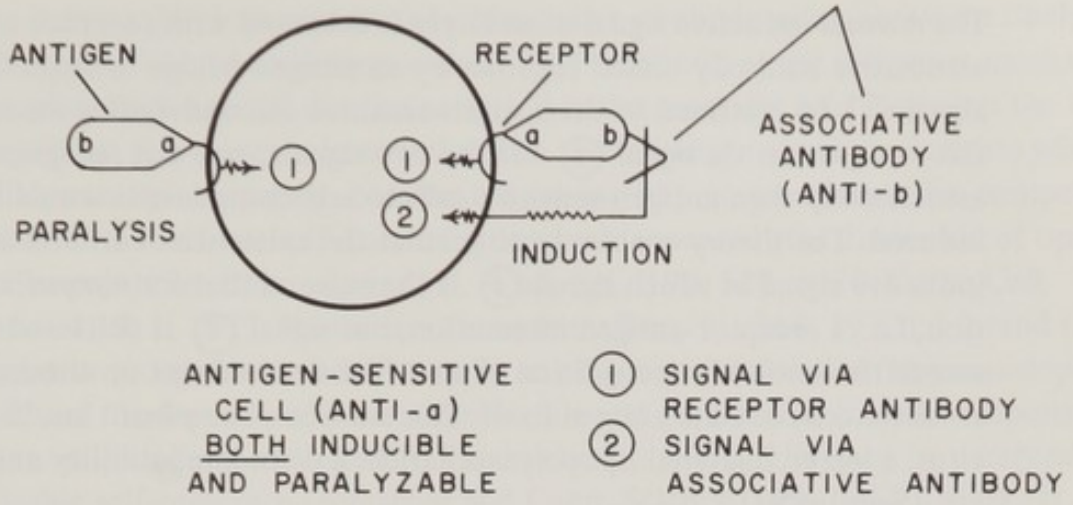

Fig. 82. A minimum associative recognition model of *normal* induction and paralysis.

– The paralytic interaction involves the binding of the antigen to the receptor on the antigen-sensitive cell with a resultant signal (1) to that cell.
– The *normal* inductive interaction has an obligatory requirement for the recognition of at least two determinants on an antigen, one by the receptor on the antigen-sensitive cell and the other by associative antibody via which another signal (2) is delivered to the cell, i.e., signals (1) plus (2) equal an inductive signal. We call this associative recognition.

The term *associative antibody* has been introduced to clarify the predicted confusion being engendered by the term, carrier-antibody, which we had coined previously and were so roundly cudgelled for doing so by Sela, four years ago at the Brook Lodge Conference on Immunological Tolerance.

This minimum model has three important elements:

– The paralytic signal, (1) , is included in the inductive signal, (1) plus (2) . This means that no cell can be induced which could not be paralyzed.
– The paralytic signal (1) passes via the receptor on the antigen-sensitive cell as a consequence of a conformational change upon interacting with antigen (Section V A 4 d). Signal (2) passes via associative antibody to the antigen sensitive cell as the consequence of its recognition of antigen. Signal (2) itself is *not* chemically identical to associative antibody. (I am trying to clarify a misconception concerning our model introduced recently by several groups of workers, e.g., Dutton *et al., Progress in Immunology*, **I**, 355, Academic Press, New York, 1971; Feldmann, *J. Exp. Med.*, **136**, 737, 1971; Anderson, Sjöberg, and Möller, *Transplant. Rev.*, **11**, 131, 1972.) Braun (Session I) has given us some provocative ideas on what the signals might be.

– The *normal* inductive signal most likely is delivered with receptor and associative antibody linked together by an antigen-bridge in order that signal (2) be confined to the antigen-sensitive cell undergoing associative recognition via signal (1) . If the two signals were not recognized associatively then antigen-sensitive cells to self-components would be induced. The theory enables us to predict the existence of an *abnormal* inductive signal in which signal (1) is the same as that for *normal* induction, i.e., a receptor-antigen interaction, but signal (2) is delivered via associative antibody recognition of a surface determinant on the antigen-sensitive cell, not the antigen itself. This surface determinant could be a virus, a lectin, bacterial lipopolysaccharide, a histocompatibility antigen, etc. (Section VI).

A. t Cells Regulate Induction Versus Paralysis Solely

The role of associative antibody is to regulate the self-nonself distinction. Since Bretscher and I (above references) have spelled this out several times I will not justify the argument. However, later (Section VI F) when I discuss allophenic (tetraparental) mice I will have to return to this problem.

The decision as to whether an antigen is self (S) or foreign (F) depends upon the past history of the animal with respect to that antigen. For example, if a self antigen were present when the immune system arose during ontogeny, and remained continuously present, then the animal would *maintain* tolerance to it. An antigen which was not continuously present but appeared late, for example, when the animal was adult would be treated as foreign.

The associative recognition model accounts for the self-nonself discrimination in the following way:

The time as well as the rate of expression of the immune system must be optimally determined during ontogeny. Antigen-sensitive cells anti-S which arise, are paralyzed by interaction with self components because the level of anti-S associative antibody is too low. The probability of having a large enough number of anti-S t cells at any moment is very low. Anti-F t and B antigen-sensitive cells accumulate in the absence of a foreign antigen (F). As long as S is continuously present, the animal maintains tolerance because paralysis of anti-S antigen-sensitive cells results from an interaction via the receptor with antigen when there is an insufficiency of associative antibody. Once the level of associative antibody is high enough, e.g., in an adult confronted with a foreign antigen, establishing of tolerance (paralysis experimentally) as well as induction has a special property when compared with maintenance of tolerance. The *maintenance of tolerance is independent of antigen-concentration whereas the establishing of tolerance is dependent on antigen concentration (The Mitchison Phenomenon).* This is a property predictable from the model.

I stress that I am *not* treating associative antibody as an accessory "helper" or "trapping" device superimposed on the "true" and as yet *unknown* mechanism of the self-nonself discrimination. It is the generally held view that the t cell "helps" but plays no *fundamental* role in the induction-paralysis decision which needs meaningful scrutiny, because it explains why no comprehensive competing models of the self-nonself discrimination have appeared (for discussion of opposing viewpoints see Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1970; Cohn, *Cell. Immunol.*, **5**, 1, 1972; Bretscher, *Transplant. Rev.*, **11**, 218, 1972). I intend the associative recognition model to apply to all antigens (so-called "thymus-dependent" and "independent") and all inducible and paralyzable immune responses ("humoral" or "cell-mediated"). Further both t and B cells must be paralyzable for stable self-tolerance (Bretscher and Cohn, Science, **169**, 1042, 1970). (It amazes me that Miller (Australian Academy of Science First Burnet Lecture, **2**, 2, 1972), could conclude, "It may in fact turn out that tolerance to self-components is a property confined exclusively to the t cell population, tolerance in B cells being merely a laboratory artifice." I wonder what theory of the self-nonself discrimination Miller had in mind that lead him to this conjecture.)

In this discussion I wish to make a rather extended extrapolation. It is that the regulation of the self-nonself distinction is the *sole* role of associative antibody. In terms of the model, associative antibody could come from any cell-lineage although it would probably be distinct from the one making effector antibody. Further, associative antibody could act either free or bound to the unipotential cell which makes it, or to a third party cell as cytophilic antibody. However, we have argued for many reasons (see above references) that:

- Associative antibody is thymus-derived.
- It acts as cytophilic antibody on a third-party cell.
- It is short-lived, compared to other immunoglobulin classes, in the absence of the antigen-sensitive cell making it; thus minimizing autoimmune accidents.

Consequently, associative antibody is predictably a *special* protein distinguishable from any of the known immunoglobulin classes (Fig. 83).

One of the several reasons for the precise nonminimal model (Fig. 83) is illustrative of a general problem. If we were dealing with an interaction between two antigen-sensitive cells, B and t, then the B cell would be induced by signal ② coming from the t cell but the t cell would be paralyzed because the B cell cannot deliver signal ② . There is an *asymmetry in their relationship.*

Several solutions to this contradiction are possible, e.g., the t cell upon induction might become a unispecific effector end cell (noninducible and nonparalyzable). However my favored solution is that this end cell secretes associative antibody which acts cytophilically on a third party induction effector cell (Fig. 83).

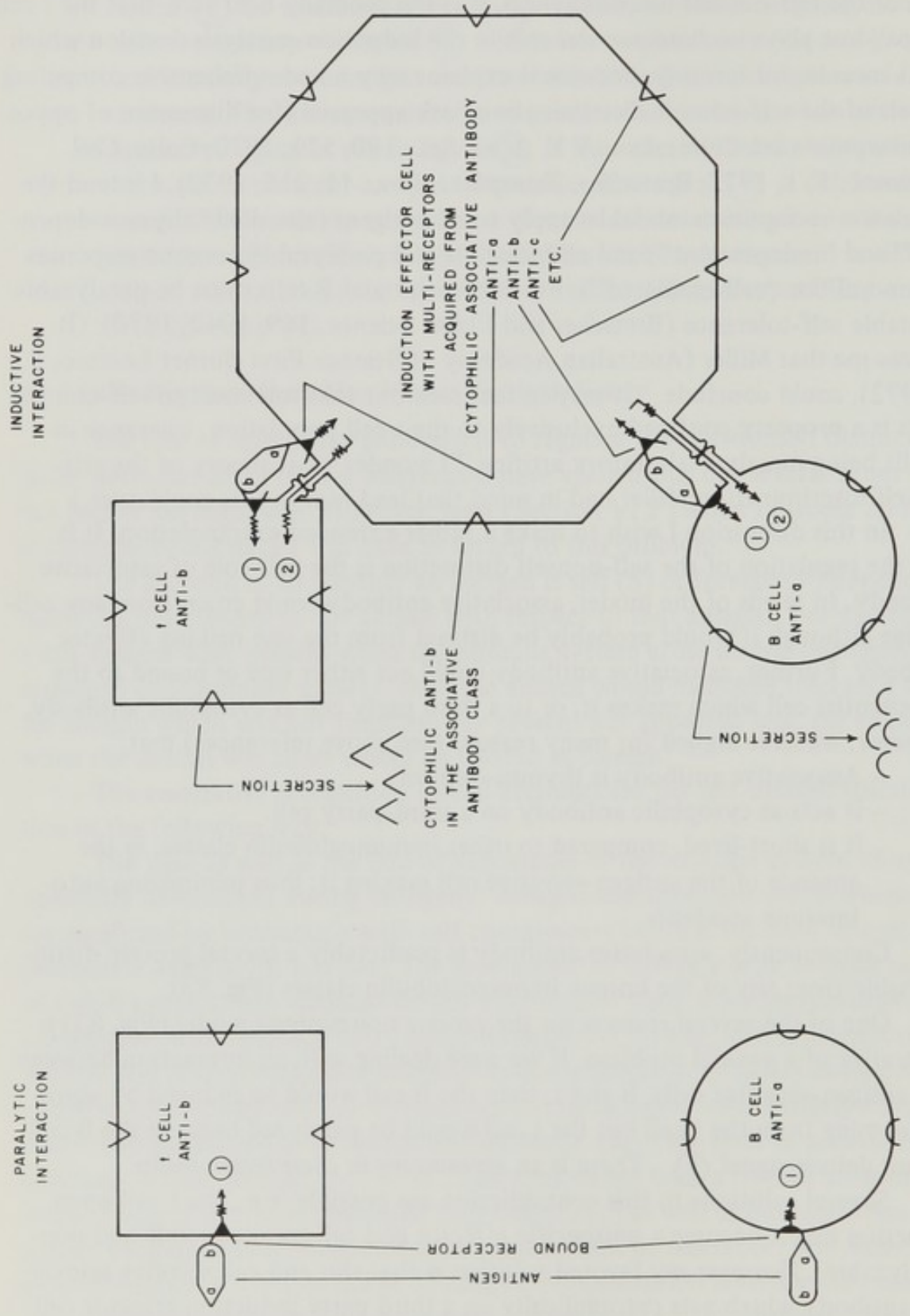

Fig. 83. A precise (not minimal) associative recognition model of *normal* induction and paralysis.

The first part of the generalization of this solution is that effector function would never be carried out by the antigen-sensitive cell itself, but rather always by secreted antibody, in some cases acting as cytophilic receptors for a third party effector cell. The only case for which this generalization would be challenged is that of so-called cell-mediated immunity. Most workers in the field postulate that the killer or effector cell is the thymus-derived antigen-sensitive t cell itself, which interacts directly with the target antigen via its synthesized receptor. According to the associative recognition theory (Figs. 82 and 83) such an interaction would tend toward paralysis of the antigen-sensitive cell. Thus the induction of a cell-mediated response would be counteracted by the paralyzing of the system when its effector mechanism functioned. Since this is unreasonable, the assumption that cell-mediated antibody acts cytophilically was introduced as a likely solution (see further discussion Cohn, "Immune Surveillance," pg. 377-379, Academic Press, New York, 1970; Cohn. *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971; Cohn, *Cell. Immunol.*, **5**, 1, 1972; Section III C 1).

B. B Cells Mediate All Effector Functions

The second part of the generalization I have already referred to by arguing that associative antibody must be of a special class distinct from all effector antibody (cell-mediated included). Since the function of associative antibody is to regulate the self-nonself discrimination, its level must be minimized in order to avoid autoimmune accidents (due to contact with antigens crossreacting with self) whereas that of effector-antibody must be maximized to cope optimally with pathogens. This requirement for independent regulation suggests that the cells expressing associative antibody and those expressing cell-mediated antibody would be of different lineages. The ontogeny of the lineages (one mediating associative recognition and the others effector functions) is not critical to the theory. However, by analogy with all other effector antibody, a bold hypothesis would be that the sole role of the t cell would be associative recognition mediated via associative antibody (coded in part by Ir-V_H^T) and the sole role of B cell would be effector function mediated via the various classes of immunoglobulin (coded in part by Ir-V_H^B). In other words in the case of cell-mediated immunity the t cell would be the controlling element in the induction-paralysis decision but would play no role in the eliciting or expression of the already induced effector function. There are three experimental arguments favoring this extrapolation.

Antibody found in the serum will coat target cells (erythrocytes, fibroblasts, tumor cells, etc.) and activate unimmunized spleen cells (effector cells) to recognize the target and effect a cytotoxic reaction measured by the chromium release assay (Möller, *In* "Immune Surveillance," pg. 96-101, Academic Press, New York, 1970; McLennan, Loewi, and Harding, *Immunology*, **18**, 397, 1970;

Perlmann and Perlmann, *Cell. Immunol.* **1**, 300, 1970). Furthermore it is known that B cells of donor origin participate in the GVH spleen enlargement assay (Cerottini, Nordin, and Brunner, *J. Exp. Med.*, **134**, 553, 1971). These experiments permit the argument that some class of serum antibody can arm effector cells. The present claim is that the "killer" effector cell is a macrophage (Evans and Alexander, *Nature,* **236**, 168, 1972; Lowmann-Matthes, Schipper, and Fischer, *Eur. J. Immunol.,* **2**, 45, 1972; Dennert and Lennox, *Nature,* **238**, 114, 1972). There are arguments against it also (Wagner, Feldmann, Boyle, and Schrader, *J. Exp. Med.,* **136**, 33, 1972). An antiidiotypic serum directed against a given serum anti-H-2 specificity will block an MLC in which recognition via that given anti-H-2 specificity would be required to effect function (Ramseier and Lindenmann, *Eur. J. Immunol.,* **2**, 109, 1972). This experiment says that serum anti-H-2 has the same idiotype as the effector antibody in the MLC. In a formal sense neither of these classes of experiments rules out the thymic origin of a special class of serum cell-mediated antibody. They show however that certain so-called cell-mediated assays are effected by cytophilic serum antibody. A properly absorbed, hence highly specific anti-θ serum will not block the cell-mediated effector function assayed by the cytotoxic chromium release assay (Dennert and Lennox, *Nature,* **238**, 114, 1972; Dennert and Lennox, manuscript in preparation). Further a t cell population can be induced to high levels of cooperating activity and yet show no cytotoxic activity (Dennert and Tucker, *J. Exp. Med.,* **136**, 656, 1972). These experiments not only separate associative recognition and cell-mediated activity, but they make unlikely a t cell effector function in cell-mediated immunity.

For these above cited experiments to map in a formal way cell-mediated immunity as a B cell effector function, identification of the immunoglobulin class and the direct demonstration that it is a B cell product would be required (for a discussion of the limitations of experiments implicating t cells in cell-mediated effector function, see Dennert and Lennox, manuscript in preparation).

I have turned to the analysis of this question and pushed the arguments to the limit because cell-mediated assays are being used to characterize the *Ir-1* product. I do not doubt that *the Ir–1 locus is expressed as the t cell receptor involved in associative recognition for all inducible and paralyzable functions* (cell-mediated included). What I am throwing into doubt is that t cells recognizing antigen via the *Ir-1* coded receptor (associative antibody) are responsible for the cell-mediated effector function. Therefore, any assay of the cell-mediated category must distinguish whether the measurement is of induction (a t cell function) or effector activity (quite possibly a B cell function). In principle then, assays of the induction of cell-mediated reactivity are neither more nor less direct measurements of the t cell associative recognition function mediated via the *Ir-1* coded receptor than assays of the induction of serum antibody.

C. Comments on t-and B-Cell Function

Before discussing associative antibody, there are several comments to be made concerning the postulate that the t cell lineage is responsible uniquely for the self-nonself discrimination mediated via associative antibody whereas the B cell lineage is responsible uniquely for effector antibody which mediates all of the protective or defense functions of the immune system.

1. THE t CELL IS NOT AN EFFECTOR CELL

Thanks to the imagination of the editors, R. Smith and M. Landy, the jacket of "Immune Surveillance, Academic Press, New York, (1970), has the model for cell-mediated killing proposed by Möller, Fig. 20, pg. 97. If I may extract the essence of his idea, it was that a certain class of antibody made by B cells could cytophilically interact with t cells and render the latter capable of acting as cytotoxic effector cells. I pointed out earlier that Möller had shown that serum antibody could arm effector cells in lymphoid tissues to be cytotoxic for a target cell. It is not clear to me today why the essential points of Möller's idea were lost. First, Möller's model made the effector arm of cell-mediated killing a B cell function (see for discussion, Cohn, *N.Y. Acad. Sci.* **190**, 529, 1971). Second, it made the t cell itself an effector cell deriving its receptors for killing from cytophilic effector antibody made by B cells. This raised immediately the key question as to whether t cells were antigen-sensitive cells at all, i.e., did they express receptors which they themselves synthesized. The only expectation of the associative recognition model is that associative and effector antibodies would be expressed by different cell-lineages. Therefore, arguments that t cells are not simply effector cells, but rather antigen-sensitive cells expressing their own synthesized receptors are needed, all the more so since we will come to the controversy as to what the chemical nature of the t cell receptor is.

The best argument is that t cells are specifically paralyzable. Therefore, even if they were also passively armed "killer" cells, they are antigen-sensitive cells which express a limited number (probably one) of receptors, i.e., the expression of specificity is clonally distributed (Section V B 1).

There are other suggestive bits of direct evidence. These consist in part of experiments showing 1) specific antigen-dependent suicide of cooperating function (Basten, Miller, Warner, and Pye, *Nature,* **231**, 104, 1971); 2) induction of cooperating but not cell-mediated activity in thymus-cell suspensions transferred to irradiated hosts (presumably without B cell function) (Dennert and Tucker, *J. Exp. Med.*, **136**, 656, 1972); and 3) the adherent cell (referred to in the literature loosely as "macrophage") not the t cell to be the cell-mediated killer effector cell (Dennert and Lennox, manuscript in preparation). The strong conclusion is that the t cell acting via its synthesized receptors is *not* the effector cell for cell-mediated reactions. The weaker conclusion (based largely on other

studies) is that the t cell is not the effector cell for associative recognition; one of the better arguments being that induction (not effecting) of cooperating function in t cells is radiation sensitive (discussed with Fig. 84). I have assumed therefore that t cells are uniquely the antigen-sensitive cells expressing associative antibody that have been postulated in making the associative recognition model.

The nonnegotiable part of my argument is that the t cell as such (defined as the antigen-sensitive cell functioning in associative recognition) is not the effector cell functioning in cell-mediated immunity. The negotiable part of my argument is that this latter cell could be another lineage of thymus-derived antigen-sensitive cell which upon induction differentiates to become a noninducible and nonparalyzable end cell. If this end cell were the killer effector cell, its receptor could be of any immunoglobulin class. If this end cell secreted cytophilic antibody which armed killer effector cells then a class of immunoglobulin unique in the constant region of its heavy chain would have to be envisaged. This new heavy chain class could be coded at the histocompatibility or allotype linked loci (or elsewhere). My extrapolation from what facts we have is that cell-mediated reactivity is a B cell derived effector function.

I am not convinced of the morphological identification of either the induction or killer effector cells. The two types of effector cell are claimed to be in the adherent population. The induction effector cell is constantly referred to as a macrophage. While for certain antigens, like SRBC, phagocytic breakdown to immunogenic fragments might enhance responsiveness, this is secondary to the induction mechanism *per se*. In fact we should be cautious not to assume that either the induction or killer effector cells act via phagocytic activity as a primary mechanism. Candidates for the former are the reticulum or dendritic cells, and for the latter, the macrophage or a lymphocyte-like cell. I remind you that the killer effector cell could be the secretor of the many pharmacologically active mediators and cytotoxic agents that t cells themselves are normally accused of making.

2. PARALYSIS RESULTS FROM INACTIVATION OF ANTIGEN-SENSITIVE CELLS

A very tricky and illustrative methodological problem is raised by this discussion of the role of the t cell. We are trying to understand the regulatory mechanism for the self-nonself discrimination. In the laboratory, we study induction and paralysis and we make it an act of faith that experiments on the genetic and nongenetic factors regulating responsiveness to experimental antigens will tell us by extrapolation how the self-nonself discrimination is made. The associative recognition model is an example of one such extrapolation. Paralysis is a "negative" phenomenon. Operationally, paralysis is a specific unresponsive state resulting from prior contact with antigen. It is assayed by trying to induce. Specific unresponsiveness resulting from the inactivation of antigen-sensitive cells could have two origins, one operating indirectly via the induced effector

antibody and the other (illustrated in Figs. 82 and 83) directly via the receptor on the antigen-sensitive cell.

I have been arguing that associative antibody cannot function normally as an effector antibody of any kind such as that which mediates complement-lysis or cell-mediated killing. Incomplete competing models have been proposed by Azar and Good (*J. Immunol.* **106**, 241, 1971), and by Kreth and Williamson (*Nature*, **234**, 454, 1971). Azar and Good postulate that paralysis is mediated by complement-lysis. This implies that induction of antibody synthesis must precede paralysis and lead to the formation of a complement-fixing antigen-antibody aggregate on the surface of the specific lymphocyte recognizing a determinant on that antigen via its receptor. The t cell would have no fundamental role in the induction-paralysis decision unless it is assumed that t cell derived associative antibody is the only and unique class mediating the postulated complement-lysis of antigen-sensitive cells. Kreth and Williamson taking their cue from Mitchison, *In* "Mediators of Cellular Immunity," pp. 77, Academic Press, New York, (1969), assign two functions to each t cell, associative recognition and cell-mediated killing, both mediated via the t cell receptor.

One of the best ways to test such proposals is to try and complete the argument: Suppose that the t cell derived, *Ir-1* coded associative antibody carried out a killer function in conjunction with either complement or the cell-mediated effector cell, and an associative recognition function in conjunction with the induction effector cell. During an immune response any antigen-sensitive cell (t or B) which had interacted with antigen would be induced, if by chance it contacted via antigen the associative antibody on the surface of the induction effector cell, and be killed if it contacted the same associative antibody on the surface of the cell-mediated effector cell or of complement. Normal induction would lead eventually not to memory, but to irreversible suppression because effector function would be more active than associative recognition function in the race between induction and killing. The difference in the regulation of effector versus associative antibody is a part of the normal induction mechanism for coping with pathogens (Section III B). The reason that proposals equating associative recognition and effector functions are unsatisfactory, is not just a question of whether they will turn out to be right or wrong. It is simply that they give us no insight into the mechanism of immune responsiveness that goes beyond a restatement of the facts themselves. This is why no attempt is made to analyze in which way the proposal permits the induction-paralysis and self-nonself discrimination to be made. For example, we are never told how the t cell interacting with an antigen-sensitive cell (via bound antigen or a cell membrane determinant) is signaled to induce or to kill. In fact, how cell-mediated immunity itself is induced, is left in limbo. If Kreth and Williamson had answered how the "induction-paralysis" decision was made as a consequence of their proposal, they might not have concluded that "killing (cell-mediated) and stimulation (induction) are two mani-

festations of the same fundamental process (inserts in parenthesis are mine)." (See Cohn, *Cell. Immunol.*, **5**, 1, 1972, for reasons why I insist on the ground rule that the induction-paralysis question be faced whenever such proposals are made.)

I have been stressing that associative and effector functions need independent regulation, thereby implying that they are mediated by different molecules produced by different cells. Consequently, I have assumed that paralysis is a central mechanism of cell inactivation which occurs independent of induction of effector antibody and is mediated via the interaction of antigen with the receptor. This is key to the self-nonself discrimination and what the associative recognition model was designed to deal with. However, the experiments are often difficult to interpret because there could exist abnormal experimental situations in which effector antibody via complement-lysis or cell-mediated killing might inactivate antigen-sensitive cells that have bound antigen, which in a sense is now recognized as a foreign surface determinant. This leads to specific suppression of the humoral response. The so called "suppressor T cells" about which there is so much flurry is a likely example of this. I need not develop what happens to the interpretation of this phenomenon if cell-mediated killing is a B cell function. Rather, I invite those who have been suggesting in a vague tantalizing way that a controlled induction of "suppressor T cells" and of "inducing T cells" provide the central regulatory mechanism for the normal self-nonself discrimination (Allison, *Lancet,* **II**, 1401, 1971; Allison, Denman, and Barnes, *Lancet,* **II**, 135, 1971; Katz and Benacerraf, *Adv. Immunol.*, **15**, 2, 1972.) to present us with a comprehensive competing general theory, not a restatement of the facts. My point is made all the more strong when our GURU (Jerne, *In* "Ontogeny of Acquired Immunity," pp. 5-7, Elsevier, North-Holland, 1972), stresses these suppressive effects as a major normal mechanism of regulation of "induction and tolerance" but fails to tell us how the decision between them is made. Until this is done I feel that *all of these suppressor phenomena are secondary* and it is sufficient to assume that the sole role of t cells is in associative recognition and that the *Ir-1* locus codes uniquely for associative antibody.

[In addition, there is a class of phenomena which might be called "specific tolerance" that does not involve inactivation, but on the contrary the induction of antigen-sensitive cells. This category of "tolerance" is assayed by the survival of a graft. Survival depends upon the production of so-called protective or enhancing antibody which blocks the cell-mediated rejection mechanism. This is simply another example of a "tolerance" phenomenon mediated via effector antibody, and for the reasons discussed above I will not deal with it. Although these enhancing or facilitating effector mechanisms are very important both practically and theoretically (see "Immunologic Intervention," Academic Press, New York, 1971), they are but a secondary consideration for an understanding of the expression of Ir loci (see Section VI F)].

3. t AND B CELL BEHAVIOR IN BIOZZI MICE

There is one experiment which Biozzi described that particularly pleased me (Session V). It was the demonstration that generally low responder mice were more resistant to a transplantable tumor than the high responder mice. This is a confirmation of the prediction which came out of the Brook Lodge Conference on Immune Surveillance, in 1970, that elimination of the humoral response would permit a more effective cell-mediated rejection of a neoplastic cell which had escaped the immune system. While I see this observation as an example of the power of genetic methodology in dissecting a complex effector function, immune surveillance, it is also clear to me that this "fact" will be misleading. The interpretation placed on it is that the low responder has a generally defective B cell system but a normal t cell system. Therefore, the high responder protects the tumor by enhancement, whereas the low responder is unable to do this, so that its normal cell-mediated effector mechanism, postulated to be a t cell function, can operate efficiently. This finding then, is taken as selfevident, since it is generally believed that the cell-mediated effector mechanism is a t cell function.

If for the reasons discussed, the cell-mediated effector mechanism is considered to be a function of B cell derived antibody, then tumor rejection in Biozzi mice reveals another relationship. Assuming for purposes of illustration that the t cell associative recognition function is identical in the Biozzi low and high responder mice, then a nonspecific lowering of B cell function could reveal itself as decreased responsiveness by assays of serum antibody, with no apparent loss of cell-mediated effector function. The reason is that Biozzi's serum antibody assays are quantitative while the cell-mediated assays are qualitative, i.e., above a certain threshold of activity the tumor is rejected, below it nothing is observed.

Two reasons for this are envisaged immediately. First, while the rate of synthesis of cell-mediated antibody might be decreased, the feedback inhibition of its synthesis by serum antibody is also diminished. Consequently the effective steady-state level of cell-mediated reactivity might not be very much affected. Second, in the balance between enhancement and killing, not only is the ratio of the two important, but also the absolute levels of each. Given a ratio and a level in which enhancement is observed (the high responder), if the ratio were kept constant, but the levels of enhancing and cell-mediated killing antibody were reduced equally, then killing would be favored. Since cell-mediated killing probably is carried out via multi-point interactions (ladder formation) between the killer and target cell whereas enhancement is not, the effectiveness of enhancement will fall off more rapidly than cell-mediated killing, as the level of both activities is proportionately reduced. In other words if only 1-10% of the determinants on the target cell were unblocked, the killer cell may still function effectively (see Section IV B for more detailed argument).

The Biozzi low and high responder mice have many gene differences between them. Since responsiveness to SRBC is linked to the heavy chain allotype, one of these differences is due to the Ir-V_H^B locus. However, there is in reality no evidence that the t cell associative recognition activities of low and high responders are the same, i.e., that they do not also differ in their *Ir–1* loci. The phenotype, general low effector antibody response, could be due to either locus alone (or for that matter to many other loci). As Herzenberg wisely remarked (Session V) it would be very informative to put each of the low and high responder Ir-V_H^B loci and Ir-V_H^T loci into a congenic background. Since it is predictable that a deletion of germ-line V genes (V_H^B, V_H^T, V_K), or an unbalanced family of them resulting from unequal crossover events could result in *general* unresponsiveness by assays of serum antibody, the Biozzi mice hopefully might permit us to analyze the origin of diversity via genetics. (See exemplary studies in rabbits by Mage, *Ann. N. Y. Acad. Sci.*, **190**, 203, 1971; Chou, Cinader, and Dubiski, *Eur. J. Imm.*, **2**, 391, 1972.)

IV. THE t CELL RECEPTOR IS AN IgM-LIKE IMMUNOGLOBULIN

Now let us return to what is known about the immunoglobulin nature of associative antibody by considering first, the positive claims concerning it. I will deal with the contradictory findings later. (Section IV B.)

A. Pro

All of the above (as well as some additional), predicted properties of associative antibody appear to have been found by Feldmann and his coworkers (Feldmann and Basten, *Nature*, **237**, 13, 1972; Feldmann, *J. Exp. Med.*, 1972, in press; Feldmann, Cone, and Marchalonis, *J. Exp. Med.*, 1972, in press.) The finding is that associative antibody is secreted by induced thymus-derived cells, acts cytophilically on adherent cells (induction effector cells) from spleen or peritoneum, is an immunoglobulin molecule of molecular weight 180,000, carrying determinants crossreacting with effector IgM made by B cells. This agrees with the studies of Hammerling and Rajewsky (*Eur. J. Immunol.*, **1**, 447, 1971), and of Marchalonis and coworkers (reviewed in Marchalonis, Cone, Atwell, and Rolley, "Biochemistry of Gene Expression in Higher Organisms," Lee and Pollak, eds, Australia and New Zealand Book Company, Sydney, 1972, in press) and of Warner (Session IV) that thymus-derived cells have on their surface a molecule related to IgM immunologically. The studies of Lesley, Kettman, and Dutton [*J. Exp. Med.*, **134**, 618, 1971 (See Table II)], indicate that associative antibody carries kappa subunit determinants. This is the state of the evidence, which in the absence of a controversy as to the facts would be considered rather strong, that the *Ir-1* locus codes for an IgM-like heavy chain participating uniquely as one of the subunits of associative antibody, the other being kappa or lambda.

B. Con

The above and other experiments upon which the arguments have been based that the t cell receptor is an immunoglobulin, were hotly disputed here. The counter arguments are two-fold:

- A search for membrane-bound immunoglobulin on thymus cells and derivatives of them by biochemical methods reported to be successful, has been negative.
- When assays, other than cooperation, presumed to measure the induced function of thymus-derived cells are used, no (or conflicting) evidence can be obtained that an immunoglobulin receptor is involved.

Uhr and Unanue (Session IV), describing terrifyingly negative experiments, cannot confirm the findings of Dutton, Feldmann, Hammerling, Marchalonis, Rajewsky, and Warner. Thymus cells or thymus-derived cells (bearing θ surface markers) do not seem to possess the claimed kappa bearing IgM-like receptors. My guess would be that this difference will depend on small but critical experimental details which will become clear when each group repeats in a cookbook way, the work of the other. Uhr's demonstration that a $\geqslant 90\%$ destruction of θ bearing cells with anti-θ and complement did not have any detectable effect on the synthesis of immunoglobulin by a cell suspension from the thymus, suggests that the differences between the two groups lies in the isolation procedures for membrane bound t cell immunoglobulin. Clearly, for the moment, Uhr and Unanue are rocking the boat but are not capsizing it. No negative experiment could do that.

Simonsen came forth with another level of argument concerning "the elusive t cell receptor. He and his coworkers (Crone, Koch, and Simonsen, *Transplant. Rev.*, **10**, 36, 1972), review their work as well as that of others. If the receptor on thymus-derived cells is studied by cell-mediated assays, e.g., graft versus host, mixed lymphocyte blast transformation, cytotoxic effects on target cells, or macrophage migration inhibition, no (or conflicting) evidence can be obtained that immunoglobulin is involved.

Simonsen has pointed out why these negative experiments using cell-mediated assays for the function of thymus-derived cells might be misleading and concludes finally that the only convincing argument will be the isolation and chemical characterization of the postulated nonimmunoglobulin receptor on thymus-derived cells. I agree with him but this only returns us to the previous controversy.

It is not fair to leave these extensive negative and conflicting studies at that. The main argument is that antiimmunoglobulin sera of many kinds do not inhibit (or inhibit erratically) induction of cell-mediated reactivity. In most studies of this kind the distinction between the induction and effector mechanisms is not made. However, it could be argued reasonably that the blocking of either mecha-

nism by antiimmunoglobulin sera should lead to an observable effect on the final assay. Three factors may be at play although not equally in all assay systems used. First, while the sensitivity of the cell-mediated assays is generally very high, the quantitation is generally very poor. In most cases it is not known how much of a decrease in the number of induced antigen-sensitive cells, nor for that matter in the level of effector antibody, would be detected. What is sometimes known is the relationship between the input population being assayed and the level (not rate) of the final response (induction plus effector function). This means that an antiserum inhibiting by a factor of 2-10-fold the *rate* of induction or effector function might not be seen in the *level* of the final assay made at a late time. This does not explain all of the negative results. The second factor is how well an antiimmunoglobulin molecule could block cell-cell interactions. The binding constant, K_{diss}, for an antiprotein-protein interaction would be of the order of 10^{-6} M. The conditions of most experiments would leave on an average 1 to 10% of the cell-associated receptors free. If these temporarily free receptors react with target determinants (for cooperation or killing) the cells might then zip themselves together irreversibly, first via specific receptor interactions, then via secondary "nonspecific" interactions which make the bound receptors unavailable to inhibition by antisera. What would be seen in a careful experiment is initial inhibition by antiserum of the rate of the response, followed by a gradual escape from inhibition. If a late time point only is examined the antiserum (or inhibitor) would seem to have no effect on the final result. An illustrative example of this escape phenomenon is provided by Faarnes, Choi and Good (*J. Exp. Med.*, **137**, 171, 1973) who interpret their findings as "antigenic modulation [?]." Third, antiimmunoglobulin sera might have some enhancing activity which masks inhibitory effects. For example, even across species, cytophilic antibody might arm effector cells which recognize a target that has bound antibody.

The experiments of Crone *et al.*, (*Transplant. Rev.*, **10**, 36, 1972), are a model for the arguments that the t cell receptor is *not* an immunoglobulin. The experimental system is a GVH reaction assayed by the enlargement of the spleen of a chicken embryo following injection of adult lymphoid cells of another histocompatibility type. The two major histocompatibility alleles of the chicken are B1 and B2. The key result is that rabbit antilight chain sera do not block the *in vivo* GVH reaction whereas chicken anti-B1 or anti-B2 sera made by cross-immunizing B1/1 and B2/2 birds, do block the GVH reaction when these antisera are directed against the donor adult cells. The failure of rabbit antilight chain sera to block an *in vivo* GVH test could have many origins, some of which I discussed above and others of which Crone *et al.*, mention. I regret that they did not use *in vitro* complement-lysis rather than blocking to test the effect of antilight chain sera on the GVH reactivity of donor cells. In any case, this is a negative finding not made more meaningful by the fact that a GVH reaction can be blocked by anti-B1 or anti-B2 sera.

The interpretation of this important latter finding depends upon the specificities of the inhibiting antibody in the anti-B sera. There could be antibody directed against the idiotype of the t and B cell receptors, as well as against any other polymorphic surface determinants. The inhibition by antiidiotype could act at the level of induction (t cell) or of effector-function (B cell?) by blocking associative and/or effector antibody. An antiidiotype serum might block a GVH reaction whereas an antilight chain serum might not, because the former would react with the variable region of the light-heavy combination, thus blocking the combining site, whereas the latter directed largely against the constant region of the light chain only, would not block interactions with antigen. Further, as Crone *et al.*, point out, the chicken embryo is producing immunoglobulin with light chain determinants that neutralize the antilight chain serum.

We should note that in every case antisera raised by isoimmunization have been effective in blocking a cellular response to antigen (see examples Green, Session I; Section V B 1; Ramseier and Lindenmann, *Transplant. Rev.*, **10**, 57, 1972, Section III B). It is only in the case studied by Ramseier and Lindenmann that the antisera are known to contain antiidiotypic activity directed against antihistocompatibility immunoglobulin probably of B cell origin. In the case of Green (Session I; Section V B 1) and of Crone *et al.*, I surmise from indirect arguments that the activity of their antisera is due also to antiidiotypic and/or antiallotypic specificities.

Simonsen's velvet suggestion hiding the mailed conclusion is that there are special "archaic or primitive" receptors on t cells responsible uniquely for recognition of major histocompatibility determinants. This seems hopelessly out of date, particularly if the B locus in chickens is analogous to the H-2 or HL-A loci (Section V C). The suggestion means that each allele must produce a product which recognizes the product of all other alleles, but not itself. This formulation ignores 1) the fact that t cell receptors recognize with a high degree of specificity a wide range of nonhistocompatibility determinants (Section V B); 2) the different selective pressures during evolution acting on a histocompatibility antigen postulated to act as a receptor, and a receptor postulated to act as a histocompatibility antigen (Section V C); 3) Bodmer's criticism of the Jerne theory applicable in this case also, namely that it is unrealistic to require strict parallel evolution at the population level of genes coding rare determinants on histocompatibility antigens, and genes coding recognition of them (Bodmer, *Nature*, **237**, 139, 1972); 4) the requirement for specific tolerance and induction, i.e., clonal selection, of histocompatibility antigens best seen, for example, if they were receptors of t cells of hybrids; 5) the total behavior (associative recognition as well as effector function) of the immune system in an attempt to solve one observation, the immune recognition of major histocompatibility determinants.

We must clarify in our discussions how we view the relationship between the major histocompatibility genes, e.g., K and D, in the mouse and Four and LA

in man, and the t cell receptor genes *Ir-1*, or else we will face confusion. If the "archaic or primitive" t cell receptors under discussion are postulated to be the major histocompatibility antigens only, e.g., K and D in the mouse, distinct from the *Ir-1* locus, then we are posing a special problem in cell-mediated effector function unrelated to the expression of *Ir-1* coded associative antibody. If the K and D genes are assumed to be part of the *Ir-1* locus (which must then code for nonimmunoglobulin receptors), we are led into an unnecessarily complicated formulation because the K and D genes are expressed in all cells unlike the *Ir-1* coded receptors which are expressed in t cells only, as functioning associative antibody. It seems to me that proponents of the hypotheses that 1) the major histocompatibility antigens expressed for all cells are also "archaic" receptors on t cells specific for their own allelic products and 2) cell-mediated killing and associative recognition are effector functions of the antigen-sensitive t cell, should treat us to a formally argued case.

C. Other Views of the t Cell Receptor

How important is the almost universal agreement that the receptors on t cells are hard to find by the variety of technics successful with B cells? I cannot help feeling that the reason that negative experiments have carried so much weight at this Brook Lodge Conference and our previous one ("Immunologic Intervention," pg. 175-181, Academic Press, New York, 1971) is psychological. We have been caught napping. Nothing had led us to expect that receptors on thymus-derived antigen-sensitive cells would be so difficult to reveal, compared to those on bone marrow-derived antigen-sensitive cells, nor that they would be coded by different gene loci. Consequently, emboldened by surprise, many have started entertaining the remaining two guesses concerning the nature of the t cell receptor coded by *Ir-1* and involved in cooperation. These are either that associative antibody (t cell receptor) is a specific recognition molecule, which is not immunoglobulin, or that the *Ir-1* product is a surface component which modulates the specificity of an immunoglobulin receptor. These were suggestions 3) and 4) on my initial list.

Some of you, admittedly hedging, like the former because you feel that it relieves you of having to defend the position that the thymus-derived antigen-sensitive cell is multipotent. The silent argument is that if immunoglobulin is not involved in recognition, then new ground rules can apply, e.g., clonal selection need not operate (Benacerraf, Session I; Benacerraf and McDevitt, *Science,* **175**, 273, 1972). Some of you like the latter because you feel it provides a rationale for the linkage and mapping of *Ir–1* within the H-2 region (McDevitt *et al., J. Exp. Med.,* **135**, 1259, 1972). In fact, Benacerraf and McDevitt (*Science,* **175**, 273, 1972), have tried to generalize this, by implying that all Ir loci are linked to histocompatibility loci because of a fundamental functional relationship between the two types of loci requiring linkage for the regulation of their expression. I

termed these two suggestions' "guesses," the first because I am putting a personal evaluation on the likelihood of its being correct, and the second because it has not been developed as a clearly reasoned hypothesis.

In the discussion of McDevitt's analysis of *Ir-1* genetics (Session II) Herzenberg and Bodmer argued that the difference between the hypotheses that *Ir-1* codes an immunoglobulin or a nonimmunoglobulin t cell receptor is a semantic one. In part this is true and is the reason I have pointed out that the nature of the t cell receptor is not where the fundamental differences between us are to be seen. I, however, am explicit in distinguishing the two hypotheses, not because the distinction is large, but to stress the biochemical studies on the t cell receptor. An immunoglobulin is defined here as an antibody utilizing either kappa or lambda or the allotype-linked heavy chain as a subunit. I will deal later (Section V C) with Bodmer's suggestion concerning the possible evolution of the *Ir-1* product, upon which he bases the argument that the difference is semantic.

The assumption that *Ir-1* codes for a nonimmunoglobulin t cell receptor will plague us, in principle, as long as it is not isolated and characterized by a universally agreed upon procedure. I could leave this assumption at that, but as you will see when I discuss the physiological and specificity requirements of this receptor, as well as the evolution of immunoglobulin, that this assumption is premature in that it is totally equivalent to reinventing an immunoglobulin. However, it is a fun idea to consider, because it lets you see to what extent immunoglobulin structure can be accounted for on general principles. Does a large range of recognition potential require nonidentical variable region subunit interactions? Are "domains" separating recognition, e.g., Fab, from function, e.g., Fc, required? Is somatic selection on replacements in precise hypervariable regions required to generate diversity? Are the *Ir-1* germ-line genes selected for the same reasons as the known structural V genes, e.g., for given specificities of survival value?

The assumption that *Ir-1* codes for a modulating element acting on an immunoglobulin receptor is trickier. It requires that associative antibody either not act as a secreted or cytophilic molecule, or else that the modulating element be secreted and function complexed to it. If this were not so the specificity of the receptor on the induced t cell and the associative antibody acting on the induction effector cell would be different. Further, this model needs 1) some precision as to the mechanism of the modulation, since from what we know of protein structure it makes little sense and 2) a rationalization of the *inherited* specific *dominant* low-high response that does *not* reside in the receptor, but in the modulating element coded by *Ir-1*. Picture the receptor molecule or associative antibody as a trimer of L, H, and the modulating subunit. How is the difference between a high and a low responder determined by the germ-line gene coding the modulating element? Every time that an anti-(T,G)-A--L receptor appears on a t cell, is there a mechanism to express the corresponding *Ir-1* gene

which modulates the low-high response difference, or are all *Ir-1* genes expressed in every t cell; in which case how is specificity determined? These need thoughtful discussions which are certain to be profitable.

All this having been said "comme exercises de style" the most likely product of the *Ir-1* locus is a heavy chain, IgM-like, which complements with the known kappa and lambda subunits to result in an immunoglobulin receptor. This means that the germ-line V_H gene array expressed in B and in t cells will be from unlinked loci. How similar they will be depends on several factors which I will return to later (Section V B).

V. THREE PROPERTIES OF THE t CELL RECEPTOR

I pointed out that the major differences between us lay not in the nature of the *Ir-1* coded t cell receptor but in the interpretation of its role and expression. In order to face this problem I will argue now that this t cell receptor is expected to have three properties relevant to the discussions here:

- Its expression would be regulated to optimize the self-nonself discrimination.
- Its degree and range of specificity would be of the same order as the receptor found on B cells: the corollary being that the *Ir-1* locus show allelic exclusion.
- It must be on the surface, is expected to be highly polymorphic and therefore should be revealed by the general techniques used to analyze histocompatibility determinants.

A. Asymmetry in t-B Relationship

The first property leads us to consider the asymmetry in the relationship between associative and effector antibody. This will permit us to appreciate why mutations at the *Ir-1* locus and Ir-V_H^B locus are expressed differently and to predict how mutants at Ir-V_κ or Ir-V_λ might behave.

The basic assumption is that the mechanism of induction and paralysis of the expression of associative antibody will be the same as that for effector antibody. I illustrated that in Fig. 83. The asymmetry in the relationship between associative antibody made by thymus-derived cells and the other immune components is best illustrated by the following diagram (Fig. 84) which I have adapted from Bretscher's recent review (*Transplantation Reviews*, **11**, 218, 1972).

First, note the symmetry. Two antigen-sensitive cells are diagrammed, the B cell and the t cell. The B cell upon induction differentiates to become an immunoglobulin secreting plasmacyte, P. Since associative antibody is postulated to act cytophilically, then a secreting cell, T, must be the product of differentiation of the t cell. This T cell is pictured to be entirely analogous to the plasmacyte, P, resulting from B cell induction. The induction of B and t cells is known

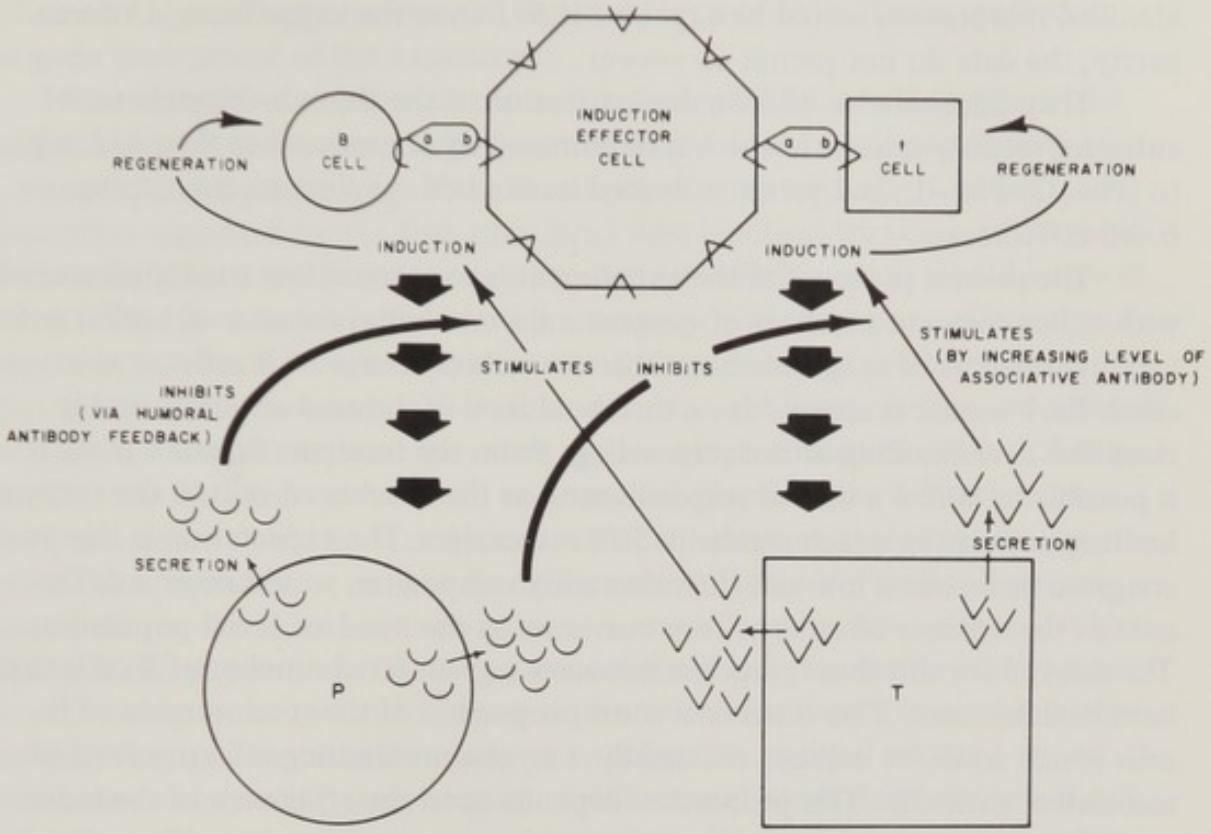

Fig. 84. The asymmetry in the relationship between B and t cells.

to be x-ray sensitive, whereas the function of P and T cells is x-ray resistant, as would be expected, since induction involves division, while secretion by the end cell does not. Both B and t cells require associative antibody for induction but *not* for paralysis.

The asymmetry arises because associative antibody obligatory for induction of both t and B is a product of the induced t cell. Consequently t cells are required for B cell induction but not *vice versa.* In Fig. 84 this is seen as an arrow labeled "stimulates" from T to both the induction of B and t cells. *No* corresponding arrow goes from P, the end cell of B differentiation. Instead an arrow marked "inhibits" is drawn which indicates the well-known feedback inhibition by effector antibody of induction.

Given this asymmetry we can now look at the predicted and experimental relationships between B and t cells in animals differing at *Ir-1* and non-*Ir-1* loci.

1. SHEARER'S EXPERIMENTS

The cellular studies were undertaken by Shearer to provide an independent test of the hypothesis drawn from the genetic data (Section II C.) that *Ir-1* genes are expressed in t cells only and Ir-V_H^B genes are expressed in B cells only. It might be possible to rule out that t and B cells have the potential to express

identical receptors as would be expected if *Ir-1* were the kappa locus. Unfortunately, the data do not permit an answer.

They do, however, allow a demonstration of the thymus-dependence of antigens, responsiveness to which is determined by Ir genes other than *Ir-1*, e.g., to (Phe, G)-Pro--L, and permit a limited number of conclusions concerning B cell differences.

The general protocol of the experiment is to reconstitute irradiated animals with either constant numbers of syngeneic thymus-cells (a source of t cells) and varying numbers of syngeneic bone-marrow cells (a source of B cells) or *vice versa.* Each mouse is assayed for a threshold level of induced antibody and is classified as responding or not responding. From the resultant titration curve it is possible to define a unit of responsiveness as the number of cells in the varying limiting population which results in 50% responders. The expectation is that two congenic mice, one a low and the other a high responder, would show a difference in the number of units of responsiveness in the t and/or B cell population. The assayed for threshold response measures a given fixed number of B cells that have been induced. This number is some proportion of the total number of B cells which could be induced maximally, i.e., at a nonlimiting effective level of associative antibody. This proportion depends upon the *efficiency* of the induction. Essentially the mouse is reconstructed by changing the ratios of t to B cells, such that the response is the same for the two strains. The problem is to interpret what the ratio means.

Critical for any interpretation is the proof that the invariant population is not limiting. This could be demonstrated by showing that an increase of 10-fold in the invariant population does not affect the titration curve of the variant population. In other words the contribution of the invariant population to the *efficiency* of induction must be experimentally set so as to be *the same* for the two strains. This I might add has not been unequivocally shown in the way the experiments were done.

Now let us consider two congenic strains, l(low) and h(high), showing a low-high responsiveness difference and for which we would like to determine whether the t and/or B cell populations are different with respect to either numbers of cells or affinity of the receptors.

Each t cell population, t_l and t_h, is held invariant at a *proven* nonlimiting level when t_l is titrated against B_l and t_h is titrated against B_h. If the titration curves of B_l and B_h are different, then there is strong evidence that these two populations are distinct, with respect to numbers of cells or affinity of receptors specific for the test antigen. The conclusion then is that one factor in the low-high responsiveness difference in the two congenic strains, l and h, is due to the B cell populations. Identical curves are only suggestive that the populations are identical, because large differences in affinity of B cell receptors could be masked by the use of polymeric antigens which make the receptor-antigen interaction of

sufficiently high affinity, so that the threshold level of induction is reached in the given time period of the experiment.

Now consider the case where each B cell population is held invariant at a nonlimiting level proven by titration against its respective t cell population, i.e., B_l versus t_l and B_h versus t_h. If the B cell populations were identical (an assumption suggested by the first titrations) then identical titration curves would be obtained if the t cell populations were identical. Such a result would be good evidence that the t and B populations are the same with respect to efficiency of induction, but the low-high responsiveness difference would not be explained. What is important, however, is that if two *identical* t cell populations are titrated, one against a low affinity and the other against a high affinity B cell population, two different titration curves could be generated. Further, the difference between the curves would be very dependent on antigen concentration. At the optimal concentration of antigen for induction, the low affinity B cell population would require more t cells to arrive at a unit of responsiveness than the high affinity B cell population. This is true even if the B cell population is shown to be nonlimiting at the optimal antigen concentration for its syngeneic t cell population. If antigen concentrations above or below the optimal are used the differences are only exaggerated, because of differential paralysis of the two B cell populations. The effect of antigen concentrations have not been studied and in any case would be difficult to control properly.

Two different t cell populations can be set at a nonlimiting level by titrating t_l versus B_l and t_h versus B_h and used to distinguish B_l from B_h, whereas the reverse is not true. This is a consequence of the asymmetry in their relationship. As long as associative antibody is not limiting the response in the reconstructed mouse, l or h, any B cell differences between them revealed by the titration are likely to be real, i.e., due to differences in numbers of cells or affinity of receptors for the test antigen. Essentially, at nonlimiting t cell levels, the level of induction will depend on B cells only. By contrast, if the two B cell populations, l and h, are set so as to be nonlimiting in the response of each reconstructed mouse strain, the two t cell populations could be identical, but titrate differently because the efficiency of induction would depend upon the differences in affinity of receptors, i.e., the efficiency of induction would be lower in the case of the B cell population with lower affinity receptors.

I recall that cross titrations t_l versus B_h and t_h versus B_l cannot be studied this way because the phenomenon of abnormal induction intervenes (Section VI). However, this same phenomenon illustrates in principle the way to analyze t and B cell responsiveness in congenic lines without the complications imposed by the efficiency of induction due to associative recognition. The level of the inductive signal (2) can be set so as to be nonlimiting and independent of the test antigen. I point this out as an aside, which will provide in the future the way to analyze t and B cell differences.

Although in principle Shearer's analysis could establish whether an *Ir-1* controlled responsiveness difference is expressed in B cells, his preliminary data (Session I, Figure 10, Tables 17, 18, and 19) are very complex. In part, this is due to the fact that the differences between the strains C57BL/6, C3H, SJL are multigenic and SJL in particular has a peculiarly "over reactive" cell-mediated system (East, *Prog. Exp. Tumor Res.*, **13**, 84, 1970; Herzenberg, Jacobson, Herzenberg, and Riblet, *Ann. N.Y. Acad. Sci.*, **190**, 212, 1971), which may distort responsiveness by a superimposed abnormal suppression mechanism. In part the assays have not been carried out after carefully demonstrating that the t or B cell contribution was not limiting. I might add that the use of a threshold assay for responsiveness would not be as meaningful as a quantitative one following serum levels. I am aware of the difficulties due to death of animals at very low bone-marrow restoration. However these are not the critical points in the curves. Further, it is not *a priori* obvious to me that a series of interactions as complex as these would permit a simple statistical analysis of the data and in fact the titrations do not follow a Poisson distribution of threshold values of responsiveness (Fig. 10; Tables 17, 18, 19). Lastly, in the case of *Ir-1* limited responsiveness, I am not certain that even a convincing demonstration of a difference in B cell level or affinity for the test antigen would permit us to conclude unambiguously that its origin was the expression of *Ir-1* in B cells. On the one hand, even in mice congenic except for the *Ir-1* locus, if the high responders had a level of B cell responsiveness equal to that of the low responders we could conclude that prior immunogenic selection by crossreacting antigens, present in the environment, had operated on both types of animals prior to the experiment to mask the postulated *Ir-1* coded difference in the B cells. (Incidentally, cryptic immunization explains in part why Ir polymorphisms at other than *Ir-1* loci are revealed so rarely even though they must occur as frequently.) On the other hand if the t cell level of responsiveness were mirrored by the B cell level we could conclude that the test antigen consisted by chance of determinants which are only, and always, found coupled so that high t cell levels would select for high B cell levels and low t cell levels would be accompanied by low B cell levels. Thus the *Ir-1* locus might not be expressed in B cells, yet give that impression for an unrelated reason.

Under what conditions might one see a specific dominant responsiveness difference at Ir loci other than *Ir-1?* Generally, it is the level of associative antibody which limits the response to an antigen. This is a consequence of its role in regulating the self-nonself discrimination and is another reason why *Ir-1* controlled differences are most often found. In order to search for non-*Ir-1* controlled differences, associative antibody must not be limiting the rate of induction of B cells specific for the test determinant. One general way to accomplish this would be to use a complex antigen, the total response to which may be the same in all strains. However, if the effector antibody response to different

determinants on that complex antigen is assayed, so that responsiveness in restricted B cell sub-populations are analyzed, strain differences, not *Ir-1* linked, may emerge. As one example, the response to (Phe,G)-Pro--L as described by Shearer (Session I) has the following characteristics:

- SJL mice appear to have a 5-fold larger number of B cells anti-Pro, L than anti-Phe,G when assayed in the presence of excess t cells, whereas DBA mice have exactly the reverse pattern for their B cells. This agrees with the finding that SJL mice are high responders to the Pro, L determinant and low responders to the Phe,G determinant whereas DBA is just the opposite.
- SJL t cells assayed in the presence of excess B cells show no difference in numbers whether humoral anti-Pro,L or anti-Phe,G are measured. The same is true of DBA t cells.

The failure to show differences in cooperating t cells specific for (Phe,G)-Pro--L may, but does not necessarily mean that the two strains SJL and DBA have an identical *Ir-1* gene product for recognition of this antigen. Even if one strain recognized only Phe,G and the other only Pro,L at the t cell level, no difference might appear in Shearer's assay because associative recognition of one determinant permits cooperation for all others. It is the total effective level of associative recognition which counts, and Shearer's assay cannot distinguish the two strains. The initial responsiveness differences of the two B cell populations to the given determinant, Phe,G or Pro,L, can be seen, because the effective level of B cells due to numbers and/or receptor affinities is limiting in the respective low responder strains.

These results indicate that under conditions where the t cell contribution is nonlimiting, the Shearer-assay for B cell differences is valid. Either the mouse is reconstituted experimentally to make the t cell contribution nonlimiting or one chooses a situation in which the t cell contribution is normally nonlimiting, e.g., responsiveness to (Phe, G)-Pro--L in mice or GAT in guinea pigs (Section V A 2).

2. THE BENACERRAF EXPERIMENTS

Benacerraf (Session I, Tables 2 and 10) stresses a rather startling finding for which he gives no precise interpretation. The finding, as he describes it, is that guinea pigs show a response difference to the antigen, GA. This difference is controlled by an Ir gene linked to the major histocompatibility locus. Presumably then responsiveness is controlled by the *Ir-1* locus which determines the recognition specificity of t cell associative antibody. Both, low and high responder animals to GA, respond to GAT equally well. What is startling is the claim that only those animals which are high responders to GA because they possess the proper *Ir-1* allele, produce serum anti-GA when immunized with GAT. If Benacerraf's interpretation were correct; that serum "anti-GA is only observed in guinea pigs possessing the GA gene" which is in the *Ir-1* locus, then t and B cell receptors

share in all probability a common subunit. In fact this result would permit a strong argument that *Ir-1* is the kappa locus. The reason is that the total response to GAT is roughly the same in all guinea pigs. Consequently, in this case t cell derived associative antibody to GAT cannot be limiting the B cell response to GA determinants. If an *Ir-1* linked GA gene were limiting the B cell response then *Ir-1* must be expressed in B as well as t cells. Under my present formulation, *Ir-1* would then have to code for kappa.

Unfortunately, a close look shows why this experiment, as it stands, cannot be used to test the hypothesis that the *Ir-1* locus codes for kappa. Two Ir loci could be involved. The genetic study showing the linkage to the major histocompatibility locus of the response to GA is carried out with strains 2 and 13 guinea pigs (Table 2). The study of the response to GAT is carried out with Hartley guinea pigs. In this latter case no genetic mapping is carried out. Instead the ability to elicit a cell-mediated reaction to GA in GAT immunized animals is used as a criterion for the presence or absence of the *Ir-1* GA-response gene (Table 10). An absolute correlation is found between a positive delayed hypersensitivity reaction to GA and anti-GA activity in the serum of the GAT immunized guinea pigs. Since there is doubt that the induction of cell-mediated reactivity is an assay of *Ir-1* coded associative antibody in any way fundamentally different from an assay of induced serum antibody, the absolute correlation is expected, but it does not tell us which Ir locus is limiting responsiveness to the GA determinant in GAT immunized animals, *Ir-1*, Ir-V_H^B, Ir-V_κ, or Ir-V_λ. We know however that the *Ir-1* locus cannot be limiting the anti-GA response difference to GAT in Hartley guinea pigs because the total anti-GAT response is the same in all animals. Therefore, the response difference induced with GAT and assayed with GA might or might not be an *Ir-1* controlled event. All of the key physiological experiments have yet to be done. Strain 2 and 13 guinea pigs immunized with GAT and assayed with GA should show no differences if *Ir-1* is expressed only in t cells. Hartley guinea pigs immunized with GA and tested on GA would behave like strains 2 and 13 if only *Ir-1* differences were involved, but if two unlinked gene loci are limiting responsiveness, e.g., *Ir-1* and Ir-V_H^B, the following genotype-phenotype relationships among Hartley guinea pigs are expected:

Antigen used in immunization	Phenotype	Genotype	
	Serum or "cell-mediated" anti-GA response	GA allele at	
		Ir-1	Ir-V_H^B
GAT	High	Low or high	High
GA	High	High	High
GA	Low	High	Low
GA	Low	Low	High
GA	Low	Low	Low

A high response to GA upon immunization with GAT requires the high response GA allele at Ir-V_H^B. A high response to GA upon immunization with GA requires the high response GA allele at both *Ir-1* and Ir-V_H^B. A low response to GA upon immunization with GA requires the low response GA allele at either or both of the Ir loci. If a low responder to GA is a high responder when immunized with GAT and assayed on GA then the high responder GA allele must be present at the Ir-V_H^B locus. This is a classical result in which a low responder to GA can be induced by a crossreacting antigen, GAT, to give a high anti-GA response. This is the "hapten-carrier" situation.

The most important case however would be the postulated low responder to GA which has the high responder GA allele at the *Ir-1* locus and the low responder GA allele at the Ir-V_H^B locus. Such an animal would be a high responder to GA if its t cell response were assayed but is a low responder when its B cell response is studied (see Section V B 3).

The formal experiments determining whether or not two Ir loci determine responsiveness to GA in Hartley guinea pigs will require genetic mapping and proper crosses.

One reason that I have gone into great detail in discussing Shearer's (Section V A 1) and Benacerraf's (Section V A 2) experiments is to illustrate why they do not provide an argument independent of the as yet incomplete genetic one (Section II C) that *Ir-1* is not the kappa locus (see Section V B 3 for a discussion of the other experiments on specificity of t and B cell receptors.)

3. NON-Ir-V_H^T LOCI

Whenever the *Ir-1* product is not limiting then response differences due to other Ir loci can be discerned. At present the only other Ir locus known experimentally to regulate responsiveness is Ir-V_H^B. There are two examples of responsiveness claimed to be neither H-2 nor Ir-V_H^B-linked. These are the response in mice to (T,G)-Pro--L and (Phe,G)-Pro--L McDevitt (Session III) and to streptococcal A carbohydrate (Braun, Kindred, and Jacobson, *Eur. J. Immunol.*, 2, 138, 1972). I have my doubts. In the case of (T,G)-Pro--L, if two factors were involved in the crosses, *Ir-1* and Ir-V_H^B, then the linkage to either might have been missed. In the case of the streptococcal A carbohydrate, the conclusion is based on a strain survey, not a genetic cross. In this case, inbred strains derived originally from a cross may have undergone a crossover between V_H^B and C_H^B, such that a responder and a nonresponder could have an identical allotype. A good example has been pointed out in the response to α-1,3 dextran (Blomberg, Geckeler, and Weigert, *Science*, 777, 178, 1972); (Session III, Table 56). Although a genetic cross shows unambiguously that α-1,3 dextran responsiveness is heavy chain allotype-linked, the strain survey reveals two ambiguities, CBA versus BALB/c and BAB/14 versus C57BL/6. CBA and BALB/c have the same

heavy chain allotype, but CBA is a low responder and BALB/c a high responder. BAB/14 and C57BL/6 have the same allotype but BAB/14 is a high responder and C57BL/6 a low responder. Since an identical strain distribution of responsiveness is found for the streptococcal A carbohydrate, the Ir-V_H^B locus is probably limiting and when proper crosses are carried out, responsiveness will be shown to be allotype linked.

The assumption that *Ir-1* does not code the kappa locus and a consideration of the asymmetry in the t-B relationship leads us to conclude that *Ir-1* gene polymorphisms will affect t cell responsiveness, directly, and only under special circumstances B cell responsiveness, indirectly. Ir-V_H^B polymorphisms will affect B cells only. Polymorphisms at the putative Ir-V_κ or Ir-V_λ loci will affect both t and B cells directly. The most likely experimental case would appear as non-*Ir-1*, non-Ir-V_H^B and t or B cell deficient. However, it would be rather difficult to reveal such a situation.

The anti-Phe,G or anti-Pro,L response in mice immunized with (Phe,G)-Pro--L or the anti-GA response in guinea pigs immunized with GAT should be compared with the response to α-1,3 dextran since all of these responses are limited by non-*Ir-1* genes. The GAT response in Hartley guinea pigs is analogous to the (Phe,G)-Pro--L response in DBA and SJL mice. The B cell populations of each strain look predominantly at different determinants, e.g., GA or non-GA, Phe,G or Pro,L, because the initial family of germ-line V_H^B genes expressed in B cells is different in each strain. In the case of α-1,3 dextran the total response is very restricted so that the dissection of it into restricted subpopulations is not necessary as it was in the case of responsiveness to (Phe,G)-Pro--L or GAT. This would be a detail except for two crucial points.

a. A Caution Concerning "Thymus-Independence"

Although responsiveness to (Phe,G)-Pro--L is not *Ir-1* linked we know from experiment that the associative antibody product of the *Ir-1* locus is required for the induction of B cells. This is the basis of the Shearer-assay and we classify (Phe,G)-Pro--L as "thymus-dependent". The response to α-1,3 dextran has not been shown to the thymus-dependent by the usual experiments. I will discuss this totally confused question of thymus-dependence and independence later (Section V B 2.). For the moment, I would like to state simply that the observed limitation by the Ir-V_H^B locus does not mean that the *Ir-1* coded associative antibody product is not required for induction. It only means that the *Ir-1* product, i.e., the t cell contribution or associative antibody, is not limiting in the low responder strain. The reason that responsiveness is limited by the Ir-V_H^B low response allele is because the *effective* level of associative antibody is similar in both low and high responding strains as a consequence of the very foreign and highly polymeric nature of the antigen that permits polygamous binding to associative antibody (Section V A 4)

b. Somatic Selection Pressures on B Cells to Improve Response

In a case where the total response to a complex antigen is the same in two animals, but the ratio of responsiveness to each of the various determinants is different, no selection pressure is put on the low response to one determinant in order to improve it. In the case of a restricted total response, somatic selection will eventually convert the low responder into a high responder. This is seen in the case of responsiveness to α-1,3 dextran. On prolonged immunization the response remains in the IgM class. No switch to IgG occurs. The high responders always produce the λμ class of antibody, whereas the low responders produce the κμ class of antibody. However, eventually the rate of synthesis of induced antibody in the initially low responder approaches that in the high responder (Weigert, unpublished data). This implies a somatic selection process acting on B cells to amplify responsiveness by increasing the numbers of B cells and/or the affinity of their receptors. The difference in responsiveness to various determinants on one antigen may attain such extremes that a complex antigen will elicit an almost homogeneous response, e.g., the pneumococcal C carbohydrate. Given a situation in which associative antibody is not limiting and a small number of germ-line $V_L V_H$ combinations, the level of a B cell response to any determinant depends on the number of mutational steps required to generate the required specificity from any one of the starting combinations. Since the animal regulates the total effector response to an antigen, not to the individual determinants, if several are recognized, some well, and others poorly, no selection pressure for any one is exerted. However, when the total response is highly restricted, a low responder is under strong selection pressure by antigen to improve the response. This is a central question for the origin of diversity.

A similar selection process would be expected to operate on t cells also, so that eventually the difference should disappear between low and high responders with respect to an *Ir-1* controlled response, e.g., to (T,G)-A--L. Yet except for slight hints (Section V A 4 b), this has not yet been found. The low responder in general establishes a steady state level of IgM anti-(T,G)-A--L synthesis under conditions where the high responder switches to IgG synthesis. If the associative recognition theory is correct then a superimposed regulatory mechanism is being revealed which permits high responders to (T,G)-A--L to switch, and prevents low responders from doing so. Further this mechanism should account for the failure to switch in the case of responsiveness (high or low) to certain antigens like α-1,3 dextran, lipopolysaccharide (LPS), polyvinylpyrrolidone, etc. It is this feedback mechanism which relieves the selection pressure for improved responsiveness by superimposing a further and more severe limitation to responsiveness than the numbers or affinity of t cells specific for the test antigen. However, the selection pressure can be restored under special conditions (Section V A 4 b).

4. t CELL IS OBLIGATORY FOR INDUCTION OF ALL CLASSES OF B CELLS

It is generally believed that associative recognition, if at all obligatory to induction, is required for the induction of the IgG class but not the IgM class of B cell. Therefore, I will discuss what the conditions are for the induction of IgM and IgG B cells under the associative recognition model. Induction depends upon the ratio and absolute numbers of signals ① and ② to a cell, as well as the inherent sensitivity of the cell to these signals. Both numbers and sensitivity may be involved in the explanation of the four observations which I have discussed scattered throughout the text. These are:

- Immunogens with repeating determinants, e.g., many hapten determinants coupled to a monomeric carrier or polymers, initiate preferentially IgM synthesis.
- Immunogens with no repeating determinants, e.g., monomeric proteins, initiate induced synthesis of antibody in both IgM and IgG without the preference cited above.
- Low responding animals due to *Ir-1* differences, i.e., effective associative antibody levels, are induced to respond in the IgM class uniquely, whereas high responders switch to synthesis in other classes,,e.g., IgG (Section V A 4a).
- *Abnormal* induction permits a low responder to a polymeric antigen to switch from IgM to synthesis in other classes (Section VI A).

If all cells were inherently equally responsive to signals ① and ② , then the differences between the induction of IgM and IgG B cells could only reside in the affinity of their receptors. With any given immunogen, if the affinity of the IgM receptor was higher than that of the IgG receptor, then the *effective* concentration of associative antibody required for induction of IgG synthesis would be higher than that required for IgM synthesis. This is a minimum assumption accounting easily for the differences between polymeric and monomeric immunogens. However, in order to account for the finding that abnormal induction (increased dose of signal ② at a constant level of signal ①) induces an IgM to IgG switch in low responders, a helpful (but not required), additional assumption would be the inherently greater sensitivity of IgM cells to the two signals and their ratios (Section VI A).

I will therefore begin the discussion assuming that the inherent sensitivity of IgM and IgG B cells is the same and appeal to the intuitively obvious. The number of inductive signals that an antigen-sensitive cell receives, will depend upon the number of receptors (R) it has, the number of associative antibody molecules (A) on the effector cell, the concentration of antigen (P) and the binding constants of the receptor, K_R , and the associative antibody, K_A , for antigen, P. For *normal* induction the number of inductive signals (① plus ② , see Figs. 82 and 83) equals the number of ternary complexes, RPA, formed. The

binding constants, K_A and K_R are contributing symmetrically to the formation of the inducing complex, RPA, i.e., the interaction of lower affinity dominates. The tighter the binding of receptor and associative antibody for antigen, the smaller is the amount of associative antibody required to deliver a given number of inductive signals to the antigen-sensitive cell. The binding of receptor and associative antibody is enormously tighter if the antigens are polymers of repeating identical units, because of "polygamous" or "multivalent" binding. The level of associative antibody required to permit induction in the case of a polymer, is orders of magnitude below that necessary for induction in the case of a monomer. In other words, at any given *actual* level of receptor and associative antibodies the *effective* concentration is much higher for polymers than monomers. Given a constant genetic background, the actual level depends upon how foreign the repeating subunits are. There will be present in the animal prior to immunization much higher levels of associative antibody directed against very *foreign* polymers such as the bacterial products POL, LPS, or SIII than against very *self-like* polymers such as (T,G)-A--L or (H,G)-A--L. *Self*-polymers like myelin, collagen, or histocompatibility antigens encounter virtually no associative antibody because they maintain tolerance. Thus there are *two factors,* but given "polygamous" binding for all repeating polymers their degree of foreignness (if I may be imprecise) determines the conditions under which the animal will respond, because the levels of associative antibody directed against foreign, are much higher than against self-like or self-polymers.

Now I can come to the one aspect of the genetics which is paradoxical. Very foreign stable polymers, e.g., POL, PVP, α-1,3 dextran, appear experimentally not to require associative recognition for induction (so-called "thymus-independence"). Further they induce a stable state of IgM synthesis with no switch to other classes. This correlation has led to the generalization that induced IgM synthesis is also thymus-independent. Benacerraf (Session I) and Katz and Benacerraf (*Adv. Immunol.,* **15**, 2, 1972), provide us with the most recent description of this point of view. *Our whole picture of how the products of Ir loci function hinges on the interpretation of the term thymus-independence.* In the light of this I will have to make a number of remarks concerning this concept, first as it relates to IgM synthesis and then to the general problem. I refer the reader to a detailed analysis of the problem by Bretscher (*Transplantation Rev.,* **11**, 218, 1972), and to a general discussion of methodology by me (*Cell. Immunol.,* **5**, 1, 1972).

Now let us deal with the IgM to IgG switch, assuming that the effective affinity of receptors is the sole factor. If, for a repeating polymeric antigen, the affinity of the receptor on an IgM B cell is greater than that on an IgG B cell (due to multivalent or polygamous binding), then the level of associative antibody required to induce the former will be less than that required to induce the latter at an optimal antigen concentration. The tighter the binding of antigen to

receptor antibody, the lower the level of associative antibody needed to form a threshold number of inducing complexes, RPA, on the antigen-sensitive cell. Therefore, IgM B cells will be induced initially when associative antibody levels are low, and IgG B cells will be induced later when the associative antibody level rises by induction to above some critical value. [The difficulty in analyzing this situation in detail is the still nagging doubt concerning whether or not the switch, IgM to IgG, occurs at the cellular level. This issue seemed to be closed by the various demonstrations that anti-IgM blocked both the induction of IgM and IgG, whereas anti-IgG blocked only IgG induction. However, if Feldmann, Marchalonis, and Warner are correct, that associative antibody is IgM-like, then these experiments are open to another interpretation—namely that the anti-IgM serum is acting at the level of cooperation, e.g., of the t cell, and is expected to block induction in both IgM and IgG classes whether or not a cellular switch operates. If this latter interpretation were to turn out to be correct, the idea that the induced IgM response is thymus-independent would be disproven formally. However the question of whether the IgM to IgG switch is a cellular event would be left open. In any case, even if a cellular switch operates it will be important only at the early stages of antigenic selection on the immune system (see discussion Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971). Thereafter, populations of IgM and IgG B cells specific for any test antigen would be present before challenge. Consequently, for illustrative purposes I will treat the situation as though there are two populations of B cells, one expressing IgM and the other IgG (or other class) receptors. The same arguments can be applied to a cellular level switch by making assumptions about the signals delivered to the B cell.]

The effective affinity of associative antibody for antigen must be equal to or greater than that of an IgM B cell receptor in order for a switch from IgM to IgG occur. If the effective affinity of associative antibody for antigen equalled that of the lower affinity IgG receptor, not the higher affinity IgM receptor, then these two classes of cells would be induced roughly simultaneously at an optimal antigen concentration. The lower affinity interaction dominates the decision as to whether induction occurs.

In the case of monomeric antigens the ratio of induced IgM/IgG antibody is much lower than that found with polymeric (polyvalent) antigens (Mäkelä, Pasanen, and Sarvas, *In* "Cell Interactions and Receptor Antibodies in Immune Responses," pg. 243, Academic Press, N.Y., 1971; Mäkelä, Koskimies, and Pasanen, *In* "Progress in Immunology," **I**, 655, Academic Press, N.Y., 1971). This is to be expected since, in the absence of polygamous binding by the receptors on the antigen-sensitive cells, the induction of IgM or IgG B cells will depend upon the intrinsic binding constant of the combining site for a determinant on the antigen. The effective concentration of associative antibody is the same for the two classes. In cases where it has been found that IgG receptors have a higher intrinsic binding constant per site than IgM receptors, I assume, if a cellular level

switch did operate in the past, subsequently the IgG B cells were selected upon somatically for better fit to a large series of naturally occurring antigens. The test antigen must be closely related to one of them. In this case we must be dealing with an IgM and an IgG population.

If associative antibody is maintained at a steady state level below that required for IgG induction but high enough for IgM induction, a stable state of IgM synthesis occurs. In the absence of feedback inhibition by IgG antibody the rate of induction of IgM synthesis is proportional to the effective level of associative antibody. This situation would arise in the case of a polymer like α-1,3 dextran if, for example, the IgM produced by induction were to inhibit by feedback the induction of associative antibody, e.g., by reacting with antigen to reduce its effective concentration. If, in addition, there was extensive treadmilling (turnover of associative antibody catalyzed by antigen) the steady state level of associative antibody would be depressed to a value below that required for the induction of IgG B cells.

a. Grumet's Experiments

Now let us apply this interpretation to the case of the *Ir–1* controlled (T,G)-A--L response by explaining 1) why the induction of associative antibody raises the level in high responders past the threshold permitting the switch to IgG synthesis and 2) why the induction of associative antibody reaches a stable state in low responders below that required to switch.

I will idealize Grumet's experiments (*J. Exp. Med.*, **135**, 110, 1972; Session I) somewhat, to emphasize the key points which become understandable in the light of the associative recognition model.

First, at a chosen low concentration of antigen both the congenic low and high responders initiate IgM synthesis only. In this sense they behave similarly. The probability of forming an inducing complex, RPA, on an antigen-sensitive cell at a given effective concentration of associative antibody will depend on K_R. At the suitably low concentration of antigen only IgM B cells can be induced in the high responder because the rate of induction of associative antibody is limited by antigen, and subsequent feedback inhibition by IgM antibody establishes a short-lived steady state below that required for the induction of IgG B cells. The chosen low concentration of antigen is still high enough to induce low responders which have some associative antibody, albeit of lower affinity and/or level, because polygamous binding brings the effective level above an inducing threshold. Presumably, if the concentration of antigen were sufficiently low a condition would be found in which high responders make, whereas low responders do not make detectable IgM. A relatively high level of associative antibody is required to switch, and below this level, chosen concentations of antigen which do not permit the high responder to switch, can still induce IgM synthesis in low responders. What I am trying to stress is that if the assays of IgM anti-

(T,G)-A--L were precise enough and a true measure of rate of induction, differences between low and high responders would be found even in their IgM response.

Second, at a chosen intermediate concentration of antigen, as expected both low and high responders initiate IgM synthesis. However, the high responder switches to IgG synthesis at an intermediate rate whereas the low responder establishes its maximum steady state rate of IgM synthesis. The switch in the high responder simply says that the induced level of associative antibody rose above that required for the induction of IgG B cells. We infer that a steady state level of associative antibody was established in low responders, because as I pointed out the rate of IgM synthesis is proportional to the effective associative antibody level. The steady state is attained because the IgM antibody feedback inhibits the induction of associative antibody, as I discussed for the case of the α-1,3 dextran response. Grumet (Session I) has presented us with evidence for an unidentified suppressive plasma factor in low responder animals. If this factor is induced and specific for (T,G)-A--L it could only be antibody of the IgM class as predicted. Since IgM antibody would feedback inhibit also in the high responder, why does it establish a steady state level of associative antibody so much higher that IgG synthesis is induced and this latter class of antibody now mediates feedback control, but at the new high level? If one *Ir-1* allele coded a receptor of high affinity for (T,G)-A--L, whereas the other coded a receptor of low affinity, then the initial rate of induction in the former would be high while in the latter it would be low. Consequently, the steady state levels at a constant feedback inhibitory rate would be different in the two cases. Several variations of this formulation are possible.

Third, at a chosen high concentration of antigen, both low and high responders initiate IgM synthesis. The high responder switches to maximal IgG synthesis, whereas the low responder shows instead a very diminished steady state level of IgM synthesis compared to that found at the intermediate concentration of antigen. This latter effect must be due to the rate of paralysis in the low responder becoming appreciable, thereby diminishing the rate of induction. The lower the initial effective concentration of associative antibody, the more the system tends towards paralysis in the competition between induction and paralysis, seen best in this case at high antigen concentrations. I stress that I have distinguished between *feedback* inhibitory effects via induced IgM effector antibody and *paralysis* via the receptor-antigen interaction (Signal ① Figs. 82 and 83).

b. Somatic Selection on t Cells

It would be expected that continued somatic mutation and selection by antigen at the t cell level would raise the effective level of associative antibody high enough so that the response difference would disappear. This would be anal-

ogous to what happens at the B cell level illustrated by the α-1,3 dextran response. There is an indication that this does occur. After repeated injections of low doses of (T,G)-A--L (0.1 μg) the high responder switches at 30 days, while the low responder starts to switch at 45 days. The experiment unfortunately was not carried out far enough, because it is precisely under conditions of low antigen concentration that the selection pressure in the low responder would be revealed best. At low concentrations of antigen which still induce, the selection pressure for increased affinity of the associative antibody is maximized and a switch can occur eventually in low responders. Once this happens a low responder should behave similar to a primed high responder. At higher concentrations of (T,G)-A--L, the low affinity t cells of low responders can be induced to associative antibody synthesis, so that no selection pressure is established for high affinity interactions. Under these conditions a stable steady state effective level is maintained by feedback in low responder strains below that necessary to switch to IgG synthesis. The situation here is analogous at the B cell level to a heterogeneous total response in which no selection pressure for a better response to any one determinant is imposed, e.g., (Phe,G)-Pro--L in mice or GAT in guinea pigs.

c. The IgM to IgG Switch is "Thymus-Dependent"

Inherent in my analysis of Grumet's experiment is the reason I disagree with his conclusion that the *Ir-1* locus exerts its effect *only* at the level of the switch from IgM to IgG, i.e., there is a *selective defect* in the induction of IgG synthesis in low responders controlled by *Ir-1*. The implication, as I pointed out above, is that the *Ir-1* gene product is not required for the induction of IgM synthesis. The induction of IgM synthesis is believed to be thymus-independent (Section V B 2). Such a view would not permit us to understand the all or none response to GAT in mice (Benacerraf, Session I). In terms of the associative recognition model, GAT is behaving more like a monomer because it is a random copolymer unlike (T,G)-A--L, which repeats an identical subunit. An *Ir-1* allele which reduces the level or affinity of associative antibody leads to a dramatic effect if the antigen is a monomer resulting in an all or none difference. If the test antigen is a regular repeating polymer like (T,G)-A--L, polygamous binding can still operate to correct (somewhat) gene defects, thereby permitting the initial IgM response. A similar situation obtains if one uses limiting concentrations of a monomer such as BSA. Small differences in affinity of associative antibody are titrated and these are the result of *Ir-1* polymorphisms. Thus we can see both from the work with (T,G)-A--L and GAT that *Ir-1* gene polymorphisms *do* affect the IgM response. The commonly held view based on "negative" experiments, that associative recognition is not required for the induction of IgM synthesis is one further example of a misleading fact because of the absence of a conceptual framework. (See also Section V B 2.)

d. Speculations on Receptors

Something should be added about the mechanism implied by this formulation. Uhr and Marchalonis have evidence that the IgM receptor on B cells and the associative antibody IgM-like receptor on t cells is monomeric (8S), not pentameric (19S). Let us accept this as correct and that the same applies to all other immunoglobulin receptors on B cells, e.g., IgG, IgA, etc. Upon introduction of an immunogen the order in which induction occurs is first t cells, then IgM B cells, followed by IgG (or other class) B cells (Cunningham, and Sercarz, *Eur. J. Immunol.*, **1**, 413, 1971). As I pointed out earlier (Section V A 4) two factors determine the order:

First there is the effective level of associative and receptor antibody. This depends largely on the ease with which polygamous binding to antigen can occur.

Second there is the inherent sensitivity of antigen-sensitive cells to signals ① and ② .

If receptors are monomeric, i.e., not covalently bound together on the cell surface, then the ease with which polygamous binding to antigen can occur depends upon their topological arrangement and/or mobility in the membrane. A topological arrangement implies either that the receptors complement with each other or that they are complexed to a "complementing subunit" in the membrane.

If the argument is correct that the t cell and IgM B cell can be induced preferentially to an IgG B cell at any given level of polymeric antigen and/or associative antibody because the "ease" of forming a polygamous bond with t cell or IgM B cell receptors is greater than that for an IgG B cell, then the same argument must apply also to paralysis. Since the interaction with the receptor on an antigen-sensitive cell is paralytic signal ① , in the absence of associative antibody a faster rate of paralysis of t cells and IgM B cells is expected at a low concentration of a polymeric antigen. This point is independent from the one which comes from a consideration of the asymmetry in the t-B relationship. Even were the rates of paralysis identical in all cells, t and B, it would appear from the usual experiments as though the t cell were inherently more sensitive to paralysis. The standard argument for inherently greater sensitivity of t cells to paralysis is made from the experimental data without understanding the consequences of the asymmetry (see discussion Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971; Bretscher, *Transplant. Rev.*, **11**, 218, 1972).

Since monomeric antigens can paralyze, *signal ① must be the consequence of a conformational change in the receptor* upon interacting with antigen via the combining site. This could be sensed by the cell via several mechanisms. Since it is certain that the conformational change must affect the variable domain, it is possible to postulate that the cell has an interaction sensing unit which is complemented with an invariant part of the variable domain. This assumption implies that all V subgroups coded by a given locus have in common

some tertiary configuration which undergoes the conformational change that is read by the cell. It is also possible, at the present state of knowledge, that the signal resulting from a receptor-antigen interaction travels from the variable domain to other domains in the receptor molecule from which it passes into the cell. This makes domain-theorists understandably unhappy because it implies that the domains interact with each other and are not simply beads on a string. Proper structural studies will settle this question.

Another category of mechanism for sensing the receptor-antigen interaction is via lattice formation. All known effector-functions are mediated via lattice formation or aggregation of the immunoglobulin, e.g., complement-lysis or histamine-release (see for discussion, Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971). We might extrapolate then, and consider that the conformational change in the variable domain following interaction via the binding site leads to aggregation, which in turn, is sensed by the system (adenyl cyclase?) transmitting signal ① . I hesitate to equate this postulated ligand-induced conformational change leading to aggregation with the visual patch (not cap) formation of receptors seen upon addition of fluorescent-labeled antiimmunoglobulin to B cells (Raff, Session IV). However, if one does equate them, two very important predictions can be made:

- Patch formation will occur upon specific binding of a monovalent ligand to B cells via the combining site of the receptor (the postulated signal ①).
- Antiidiotypic (or even anti-Fab) bivalent immunoglobulin will deliver signal ① which in the presence of signal ② (abnormal induction, Section VI) will lead to an induction of the B cell. The monovalent Fab fragment of antiidiotypic antibody will neither paralyze nor induce.

B. Range, Degree and Dictionary of Specificities of t and B Cell Receptors

The second property of the *Ir-1* coded receptor to consider is the selective pressures determining its specificity. Do we expect the *range* or *degree* of specificities recognized by the B or t cell populations to be different? Do we expect the *dictionary* of specificities to be different? I like this term "dictionary of specificities" chosen by Benacerraf and McDevitt because it permits us to make a key distinction which will become clear, namely, that between *range, degree* and *dictionary* of specificities. The presently accepted position is that the *range* and *degree* of specificities potentially recognizable by B and t cell populations are widely different (see for example, Gally and Edelman, *Ann. Rev. Genetics*, **6**, 1, 1972). Why is this unlikely? The driving selective force to increase the *degree* of specificity is the self-nonself discrimination. The *range* and *degree* of specificity are antithetical properties of a molecule. A low degree of specificity implies a wide range of recognition potential by a given receptor. The counter

selective pressure is that the higher the degree of specificity, the more antibodies needed to cover the range of recognition required in order to have an immune system which can anticipate a large variety of antigens. Consequently, the actual degree of specificity we see in an antibody molecule today is the resultant of these two counter selection pressures. This argument applies to any part of the immune system which must make the self-nonself discrimination, whether it be involved in effector or associative recognition functions. As a minimum, a functioning immune system must have a degree of specificity in both the t and B cell populations permitting it to make the self-nonself distinction, and a range of specificities large enough to be able to recognize at least one determinant on a random antigen. We know what this means for the B cell population, because degree of specificity (crossreactions) and range of specificity (soup to nuts), in serum antibody have been studied for fifty years now. The minimum requirements for t are similar to those for B cell receptors.

Of course, any immune system meeting the mimimum requirements for range and degree of specificity in both t and B cells, would operate efficiently if the number of B cell specificities, N_B, were much smaller than the number of t cell specificities, N_T, i.e., $N_B \lll N_T$. The excess N_T would permit the self-nonself discrimination and the maximum use of the N_B specificities. However, there is a point above which a given specificity in the excess N_T would be redundant at the level of the antigen which has many determinants, and there would be no (or a negative) selective pressure maintaining it. If, on the other hand, $N_B \ggg N_T$, the symmetrical situation exists in which there is no selective pressure to maintain the excess N_B specificities. The key point is that the minimum requirement implies a sophisticated degree and range of specificities in both cell populations, and there is no selective pressure for large overshooting by one or the other population, i.e., $N_B \ggg N_T$ or $N_T \ggg N_B$. Certainly, the widely accepted concept that t cells are of low degree of specificity ("primitive" not "primordial") does not take the selective pressures of the self-nonself discrimination into account.

There is another factor to consider. Since the induction of t cells (like B cells) requires associative recognition of antigen via the receptor on t cells and associative antibody, the specificity of this class in inducing itself is expected to have the same degree as that in the B cell class. The animal gains nothing in having its receptor antibody of high affinity (high degree of specificity) and its associative antibody of low affinity (low degree of specificity) or *vice versa.* The probability of an inductive encounter depends on the geometric mean of the product of the two affinities, K_A and K_R, receptor and associative antibody for an antigen.

However, formally speaking, the *dictionary* of specificities could be different. The B or t cell receptors could each have the same range of specificities, the

same degree of specificity (average binding constant), but as an extreme example a nonoverlapping dictionary. It would be equivalent to putting a random half of the words in the dictionary into the B cell category and the other half in the t cell category. Since the t cell receptor coded in part by *Ir-1* differs as a minimum, by a heavy chain (V_H^T) the dictionary is expected to be different. Of course, it is very unlikely that the dictionary is nonoverlapping (the extreme case). It is more likely that the overlap is considerable because the most important antigens of survival value are repeating polymers. The fact that t and B cell receptors are different molecules leads to an ambiguity in discussing the dictionary of specificities. If one could examine separately the t and B cell receptors binding any experimentally defined determinant, distinguishable differences would be seen. For example, there are many different variable regions of immunoglobulins which have anti-DNP activity of roughly the same binding constant. For the purposes of our discussion, the function to consider is effective recognition of "determinants," not differences in sequence of the antibodies recognizing them.

This second point concerning the degree, range and dictionary of specificities might be made clearer by another analogy. Cows express lambda as virtually the only light-chain class, whereas mice express almost uniquely kappa. Man expresses both classes roughly equally. Yet we have not concluded that the range and degree specificity of cow immunoglobulin is different than that of mice. On *a priori* grounds because the lambda and kappa V region sequences are different, we know that the dictionary of specificites recognized by cow and mouse is different. However, the dictionary of recognizable determinants is selected at the level of antigens and two animals are equally successful if one recognizes the N-acetygalactosamine and the other the phosphorylcholine determinants of the antigen, C carbohydrate. The same analogy applies in comparing the heavy chain V_H regions of two animals, e.g., rabbits *versus* mice or even allelic V regions of high and low responders to a given antigen. The range and degree of specificity in the t and B cell populations for an optimally functioning immune system are expected to be roughly the same; the dictionaries might be different.

1. Ir-V_H^T MANIFESTS ALLELIC EXCLUSION

This specificity argument leads to the prediction that the expression of *Ir-1* will show allelic exclusion. The surprise is that a hint of this being true, appeared at this conference in the form of a potentially very important experiment presented by Green (Session I).

Using guinea pig lymphocytes as antigens, two antisera were made in the inbred strains 2 and 13, 2 anti-13 and 13 anti-2. These strains differ in responsiveness to several antigens controlled by alleles at the guinea pig equivalent of the *Ir-1* locus. The result I wish to comment on is that in $(2 \times 13)F_1$ hybrids the

2 anti-13 serum blocks responsiveness controlled by the strain 13 Ir allele, but permits expression of the strain 2 Ir allele, whereas the 13 anti-2 serum does the opposite. The interpretation advanced by Green and Benacerraf is that the antiserum directly or indirectly blocks the recognition of antigen by the t cell receptor. I accept this interpretation but it is too vague and I would like to comment on the mechanism. For the discussion, I will assume the guinea pig and mouse have the same structure comprising the major histocompatibility linked Ir locus, and I will apply the nomenclature developed for mice to this experiment with guinea pigs.

First I would like to indicate why I do *not* think that the H-2 determinants on K and D are involved. Whatever evidence we have (Cullen, Schwartz, Nathenson, and Cherry, *Proc. Nat. Acad. Sci.*, **69**, 1394, 1972; Cullen, Schwartz, and Nathenson, *J. Immunol.*, **108**, 596, 1972), is consistent with the assumption that K and D are each single cistrons coding for single polypeptide chains which are not covalently joined with each other. For example, during the discussion of this question (Session IV), Lilly presented evidence that one can "cap" K and leave D evenly distributed on the cell surface or *vice versa*. If we assume that they are t cell receptors themselves the inelegant situation arises that since all antigen-sensitive cells (B and t) express both alleles of K and D, every cell would express the same four receptor specificities plus at least one unique receptor coded by *Ir-1*. Most important however, the F_1 hybrid antigen-sensitive cell expressing products of both alleles of K and D could not tell which had reacted with the antisera unless the assumption made by Green (Session I) is added—namely, that K and D are part of and modulate the receptor structure. This is ruled out because it requires that each allelic K and D product complement with the receptor coded by the *Ir-1* locus on the same chromosome, i.e., the interaction occurs *in cis* only. Therefore the K and D proteins could not be part of the t cell receptors being blocked by the 13 anti-2 and 2 anti-13 sera. If the two antisera, 13 anti-2 and 2 anti-13, are directed against the t cell receptor itself, the determinant on that receptor being recognized is created by the allelic polymorphic *Ir-1* coded difference, as a result of amino acid replacements. These replacements also determine the germ-line combining specificities coded by the receptor. If the receptor is an immunoglobulin we could look upon these antisera as recognizing either idiotypic determinants and behaving like that described earlier (Session III) for the α-1,3 dextran and C carbohydrate (phosphorylcholine) responses or allotypic determinants in the V and/or C regions like those found in the rabbit. The results with the hybrid $(2 \times 13)F_1$, show that the expression of responsiveness is separable by selective inhibition of the allelic products. For me the most likely explanation is that the *Ir-1* locus shows allelic exclusion and clonal expression. Clearly if we had been dealing with B cell receptors such as those determining phosphorylcholine responsiveness and had shown that anti-

idiotypic or antiallotypic sera blocked specifically the antibody response determined by one or the other allele in F_1 hybrids, we would have considered as likely explanations that allelic exclusion and clonal expression had operated (see also Cosenza, and Kohler, *Science,* **176**, 1027, 1972, Hart, Wang, Pawlak, and Nisonoff, *J. Exp. Med.,* **135**, 1293, 1972).

For Green's experiment, however, blocking is an inadequate test of the allelic exclusion hypothesis. Complement-mediated cytoxicity resulting in killing of cells expressing one or the other allele would be essential. Unfortunately this test cannot be used unless the antisera are properly absorbed to rid antihistocompatibility activities.

In principle Crone *et al.*,(Section IV B) have reagents for analyzing the *Ir-1* locus of chickens similar to those of Green for guinea pigs. They, too, are in a position to look at allelic exclusion in the expression of t cell receptors. In view of Simonsen's insistence that t cells express many different receptors, it would be a poetic touch for him to demonstrate allelic exclusion in the expression of *Ir-1.*

It is basically because experimentally only hints are available that *Ir-1* shows allelic exclusion that I approached the problem earlier from a general viewpoint by asking what selective forces determine the self-nonself discrimination. If an antigen-sensitive cell expressed receptors of many different specificities and upon induction amplified all of them, the net result would be equivalent to producing an antibody of a low degree of specificity. The driving selective forces for allelic exclusion are the same then, as those I have analyzed above for antibody specificity namely the self-nonself discrimination. (For further discussion see Bretscher and Cohn, *Science,* **169**, 1042, 1970; Cohn, *Ann. N.Y. Acad. Sci.,* **190**, 529, 1971; Cohn, *Cell. Immunol.,* **5**, 1, 1972).

I remind you also of the importance of an experimental demonstration that allelic exclusion operates at the *Ir-1* locus. Since the H-2 K and D cistrons do not show allelic exclusion, both allelic products being present on antigen-sensitive cells, all models of the Gally-Ohno type are ruled out in which whole or parts of chromosomes are rearranged so as to convert heterozygotes into homozygotes. We would have to consider regulatory mechanisms operating at the level of the gene (see Cohn,, *Symp. Intl. Soc. Cell. Biol.,* 7, 1, 1969).

2. "THYMUS-INDEPENDENCE" IS NOT A FUNDAMENTAL DISTINCTION

We are now ready to analyze what turns out to be the key difference between us. How can one interpret so-called thymus-independent antigens? Is there a class of responses for which the *Ir-1* gene product, associative antibody is not required? I wish to deal with this problem in the specific form under which it is camouflaged here, leaving the general discussion to Bretscher's review (*Transplant. Rev.,* **11**, 218, 1972). We must try to provide an understanding of the immune system which goes beyond the facts in order not to be mislead by them. In the 1930's it was concluded that enzymes were not proteins, because

catalytic activity could be measured in the absence of detectable protein. Today, we are concluding that associative recognition is not obligatory for induction because we can measure induced effector antibody activity in the absence of detectable associative antibody (cooperating function).

There are three positions on the question of thymus-dependence and independence, i.e., on the role of associative recognition.

- The response to all antigens is *fundamentally* thymus-dependent in that associative recognition is obligatory for induction but *not* for paralysis. This is the associative recognition model which Bretscher and I have developed.
- The response to all antigens is *fundamentally* thymus-independent in that there is an as yet unknown mechanism for the self-nonself discrimination. Associative recognition is a helper device superimposed on this unknown mechanism. This is the Mitchison-Möller-Miller position.
- The response to some antigens is in the first category, being *fundamentally* thymus-dependent and to some in the second category, being *fundamentally* thymus-independent. This is the Benacerraf-Grumet-Sela position.

The role of the *Ir-1* locus in regulating the induction-paralysis decision cannot be analyzed under the Mitchison-Möller-Miller position because it is a negative statement to the effect that the associative recognition model is wrong. It does not tell us how the induction-paralysis decision is made. In this sense it is not a theory. They owe us a competing concept.

Katz and Benacerraf (*Adv. Immunol.*, **15**, 2, 1972), accept an ill-defined form of the associative recognition model for thymus-dependent antigens, but lack a model for thymus-independent antigens. Consequently they are forced into the position of simply restating the facts or interpreting literally (with no generalization) the experiments defining thymus-independence rather than extrapolating whatever form of the associative recognition model they do accept from thymus-dependent to thymus-independent antigens. If there is one central mechanism for the self-nonself discrimination, then their position is contradictory; if there are actually two mechanisms then we need an analysis of both of them not a literal description of the experiments. This position was expressed in several ways here. It is well to recall them before discussing so-called thymus-independence viewed via the associative recognition model.

Benacerraf (Session I) interpreted my remarks as postulating that all antigenic determinants can act equally well as "haptens and as carriers." This is a misunderstanding, for from the general nature of the theory I am analyzing I agree with him that the *dictionary of specificities* expressed by t and B cells are in principle, different simply as a consequence of the assumption that their receptors are coded in part by different loci, Ir-V_H^T and Ir-V_H^B. Clearly, for any given determinant on an immunogen the response in the effector or associative

antibody class depends on the number of cells and the affinity of their corresponding receptors relative to those directed against other determinants. This has been the basis of my analysis of specific dominant responsiveness. The confusion is due to the jargon, haptens and carriers, which makes what could be a provocative postulate (even if I did not make it) devoid of meaning. I have deliberately avoided these terms thus far. A hapten for me is a nonimmunogenic molecule which when coupled to an immunogenic one (a carrier) directs the induction of antibody complementary to it. Benacerraf redefines these terms by his illustrative choice of experimental evidence for the hypothesis that given determinants "cannot function equally as haptens and as carriers." He points out that "polysaccharides and D-amino acids, for instance, are adequate as haptenic determinants but cannot function as carrier determinants." Thus in effect Benacerraf defines two types of determinant on the immunogenic molecule. The "hapten" is a determinant against which induced effector antibody but not associative antibody (cooperating activity) is measurable and the "carrier" is a determinant against which induced associative antibody (cooperating activity) (as well as effector antibody) is detectable. Thus thymus-independent antigens, of which polysaccharides and D-amino acid polymers are examples, act as haptens in the Benacerraf sense, i.e., they induce the formation of effector antibody (a B cell response) but have not been detected to induce associative antibody (cooperating activity). From this, Benacerraf (Session IV) concludes that hapten determinants are not recognizable by t cells, only by B cells. In his own words, "there is one further safe generality; that *certain types* of molecules do not stimulate thymocytes. These are the so-called thymus-independent antigens such as most complex polysaccharides." This position (far from "safe") would be hard to defend were it not for the fact that thymus-independent antigens induce IgM synthesis. The reason is that a B cell interacting with one determinant cannot tell whether that determinant is on a thymus-dependent or independent antigen (i.e., whether another determinant on that antigen has been recognized by t cell associative antibody) unless some signal is transmitted to it. Consequently, it is assumed that the B cell interacting with any thymus-dependent or independent antigen is induced to make IgM antibody in the absence of associative antibody, but in its presence switches to IgG. I recall that the signal leading to IgM synthesis in this model is the one we have postulated leads to paralysis (associative recognition model, Figure 82). *Consequently, this formulation remains unsatisfactory for it ignores how the self-nonself discrimination is regulated for the induction of IgM antibody by any type of antigen, thymus-dependent or independent.* For me, this is a conceptual hiatus which, if faced with a general theory allows one to be suspicious of such "facts" as thymus-independence. For Benacerraf (Session IV), requiring that a theory explain induction and paralysis for both thymus-dependent and independent antigens is an unnecessary extrapolation beyond "facts"

and is equivalent to asking to which "church" or "faith" one belongs. So he pleads the "Fifth Amendment" on the assumption that a sphinx is of no use if it does not have a secret.

There are two ways of dealing with thymus-independence, given the obligation to face the problem of the self-nonself discrimination. You can argue either 1) that there is a second special lineage of nonthymus-derived cell, e.g., bone-marrow derived, possessing all of the cooperating properties which I have ascribed to the t cell or 2) that the antigens classified as thymus-independent have a common structural characteristic which enables them to be immunogenic at levels of associative antibody below that detectable by existing methods. The choice between these two suggestions is based on likelihood considerations.

The first suggestion requires that the dictionary of specificities of the two postulated cooperating cell-lineages be different, i.e., receptors coded by as yet unknown gene loci, and that in general one reinvent the t cell. Further, it remains odd that all antigens showing the property of thymus-independence should be repeating polymers of very foreign subunits. If two distinct cooperating cell lines operated there is no reason that the thymus-independent antigens should be categorizable as polymeric. Consequently, I think this suggestion very unlikely.

The second suggestion is that polymeric antigens of very foreign subunits would not appear to require associative antibody for induction only because the experimental procedures which reduce the level of associative antibody, do not in fact eliminate it. The fact that polysaccharides, PVP, LPS, POL, and D-amino acid polymers will induce animals which have been surgically (thymectomized, X-rayed, bone-marrow restored) or genetically ("nude" mutation) t cell depleted, need only mean that these antigens are efficient immunogens in that the actual level of associative antibody permitting induction by them can be very low. This is an expected property of very foreign repeating polymers. These surgical and genetic procedures reduce, but have not been shown to eliminate associative antibody. It is simply not possible to conclude as does Feldmann, *In* "Immunological Intervention," pg. 74. Academic Press, New York, 1971, that, to the antigen POL one "can get a 100 percent normal response in the *total* absence of T cells." Consequently, apparent thymus-independence should not be used as an argument that associative antibody cannot or does not recognize thymus-independent antigens.

Under the associative recognition model, the reason that *Ir-1* control of thymus-independent antigens has not been revealed experimentally is because in general the polymorphisms do not create an all or none level of associative antibody. Consequently, immunogens efficient because they are polymers and bind polygamously to associative and receptor antibodies still can induce an IgM response in low responders. If *Ir-1* control were demonstrated for a thymus-independent antigen, defined *a priori* like the flagella protein, POL, then we would simply reclassify it as thymus-dependent. No clean distinction between

them exists. I recall that in recent times KLH has changed from a thymus-independent to a thymus-dependent antigen only as the result of a redefinition of the terms.

Sela (Session IV) has introduced another category of hypothesis distinguishing thymus-dependent from thymus-independent antigens. He proposes that the former are degraded and lost (metabolizable), whereas the latter are stable and persistent (nonmetabolizable).

Of course, rate of degradation affects the persistence of the inductive signal and the extent of the treadmill effects I discussed above, but these are clearly secondary considerations which need sorting out. The primary implication of the Sela hypothesis is that the role of the *Ir-1* coded product is to stabilize metabolizable thymus-dependent antigens so they become immunogenic. Sela is proposing in effect a rather subtle form of the associative recognition hypothesis for induction by thymus-dependent antigens. (I am putting aside the difficulty of inventing a precise mechanism for stabilization in order to make a methodological point.) The stabilized thymus-dependent and the thymus-independent antigens are equivalent. Since under this model thymus-independent antigens have bypassed the requirement of associative recognition (stabilization) for induction, it is obvious what makes Sela's concept of thymus-dependence and independence incomplete. The signal distinguishing induction from paralysis is not analyzed! When this is done it becomes clear that a stabilization model is unsatisfactory for it does not account for any aspect of the self-nonself discrimination, e.g., dose-dependence of establishing tolerance, dose-independence of maintaining tolerance, breaking tolerance, etc.

One reason that L-amino acid polymers are thymus-dependent whereas D-amino acid polymers are seemingly thymus-independent, is simply that the latter are more foreign to the animal and the effective preimmunization level of associative antibody is higher for the D- than the L-polymer. The other reason is that the size of the active immunogen might be smaller in the case of L-polymers due to degradation prior to encountering the immune system. One important example testing the Sela rule is the D-glutamyl polymer which is nonimmunogenic until coupled to an immunogenic substance. This hapten can however paralyze (Roelants, and Goodman, *Nature,* 227, 175, 1970). Therefore, it can interact with B cell receptors effectively. The D-glutamyl polymer is nonimmunogenic because the level of associative antibody specific for it (a t cell function) is too low. Consequently it takes more than being a D-polymer (nonmetabolizable?) to render it thymus-independent. Again, the general correlation that nonmetabolizable D-polymers behave as thymus-independent whereas metabolizable L-polymers behave as thymus-dependent antigens is a misleading "fact" in the absence of a conceptual framework.

3. AVAILABLE SPECIFICITY STUDIES DO NOT PERMIT CONCLUSIONS ABOUT THE DICTIONARY EXPRESSED BY t AND B CELL RECEPTORS

Now it should be clear why the conclusion suggested by the genetic studies (Section II C) that t and B cells express receptors of different structure (coded by different loci) cannot be derived from the available specificity studies (See also Section V A 1). If they had disproven that the t and B cell dictionaries are the same, we would have had an argument, independent of the genetics, against *Ir-1* coding the kappa locus (assuming of course no additional unknown loci coding for the t cell receptor).

First of all, the assays for the recognition potential of associative antibody class are in general inadequate. The immunization of an adoptively transferred thymus cell suspensions with a determinant of known structure, H, linked to an immunogenic molecule, P, induces associative antibody formation to many determinants on the immunogen, HP. Which antibodies and in what proportion they appear is determined by many already discussed factors, genetic and non-genetic. However, even if the associative antibody response to the determinant, H, were considerable, ~10% of the total, the subsequent assay for its presence by cooperating activity might not reveal it. If the challenge antigen were HA in which A was a foreign but virtually nonimmunogenic molecule, the induction of B cells anti-A by cooperation with associative antibody, anti-H, might not be revealed, because the test system in all likelihood would tend towards paralysis. Nevertheless, there are suggestions that determinants containing DNP are recognized by associative antibody (Iverson, *In* "Cell Interactions," Silvestri, ed., pg. 192, North-Holland Publishing Co., Amsterdam, 1972).

Second, thymus-independent antigens might not show cell-mediated reactions for many reasons other than the present view which I believe to be incorrect, namely that cell-mediated reactions are a t cell effector function. For example, if thymus-independent antigens do not switch from IgM to the Ig-class mediating killer activity, no cell-mediated activity would appear. This may be why cell-mediated reactions appear to be controlled by the *Ir-1* locus as "all or none" not "high or low" responsiveness as is found for serum antibody (Benacerraf, Session I; Section V A 2). Low responders simply do not switch so that the antibody class responsible for cell-mediated activity may not be induced in low responder strains just as is the case for the IgG class in which the *Ir-1* controlled response is also "all or none" (Section V A 4 a). The whole concept that cell-mediated assays measure the activity of the *Ir-1* gene product in a more direct fashion than serum antibody assays is being challenged by me. At the moment, the simplest assumption is that *Ir-1* codes uniquely for associative antibody which only has cooperating function.

Third, the widely accepted conclusion that t cells express "primitive" receptors, i.e., of low degree and narrow range of specificities, is based on the apparent lack of examples of t cells, but not B cells, being able to respond to an

antigenic determinant. The usual finding is that B cells can, but t cells cannot or poorly respond, e.g., to PLL, (T,G)-A--L, etc. This general result is not revealing anything about the inherent properties of the receptors on the two populations of cells. This finding is an inevitable consequence of the known assays for induced t cell associative antibody that, in the end, depend upon cooperation leading to a B cell response. One is not likely to detect t cell responsiveness and B cell unresponsiveness with the available assay systems used as they are. It is predictable that a proper search will reveal determinants to which t cells but not B cells respond (See Section V A 2). This will eliminate the *a priori* unlikely suggestion that t cell receptors are "primitive."

I stressed at the beginning that the self-nonself discrimination cannot be encoded in germ-line genes but must be acquired. Since this process provides the selection pressure for specificity it is unlikely that a general rule concerning the dictionary of specificities can be made about the recognition potential of associative compared to effector antibody. In any case such general rules (Benacerraf, Session I; Katz and Benacerraf, *Adv. Immunol.*, **15**, 2, 1972), as:

- t cells unlike B cells recognize poorly, if at all, synthetic determinants (DNP, arsanilate, etc.) or those on polysaccharides, synthetic polymers (PVP, D-amino acids) and certain proteins like flagella (POL), or
- t cells are specialized to recognize native and altered proteins and polypeptides,

are neither derivable from the specificity data nor, in the absence of a theory, are they meaningful generalizations.

If one insists on such formulations, based on "facts", it should be because there is a rationalization for them. Why was the *Ir-1* locus selected upon so that its product does not recognize the determinants above listed, whereas the loci coding B cell receptors were selected to recognize them? Why was the *Ir-1* locus selected upon so that its product remained "primitive," i.e., for t cell receptors which are less specific than the B cell receptors (Unanue, Session IV; Gally and Edelman, *Ann. Rev. Genetics*, **6**,1, 1972)?

C. The Linkage of the Genes K and D as Well as Ss and Slp to *Ir-1* Is Fortuituous

A discussion of this proposition is certain to be tricky. There is a difference between the two statements:

- If several genes interact to improve fitness (directly or indirectly) then natural selection will favor closer linkage between them.
- If several genes are closely linked then natural selection has operated because they interact to improve fitness.

The first is an established principle of evolution and the second is an intuitive feeling of likelihood made stronger if a precise mechanism of interaction can be proposed. The term "interact" is important because it implies a functional

relationship albeit an indirect one. The intuition concerning the second statement has had a profound effect on our thinking here. It has been expressed by the vague idea that if two components have in common being on the surface and coded by linked genes then they "interact to improve fitness." Thus far this intuitive feeling has led to no specific experiment or generalization applicable to the IX linkage group. So I propose to look at it closely.

The *Ir-1* locus is the *only* Ir locus controlling specific dominant responsiveness for which the linkage relationships to other markers are known. No generalization then on experimental grounds of a relationship between Ir loci and linked histocompatibility loci is warranted. The region is mapped starting from the centromere.

●———K———*Ir-1*———Ss———Slp———D———.

Each of the "genes" K, D, Ss and Slp seems to code for a single polypeptide chain and I will assume this to be so (Cullen, Schwartz, Nathenson, and Cherry, *Proc. Nat. Acad. Sci.*, **69**, 1394, 1972; Shreffler, and Passmore, Proc. Symp. Immunogenetics of the H-2 System, Karger, Basel, pg. 58, 1971).

K and D are uniquely membrane bound proteins whereas Ss and Slp are secreted serum proteins. Both alleles of K and D are expressed to different levels in the membranes of all cells whereas *Ir-1* is expressed as a receptor only in t cells and likely shows allelic exclusion. The kidney probably synthesizes both Ss and Slp. Slp is expressed only in males for it is induced by testosterone. So we appear to be dealing with four cistrons K, D, Ss and Slp and one functionally unified gene locus, *Ir-1*, each expressed and regulated differently. There could of course be many as yet unknown genes in this region. The only common element is that the K, D and *Ir-1* products are surface components. Let us look at that feature.

I have been bothered that only two membrane proteins, K and D, should appear to have all sorts of special properties as immunogens not shared by any other surface antigens. Consequently, I feel relieved that the peculiar immunogenic properties of K and D are being reinvestigated. One peculiar property was that an inductive xenogeneic stimulation is less strong than an allogenic one. This is no longer a general rule (Bach, Session IV; Widmer and Bach, *J. Exp. Med.*, **135**, 1204, 1972). Another peculiar property was that a very large proportion of antigen-sensitive cells seemed to respond to allogeneic stimuli provided by determinants on the allelic K and D proteins. This is no longer certain since the assay systems do not distinguish induction from effector processes. Consequently, it may be the effector, not the antigen-sensitive cells which are being counted in the assay (discussed in Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971). In addition *abnormal* induction could be operating (Section VI). Further, many other surface components besides K and D contribute to the MLC assay. This latter finding has been revealed by the work of Bach, Klein, Shreffler, and van Rood

(Session II). The genes mapping in the mouse between K and D code surface determinants which are the major inducers of responsiveness in the MLC. This makes more sense for two reasons.

First, the greater the number of differences (degree of foreigness) between the responding and target population the greater is the expected MLC response. If these new determinants are immunoglobulins coded in part by the Ir-V_H^T locus, then there could be at least as many polymorphisms as one sees in the Ir-V_H^B locus. You may recall that many differences due to structural V_H^B genes have been observed by analysis of the genetics of the idiotype of effector antibody (Session III). Further, allotypic markers analyzed both serologically and by sequence, label the V_H locus of rabbit and the V_κ locus of mouse, e.g., the I_B peptide (Section II C). It is by analogy with Ir-V_H^B (as well as on general grounds) that I expect the *Ir-1* coded t cell receptor to be highly polymorphic and therefore revealed by technics used to detect histocompatibility determinants. We have seen examples in the work of Ramseier and Lindenman (Section III B), Green (Section V B 1), and Simonsen (Section IV B).

Second, if the MLC assay is essentially an *in vitro* induction system, then in addition to *normal* induction of t and B cells via associative recognition of the cell surface determinants, *abnormal* induction should be operating. This latter might in fact be the more powerful stimulus with certain cell combinations (Section VI E).

I have treated the *Ir-1* locus expressed in t cells as a series of contiguous V_H^T genes linked to a C_H^T gene analogous in structure to the known heavy chain locus $V_H^B C_H^B$ expressed in B cells. It is therefore no surprise that the *Ir-1* locus itself consisting of many genes is held together by selection and not scattered over the genome. If the analogy is valid, the mechanism of joining of V to C by translocation occurring only in *cis* as well as allelic exclusion might require that the family of V genes be contiguous and physically adjacent to the corresponding C genes.

Now let us deal with the evolution of *Ir-1* itself. Jerne (*Eur. J. Immunol.*, **1**, 1, 1971), proposed that the V genes were selected upon to code for specificities directed against all of the unique major histocompatibility determinants coded by the allelic loci of a given species. I have analyzed elsewhere why this theory of diversity is unlikely (Cohn, *Ann. N.Y. Acad. Sci.*, **190**, 529, 1971). However, at the moment, we are considering Jerne's idea not as a theory of diversity but as a theory of germ-line V gene evolution. Bodmer (*Nature*, **237**, 139, 1972), has pointed out why it is untenable as a theory of evolution and proposes a way of saving the idea. Starting as Jerne does with a primordial system of cell to cell recognition, Bodmer proposes that V genes evolved from genes coding for products which recognize self-antigens common to all individuals of the species. In other words, instead of selection for recognition of a self-determinant rarely seen by an individual, the selection is for recognition of determinants pre-

sent in all members of the species because they are essentially invariant. From this primordial set of recognition units, the known modern day V loci evolve by duplication and translocation. These modern V loci eventually escape the requirement for self-antigen recognition and are selected upon for optimal recognition of foreign antigens.

In developing a theory of diversity, I have suggested that the selective pressures on the modern V loci are for the coding of specificities of immediate survival value (Section II A). The speculations on the evolution preceding this modern period might seem somewhat *ad hoc* at the moment were it not for the fact that there is the undercurrent that the *Ir-1* locus is this postulated "archaic or primitive" cell to cell recognition system maintained into modern times by some selective pressure and upon which the mechanisms of induction and paralysis have been imposed. I look forward to the arguments which will be used in the debate as to whether associative antibody coded by *Ir-1* is an immunoglobulin as broad in range and as specific as effector antibody (Section V B).

What do we get out of the fact that *Ir-1* is found today next to K, D, Ss and Slp? The immunological relatedness of K and D and Ss and Slp indicate that they arose by gene duplication (Shreffler, David, Passmore, and Klein, *Transplant. Proc.*, **III**, 176, 1971). This implies that each pair, K and D or Ss and Slp, started evolution functionally related. Their structural similarity today places them as still probably functionally related. There is no evidence and no proposal as to why the *Ir-1* locus trapped between K and Ss should be considered as functionally related to them nor for that matter why the pairs K and D versus Ss and Slp should be considered in any way functionally related. If *Ir-1* is a heavy chain locus it seems unlikely that even the Ss Slp genes would sit between the V and C genes of *Ir-1*. Therefore the findings that the MLC "locus" in man is spread outside the interval between Four and LA (Session II) must imply that this locus is coding for antigenic determinants unrelated to *Ir-1*. In other words the MLC assay is looking at gene products which include those of *Ir-1* and many others unrelated to it.

Using the analogy with Ir-V_H^B which has evolved by gene duplication, Ir-V_H^T likely followed the same path. Gene duplication by unequal crossing over built up the family of V_H^T genes to make the *Ir-1* locus. Polyploidy followed by chromosomal rearrangements likely led to the unlinked duplication of this locus which diverged to become the allotype marked heavy chain and possibly also the kappa and lambda loci. This implies that these unlinked duplicated Ir loci contain linked "K"- and/or "D"-like genes which diverged to become the minor histocompatibility genes. A search for this would be rewarding. The finding that associative antibody is "IgM-like" is therefore exciting because it is expected from what we know about the evolution of immunoglobulins. The first heavy chain class to appear as effector antibody was IgM (a B cell product). Its postulated

direct precursor, the Ir-V_H^T product (a t cell product) is expected therefore to be "IgM-like."

Now the question will be raised as to why the *Ir-1* locus, if fortuitously between K and Ss has remained there throughout mammalian evolution. First of all, we only have evidence for *Ir-1* being linked to a major histocompatibility locus in mouse, rat, and guinea pig. However, I would not be surprised if it were a general rule. I could argue reasonably that the positioning of *Ir-1* between K and Ss is a frozen accident maintained either because of a superimposed mechanism or because the chance of separating closely linked genes is low. As an example of a superimposed mechanism, the genes on the X chromosome have remained linked throughout the evolutionary existence of its role in sex determination. The genes on this X chromosome do not appear to be any more functionally related one to the other than to an autosomal gene. What holds them together? Once the chromosome carrying a "fortuitous" (in a functional sense) collection of genes, acquired the gene which determines sex then a selection was imposed for dosage compensation. This superimposed mechanism fixed the collection because any X-linked gene which wandered to an autosome would upset the dosage compensation and be selected against (Ohno, *In* "Sex Chromosomes and Sex-linked Genes." Springer-Verlag, Berlin, 1967). We might look for an analogous superimposed mechanism operating on the IX linkage group. It is possible that the T locus expression might be an example of such a mechanism (Bennett, Boyse, and Old, *In* "Cell Interactions," Silvestri, ed., pg. 247, North-Holland Publishing, Amsterdam, 1971). Linkage via superimposed mechanisms is not however the functional relationship we are groping for.

If one starts with the *Ir-1* locus trapped between K and Ss to which it is unrelated, the tendency is to keep them together because the two mechanisms translocation and inversion which would break up the linkage have a very low probability of being established in the species. Translocation often leads to mispairing at meiosis and the defective offspring (often sterile) are eliminated with loss of the already rare event. Inversion most often inactivates the function. In addition, most of the breaks within the *Ir–1* region itself that would separate it from K, D, Ss or Slp, would destroy its function. Further if there is functional relatedness keeping the K and D cistrons linked by selection, then the unrelated *Ir-1* locus and the Ss-Slp genes would be maintained also as linked. In a sense the K-D relationship would be viewed as a superimposed mechanism keeping *Ir-1* and *Ss-Slp* together.

My last point is related to the meaning of the fact that the *Ir-1* product can be revealed (or assayed) both as a receptor for antigen on t cells and as a surface component which is polymorphic (histocompatibility antigen). Where do we go with this fact? How do we unravel the "Gordian knot mystique" surrounding our repeated probing of the concept "histocompatibility versus receptor (recognition)

genes"? Would the finding that all of the other Ir loci, e.g., Ir-V_H^B, are linked to genes coding polymorphic surface determinants lead us anywhere?

There must be hundreds of polymorphic membrane components which could be detected as "histocompatibility" antigens by the many assays discussed here. Since there are only twenty chromosomes in a haploid set in the mouse, it is obvious that any randomly chosen gene will show linkage to some histocompatibility gene. The linkage of specific dominant Ir genes to something labeled H-1, H-2, H-3, etc., is not in and of itself a reason to functionally relate them. Admittedly there is something special about the products of the major histocompatibility genes as antigens. They are highly immunogenic. However, this fact has not led as yet to a precise reason to relate the functioning of *Ir-1* to *H-2K* or D. If the product of the *H-2K* and D cistrons is expressed in all cells in relatively high concentration and is homogeneous in an individual, then it is no surprise that they would be good immunogens. If one immunizes an animal with a heterogeneous mixture like serum immunoglobulin, the total response is excellent but the chances of making a given antiidiotypic specificity are very poor. However, any given myeloma protein induces an excellent antiidiotypic response. The MLC looks at the totality of differences between two cells and this could be large (analogous to a response to serum immunoglobulin). However the level of response to individual components will depend upon what proportion they are of the total, and how large the polymorphic difference or degree of foreigness. What makes certain histocompatibility antigens major and others minor, depends upon many factors unrelated to specific properties of the immune system.

If the Ir loci are the structural genes for immunoglobulin subunits then we might ask what surface components would be required for their expression and for this reason, the genes coding them might be linked as a "regulon," a unit of genes regulated together. These might be such components as are required for the insertion of the receptor into the membrane and for sensing when an interaction with antigen had occurred. However, on the one hand it is unlikely that surface components expressed in all cells, e.g., K and D, would be examples of this and on the other hand, when we examine known cases of complementing subunits or functionally related proteins, most often they are not coded by linked genes. A perfect example is the B cell immunoglobulin receptor made up of subunits, light and heavy, coded by unlinked genes.

For me the take home lesson is the following:

The chromosomal segment "K-Ir-1-Ss-Slp-D" has taken on a new look of great simplicity. K, D, Ss and Slp appear to be single cistrons. Their unusual property is the high degree of polymorphism of the K and D genes. The evolutionary selection for many antigenic variants of the K and D products is related to their functioning as Bodmer has discussed (*Nature,* **237**, 139, 1972). By contrast, the *Ir-1* locus is selected so that its product functions as a receptor. This latter selec-

tion for receptor function of necessity leads to polymorphic antigenic differences between the products. However, for the functioning of the *Ir-1* locus these antigenic differences are secondary in the selection, whereas for K and D they are the primary selective target. No one has as yet given us a reason to equate these roles in order to explain the linkage of K, D and *Ir-1*.

VI. THE GENETICS OF ABNORMAL INDUCTION

The genetics of immune responsiveness can be somewhat masked by more indirect effects and I wish to deal with a most important example before closing.

The associative recognition model for *normal* induction (Figs. 82 and 83) permits the prediction of the existence of abnormal induction (Fig. 85) (Cohn, Immunologic Intervention, pg. 72, Academic Press, N. Y., 1971; Bretscher, *Cell. Immunol.*, **6**, 1, 1973). In the *abnormal* case, signal (1) comes via the receptor-antigen interaction as in the case of *normal* induction, but signal (2) comes via the associative antibody interaction with a surface determinant on the antigen-sensitive cell. Thus, the self-nonself discrimination is sabotaged because the interaction of a receptor with a self-component which would be normally paralytic (signal (1)) is converted into an inductive event by the associative antibody interaction with a cell surface component generating signal (2) . Experimentally this phenomenon was first seen by McCullagh (*J. Exp. Med.*, **132**, 916, 1970), as a breaking of unresponsiveness to sheep red cells by allogeneic lymphoid cells. Later Katz and Benacerraf (reviewed in *Adv. Immunol.*, **15**, 2, 1972), and Kreth and Williamson (*Nature,* **234**, 454, 1971), extended McCullagh's findings by showing that, by addition of allogeneic lymphoid cells, antibody synthesis could be induced to a determinant on an antigen under conditions where it was essentially nonimmunogenic. The phenomenon of *abnormal* induction was given the names "graft versus host," "allogeneic," or "cell surveillance" models of induction. I find this unfortunate, because it confuses the cell-mediated killing and induction mechanisms (Section III C 2). What is a total confusion for me is a basic principle for Kreth, Williamson, and Nature's correspondent (*Nature,* **234**, 439, 1971). I apologize for stating the problem this way, but it illustrates the role of competing concepts.

There are several reasons that I am analyzing *abnormal* induction as my closing point.

A. Grumet's Coaxing of IgM to IgG Switch in Low Responders

Grumet (Session I) has shown us that by setting up an abnormal induction situation in low responders which only make an IgM response, the synthesis of IgG is induced. This result is predictable because the interaction of the antigen with receptor takes place in the presence of a nonlimiting level of signal (2) , more than equivalent in effect to a high level of associative antibody induced by

normal induction in high responders. The switch from IgM to IgG induced *abnormally* (via signal ②) proves that what limited IgM synthesis to a steady state value was the number of doses of signal ② , i.e., the effective level of associative antibody.

This experiment is very important, not only because it is a key lead to the mechanism of induction, but because it disproves the most commonly held view as to the role of the t cell, namely that cooperation or associative recognition is uniquely a "helper" mechanism for "concentrating," "presenting," "handling," "magnifying," "focusing," "trapping," and/or "topologically arranging" antigen. If a so-called thymus-dependent antigen (T,G)-A--L can be converted into an immunogen in low responders, inducing IgG antibody by delivering signal ① via the receptor-antigen interaction and signal ② via the associative antibody–histocompatibility determinant interaction, then the t cell cannot be "helping" via antigen presentation (Fig. 85). The system has uncoupled the normal recognition of antigen by both receptor and associative antibody.

In hindsight given the associative recognition model we should have predicted, but alas did not, that haptens (nonimmunogenic molecules) would be converted into immunogens via abnormal induction, e.g., Katz, Davie, Paul, and Benacerraf, *J. Exp. Med.*, **134**, 201, 1971. The prediction yet to be verified, that low molecular weight haptens, e.g., DNP-lysine, can paralyze, was made however (Cohn, "Immunological Tolerance," pg. 298, 1969. Academic Press, New York). In foresight, the extension will be that very low molecular weight substances as DNP-lysine will be made immunogenic, and thymus-independent antigens will be encouraged to switch from IgM to IgG synthesis if signal ② is delivered via abnormal induction. The use of monovalent interactions with receptor, e.g., DNP-lysine, coupled with abnormal induction, will permit a quantitative study of the inherent sensitivity of IgM and IgG B cells to induction, an assumption which lies as yet unanalyzed in considering the switch.

The fact that such precise experiments can be suggested and their outcome predicted is a rather nice justification of the associative recognition model.

B. Bretscher's Analysis of Generalized Autoimmunity

Bretscher has developed elegantly the concept of abnormal induction to explain the phenomenon of generalized autoimmunity (Bretscher, *Cell. Immunol.*, **6**, 1, 1973). His point is that when a new foreign surface determinant appears on a t cell, then under certain conditions abnormal induction occurring via recognition of this surface determinant by associative antibody induces t cells with anti-t cell specificity. Once this occurs the system cascades out of control to induce associative autoantibody to B cells and eventually B cells anti-self are induced, e.g., anti-RBC, antinuclear membrane, antinucleic acid, etc. Thus the immune system is sabotaged by abnormal induction so that the major somatic selective pressure is by self-antigens as in the case of NZB mice. It is then under-

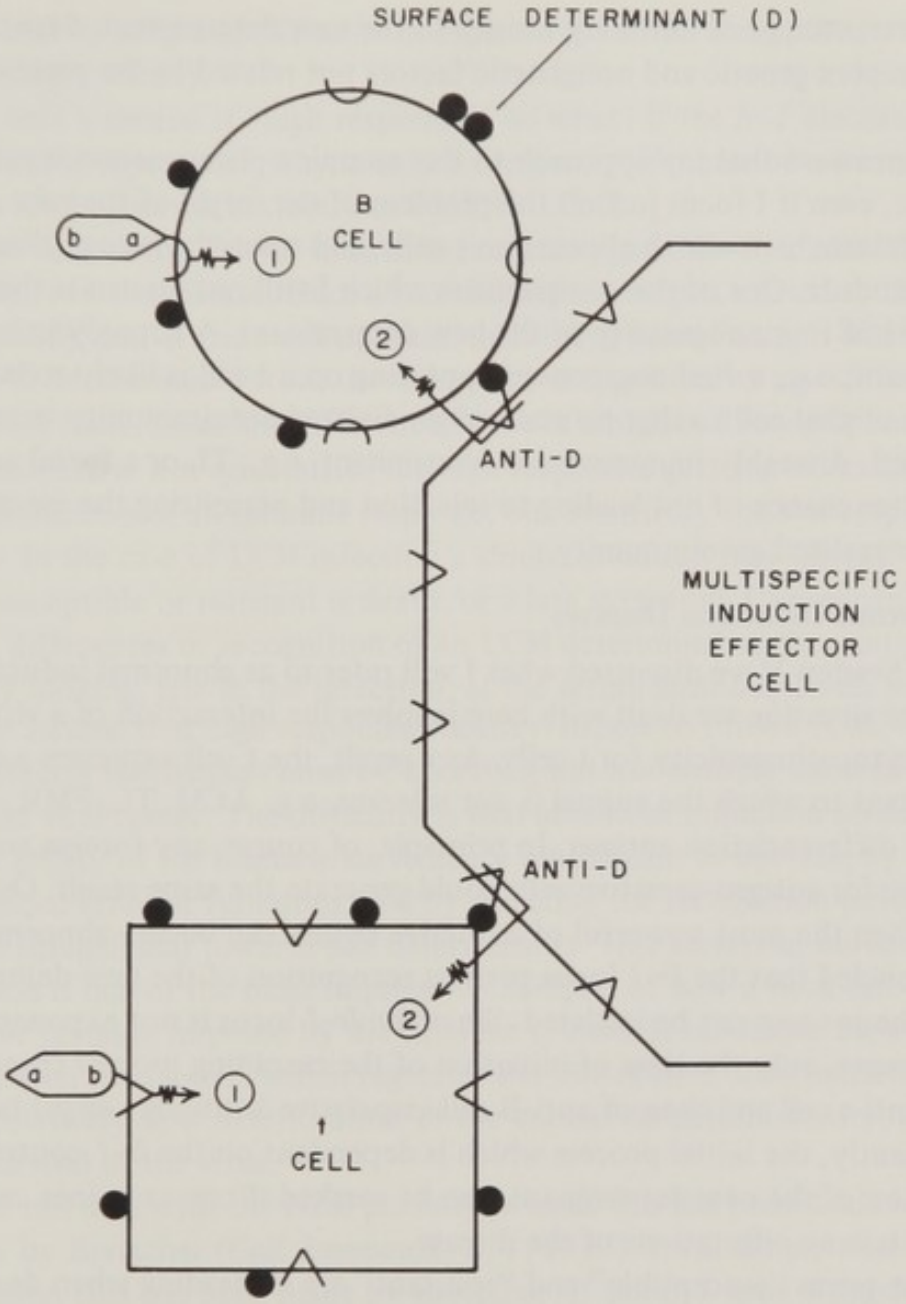

Fig. 85. The associative recognition model of *abnormal* induction.

standable that these animals respond better to certain self-like antigens, e.g., SRBC and BSA, than to very foreign antigens, e.g., KLH (Cerottini, Lambert, and Dixon, *J. Exp. Med.*, **103**, 1093, 1969). This is a good moment to point out that generalized autoimmunity via abnormal induction is an experimental proof that the self-nonself discrimination is not germ-line gene encoded, but acquired or learned.

The problem we faced in Session V is the origin of the new foreign t cell determinant and its recognition by the *Ir-1* coded associative antibody. The expression of a predominantly particular autoimmune response, e.g., anti-RBC,

antinuclear, etc., or of antibody mostly to the new determinant, depends on other complex genetic and nongenetic factors not related to the present discussion.

I am aware that my approach to this complex phenomenon is rather simplistic, even if I focus just on the problem of the origin of the new and foreign surface determinant which appears on t cells, and upon the recognition of it by an *Ir-1* product. One of the complexities which I will not discuss is the effect of the degree of immunogenicity of the new determinant. A strongly immunogenic determinant, e.g., a viral component appearing on a t cell is likely to lead to the rejection of that cell so that no state of generalized autoimmunity would be established. A weakly immunogenic determinant, e.g., TL or a foetal antigen, has a better chance of not leading to rejection and permitting the cascade towards generalized autoimmunity.

C. Abnormal Induction Diseases

In Session V we discussed what I will refer to as abnormal induction diseases. The situation we dealt with here involves the interaction of a virus of limited cytopathogenicity for t cells. As a result, the t cell expresses a surface determinant to which the animal is not tolerant, e.g., LCM, TL, FMR, G, or any foetal or differentiation antigen. In principle, of course, any foreign substance cytophilic for antigen-sensitive cells could generate the same result. Once this occurs, then the most powerful of inductive signals can occur—abnormal induction—provided that the *Ir-1* locus permits recognition of the new determinant, so that the process can be initiated. Since the *Ir-1* locus is not expressed usually as all or none, only the time of initiation of the cascading process (production first of anti-t cell and then of anti-B cell associative antibody) might be affected. Consequently, the initial process which is dependent on the *Ir-1* controlled recognition of the new determinant, can be masked if one examines carefully only the late manifestations of the disease.

The terms "susceptible" and "resistant" are misleading when dealing with *Ir-1* controlled effects. Since this locus determines associative antibody specificity uniquely, high level or affinity recognition would be dominant to low level or affinity recognition. It would therefore be assumed as likely that when "susceptible" is dominant to "resistant," "susceptible" means a high responder and "resistant," a low responder, and *vice versa.*

The dominance of susceptibility to LCM (Oldstone, Session V) is as expected, the result of a high responder allele of *Ir-1*. In the case of Friend virus, the situation is more complex as "susceptibility x resistant" crosses yield an intermediate phenotype (Lilly, Session V). In this situation either *Ir-1* allele could be the high responder equivalent. If resistance were dominant as Pincus (Session V) contends, the this *Ir-1* allele would be the high responder equivalent. (I am introducing a likelihood factor and turning around the more logical

approach used by Lilly and Oldstone in order to get a new insight into this complex phenomenon.)

The next question is "high responder" to what? If the *Ir-1* discrimination is for the viral antigen itself, why is only a marginal difference between strains seen at the level of serum antibody to FMR or LCM?

In the case of the Friend virus infection, the *Rgv-1* (*Ir-1*) allelic difference is revealed late in the disease. Once abnormal induction gets started so that associative antibody anti-B and t cell appears, then induction of an anti-FMR B cell response will be uncoupled from the obligatory recognition by associative antibody to FMR itself. Since the difference between a high and low responder *Ir-1* allele is quantitative not qualitative, the high responder gets started earlier in the abnormal induction of an immune response, but eventually the low responder catches up. In the case of LCM infection a similar situation may obtain. The assay for susceptible or resistant is death, or a late symptom. Consequently, initial *Ir-1* differences or recognition of an LCM determined surface antigen are not seen by looking, late in the infection, at the serum antibody levels to LCM.

If we assume that high responder means resistant to Friend virus, then the immune effector mechanism must be operating but not entirely successfully in rejecting the viral tumor. The difficulty is that *abnormal* induction tends to reduce the ability of the immune surveillance mechanism to operate, by deregulating balanced effector functions, and by directing the recognition potential away from foreign, and towards self determinants. This abnormal induction phenomenon is one of the most impressive examples of how a virus can escape the selective pressure imposed by the immune system. It sabotages the associative recognition mechanism which regulates the self-nonself discrimination. This permits a relatively slow deterioration of the animal via autoimmunity, and eventual survival of the virus.

I will not deal with the NZB phenotype since this has been dealt with beautifully by Bretscher (*Cell. Immunol.*, **6**, 1, 1973). I will simply extend one prediction that has been made by Bretscher. If the *Ir-1* gene is regulating the susceptibility-resistance phenotype, via recognition of a viral induced foreign surface determinant and subsequent abnormal induction, then the common feature should be the early appearance of antibodies to t cell antigens. This will prove to be an important diagnostic tool.

D. Other Views of How Tolerance to Self Is Broken

Many workers have noticed the relationship between viral infection and autoimmunity (e.g., Allison, Denman, and Barnes, *Lancet*, **II**, 135, 1971). However, these workers have assumed that the major (if not only) mechanism of the breaking of tolerance was "through a carrier effect" which "can stimulate B cells to produce autoantibody." Since they assume that the distinction between

"thymus-dependent" and "thymus-independent" antigens is *fundamental* and that associative recognition is not obligatory for *normal* induction, they miss the phenomenon of *abnormal* induction. Further they fail to explain the origin of self-tolerance; they assume it. Given the associative recognition model, if an antigen crossreacting with self is injected, a competition between the self and crossreacting nonself component is set up. Only under special conditions can one break tolerance this way. The experimental model (Benjamin, and Weigle, *J. Exp. Med.*, **132**, 66, 1970), shows that the breaking of paralysis is a function of the competition between the antigen used to paralyze, and the one used to break paralysis. The competition depends in part upon 1) an optimum degree of cross-reaction (neither too similar nor too unrelated, e.g., totally) and 2) the relative concentrations of the two components. Consequently, the breaking of tolerance via normal associative recognition is relatively easy if the self-component is present in trace amounts, e.g., a hormone like thyroglobulin or an idiotypic determinant, because the challenging crossreacting immunogen is administered at a concentration many-fold the self-component. This point, that the self-component competitively blocks the breaking of tolerance by the crossreacting antigen is constantly missed. If it were true that the breaking of tolerance only required contact with an antigen crossreacting with self, no animal could survive, because it lives in a sea of such antigens. It is because competition by the self-component is missed, that many superimposed or secondary mechanisms are invented such as "suppressor T cells" of autoantibody formation (Allison, *Lancet*, **II**, 135, 1401, 1971), or the survival in the animal of only very low affinity (nonreactive) anti-self antigen-sensitive cells which can be induced by "concentrated" antigen resulting from "helper" t cells interacting with the cross-reacting antigen. (Taylor, and Iverson, *Proc. Roy. Soc. Lond.* (B), **176**, 393, 1971). Incidently, generalized autoimmunity resulting from *abnormal* induction rules out this latter view on experimental grounds.

If the crossreacting component is an altered t cell surface due to the appearance of a new foreign determinant (viral or viral induced) the probability of *normal* as compared to *abnormal* induction of autoantibody to t cells will depend upon how massive the viral infection is. Both modes of induction, normal and abnormal, are favored over paralysis, as the number of t cells expressing the foreign determinant increases. However, the probability of breaking through and cascading towards generalized autoimmunity via abnormal, as compared to normal induction, is greater at the low levels of infection. The rates of the two processes approach each other as the level of infection increases. I am not arguing that tolerance to self cannot be broken by normal induction alone. I am simply pointing out that special conditions are required to manifest an autoimmune reaction by this mechanism, and these should be analyzed in each case. An important example to think about is the induction of systemic lupus erythematosus by procaine amide (Whittingham and Mackay, *Aust. Ann. Med.*, **4**, 358, 1970).

Although it is an aside, I would like to discuss the experimental example interpreted as breaking of tolerance by Green (Session V). The response in guinea pigs to limiting concentrations of a self-component, serum albumin, altered in various ways, is determined by the guinea pig equivalent of the *Ir-1* locus in a specific dominant fashion. The interpretation proposed by Green is that there exists in the responder a germ-line immune response gene, coding for the recognition by t cells of a determinant on native serum albumin. If this gene product is revealed in some way, e.g., by breaking tolerance, then antibody to the altered albumin can be induced in the responder (not the nonresponder), by cooperation between the t cells recognizing the native determinant and the B cells recognizing the hapten or altered determinant. This interpretation is internally contradictory. The breaking of tolerance, required to reveal the postulated Ir gene coding recognition of a self-determinant necessitates associative recognition of both the altered determinant or "hapten" and the native determinant by t cell associative antibody. This is the only way to induce the responder's t cells in which are expressed the postulated *Ir-1* gene product, anti-self-serum albumin. If t cells can recognize the altered determinant or hapten in order to cooperate in inducing associative antibody which breaks tolerance to the native determinant, then they must be able to cooperate in inducing B cells antihapten. Consequently, there is no reason to postulate t cell recognition of a native determinant for the induction of B cells anti-hapten. Further the difference between responder and nonresponder is seen only at the lowest challenging concentrations of altered albumin. The animal has a normal amount of native serum albumin (of the order of a gram), and the challenge of altered albumin in order to see a response difference is a microgram (10^6-fold difference). The massive level of the native component would block any breaking of tolerance to itself by the trace of crossreacting immunogen. Consequently, I would surmise that the Ir gene coding the response difference permits recognition of a determinant common to all the immunogens used. If it is unthinkable that the various alterations of serum albumin result in such a common foreign determinant (Green, Session V), two other explanations are possible. First, there might be an immunogenic substance present in the complete Freunds adjuvant to which the altered albumin is absorbed and which acts as a "carrier." Second, an immunogenic substance in the adjuvant might be absorbed on, or cytophilic for, the surface of antigen-sensitive cells which are now induced to produce antihapten via abnormal induction. This is the way that many lectins, e.g., Con A, PWM, etc., and bacterial products, e.g., LPS, probably act (see below, Section VI E).

E. Experimental Abnormal Induction Situations

There are a whole range of experimentally studied situations which operate via abnormal induction, besides the viral induced autoimmune and immune com-

plex diseases. These are the allogenic cell-induced neoplasias, the lectin-stimulated (PHA, Con A, etc.) and LPS-stimulated responses in t and B cells (see discussion by Bretscher, *Cell. Immunol.*, **6**, 1, 1973; Bretscher, *Transplant. Rev.*, **11**, 218, 1972). Clearly, if a foreign determinant is put on a t or B cell, e.g., lectins or LPS, the identical mechanism of abnormal induction can operate, as I discussed for certain viruses. The immunological behavior of lectins and LPS is, therefore, somewhat complicated by this, but in a way easy to understand. For example, LPS permits a "nude" spleen to respond to SRBC (Sjöberg, Anderson, and Möller, *Eur. J. Immunol.*, **2**, 326, 1972; Watson, Epstein, Nakoinz, and Ralph, *J. Immunol.*, 1972, in press), and a normal spleen to respond to a non-immunogenic substance (Schmidtke and Dixon, *J. Exp. Med.*, **136**, 392, 1972). If LPS were cytophilic for the B cell, then abnormal induction of anti-SRBC would occur as a consequence of the B cell receptor–SRBC interaction (Signal ①) and the associative antibody-LPS interaction (Signal ②). I recall that I have discussed the so-called thymus-independence of LPS which is due to the highly effective associative antibody recognition of it, compared to SRBC. "Nude" mice are not devoid of associative antibody, they are depleted so that the response to very foreign polymers (in particular LPS) is still discernable. In principle, the lectins and LPS are stimulating a response to antigens by a mechanism identical to that resulting from the injection of allogeneic cells.

I postulated a possible abnormal induction contribution to the MLC reaction (Section V C). Such a reaction would in part be x-ray and mitomycin resistant. Consider two cell populations, the responder and the target (mitomycin or x-ray treated); it is uniquely the incorporation of thymidine into DNA in the responding population that is under assay. The stimulation of DNA synthesis depends upon the recognition by the receptors in responding population of foreign determinants on the target. Three interactions can be envisaged to occur in the MLC.

A simple receptor–determinant interaction would be a paralytic signal ① . Whether the cell undergoes a division before being inactivated is unknown, but this interaction alone probably does not contribute much to the assay.

A normal inductive stimulation of the responding population might be delivered via normal associative recognition of determinants on the target cell. *In vitro* induction probably contributes significantly to the assay. However, since the level of the response of lymphocytes to target lymphocytes (MLC) is so much greater than that of lymphocytes to target erythrocytes, fibroblasts metabolically blocked target cells, e.g., formaldehyde treated, or simpler antigens, I am suspicious that the abnormal inductive mechanism also operates, i.e., *the target cell contributes more than just antigenic determinants to the induction of the responder cell.*

In order for abnormal induction to operate, the target cell must not only supply foreign determinants, but also some associative antibody against the

responder surface determinants. Mitomycin or x-rays probably do not affect the preculture level of associative antibody, which could leak from the target antigen-sensitive to the induction effector cell, or be already present on the latter. Thus the responder antigen-sensitive cell receiving signal (1) via its receptor interacting with any ligand in the medium, immunogenic (e.g., serum, target cells) or nonimmunogenic (e.g., sugars, peptides, lipids, self-components, etc.) would be induced, because signal (2) would be delivered via an associative antibody-surface determinant interaction. This might be a major effect.

The first reaction by some of you would be to point out that the MLC interaction between two parental cultures, one responder and one target, would include both normal and abnormal induction, whereas that between responder parental and target F_1 hybrid cells would show only normal not abnormal induction. Thus the contribution of each effect could be evaluated. The published data are too contradictory at the moment for me to sort out the results. However, I remind you of the Ramseier-Lindemann experiment in which F_1 hybrid cells do respond to idiotypic determinants on parental cells, and this response might initiate abnormal induction. Maybe the nonreproductibility of the results (parental versus F_1) is in part due to the variable contribution of abnormal induction.

F. Allison's Analysis of Tolerance in Allophenic Mice

The finding is that allophenic (tetraparental), but *not* F_1 hybrid mice demonstrate a detectable immune response to self-histocompatibility antigens assayed both in their ability to mount a "cell-mediated" killing reaction towards parental cells and to block this reaction with serum antibody (Wegmann, Hellstrom, and Hellstrom, *Proc. Nat. Acad. Sci.*, **68**, 1644, 1971; Phillips *et al.*, *Nature*, **234**, 146, 1971). This is often cited as the key experiment showing that balanced enhancement rather than a central failure of the immune system, mediates normal tolerance to self. We must face this experiment because it challenges our view as to how the *Ir-1* locus is expressed in regulating the self-nonself discrimination (Section III A) and it weakens the previously presented argument, that specific dominant responsiveness is *not* due to tolerance (Section I C). Allison, (*Lancet*, **II**, 1401, 1971), has rationalized this difference between allophenic (tetraparental) and F_1 hybrid mice in terms of two postulates:

- An antigen-sensitive cell cannot be induced to determinants on its own membrane.
- Histocompatibility antigens not present on each antigen-sensitive cell are tolerated because of *induced "autoimmune" reactivity* (blocking antibody to self or selective cell-mediated feedback killing of those antigen-sensitive cells which possess receptors specific for self histocompatibility determinants).

These two postulates apply to surface cell-bound antigens only; for soluble antigens they are inapplicable and Allison provides us with two other ground rules for these, with which I will not deal.

The F_1 hybrid antigen-sensitive cell population is homogeneous with respect to its surface antigens, and by the first postulate cannot be induced by them. The allophenic (tetraparental) antigen-sensitive cell population is a mixture of two antigenic types, so that the restriction imposed by the first postulate does not apply, and they mutually induce each other as stated in the second postulate.

The first postulate is too arbitrary, as can be seen by asking how the combining site of the antihistocompatibility receptor on an antigen-sensitive cell knows when it has interacted with a determinant which obeys the rule. The second postulate is simply a restatement of the fact of balanced enhancement, which is taken as the only mechanism of self-tolerance to histocompatibility determinants, (Since t cells have different surface antigens than B cells, this view requires, as a normal mechanism, balanced enhancement to prevent the two populations from mutual annihilation. However, this only creates the dilemma that balanced enhancement between t and B cells sabotages the self-nonself discrimination in general, and would lead inevitably to generalized autoimmunity). Experimentally, Wegmann *et al.*, showed that when a kidney fibroblast of the parental type is the target cell, allophenic but *not* F_1 hybrid or parental immune cells, killed it. Consequently the second postulate restating the fact that the mixture in the allophenic of the two parental types of antigen-sensitive cells are induced by each other, cannot be extrapolated (on experimental grounds) to explain normal self-unresponsiveness to the fibroblasts, for example, in F_1 hybrid or parental animals.

Given the associative recognition model, the hybrid is the normal, and the allophenic, the special case. The level of the autoimmune reaction in allophenics is extremely low. Since normally there is always competition between induction and paralysis, as any assay is pushed to greater and greater sensitivity, some level of induction to self-antigens, even in F_1 hybrids may be detected in the future. Balanced enhancement may be a second line of protection against autoimmune disease in special cases in adults because of the difficulty of establishing (dose-dependent) as compared to maintaining (dose-independent) tolerance. The normal physiological importance of balanced enhancement as a primary protective mechanism against autoimmune disease, has yet to be evaluated. Certainly in the case of adult, and even neonate chimeras or tumors which escape immune surveillance, balanced enhancement operates as a major mechanism to protect the tissue. In the case of normal development, I would estimate that it does not operate as a major mechanism.

Allison is correct in being impressed with the difficulty of rendering animals unresponsive to histocompatibility antigens. It is a curious fact that Medawar's renowned experiments on establishing "tolerance" to foreign skin

grafts, by injection of cells into neonates, were successful most often when the tissues used to tolerize were a good source of immune cells, e.g., spleen, lymph nodes, or bone marrow. Further, in many cases, it has been shown that the graft was not rejected because of balanced enhancement. This is not too surprising, since the injection of foreign antigen-sensitive cells into a neonate which cannot mount an efficient rejection of them, is ideal for the setting up of an abnormal induction situation from which autoimmunity or balanced enhancement ensues. Among the surviving animals in a Medawar experiment, are those which may be truly unresponsive, as well as those which manage to set up a proper steady state between induction of both blocking antibody and cell-mediated killing, and feedback inhibition of the induction of both, by the induced blocking antibody. The establishing of such a steady state depends upon the resultant of many complex interactions, which need no discussion at the moment. As interesting as this phenomenon is, it is not central to normal self-tolerance.

Consequently, allophenic (tetraparental) mice need a special explanation and many are possible. Hybrid mice become unresponsive to self as I described earlier (Section III A). The allophenic, induced at a low level to self, is behaving like an irradiated adult made chimeric.

The immune system developed from the cells of one parent in the allophenic, may become just sufficiently mature before encountering the cells of the other parent, so that it reacts to them as foreign. This implies that the allophenic, unlike the hybrid animal is not a truly homogeneous mixture of the two parental cell types. This category of explanation for the autoimmune responsiveness of the allophenic compared to the hybrid mouse, permits us to encompass this fact in the general phenomenology of immunology without making special rules for "soluble" as opposed to "insoluble" antigens.

Now let us apply this to an analysis of the "tolerance" model of specific dominant responsiveness (Section I C). The finding is that the allophenic mouse derived from *Ir-1* determined responder and nonresponder parents, is responsive.

If we admit that the postulated self-component which the test antigen resembles, is *not* expressed in allophenics, then the experiment does not test the "tolerance" model.

If we postulate that the self-component is expressed, but the allophenic mouse is responsive to it as shown by "balanced enhancement," then two different mechanisms must operate to determine specific dominant responsiveness to the test antigen.

- The responder parent and F_1 hybrid respond to the test antigen which is recognized as foreign, because the self-component which it resembles, is not expressed in either animal.
- The interpretation of the allophenic mouse and the nonresponder parent are in contradiction. The allophenic is postulated to respond, although the self-component is expressed, because, in fact, the animal is being

induced by it and is in a steady state of balanced enhancement. This state is upset by immunization with the test antigen which breaks "tolerance" to the self-component. The nonresponder parent, which is also postulated to express the self-component does not respond (unlike the allophenic which does respond), because it must be truly unresponsive as a central mechanism.

If the allophenic had been a nonresponder, tolerance models of specific dominant responsiveness would have been strongly favored. Since the result is that they are responders, the argument that the tolerance model is incorrect, is made with greater difficulty. Since we are led to a contradiction, if we assume that the major mechanism of normal self-tolerance is "balanced enhancement" rather than a central failure of responsiveness, the low level of autoimmunity in allophenics requires special explanations. Most of them would be based on the assumption that for one reason or another only a partial state of paralysis was established and this should be (*but is not*) seen in a comparison of the responsiveness of hybrids and allophenics to the test antigen, if the tolerance model were correct.

McDevitt has suggested a way to evaluate the effect upon responsiveness of the autoimmunity detectable in allophenic not F_1 hybrid mice. The experiment is to study the response in an allophenic made between two *Ir-1* determined nonresponders which differ at their H-2 loci. If, as expected, this animal is a nonresponder (like the F_1 hybrid between nonresponders) then the low level of autoimmunity present in allophenics cannot explain the responsiveness observed in the (responder x nonresponder) allophenics and the tolerance model of specific dominant responsiveness is ruled out. If the (nonresponder x nonresponder) allophenic is a responder then the interpretation is complicated because the low level of autoimmunity could make the animal hyperreactive (generalized responsiveness) to the weak "self-like" immunogen, (T,G)-A--L, in much the same way as NZB mice are hyperresponders to certain "self-like" antigens. In this case the tolerance model of specific dominant responsiveness would be left untested.

VII. IMMUNE DISEASE STATES IN MAN

My closing remarks deal with the studies on the human material and the importance of the associative recognition theory in dealing with it. I have stressed the problem of the way in which the genes which code for the specificity of the recognition molecules, determine responsiveness. Consequently, I have dealt with specific dominant responsiveness almost uniquely. The immune response locus most important in this respect is closely linked to the major histocompatibility locus. This locus determines, in part, at least, the specificity of associative antibody. I assume that a similar situation would obtain in humans. Most of the work

with human material has involved the genetics of histocompatibility antigens, not of the Ir locus. This work has been superb, for in fact it suggested for the first time, the possible two cistron nature of the major histocompatibility locus, Four and LA, which in mouse are K and D. However, the problem I have dealt with is the expression of the Ir locus, not the histocompatibility genes, K and D or Four and LA. Most workers, as I have pointed out, equate the *Ir-1* and the histocompatibility K and D or Four and LA genes so that the association between disease and HL-A type is interpreted to mean that "histocompatibility antigens may have *functional* properties important in determining susceptibility to disease" (Ellman, Green, and Martin, *Lancet*, I, 1104, 1970). I have argued that there is no reason as yet to equate the two. Bodmer (Session VI) in his probing analysis of the association between disease and HL-A, deals with the interpretation of the Ir gene control of susceptibility. Clearly the relationships are certain to be complex in human systems. My only contribution will be to stress the importance of abnormal induction.

It is probably largely via abnormal induction that the HL-A linked Ir-V_H^T locus plays a role in disease. The various reviews stress the association between HL-A type and leukemia, Hodgkins and autoimmune disease (Lupus erythematosis, chronic glomerulonephritis, etc.) (McDevitt and Bodmer, *Amer. J. Med.*, **52**. 1, 1972; Bodmer, Session VI.) As I have discussed above, given the genesis of generalized autoimmunity via abnormal induction these findings are not unexpected. Once a virus puts a foreign determinant onto a t cell without wrecking its associative recognition function, the animal is open to self sabotage via abnormal induction. This leads to autoimmunity, which in turn renders the immune surveillance mechanism ineffectual, permitting neoplastic transformed cells to establish themselves. Since in this situation the cells most stimulated to divide are lymphocytes, the probability of a viral or other transformation in this cell type is highest. Hence the preferential incidence of lymphomas and leukemias. Further, which particular syndrome of autoimmunity is manifested, i.e., the weakest target tissue, depends on other genetic and nongenetic influences.

The early sign of a cascading generalized autoimmunity via abnormal induction would be associative and effector antibody (lymphocytotoxic and cell-mediated) to the t cell itself. This latter antibody might provide today a very early diagnostic tool and if so, should be one parameter to associate with HL-A type. In the future associative antibody (idiotype and specificity) would provide a better parameter to study than the serum effector antibody.

Given this picture, it is clear that little can be done today about controlling, in a specific way, the Ir gene product expressed by the "susceptible" or high responder allele. However, once generalized autoimmunity begins to develop the associative recognition theory gives us one point at which the cascade can be stopped. If the function of associative antibody is blocked (by antiserum to it

or by inhibiting signal (2)) then the only outcome of an interaction of the t or B cell with antigen will be paralysis. Consequently, the normal state of tolerance will be reestablished. The regeneration of the immune system after establishing generalized tolerance will result in its being maintained. While such an approach opens great hope for dealing with autoimmune and immune complex disease, as well as transplantation problems, it could not be used to deal with a leukemia which had escaped an immune system sabotaged by abnormal induction. The result of the blocking of associative antibody function would be tolerance to the virus and associated foreign antigens. Other procedures must be added.

VIII. CODA

I have based my interpretation of the genetics on two conceptual formulations, the somatic mutation theory for the origin of diversity and the associative recognition theory for the expression of diversity. In order to sort through the complex phenotype resulting from a simple genetic event, we must have a coherent understanding of the origin and expression of antibodies.

I have no doubt that the general form of the associative recognition theory for the self-nonself discrimination will prove correct (Fig. 82). Upon interaction with antigen two signals are required to induce any antigen-sensitive cell, one transmitted via its receptor and the other via associative antibody. Only one signal transmitted via the receptor is required to paralyze. In order for associative antibody to function as the controlling element in the self-nonself discrimination, *normal* induction must involve the associated recognition of at least two determinants on the antigen. If this does not occur, as in the case of *abnormal* induction, the self-nonself discrimination cannot be made and autoimmunity results. The cell cannot tell whether the two determinants being recognized by receptor and associative antibody respectively, are on one antigen. It senses only the two signals delivered via each interaction. Consequently, under special circumstances, when one signal is delivered via the receptor-antigen interaction, and the other signal is delivered via an associative antibody-cell surface interaction, *abnormal* induction results. Such a large number of complex phenomena is understandable in terms of this simple concept of *normal* and *abnormal* induction, that it is a valuable one if for no other reason than to be able to discuss a complex of facts in an orderly way.

Since it is associative antibody which regulates the self-nonself discrimination, it is expected to be of a special class and made by a cell of a unique lineage. In order to minimize the danger of autoimmune accidents, associative antibody must be regulated at a low level and be short-lived in the absence of the cell making it. By contrast, effector antibody which mediates all of the protective functions of the immune system must be long-lived and regulated at a high level. Consequently, it comes as no surprise that experiment has identified associative

antibody as being thymus-derived (made by a t cell) and effector antibody as being bone-marrow-derived (made by a B cell). The two antibodies are probably immunoglobulins which share the same light chain subunits, kappa and lambda, coded by two unlinked loci. Their respective special functional properties are conferred by the heavy chain subunit, which in the case of associative antibody is coded by a locus, $V_H^T C_H^T$, closely linked to the major histocompatibility genes, and in the case of effector antibody is coded by an unlinked locus, $V_H^B C_H^B$, marked by a serologically identifiable polymorphic determinant (allotype). The assay for associative antibody is cooperating activity in induction and for effector antibody is a number of specialized activities, e.g., complement-lysis or cell-mediated reactivity. All classes of antibody, associative or effector, probably function after secretion, following induction of antigen-sensitive cells. Some of them arm third party effector cells such as the macrophage, basophil, or dendritic cell. We are made aware by the theory that the antigen-sensitive cell itself is unlikely to ever function as an effector cell by recognition of a target via its own receptor.

There are hundreds of germ-line genes which could determine responsiveness, either specifically or generally, and which are expressed recessively or dominantly. Specific dominant responsiveness, the only paradoxical case, is determined by structural germ-line genes which code the specificity of the receptors on antigen-sensitive cells, associative antibody on t cells, and effector antibody on B cells. These immune response (Ir) genes are the V genes, V_κ, V_λ, V_H^B and V_H^T. As a consequence of the asymmetry in the relationship between t cells and B cells (t is required for the induction of both t and B but not *vice versa*), the most often observed cases of genetically determined specific dominant responsiveness are due to the V_H^T locus but if searched for properly, the V_H^B locus will be just as frequently implicated. The linkage of the Ir-V_H^T locus to the two major histocompatibility genes (*H-2K* and *H-2D* in the mouse) is a useful experimental tool, but seems to have no unique functional significance.

Specific dominant immune response genetics must be explainable as a consequence of our understanding of the more general question of the origin of diversity. One way that a germ-line V gene determining specific responsiveness is established in a population, is by having its contribution felt sufficiently for the germ-line selection to act upon it. This suggests a minimized number of germ-line V genes which are selected upon in the germ-line, because they code for specificities of immediate survival value to the animal, but which can evolve further as a consequence of somatic mutation followed by antigenic selection. Thus, under this somatic mutation theory, the total capacity of the animal to respond, increases with the time of somatic selection from some minimum capacity coded in the germ-line, to some maximum capacity coded in the soma. Immune response genetics due to polymorphisms in germ-line V genes is one consequence of this process, because the probability that an animal could respond to a given

antigenic determinant in a reasonable time period, depends upon the minimum number of mutational steps that the corresponding antibody is from any one of the germ-line $V_L V_H$ combinations.

This conceptual framework is derived from the minimum obligations account for both the self-nonself discrimination and for the ability of the immune system to recognize a large number of different determinants. It is consistent with the fundamentals of our understanding of the mechanisms of gene evolution and expression.

There is such a thing as "how-not-to-think-about-the-immune-system." If you explain one fact by one theory, or if you ignore these above minimum obligations, then the basic origin of apparently disparate phenomena is masked, a special jargon is generated which prevents us from communicating across observation-boundaries, and we are left with no way to be suspicious. For example, the literature is replete with theories of either induction or tolerance, proposed without a consideration of what determines the decision between them. Since in the absence of a conceptual framework, we take "facts" at face value or as self evident, it is no wonder that we are handcuffed to such fuzzy (right or wrong) ideas as "thymus-dependence and independence," "cooperation and killing are two sides of the same t cell coin," t cell receptors are more "primitive" (less specific) than B cell receptors, "paralysis is mediated solely via effector antibody," " associative recognition is a 'helper' device to concentrate, trap, or topologically arrange antigen," "etc." I am not dealing with the question of whether a given conceptual framework is right or wrong. The one I discussed here is for me the most likely, not a unique solution. Rather I am insisting that we consider one which enables us to understand a maximum number of facts (the self-nonself discrimination being one), to be wary, for rational reasons, of those which we do not understand and to glean an insight into the working of the immune system which goes beyond experiment. It is only in this way that at each of these conferences we will not have to solve over and over again by trial and error, the same basic problem every time that it crops up under a different "nom de plume."

ABBREVIATIONS

ALS	antilymphocyte serum
ANA	antinuclear antibody
Au	Australia antigen
BCG	Bacille Calmette-Guerin
BGG	bovine gamma globulin
BSA	bovine serum albumin
BUdR	5-bromodeoxyuridine
cAMP	3′, 5′ cyclic adenosine monophosphate
CML	cell mediated lympholysis
Con-A	concanavalin-A
CRL	complement receptor lymphocytes
DNCB	dinitrochlorobenzine
DNP	dinitrophenyl
DTH	delayed type hypersensitivity
Fab	Ig fragment with one antigen binding site; one L chain and amino-terminal half of H chain
$F(ab')_2$	two Fab fragments attached to each other
Fc	carboxy-terminal halves of two H chains from same Ig molecule
FMR	Friend, Moloney, Rauscher common virus antigen
GVH	graft-versus-host
Hb	hemoglobin

H-2	the major system of mouse histocompatibility antigens
HL-A	the major system of human leucocyte antigens
HSA	human serum albumin
HGG	human gamma globulin
Ig	immunoglobulin
IgA, IgE, IgG, IgM	standard nomenclature for human-globulin classes; also used here to name analogous proteins in other species
IudR	5′-iododeoxyuridine
KLH	keyhole limpet hemocyanin
LCM	lymphocytic choriomeningitis
LD	lymphocyte defined antigen (allogeneic difference in lymphocytes)
LDV	lactic dehydrogenase virus
LLV	lymphocytic leukemia virus
LLV-F	helper virus which naturally accompanies SFFV in the Friend virus complex
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIF	macrophage inhibitory factor
MLC	mixed lymphocyte culture
MLV	Moloney leukemia virus
NIP	3-nitro-4 hydroxy-5 iodophenylacetic acid
PCT	plasma cell tumors
PFC	plaque forming cells
PHA	phytohemagglutinin

PLL	poly-L-lysine
POL	polymerized flagellin of *Salmonella adelaide*
poly A:U	polyadenylic acid: polyuridylic acid
PPD	purified protein derivative, an extract of *M. tuberculosis*
PWM	pokeweed mitogen
RFC	rosette forming cells
SD	serologically defined antigen (allogeneic differences in lymphocytes)
SRBC	sheep red blood cells
SFFV	spleen focus forming virus, part of the Friend virus complex
SLE	systemic lupus erythematosus
Theta	isoantigen expressed on murine thymus-derived lymphocytes
TMV	tobacco mosaic virus
TNP	trinitrophenyl

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A further notable development dealt with by this conference is the recognition that lymphocytes express antigenic configurations responsible for the *in vitro* mixed lymphocyte culture reaction which are distinct from all those thus far defined serologically. The genes for the antigens inducing the mixed lymphocyte reaction, the major histocompatibility antigens, and immune response genes thus comprise a closely linked series controlling several important cell surface recognition phenomena.

The complex array of genes (including *H-2*-linked genes) which affect susceptibility to murine viral leukemogenesis were explored in detail. Finally, the emerging associations between histocompatibility antigens and several human diseases were reviewed in the light of the preceding discussions. While continuing a distinctive style of scientific communication, this final Brook Lodge Immunology Conference differed markedly from its four predecessors: they had dealt with assessment of major bodies of *established* information, whereas this conference additionally brought together a large amount of *new*, unpublished data on several exciting recent developments. The implications of these discoveries for antigenic recognition and the generation of diversity (possibly the two most challenging issues in immunology), and the prospect of finally perceiving a rational basis for individual susceptibility to an extensive spectrum of disease states, notably viral oncogenesis and autoimmune disorders, were explored in considerable depth. In addition, the principles of population genetics and the evolution of linked loci were applied to the experimental data in a lucid and highly informative manner.

As the scientific product of this conference, this latest volume in the PERSPECTIVES IN IMMUNOLOGY series reflects the forefront of our knowledge of the genes controlling the specificity and regulation of the immune response. These momentous developments in immunology and genetics are, in a way, analogous to the ushering in of the era of molecular biology by the microbial geneticists in the 1950's. This new understanding will have much to tell us about the underlying character of host susceptibility to various disease states; by uniting a great body of information under a single conceptual framework, begin to dispel the frustration of attempting to cope with what had so long been hopelessly complex.