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TRANSPLANTATION GENETICS OF PRIMATES

Hans Balner

Jon J. van Rood

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Introduction

THIS VOLUME CONTAINS the proceedings of the first histocompatibility workshop and symposium for primates held September 1971 in Rijswijk, The Netherlands. There were of course reasons to choose this time and place. The time seemed appropriate because of an increasing use of subhuman primates in transplantation research and since tissue typing is a prerequisite for meaningful transplantation research, several groups of investigators started tissue typing various primate species on their own, each using their own methods, reagents, and nomenclature.

The history of HL-A, the major histocompatibility system of man, has taught us that only workshops can solve the problems of reagent identification, definition of antigens, and international agreement on nomenclature. A workshop implies that the investigators with experience in tissue typing of a particular species will convene to type the same panel of individuals with their own reagents and compare results with obtained by others. The best location for a workshop is clearly the site where the blood samples of unrelated individuals as well as members of pedigreed families can be obtained. Proper typing facilities and sophisticated computer equipment and staff are required. It is for those reasons that the Rijswijk/Leiden combination was chosen as the location for the first primate histocompatibility workshop. The department of immunohaematology at Leiden had the experience of organizing a human histocompatibility workshop in 1965 and the required computer facilities and know-how, and the primate center TNO at Rijswijk, only 15 miles away, had the required animals and a long-standing interest in tissue typing of Rhesus monkeys and chimpanzees.

Thus, about 20 investigators from nine laboratories came to Rijswijk with their reagents. Within 2 wk, 142 rhesus monkeys (including 55 children of pedigreed families) and the 60 chimpanzees of the Rijswijk colony were typed for leukocyte and red cell antigens, using many different techniques. In all, about 800 reagents were used and their reaction patterns compared. During the 2 days separating the symposium from the workshop, the computer team spent night and day working out the raw data, making it possible to present a preliminary analysis on the second day of the symposium.

The first part of this volume contains the papers presented on the first day of the symposium. These contain no information gained during the workshop, and each group still used its own nomenclature. The Joint Report contains a preliminary analysis of the workshop data and some of the conclusions reached during the discussion of the results. Agreement was reached to recognize the RhL-A system of rhesus monkeys as the major histocompatibility system of this species. Specificities for which control by allelic genes had been proved previously were assigned their original name. Specificities that were reasonably well defined by groups of at least three sera, preferably from different laboratories, obtained workshop designations (RhW rather than RhL-A until proved to be "specificities" of an allelic series of RhL-A). Specificities defined by individual groups after the workshop will henceforth be indicated with symbols indicating the laboratory of origin and an arbitary sequence number.

For chimpanzees with no family data as yet available, most discussants felt that it was wiser to assign workshop designations only. Specificities clearly defined by groups of at least two different chimpanzee or human sera were assigned ChW symbols. Whenever convincing evidence was avail-

able that a chimpanzee specificity was the analogue of an established human specificity, the relevant HL-A sequence number was adopted (for example, ChW-4, ChW-6, and ChW-11 for the analogues of 4a, 4b, and HL-A11, respectively; 4 and 6 are the provisionally assigned HL-A numbers for 4a and 4b). All other chimp specificities were given new ChW designations with sequence numbers unrelated to previously published group designations. A detailed description of the groups and a list of equivalent designations of specificities by the various groups of investigators can be found in the Joint Report.

We would like to thank our many collaborators for their invaluable help and dedication. They are too numerous to be listed by name, but we would like to mention specifically the members of the teams who started bleeding the animals at 7 a.m., the cell processors who provided everyone with purified lymphocyte suspensions (20–30 animals per day) at around 10 a.m., the computer team who worked through many a night to run and analyze intricate programs, our secretaries, chauffeurs, and members of the catering service whose continuous efforts were the basis of the success that this first primate workshop turned out to be.

H. Balner
J. J. van Rood

Tissue Typing of Rhesus Monkeys: Application in Transplantation Research

By H. Balner, H. Dersjant, W. van Vreeswijk, and B. W. Gabb

THE STUDY OF PRIMATE LEUKOCYTE ANTIGENS is one of our major research projects. Initially, leukocyte typing of rhesus monkeys was used primarily as a tool to determine chimerism in irradiated, bone-marrow-treated animals. When a larger number of typing reagents became available, groups of sera with similar reactivity patterns were selected and the first leukocyte groups established.[1] The sera defining the first groups were of rather broad specificity but could be improved by absorptions.[2] Subsequently, typing reagents of higher specificity were produced, primarily by planned immunizations. When 12 groups of antisera were available, a population analysis suggested that the identified specificities were controlled by one genetic system. This assumption was supported by segregation studies in families while results of intersib skin grafting and preliminary data of mixed leukocyte cultures proved that this was the rhesus monkey's major histocompatability system, called RhL-A.[3] Numerous new typing reagents have since been produced, mostly by planned immunizations. Some of the newly defined specificities had a low frequency in the population and were included in previously established groups of broader specificity. Suggestive evidence was recently obtained for the existence of two series of allelic genes within the RhL-A system,[4] but this hypothesis still remains to be proved.

In this paper we present serological data, the phenotypic distribution of currently identifiable specificities, and their associations. The influence of genotypic identity for RhL-A was assessed by intersib skin grafting, and the value of matching for phenotypic identity was assessed by skin, organ, and bone marrow transplantation between unrelated individuals. In accompanying papers, an up-to-date analysis of the RhL-A system is presented by Gabb et al.[5] while results of mixed lymphocyte cultures in relation to RhL-A are described by Appelman et al.[6]

PRODUCTION AND SELECTION OF TYPING REAGENTS

Methods of raising antisera have been reported previously.[2] Rejection of skin grafts from two or three donors (selected for appropriate RhL-A differences in relation to the recipient) can lead to satisfactory antibody formation, but optimal sera are obtained if this is followed by one or two booster injections of whole blood or separated leukocytes. Sera are considered interesting if screening for cytotoxicity with lymphocytes from unrelated monkeys yields less than 25% strongly positive reactions and few weak ones. Optimal reagents are obtained when sibs differing for one haplotype are cross-immunized.[4] Attempts to reproduce such highly specific reagents are not always successful. More often than not, extra antibodies of partly unknown spe-

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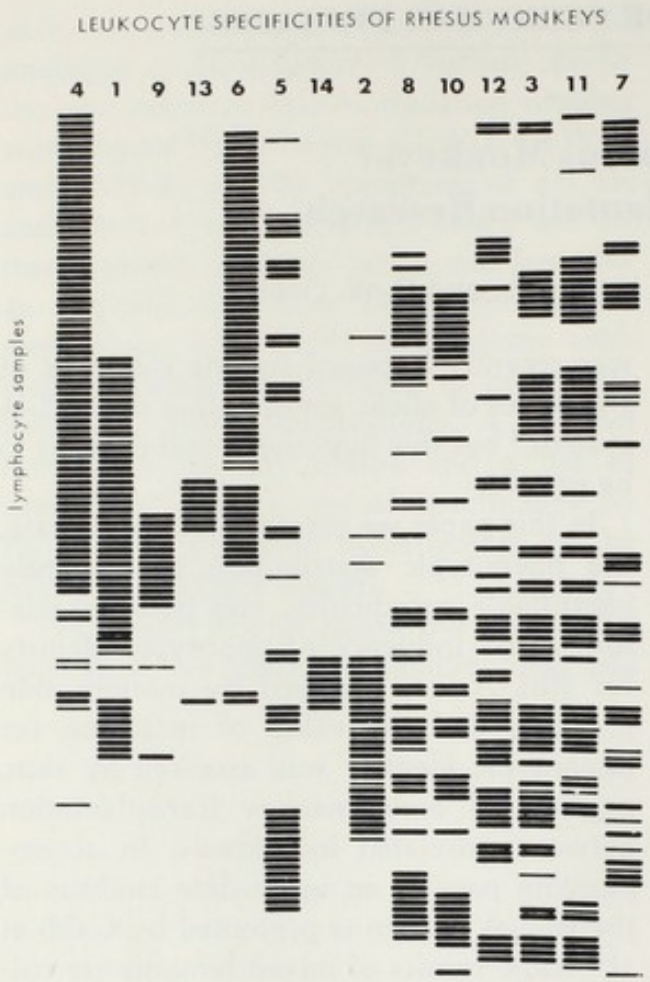

Fig. 1. Reactivity patterns of 14 groups of Rhesus isoantisera with 126 unrelated rhesus lymphocyte samples (microcytotoxicity). Bold bars: strong reactions of most sera of particular group. Thin bars: weak or partly negative reactivity.

cifity are found on reimmunization. However, there were a few animals, the sera of which maintained the original high specifity even after repeated reimmunization and harvesting. Sera were lyophylized for storage and used undiluted (cytotoxicity titers ranged from 1/8–1/32).

Typing results were analyzed by computer, and reagents with similar reactivity patterns were grouped together and considered to define provisional specificities.[7] New sera are added each year. Thus, reagents defining broad specificities are gradually replaced by groups of sera of higher specifcity. In selected cases, sera are fractionated by absorptions.

A slightly modified version of Kissmeyer-Nielsen's microcytotoxicity test, with a reproducibility of about 95%, was used.[8] Recently several of the reagents showed distinct reactivity patterns in the complement fixation (CF) test. We intend to use this advantageous method in routine typing.

DISTRIBUTION OF RhL-A SPECIFICITIES

Until early 1971, a panel of 60 isoantisera, defining 14 specificities or groups, was used. Figure 1 shows the distribution of these 14 groups on the lymphocytes of 126 unrelated Rhesus monkeys. A genetic analysis of typing results for nearly 200 unrelated monkeys and segregation patterns in rhesus families was presented by Gabb et al.[5] Results obtained with new reagents were included in that study. Figure 2 shows several associated specifcities that were gradually identified in past years. Groups

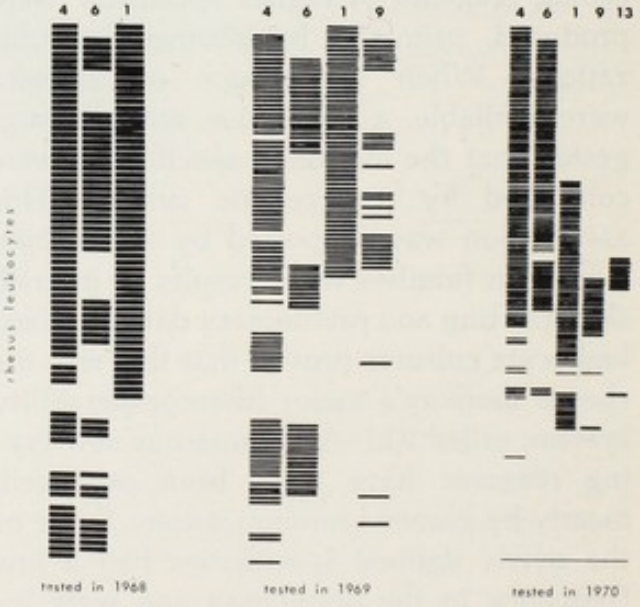

Fig. 2. Distribution of several associated leukocyte specificities in three populations of unrelated Rhesus monkeys. Numbers above columns indicate specificities (shown in Fig. 1) defined by three to six sera each. Horizontal bars: cell samples reacted positively with most sera of group. Proportion of positives for some groups became smaller in consecutive years. Reagents of broader specificity were replaced by reagents of narrower specificity.

6 and 1 were always positively associated with the broad specificity 4 (virtually "included"), while the newer groups 9 and 13 seem included in group 1. These five specificities show associations similar to those described for several families of associated antigens of HL-A.[9-11] The optimal sera defining groups 6, 10, and 13 seem to be operationally monospecific (Table 1); some of those for groups 9 and 14 contain extra antibodies to antigens of rather low frequency; and sera defining the other groups have not yet been thoroughly analyzed by absorptions. Continuation of these investigations should soon reveal the degree of heterogeneity of the sera and cross-reactivity for various antigens.

Table 2 lists the CF reactivity of several Rhesus isoantisera against platelets from 36 rhesus monkeys. When about 50 sera defining the 14 groups (cytotoxicity, Fig. 1) were screened, only 16 sera showed a distinct reactivity pattern. It can be seen that several of the group 4, 6, and 12 sera had a CF reactivity pattern included in that for cytotoxicity. Surprisingly, 9 other sera showed CF reactivity patterns positively

Table 1

ABSORPTION ANALYSIS OF SERA DEFINING **RhL-A** GROUPS **13**, **10** AND **6**

(micro cytotoxicity)

reactivity of serum 113 (group 13)		after abs. with leukos							reactivity of serum 57 (group 10)		after abs. with leukos							reactivity of serum 26 (group 6)		after abs. with leukos						
test cells	unabsorbed	329	1811	888	Z	AE	G	1140	test cells	unabsorbed	1164	N	1001	GG	769	823	1056	test cells	unabsorbed	329	388	1164	294	422	BF	AC	BI	1821
329	***	⊙	-	-	-	(+)	-	-	1164	***	⊙	-	-	-	-	-	-	329	***	⊙	-	-	-	-	-	-	-	-
1811	***	-	⊙	-	-	-	-	-	N	***	-	⊙	-	-	-	-	-	388	***	-	⊙	-	-	-	-	-	-	-
888	***	-	-	⊙	-	-	-	-	1001	***	-	-	⊙	-	-	-	-	1164	***	-	-	⊙	+	**	-	-	**	-
Z	***	-	-	-	⊙	-	-	-	GG	***	-	-	-	⊙	-	-	-	294	***	-	-	-	⊙	-	-	-	-	-
AE	***	-	-	-	-	⊙	-	-	769*	***								422	***	-	-	-	-	⊙	-	-	-	-
G	***	-	-	-	-	-	⊙	-	823*	***								BF	***	-	-	-	-	-	⊙	-	-	-
1146*	***								1056*	***								AC	***	-	-	-	-	-	-	⊙	-	-
579	***	-	-	-	-	-	-	-	1009	***	-	-	-	-	-	-	-	BI	***	-	-	-	-	-	-	-	⊙	-
BM	***	-	+	-	-	-	-	-	498	***	-	-	-	-	-	(+)	-	1821*	***									
KK	***	-	-	-	-	-	-	-	324	***	-	-	-	-	-	-	-	657	***	-	-	-	-	-	-	-	-	-
DN	***	-	-	-	-	-	-	-	728	***	-	-	-	-	-	-	-	579	***	-	-	-	-	-	-	-	-	-
589	***	-	-	-	-	-	-	-	388	***	-	-	-	-	-	-	-	672	***	-	-	-	-	-	-	-	-	-
426	***	-	-	-	-	-	-	-	376	***	-	-	-	**	-	-	-	BT	***	-	-	-	-	-	-	-	-	-
852	***	-	-	-	-	-	-	-	583	***	-	-	-	-	-	-	-	DA	***	-	-	-	-	-	-	-	-	-
ZZ	***	-	-	-	-	-	-	-	AH	***	-	-	-	-	-	-	-	598	***	-	-	-	-	-	-	-	-	-
BT	***	-	-	-	-	-	-	-	DN	***	-	-	-	-	-	-	-	600	***	-	-	-	-	-	-	-	-	-
DA	***	-	-	-	-	-	-	-	BT	***	-	-	-	-	-	-	-	703	***	-	-	-	-	-	-	-	-	-
AX	***	-	-	-	-	-	-	-	AX	***	-	-	-	-	-	-	-	888	***	-	-	-	-	-	-	-	-	-
599	***	-	-	-	-	-	-	-	381	***	-	-	-	-	-	-	-	1745	***	-	-	-	-	-	-	-	-	-
598	***	-	-	-	-	-	-	-	599	***	-	-	-	-	-	-	-	1748	***	-	-	-	-	-	-	-	-	-
730	***	-	-	-	-	-	-	-	493	***	-	-	-	-	-	-	-	1754	***	-	-	-	-	-	-	-	-	-
1746	***	-	-	-	-	-	-	-	730	***	-	-	-	-	-	-	-	1746	***	-	-	-	-	-	-	-	-	-
1745	***	-	-	-	-	-	-	-	600	***	-	-	-	-	-	-	-	1747	***	-	-	-	-	-	-	-	-	-

* animal not available for testing

related to each other but utterly independent of their cytotoxic reactivity (sera of groups 5, 7, 8, 13, and 14 of Fig. 1). As soon as sufficient data are available, a population analysis and family study will be done to detect the place of these new specificities with regard to the RhL-A system.

Another serological issue is the presence of leukocyte or tissue antigens on red cells. Previous studies indicated that some leukocyte antigens may well be similar to antigens carried by erythrocytes.[12] In collaboration with our group, Dr. P. T. Sullivan of Madison, Wisconsin, is currently typing large numbers of unrelated monkeys and members of pedigreed families for the antigens of established red cell systems.[13] Comparison of these data with the results of leukocyte typing should reveal whether linkage exists between any red cell system and RhL-A.

There is the question of similaries between RhL-A specificities and leukocyte antigens of other primate species including man. Data regarding this issue will be presented by Dersjant et al.[15] and van Rood et al.[14] Obtaining firmer answers to some of the relevant questions requires an elaborate program of absorbing rhesus sera with other primate cells and sera from other primate species with Rhesus cells.

RELEVANCE OF RhL-A FOR HISTOCOMPATIBILITY

Skin Grafting

A limited number of experiments was performed using sibs as donors and recipients. Genotypic RhL-A identity was assumed on the basis of identical reactions with nearly all available antisera and assignment of the same parental RhL-A haplotypes. In a few cases the number of informative children per family was insufficient for reliable haplotyping.[3] Figure 3 shows that identical combinations had a significantly longer survival time. These results corroborated our assumption of

Table 2

REACTIVITY OF RHESUS ANTISERA AGAINST RHESUS CELLS

Comparison of cytotoxic (CT) with complement fixing (CF) reactivity patterns of Rhesus antisera

RHESUS MONKEY CELLS	RhL-A groups: 4		6		12		7 13 8 14 14 5 5 13
	technique: CT	CF	CT	CF	CT	CF	complement fixation
	sera: 17,19	19	26,65,24,25	65,24	61,12	61,12	34 113 38 108 99 107 22 106 109
954	+++	+++	+++	+++	902 +++	+++ +++	902 +++ +++ +++ +++ +++ +++ +++ +++ +++
746	+++	++	+++	+++	747 +++	+++ +++	554 +++ +++ +++ +++ +++ +++ +++ ++ ++
657	+++	+++	+++	+++ +++	542 +++	+++	1746 +++ +++ +++ ++ +++ +++ +++ + +
294	+++	++	+++	+++ +++	640 +++	+++	583 ++ + +++
1748	+++	++	+++	+++ +++	857 ++	+++	422 +++ +++ ++
1433	+++	+++	+++	+++ +++	880 +++	+++	1753 +++ +++
1205	+++		+++	+++ +++	825 +++	+++	846 +++
747	++		+++	+++ +++	989 +++	+++	1434 +++
701	+++		+++	+++	1418	++	823 +++
1164	+		+++	+++	657 +++		989 +++
857	++		+++	+++	422 +++		1748 +++
1221	+++		+++	+++	954 +		1750 +++
1747	+++		+++	+++	918 ++		640 +++
388	+		+++		1434 +++		880 +++
422	+		+++		1753 +++		980 +++
583	+				1001 +++		
321	+++				422 +++		
546	+++				1433 +		
640	++						
303	+						
1418	+						
902	+++						
846	+						
988	+++						
1749	+						
1750	+++						
1811	+++						
542							
823							
825							
880							
918							
434							
989							
1753							
1001							

RhL-A being the major histocompatibility locus.[4]

In skin grafting experiments performed using unrelated host/donor combinations, the question was whether phenotypic identity or near-indentity (incomplete phenotypic identity or IPI) would influence graft survival. Table 3 shows that there was a tendency for IPI combinations to show longer survival times. In other experiments, ALS was simultaneously tested for its potency, causing longer graft survival times and more marked differences between the survival of grafts from various donors but similar results. Attempts are currently being made to find out which RhL-A disparities lead to significantly shorter survival times, which RhL-A specificities are the "strong" transplantation antigens.

Organ Grafting

In collaboration with Marquet, Heystek, Dicke, van Bekkum, the influence of phenotypic host-donor identity was assessed for orthotopic kidney grafts, and techniques and preliminary results were published.[16] Initially, identity was based on typing for 12 RhL-A specificities, and no immunosuppresive therapy was applied. It was found that IPI combinations were among the longest survivors. Figure 4 shows that three of ten IPI combinations showed a longer survival time, yet about 70% of the IPI combinations showed rejection times within the normal range (no immunosuppression). Current experiments in which IPI selection is based on identity for 14 or

Table 3. Influence of RhL-A Matching on Skin Allograft Survival in Rhesus Monkeys Without Immunosuppression

Host/Donor Combinations	Graft Rejection on Day 8	Day 9	Day 10	Day 11
Identical	1	2	3	2
Not Identical	5	4	1	—

Mean survival of identicals: 9.8 days (± 0.36); mean survival for nonidenticals: 8.6 days (± 0.22); $t_{16} = 2.7$; $p < 0.02$.

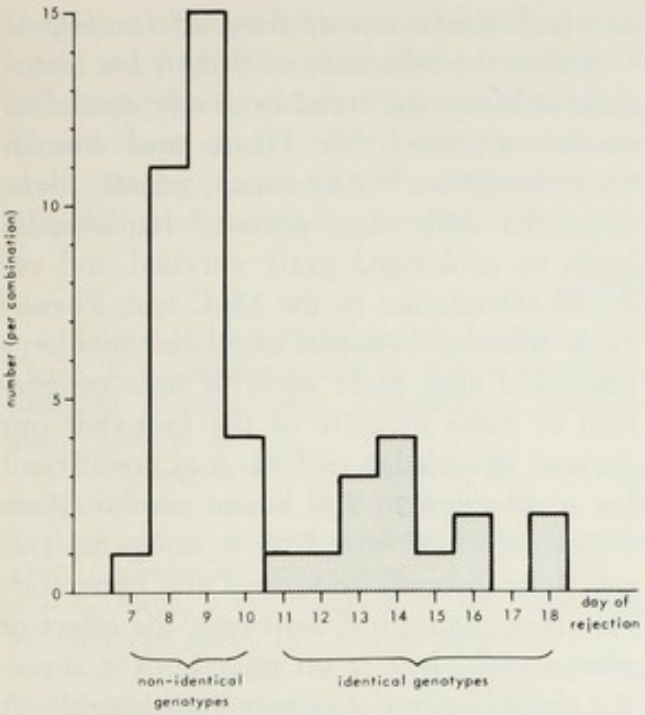

Fig. 3. Skin graft survival times between siblings of rhesus families genotyped for RhL-A. Shaded areas: host/donor combinations with identical RhL-A genotypes (mean survival, 14.3 ± 0.6 days). Empty areas: combinations for one or both parental haplotypes (mean survival, 8.7 ± 0.2 days).

more RhL-A specificities seem to confirm the previous results. Survival times for IPI combinations given standard immunosuppressive treatment (ALS plus Imuran) have so far not been found to be different from those for poorly matched combinations given the same immunosuppressive therapy. In collaboration with the Department of Surgery of Leiden University, a program of orthotopic and heterotopic heart transplantation is in progress, but the influence of RhL-A matching on survival has not yet been assessed.

Bone Marrow Transplantation

Although bone marrow grafting in monkeys has been an active research program of this institute since 1960,[17] the influence of RhL-A matching on the severe immunological complications (GvH reaction) has not been investigated previously. Because of the time-consuming selection of identical couples, the currently available data are limited. IPI combinations were chosen from unrelated individuals and grafting was done by the standard method (4×10^8 bone marrow cells per kilogram given i.v. 24 hr after lethal total body irradiation). The results of pilot experiments in which IPI was based on identity for 12 RhL-A specificities were disappointing. If no measures were taken to mitigate the severe GvH reaction, the outcome was not influenced by phenotypic identity between host and donor. More recently, experiments were done with stricter criteria for identity (14 or more RhL-A specificities) and applying optimal treatment to mitigate GvH reactions (stem-cell separation[18] and ALS treatment of the recipient prior to grafting[19]). The first experiments gave encouraging results: GvH reactions seemed to be milder although long-term survivals have as yet not been obtained. This program is being persued with the highest priority.

DISCUSSION

The study of RhL-A and its relevance for histocompatibility is important for a num-

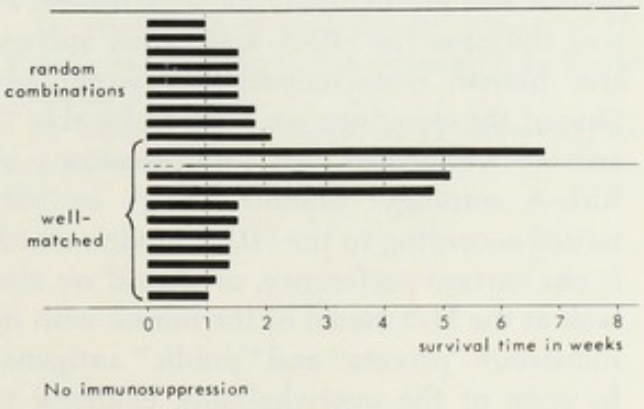

Fig. 4. Influence of leukocyte matching on kidney transplantation in rhesus monkeys. Each horizontal bar represents survival time of one animal. Surviver for 7 wk was found to be well-matched by retrospective analysis. Selection of other well-matched combinations was based on prospective typing. Mean survival for random combinations: 11 days (± 2.65). Mean survival for well-matched combinations: 18 days (± 14.4); $t_{17} = 1.57$; $p < 0.1$.

ber of reasons. First, the rhesus monkey is one of the few species for which information regarding the major histocompatibility system (MHS) has reached the stage of antigen definition and formal genetic analysis. Second, we are dealing with a primate species, which implies that the genetic organization of RhL-A is probably similar to that of HL-A. It is therefore reasonable to assume that data regarding relevance for histocompatibility are more readily applicable to clinical situations than data obtained with mice or rats. Third, now that we have reached a certain understanding of the genetic makeup of RhL-A, questions such as a possible linkage between the MHS and the genetic system governing the immune response, the phylogeny of the major histocompatibility systems of mammals, and numerous other issues will lend themselves to meaningful investigations in a species closely related to man.

Together with the contribution by Gabb et al.[5] and Appelman et al.[6] we have attempted to present a picture of our current incomplete knowledge of RhL-A. A few weeks from now our understanding of RhL-A will undoubtedly have increased, as was the case for HL-A after each successive human histocompatibility workshop. One of the questions we hope to be able to answer concerns the interpretation of RhL-A serology: Should RhL-A be patterned according to the HL-A model, which is our current preference, or should we also look at the H-2 model of the mouse with its numerous "private" and "public" antigens? In spite of the overwhelming evidence in favor of the HL-A approach (complex antibodies reacting with simple, single antigens or two linked loci), several geneticists regard the current HL-A interpretations as oversimplified.[20,21] Unfortunately, many problems that plague the analyzers of HL-A (broad specificities splitting up into narrow inclusions, cross-reactivity, the reaction-negative-absorption-positive phenomenon) are already with us and create as much confusion as they do for HL-A.

As for the relevance of RhL-A for histocompatibility, the trend is clearly similar to results obtained for HL-A and human transplantation.[22] As in man, genetic identity (sibs with same parental haplotypes) leads to prolonged graft survival and reduced stimulation in the MLC test. Phenotypic identity between unrelated monkeys improved skin graft survival in a proportion of cases in spite of the fact that our current knowledge of RhL-A is unsuffcient for a selection of full-house combinations (recognition of four RhL-A antigens, two per chromosome). In kidney and bone marrow transplantation, however, the effect of phenotypic identity on rejection (or severity of GvH reactions) was questionable. A similar lack of correlation between survival of human cadaver kidney grafts and HL-A matching has been reported by some investigators,[23,24] while others have clearly demonstrated that HL-A matching significantly improves human cadavar kidney graft survival provided that potential mismatches were taken into account and identity accepted only if all four HL-A antigens were ascertained.[22] Regarding the organization of the major histocompatibility system as well as its relevance for histocompatibility, it would seem that results obtained in Rhesus monkeys show a striking similarity with the human situation.

SUMMARY

The current state of tissue typing in rhesus monkeys is reviewed. Serological characteristics of the reagents defining the mostly complex antigens of the RhL-A system are described and the influence of matching host/donor combinations for those antigens are assessed by skin, organ, and bone marrow transplantation. Graft survival was unequivocally prolonged if skin was exchanged between identical sibs. However, phenotypic identity between unrelated individuals was of questionable value. Skin grafts showed marginal pro-

longation of survival times, and kidney and bone marrow transplantation was barely influenced by similar host/donor matching. This is best explained by our present inability to recognize all the antigens of the RhL-A system so that potential incompatibilities are still overlooked. The striking similarity between the described role of RhL-A for histocompatibility in monkeys and the known role of HL-A in human transplantation is emphasized.

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Genetics of RhL-A System of Rhesus Monkeys

By B. W. Gabb, A. Piazza, J. d'Amaro, and H. Balner

THE LEUKOCYTE ANTIGENS of rhesus monkeys have been studied in Rijswijk since 1965,[1] and an increasing number of rhesus isoantisera have been prepared and studied in unrelated individuals and in laboratory bred families. An analysis of population and family data recently led to the suggestion that 12 groups of rhesus leukocyte antisera were acting against antigens controlled by a single complex genetic locus.[2] The data for three antibody groups (9, 6, and 10) were compatible with the hypothesis that these groups detected the products of a series of multiple alleles similar to the LA or Four series of the HLA system found in man. Since our earlier report, typing of random monkeys has continued using the same modification of Kissmeyer-Nielsen's lymphocyte microcytotoxicity method.[3] In this paper we shall consider the implications of the data obtained this year from testing 198 animals with up to 96 antisera.

The results of each typing was given a computer score from 0 to 5 depending on the intensity of the reaction. For instance, a laboratory recording of — corresponded with a computer score of 0 and $< 5\%$ of "killed lymphocytes;" (+) was 1 and 5–10%; + was 2 and 10–20%; ++ was 3 and 20–50%; +++ was 4 and 50–80%; ++++ was 5 and $> 80\%$. A score of 3 or more was considered positive, while 2 or less was considered negative. Duplicate tests on 32 animals with 62 antisera indicated that the repeatability of the positive or negative classification was 89%. The usefulness of particular sera as typing reagents was assessed from the proportion of doubtful reactions (scores of 2 and 3). Obviously, more reliance can be placed on results with sera where the proportion of doubtful reactions is low. The classification of sera according to proportion of doubtful reactions (Table 1) indicates that most of the antisera used were not of exceptionally high quality. However, about half of the reagents may be considered useful as they gave reactions in the doubtful range with less than 20% of white cells from random rhesus monkeys. Sera with similar reactivity patterns were classified into 14 groups, each containing two to four sera. It was then possible to classify each tested animal for the reaction of the group, using a scale similar to that used to record the serum reactions. Scores 2 or 3 were used when the group could not be assigned with certainty due to weak or discordant reactions of the sera involved (Table 2). A classification of our scores indicates that the definition of some of the groups is probably more precise than the definition of others. The data for each of the 91 paired comparisons of the groups were tested against the hypothesis that the groups were independent of or controlled by a series of allelic genes similar to the ABO blood group system of man. Table 3 contains a summary of this analysis. Since each group (except group 3) has both significant positive and negative correlations with at least one other group, it would appear that most of the reactions might be attributed to a single complex system.

To analyze the system further, the group data were examined by a computer program that tested all combinations of groups, three and four at a time, for compatibility with control by a multiple allelic system. Any combination giving more than 2% triple positive animals was rejected. The

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Table 1. Rhesus Leukocyte Isoantisera Classified According to Ratio of Doubtful Reactions to Total Reactions

Ratio	Doubtful to Total Reactions			
	<0.1	<0.1 but <0.2	>0.2	Total
Doubtful positive				
<0.2	6	7	0	13
Total positive				
>0.2	13	18	52	83
			Total	96

printout indicated the observed and expected numbers for any combination with less than 2% of triples together with χ^2 for agreement between the data and the hypothesis. Of the 364 possible combinations of 14 groups taken three at a time, 126 combinations had a frequency of triplets less than 2%, and 42 of these combinations had a χ_3^2 for goodness of fit less than 7.815 ($p > 0.05$). Nine of these combinations had no triplets at all. Analysis of the group data taking four groups at a time indicated that 21 combinations had a frequency of triple positive cells less than 2%. Data for 11 of these combinations were compatible with control by a series of multiple alleles, but no series had a complete absence of triplets (Table 4). Most of the acceptable combinations included the low-frequency groups 13 and 14 separately or together. The only other combinations of groups that were compatible with the population data were the series 9, 6, 2, and 9, 6, 10. Examination of the family data indicated that there were two maternal haplotypes in which 6 + 2 was inherited in coupling. No such arangement has been found for the combinations 9 + 6, 9 + 10, or 6 + 10, and the present data are in agreement with our previous publication.[2]

To extend this series of alleles, comparisons with the activity of individual sera were made. The most promising comparison was with a serum L2 (from Group 5). Data for the series 9, 6, 10, L2 contain no triply positive animals and fit the expected distribution satisfactorily ($\chi_6^2 = 8.3$, $p > 0.2$). The addition of a modified group 12 (considered present only when both of

Table 2. Rhesus Leukocyte Groups Classified According to Ratio of Doubtful Allocations to Total Allocations

Group	Strength of Reaction						Ratio of Doubtful to Total	Ratio of Doubtful + to Total +
	0*	1†	2‡	3§	4‖	5¶		
14	179	0	1	0	2	13	0.005	0.0
10	144	0	1	4	13	34	0.03	0.08
13	169	0	3	4	6	11	0.04	0.2
6	115	5	3	5	16	52	0.04	0.07
2	145	3	5	8	18	18	0.07	0.18
9	133	6	7	8	14	30	0.08	0.15
5	139	6	7	10	13	22	0.09	0.22
11	94	6	5	18	22	51	0.12	0.20
12	99	4	9	18	23	43	0.14	0.21
1	94	8	6	22	30	38	0.14	0.21
3	49	9	14	25	32	68	0.19	0.20
7	58	13	10	34	43	40	0.22	0.29
4	56	7	21	23	32	59	0.22	0.22
8	79	5	20	30	31	33	0.25	0.32

*Negative with all sera of group.

†Weak reaction with one or two sera defining group.

‡Classification doubtful, probably negative for group.

§Classification doubtful, probably positive for group.

‖Strong reaction with most sera of group.

¶Strong reaction with all sera of group.

Table 3. Two-by-Two Analysis of 14 Rhesus Leukocyte Groups Tested on 198 Unrelated Animals

Group	4	1	9	13	6	5	14	2	8	10	12	3	11
1	+												
9		+											
13	+	+	0										
6	+												
5	—	—	0	0	—								
14	0	0	0	0		0							
2	0	—	—	0	—		+						
8			—		—	+							
10	0		0	0	—		0		+				
12			—	0	—			+	—	—			
3				0			0				+		
11	+	+		0		0	0	—		0		+	
7				—			—			—		+	+

+: Positive correlation between groups ($p < 0.01$); occurs 15 times.

−: Negative correlation between groups ($p < 0.05$); compatible with control by allelic series ($p > 0.05$); occurs 17 times.

0: No significant correlation between groups but compatible with control by allelic series ($p > 0.05$); occurs 21 times.

Blank: No significant correlation ($p > 0.01$); incompatible with control by allelic series ($p < 0.05$); occurs 38 times.

the group 12 sera give a 4 or 5+ reaction together) also shows a fair fit ($\chi_{10}^2 = 13.5$, $p > 0.10$; Table 5). However, there were two triple positive animals in this series. It is currently, our opinion that the slight excess of 9, 6 heterozygotes may be due to the presence of an additional antibody in most of the group 9 sera. As this extra antibody can be removed by absorption, it is hoped that group 9 will be shorter and

Table 4

	χ^2	Number of Triple + ve Animals
Sets of three		
9-13-5	3.37	0
9-13-14	6.28	0
9-6-2	5.43	0
9-6-10	6.30	0
9-14-3	7.72	0
13-5-14	3.72	0
13-14-11	3.45	0
13-14-7	2.51	0
13-2-11	2.21	0
Sets of four		
9-13-5-14	8.90	1
9-13-14-10	8.30	2
9-6-14-10	11.78	2
9-14-10-12	12.42	3
13-5-14-11	5.63	3
13-5-14-7	4.73	3
13-14-10-11	7.22	3
13-14-10-7	6.45	3

Table 5

9	6	10	L2	(12)	Number Observed	Number Expected
+	+	—	—	—	17a*	11.49
+	—	+	—	—	10b†	7.74
+	—	—	+	—	2	3.05
+	—	—	—	+	7	5.81
—	+	+	—	—	9	11.26
—	+	—	+	—	4	4.43
—	+	—	—	+	5	8.45
—	—	+	+	—	5	2.99
—	—	+	—	+	3	5.69
—	—	—	+	+	3	2.24
+	—	—	—	—	16	23.90
—	+	—	—	—	38	37.35
—	—	+	—	—	24	23.33
—	—	—	+	—	7	8.28
—	—	—	—	+	21	16.81
—	—	—	—	—	27	32.50
					Total 198	

*Includes one triply positive animal 9 + 6 + (12) +.

†Includes one triply positive animal 9 + 10 + (12) +.

The following gene frequencies were observed: 9: 0.1413; 6: 0.2054; 10: 0.1384; L2: 0.0545; (12): 0.1039; blank: 0.3565.

$\chi_{10}^2 = 13.51$; $p > 0.10$.

Table 6

13	2	11	Number Observed	Number Expected
+	+	—	5	2.42
+	—	+	6	5.29
—	+	+	13	12.07
+	—	—	9	12.27
—	+	—	26	29.52
—	—	+	72	71.63
—	—	—	67	64.81
		Totals	198	198.01

The following gene frequencies were observed: 13: 0.0518; 2: 0.1181; 11: 0.2580; blank: 0.5721. $\chi_3^2 = 4.3$; $p > 0.2$.

the fit will be better in future studies. The leading reagents defining groups 6, 10, and 13 seemed operationally monospecific. Absorption data for other groups are not yet complete.[4]

It is interesting to note that of the groups remaining, data for the series 2, 11, 13 contained no triples and were compatible with the hypothesis of control by a series of alleles (Table 6). This leads to the speculation that the rhesus leukocyte groups are controlled by two series of closely linked alleles perhaps similar to the HL-A system described in man.[5] The leukocyte groups of three rhesus families showing segregation are given in Table 7. Since the same animal was father of families B and C, it can be seen that 6 and 2 travel together in coupling in one of the maternal haplotypes of family C. Unfortunately, the other maternal haplotype is not available in the combination a/d to determine whether group 6 is absent as theory leads us to expect.

The degree of serological clarity shown in Table 7 was not found in all families. To assess the likelihood that the reagents are detecting a single system, we examined the data from 29 families with 88 children for double back-cross matings between the pairwise combinations of groups 9, 6, 10, 2, 13, and 11. The $z_1 + c_1$ corrected scores[6] calculated at $\Theta = 0.05$ for each pair of groups are shown in Table 8. It can be seen that the family data are not sufficient to prove that any pair of groups belong to a complex system or that they are closely linked, although, a Z score obtained by pooling families that were double backcross matings with respect to either 9, 6, or 10 and 2, 13, or 11 was 3.7, indicating the existence of a complex system ($p < 0.001$). The magnitude of the Z score is probably conservative as families containing members such as CR (Table 9) were scored as if

Table 7. Family Data

	9	6	10	13	2	11	Haplotype
Family A							
male 590	—	—	+	+	+	—	ab
female 580	+	+	—	—	—	+	cd
Children							
WW	+	—	+	+	—	+	ac
CV	—	+	+	+	—	—	ad
EI	—	+	—	—	+	—	bd
Haplotypes							
a	—	—	+	+	—	—	10, 13
b	—	—	—	—	+	—	?, 2
c	+	—	—	—	—	+	9, 11
d	—	+	—	—	—	—	6, ?
Family B							
male 598	—	+	—	+	—	+	ab
female 834	—	—	—	—	+	—	cd
Children							
UU	—	—	—	—	+	+	ac
BD	—	—	—	—	+	+	ac
CS	—	+	—	+	—	—	bd
EK	—	—	—	—	+	+	ac
Haplotypes							
a	—	—	—	—	—	+	?, 11
b	—	+	—	+	—	—	6, 13
c	—	—	—	—	+	—	?, 2
d	—	—	—	—	—	—	?, ?
Family C							
male 598	—	+	—	+	—	+	ab
female 728	—	+	+	—	+	—	cd
Children							
KK	—	+	—	+	+	—	bc
AS	—	+	—	—	+	+	ac
EP	—	+	+	+	—	—	bd
Haplotypes							
a	—	—	—	—	—	+	?, 11
b	—	+	—	+	—	—	6, 13
c	—	+	—	—	+	—	6, 2
d	—	?	+	—	—	—	?, 10, ?

Table 8. Z Scores Between Rhesus Groups From Double Backcross Families at $\theta = 0.05$

Group	9	6	10	2	13
6	1.3				
10	*	−0.1			
2	*	−0.9	−1.2		
13	*	2.1	2.2	0.5	
11	0.5	2.1	0.9	*	2.75

*No information.

Z score greater than 3 is necessary to establish linkage. Z score less than −2 is evidence against linkage.

Table 9

	Sera Group 6						Sera Group 10			
Male										
600	3	+	2	2	+	+	+	+	+	+
Female										
669	—	—	—	—	—	—	—	—	—	—
Children										
AC	+	+	2	3	+	+	—	—	—	—
BI	+	+	3	3	+	N	—	—	—	—
CR	2	3	2	2	+	N	—	—	—	—
EL	—	—	—	2	2	—	+	+	+	+

+: Definite positive reaction.
—: Definite negative reaction.
3: Doubtful; probably positive.
2: Doubtful; probably negative.
N: not tested.

they contained a recombinant. If the weak reactions obtained with cells from CR are interpreted as positive, the family is compatible with the hypothesis that a complex locus controls the antigens reacting with these groups of antisera. It is our opinion that six of the 49 children in double backcross matings that gave reactions discordant with complete linkage cannot be used to estimate the recombination fraction as the serum reaction patterns were ambiguous for those animals.

Segregation ratios for backcross matings ($+ \times —$) with at least one negative progeny were calculated according to the method outlined in Maynard-Smith et al.[7] Except for group 2, the ratio of + : — was not significantly different from the Mendelian expectation of 0.5. Children from $— \times —$ matings were almost always negative for the group (Table 10). Data for segregating intercross families have not been included as the numbers for each comparison were small.

SUMMARY

The reaction patterns of seven groups of antisera with 198 random rhesus monkeys and 88 children from 29 families were analyzed. The data support the hypothesis that the reagents detected the products of a single complex genetic locus, possibly similar in structure to the HL-A locus of man.

Table 10

Backcross Families				Proportion of Negative Children Allowing for Small Family Size ± SE	Number of Children From H−X− Matings	
Group	Families	Children +	Children −		+	−
9	5	6	10	0.58 ± 0.14	0	59
6	9	13	15	0.50 ± 0.12	0	33
10	15	22	28	0.50 ± 0.09	0	17
13	10	14	19	0.54 ± 0.10	0	31
2	12	10	27	0.71 ± 0.08*	0	12
11	15	17	30	0.60 ± 0.08	1	11

*Deviation from expectation of 0.5 significant at 0.05.

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Mixed Leukocyte Cultures in Rhesus Monkeys

By A. W. M. Appelman and H. Balner

THE DEVELOPMENT IN THE FIELD OF HISTOCOMPATIBILITY TESTING of rhesus monkeys has progressed considerably over the last few years. Recently evidence has been obtained that the recognizable leukocyte antigens of rhesus monkeys are probably governed by one genetic system called RhL-A.[1] As an addition to serological typing of rhesus monkeys, we considered it useful to develop the mixed lymphocyte reaction for this species. Pilot experiments performed by Lina and Dicke of this laboratory in 1969 demonstrated that the mixed leukocyte cultures (MLC) test in rhesus monkeys is feasible. However, it also became clear that several parameters of the experimental procedure would have to be carefully investigated to make the MLC in monkeys a reproducible test. In the present paper, a number of the technical variables influencing the outcome of the MLC test have been carefully studied, and the influence of RhL-A identity on the degree of MLC stimulation has been investigated in related and unrelated animals. As in other species, it was found that within the families a clear-cut relation existed between genotype identity and low stimulation in the MLC reaction. If unrelated individuals were used, phenotypic identity sometimes resulted in a lower degree of stimulation.

MATERIALS AND METHODS

Feral rhesus monkeys were imported from India in several shipments. To the best of our knowledge, these animals were not related although a degree of inbreeding within each shipment cannot be excluded. The pedigreed families have been raised in this institute since 1965.

Blood was obtained from the femoral vein in heparinized vacutainer tubes (Becton-Dickinson, 100 × 16 mm, 145 USP). Granulocytes were removed by incubation of the blood with carbonyl iron powder during 15 min at 37° in a shaking water bath. The blood was filtered through a nylon mesh to remove aggregates and diluted with 3 volumes of Hank's Eagle's (HE) culture medium.

To separate the lymphocytes, 20 ml of the diluted blood was layered over 10 ml Ficoll-Isopaque.[2] The tubes were spun at 20°C for 15 min at 650 *g* (2100 rpm). Cells were collected from the interphase layer with a pasteur pipet, and the suspension was diluted with 3–4 volumes of HE and centrifuged at room temperature for 10 min at 300 *g* (1400 rpm). The cell pellets were washed twice by resuspension in HE and centrifuged under the same conditions. They were suspended in HE supplied with 20% rhesus monkey serum to give a concentration of 10^6 leukocytes/ml. Lymphocyte recovery varied from 20 to 40%. The final cell suspension contained between 2 and 10% granulocytes. The viability of the cells was greater than 90%.

The cells were cultured in a total volume of 2 ml in 15 ×100 mm disposable glass tubes. Cells from both animals were always present in equal numbers (1×10^6 each). The tubes were closed with tightly fitting rubber caps and maintained upright at 37°C. Where indicated, 15 μl phytohaemagglutinin (Burroughs-Welcome) was added.

At the appropriate time after initiating the cultures (routinely after 4.5 days), 20 μl HE medium containing 1 μCi ^{3}H-thymidine (specific activity 15 mCi/mM) was added and incubation was continued for a period of 24 hours at which time they were removed. Subsequently, the cells were agitated on a Vortex mixer and washed with 3 volumes of ice-cold saline containing 2% acetic acid. The pellets were broken on the Vortex mixer and resuspended in 5 ml 5% trichloro-acetic-acid (TCA). After three further washings with TCA, the pellets were dried, dissolved in Nuclear Chicago-Solubilizer (0.6 *N* solution in toluene) and counted after the addition of 15 ml toluene scintillation fluid.

For family studies, the two-way stimulation method was used. All other experiments were done with both the two-way and the one-way

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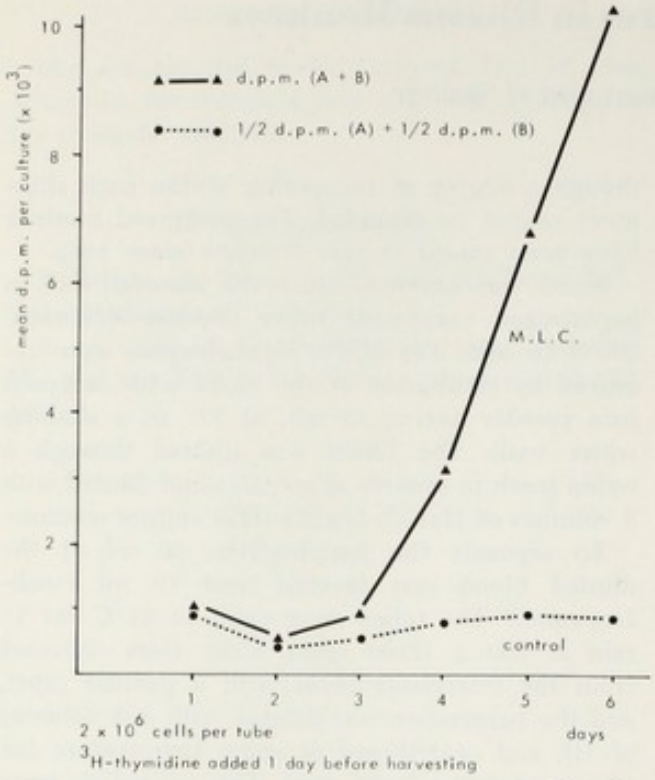

Fig. 1. MLC in rhesus monkeys. Thymidine incorporation on different days of culture.

stimulation technique as described by Bach and Voynow.[3] To inhibit DNA synthesis, the stimulating cells were treated with mitomycin-C. The final concentration was 12.5 μg mitomycin-C per 5–10 × 10^6 leukocytes.

Each MLC test was performed in triplicate and repeated once and sometimes twice on different days. Some of the repeat studies were performed with a slightly different microtechnique* (3 × 10^5 lymphocytes per ml per tube, ^{14}C thymidine to measure DNA synthesis).

The results of all experiments were expressed as total number of dpm per tube. Since variations between triplicate cultures were usually small, an average value was given. In the two-way MLC, 2 × 10^6 cells of both monkeys cultured separately served as a control for background incorporation. In the one-way technique, cultures of cells from monkey A and its own mitomycin-treated leukocytes were used as controls for reactivity of A against B.

To check the influence of phenotypic identity in unrelated monkeys, the stimulation index AB_m/AA_m was calculated for each combination and the deviation from the mean index of incompatible pairs was determined. To test the reactive capacity of the cells, phytohemagglutinin (PHA) was often used as the stimulating antigen.

*Courtesy of Dr. V. C. Eysvoogel, Amsterdam.

RESULTS

To find a practical routine method for the measurement of in vitro cell reactivity, several different culture conditions had to be assessed. Variables that were investigated include the source of serum to be used, the thymidine concentration, the culture time, and the number of cells per culture. Of the different sera tested (fetal calf, calf, human AB, and rhesus monkey), monkey serum gave the lowest control incorporation values and the highest MLC values and was chosen for further experiments. The optimal thymidine concentration for 24-hr incorporation was determined with the method described by Schellekens[4] and proved to be 16 μg/ml. At this concentration, incorporation was linear over the incubation period.

Figure 1 shows ^3H-thymidine incorporation in MLC on different days of culture. The reactivity of the cells was measured on 6 subsequent days with thymidine present during the last 24 hr. As the difference between MLC and controls was good enough after 5½ days, it was decided to take this culture time as a standard. When PHA was added, the cultures were maintained during 3 days. ^3H-thymidine was present during the last 24 hr. A linear relationship between the number of leukocytes and the thymidine incorporation was found with

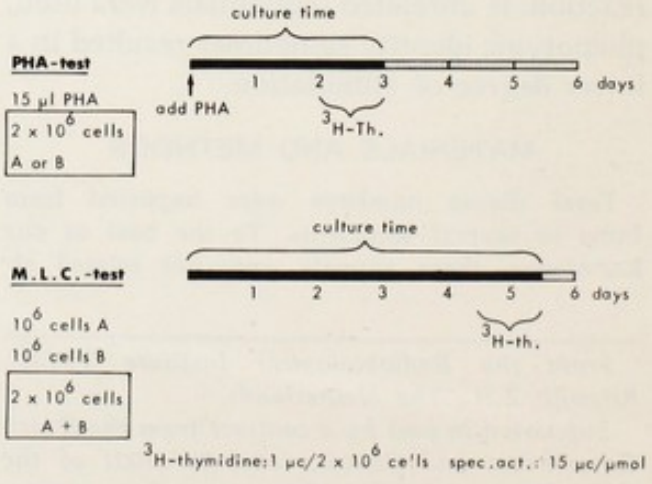

Fig. 2. Standard procedures.

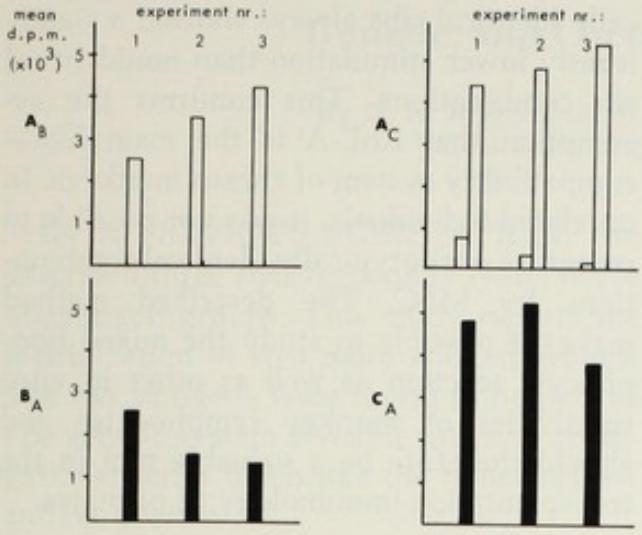

Fig. 3. Reproducibility of MLC.

cell numbers of 1, 1.5, and 2 million per tube. Routinely, 2×10^6 cells per tube were cultured. Figure 2 shows the final experimental design of routine MLC and PHA cultures.

Typical results of MLCs between unrelated individuals are shown in Fig. 3. Experiments 1, 2, and 3 were performed at monthly intervals. In the upper part of Fig. 3, A reacts against different stimulators B and C and though there are variations from experiment to experiment in each experiment, B stimulated less than C. In the lower part of Fig. 3, the same stimulating cells are cultured with different reactor cells, and the same holds true: in each experiment C reacted stronger than B. The small bars represent incorporation values when A was cultured with its own mitomycin treated cells A_m.

It has been shown for humans that MLCs between individuals phenotypically identical for HL-A, as a whole, show a somewhat lower stimulation than MLCs between nonidenticals.[5] However, in individual cases it was not possible to select unrelated identicals on the basis of MLC reactivity. This was ascribed to minor HL-A differences or to reactivity against non-HL-A antigens.

To test whether phenotypic identity would influence the degree of stimulation in monkey MLC, phenotypically identical couples were selected on the basis of RhL-A typing.[1] Cells from these animals were tested in one- and two-way MLCs against each other and against third-party cells from a donor with a totally different RhL-A phenotype. Table 1 shows that in identical combinations nine of 16 pairs gave a low stimulation index of less than 50% of the mean (against five of 16 for nonmatched pairs). A high stimulation index (greater than 100% of the mean) was found in four identical and six nonidentical combinations.

Figure 4 shows usual result of two-way MLCs performed with cells from siblings of pedigreed families. Genotypic identity was assumed on the basis of identical reactions with all available antisera and assignment of the same parental haplotypes (chromosomes). As expected, mixtures of genotypically identical siblings showed a degree of stimulation similar to that of controls, whereas higher values were obtained when mixtures from nonidentical siblings were used. No attempt was made to investigate whether stimulation was different if cells from sibs differing for one or for two haplotypes were cultured together.

SUMMARY

A reliable technique to measure the reactivity of rhesus monkey lymphocytes in mixed lymphocyte cultures is described. Within families, MLCs between genotypi-

Table 1. Unidirectional MLC Testing in Unrelated Rhesus Monkeys; Influence of Phenotypic Identity

Stimulation Index (% of Mean)	Incompatibles	Identicals
< 50	5	9
50–100	5	3
> 100	6	4

Values expressed as percent of mean stimulation index of incompatibles. Stimulation index: AB_m/AA_m.

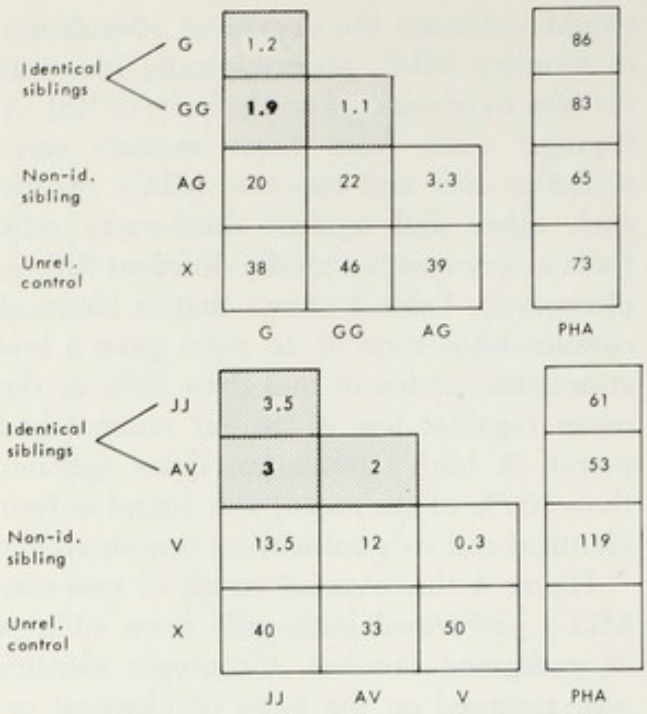

Fig. 4. Two-way MLC reactions between rhesus monkey siblings; influence of identity for RhL-A. Figures represent dpm $\times 10^{-3}$

cally identical sibs always showed a significantly lower stimulation than nonidentical sib combinations. This confirms the assumption that RhL-A is the main histocompatibility system of rhesus monkeys. In unrelated individuals, it was not possible to recognize phenotypically identical combinations by MLC. The described method makes it possible to study the mixed lymphocyte reaction as well as other in vitro reactivities of monkey lymphocytes and should therefore be a valuable tool in the transplantation immunology of primates.

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Rhesus Lymphocyte Alloantigens. II. Serologic, Genetic, and Chemical Characteristics

By G. N. Rogentine, Jr., C. B. Merritt, L. A. Vaal, E. B. Ellis, and C. C. Darrow, II

IN A PREVIOUS PUBLICATION,[1] we described three rhesus monkey lymphocyte alloantigen groups. This study reports the identification of two more antigen groups. The five antigens were found to be part of one genetic system divisible into two segregational series much like the human HL-A antigen system.

Solubilization of the rhesus antigens was achieved by papain digestion of spleen cells. The resulting product was found to be quite similar in amino acid composition to both HL-A and H-2 soluble antigens, providing further evidence for its homology to other species' major transplantation antigens.

MATERIALS AND METHODS

The lymphocytes of 170 unrelated rhesus monkeys were tested with 197 rhesus alloantisera obtained from multigrand females or by intentional immunization with skin grafts and leukocytes in a cytotoxicity test.[1] Two-by-two chi-square analyses of the sera were done on an IBM 360 computer. Fourteen pedigreed rhesus families were also typed. Genotypes of each family member (8 males, 14 females and 32 children) were ascertained from phenotype data. Rhesus spleens were subject to papain digestion to obtain water soluble lymphocyte alloantigens by a method previously described.[2] The soluble product was tested for serologic specificity by inhibition of specific antibody mediated ^{51}Cr release from target lymphocytes.[2] Amino acid analyses of acrylamide gel fractions of soluble rhesus antigens were done by standard techniques.[2]

RESULTS

Chi-square analyses of the 107 sera revealed the existence of five groups of sera all correlated with each other at the $p = 0.01$ level or better. Ten sera were in group 1, eight in group 2, 11 in group 3, nine in group 5, and five in group 6. Four other groups of sera were found. Analyses of these groups (4, 7, 8, and 9) is as yet incomplete and will be reported later. If the lymphocytes of an animal reacted with 90% or more of the sera of a given group, the cell was assigned the antigen (or antigen group) defined by these sera. The first three antisera groups and the antigen groups they define have been previously reported.[1]

The antigen frequencies in the unrelated population are listed in Table 1. The most frequent antigen is 3; the least frequent is 6. Phenotype frequencies of these monkeys are listed in Table 2. Two points are of primary importance: there exists an extensive phenotype polymorphism, and, there is no phenotype that includes antigens 1, 2, and 5. This latter observation suggests that 1, 2, and 5 are alleles.

Two-by-two chi-square analyses of antigens and Hardy-Weinberg behavior of antigen pairs[1] is presented in Table 3. Negative 2 $\times$ 2 chi-square values indicate possible alleles. Low Hardy-Weinberg chi-square values suggest allelic behavior, while high values make it unlikely. A study of this table reveals that antigens 1 and 2 behave like alleles. Antigens 3 and 6 behave similarly, while antigen 5 is less clear-cut. Using the more rigorous Hardy-Weinberg analysis, we see that antigens 3 and 5 have a highly significant chi-square value, suggesting nonallelism, whereas 1 and 5 and 2 and 5 have low chi-square values, indicating possible allelism. Thus, we tentatively conclude that 1, 2, and 5 are

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Table 1. Antigen Frequency Among 170 Unrelated Monkeys

Antigen	Frequency
1	0.200
2	0.288
3	0.411
5	0.176
6	0.082

one set of alleles while 3 and 6 are another set. This analysis is consistent with the failure to find any 1, 2, 5 phenotype in 170 unrelated monkeys.

Fourteen families studied were entirely consistent with the predictions of two sets of multiple alleles, and there was no single instance of crossing over detected. This indicates that antigens 1, 2, 3, 5, and 6 are all part of a closely linked genetic system. Haplotypes of 36 parental chromosomes are listed in Table 4, which shows that nine of 12 possible haplotypes were found in this limited number of animals, indicating again the extensive polymorphism of this system, and that no haplotypes were found that contained more than one of antigens 1, 2, and 5 or more than one of antigens 3 and 6, which is further proof for the existence of two sets of alleles in this antigen system.

The antigens were readily solubilized from spleens by the papain digestion method. Serologic specificity was retained by the soluble product (Fig. 1). Amino acid analyses of the acrylamide gel purified soluble antigen are compared in Fig. 2 to those of similar material from lymphoid tissue culture cells of man bearing HL-A antigens and spleen of mice bearing H-2 antigens.[3] A striking similarity is noted.

DISCUSSION

We have previously reported the existence of three rhesus lymphocyte alloantigen groups (groups 1, 2, and 3). At that time, unrelated population studies indicated that 1 and 2 were possibly alleles, but 3 was clearly not. Family studies indicated that 3 was closely linked to 1 and 2. We suggested that 1 and 2 were elements of one segregational series and 3 was an antigen at a second series. The analogy with the two segregational series of the human HL-A antigens is obvious. The data pre-

Table 2. Phenotype Frequency Among 170 Unrelated Monkeys

Phenotype	Frequency	Phenotype	Frequency
—	0.324	2, 3, 6	0.006
1	0.047	2, 3, 5, 6	0.006
1, 2	0.006	2, 5	0.018
1, 2, 3	0.018	2, 6	0.024
1, 3	0.094	2, 5, 6	0.006
1, 3, 5	0.024	3	0.082
1, 3, 5, 6	0.006	3, 5	0.041
1, 5	0.006	3, 6	0.012
2	0.082	5	0.053
2, 3	0.106	6	0.024
2, 3, 5	0.018		

Table 3. Antigen Pair Comparisons for Allelic Behavior

Antigen Pair		2 × 2 Table		Hardy-Weinberg	
		X^2	p	X^2	p
1	2	— 5.36	<0.05	0.69	>0.7
1	3	+14.34	<0.001	46.01	<0.0001
1	5	+ 0.06	>0.8	2.70	>0.2
1	6	— 0.82	>0.7	0.37	>0.8
2	3	+ 3.18	>0.07	27.01	<0.0001
2	5	— 0.02	>0.9	2.52	>0.2
2	6	+ 2.13	>0.6	16.05	<0.0005
3	5	+ 1.85	>0.6	16.90	<0.0005
3	6	— 0.01	>0.9	1.36	>0.4
5	6	+ 0.00	>0.9	2.84	>0.2

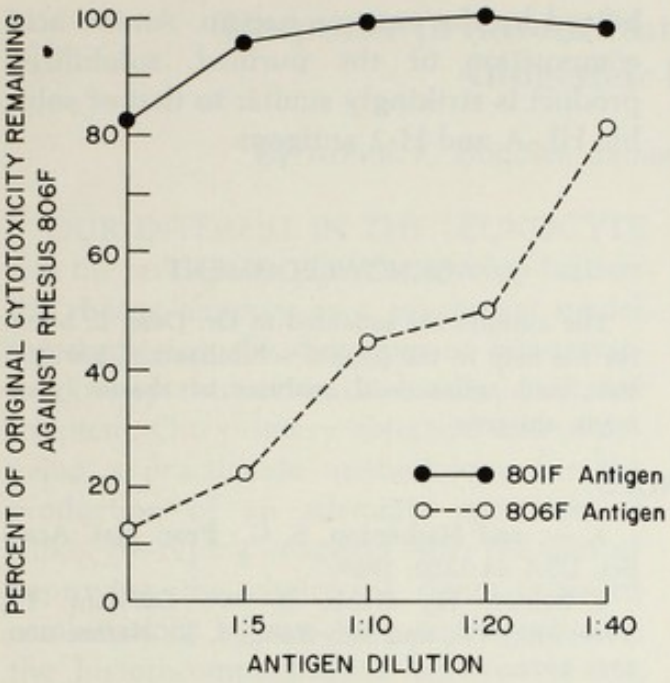

Fig. 1. Inhibition of antiserum reacting only with rhesus 806F lymphocytes by soluble lymphocyte antigens from rhesus monkeys 801F and 806F.

sented in this paper confirm our original findings and add one more antigen to each segregational series.

These findings are in accord with those previously described by Balner et al.,[4] although it was not clear whether their data fitted into two or more segregational series. Thus, these two groups of investigators have clearly defined a single major lymphocyte alloantigen system in rhesus monkeys.

Table 4. Monkey Lymphocyte Alloantigen Haplotypes From Family Studies

Haplotype	Number	Frequency
—	18	0.500
1	3	0.083
1, 3	2	0.055
1, 6	0	0
2	1	0.028
2, 3	3	0.083
2, 6	2	0.055
5	2	0.055
5, 3	0	0
5, 6	0	0
3	3	0.083
6	2	0.055
Total	36	

Our tentative finding of two closely linked segregational series of antigens, by analogy to HL-A antigens, suggests that rhesus alloantigens are also histocompatibility antigens.

The ease of solubilization of these antigens with papain and their striking similarity in amino acid composition to two other species' major histocompatibility antigens are further evidence that the rhesus lymphocyte alloantigen system is a major transplantation antigen system of that species. Balner et al.[4] have provided good evidence by skin grafting and mixed leukocyte culture techniques that their lymphocyte alloantigen system is a major transplantation antigen system. Final proof awaits successful acceleration of graft rejection after im-

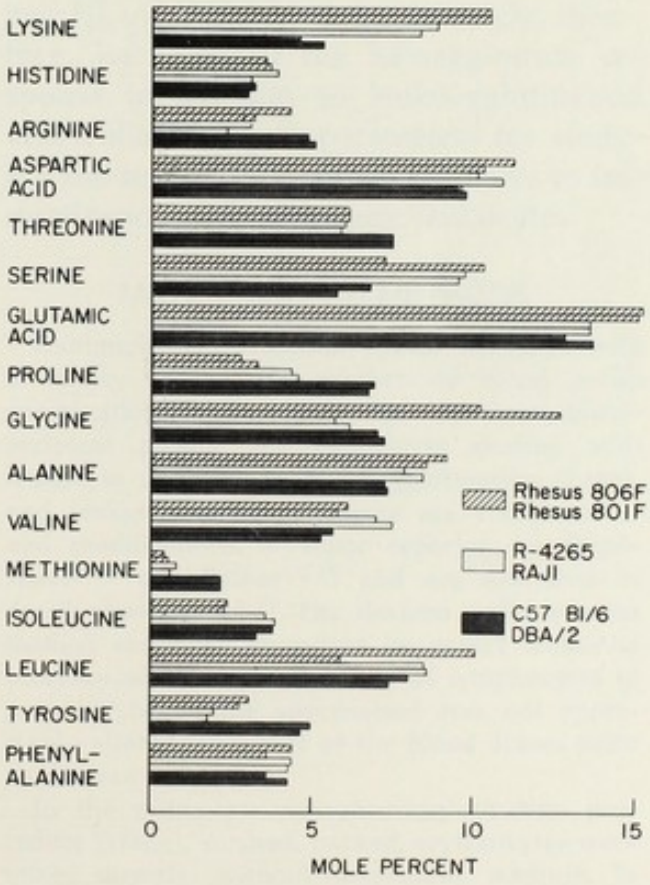

Fig. 2. Amino acid composition of solubilized purified rhesus lymphocyte antigens (806F and 801F), human lymphoid tissue culture cell HL-A alloantigens (R-4265, RAJI), and mouse spleen H-2 alloantigens (C57BL/6, DBA/2).

munization with purified soluble lymphocyte antigen. Preliminary results in our laboratory have been encouraging in this respect.

SUMMARY

Five rhesus lymphocyte alloantigen groups have been defined. They behave as a part of a single genetic system that can be subdivided into two closely linked segregational series. They can be readily solubilized by the enzyme papain. Amino acid composition of the purified, solubilized product is strikingly similar to that of soluble HL-A and H-2 antigens.

ACKNOWLEDGMENT

The authors are indebted to Dr. Dean L. Mann for his help in the papain solubilization, purification, and amino acid analyses of rhesus lymphocyte antigens.

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Response to Immunization With Rhesus Monkey Allogeneic Leukocytes

By Arthur E. Bogden, James H. Gray, and Marie Brule

OUR INTEREST IN THE LEUKOCYTE was the result of an effort to develop further the rhesus monkey as a preclinical model for studies on the dynamics of bone marrow transplantation in the totally irradiated recipient. Our primary objective was to develop a practicable methodology for the production of an adequate spectrum of leukocyte-typing reagents with the aim of improving the choice of donor-recipient combinations by matching and eliminating the histoincompatibilities between strong antigens or antigenic groups. The system was to be applicable to small populations of immunogenetically heterogeneous animals, the methodology that was developed was not to be critically dependent on the continued survival of any one particular donor and recipient pair, and the techniques for the production of isoantiserum reagents were to be within the realm of applicability to man.

Our decision to use the i.c. route for injection of intact leukocyte preparations and repeated fractional doses rather than one equivalent dose was based on the observations of Billingham,[1] Friedman,[2] and Rappaport[3] and was predicated on the practical consideration that i.c. recipients can withstand multiple inoculations (as many as 30) without untoward event. In this manner the formation of hot sterile abscesses resulting from repeated s.c. injections, and the danger inherent in multiple transfusing of recipients with known leukocyte incompatible blood, were evaded.

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Isoantibodies against human and lower primate leukocytes have been demonstrated mainly by leukoagglutination, cytotoxicity, or complement fixation. In earlier studies on the rat, we were able to demonstrate an association between red cell antigens and histocompatibility factors by describing a correlation between red cell phenotype and susceptibility or resistance to allografts of tumor tissue.[4,5] The genetic locus thus detected has been shown to be polymorphic[5-7] and fundamentally analogous to the primary histocompatibility systems in other species (e.g., B of chickens, H-2 of mice, and HL-A of man[4,8,9]). Instinctively, therefore, we included the hemagglutinin response in addition to leukoagglutination and leukotoxicity as parameters for studying the response of rhesus monkeys to immunization with allogeneic leukocytes.

MATERIALS AND METHODS

Immunologically mature rhesus monkeys with a variety of sex and erythrocyte blood group combinations[10,11] were divided into three donor-recipient panels. All procedures dealing with leukocyte suspensions, leukoagglutination (Lagg), and leukotoxicity (Ltox) tests are combinations and modifications of those reported by Engelfriet[12,13] and Balner[14,15] and are described in detail elsewhere.[16,17] The dextran sedimentation method employed permitted on actual leukocyte recovery of 80%, and the ratio of lymphocytes to granulocytes in the supernatant was not appreciably altered from that of the blood drawn prior to dextran treatment.

In the saline-free microhemagglutination procedure (Hagg), washed, packed, erythrocytes were mixed directly, without suspending medium, in heat-inactivated (56°C for 30 min) isoantiserum, and normal rhesus serum was used as the diluent for titering the isoantisera.[16] Care was taken in the choice of normal rhesus serum as diluent since hemagglutinins with a broad spectrum of reactivity were detectable in post-partum females as much as 45 days after delivery. Titer as herein

reported is the reciprocal of the highest dilution of the antiserum showing hemagglutinating activity. Lagg tests were run in triplicate; each isoantiserum was tested against cell suspensions prepared from the same animal on three different days, and the + or − reactions are the results of two of three tests.

Each immunization treatment consisted of 9 i.c. injections of 0.1 ml leukocyte suspension containing at least 2 million cells, in multiple sites (e.g., five sites on the back over scapular area toward axilla and four sites on lower abdomen inguinally) and 0.1 ml of the leukocyte suspension injected deep into the palm of the hand. Injection sites were alternated bilaterally. Thus, at least 20 million cells were injected per treatment, and treatments were repeated at 5–7-day intervals for a total of 20–30 treatments. Aseptic precautions were observed to prevent infection at injection sites.

All sera used in absorption analysis for leukotoxicity required two to six absorptions using a total of 130–200 million leukocytes to remove all toxicity. Erythrocyte contamination of leukocyte preparations ranged from 11,000 to 14,500 cells per cu mm. In absorbing Hagg activity, great care was taken to insure complete removal of saline and the buffy coat. Since it required an average of three repetitive absorptions to remove all Hagg activity, the antisera were in contact with approximately 4.5×10^6 leukocytes during the absorption process. We found that it required 30–40 times that number of leukocytes to remove the Ltox activity from an antiserum. Routinely, therefore, a drop of 10–20% leukotoxicity after erythrocyte absorption was not considered significant. For greater comparative validity, an entire panel was tested on the same day with the same antiserum preparation, and in the case of leukotoxicity the same batch of complement, with known positive and negative controls.

RESULTS

Isoantisera resulting from the first immunization series (ten treatments) gave very low hemagglutinin titers when tested in classic hemagglutination tests using saline as diluent for antiserum and for

TABLE 1

Reactivity of Rhesus Isoimmune Sera as Defined by Hemagglutination, Leukoagglutination, and Leukotoxicity -- Group II Panel

Isoantiserum	Serologic Test	Rhesus animals tested							
		I-21	I-133F	I-L12	I-14	I-68M	I-L09	I-L11	I-5
I-68M	Hagg	-	-	-	-	-	-	-	-
	Lagg	-	+	-	+	-	+	-	-
	Ltox	98	92	5	76	2	80	3	6
I-L09	Hagg	-	+	+	+	+	-	+	+
	Lagg	-	+	-	+	-	-	+	+
	Ltox	92	92	87	15	72	4	80	75
I-L11	Hagg	+	+	+	+	+	+	-	+
	Lagg	+	+	+	+	+	+	-	+
	Ltox	4	77	90	7	8	9	1	54
I-5	Hagg	-	+	+	+	+	-	+	-
	Lagg	+	+	-	+	+	+	-	-
	Ltox	98	8	15	83	52	71	87	3

Encircled reactions indicate leukocyte donor used for isoimmunization.

Ltox = % stained cells.

erythrocyte-suspending medium. When the same rhesus isoantisera were tested in the absence of saline, the hemagglutination reactions were strong and macroscopically visible as 2+ to 4+ reactions, indicating that saline concentrations greater than that found in normal monkey serum significantly reduced the hemagglutinating activity of the rhesus isoantisera. When normal saline was used for both antiserum diluent and cell suspension, the weaker antisera lost all reactivity. In the absence of saline, negatives and end points were clear-cut and reactivity of any specific isoantiserum was reproducible.

Isoantisera titered after the second series of immunizations (after 20 treatments) produced Hagg titers ranging from 1 to more than 512, indicating not only variability in response but differences in the isoantigenicity between donor-recipient pairs. Medium-to-high titered sera maintained their isohemagglutinin levels for about 20 days posttreatment, followed by a precipitous drop in titer by 40 days, with a more gradual decline thereafter. Some sera still had low but detectable isohemagglutinating activity (titers of 1–8) at 90 days. Sera with low initial titers lost activity earlier.

When the sera from 14 isoimmunized rhesus monkeys were tested against a panel of 28 erythrocyte donors, it became clear that rhesus monkeys not only differ by but also share a number of isohemagglutinogens. Only one animal of the 14 isoimmunized showed no hemagglutinin response and developed leukogglutinins and leukotoxins. Of interest was the evidence that even without absorption analysis some of the isoantisera showed selective reactivity; e.g., antisera from rhesus 1-32 reacted with erythrocytes from only nine animals of 28 tested.

The pattern of serologic responses obtained in the rhesus monkey by immunization with allogeneic leukocyte preparations, as defined by (Hagg), (Lagg) and (Ltox), is illustrated by the reactions obtained with Group II panel (Table 1). In this instance, Ltox is indicated by per cent of stained cells to illustrate the variability of reactivity between leukocytes from different animals. The incidence of the various patterns of serologic reactivity obtained with sera tested against immunizing donor's cells and against a large panel of different leukocytes and erythrocytes is summarized in Table 2. It is evident that most of the antisera obtained were multireactive, having more than one antibody.

Of particular importance is the association of Lagg and Ltox with Hagg. Of the various combinations of serologic reactions obtained, Hagg activity was associated with 92% of immunizing leukocyte donor's cells and 66% of the cells from randomly selected test panels; 32% of the test combinations were positive for all three serologic reactions; 24% of Lagg activity was associated with Hagg in the absence of Ltox, whereas only 10% of Ltox activity was associated with Hagg in the absence of Lagg; 17% of the tests showed Hagg activity without either Lagg or Ltox, and only 5% of the tests showed both Lagg and Ltox activity in the absence of Hagg; 6% of the tests showed only Lagg activity, 3% only Ltox activity, and 3% were negative for all three reactions. Of interest was the iso-

TABLE 2

Incidence of the Various Combinations of Hemagglutinating, Leukoagglutinating, and Leukotoxic Reactions Occurring With Rhesus Isoimmune Sera

Reaction Patterns			Pattern Frequency (%)	
Hagg	Lagg	Ltox	Cell Donors[1]	Test Panel[2]
+	+	+	71	32
+	+	-	7	24
+	-	+	14	10
-	+	+	0	5
-	-	+	7	3
-	-	-	0	3
-	+	-	0	6
+	-	-	0	17

[1] Calculations based upon 14 donor:recipient combinations.

[2] Calculation based upon 119 combined tests.

immune response that produced Hagg and Lagg but no Ltox, indicating that leukoagglutination may not always be the manifestation of a leukotoxic antibody in the absence of some factor of complement. Tested against a panel of cells, this particular isoantiserum showed selective Lagg activity not always concomitant with Hagg. From an analysis of the various reactions obtained, one can conclude that although there may be an overlap in reactivities by the three serologic test systems in detecting the same antigen on two different cell types, there are isoantigenic differences detectable only by a particular serologic test system.

Of particular significance to this report were those overlapping reactivities that were both leukotoxic and hemagglutinating. The overwhelming association of Hagg and Ltox raised the possibilities of a histocompatibility hemagglutinogen in the rhesus animal and of a common isoantibody therefore reacting with its homologous antigen in two different test systems. To resolve this question, each isoantiserum was re-

TABLE 3

The Effect of Absorption With Erythrocytes on the Leukotoxic Activity of Rhesus Isoimmune Serum

Isoantiserum Donors	Absorbing Cell Donors	Percent Leukotoxicity							
		RHESUS ANIMALS TESTED							
		I-21	I-133F	I-L12	I-14	I-68M	I-L09	I-L11	I-5
I-5	Unabsorbed	98	8	15	83	52	71	87	3
	I-14	73	1	2	74	50	63	77	1
I-L09	Unabsorbed	92	92	87	15	72	4	80	75
	I-133F	26	66	67	60	62	6	77	48
I-L11	Unabsorbed	4	77	90	7	8	9	1	54
	I-L12	9	38	67	4	1	9	3	67
I-68M	Unabsorbed	98	92	5	76	2	80	3	6
	I-21	68	36	1	46	3	39	3	4
		RHESUS ANIMALS TESTED									
		I-20	I-19	I-N43	I-L08	I-34	I-66F	I-18	I-66M	I-L10	I-23
I-L10	Unabsorbed	72	22	36	78	40	77	76	94	3	65
	I-L08	11	3	7	77	53	70	33	74	1	82
I-23	Unabsorbed	60	80	6	12	100	95	94	70	80	1
	I-34	5	79	1	8	91	79	73	7	10	3
		RHESUS ANIMALS TESTED									
		I-24	I-28	I-33	I-35	I-29	I-26	I-27	I-32	I-31	I-30
I-31	Unabsorbed	7	59	8	43	8	57	9	47	5	4
	I-35	2	41	8	75	1	25	9	23	2	3

Values in squares indicate leukocyte donor used for isoimmunization.

peatedly absorbed with erythrocytes from the specific immunizing leukocyte donor until negative for Hagg activity. The effect of erythrocyte absorption on the leukotoxicity of rhesus isoimmune serum is illustrated in Table 3 and is summarized as follows: no significant change in the Ltox activity with the leukocyte donor nor with panel cells (e.g., isoantiserum 1-5); no significant change in the Ltox activity with the leukocyte donor but selective, complete, or partial removal of Ltox activity with panel cells (e.g., isoantisera I-L10 and 1-23); partial removal of Ltox with leukocyte donor but selective, partial removal of Ltox with panel cells (e.g., isoantisera I-L09 and I-L11) and an across-the-board partial absorption of leukotoxicity with all panel cells as seen with isoantiserum I-68M; and an increase in Ltox with leukocyte donor but partial removal of Ltox with panel cells (e.g., I-31), and partial removal of Ltox with leukocyte donor but with an increase in Ltox with certain panel cells (e.g., I-L09 and I-L10).

Two methods of antiserum absorption were employed for the analysis and subsequent development of highly selective leukotoxic typing reagents: absorption analysis, a methodical absorption with the leukocytes from individual, positive-reacting donors of the primary test panel, and selective absorption, absorption with leukocytes from positive-reacting donors, singly or pooled, arbitrarily selected on the basis of having only moderate (less than 60% cytotoxicity) reactivity with the unabsorbed serum. Results of absorption analysis are illustrated by rhesus I-18 antiserum which was cytotoxic to the leukocytes from every animal of a test panel. Methodical absorption of this serum produced two highly selective reactivities, one polyspecific that was leukotoxic to seven of 37 animals, and one, possibly monospecific, that was leukotoxic to only two of 37 animals tested. The use of selective absorption is exemplified by serum from rhesus I-30 which, when tested unabsorbed against a primary panel of ten leukocyte donors, showed a range in leukotoxicity from 16 to 95%. Absorption of rhesus I-30 antiserum with leukocytes having 35% leukotoxicity (I-35) removed the cytotoxicity for three leukocyte donors in the primary test panel and showed significant reactivity with only 16 of 31 leukocyte donors when tested against a large panel (Table 4). The positive-reacting cells in this test panel also showed a wide range of per cent cytotoxicity, with seven donors in the panel showing a cytotoxicity of 60% or less. Leukocytes from these seven animals were pooled and used for reabsorbing rhesus I-30 antiserum. When again tested against the major panel, this serum now reacted with only four of the 31 leukocyte donors and may well be monospecific (Table 4).

The isoantigenic profile of our in-house rhesus monkey colony, as determined by

TABLE 4

Leukotoxic Activity (% Leukotoxicity) of Rhesus I-30 Antiserum Before and After Absorption With a Selected Pool of Leukocyte Donors

Leukocyte test panel	Absorbed with WBCs from I-35	Absorbed with WBCs from I-14, I-19, I-20, I-68M, I-L08, I-L12 and I-32
I-8	0	0
I-9	84	0
I-10	0	0
I-11	0	5
I-13	0	0
I-14	59 *	0
I-15	1	2
I-16	3	1
I-17	1	11
I-18	0	11
I-19	13 *	0
I-20	57 *	4
I-21	0	5
I-27	88	81
I-29	95	79
I-30	0	0
I-32	60 *	3
I-68M	54 *	0
I-L12	17 *	0
I-N43	0	1
I-L11	86	0
I-L10	86	0
I-L08	19 *	0
I-L09	0	0
I-2A7	70	2
I-3A1	95	69
I-3F6	0	0
I-5E9	88	32
I-9D9	0	0
I-2A9	68	0

* Pooled leukocytes used for antiserum absorption.

12 leukocyte typing reagents developed by these two methods, is summarized in Table 5. The leukocyte antigens or antigenic groups detected by these reagents have been designated RhL_1, RhL_2, RhL_3, etc., in numerical sequence. The animals reacting with the largest number of reagents are grouped at the top of the table and animals with lesser reactivities in descending order. The highly selective quality of the reagents and the differences in their reactivity are clearly indicated. It is of interest that four animals did not react with any of the reagents, 12 reacted with only one reagent, and two animals each reacted with six and seven reagents. The largest group of animals, 19 showed positive reactivity with two to five reagents. Table 5 illustrates the hemagglutinogen profile as defined by heteroimmune typing reagents having the specificities elaborated by Owen.[10,11] There appears to be no correlation between the presence or absence of any erythrocyte antigen, or multiple of antigens, and a specific leukocyte antigen or antigenic group.

TABLE 5

Antigenic Profile of a Rhesus Monkey Colony

Rhesus panel	Leukocyte antigens (RhL)	Erythrocyte antigens
I-27	1,5,6,7,8,11,12	B,C,E
I-29	1,5,6,7,8,11,12	B,C,D
I-L12	1,2,7,9,10,11	B,C,D
I-5E9	1,5,7,8,11,12	B,C
I-11	2,6,7,9,10	B,C
I-19	1,4,5,7,11	B,E
I-L08	2,3,7,9,10	B,C,D
I-3A1	1,5,7,8,12	B,E
I-27J	1,5,7,8,12	B
I-90J	1,3,5,7,8	A,B
I-2A7	3,6,10,12	B,C,E
I-13	2,7,9,10	A,B
I-8	1,6,7	A,E
I-9	3,10,12	B,D
I-W77	2,6,7	A,D,E
I-20	6,12	B,C
I-38	6,12	B,C,E
I-4B0	6,12	B,D
I-L09	3,10	A,B,D
I-9D9	3,10	B,C
I-68M	3,12	A,B,C
I-16	4	A,B,D
I-15	6	B,E
I-18	6	A,C,E
I-21	6	A,B,C
I-N43	11	B,D
I-14	12	A,B,C,D,E
I-32	12	A,C
I-L10	12	B
I-L11	12	B,C
I-2A9	12	B
I-5C8	12	A,B
I-23H	12	A
I-10	Negative	A,B,C
I-17	Negative	A,E
I-30	Negative	B,C,E
I-3F6	Negative	B,C

DISCUSSION

It is apparent that the intracutaneous route and injection into multiple sites at 5–7-day intervals is an effective isoimmunizing procedure in the rhesus monkey, permitting treated recipients to withstand the stresses of multiple innoculations without untoward event. The isoimmunization protocol produced Hagg, Lagg, and Ltox antibodies, and the serologic reactions were strong and repeatable in in vitro test systems. From the diversity of the pattern of serologic reactivities obtained, it is obvious that immunization of rhesus monkeys with rhesus leukocytes produced isoantisera of multiple reactivities. Use of the three serologic test systems (Hagg, Lagg, and Ltox) revealed that a particular isoantiserum could manifest any one or all reactivities, depending not only on the isoantigenic mosaic of the donor-recipient pair used for immunization but apparently on the isoantigenic expression of the panel of cells being tested. The very significant association of the Hagg and Ltox activities indicates the possible existence of a histocompatibility hemagglutinogen system. The presence of leukotoxic hemagglutinins was indicated by the changes in the Ltox activities of isoantisera after absorption with erythrocytes. The possibility of stearic hinderence between hemagglutinins and leukotoxins was also indicated. Whatever the phenotypic expression, the result of these studies indicate that rhesus leuko-

cytes and erythrocytes share common isoantigens, and where such an antigen is a leukotoxinogen, specific leukotoxicity can be removed by absorption with erythrocytes.

Since the number of strong histocompatability antigens appears to be limited, we can assume that the randomly produced reagents in these studies recognized primarily the strong antigens or antigenic systems. One must also consider that they may have detected those antigens by which the rhesus animals most commonly differ. Lack of reactivity with a number of reagents could therefore mean that an animal is not only lacking the corresponding strong antigens but that whatever other antigens are present on its leukocytes are those weaker and/or most commonly shared by the rhesus population in general and not a major factor in the selection of histocompatable donor-recipient pairs. It may well be, for example, that those animals that reacted with none of the reagents are as histocompatable as the two animals that reacted with seven of the same reagents.

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Leukocyte Antigens of Baboons

By H. J. Downing, P. Brain, M. G. Hammond, G. H. Vos, and G. R. Webb

THE BABOON IS BEING USED in large numbers for transplant programs and it is therefore desirable to be able to identify its tissue antigens. It has been shown that the leukocytes of baboons will react with human leukoagglutinating sera.[1] Using 26 sera, Murphy et al. found that the greater the number of differences in the leukocyte antigens between the donor and the recipient of a skin graft, the shorter was the period of survival of the graft. This suggested that these human antisera were recognizing tissue antigens of the baboon. This was supported by their observation that homogenates of baboon kidneys reacted with the same antisera as did the leukocytes from the same baboon. Unfortunately, however, the sera used had not been previously characterized in man. Even if a serum had been characterized in one species it is difficult to apply it to another species. For example, if a human antiserum that detects an antigen, say, HL-A2, in man, reacts with the leukocytes of some baboons, it does not necessarily mean that these individual baboons posses the equivalent of the HL-A2 antigen. Even in different human populations it has been found that a serum that identifies a certain antigenic complex in one of these populations does not necessarily do so in the other.[2,3] An illustration of this is the serum Willett which has been described as having an agglutinin activity that corresponds exactly with its cytotoxicity activity.[4] This is certainly true for a white population, but it is not the case for Indians and blacks where the agreement between the two tests falls to 20%. Similarly, two sera that give a close correlation in a white population need not necessarily correlate with one another when tested in another population. In a white population the two sera Willett and S71 gave a close agreement with a χ^2 of 27.7, while in a black population there was very little association between these sera, and the χ^2 was reduced to 2.2. The reason for this is that many sera thought to be monospecific contain second antibodies against determinants that are very rare in one race but common in another, and this is far from being a rare occurrence. In a survey of white, Indian, and black populations, Brain and Hammond[3] found that although many leukocyte antisera appeared in the same tightly associated groups in all three populations, other sera closely associated in one race group were not associated in one of the other race groups. Where the same groups of closely associated sera are found in all three populations, it can be concluded that each of these groups of sera identifies a complex of antigenic factors frequently inherited in association.[5]

On the basis of this principle, human leukoagglutinating sera were used to study the leukocyte groups of baboons.[6] The results are illustrated in Fig. 1. The numbers are the reference number of the sera, the diameter of the circles represent the number of positive tests expressed as a percentage of the total, and the thickness of the lines represents the degree of association as measured by the χ^2 test. This method of drawing maps was first used by Dausset. There is little resemblance between the χ^2 maps for the two species except for one cluster of sera that detect HL-A7 in man and form a corresponding cluster in baboons. This strongly suggests that baboons have a leukocyte antigen that resembles HL-A7, but as this study did not reveal any other antigen shared by humans and ba-

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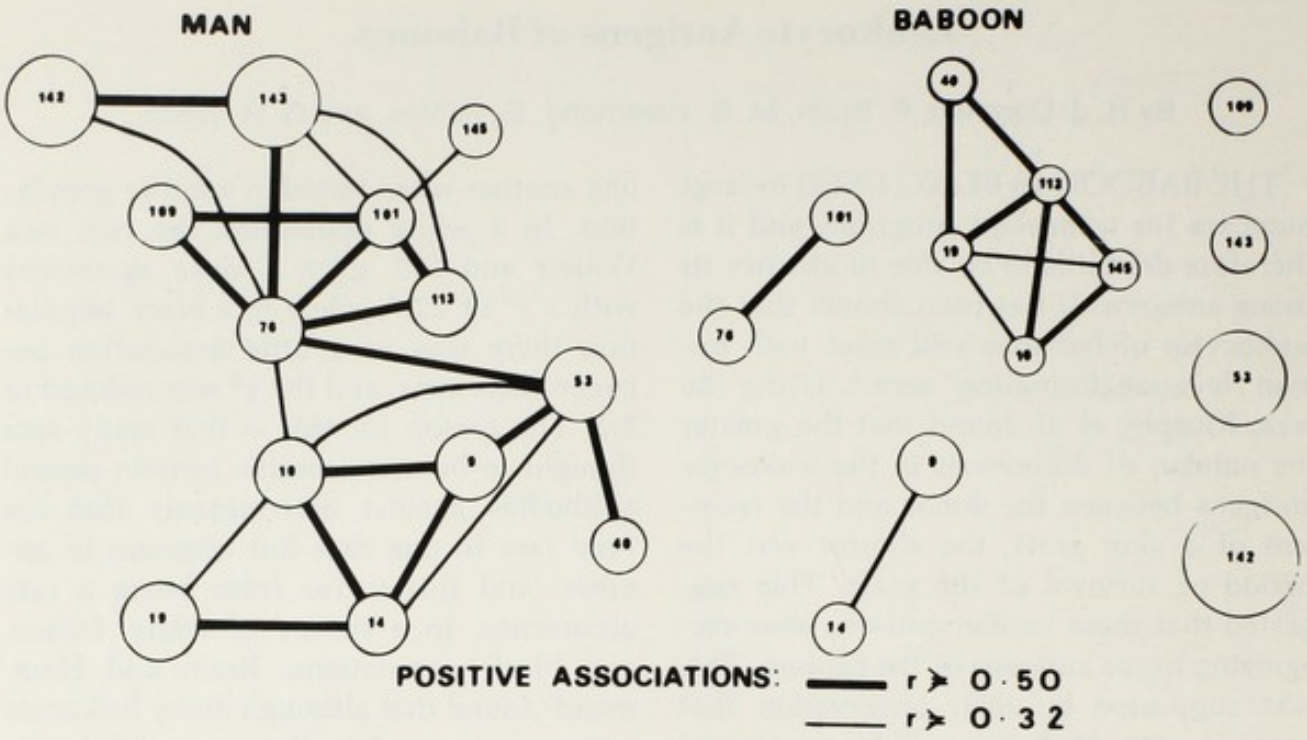

Fig. 1. Relations of 13 sera in man and baboon recognizing 7c complex in man.

boons, an attempt was made to develop isoantibodies in baboons.

Baboons were immunized by skin grafts[7] followed by s.c. booster injections of leukocytes in Freund's adjuvant. Ten days later, samples of blood were taken from the 16 baboons concerned and the sera tested by cytotoxicity against a panel of baboon lymphocytes stored in liquid nitrogen. Of the 16 baboons, 15 gave positive results and were plasmapheresed to give bulk supplies of plasma. The remaining baboon was given a further injection of lymphocytes but again failed to develop antibodies. The

Table 1. Pairs of Baboons With Similar Leukocyte Antigens as Determined by Cytotoxicity Test

	ANTISERA														
CELLS	B2	A3	E5	28	C9	B8	C7	C6	D4	E2	T2	T7	T8	T11	T13
B1	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
T2	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
4	+	−	+	−	−	+	+	−	+	−	−	−	+	+	+
5	−	−	+	−	−	+	+	−	+	−	−	−	+	+	+
7	+	+	−	+	−	−	+	+	+	+	+	+	+	+	+
C9	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
T3	+	−	+	−	−	+	+	+	+	+	−	−	+	+	+
T7	+	−	−	−	−	+	+	+	+	+	−	−	+	+	+
9	+	−	+	−	+	+	+	−	−	−	−	−	+	+	+
C3	+	−	+	−	+	+	+	−	+	−	−	−	+	+	+
C1	+	+	−	+	−	+	+	−	−	−	−	−	+	+	+
C5	+	+	−	+	+	+	+	−	−	−	−	−	+	+	+

results of the cytotoxicity tests between the 15 sera and the lymphocytes from 45 baboons were analyzed by a computer and the χ^2 relationships between sera and between cells determined. The χ^2 values for the sera are shown in Fig. 2. Sera C7 and B8 are from baboons immunized by tissues from the same donor E3 and show a high degree of association. Although baboons A3 and E5 were immunized by donor C3, the sera from these baboons are not associated. Serum E5, however, is related to C7 which is also related to serum T11. Tissues from baboon 7 were used to immunize five baboons, and the sera from these baboons fall into two unrelated groups. Serum T8 is associated with sera T13 and T11, thus forming a group of six sera as shown at left of Fig. 2. The sera from the other two baboons, T2 and T7, that received tissues from baboon 7 are associated with one another but belong to a separate group of 4 sera (T2, T7, 28, and C6) as shown at right of Fig. 2. Outside these two groups of sera are four other sera (plus one serum not shown in Fig. 2) that are not related to any other serum.

Absorption studies have not been performed on any of these sera as the sera were produced by random immunizations and are unlikely to be monospecific. Instead, the sera have been used to compare the lymphocytes from the various baboons in our colony. From the analysis of these results, six pairs of baboons have been selected and are shown in Table 1. The two baboons in the first pair gave identical results with all 15 sera while the members of

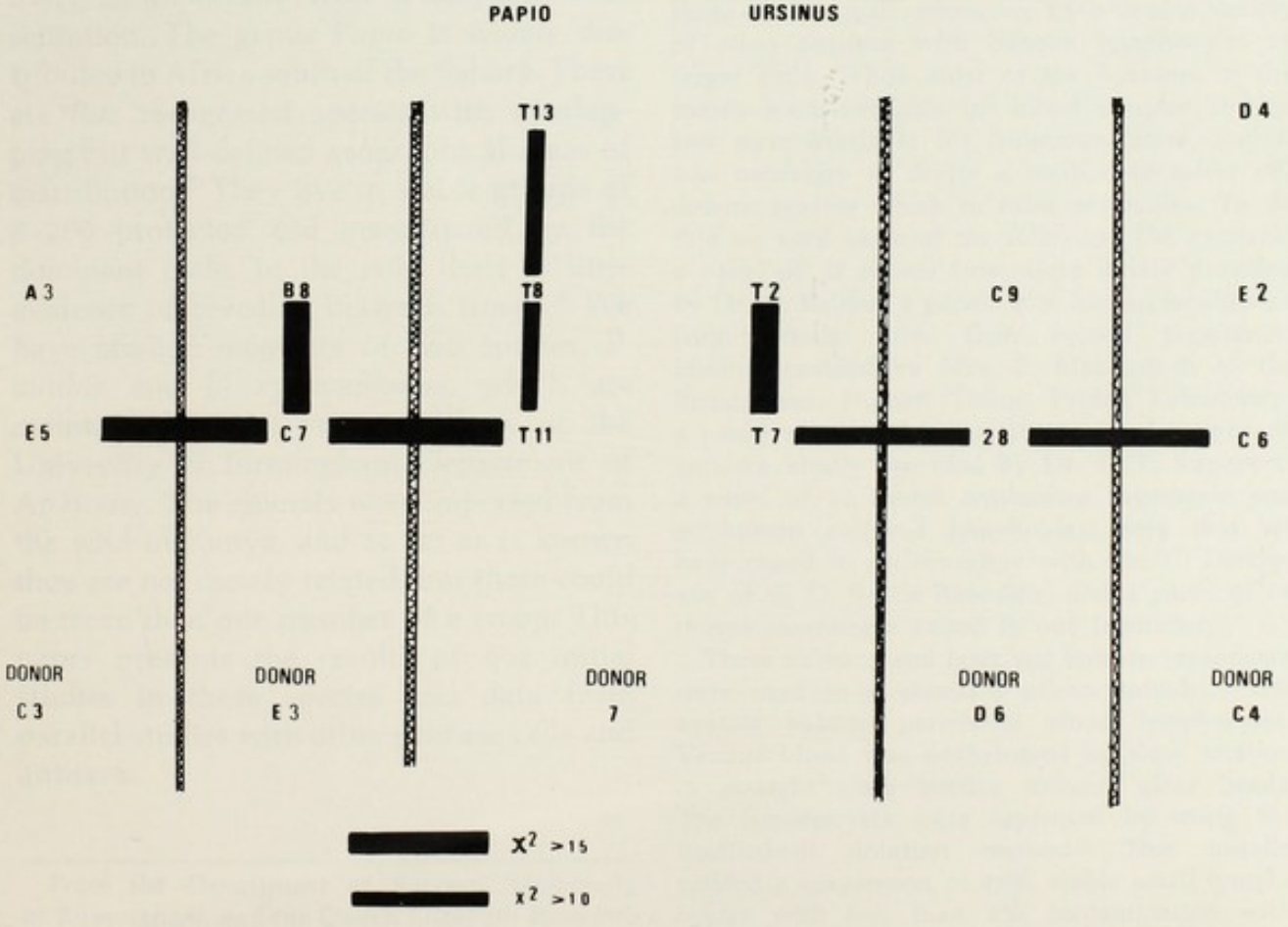

Fig. 2. Relationship between 14 cytotoxic sera produced in baboons by isoimmunization.

the other pairs differ from one another with respect to only one serum.

The next stage of the immunization program will be to exchange skin grafts between the members of each pair in an endeavour to produce more specific sera. These sera will be tested by absorption to see if any of them are monospecific. This work is being performed in one species of baboon, *Papio ursinus*, and it would be of interest to test these sera in other species of baboon. For this reason we hope to collaborate with other laboratories working in this field, especially Dr. Barnes and his colleagues at the University of Birmingham. They have already tested our first batch of sera.

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Leukocyte Antigens in Baboons: A Preliminary to Tissue Typing for Organ Grafting

By A. D. Barnes and R. J. Hawker

THE DEVELOPMENT OF CYTOTOXIC ANTISERA is described in two species of baboon, *Papio cynocephalus* and *Papio anubis*. The antigens identified are distributed between the two species and have been detected by two operationally monospecific and two multispecific antisera. These studies are being extended in view of the increasing interest in this genus in transplantation studies. Various species of baboon *(Papio)* are used to study models of human disease and organ grafting in particular.[1,2] They are a convenient size for surgery, and in reasonable supply, and can be bred in captivity, an important factor in long-term conservation. The genus *Papio* is widely distributed in Africa south of the Sahara. There are five recognized species with overlapping but well-defined geographical areas of distribution.[3] They live in stable groups of 8–200 protected and inseminated by the dominant male. In the wild there is little evidence of breeding between troops.[4] We have studied members of two species, *P. anubis* and *P. cynocephalus*, which are maintained in the primate colony of the University of Birmingham Department of Anatomy. The animals were imported from the wild in Kenya, and as far as is known they are not closely related, but there could be more than one member of a troop. This paper presents the results of our initial studies in these species and data from parallel studies with other primate cells and antisera.

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MATERIALS AND METHODS

The two species of *Papio* are readily distinguished morphologically.[3] *P. anubis*, the olive baboon, has a ruff of hair around the cheeks, giving a rounded appearance to the face. The individual hairs are brown at the base with one or two light rings and are black at the tips. The dorsal and ventral hair is of uniform color. The hair on the paws is black. *P. cynocephalus*, the yellow baboon, has a black face. Its long, silky, yellow hairs form fringes along its back and limbs. The hair on the paws is not black. Also available in the colony, but not forming the main part of this study, are two species of *Macaca (M. mulatta* and *M. nemestrina)*.

We were particularly interested in baboon lymphocytotoxic isoantisera, but to help develop these in the small colony we have used a variety of other antisera with baboon lymphocytes as target cells. While most of the baboons in the colony were available for blood samples, only a few were available for isoimmunization, and it was necessary to devise a method to select cell donors against which to raise antibodies. To do this we used some of the following 151 antisera: a panel of 43 rhesus isoantisera kindly provided by Dr. H. Balner; a panel of 28 human isoantisera from women after their second pregnancy, kindly provided by Mrs. P. Mackintosh of the Birmingham Human Tissue Typing Laboratory; a panel of 30 rabbit antistreptococcal membrane antisera kindly provided by Dr. F. T. Rapaport; a panel of 11 rabbit antihuman thymocyte and antihuman cultured lymphoblast sera that we have raised in collaboration with Mr. C. Dickerson of G. D. Searle Research; and a panel of 39 rhesus isoantisera raised in our laboratory.

These antisera and later our baboon isoantisera were used in a standard microcytotoxicity test against baboon peripheral blood lymphocytes. Venous blood was defibrinated by slow rotation in straight glass bottles without glass beads. The lymphocytes were separated by using the ficoll-triosil flotation method.[5] This usually yielded a suspension of 95% viable small lymphocytes with less than 3% contamination with immature red cells. It was necessary to wash the cells well to remove serum which acted as an inhibitor in the cytotoxic system. Glass tissue typ-

Table 1. Antisera Used to Select Pairs of Baboons for Skin Grafting

	Number of Sera Used to Select Baboons	% Sera Giving With Baboon	> 60% Cytotoxicity With Rhesus
Rhesus Isoantisera (Balner)	35	18.6	95.3
Rhesus Isoantisera (Birmingham)	23	5.1	53.8
Human Isoantisera (Birmingham)	6	17.8	17.8
Rabbit/streptococci (Rapaport)	10	3.3	13.3
Rabbit/human (Birmingham)	8	100.0	100.0

ing plates with 50 circles (Compatype, G. D. Searle) were primed under paraffin oil with 2 μl of heat-inactivated antiserum and stored at −20°C. To these was added 2 μl of a lymphocyte suspension in phosphate buffered saline (PBS) adjusted to 2 × 10^6 cells/ml (4000 cells) and 2 μl of rabbit complement. The rabbit serum was titrated for toxicity and complementary activity and was diluted if necessary. The plates were incubated at 37°C for 60 min; 2 μl of 8% eosin-y in PBS was added and after 3 min at room temperature the reactions were fixed with 40% formaldehyde neutralized with calcium carbonate.[6] The plates were read up to 48 hr later under a phase-contrast microscope. They were scored as follows: less than 30% dead cells, negative; 30–50% dead cells, weak positive; more than 60% dead cells, positive. The plates always included positive and negative controls. When the cell death in the negative controls was more than 20% or in the positive controls less than 100%, the results of the plate were disregarded. All readings were made without knowledge of the sera and to the nearest 10%. The reproducibility of the test system was checked frequently with duplicate plates.

RESULTS

In the first series of experiments the lymphocytes from 38 baboons were reacted against a panel of 82 heterologous antisera from the sources stated (Table 1). As might be expected, many of the reactions were weak because the antibodies were not primarily directed against baboon antigens. A computer simplification of the data was made to select animals that reacted similarly to the panel of antisera. Four of the animals that showed one or two reaction differences from another member of the colony were made available from isoimmunization.

The antibody producers received skin allografts s.c. in the groin from the selected donors. The grafts were repeated at weekly intervals for up to 7 wk (Fig. 1). No attempt was made to study the survival time of the skin grafts, but none developed into epidermal cysts. Serum were examined weekly for the presence of isoantibodies against the donor's peripheral blood lymphocytes.

In contrast to the weak reactions of baboon cells with the nonspecific antisera, the serum of the isoimmune baboons was highly cytotoxic after the fifth skin graft. Two of the antisera caused 100% lymphocytotoxicity even when diluted more than 1:100 (Fig. 1). The specificity range of the stronger antisera (A and B) was much wider than that of the antiserum that was slowest to appear (D). Antiserum C proved

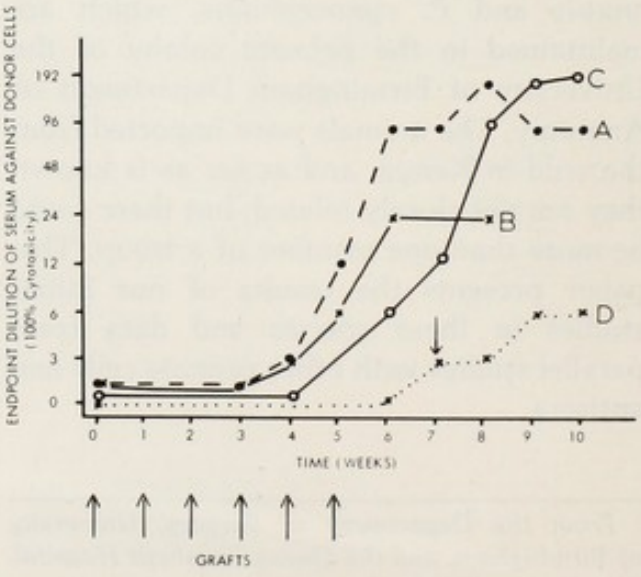

Fig. 1. Cytotoxic titers of baboon sera following repeated skin allografting.

to be a strong antiserum of narrow specificity. With these isoantisera it was possible to alter the scoring system so that a strong positive reaction was more than 90% kill of the lymphocytes.

A panel of 32 baboons (16 *anubis* and 16 *cynocephalus*) was tested against the series of antisera raised in the four immunized baboons (Table 2). The results showed that as immunization continued and the titer of the antiserum rose, its specificity widened. The antisera appeared to distinguish distinct antigens in the panel studied in that some animals were negative to sera from all four donors while others reacted to one or more. Only rarely did animals react to three antisera, and then one reaction was weaker than the others.

The pattern of reactivity against the isoantisera is similar in the two species of *Papio*. Antisera A and D were raised by skin grafting *P. cynocephalus* (m. 2146) with skin from *P. cynocephalus* (m. 2136) and *P. cynocephalus* (m. 2144) with skin from *P. cynocephalus* (m. 2146). Antisera B and C were reciprocal heterologous antisera of *P. cynocephalus* (m. 2136) anti-*P. anubis* (m. 2105) and P. *anubis* (m. 2105) anti-*P. cynocephalus* (m. 2136), respectively. The four antisera appeared to react similarly in the two species.

The same panel of baboons was also examined on the same plates using the baboon isoantisera kindly provided by Dr. Downing. These were raised in *P. ursinus* by immunization with skin grafts. The composite results, shown in Fig. 2, were graded as strong-positive when more than 90% of the cells are dead, positive when 60–90% are dead, and negative when less than 60% are killed. Some of the sera gave a wide range of cytotoxicity but others gave fewer positive reactions.

We attempted to determine the range of specificity of the baboon antisera. Heat-inactivated antiserum (100-μ 1 volumes) was absorbed for 2 hr at room temperature with 10^7 washed peripheral blood lympho-

Table 2. Cytotoxic Reactions of 16 Baboons of Each Species With Four Groups of Antisera

Antisera	Baboons															
P. cynocephalus																
A.5	60	60				90	100	100			80	60		60		100
A.7	70	100				100	100	100	*		100	100		70		100
A.10	90	100	*	60	60	100	100	100	*	*	100	90		80	60	100
B.5	*			60		*		*		70					*	*
B.8	60			90	80	70		*		100						
C.6	*						100	*	60	100		80		100	100	
C.8	90			80	*		100	60	80	100		100		90	100	*
C.10	100	60	*	100	100		100	100	100	100		100		100	100	
D.10													100			
P. anubis																
A.5	70	*	*				*			100				100	*	
A.7	90	100	60	*	60		90			100	*			100	60	
A.10	100	100	100	*	60	*	100	*	60	100	80	*	80	100	60	*
B.5	*	*		70	*		90	100		100		*	60	60		
B.8	*	60		90	100		100	100		100		*	70	60		
C.6	90		100		60				100			100	100	*		
C.8	100		100		90				100		*	90	80	100		
C.10	100		100		100	60	*		100		*	100	100	100		
D.10		90														

*30–60% kill.

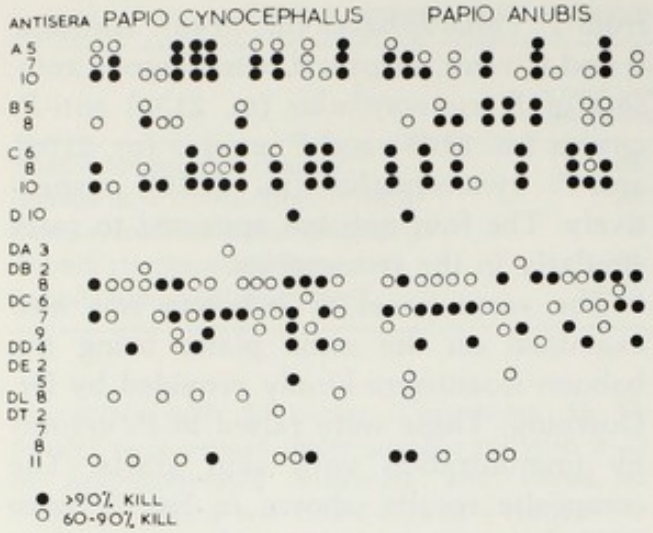

Fig. 2. Cytotoxic reactions of 32 baboons against Birmingham (A, B, C, D) and Durban (DA-DT) sera. Only strong reactions are recorded.

cytes. The absorption was repeated if the cytotoxicity of the initial serum was greater than 1/192. The high titer antisera were tested at several dilutions in PBS against a range of target cells.

Specificity of Antiserum D10 (m. 2144, anti-m. 2146), *P. cynocephalus* isoantiserum, reached a titer of 1:6 after seven sets of skin allografts. The donor and recipient *cynocephalus* baboons were from one importation and could have been members of one troop. The antiserum reacted positively against the cells of only one other baboon (m. 2055) which was *P. anubis*. The serum was absorbed with the cells of m. 2055, m. 2146 and a group of nonreactive *P. cynocephalus* and *anubis* cells. The reactivity against m. 2055 and m. 2146 cells was unaffected after absorption by other than m. 2055 or m. 2126 cells (Table 3). The serum gave 6% positive reactions in our colony and is so far operationally monospecific, but this must be checked by studies in larger populations. Preliminary dilution experiments support this finding as the titer against both cell types was the same.

The titer of Antiserum C8 and C10 (m. 2105, anti-m. 2136) *P. anubis*, anti-*P. cynocephalus* sera rose rapidly after the sixth skin graft. The sera gave a much higher frequency of positive reactions in both species than antiserum D10. The absorption studies so far suggest that serum C8 is operationally monospecific as shown by its absorption pattern (Table 4). Cells that fail to react to antiserum C8 do not alter its reaction to the positive cells of m. 2136, 2140, 2104, 2113, or 2056. Absorption with any one of these cell types removed all the reactivity against any of the other cell types with one exception. A later antiserum obtained from this animal (C10) gave positive reactions with the previously negative cells of m. 2107. Although absorption of C10 with cells from m. 2056 or m. 2140 removed or reduced the activity against m. 2107, absorption of C10 with m. 2107 did not alter the reactivity to the other cells (Table 4).

Unlike the previous antisera antiserum A10 (m. 2146, anti-m. 2136) *P. cynocephalus isoantiserum* appears to be multispecific (Table 5). Absorption with cells from the skin donor removes all the reactivity of the serum, but absorption with cells from the other positive reactors (m. 2144 and m. 2105) has a variable effect. As the serum was made by grafting a D positive baboon with skin from an A and C positive donor, it would be tempting to assume the antiserum was anti-A and anti-C. This is not supported by absorption as m. 2144 is A

Table 3. Absorption of Antiserum D10 (m. 2144, anti-m. 2146) (*P. cynocephalus* Isoantisera)

Sera	Cytotoxicity (%) m. 2146 Cells	m. 2055 Cells
Neat D10	100	90
ABS with nonreactive *P. cynocephalus*	100	80
ABS with nonreactive *P. anubis*	90	80
ABS with m. 2146	0	0
ABS with m. 2055	0	0

Table 4. Absorption of Antisera C8 + C10 (m. 2105, anti-m. 2136) (*P. anubis, anti-P. cynocephalus*)

Sera	Cells					
	2136	2140	2104	2113	2056	2107
C8 Neat	100	100	100	90	100	0
ABS nonreactive *P. anubis*	100	100	100	80	100	0
ABS nonreactive *P. cynocephalus*	90	100	100	90	100	0
ABS 2136	0	0	0	0	0	0
ABS 2140	0	0	0	0	60	0
ABS 2104	0	0	0	0	0	0
ABS 2113	0	0	0	0	0	0
ABS 2056	0	0	0	0	0	0
C10 Neat	100	100	100	100	100	100
ABS 2056	0	0	0	0	0	0
ABS 2140	0	0	0	0	0	60
ABS 2107	100	100	100	100	100	0

positive and m. 2105 A and B positive. The findings are best explained by assuming antisera A are anti-C, A, and E. The last is not defined positively by a serum. Further support for the multispecificity of this serum comes from the effect of dilution. The serum has a titer of 1:96 against donor cells but only 1:12 and 1:24 against m. 2144 and m. 2105, respectively.

Antiserum B (m. 2136, anti-m. 2105) *P. cynocephalus*, anti-*P. anubis* had a wide range of specificity as shown by absorption and dilution studies and is being studied further.

Table 5. Absorption of Antiserum A10 (m. 2146, anti-m. 2136) (*P. cynocephalus* Isoantisera)

Sera	Cytotoxicity (%)		
	2136 Cells	2144 Cells	2105 Cells
Neat A10	100	100	100
ABS 2136	0	0	0
ABS 2144	100	0	60
ABS 2105	90	100	0
ABS 2146	100	100	100
ABS 2144 + 2105	100	0	0

DISCUSSION

Any conclusions drawn from these results must be very tentative because the population studied was small. We recommend the method used to select pairs of primates to immunize as we obtained two excellent antisera from these few experiments. Without some preliminary selection it is doubtful whether we would have obtained these results. The method described was also used to obtain the macaque sera we used in this workshop.

The cytotoxicity test described for *Papio* is equally applicable to *Macaque*. It is important to have a suitable rabbit complement source. The serum of some rabbits is toxic to monkey lymphocytes. No advantage was found by adding the complement after an initial incubation, but it was important to wash the target cells well with PBS before putting them on plates. Even though only two of the antisera are monospecific, it is of interest that few of the monkeys react positively with more than two of the Birmingham antisera. The reaction pattern is not so clear with the Durban antisera raised in a third species of *Papio*. Durban antiserum E5 shows a certain similarity to Birmingham D10. Durban antisera B8 and C7 gave a wide reaction pattern, in many cases weak, which may indicate an interspecies reaction.

The similarity of strong reactions in the two species of *Papio* is interesting as we have not observed strong reactions when our rhesus isoantisera are reacted with target cells from the closely related pigtail monkey (*M. nemestrina*). It would appear that further studies of leukocyte antigens of these and the other three species of *Papio* would be rewarding and might provide information helpful in the understanding of the evolutionary development of the genus. The social structure of baboon troops is convenient for immunogenetic studies as there is a degree of inbreeding within a troop. Unfortunately we have little

information on the origin of our baboons, but it may be of significance that the antiserum with the lowest frequency of reaction (D) was produced by grafting two baboons from one importation who could be members of one troop.

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Leukocyte Antigens of Chimpanzees (ChL-A)

By H. Balner, W. van Vreeswijk, H. Dersjant, J. d'Amaro, A. van Leeuwen, and J. J. van Rood

OUR PROGRAM FOR STUDYING the major histocompatibility systems of subhuman primate species includes investigations of the leukocyte antigens of chimpanzees. In 1966, our first nine chimpanzees were typed with a small panel of isoantisera, and the reagents showed interesting reactivity patterns when used against human lymphocytes. Some of the sera had distinct anti-4a, anti-4b and anti-7c specificity for chimpanzee and human lymphocytes.[1] (This is the original van Rood nomenclature. For the current, official nomenclature of HL-A antigens, see Ref. 8.) The chimpanzee colony was gradually enlarged and the number of isoantisera increased. In 1969, the first five specificities or leukocyte groups of chimpanzees were described. These groups were defined by unabsorbed chimp isoantisera, and the sera of each group showed similar reactivity patterns against 47 chimp lymphocyte samples.[2-4] Independently and at about the same time, investigators at Duke University described six chimpanzee groups.[5] Because of the small numbers of animals tested in both investigations, population analyses were not attempted. During 1970, our group typed 198 chimps with a slightly altered panel of antisera. This led to the identification of eight "broad" or complex specificities. A preliminary genetic analysis suggested that these specificities were probably controlled by one immunogenetic system, provisionally called ChL-A.[6] The first results obtained with the complement fixation technique seemed to confirm the existence of these specificities.[7]

This paper presents the most recent typing results obtained by testing more than 60 chimpanzees with an enlarged panel of isoantisera using both the cytotoxicity and complement fixation technique. It will be shown that when complement fixation is employed, numerous reagents identify rather narrow specificities that are usually included in the broader specificities identified by the same or related sera when the cytotoxicity technique is applied. A number of reagents had been obtained from Metzgar's group at Duke University. Comparison of reactivity patterns of the sera defining our chimpanzee groups with those of Metzgar's sera (which were mostly reagents defining their groups) revealed interesting similarities.

In an accompanying paper,[8] van Rood et al. describe the reactivity patterns of our chimp isoantisera with human cells as well as the reactivity of human anti-HL-A sera with the Rijswijk panel of chimpanzees. In that communication, the striking similarities between some of the specificites of the ChL-A system with those of the human HL-A system[6,7] are again emphasized, and the phylogenetic and practical implications of those similarities are discussed.

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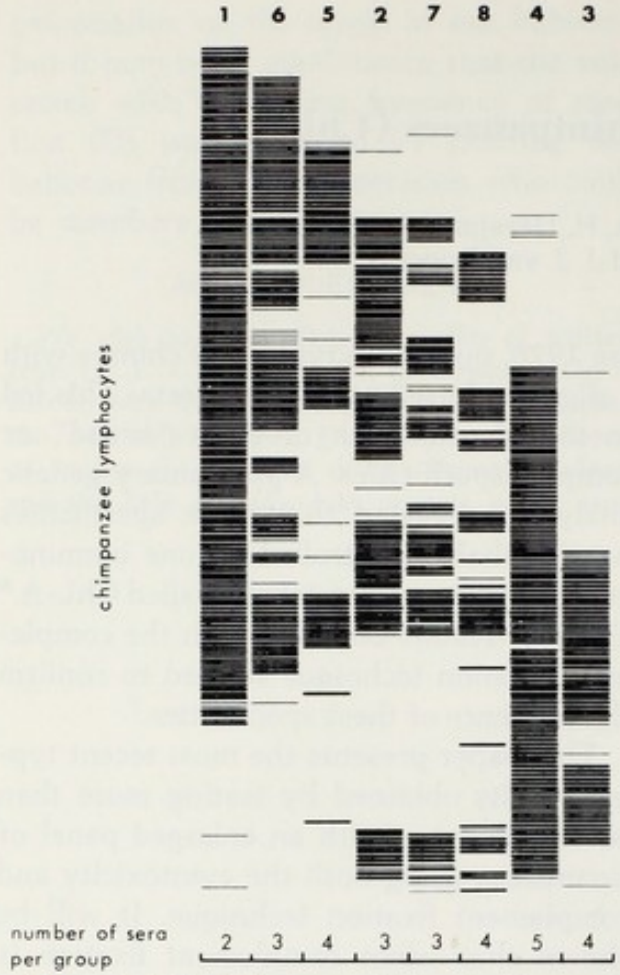

Fig. 1. Cytotoxic reactivity patterns of 8 groups of antisera (28 sera from 20 chimpanzees) with lymphocyte samples from 198 champanzees. Bold bars: most sera of group reacted strongly positive. Thin bars: weak or partly negative reactivity. Cell samples have been arranged for optimal visualization of relationship between groups.

MATERIALS AND METHODS

A total of 73 chimpanzees (61 of the Rijswijk Primate Center and 12 from Behringwerke, Marburg) were typed with most of the available reagents. A slightly modified version of Kissmeyer-Nielsen's microcytotoxicity method[9] and the microcomplement fixation test (performed at the Department of Immunohematology, Leiden University Medical Center) as described by Colombani et al.[10] were used. Methods to produce chimp isoantisera and to select, by computer analysis, sera with similar reactivity patterns have been described.[11] Sera are stored at room temperature after freeze-drying and are used undiluted. A selection of our typing reagents was sent to Metzgar and associates at Duke University for testing on their chimp panel using the NIH cytotoxicity method. Several of their reagents were sent to us and tested with lymphocytes of the Rijswijk chimps.

RESULTS

Figure 1 shows the reactivity pattern of our standard panel of 27 chimp isoantisera with cells from 198 chimps. These data were used for a genetic analysis which revealed that the eight broad leukocyte specificities of chimps are probably controlled by one genetic system, provisionally referred to as ChL-A.[6,7] More recently, cells

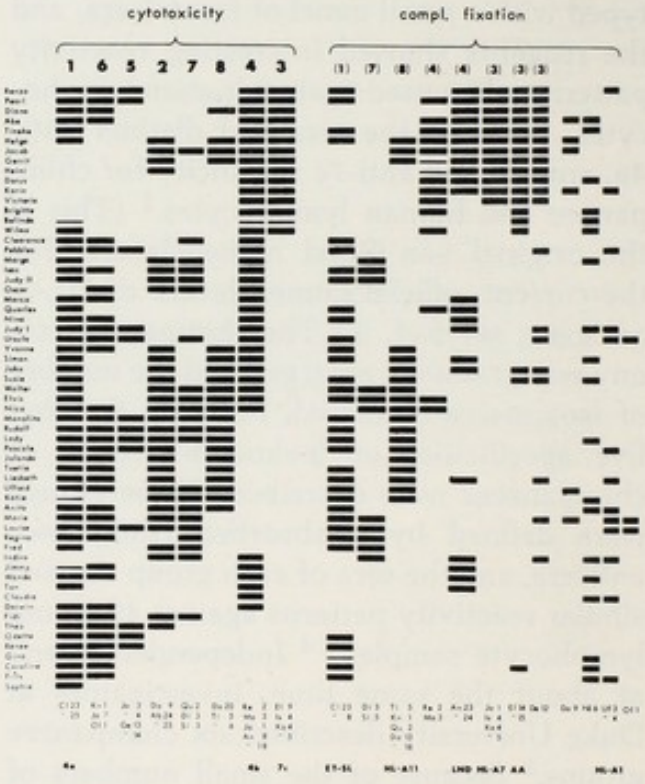

Fig. 2. Reactivity patterns of chimpanzee isoantisera with cells from 61 unrelated chimpanzees. At left: distribution of ChL-A specificities. Data on cytotoxicity for lymphocytes were obtained with 25 of 28 sera used also for data of Fig. 1. Group numbers are indicated above columns. At right: data obtained by complement-fixation on chimpanzee platelets. Numbers in parentheses above columns indicate ChL-A groups corresponding to complement-fixing reactivity patterns. Bold bars: strong serum reactivity Thin bars: weak serum reactivity. At bottom of each column are serum codes and reagent specificities for human lymphocytes in bold type.

from 61 unrelated chimps were typed with a slightly enlarged panel of isoantisera, using the cytotoxicity (CT) and complement fixation (CF) techniques (Fig. 2). In the CF test, many of the reagents had similar reactivity patterns that were usually included in the broader specificities previously defined by CT with the same or related sera (different batches from same animal).

In spite of the small number of animals tested, it seemed worthwhile to analyze the group 4 or group 3 also when the CF tech- particularly since several new sera showed a rather low incidence of positive reactions (Fig. 2). The relationship between individual sera and between groups of positively associated sera was tested for independence and for allelism according to Andresen's method.[13] If reactivity patterns compared by pairs show a negative association with a high χ^2 value for independence and a low χ^2 value for allelism, the specificities in question may represent the products of allelic genes. The outcome of the analysis revealed that group 1 could be allelic with group 4 or group 3 also when the CF technique was used (Fig. 2). This series of possibly allelic specificities had been described previously for the analogous groups determined with the cytotoxicity technique (Fig. 1 and 2). Because of impressive similarities between chimp groups 1, 4, and 3 and human antigens 4a, 4b, and 7c, respectively, we previously proposed to regard this series as the chimp's analogue of the second (sub) locus of HL-A.[6] Further analysis of the CF reactivities shown in Fig. 2 reveals that specificity 8 is negatively associated with and could be allelic with specificities Ni_6 and Uf_4 (Table 1). The specificities defined by Ni_6 and Uf_4 did not show an allelic relationship with each other, but if we take into account that the chimp sera defining specificity 8 quite accurately identify HL-A11 in man and chimpanzees and that Uf_4 is a reasonable reagent to define HL-A1 in man,[14] we are tempted to speculate that the sera of group 8, Uf_4 and Ni_6 may define constituents of a second series of the still hypothetical ChL-A system. If confirmed, such a second allelic series of chimpanzee antigens would most logically be the analogue of the first or LA series of the human HL-A system.

Table 1. Analysis of Relationship Between Selected Pairs of Antisera Shown in Fig. 2; Complement Fixation, Rhesus Monkey Platelets

Specificities*	X^2 Independence	X^2 Allelism*	2 × 2 Table Data ++	+−	−+	−−
$Do_{18} + Ni_6$	10.01	1.41	5	19	23	14
$Do_{13} + Ni_6$	4.20	0.08	5	14	23	19
$Kn_1 + Ni_6$	5.70	0.50	4	14	24	19
$Do_{18} + Uf_4$	3.90	0.56	2	22	11	26
$Do_{13} + Uf_4$	4.20	0.01	1	18	12	30

*Based on gene frequencies for each specificity calculated from one minus square root of frequency of negatives. For detailed description of applied analysis, see Ref. 13.

None of 61 chimpanzees in Fig. 2 was positive for more than two of three specificities: No_6 and Uf_4.

Tables 2 and 3 compare the reactivity patterns of our own sera with those of selected sera sent to us by Metzgar's group at Duke University. Table 2 compares reactivity patterns when the one-stage Kissmeyer-Nielsen CT test was used. It can be seen that many positive associations were found between the patterns of our groups 1, 2, 3, 4, 6, and 8 and several selected sera of the Duke investigators and the corresponding Duke groups. Table 3 shows a similar comparison when the CF reactivity patterns of selected Rijswijk sera were compared with the CT reactivity of the available Duke reagents. Again, several striking similarities can be observed. Metzgar and associates did a similar comparative study using the two-stage CT method[15] and found significant similarities between their reagents and ours.[12]

Absorption studies so far have been performed only with sera defining groups 2, 3, and 5, using 100×10^6 leukocytes to absorb 0.1 ml serum. Virtually all cytotoxic

reactivity was removed by absorptions of each serum with about 10 positive cell samples for each serum, and these sera were tentatively accepted as being operationally monospecific.[3] Cross-reactivity for various shorter specificities can of course not be excluded. The fact that numerous sera of group 3 had a slightly lower incidence of positives in the CF test (Fig. 2) could be due to a lesser sensitivity of the CF method. Sera defining groups 1 and 4 by cytotoxicity (the equivalent of the human 4a and 4b) were not operationally monospecific when absorbed with chimp cells positive for groups 1 and 4, respectively. However, absorptions with human cells carrying 4a and 4b removed virtually all anti-4a and anti-4b reactivity of chimp sera for human lymphocytes. The unexpected lack of cross-reactivity of the anti-1 and anti-4 chimp sera when absorbed with chimp cells has been discussed previously.[4,6] Because several of the anti-1 and anti-4 sera show narrower reactivity patterns in the CF test (Fig. 2). further absorption studies are planned to investigate whether the sera defining these included specificities are monospecific or cross-reacting when absorbed with chimp cells.

The sharing of tissue antigens between chimpanzees and other primate species will be dealt with in this volume by van Rood et al., with regard to human lymphocytes,[8] by Dersjant et al. for rhesus monkey cells,[16] and by Metzgar et al. for orangutan and gorilla cells.[12] Previously, investigators at Duke University and in our group have shown striking similarities between ChL-A specificities and HL-A. These observations have already led to rather extensive use of chimp isoantisera for human tissue typing.[6-8]

Cross-species typing between chimpanzees and rhesus monkeys[16] show interesting cross-reactivities between antibodies defining the antigens of the major histocompatibility systems of chimps (ChL-A) and those of rhesus monkeys (RhL-A), al-

Table 2. Comparison of Reactivity Patterns of Sera-defining Rijswijk Chimpanzee Leukocyte Groups and Sera Obtained From Investigators at Duke University

Rijswijk Groups (ChL-A)*	Duke Sera†	Groups	2 × 2 Tables‡ −−	−+	+−	++	T	HL-A-related Specificity in Man
	Bogam 3.3		9	2	4	45	5.3	
1	Peck 27.3	I	9	2	5	44	5.0	4a
	Larr 18.1	I	7	4	5	44	4.0	
2	Joni 9.2	VI	15	14	4	27	3.2	
3	Mart 30.9	IV	42	1	0	17	7.4	HL-A7 and/or AA
4	Wanda 9.2	III	20	0	18	22	4.2	4b
	S4 28.3		11	9	3	37	4.1	
5								
6	Larr 4.1	I	23	2	17	18	3.5	
7								
8	Lucy 16.2		24	12	2	22	4.5	HL-A11

*Kissmeyer-Nielsen microcytotoxicity technique; see Fig. 1.

†For details regarding reactivity patterns of these sera and comparison with Rijswijk sera using NIH technique, see paper by Metzgar et al. in this issue.

‡Sixty chimpanzee cells tested and 2 × 2 comparisons made between Rijswijk groups and individual Duke sera. Correlations are given as T values (square root of chi-square values).

Table 3. Comparison of Reactivity Patterns of Selected Rijswijk (C′ Fix) and Duke Sera (Cytotox)

Rijswijk Groups (CHL-A)	Rijswijk Sera*	Duke Sera†	Duke Groups	2 × 2 Tables‡ −−	−+	+−	++	T	HL-A-related Specificity in Man
1	Cl_8	Peck 27.3	I	14	12	0	31	4.7	4a
	Cl_{26}	Bogam 3.3	II	12	3	1	44	6.3	
8	Qu_0	Lucy 16.2		18	4	2	25	5.3	HL-A11
	Kn_1	Lucy 16.2		25	16	0	16	4.2	
3	El_{14}	Mart 30.9	IV	37	2	3	15	6.0	HL-A7 and/or AA
	Is_{14}	Mart 30.9		40	6	0	11	5.7	
	Jo_1	Mart 30.9		40	6	0	11	5.7	
	Ka_5	Mart 30.9		40	6	0	11	5.7	
4	Ma_3	S4 28.3	III	20	14	0	23	4.6	4b
	Re_2	S4 28.3		20	14	0	23	4.6	
New	Uf_3	Duncan 13.3	VIII	34	8	2	13	4.7	HL-A1
	Uf_4	Duncan 13.3		35	9	1	12	4.7	

*Tested by microcomplement fixation (Colombani technique).

†Tested by microcytotoxicity (Kissmeyer technique).

‡Fifty-seven or 60 chimp cells tested and 2 × 2 comparisons made between individual sera (not groups); correlations given as T values (square root of chi-square values).

though, cross-species absorption studies will have to be performed before the degree of antigenic similarity between the two species can be defined with more confidence.

SUMMARY

Characteristics of chimp leukocyte antigens have been investigated further. Screening of 56 isoantisera for CF reactivity confirmed the presence of several leukocyte groups previously established with the CT test, and several new specificities of rather low frequency were provisionally defined with the CF technique. The reactivity patterns of the typing reagents used at this institute were compared with those obtained from investigators at Duke University. It was found that most of the chimpanzee specificities described so far can be identified by isoantisera prepared independently at Duke and at Rijswijk. This finding can be regarded as additional evidence that the currently recognizable chimp groups are likely to be among the most important antigens of the chimp's major histocompatibility system. In anticipation of confirmation by family studies and in analogy with the human HL-A system, the major histocompatibility system of chimpanzees has been designated as the ChL-A system.

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Characterization of Chimpanzee Leukocyte Alloantisera

By R. S. Metzgar, H. F. Seigler, F. E. Ward, E. D. Hill, and T. Mohanakumar

DETAILED CHARACTERIZATION of the major histocompatibility system of man (HL-A) has progressed rapidly during the last decade. The organization of the HL-A locus became clearer through extensive family studies, and the relationship of the system to histocompatibility was aided by the correlation of leukocyte typing with mixed lymphocyte reactivity (MLR) and with skin graft survival times between siblings. Definition of the major histocompatibility locus in chimpanzees has been handicapped by the paucity of families in this species. There are very few chimpanzee families with two full siblings and none, to our knowledge, with more than two, so that characterization of chimpanzee alloantigens and their relationship to histocompatibility will initially have to come from studies of unrelated animals. Preliminary studies from our laboratory[1,2] and from Balner et al.[3,4] established the existence of chimpanzee leukocyte groups with chimpanzee alloantisera. Recently Balner et al.[4] established the existence of eight chimpanzee leukocyte groups after testing cells from 195 animals. We have completed cytotoxicity testing of 148 chimpanzees with our chimpanzee antisera as well as with serum samples representative of the chimpanzee antigens described by Balner.

The cytotoxicity method used for this study is a modification of the Terasaki microtechnique described by Mittal et al.[5] One microliter of the antiserum was mixed with ½ μl of purified lymphocytes (4000/cu mm) and incubated at room temperature for 35 min. Rabbit complement (5 μl) was then added and the mixture incubated for 35 min at 37 C. Eosin (5 μl) and formalin (2 μl) were added and the reaction read on an inverted phase microscope.

Some of the chimpanzee serum donors used in this study received cells from individual human donors during the course of their immunization. These animals also later received cells from a chimpanzee donor rather than from a human. The animals that received human cells in addition to chimpanzee cells are given in the paper by Ward in this issue.[6]

Figure 1 is a dot chart of the cytotoxicity reactions of 49 chimpanzee alloantisera with lymphocytes from 115 chimpanzee donors. All animals were tested in duplicate and 66 of the animals were repeated on three different occasions. Eleven antisera are those provided by Balner and are representative of some of the chimpanzee groups that he and his colleagues described.[4] The *r* values, from pairwise comparison of all the antisera used in Fig. 1 with 115–148 chimpanzee cell donors, are given in Fig. 2.

Group 1 is related to antigen 4a and has been previously referred to as Duke 1. It correlates well with sera-defining antigens 5 (Jac 4) and 6 (Jac 7) of Balner. It is also positively associated with Groups 2 and 5 and negatively associated with Groups 3, 4, and 7. Preliminary absorption studies with three antisera in Group 1 (Larr 10-68, Peck 3-70, and Kong 2-70) indicate that this group of antisera is probably detecting a single antigenic specificity. Each of these antisera were absorbed with cells from four different positively reacting chimpanzee donors and then tested with cells from 35 positive animals. Absorption of any of the three antisera with cells from any of the donors removed the reactivity for all 35 Group 1 positive cells tested. Antisera Peck 3-69 and 3-70 of this group when tested on

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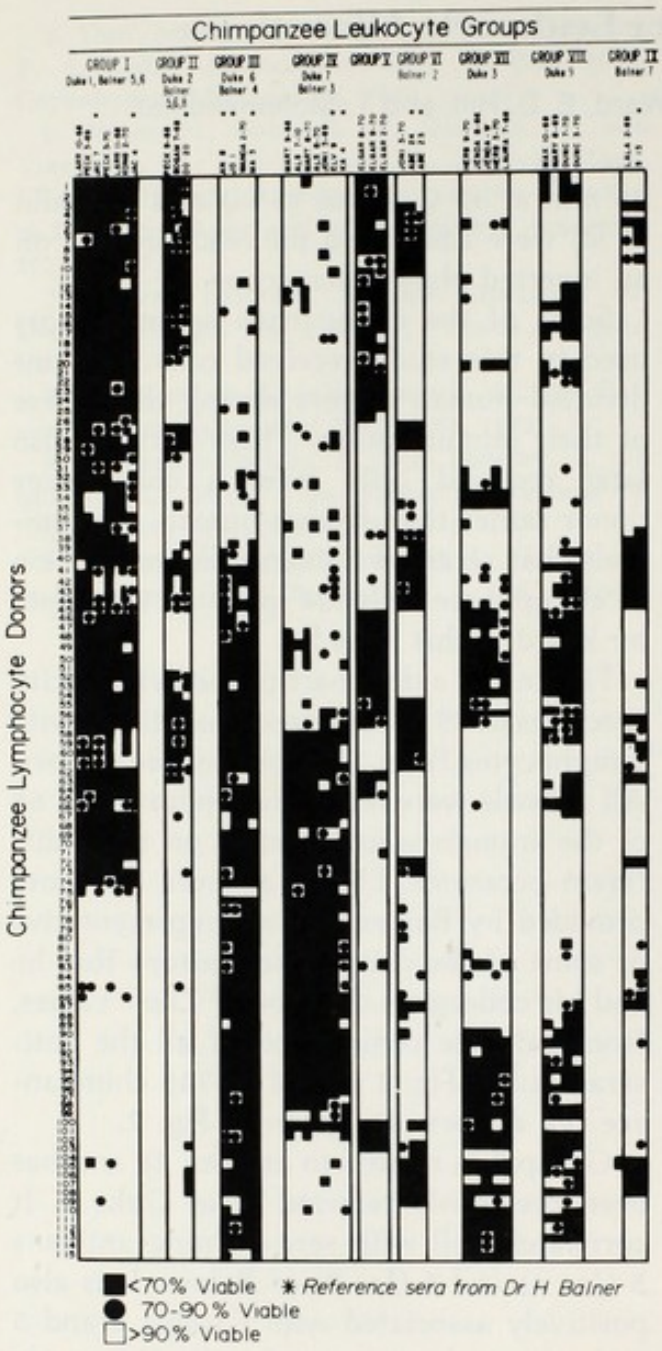

Fig. 1. Cytotoxicity reactions of chimpanzee alloantisera with chimpanzee lymphocytes.

human cells gave a high correlation (*r* value, 0.9) with the 4a EDTA leukocyte agglutination typing of van Rood.[6]

Group 2 antisera can be classified as a short 4a and have been previously referred to as Duke 2. Our antisera Peck 9-68 and Bogam 7-68 are positively associated with serum Do 20 of Balner's antigen 8 and are also associated with Groups 1 and 5. Sera from this group give a negative correlation with Groups 3, 4, 6, and 7. Antiserum Peck 9-68 from this group has been initially absorbed with cells from four different chimpanzees that reacted with this group of antisera and tested with cells from 16 positive donors. Absorption with cells from any of the four donors removed all reactivity for the 16 other donors tested.

Group 5 is composed of sera from three different bleeding dates from chimpanzee Elgar. These sera are positively associated with Groups 1 and 2 but react with cells from three different donors (Nos. 101, 102, and 103) that fail to react with the long 4a sera (Group 1). This group is also negatively associated with Groups 3 and 4. Antiserum Elgar 8-70 of this group has been absorbed with cells from three negative donors, each of which removed all cytotoxic activity when tested with cells from 13 other positive donors.

Groups 3 and 4 are related to antigens 4b and/or 7c. Three of the antisera in Group 3 are from Balner and are representative of his antigen 4. Thus far we have only one antiserum that correlates with this group. Absorption of this antiserum (Wanda 2-70) with any one of five cells positive for Group 3 antigen removed reactivity for 22 other donors positive for this group. One antiserum (Mart 8-70) in Group 4 has been shown to be multispecific. Absorption of this antiserum with cells from two of the donors used to absorb antiserum Wanda 2-70 in Group 3 left good reactivity for certain other donors positive for Group 4. Thus the relationship between Group 4 (short 4b) and Group 3 (long 4b) apparently is not one of inclusion. We have not as yet been able to correlate the reactions of the absorbed Mart 8-70 antiserum with any of the existing chimpanzee groups.

Group 6 includes two antisera defining Balner's antigen 2 and one antiserum from our panel. This group shows a positive association with Group 9 and a negative

association with Group 2. Groups 7 and 8 give a positive correlation with each other and with Group 3. None of the Balner sera tested gave a high correlation with either of these two groups. One antiserum in each group (Group 7, Jenda 8-66; Group 8, Max 10-68) after absorption with cells from five different donors was negative when tested back with 17 donors positive for the particular antigen group. Group 9 is represented by an antiserum defining antigen 7 of Balner and one of our alloantisera. This group shows a positive correlation with Group 6. No absorptions have been per-

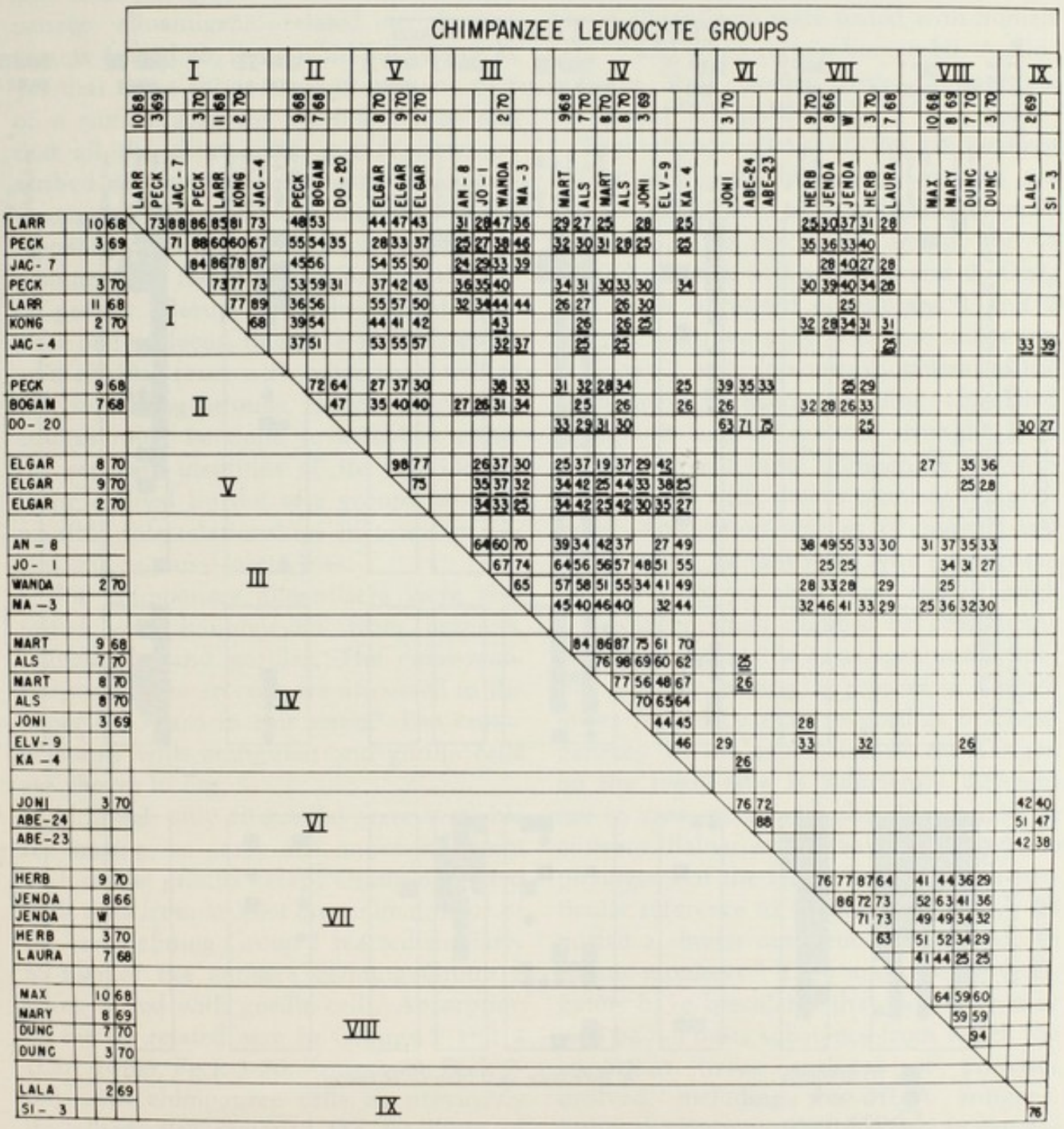

Fig. 2. Correlation coefficients (*r* values) × 100 between chimpanzee alloantisera. Underlined numbers are negative correlations. Chimpanzee antisera are those used in Figs. 1 and 3.

formed as yet with antiserum Lala 2-69.

Thus we detected at least nine different antigen groups that correlate with seven of the eight antigens defined by Balner et al.[4] in a recent publication. We prefer to consider the similarly reacting antisera as defining antigen groups rather than individual antigenic specificities, since the absorption data are limited and adequate family studies are not currently available. The groups related to 4a (Groups 1 and 2) show the usual positive association with each other and a negative association with Groups 3 and 4 related to 4b. Only two of

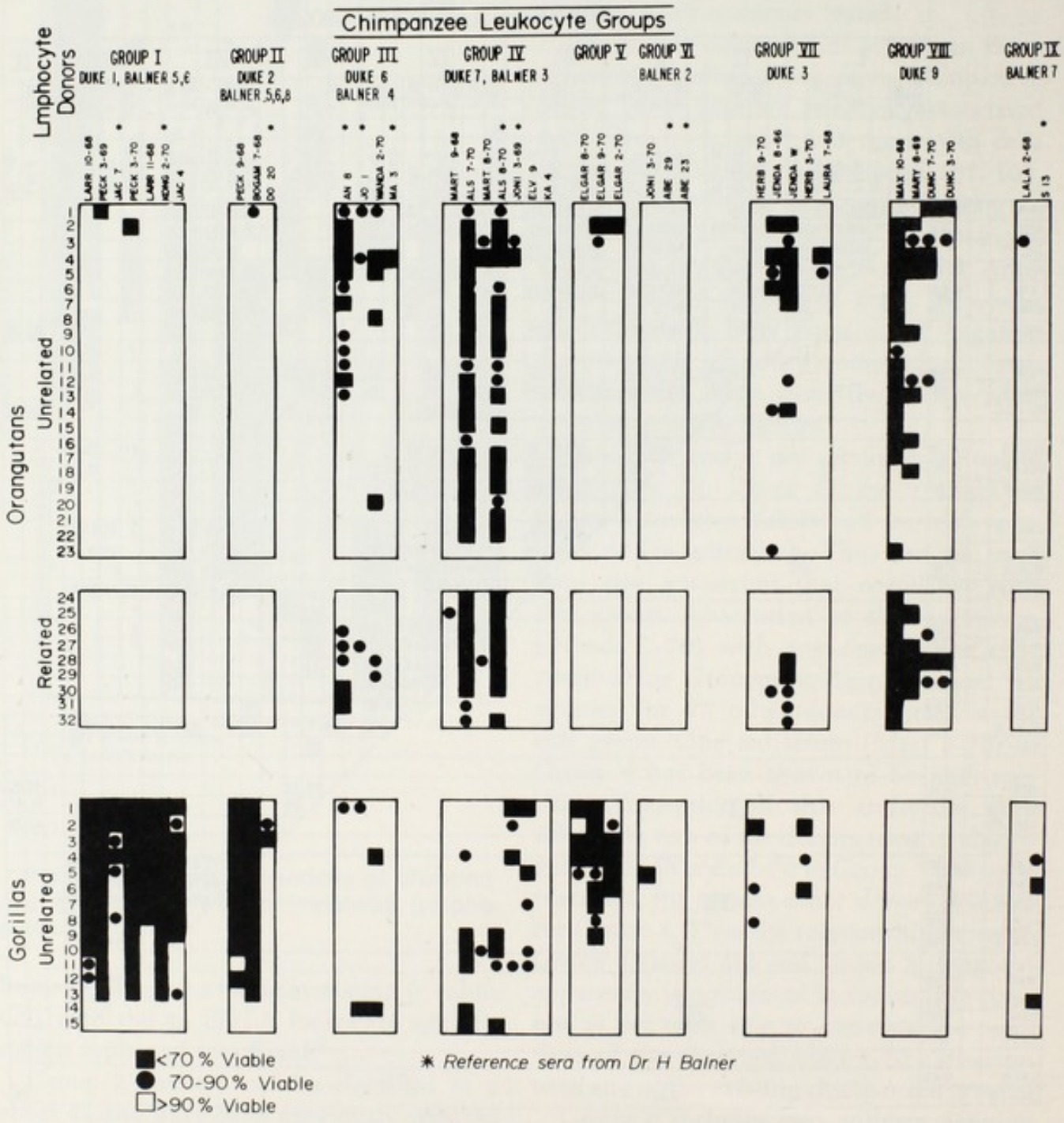

Fig. 3. Cytotoxicity reactions of chimpanzee alloantisera with orangutan and gorilla lymphocytes.

the 115 chimpanzee donors shown in Fig. 1 failed to react with any sera of Groups 1, 2, 4, and 5. Cells from these two donors might be classified as 4a and 4b negative although one antiserum from the 4b Group 3 reacted and cells from these two animals were not used for absorption to rule out the CYNAP phenomenon. Although the data clearly establish the existence of chimpanzee alloantigens related to human groups 4a and 4b, there is little evidence as yet that these chimpanzee groups are part of a similar sublocus in this species and that all the chimpanzee groups now described are related to the same major histocompatibility system. If one looks at the reactions of Groups 6–9, for example, and assumes that these are not part of the 4a-4b groups (Groups 1–5) that might comprise one sublocus, there are still numerous animals that react with more than two of the remaining groups. More absorption studies must be done to establish either the separate identities of the specificities being defined by the sera groups or their possible interrelationships in terms of inclusions or cross-reactivities.

The chimpanzee alloantisera were also tested with lymphocytes from humans, orangutans, and gorillas. The cross-reactions with human cells are discussed in the paper by Ward in this issue.[6] The cross-reactions with orangutan and gorilla cells are shown in Fig. 3.

Although only 15 gorillas were available for testing, at least one antiserum from each of the groups except Group 8 reacted with cells from at least one animal. Four of the sera defining Group 1 reacted similarly to two of the antisera defining Group 2 when tested with gorilla cells. Absorption of the 4a related sera in Groups 1 and 2 (Larr 10-68, Peck 3-70, Kong 2-70, Peck 9-68) with chimpanzee cells as previously described, also removed the reactivity of these antisera for gorilla cells. In two of the gorillas tested (Nos. 14 and 15) that were negative for all sera defining chimpanzee 4a, Groups 1 and 2 reacted with some of the sera defining Groups 3 and 4 related to 4b. The alloantisera defining groups in the chimpanzee population for the most part do not react as similar groups when tested with the limited numbers of orangutans and gorillas available. These findings have also been noted when certain chimpanzee group alloantisera were tested with human cells[1,2] and when certain human HL-A alloantisera were tested with chimpanzee, orangutan, and gorilla cells.[2,6,7]

The cross-reactions of the chimpanzee alloantisera with the orangutan cells are interesting because most of the 32 animals tested failed to react with any of the 4a related antisera in Groups 1 and 2. Only cells from unrelated orangutans 1 and 2 reacted and then only with one antiserum in Group 1 or 2. In contrast, several of the chimpanzee antisera defining 4b-related Groups 3 and 4 reacted strongly with orangutan cells, and chimpanzee 4a antisera reacted with rhesus monkey lymphocytes. Absorption studies of human and chimpanzee 4a antisera with orangutan cells have not yet been done, and although a relatively small number of orangutans have been tested, the 4a antigen in this species is either lacking, is present in a much lower frequency than in gorillas or chimpanzees, or the expression of the antigen on the membrane is sufficiently different not to cross-react with the chimpanzee 4a antisera. Balner et al.[4] have discussed the phylogeny of the HL-A antigens with particular reference to 4a and 4b and have reported a similar antigenic configuration in rhesus monkeys.[8] They and other investigators have speculated that 4a and 4b may well be the basic substance from which the important tissue antigens of primates evolved, including the HL-A antigens. Either the absence or a difference in expression of 4a associated specificities in this group of higher apes would represent a

significant deviation in the phylogeny of certain HL-A associated antigens. We have recently isoimmunized eight orangutans and are in the process of characterizing the allospecificities being defined in this species. It will be interesting to see the nature of the cross-reactions of these antisera with gorilla, chimpanzee, and human cells and their relationship to 4a antigens. Orangutan lymphocytes failed to react with cells from antisera defining Groups 6 and 9 and only one animal gave a significant reaction with Group 5 antisera. Several Group 8 sera that failed to react with cells from the 15 gorillas reacted strongly with orangutan lymphocytes.

The data presented here, in other papers at this symposium, and in previously published work[1-4] establish the existence of chimpanzee alloantigens, some of which may be similar to some of the HL-A antigens of man. The nature of the similarities and differences is one of the main concerns of this symposium. It is quite clear, however, that several antisera apparently defining the same antigen(s) in one species can react quite differently when tested with lymphocytes from another closely related species. Similar results have been noted when defined human HL-A antisera were tested with certain isolated racial groups.[9,10] This finding implies heterogeneity in antibody specificity between individual sera defining the same antigen and variation in expression of an antigen defined in one species of cells from different but closely related species. The antibody heterogeneity could be due to the closeness of fit or avidity of the antibody active site with the antigenic determinant, and the antigen heterogeneity could be due to the qualitative or quantitative differences in the expression of the antigen on the cell membrane. Differences in antigen expression between species could also be influenced by the presence of different species-specific membrane antigens that may exert an effect on alloantigens by steric hindrance.

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HL-A and ChL-A: Similarities and Differences

By J. J. van Rood, A. van Leeuwen, and H. Balner

IN PREVIOUS PUBLICATIONS we have shown that chimpanzees carry equivalents of the 4a (W4) and 4b (W6) antigens, ChL-A1 and 4, respectively.[2] Chimpanzees make useful cytotoxic anti 4a and anti 4b antibodies which can be used in human histocompatibility typing. Furthermore, it was shown that they carry the 7c antigen. Similarly, Dorf and Metzgar reported the presence of HL-A1, HL-A7 and 4a (W4) antigens on the lymphocytes of their chimpanzees.[1,2] In this publication we will focus our attention especially on the so-called short antigens.[1] The main questions we want to ask ourselves are: To what extent are the antigens carried by chimpanzees really serologically undistinguishable from the corresponding HL-A antigens? What are the implications of the discrepancies we have already encountered?

MATERIALS AND METHODS

Chimpanzees: Our colony consists of 62 animals. Part of this study was done with chimpanzees from the Behringwerke, Western Germany. Previous work included the typing of chimpanzees from the various U.S. primate centers.

Humans: All chimpanzee sera were prescreened against a highly selected panel of 46 unrelated donors and, if promising, tested against 100 or more individuals, including some family members.

Sera: The chimpanzees were tested with our regular panel of human typing sera (i.e., 29 agglutinating sera, 81 cytotoxic sera, and 23 complement fixing sera) as well as with a panel of 72 chimpanzee isoantisera.[5] Most of the chimpanzee sera were produced by Balner and co-workers; some were provided by Metzgar and Johannsen from Behringwerke. The 72 chimpanzee sera were tested also in humans, both in the cytotoxicity and the microcomplement fixation test.

Techniques: EDTA agglutination, microcytotoxicity (one stage and two stage), microcomplement fixation and absorption tests were performed as described.[3,4] Cross-species typing results were checked by absorptions,[6] as indicated in the text and tables, i.e., human sera were absorbed with chimpanzee lymphocytes, retested against the absorbing cell and against human lymphocytes reactive with the serum under study.

RESULTS

Typing Chimpanzee Lymphocytes With Human Sera

Table 1 summarizes the results of testing 138 human sera against the chimpanzee panel with three direct techniques (agglutination, cytotoxicity, and complement fixation) and checking by the (indirect) technique of absorption. In the LA series evidence points to the presence of HL-A 1 and HL-A 11. W28 (Ba*) and W19 (Li) might be present, but evidence is incomplete. For the remaining antigens it can only be said that the direct tests thus far do not indicate their presence or absence conclusively.

For the Four series, apart from 4a (W4) and 4b (W6) which are clearly represented, the data strongly indicate the presence of two or three antigens of the 7c° complex. The 7c complex consists of HL-A 7, and W22 (AA) and W27 (FJH).[7] Furthermore, it is possible but not proven that HL-A 12, W15 (LND) and W17 (Orlina, Mapi) occur in the chimpanzee, while evidence for the presence of HL-A 13, W5 (R) and W14 (Maki) is only preliminary. The results of the direct tests do not provide evidence for the presence of other antigens of the Four

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series, which does not, of course, prove their absence.

Typing Human Lymphocytes With Chimpanzee Sera

In the chimpanzee we were able to recognize eight different specificites most of them probably complex and some included in each other.[5] When the 30 chimpanzee isoantisera used to detect the ChL-A specificities 1–8 and 42 unclassified chimpanzee isoantisera were tested against the panel of highly selected cells obtained from 46 humans (using one-stage cytotoxicity and complement fixation techniques) the following results were obtained (Table 2): ChL-A 1 sera recognize 4a (W4); ChL-A 4 sera recognize 4b (W6), while one anti-ChL-A 4 serum recognized W15 (LND) in the complement fixation test; ChL-A 8 sera recognize HL-A 11, while a few sera defining ChL-A 2 and ChL-A 7 recognize HL-A 11 plus part of HL-A 3; ChL-A 3 sera recognize W22 (AA), with part of HL-A 7; ChL-A 5 and 6 sera have thus far been negative. In addition, the chimpanzee

Table 1. The Presence of HL-A Antigens in the Chimpanzee

HL-A Specificity	Reactivity of Human Sera With Chimpanzee Cells: Cytotoxicity (One Stage)	Cytotoxicity (Two Stage)	Complement Fixation	EDTA Agglutination	Absorbing*	Immunizing†
LA series						
1	3++‡	2 (all positive)	5++		+++	+++
2	3–	1–	4–	–		
3	3–	1++	1++			<3
9	3–	1–	3+++			
10	4–	1–				
11	2+++	2+++	1++		+++	+++
Ba* (W28)	2–3++	1++			+++	
Li (W19)	1++					
FOUR series						
5 SL	2++					
5	3–	1–	2–		–	
7	3–4++	2–	2–		+++?	
8	3–	1–	1–			
12	3–	1++	5++		+?	
13	2–3–	2–	1+			
R (W5)	2–	2–	2++			
Orlina (W17)	3+++	1++	1–		++	
AA (W22)	2+++	1++	1++		++	+++
FJH (W27)	3–	1++	2+			
BB (W10)	2–	1–	1+			
Maki (W14)	2–	1++	1+			
LND (W15)	2++	1++	4++		++	+++
4a (W4)	3++	2++		3++	++	++
4b (W6)	3++	1++		3++	++	++

‡The number indicates how many sera were used to detect this specificity; it implies that positive and negative reactions were found. Italics means that the sera used give regularly consistent, i.e., similar results; +++, serologic evidence strongly positive; + serologic evidence weakly positive.

*On the basis of absorbing human anti-HL-A sera with chimpanzee cells and testing the absorbed serum against human cells reactive with the unabsorbed serum.

†On the basis of testing human cells with chimpanzee isoantisera (Table 2).

Table 2. Reactivity of Anti-ChL-A Sera With Human Lymphocytes

Anti-ChL-A	ChL-A Series	Cytotoxicity (One Stage)	Complement Fixation
1	Four	4a (W4)	Positive
2	LA	11 + <3	Negative
3	Four	W22 + <7	W22
4	Four	4b (W6)	LND
5	Four	Negative	Negative
6	Four	Negative	Negative
7	LA	11 + <3	Negative
8	LA	11	Negative

The ChL-A series LA and Four are thought to be the chimpanzee equivalents of the HL-A series LA and Four.

sera sent to us by Johannsen (Behringwerke) and Metzgar (Duke University) recognized, in part, the same HL-A antigens, furthermore Metzgar's Duncan 0370 shows an excellent correlation with HL-A 1. The data in Table 1 and 2 indicate that isoimmune human sera can recognize isoantigens on chimpanzee cells and vice-versa that chimpanzee sera can recognize HL-A antigens on human cells.

Detailed Analysis of Several Specificities

Tables 3, 4, and 5 detail the degree of similarity between three HL-A specificities (HL-A 11, W15 (LND), and 7c and their counterparts on chimpanzee cells.

HL-A 11: Table 3 shows that the reactivity of a human anti HL-A 11 serum (No. 4306) is nearly identical to a chimpanzee isoimmune serum (No. 6391) when tested against almost 200 unrelated individuals. When these sera were tested against part of the chimpanzee colony they showed a similar reaction pattern, although the human serum was weaker. Thus chimpanzees might carry an equivalent of the HL-A 11 antigen with a gene frequency of approximately 0.22. On the basis of typing with both the human and chimpanzee sera, HL-A 11 shows the best correlation with ChL-A 8 but chimpanzee alloantisera reactive with ChL-A 2 and 7 were also often reactive with HL-A 11 in humans (Table 2).

W15 (LND): Table 4 shows that the human anti W15 (LND) serum 8188 and the chimpanzee serum 4278 (An 23) give an excellent correlation when tested against human lymphocytes. When they were tested against the chimpanzee panel it turned out that the chimpanzee serum had a much broader reactivity than the human anti-W15 serum, in fact its reactivity pattern is only slightly shorter than that of anti-ChL-A 4 (4b) sera. Thus, although the W15 (LND) is present on chimpanzee lymphocytes it cannot be equated with one of the ChL-A antigens we have been able to recognize thus far.

Table 4 also shows that we had available another anti-W15 serum (No. 12371) which gave a good correlation with 8188 when tested in humans. The few extra reactions were due to weak anti-W5 (R) reactivity. When the two sera were tested against the chimpanzee panel it turned out that serum 12371 was quite often positive, while 8188 was positive only occasionally. The extra antibody in serum 12371 did not show a correlation with the antibody recognizing the non-W15 chimpanzee antigen in serum 4278. Most of the chimpanzee cells reactive with 12371 in the cytotoxicity test were not able to remove by absorption the reactivity

Table 3. The Presence of HL-A 11 on Chimpanzee Lymphocytes

Human Panel	Human 4306 Anti-HL-A 11			Chimpanzee Panel	Anti-HL-A 11 Human 4306			
Tineke 5		+++	—	Tineke 5		+++	+	—
chimpanzee 6391	+++	15	1	chimpanzee 6391	+++	6	9	2
	—	0	174		—	1	2	21

Table 4. The Presence of LND (W15) on Chimpanzee Cells

Human Panel	Anti-LND Human 8188			Chimpanzee Panel	Anti-LND Human 8188		
Anita 23		+++	—	Anita 23		+++	—
chimpanzee 4278	+++	22	1	chimpanzee 4278	+++	1	12
	—	1	120		—	0	36
Anti-LND		+++	—	Anti-LND		+++	—
human 12371	+++	10	2	human 12371	+++	2	13
	—	0	100		—	0	46
Chimpanzee Panel	Anti-LND Human 8188 (Absorption)			Chimpanzee Panel	Anti-LND Human 12371 (Absorption)		
Anti-LND human 8188		+++	—	Anti-LND human 8188		+++	—
(cytotoxicity)	+++	1	1	(cytotoxicity)	+++	1	6
	—	0	26		—	0	2

against human cells. Interestingly, the single chimpanzee cell sample able to remove the antibody from both sera was from the chimpanzee that induced anti-W15 (LND) reactivity in the serum of another chimpanzee (serum An 23).

The 7c Antigen: We have previously reported that chimpanzee cells were reactive with agglutinating anti-7c sera and were able to absorb out the agglutinating anti-7c antibody.[8] Anti-7c antibodies cross-react with HL-A 7, W22 (AA) and W27 (FJH).[7] Testing with cytotoxic anti-7 sera gave erratic results: the reactions were generally weak and not consistent, i.e., no chimpanzees were found that gave a positive reaction with all three or four anti-7 sera used.

When it was observed that a chimpanzee had made a reasonably good anti-W22 (AA) serum (No. 3600 Elv 14) it was decided to study the matter further (Table 5). It could be shown that the chimpanzee serum 3600 gave an excellent correlation on chimpanzee cells with agglutinating human anti-7c sera. Because the reactions of chimpanzee cells with several human anti-W27 (FJH) sera were generally nega-

Table 5. The Presence of 7c (HL-A 7 + AA + FJH) on Chimpanzee Cells

Human Panel	Anti-AA Human 710			Chimpanzee Panel	Agglutinating 7c		
Elvis 14		+++	—	Anti-HL-A 7 human 1081		+++	—
chimpanzee 3600	+++	7	2	(absorption)	+++	9	0
	—	0	49		—	5	24

Chimpanzee Panel	Anti-AA Human 710		
Elvis 14		+++	—
chimpanzee 3600	+++	11	12
	—	5	20

Chimpanzee Panel	Agglutinating 7c			Chimpanzee Panel	Agglutinating 7c		
Elvis 14		+++	—	Anti-HL-A 7+FJH		+++	—
chimpanzee 3600	+++	12	1	human 2398	+++	1	6
	—	2	24	(absorption)	—	3	4

tive (Table 1) and because chimpanzees can make anti-W22 (AA), the obvious question was whether or not 7c in chimpanzees equates with W22 (AA). Table 5 shows that a human anti-AA serum (No. 710) did not recognize the same antigen in chimpanzees as the chimpanzee anti-AA serum (Elv 14), although the two sera show an excellent correlation when tested against human lymphocytes. Two anti-HL-A 7 sera were used to determine whether HL-A 7 is present on chimpanzee lymphocytes. One serum, No. 1081, is a "pure" anti-7, the other a serum that cross-reacts with 7 and W27 (FJH) (No. 2398).

The sera were only weakly reactive in the one stage cytotoxicity test against the chimpanzee panel. When they were absorbed with chimpanzee cells it turned out that they recognized a polymorphism, i.e., some cells were able to remove the antibody, others were not. About two thirds of the cells able to remove the antibody activity from serum 1081 were the ones that carried 7c.

Because 1081 and 2398 are identical except for anti-W27 (FJH) activity in 2398 and because W27 (FJH) appears to be lacking in chimpanzees we had expected the two sera to react identically in chimpanzees. However, Table 5 shows that serum 2398 could recognize an antigen allelic to that recognized by 1081.

It should finally be noted that all chimpanzee sera with anti-7c activity had anti-ChL-A 3 activity when tested on chimpanzee cells (Table 2).

DISCUSSION

Before discussing these data it is first necessary to answer the question: what criteria have to be fulfilled before one can conclude that an HL-A antigen is present in another species, in this case in the chimpanzee? It seems realistic to accept that there are stages of certainty.

Stage 1: When, after isoimmunization, an antibody is formed that recognizes an HL-A antigen, one can conclude that the equivalent of that antigen is present in the chimpanzee. One restriction which should be made is that such sera must be tested against large numbers of cells to exclude the possibility that the serum recognizes a variant. On the basis of chemical analysis, in the future, it may be possible to conclude that the *same* antigenic substance is present. Possible candidates are (Table 1) HL-A 1, 11, HL-A 7, W22 (AA), W15 (LND), 4a (W4) and 4b (W6).

Stage 2: When chimpanzee cells can remove antibody activity by absorption from human oligospecific anti-HL-A sera (as shown by testing after absorption against appropriate human cells), then this is strong suggestive evidence, but not final proof, for the presence of the corresponding antigen on chimpanzee cells. These absorption experiments do not exclude the possibility that absorption is due to cross-reactivity of different antigens. In this category could fit W28 (Ba*) and W17 (Orlina or Mapi).

Stage 3: When two or more anti-HL-A sera give concordant results when tested with chimpanzee cells, i.e., when they are regularly both positive or both negative, one can conclude that it is possible the same HL-A antigen under study (or an equivalent) is present in the chimpanzee. This criterion carries special weight when such concordance is observed while using different techniques like cytotoxicity and complement fixation. HL-A 3, 9, 12, and W14 (Maki) might classify in this stage.

Stage 4: When a single human anti-HL-A serum detects polymorphism in chimpanzees or two or more sera give positive but not concordant results it should never be regarded as proof for the presence of the corresponding antigen in chimpanzees. For all we know it might be a completely unrelated antigen. For the time being, we interpret in this fashion the reactions ob-

tained with the anti-Li and anti-SL sera.

It will be clear that an antigen can move from stage 4, to 3, to 2 to 1. It will also be clear that we do not regard our studies as finished and that this paper should be regarded as a progress report.

The data on HL-A 11 are consistent with the assumption that an HL-A 11 antigen very similar to that in man is present in the chimpanzee. As in man, it seems that the chimpanzee anti-11 antibodies cross-react with HL-A 3. In contrast to man, HL-A 11 seems to be a stronger antigen than HL-A 3 in chimpanzees. It should be remembered though that our data do not show conclusively that HL-A 3 is present in the chimpanzee. It is interesting that seven of the nine anti ChL-A 2, 7, and 8 sera recognize HL-A 11 (with part of HL-A 3) when tested against human cells. Because ChL-A 7 and 8 have an antithetic distribution and are included in ChL-A 2,[5] one could speculate that ChL-A 7 and 8 comprise at least two HL-A related antigens one of which is shared and is identical or similar to HL-A 11. Against this explanation argues the observation that HL-A 11 correlates best with ChL-A 8. There is as yet no proof for the alternative explanation that ChL-A 7 is equivalent to HL-A 3.

The W15 (LND) story seems to be rather straightforward. The antigen appears to be present in the chimpanzee in low frequency. The only anti-W15 serum that has been produced cross-reacts with a chimpanzee antigen that we have not yet detected in man but constitutes the greater part of ChL-A 4 in chimpanzees that we had equated with 4b. This is in itself interesting, because it might imply that anti-4b sera, *if* they contain cross-reacting antibodies, react with one set of antigens in man and with an at least partially different set of antigens in the chimpanzee.

The finding that the two human anti-LND sera which seemed to be nearly identical when tested in humans were dissimilar when tested in chimpanzees confirms and extends the observations by Shulman et al. that the use of other species to study the reactivity of human typing reagents can be very informative.[9]

In our opinion, the 7c data are the most revealing, in that they warn us of how incomplete our insight is into the fine structure of the HL-A antigens. Our data show that in the chimpanzee 7c cannot be equated with AA (W22). On the other hand, AA or an equivalent must be present in the chimpanzee, otherwise the anti-AA antibody could not have been formed.

We also have no explanation for the behavior of the anti-HL-A 7 sera. It could be that the anti HL-A 7 plus anti-W27 (FJH) serum recognizes a chimpanzee equivalent of W27 that we have so far missed with the pure W27 (FJH) sera. Similarly, the behavior of the pure anti-HL-A 7 serum could be explained by the assumption that the serum contains an anti-W22 (AA) antibody which gives 100% Cynap reactions in humans but recognizes part of the W22 (AA) in chimpanzees.

These assumptions would also imply that HL-A 7 is not present in the chimpanzee. Although a pure anti-HL-A 7 chimpanzee serum has not been found thus far, it is relevant to point out that some of the anti-ChL-A 3 sera, when tested in humans, contained a weaker and generally incomplete anti-HL-A 7 next to the anti-W22 (AA). Another possibility is that HL-A 7 is present in the chimpanzee together with W22 (AA). This could explain the similar reactivity of the pure anti-7 and chimpanzee anti-AA sera but not the lack of correlation with the human anti-AA.

It is unknown whether the ChL-A antigens are chemically identical to the HL-A antigens. Our strongest argument, that at least some of them are identical, the immunization studies, is still an indirect one: the specificity of the antibody is certainly determined by the immunizing antigen, but

at least as much by the repertoire of the immunocompetent cell clones forming the antibody. If for instance ChL-A8 is found to be chemically different from HL-A 11, this would imply that two different antigens gave rise to the same antibody specificity. That could be interpreted as an argument that the anti-HL-A and anti-ChL-A antibodies have a biological function.

As stated in the introduction, our interest in investigating the similarities of the HL-A and ChL-A systems had its origin also in the need for specific strong typing sera for man. Although excellent sera can be obtained from humans, we have wondered whether the chimpanzee might not be preferable as an anti-serum producer and not only for the obvious ethical reasons. Our line of reasoning was the following. As discussed by Jerne, the experience gained with the MLC test implies that there may be an innate cellular immunity against all but one's own (HL-A) antigens.[10] When an individual is immunized (or rather hyperimmunized?) against HL-A, antibody production will follow against one or more HL-A antigens. The specificity of this antibody response however may be "confused" by the memory of the innate cellular immunity which, after all, is directed against all HL-A antigens.

It might be that this is the basic reason why hyperimmune antibodies are often of such broad specificity. The chimpanzee, although having a "confused *chimpanzee* memory" would hopefully have an unconfused memory for most of the HL-A antigens and thus produce antisera which, when tested on human cells, might be of higher resolution than the average human antiserum. It is too early to decide whether this is indeed the case, but considering small numbers of chimpanzees immunized and tested thus far, the yield of interesting strong typing reagents for man certainly warrants continuation of this approach. These considerations also argue for the use of partially purified HL-A antigens in the chimpanzee and other primates as had already been done by Metzgar and Miller.[11]

SUMMARY

A colony of 62 chimpanzees was tested with 210 human and chimpanzee alloantisera by cytotoxicity, complement fixation and agglutination. Results were checked by absorptions and by retrospective analysis of immunization data. Criteria have been formulated and must be met before one can decide whether an HL-A antigen is present in another species. For chimpanzees, evidence available to now indicates the probable presence of HL-A 1, 11, 7, W22 (AA), W15 (LND), W4 (4a), and W6 (4b), and the possible presence of HL-A 3, 9, 12, W 14 (Maki), W28 (Ba*), and W17 (Orlina, Mapi). The theoretical advantages of raising sera for human tissue typing in a related species are discussed.

ACKNOWLEDGMENT

The skilled assistance of all members of the maintenance department of the Primate Center T.N.O. was indispensable and highly appreciated. The excellent technical performance of the serological test by Mrs. A. Puliga, Mrs. R. Castelli, Mrs. A. van Berkel, Miss H. de Groot, Miss E. Blokland, Mrs. L. Roman, Miss R. de Graaf, and Miss C. A. Zandbergen is gratefully acknowledged.

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Cytotoxicity Reactions of Chimpanzee Antisera With Human Lymphocyte Donors Phenotyped or Genotyped for HL-A

By F. E. Ward, H. F. Seigler, and R. S. Metzgar

THE ABILITY OF CHIMPANZEE ALLOANTISERA to detect human lymphocyte polymorphisms has been previously described.[1,2] Most of these studies used cells from unrelated donors phenotyped for HL-A, although the ability of some of the antisera to determine HL-A haplotypes in certain human families has been reported.[3] In the latter study, most of the chimpanzee antisera that detected cross-reacting alloantigens in the human population seemed to be detecting HL-A related antigens.[3] Since some of the chimpanzee antiserum donors used in our studies had initially received cells from human donors before getting cells from a chimpanzee donor (xenoalloimmunization), we were interested in expanding the family studies to see whether any non-HL-A antigens could be detected and to determine what specificities were being defined in the cross-reacting HL-A patterns with HL-A phenotyped unrelated donors. The work to be reported here is the result of the recent cytotoxicity testing of chimpanzee alloantisera and chimpanzee xenoalloantisera with unrelated individuals phenotyped for HL-A and 56 families genotyped for HL-A.

Seventy-two unrelated individuals from our HL-A reference cell panel and 56 different families, genotyped for the HL-A system, were tested with up to 118 antisera produced in chimpanzees. Families were tested only with chimpanzee antisera produced in our laboratory, whereas the 72 unrelated individuals were also tested with reference chimpanzee antisera kindly supplied by Balner. A majority of the families were tested only once, but six families, including the BER family presented below, were tested in duplicate on two or more occasions. A two-stage semimicro cytotoxicity dye exclusion method was employed.[4] Some of the chimpanzee antiserum donors used in this study received only chimpanzee antigens, whereas others initially received cells from individual human donors and later cells from chimpanzee donors. Identification of all defined HL-A antigens in the unrelated donors and family members was by lymphocytotoxicity, employing human alloantisera. The 4a and 4b typing of some of the unrelated donors was done by van Rood using EDTA leukoagglutination and his human sera.

The reactions obtained by testing the 72 unrelated individuals with 118 chimpanzee antisera are shown in Fig. 1. Phenotypes of these individuals for defined HL-A specificities are indicated towards the bottom of the figure. Antisera raised in animals that initially received human cells are indicated by an asterisk.

Metzgar et al.[5] have recently defined nine antigenic groups (Groups I-IX) in chimpanzees using some of the 118 antisera (Table 1). Chimpanzee antisera that defined a particular group in the chimpanzees did not behave similarly when tested with human lymphocytes. However, two of the antisera in Group I were correlated with antigen 4a defined by EDTA leukoagglutination with human sera.

Although chimpanzee alloantisera generally were less frequently reactive with the

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Fig. 1. Reactions of chimpanzee antisera with unrelated human cells phenotyped for HL-A.

TABLE 1.

Antigen Groups Defined in a Chimpanzee Population by Chimpanzee Antisera

GROUP I
4a, Duke 1, Balner 5 or 6:
*Peck 3/27/70
Larr 11/4/68
*Kong 2/9/70
*Larr 10/18/68
Peck 3/28/69

GROUP II
4a Related, Duke 2, Balner 5, 6, or 8:
*Peck 9/17/68
Bogan 2/9/70
Bogan 7/68

GROUP III
4b, Duke 6, Balner 4:
*Wanda 2/9/70

GROUP IV
7c, Duke 7, Balner 3:
Als 8/7/70
Als 7/31/70
Martha 9/30/68
Joni 3/28/69
Martha 8/7/70

GROUP V
*Elgar 9/24/70
Elgar 8/27/70
*Elgar 2/17/70

GROUP VI
Balner 2:
Joni 3/27/70

GROUP VII
Duke 3:
*Jenda 8/5/66
Jenda W
Herbert 9/23/70
Laura 7/68
Herbert 3/24/70

GROUP VIII
Duke 9:
*Max 10/10/68
Mary 8/20/69
Duncan 7/10/70
Duncan 3/13/70

GROUP IX
Balner 7:
Lala 2/18/69

*Probably monospecific in chimpanzee population

human lymphocytes than were sera from animals that at one time received human cells, (e.g., Herbert 2/16/70 vs. Peck 3/27/70), most of the alloantisera reacted with human cells, and the positive reactions were strong and highly reproducible. Also, most of the alloantisera that were negative with unrelated cell donors were found to react at a low frequency in the family studies.

Marked differences were observed in per cent reactivity of chimpanzee antisera with chimpanzee cells compared with their reactivity with human cells. For example, Kong 2/9/70, an alloantiserum which defined 4a in chimpanzees,[5] reacted frequently with chimpanzees, but very infrequently with human lymphocytes. On the other hand, Peck 3/27/70, a xenoalloantiserum that also defined 4a in chimpanzees reacted frequently in humans and detected the human 4a antigen. Whether or not frequency of reactivity is related to the observation that Peck is a xenoalloantiserum and Kong an alloantiserum remains to be determined.

Some chimpanzee antisera, however, reacted similarly with human cells. Based on chi-square associations between chimpanzee antisera tested on the unrelated human cells, we were able to define eight antigenic groups (Group A-H) (Fig. 2). The corresponding chi-square values are shown in Table 2.

Group A, defined by four antisera, two from Balner and two samples from chimpanzee Larr, appeared to define a new specificity. Group C, which was defined by three different samples from chimpanzee Wanda and by Balner's antiserum Abe 8, appeared to define a new specificity although it may possibly include HL-A 11. Neither Group A nor C was correlated with any of more than 200 human alloantisera with which the unrelated cell donors had been tested nor with any of the defined HL-A specificities. Two additional Groups, F and G, each defined by one chimpanzee antiserum that correlated with a single human alloantiserum, seemed to define new specificities.

Ten antisera that correlated with HL-A 2 but which reacted with many HL-A 2 negative cells defined Group B. Group D was defined by three antisera that were highly correlated with HL-A 2 and only rarely reactive outside of HL-A 2. All donors of HL-A 2-related antisera had received human material during immunization, consistent with the report that the HL-A 2 antigen is absent in chimpanzees.[3]

Lala 2/18/69 and four of Balner's antisera were found to correlate with HL-A 11 and were assigned to Group E. Although chimpanzee Lala received human material during the course of her immunization, the last time was in March 1967. She later was

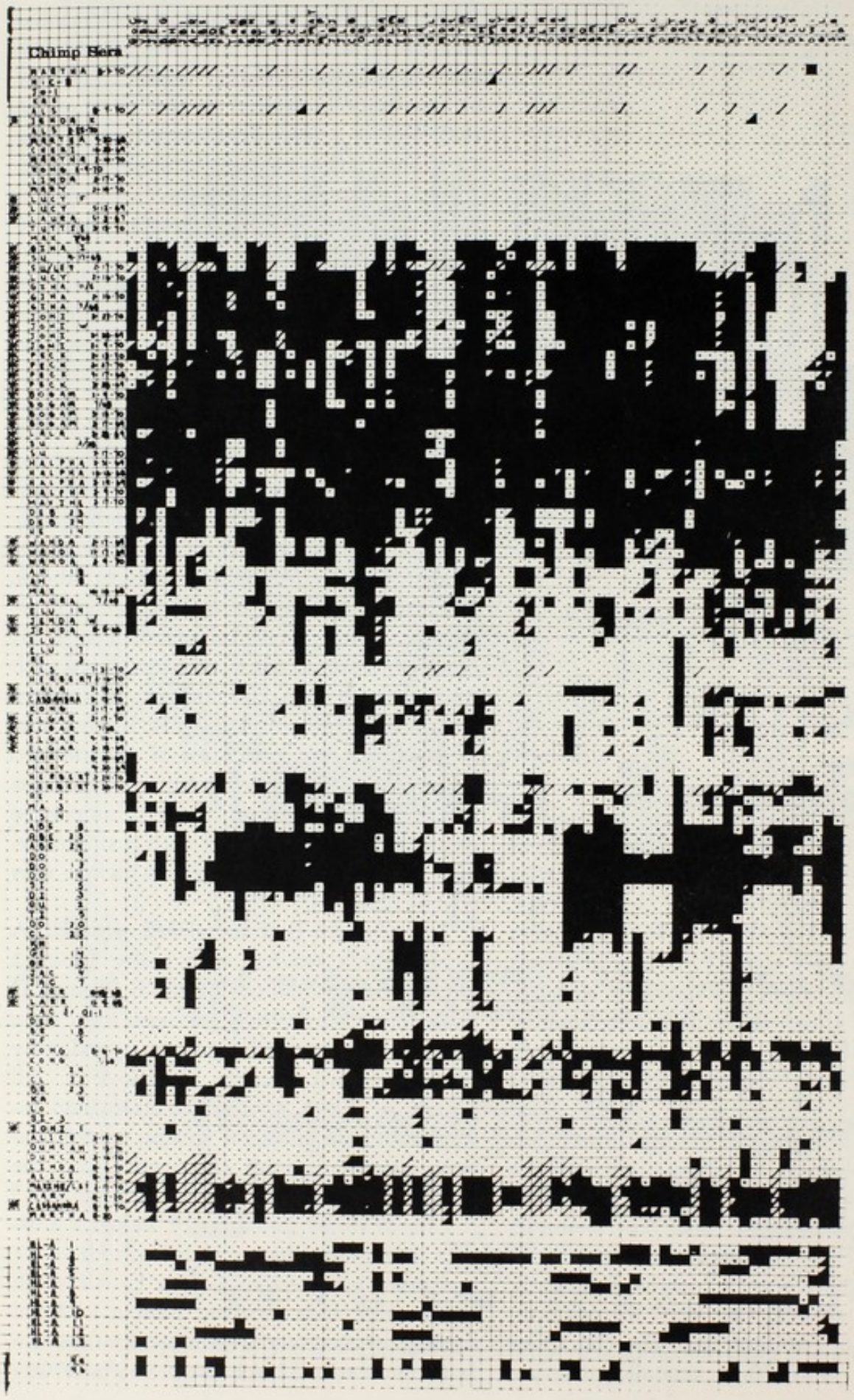

Fig. 2. Human white cell antigen groups defined by chimpanzee antisera.

TABLE 2.

Chi-Square Values Between Chimpanzee Antisera
Which Defined Antigen Groups in a Human Population

Group A [New]

	Jac 4	Jac 7	Larr 11/4/68
Larr 10/18/68	45	31	26
Jac 4		46	37
Jac 7			23

Group B [Long HL-A 2]

	Joni 3/27/70	Lucy 2/16/70	Gina 7/68	Gina 11/5	Joni 3/28/69	Lala 3/28/69	Bogam 2/17/69	Joni 2/9/70	Joni W
Gina 2/16/70	31	43	40	39	20	20	22	13	15
Joni 3/27/70		19	28	29	30	14	8	26	32
Lucy 2/16/70			39	38	11	15	15	7	5
Gina 7/68				58	21	24	19	15	19
Gina 11/5					21	24	16	15	21
Joni 3/28/69						12	3	22	28
Lala 3/28/69							0	6	12
Bogam 2/17/69								3	4
Joni 2/9/70									24

Group C [New]

	Wanda 11/17/69	Abe 8	Wanda 2/17
Wanda 2/9/70	23	29	12
Wanda 11/17/69		32	27
Abe 8			27

Group D [HL-A 2]

	Su 9/17/68	Su Abs
Gina I	42	25
Su 9/17/68		30

Group E [HL-A 11]

	Ti 5	Di 3	Qu 2	Do 20
Lala 2/18/69	26	13	20	17
Ti 5		36	50	27
Di 3			46	19
Qu 2				29

Group F [New - LHE]

	LHe 20/5/68*
Alice 8/6/70	20

Group G [Ao28]

	Ao 28*
Elgar 2/18/69	25

Group H [4a]

	4a*
Peck 3/27/70	8
Peck 3/28/69	10

*Human Antiserum

immunized with chimpanzee cells and it is possible that the anti-HL-A 11 reactivity of the 2/18/69 bleeding was produced in response to chimpanzee antigen. Since Balner's Ti 5, an alloantiserum, also defined only HL-A 11, it seems possible that chimpanzees may have the HL-A 11 antigen or one that is very similar.

Two antisera from chimpanzee Group I, Peck 3/27/70 and Peck 3/28/69, were correlated with antigen 4a in our unrelated human panel and were designated as Group H. As was noted earlier, other antisera in chimpanzee Group I were not correlated with the human 4a antigen. Thus, the 4a antigen in man may be similar but not identical to the 4a found in chimpanzees.

We did not find a correlation between Group IV (7c) antisera and HL-A 7 defined by human cytotoxic antisera, although such an association has been suggested,[6] nor was there a correlation found between human antigen 4b defined by human leukoagglutinating antisera and the one cytotoxic chimpanzee 4b related serum of Group III. Rather, Wanda 2/9/70 which defined Group III (4b) in chimpanzees was associated with human Group C. Recently, Metzgar and Miller reported producing a high titered cytotoxic 4b antiserum in a monkey by immunization with papain-soluble 4b antigen extracted from human spleen.[7] Characterization of the monkey 4b antiserum was done on the cell donors typed by van Rood. It will be interesting to see whether or not this monkey 4b antiserum cross-reacts with chimpanzee cells.

From the 56 families tested, two have been chosen for detailed presentation to illustrate the results obtained with chimpanzee antisera. In the BER family (Table 3) four HL-A antigens in the first segregating series

TABLE 3.

BER FAMILY

	Father	Mother	Sib 1	Sib 2	Sib 3	Sib 4	Sib 5	Sib 6	
HL-A	AB	CD	BC	BC	AC	AC	AC	BD	
			A -	HL-A 3	HL-A 5				
			B -	HL-A 11					
			C -	HL-A 1	W27				
			D -	HL-A 2					
A	+	0	0	0	+	+	+	0	3 chimp sera
B	+	0	+	+	0	0	0	+	11 chimp sera
C	0	+	+	+	+	+	+	0	0 chimp sera
D	0	+	0	0	0	0	0	+	2 chimp sera
A+C	+	+	+	+	+	+	+	0	3 chimp sera
B+D	+	+	+	+	0	0	0	+	10 chimp sera
A+D	+	+	0	0	+	+	+	+	0 chimp sera
	+	+	+	+	+	+	+	+	40 chimp sera
	0	0	0	0	0	0	0	0	34 chimp sera

and two in the second segregating series were easily identified by numerous alloantisera. Two groups of HL-A-identical siblings were among the offspring: Sibs 1 and 2 and Sibs 3, 4, and 5, respectively. Sixty-nine of the 103 chimpanzee antisera tested in this family yielded positive reactions; 29 of these showed segregating patterns that coincided with HL-A segregation patterns and the remaining 40 were positive for all eight family members. Sera in Group D (HL-A 2) segregated with the D haplotype, while sera in Group B (HL-A 2+), in Group H (4a), and Lala, the serum representing Group E (HL-A 11), were among those positive with all family members. Here, as in other families, HL-A-identical siblings reacted positively with the same chimpanzee antisera in repeated typings, and offspring showed positive reactions only with antisera for which at least one parent was positive. Only HL-A patterns were observed in each family studied.

In the L TAY family (Table 4) only four siblings were available for tissue typing. Using human alloantisera, three HL-A antigens could be distinguished, HL-A 2, HL-A 7, and W18. HL-A 2 and HL-A 7 appeared to be carried on one HL-A chromosome while W18 was determined by its homologue. The inheritance of the remaining pair of HL-A-bearing chromosomes could only be tentatively assigned based on reactions with human sera as only one additional segregation pattern (A+C) was clearly defined. With the exception of pattern D, all HL-A segregation patterns detected with human sera were obtained with chimpanzee sera. In addition, pattern C, not found with human sera, was clearly defined by four chimpanzee antisera, two of these four being alloantisera. None of the

TABLE 4.

L TAY FAMILY

	Sib 1	Sib 2	Sib 3	Sib 4	
HL-A	BC	AD	BD	AC	
		A - HL-A 2 B - C - D -	HL-A 7 W18		
A	-	+	-	+	15 human sera, 2 chimp sera
B	+	-	+	-	14 human sera, 1 chimp serum
C	+	-	-	+	0 human sera, 4 chimp sera
D	-	+	+	-	2 human sera (weak), 0 chimp sera
A+C	+	+	-	+	8 human sera, 3 chimp sera
A+D	-	+	+	+	1 human serum, 8 chimp sera
B+C	+	-	+	+	1 human serum (weak), 2 chimp sera
B+D	+	+	+	-	2 human sera, 1 chimp serum
	-	-	-	-	36 human sera, 18 chimp sera
	+	+	+	+	25 human sera, 28 chimp sera

four antisera were included in Groups A-H nor in Groups I-IX. Gina I from Group D segregated with the A pattern, while the other serum in Group D, Su 9/17/68, and all sera in Groups B, G, and H reacted with all family members. The chimpanzee antisera have been useful in establishing and confirming HL-A inheritance in families.

Detection of HL-A segregation patterns with alloantisera, as well as xenoalloantisera, is evidence for similarity between HL-A and chimpanzee antigens, although the exact nature of this cross-reactivity is not clear. Perhaps as significant as the similarity between human HL-A antigens and chimpanzee antigens, as evidenced by the human 4a, chimpanzee 4a relationship, are the numerous serological reactions that segregate with HL-A antigens in families but do not correlate with any of the recognized HL-A specificities nor with any of the human cross-reacting HL-A antisera when tested with human cells. Antisera that behave similarly when tested with chimpanzee cells give very different reaction patterns with human cells; the reverse is also true. Further investigation is required to establish the nature of these intriguing differences.

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Cross-reactions of HL-A Antibodies. IV. Absorptions and Elutions With Primate Platelets

By Martin E. Dorf, Setsuko Y. Eguro, and D. Bernard Amos

MANY OF THE ANTISERA used for tissue typing are more reactive than those selected to define the recognized HL-A specifiicities. This additional reactivity can be attributed to cross-reactivity and/or multiple antibodies. We have previously shown that the HL-A 3 activity from several antisera can generally be absorbed by HL-A 3, HL-A 11, or HL-A 1 cells and by a few individuals having none of these specificities.[6] We have also separated the cross-reactive and specific components of certain antisera using quantitative absorption or elution techniques.[7] This report described absorptions and elutions of another human alloantiserum "Anderson," which detects three identifiable HL-A specificities and also reacts with certain nonhuman primate leukocyte antigens.[5,9]

Seven bleeding samples of serum Anderson obtained from the Transplantation Immunology Branch of the National Institute of Allergy and Infectious Diseases were tested against a panel of 36 lymphocyte donors and titrated by two-stage Amos cytotoxicity assay (Table 1).[6] All bleeding samples reacted with the HL-A 11 cells, but samples drawn later in the immunization cycle showed additional reactivity. Thus bleeding A appeared to be a low-titered, monospecific anti-HL-A 11, but later serum samples also reacted weakly with HL-A 10 and HL-A 1 cells. The immunizing donor was HL-A 1 negative and HL-A 11 positive, although not tested for HL-A 10. When tested with cells from 46 chimpanzee donors, Anderson bleeding G reacted with a frequency of 98%. Two levels of reactivity were apparent when chimpanzee cells were tested with dilutions of this sample. Cells from some animals reacted at titers greater than 1:9 as did the human HL-A 11 cells. Cells from other animals did not react with Anderson serum diluted beyond 1:3; neither did human HL-A 1 and HL-A 10 cells.

Serum Anderson was absorbed seven times for 15 min at room temperature with 2×10^9 human or primate platelets/ml per absorption. Samples absorbed with platelets from three HL-A 10 and three HL-A 1 donors showed incomplete absorption when tested with HL-A 11 cells, but complete absorption when tested with lymphocytes from either HL-A 10 or HL-A 11 donors (Fig. 1). A single absorption with platelets from HL-A 11 individuals completely removed cytotoxic activity against HL-A 11, 10 or 1 lymphocytes. Large variations in the absorptive capacities of platelets from HL-A 1 donors were noted. This was in sharp contrast to the minimal differences noted following absorption with three different HL-A 10 donors. Platelets from a homozygous HL-A 3 donor could remove the anti-HL-A 1 cytotoxins from serum Anderson, but there was little or no specific absorption of anti-HL-A 11 or anti-HLA 10 activity. Control absorptions were performed with platelets from a donor with HL-A 2, 9, 7, and W15 and from the serum producer Anderson (HL-A 2, 12). There was a considerable amount of "nonspecific" absorption with the latter platelet preparation in comparison to the other negative control. Unfortunately, the blood from

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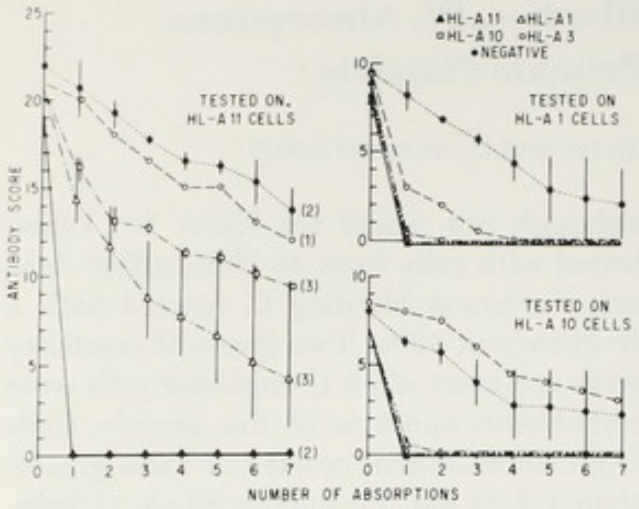

Fig. 1. Serial absorptions using 2 × 10^9 platelets per absorption. Numbers in parentheses indicate the number of different platelet donors with the stated HL-A specificities used for absorptions. The second HL-A antigen present on cells of these donors was not HL-A 1, 10, or 11. Vertical lines indicate the maximal range of residual antibody activity remaining following absorptions. Antibody scores are determined by summing the reaction scores (+ to ++++) during titrations of absorbed sera with at least three donors with the stated HL-A specificity, the results are then averaged.

which donor Anderson's platelets were extracted was delayed 1 wk in transit, and arrived in unsatisfactory condition.

Additional absorptions were performed with platelets from four chimpanzees, two gibbons, and an orangutan (Fig. 2). Platelets from chimpanzees Melilot and Sandra could completely absorb all cytotoxic activity from serum Anderson. In contrast, platelets from chimpanzee Edgar removed cytotoxins for HL-A 10 and HL-A 1 cells, but not for HL-A 11 lymphocytes. Absorptions with platelets from chimpanzee Homer readily cleared HL-A 10 cytotoxins, removed anti-HL-A 1 activity with some difficulty, but gave only slight absorption of HL-A 11 activity. Platelets from gibbon Ajay absorbed serum Anderson in the same manner as those from chimpanzee Homer. The absorption patterns with cells from a second gibbon, Blackey, resembled those of chimpanzee Edgar. Orangutan platelets were the least efficient for absorption of HL-A 11, HL-A 10, or HL-A 1 activity from this serum.

Table 1. Reaction of Serum Anderson With Human Lymphocytes

Serum Code	N I H Catalogue Number	Lymphoctye Donors [d] FR	Rld	KS	AlR	SO	CaB	BM	PC	GCo	JL	DR	ROT	SuG	DBA	RA	PEW	LT	MDo	PC	PC	EJR	EsH	LaH	JBl	JBJ	TeJ
A	6-05-20-01	2[a]	1	1	2	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	6-06-24-01	4	2	4	4	4	4	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
C	6-07-01-01	4	4	8	8	4	8	4	0	1	1	1	0	1	2	0	0	0	1	1	0	1	2	0	0	0	0
D	6-07-29-01	16	8	16	16	16	16	8	2	1	1	2	2	4	4	2	2	1	2	2	2	2	2	0	0	0	0
E	6-08-12-01	16	8	16	16	16	16	16	1	1	2	2	2	4	4	2	4	2	2	2	2	2	4	1	0	0	0
F	6-08-19-01	16	8	16	16	32	16	16	4	1	1	2	2	4	4	2	4	1	2	2	4	2	4	1	0	0	0
G	6-08-25-01	32	16	16	16	32	16	32	?	2	2	2	2	4	4	2	4	2	4	4	4	4	4	1	0	0	0
HL-A Groups of Donors																											
	HL-A 11	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	(+)[b]	-	-	-	-	-	-	-	-	-
	HL-A 1	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	(+)[b]	-	-	-	-	-	-	-	-	-
	HL-A 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	HL-A 3	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	++[c]	+	+	+

[a] Reciprocal of highest dilution giving a positive reaction

[b] HL-A 1-11 variant antigen

[c] Homozygous HL-A 3 donor

[d] Ten additional donors, negative with all bleedings of serum Anderson and lacking HL-A, 11, 10, 1 or 3 are not illustrated.

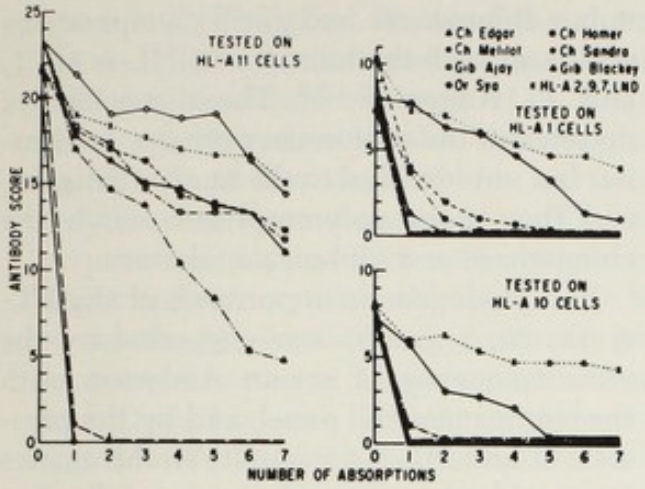

Fig. 2. Absorptions with primate platelets; see legend for Fig. 1. Lymphocytes from chimpanzees Melilot and Sandra were positive with cytotoxic HL-A 11 antisera and negative with HL-A 1 antisera. Cells from chimpanzee Homer reacted with HL-A 1 antisera but not with anti-HL-A 11 sera.

Following absorption, acid eluates were prepared as previously described from platelets of each human donor.[8] The eluate activity recovered from HL-A 11 cells had the same specificity and relative strength of reactivity with HL-A 11, HL-A 10, and HL-A 1 cells as did the unabsorbed serum (Fig. 3). The eluate from HL-A 10 platelets reacted with HL-A 11 and HL-A 10 cells, but not with lymphocytes from HL-A 1 donors. The reverse situation occurred with the HL-A 1 eluate which reacted with HL-A 11 and HL-A 1 lymphocytes, but not with HL-A 10 cells. The eluate prepared from an HL-A 3 donor had anti-HL-A 11 activity plus weak anti-HL-A 1 activity; it failed to react with HL-A 10 cells. This is consistent with the previously established cross-reactive group consisting of HL-A3, 11, and 1.[6,9] The eluates prepared from the negative cells contained no antibody activity (Fig. 3).

The anti-HL-A 10–11 or HL-A 1–11 activity recovered following elution from HL-A 10 or HL-A 1 platelets, respectively, (Fig. 2) seemed inconsistent with absorption patterns indicating that HL-A 10 or HL-A 1 platelets could completely absorb antibody activity for themselves or each other (Fig. 1). It is difficult to explain the loss of most of the antibody activity directed toward one of the cross-reacting antigens (HL-A 1 or 10) following absorption and elution with platelets of the other cross-reacting specificity. Perhaps the quantity of antibody was below the threshold of detection by our regular cytotoxicity assay. If this were true, we would expect platelet eluates from HL-A 1 and HL-A 10 cells to demonstrate synergy when recombined. A synergistic effect can be demonstrated using HL-A 1 and HL-A 10 target cells (Table 2). This may reflect cooperation between very low affinity and higher affinity antibodies. In addition, using a more sensitive cytotoxicity procedure[1] requiring more complement, lower temperature, and longer incubation times, we could demonstrate moderate quantities of anti-HL-A 10 activity remaining in sera absorbed with HL-A 1 platelets even after seven serial absorptions. Weak anti-HL-A 10 activity was also found in the eluate from HL-A 1 platelets. Likewise, serum Anderson absorbed with HL-A 10 platelets reacted with HL-A 1 lymphocytes using the more sensitive assay.

Eluates prepared following absorption with subhuman primate platelets were also

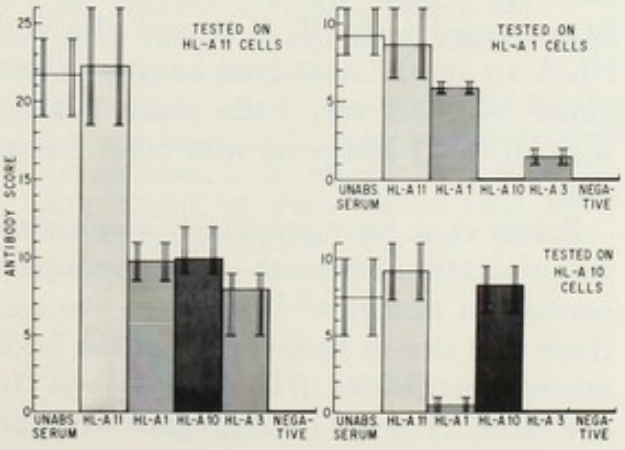

Fig. 3. Reactivity of eluates following absorptions with human platelets. Vertical bars indicate ranges.

Table 2.

Synergistic Effect of Mixing Eluates of Anderson Serum

Test Cell	Platelet Eluate From HL-A 1	Platelet Eluate From HL-A 10	HL-A 1 + HL-A 10	Unabsorbed Serum
HL-A 1	7.0*	0	9.2	9
HL-A 10	0	5.0	9.5	8
HL-A 11	16.2	14.0	16.5	22

* mean antibody score in duplicate tests. In tests with eluate from HL-A 1 or HL-A 10 platelets 1 μl of eluate was added to the wells. In tests with the mixture 0.5 μl of each was added.

tested for cytotoxic activity (Fig. 4). The eluates from chimpanzees Melilot and Sandra reacted with all human cell donors having either HL-A 11, HL-A 10, or HL-A 1. The amount of eluate activity recovered from Melilot and Sandra was slightly less than that recovered in eluate from HL-A 11 platelets. Platelet eluates from these two chimpanzees demonstrated quantitative differences in eluate activity when tested on HL-A 1 and HL-A 10 cells, suggesting polymorphism of the chimpanzee HL-A 11 homologue. The eluate from chimpanzee Edgar was similar to that recovered from HL-A 10 cells. It reacted with HL-A 11 and HL-A 10 lymphocytes, but not with cells from HL-A 1 donors. The eluate obtained following absorption with platelets from chimpanzee Homer demonstrated weak activity for HL-A 11 cells, but none for HL-A 10 or HL-A 1 cells. The eluate from gibbon Ajay reacted with HL-A 11 and also with HL-A 10 and HL-A 1 lymphocytes. Orangutan Sya had very little eluate activity with HL-A 11 and none with other human cells.

Balner et al. have previously emphasized the importance of the 4a–4b-like groups in nonhuman primates.[2,3] Similarly, we conclude that simian homologues of the Lc-20 related specificities (HL-A 11, HL-A 10, and HL-A 1) of the LA segregant series are broadly distributed among primates. To date, no chimpanzee leukocyte antigens are known to react with the HL-A 2 and HL-A 9, but chimpanzee and gorilla lymphocytes will react with the human anti-HL-A 10, 1, and 11 reagents.[2,4,5,8] These specificities detected in the subhuman primates are similar but not identical to the human antigens, and they show polymorphism within the chimpanzee and gibbon populations.

The phylogenetic importance of the HL-A 11, 10, 1 groups was suggested by the 98% frequency of serum Anderson with the chimpanzee cell panel, and by the presence of anti-HL-A 11 activity in the eluates from subhuman primate platelets following absorption of serum Anderson. The absorption and elution studies performed with platelets from chimpanzees Melilot and Sandra indicate that some chimpanzees have an HL-A 11-like antigen. This was previously suggested by Dorf and Metzgar,[5] who absorbed the anti-HL-A 11 activity from a human alloantiserum with chimpanzee cells, and by Balner et al.[4] who produced a chimpanzee alloantiserum that detected HL-A 11 when tested on human cells. Recently, we produced a cytotoxic chimpanzee isoantiserum that only reacts with 80% of the HL-A 11 cells from

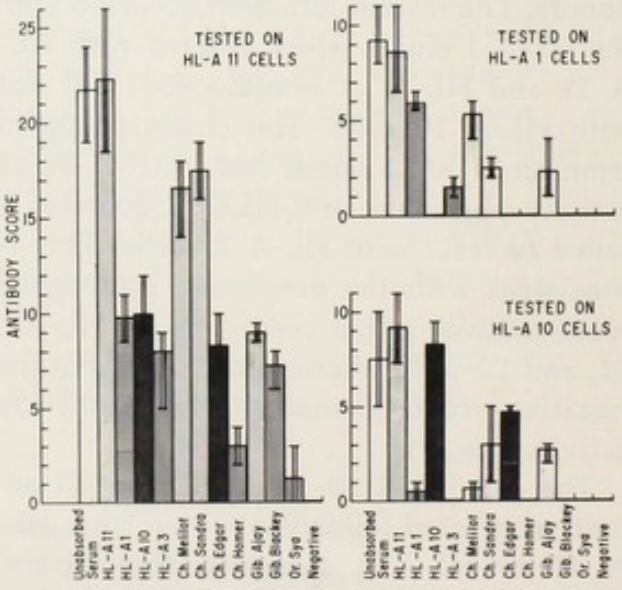

Fig. 4. Reactivity of eluates from primate platelets following absorptions illustrated in Figs. 1 and 2.

our human cell panel. Tests of the eluates obtained from Melilot and Sandra showed considerable differences with HL-A 10 and HL-A 1 cells, indicating that the chimpanzee HL-A 11-like determinants are more polymorphic than those in man. The absorption and elution data with chimpanzee Edgar indicate considerable similarity between the HL-A 10-like determinants present on chimpanzee Edgar's platelets and those found in man. All the primate cells tested could absorb anti-HL-A 10 activity from serum Anderson, again suggesting a possible phylogenetic significance for this specificity.

The elutions of serum Anderson with human platelets (Fig. 4) support the concept that HL-A antigens constitute a mosaic of determinants, some of which appear specific for a particular antigenic group, plus others which are shared with cross-reacting antigens. Thus, HL-A 11 would consist of a series of determinants: an 11-specific antigenic determinant, plus one which cross-reacts with HL-A 10, and another which is shared by HL-A 1 but not by HL-A 10. These determinants need not be separate molecular entities, but may consist of overlapping determinants. We have previously reported individuals of one black family possess a "variant" HL-A 1-11 allele, i.e., such persons only reacted with cross-reacting HL-A 1 and HL-A 11 sera, but generally failed to react with or absorb the type specific HL-A 1 or HL-A 11 antisera.[6]

SUMMARY

Lc-20 antiserum Anderson was fractionated into three components. The first component, obtained by absorption, was a specific anti-HL-A 11. The other components, isolated by elution, were cross-reacting anti-HL-A 11-1 or HL-A 11-10. Chimpanzees, gibbons, and orangutans have HL-A 11-like or HL-A 10-like determinants since platelets from subhuman primate species can partially absorb Anderson serum. Eluates prepared following these absorptions indicate considerable polymorphism within and between these primate species.

ACKNOWLEDGMENT

We wish to express our gratitude to Dr. R. L. Walford and Dr. R. S. Metzgar for their kind cooperation and supply of materials.

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HL-A Antibodies in Chimpanzees After Specific Treatment With Human Leukocytes and Antihuman Lymphocyte Globulin

By R. Johannsen and F. R. Seiler

THE PRODUCTION OF ANTISERA directed against human leukocyte antigens (HL-A) in chimpanzees has been described by various authors during the last few years.[1-4,6,9,10,11,13] If human cells were taken as an antigenic source, one ran the risk that other antigenic features of the heterologous cell may have been recognized before those of HL-A.[9,11,13]

Therefore, it was attempted to produce a state of specific nonresponsiveness in the animals to species-specific antigens and to boost with cells of a selected donor who had additional HL-A antigens. It has been demonstrated that pretreatment with human lymphocytes and antihuman lymphocyte globulin can possibly induce a specific state of nonresponsiveness for human-specific antigens in chimpanzees. The recognition of additional HL-A specificities on the subsequent immunogenic cell seems not to be reduced when total buffy coat leukocytes are used for booster injections.

MATERIALS AND METHODS

Reagents: Chimpanzee sera and plasma were obtained after the donor had received multiple injections of human leukocytes or lymphocytes. None of these reagents were absorbed with red cells prior to their use in chimpanzee-human cross species typing. For typing of human and chimpanzee lymphocytes human isoantisera were used. Most of the reagents were obtained from J. J. van Rood, Academic Hospital, Leiden; others from F. Kissmeyer-Nielsen, Kommunehospital, Aarhus, the NIH Serum Bank, Bethesda, Md., and from the Behringwerke AG. The HL-A specificites of the reagents described in this communication are those which have been internationally agreed upon.

Typing Procedures: For chimpanzee, as well as human lymphocytes, the microcytotoxicity test described by Kissmeyer-Nielsen and Thorsby[8] was used. Pure lymphocyte suspensions were prepared using the flotation method on Ficoll-Isopaque as reported by Böyum.[5]

Immunization of Chimpanzees: Two groups of of chimpanzees (Table 1) were treated once intravenously with isolated human lymphocytes from a single donor (P) and with 20–50 mg/kg antihuman lymphocyte globulin[7,12] (AHLG Behringwerke, Lot 34-12-84). The AHLG was prepared from an "early serum" and was found to be immunosuppressive in vivo (skin transplant survival 25/26 days in *Macaca speciosa*, as tested by Balner, T.N.O. Rijswijk, Netherlands).

One week later, the first (leukocyte) group, including three animals, received subcutaneously total buffy coat leukocytes prepared from 10–20 ml heparinized blood of a selected donor (G). Additional to cells of donor (P) these cells had HL-A 3 and HL-A 11.

The second (lymphocyte) group, including four animals and one control animal without AHLG, was treated subcutaneously with 10^6–10^8 isolated human lymphocytes from another selected donor (L), who had additional HL-A 10. (For animals Oskar and Tom the cells were also incompatible for 4a.)

Analysis of Chimpanzee Antisera: Chimpanzee sera and plasma were tested against a human lymphocyte panel of about 50 cells with known HL-A types. The reactions observed were correlated with the various known HL-A antigens and 2 × 2 tables were constructed. The results of comparison analysis were confirmed by absorption studies.

RESULTS AND DISCUSSION

Figure 1 depicts the lymphocytotoxic titers during the course of immunization. The antibody response in animals treated with human lymphocytes is found to be very different from the response in animals boosted with human leukocytes. Practically no cytotoxic humoral response, neither to HL-A nor to human lymphocytes, can be

From Behringwerke AG, Marburg-Lahn, Western Germany.

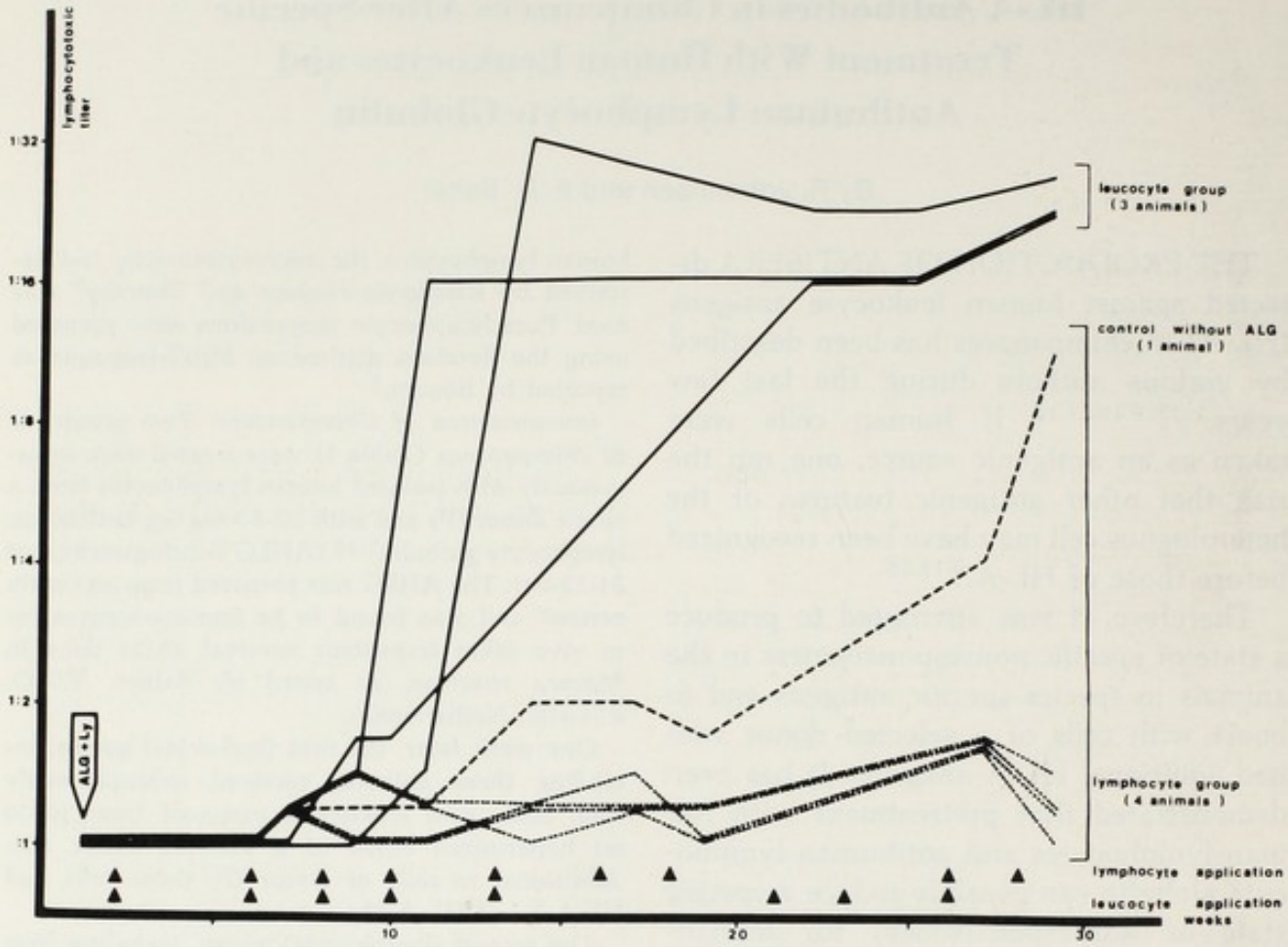

Fig. 1. Lymphocytotoxic titers during the course of immunization of chimpanzees with human leukocytes G and lymphocytes L after treatment with lymphocytes P and AHLG.

demonstrated in the four animals of the lymphocyte group after ten booster injections over a period of more than half a year. This was confirmed by agglutination, rosette inhibition, and thrombocyte aggregation-tests. The control animal without AHLG in the lymphocyte group (Rudolf) produced cytotoxic antibody activity that appeared to be nonspecific for HL-A. In contrast, each of the three animals of the

Table 1. Matching of Chimpanzee Recipients With Human Lymphocytes P for Induction of Nonresponsiveness and for Subsequent Immunization With Leukocytes G and Lymphocytes L

Chimps	Darwin	4a	4b				Fidi	4a	4b	7b			11
	Sophie	4a		Ba*			Oskar		4b	7b			11
	Heini		4b	Ba*		11	Theo	4a	4b	7b	1		11
							Tom		4b	7b	1		11
							Rudolf (control)	4a		7b			11
1 Injection	Lymphocyte P + AHLG	4b	6b	7b	2	7	Lymphocyte P + AHLG	4b	6b	7b	2		7
2–10 Injection	Leukocyte G	4b	6b	7b	3	11	Lymphocyte L	4a	4b	7b	10		

Animal Rudolf (control) was treated without AHLG. Numbers indicate HL-A specificities.

Table 2. Comparison Analysis of Sera From the Three Animals of the Leukocyte Group

First	Second	Serum	++	+−	−+	−−	N	x^2 Corrected	r
So 5115	HL-A 3 + 11	Unabsorbed	23	7	0	22	52	27,2	0,72
		Absorbed	21	0	2	29	52	40,7	0,88
Da 5116	HL-A 3 + 11	Unabsorbed	23	5	0	21	49	29,3	0,77
		Absorbed	22	0	1	29	52	44,2	0,92
He 4957	HL-A 3 + 11	Unabsorbed	23	10	0	19	52	21,0	0,63
		Absorbed	19	0	4	29	52	34,3	0,81

Each of the sera shows good correlation to human lymphocytes positive for HL-A 3 and/or HL-A 11.

group, boosted with leukocytes, developed strong cytotoxic antibodies (Table 2).

Good correlation to HL-A 3 and HL-A 11 is indicated by 2 × 2 associations and chi-square values. Identical results with these sera were obtained in the laboratories of F. Kissmeyer-Nielsen and J. J. van Rood.

It is remarkable, that one of the three animals (Heini) was positive for HL-A 11 himself, as tested with human isoantisera. Nevertheless, this animal has produced antibodies reacting with HL-A 11 positive cells from humans and chimpanzees, but not with his own. This finding could indicate a difference between HL-A 11 in humans and the corresponding antigens in chimpanzees. Bispecific antisera for HL-A 3 and HL-A 11 could be obtained by absorption with human cells (Table 3).

As can be seen in Table 3 the extra reactions were eliminated only with 4b-positive cells: (1) If the absorbing 4b-positive cell was negative for HL-A 1, 3, 9, and 11 then antisera directed only against HL-A 3 and 11 were obtained. This is also shown in Table 2. (2) 4b-negative cells being also negative for HL-A 1, 3, 9, and 11 did not remove a significant amount of the antibody activity. (3) If the 4b-positive cell was also positive for one of the cross-reacting antigens HL-A 1, 3, 9, or 11, no antibody activity remained. (4) 4b-negative cells being positive for HL-A 1 and 3 absorbed only antibodies directed against HL-A 3 and 11 but not the extra antibodies. As far as tested the isolated extra antibodies reacted only with 4b-positive cells. (5) Preliminary absorption studies with 4b-positive chimpanzee leukocytes have shown, that there was a significant but, in no case, a complete removal of the extra antibodies. These data indicate a correlation of the extra antibodies with a part of 4b. The two most probable reasons for the appearance of these antibodies are: (1) The extra antibodies demonstrate a species-specific difference between human and chimpanzee as far as 4b is concerned. (2) The extra antibodies are directed against an antigenic component of 4b present on the immunogenic cell but not present on the cell used for the induction of nonresponsiveness. Ex-

Table 3. Absorption of Sera of the Leukocyte Group With Human Leukocytes

Absorbing Cell	Antibodies to HL-A 3 and 11	Antibodies Extra
4b positive, HL-A 1,3,9, and 11 negative	+	−
4b negative, HL-A 1,3,9, and 11 negative	+	+
4b positive, HL-A 1,3,9, and 11 positive	−	−
4b negative, HL-A 1 and 3 positive*	−	+

*HL-A 9 and 11 not yet tested.

Absorbing cells, being positive for HL-A 1, 3, 9, or 11, eliminate antibodies directed against HL-A 3 and 11.

Absorbing cells, being positive for 4b, eliminate extra antibodies.

periments now under investigation may prove the heterogeneity of the 4b-complex, which can be expected from the above data.

The fact that, during our immunization schedule, no humoral antibody response to lymphocytes could be detected in four of the animals, gives rise to the following questions: (1) Does the nonresponsiveness depend on the different immunogenicity of the types of cells (lymphocytes, leukocytes) used? (2) Does the nonresponsiveness depend on the different immunogenicity of the various HL-A patterns of the donor cells? (3) Does AHLG play any role in the initiation of nonresponsiveness?

The delayed response in the control animal to lymphocytes compared with the response to leukocytes in three animals indicates a different immunogenicity of the lymphocytes and leukocytes chosen (Fig. 1). No comment can be given to the second question because no appropriate controls were included in the leukocyte group.

The efficacy of AHLG for the suppression of antibody formation is clearly shown in Fig. 1 which compares the titer curve of the control animal with the curves of the other four AHLG-treated nonresponding animals of the lymphocyte group. Moreover, there is some evidence, that AHLG does not induce an unspecific immunosuppression but rather acts more specifically in suppressing the response to human-specific features by coating the relevant antigens. Simultaneously, the antibody production against additional iso-antigens is permitted.

There seems to exist a state of nonresponsiveness for the antigens of the tolerogenic human cell (P) but no suppression of specific antibody response against the additional isoantigens HL-A 3 and HL-A 11. Investigation in progress may clarify whether the applied immunization schedule and the coating of cells with AHLG have also led to a specific suppression of cellular immunity and induced a situation of tolerance.

SUMMARY

Treatment of chimpanzees with human lymphocytes from a single donor and AHLG can induce a specific state of humoral nonresponsiveness for the antigens of the heterologous cell. In four animals, this immunological state was established for a period of more than half a year and could not be abrogated by lymphocytes of another donor with additional HL-A specificites. The control without AHLG developed cytotoxic antibodies after three booster injections.

In comparison three animals, boosted with leukocytes after an identical pretreatment, developed cytotoxic antibodies that were specific for the additional HL-A specificities on the immunogenic cell. These antibodies are highly correlated with HL-A 3 and HL-A 11. Moreover, there are distinct extra antibodies reacting only with a part of 4b. Absorption studies confirm the complex nature of this antigen and its possible involvement in species-specific features.

ACKNOWLEDGMENT

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Reactions of Human HL-A Sera With Orangutan and Gorilla Lymphocytes

By H. F. Seigler, R. S. Metzgar, F. E. Ward, and D. M. Reid

DURING THE PAST 20 yr studies of the mouse H-2 locus and the HL-A locus in man have led investigators to note certain similarities between these genetic systems. Serologic, biochemical, and biologic analogies of these systems have stimulated a great deal of interest in the phylogeny and evolutionary development of histocompatibility antigens.

Investigations of the interrelationships between the tissue alloantigens of man and the great apes seemed to be a logical approach. Although the first evidence of cross-reactivity between human and chimpanzee membrane antigens utilized xenoantibodies,[1] most of the studies were done with the readily available defined human alloantibodies. In the interim, the necessary intraspecies alloantibodies and cross-species xenoantibodies were being developed in primates. Metzgar and Zmijewski[2] first described the cross-reaction of chimpanzee membrane isoantigens with human alloantibodies. This was followed by a more detailed description of the reactions between defined human alloantisera and chimanzee leukocytes by Balner et al.[3] They also noted an allelic relationship between 4a and 4b in the chimpanzee as well as a similar system in the rhesus monkey.[3] Metzgar and Seigler[4] also observed cross-reactions between human alloantibodies and orangutan, gorilla, chimpanzee, rhesus monkey, and African green monkey tissue culture cells by mixed agglutination. Utilizing both direct testing and absorption experiments with human alloantibodies, Dorf and Metzgar[5] noted a negative relationship in the chimpanzee of HL-A 7 (related to 4b) and 4a, once against suggesting an allelic relationship of these two antigens. They also noted that when human sera did cross-react with chimpanzee cells, no more than two antisera defining specificities from each HL-A locus reacted with cells from a single animal.

This study demonstrates the cross reactions of orangutan and gorilla lymphocytes with human alloantibodies, defining HL-A 1 through 13 as well as several additional HL-A related specificities. Thirty-two orangutans and 15 gorillas were tested by a two-stage semimicro cytotoxicity test[6] with 60 human alloantisera. The antisera were absorbed once with an equal volume of orangutan red blood cells. Lysis was determined by trypan blue dye exclusion. The results of the cytotoxicity testing with both species are presented in Table 1.

Orangutan cells showed no strong reactivity with two HL-A 1 antisera thought to be monospecific. One oligospecific antiserum which contained HL-A 1 activity had five strong reactions. Similarly, there was nonreactivity- of defined HL-A 2, 3, 4c, and 5 sera with orangutan cells. However, seven of the 32 animals reacted with a single oligospecific antiserum containing HL-A 2. Three HL-A 7 antisera were tested with orangutan cells. One of these failed to react whereas the other two antisera detected polymorphisms. Two of the orangutan donors were positive with both of the reactive HL-A 7 antisera. Two of the three anti HL-A 8 sera tested detect only HL-A 8 in a white population. One of these showed cross-reactions with orangutan cells which were included in the reactions of the oligo-

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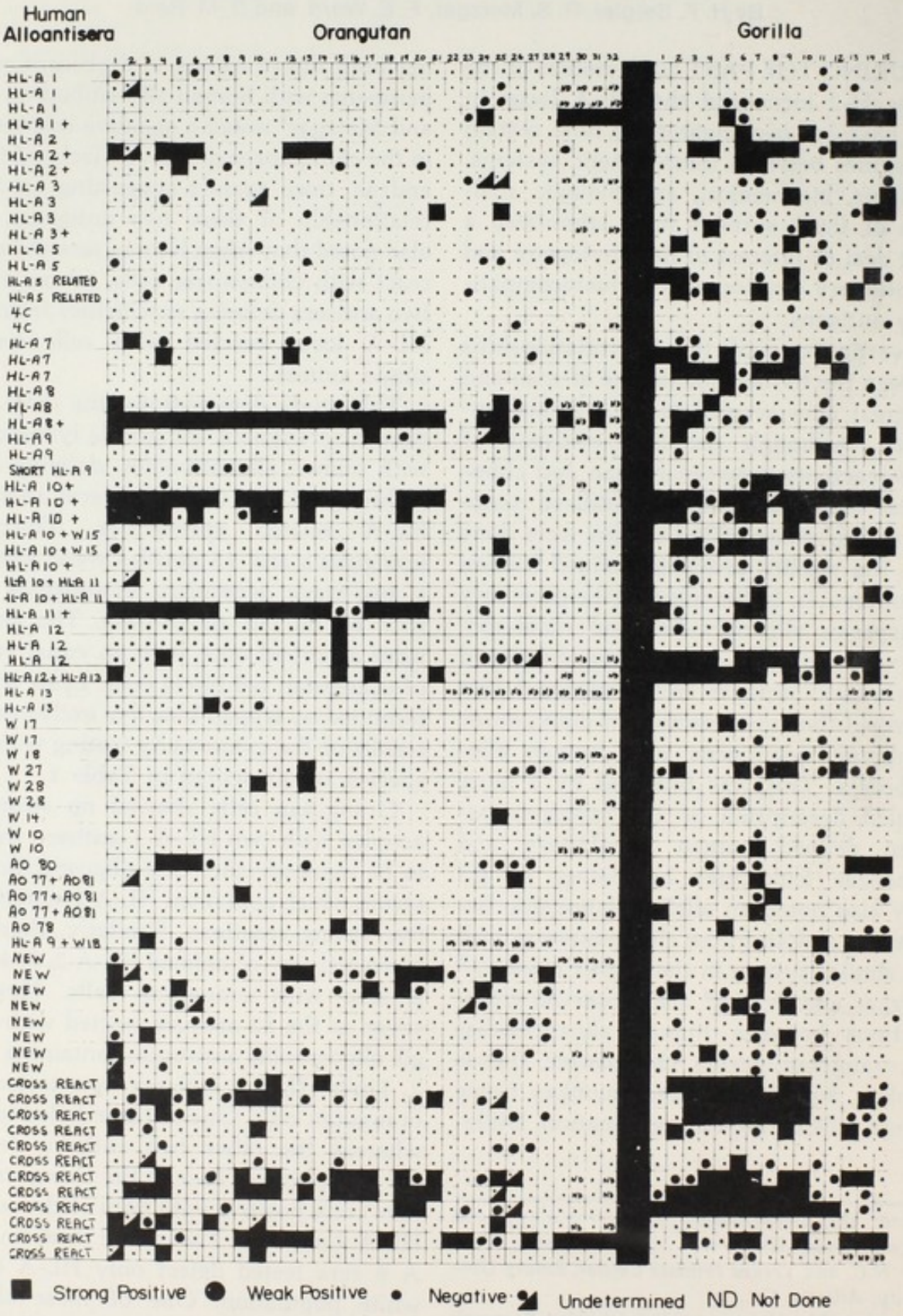
Table 1.

LYMPHOCYTOTOXICITY OF ORANGUTAN AND GORILLA CELLS WITH HUMAN ALLOANTISERA



specific HL-A 8 antiserum. Similar results have been observed with both anti HL-A 7 and HL-A 8 antisera when they have been tested with genetically isolated human populations. There were four weak reactions (30–50% lysis) with the short HL-A 9 antiserum, which were not included within those of the long antiserum. Two of the oligospecific HL-A 10 antisera cross reacted strongly with orangutan lymphocytes. The reactions of one of these sera were included in the other's reactions. These two HL-A 10 antisera are two different bleeding dates from the same donor. The additional antibodies in these sera do not correlate in humans with any of the defined HL-A specificities. For the most part only weak reactions were observed with the other HL-A 10 sera. The oligospecific HL-A 11 antiserum reacted with 20 of 32 orangutan donors whereas the two antisera detecting both HL-A 10 and HL-A 11 were infrequently reactive.

Three antisera defining only HL-A 12 in humans and one antiserum that detected HL-A 12 plus HL-A 13 were tested. One of the orangutan donors reacted with all four of these antisera and this cell was the only reactor with two of the specific HL-A 12 antisera. The two remaining HL-A 12 antisera reacted with several other donors. However, the reactions with the HL-A 12 plus HL-A 13 antiserum did not include those cells that reacted with the HL-A 13 antiserum. Strong cross-reactions were present with some of the sera-defining antigens described in the last human histocompatibility testing workshop as well as the new A O specificities. However, similar reaction patterns between these antisera were not noted. Sera that react with 75–80% of human donors (designated cross-react on Table 1) gave markedly different results with the 32 animals tested. Some of the antisera reacted strongly and frequently.

In summary, orangutan cells cross react with certain human alloantibodies. Similar reaction patterns were noted between three antisera defining HL-A specificities 8, 10, and 11 and orangutan cells. This association can be attributed to either a common extra antibody since each of the antisera are known to be oligospecific, or if one assumes that the orangutan cross-reactions were due to the major HL-A specificity being defined by these sera, then HL-A 8, 10, and 11 are closely associated in this species. The inclusion phenomenon observed in humans for a short HL-A 9 antiserum and standard HL-A 9 was not observed in the orangutan. One orangutan donor was strongly reactive with all of the anti HL-A 12 antisera tested and indicates that HL-A 12 is present in this animal. Since the patterns of cross-reactivity for the other HL-A specificities are dissimilar, interpretation at this time is difficult.

The gorilla cells gave more frequent cross-reactions with the human sera than did the orangutans. Five animals reacted with the four HL-A 1 antisera; however, only one animal reacted with more than one. The oligospecific HL-A 1 antiserum showed more frequent and stronger reactions. HL-A 2 antisera were more consistently positive with gorilla cells than orangutan cells. Three of the 15 animals reacted strongly with the monospecific HL-A 2 antisera and 12 of 15 reacted with at least one of the oligospecific HL-A 2 antisera. This particular specificity has not as yet been detected in chimpanzees.

Anti-HL-A 3 was also frequently reactive with this species: eight animals were positive with these sera and one donor reacted with all three HL-A 3 antisera. Ten animals reacted with the HL-A 5 or 5-related sera and half of these donors reacted with two or more of them. Seven gorillas were positive with at least two of the three HL-A 7 sera. This degree of concordance lends credence to the possibility that HL-A 7 or an HL-A 7-like antigen occurs in this species. The monospecific and oligospecific HL-A 8 reagents showed strong reactions but no clear-cut repetitive

patterns. No animal reacted with the short HL-A 9 antiserum. However, two animals reacted with both of the long HL-A 9 sera and four additional animals reacted with at least one of them. The HL-A 10+ sera which were highly reactive in the orangutan reacted in a block-like fashion in the gorilla. The gorillas reacted infrequently with the HL-A 11+ antiserum. One monospecific HL-A 12 serum reacted positively with 12 of 15 animals as did the HL-A 12 plus HL-A 13 serum, but two other monospecific HL-A 12 sera and one which defined HL-A 13 reacted infrequently. The observed patterns of cross-reactivity in the gorilla are difficult to interpret in terms of defined human HL-A antigens.

The gorilla cells were more positive than orangutan cells with sera characterized in the last human histocompatibility testing workshop (W designations) and with new AO antisera. However, no clear grouping of the reactions of these sera was seen in this small number of animals.

The cross-reacting sera behaved differently with gorilla cells than with orangutan cells. They were more frequently positive and reacted more often in block fashion similar to that observed in the chimpanzee and human populations. The gorilla demonstrated good reactions with all defined HL-A antisera with the exception of anti-HL-A 13.

The failure of primate lymphocytes to react similarly with several monospecific alloantisera defining the same specificity but produced in another species, makes interpretation of the data extremely difficult. Balner and van Rood have tested chimpanzee cells with human alloantisera and human cells with chimpanzee alloantisera; these data suggest both similarities and differences between HL-A and chimpanzee alloantigens. Ward et al.[7] reported at this conference on 56 human families genotyped with human sera for HL-A. Only HL-A patterns were detected by a panel composed of approximately 90 chimpanzee alloantisera. The nine chimpanzee leukocyte groups defined by Metzgar et al.[8] do not appear to define the same groups in a human population, and it is not yet clear whether or not human sera define the same groups in both humans and chimpanzees. Amos and Ward,[9] as well as others, have observed differences in associations of human alloantisera prepared in a predominantly white population when used for testing isolated populations, similar to the results presented here. All of these data suggest some similarities in both the cross-reacting antigenic specificities and alloantibodies produced in closely related species, but point to differences for both as well. Selected cross-species immunizations and absorptions experiments must be done to clarify the nature of the observed cross reactions between man and nonhuman primates and to provide a better understanding of the phylogeny of HL-A.

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Cross-reactions Between Human, Chimpanzee, and Streptococcal Antigens

By Felix T. Rapaport, A. S. Markowitz, Audrey P. Raisbeck, John H. Ayvazian, and Hans Balner

IT IS GENERALLY AGREED that most, if not all, mammalian species possess a genetically controlled histocompatibility system that plays an important role in conditioning responses to allogeneic tissues. The first of such systems to be defined was the murine H-2 system.[1] More recently, similar immunogenetic systems have been reported in man[2,3] and rats,[4,5] while suggestive evidence for such a system has been obtained in guinea pigs,[6] pigs,[7] and dogs.[8-11] Shulman et al.,[12] Metzgar and Zmijewski,[13] and Balner et al.[14,15] have demonstrated a similar situation in chimpanzees and in rhesus monkeys.[16] Evidence has also been provided that a number of antigenic components of the chimpanzee ChL-A system may be similar to or cross-react with human HL-A isoantigens.

Many of the histocompatibility systems detected thus far have been shown to exhibit extreme degrees of polymorphism. In humans, this finding has been expressed by the occurrence of numerous cross-reactions between different HL-A antigens, so that a number of "families" of such cross-reacting antigens or CREGs, has been identified.[17] The cross-reactions observed between histocompatibility antigens within the same species have recently been shown to extend to isoantigens of other species, as reported for rabbits and mice by Abeyounis and Milgrom,[18] for rats and mice by Sachs, Winn, and Russell,[19] and for rhesus monkeys, chimpanzees, and man by Balner et al.[14] In addition, a growing body of evidence points to the presence in dogs, rabbits, guinea pigs, rats, and mice of histocompatibility antigens that can cross-react with antigen(s) of the Group A streptococcal membrane.[20-24] These observations, which have recently been confirmed by Vogel, Heymer, Smith, and Haferkamp,[25] provide suggestive evidence that a significant proportion of histocompatibility antigen(s) which have thus far been considered to be species-restricted may actually be highly ubiquitous throughout nature. There may therefore exist in nature a wide-ranging and occasionally unexpected spectrum of cross-reacting determinants capable of affecting immunological responses. The tempo and intensity of such responses may be directly dependent upon the degree to which the host's cell surface components cross-react with or share common determinants with the antigenic structure(s) to which the host is exposed; i.e., this factor may be an important component of the antigenic recognition process.

The present report describes a series of preliminary studies designed to further study these considerations, with particular reference to the detection of possible cross-reactions between human and chimpanzee histocompatibility antigens and Group A streptococcal membrane antigens. For this

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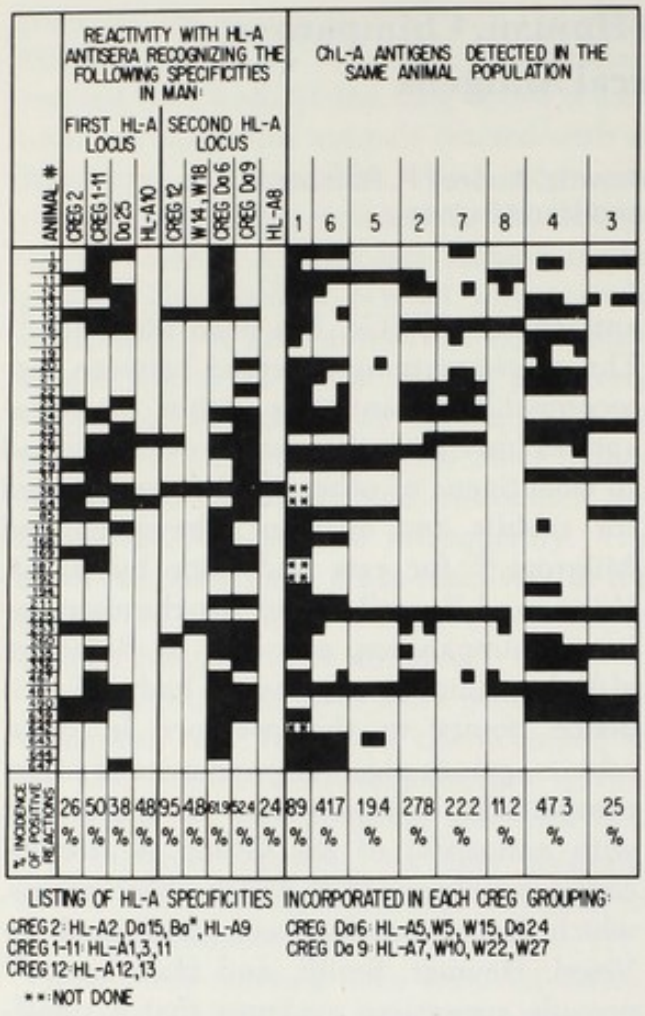

Fig. 1. Comparison of the distribution of the ChL-A leukocyte groups with patterns of chimpanzee reactivity to human HL-A antisera.

purpose, the lymphocytes of 42 chimpanzees of the LEMSIP Colony at Sterling Forest, N.Y., were tested with the battery of 45 human HL-A typing antisera of Dausset et al.[26] This battery of sera is capable of detecting human antigen(s) HL-A 1, 2, Da 15 (Ba*), HL-A 3, 9, 11, 12, and 13 at the first HL-A locus, and antigen(s) HL-A 5, 7, Da 18 (W14), 20, 23, 24, Te 57, Te 58 (W18), AA, W22, Bt22, BB (W10), FJH (W27), and Maki (W14) at the second locus. The lymphocytes of 37 of the animals were also tested with a battery of antisera recognizing ChL-A antigens 1,2,3, 4,5,6,7, and 8 of Balner et al.[14,15] The results of these studies were correlated with the reactions of lymphocytes obtained from the same donors, when tested with a battery of Group A Type 12 streptococcal membrane antisera produced in New Zealand rabbits by a standard technique that has been described in detail in previous reports.[27] The technique of lymphocytotoxicity of Kissmeyer-Nielsen et al,[25] as modified by Balner et al,[14] was used for all serological tests. Each of the tests with human HL-A antisera included suitable positive and negative control reagents. Positive controls consisted of polyvalent human antisera obtained from multiparous donors; negative controls consisted of pooled sera obtained from nine normal nonimmunized male AB donors. None of the chimpanzee cells gave cytotoxic reactions with the negative controls; all cells reacted with the positive controls.

In view of the increasing evidence of cross-reactivities between different HL-A antigens,[17,30] the responses of chimpanzee leucocytes to HL-A antisera have been grouped in this study according to the *major cross-reacting groups* or CREGs detectable in human subjects with such antisera. The CREGs include CREG 2, consisting of seven antisera detecting HL-A antigens HL-A2, 9, Da 15, or Ba*; CREG 1–11, which includes seven antisera recognizing antigens HL-A 1, 3, and 11; CREG 12, which includes four antisera detecting antigens HL-A 12, and 13; CREG Da6, which includes eight antisera detecting HL-A 5, Da 6, W5(Da20 or R*), W15 (Da23 or LND), and Da 24, and CREG Da9, which includes six antisera detecting HL-A7, W10 (BB), W22 (AA, Bt22), and W27 (FJH). The chimpanzee cells tested were considered "positive" for a given CREG if at least three of the sera of that CREG elicited a strong cytotoxicity reaction.

Figure 1 illustrates the patterns of reactivity of chimpanzee lymphocytes to human HL-A antisera, and correlates the results with the ChL-A phenotypes of the

same animals, which have been reported previously by Balner et al.[15] Lymphocytes from six animals gave no reaction with any of the HL-A typing sera. Examination of the results obtained with HL-A antisera detecting antigens of the first locus in humans indicates that antisera detecting CREG 2 antigens had a 26% incidence of cytotoxic reactions in chimpanzees; this was 50% for CREG 1–11 antigens; 38% for Da25; 4.8% for HL-A 10. The highest incidences of lymphocytoxicity obtained with antisera recognizing HL-A antigens of the second locus in man occurred with sera detecting CREGs Da6 (61.9%) and Da9 (52.4%). There appeared to be a contrasting distribution in the incidence of reactivity to CREG Da6 and CREG Da9 antisera in the chimpanzee population under study. Antisera detecting CREG 12, W14, and W18, and HL-A8 had an incidence of cytotoxic reactions of 9.5%, 4.8%, and 2.4%, respectively, in the same chimpanzees.

Dausset has recently suggested the possibility that each CREG may consist of a fundamental molecular structure, which subsequently undergoes minor modifications, thereby producing a number of additional cross-reacting specificities.[30] It therefore appeared to be of interest to attempt to correlate the reactivity of chimpanzee cells to such CREG antisera with the results of tests performed in the same animals with antibodies produced in response to sensitization with streptococcal membrane antigens. For this purpose, the lymphocytes of the 42 LEMSIP Colony chimpanzees reported in this study were tested with a battery of 30 rabbit anti-Group A type 12 streptococcal membrane antisera. The chimpanzee cells were tested with a preimmunization and a postimmunization serum sample obtained from each rabbit. Strong lymphocytotoxicity reactions were elicited by postimmunization serum samples in 18 chimpanzees. Five of the cells tested (chimpanzees 1, 12, 19, 27, 639) reacted strongly with only one antiserum; three others (chimpanzees 116, 169, 225) reacted with two sera; two (chimpanzees 15, 490) cell samples reacted with three sera; two cell samples (chimpanzees 253, 355) reacted with six sera; one cell sample (chimpanzee 23) reacted with nine sera; two cell samples (chimpanzees 64, 85) reacted with 11 sera; two cell samples (chimpanzees 11, 16) reacted with 13 sera, and one cell sample (chimpanzee 26) reacted with 21 of the 30 antisera. None of the preimmunization sera elicited a reaction with the same cells. The lymphocytes obtained from another 17 animals were not affected by either pre- or postimmunization serum samples. Positive cytotoxicity reactions were observed with both pre- and postimmunization rabbit sera in another seven animals. In view of the probability that such reactions were a consequence of humoral factors other than antistreptococcal antibodies, this group has not been included in the analysis of the results.

Figures 2 and 3 illustrate the incidence of responses to HL-A and ChL-A typing antisera in chimpanzees whose cells reacted with streptococcal antibodies and compare them with the results of similar tests performed in animals whose cells were not affected by such antibodies. Human HL-A antisera detecting antigens of the first HL-A locus elicited strong cytotoxicity reactions in lymphocytes obtained from all but two of the chimpanzees (89%) whose cells also reacted with streptococcal antisera; in general, each of the cell samples reacted with sera detecting at least two HL-A CREGs and/or antigens Da25 and HL-A 10 at the first locus (Fig. 2). In contrast, the same HL-A antisera only elicited cytotoxic reactions in 4 of the 17 chimpanzees (16%) whose cells did not react with streptococcal antisera; none of these cells reacted with more than one CREG or an antigen of the first locus. The absence of

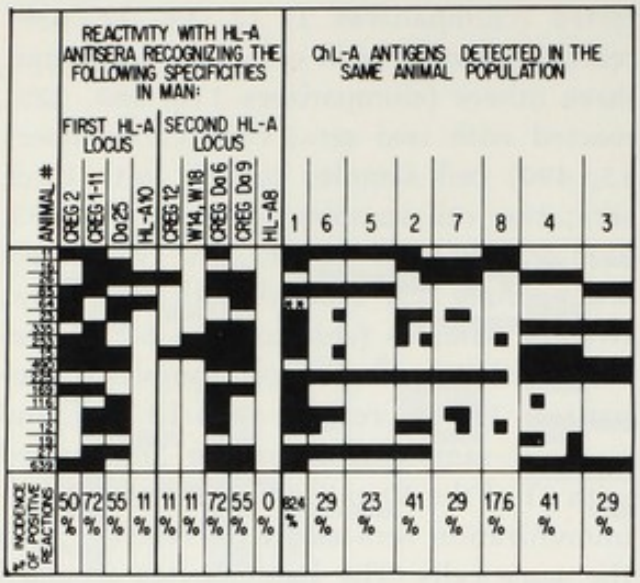

Fig. 2. Cytotoxic reactions to HL-A and ChL-A typing antisera observed in chimpanzee cells which also reacted with streptococcal antibodies.

reactivity to antisera of the first HL-A locus was particularly evident with regard to reagents detecting components of CREG 2 and CREG 1–11 (HL-A 1, 3, 11). There was also a relative decrease in the incidence of reactivity to CREG Da6 sera in these animals. The incidence of specificities ChL-A 2, 5, 7, and 8 appeared to be decreased in nonreactors to streptococcal antisera, but the incidence of specifrcities ChL-A 1 and 4 (the analogues of the human 4a and 4b specificities, respectively) seemed unchanged.

These results provide suggestive evidence that antisera produced in response to human and chimpanzee alloantigens and to Group A streptococcal membrane antigens may recognize similar antigenic configurations located on the surface of the lymphocytes of some, but not all, chimpanzees tested in the course of this study. The apparent association between reactivity to first locus HL-A antisera and streptococcal antisera appeared to be particularly striking in this regard. Taken together, the results support a number of reports of possible cross-reactions between streptococci and human isoantisera,[31,32] as well as the correlation observed by Mickey, Kreisler, and Terasaki[33] between the incidence of antigen HL-A 2 of the frrst HL-A locus and glomerulonephritis (a nonsuppurative sequela of Group A streptococcal infection) in human subjects.

The observations presented in this report are evidently of a preliminary nature, and must await confirmation by absorption studies specifically designed to prove the presence of CREG and streptococcal membrane cross-reacting antigens in chimpanzee cells, and to identify possible RENAP (reaction negative, absorption positive) reactions. The results would appear to provide a further illustration, however, of the potential scope and biological implications of the polymorphism of the histocompatibility antigens in mammals. They also lend additional support to the concept that the antigenic structures implicated in transplantation may be distributed widely throughout nature.[34]

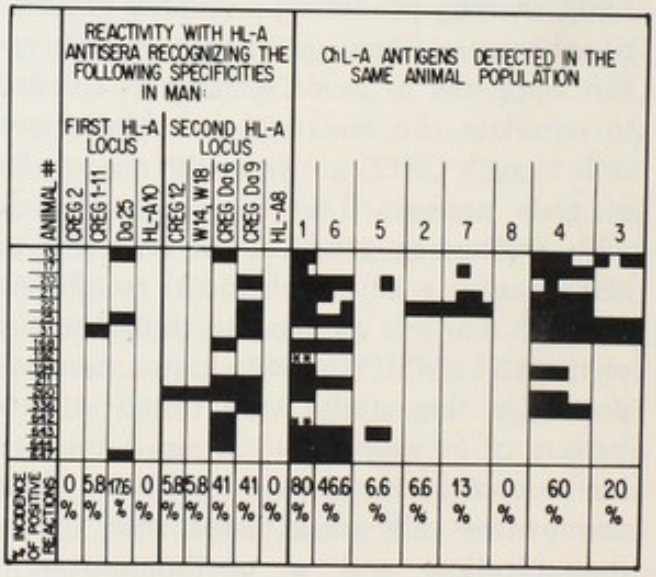

Fig. 3. Cytotoxic reactions to HI-A and ChL-A typing antisera observed in chimpanzee cells that failed to react with streptococcal antibodies.

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Cross-species Tissue Typing Between Rhesus Monkeys, Speciosa Monkeys, and Chimpanzees

By H. Dersjant, W. van Vreeswijk, and H. Balner

THE INTEREST in the human HL-A antigens and the complexity of their interrelationships has focused attention also on their phylogeny. The big apes would obviously be the first animals to be considered for comparative studies that might show to which degree they share antigens with man; however, lower primates and even lower animal species may also be useful for comparison. It has already been shown that chimpanzees have several tissue antigens in common with man and that chimpanzee isoantisera can be useful reagents for human tissue typing.[1-4] The relatively long survival of chimpanzee xenografts in man lends further support to the existence of a rather high degree of antigenic similarity between chimp and man. Dorf and Metzgar[5] showed that serological relationships also seem to exist between man and the gorilla.

As for the lower primates, there is some suggestive evidence that certain leukocyte specificities of rhesus monkeys may be related to the human 4a and 4b specificities.[2] Since there is already a vast knowledge of the main histocompatibility systems of chimpanzees and rhesus monkeys,[6] it seemed worthwhile to study the serological cross-reactivity between various primate species. In this volume, van Rood[7] and investigators from Duke University[8,9] will discuss such cross-reactivities between apes and man. We shall deal with similarities between three subhuman primate species, namely, chimpanzees, rhesus monkeys *(Macaca mulatta)* and speciosa monkeys *(M. arctoides)*. Although not much work has been done hitherto on the leukocyte antigens of speciosa monkeys, this species was also included in this cross-typing study because of its availability and its rather close phylogenetic relationship to the rhesus monkey.

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MATERIALS AND METHODS

Methods to produce isoantisera in apes and monkeys have been described elsewhere.[1,10]

For chimpanzee as well as for rhesus and speciosa lymphocytes, a slightly modified version of the microcytotoxicity test as described by Kissmeyer-Nielsen and Thorsby was used.[11] Pure lymphocyte suspensions were prepared using the Ficoll-Isopaque flotation method according to Böyum.[12] Chimpanzee cells were suspended in Hanks' medium, rhesus and speciosa cells in normal rhesus and speciosa serum, respectively. The incubation time was 30 min for tests in which chimp sera were used and 15 min for rhesus and speciosa sera, since previous experiments had shown the incubation time to be more related to the serum used than to the species of the target cell donor. In all tests, normal unabsorbed rabbit serum was used as complement.

RESULTS

Cross-typing of Chimp Cells With Rhesus Sera

In Fig. 1 a comparison is made between the chimp groups on 38 chimpanzee cells and the reactivity with our normal panel of rhesus sera defining the RhL-A specificities consisting of two to four sera per group. Nine rhesus groups showed polymorphism with chimpanzee cells, one set (Group 14) reacted positively with all cell

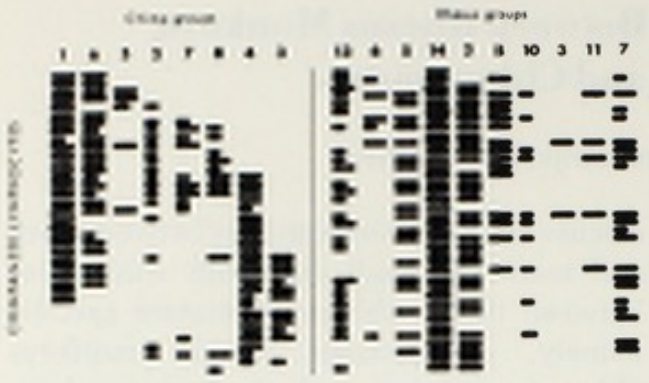

Fig. 1. Comparison of reactivity patterns of chimp sera (eight groups) and rhesus sera (ten groups) with 38 chimp cells (cytoxicity). Rhesus groups 1, 4, 9, and 12 have been omitted since they showed no positive reactions with chimp cells. Longer symbols, all sera of a group reacting positively; shorter symbols, part of the sera of a group reacting positively.

samples tested. No clear-cut correlation can be seen between the reactivity patterns of the rhesus sera and those of the known chimpanzee leukocyte groups. In some cases, only part of the sera of a particular rhesus group reacted with chimp cells, a phenomenon which is known to occur also in cross-typing between ethnologically different populations. Although a number of rhesus leukocyte groups seems to be well represented on chimp cells, their frequency is totally different from that on rhesus lymphocytes. These differences are depicted in Table 1. As can be seen from this table, Groups 2, 13, and 14 have a low frequency in the rhesus monkey but show a very high frequency in chimps. Groups 3 and 11 show the reverse. Sera from groups 1, 4, 9, and 12 did not show any reactivity with chimp cells. So far, no absorption studies have been done; however, they have already been planned for the near future.

Cross-typing Rhesus Cells With Chimpanzee Sera

As can be seen from Fig. 2, chimpanzee groups are not so well represented on rhesus cells as are rhesus groups on chimpanzee cells. Only the sera defining Groups 1, 2, 4, and 3 showed reactivity with rhesus cells. The sera from chimp Group 2 did not all react in the same way. The frequency of the four chimp groups in monkeys does not differ significantly from that in chimpanzees.

Although only few reactivities seem to be present on rhesus cells it is noteworthy that the chimp Groups 1 and 4 which are related to the human 4a and 4b, respectively, show the same allelic distribution on rhesus cells as they do in chimps. Chimp Group 2 seems to be included in Group 1. The rhesus groups 4, 1, 9, 13, and 6 which are positively associated in rhesus monkeys show some correlation with the 4a-like chimp 1 and 2 specificities. (It should be recalled that an association between rhesus Group 1 and the human 4a had been previously suggested on the basis of absorption studies.)[2] The analogue of 4b in chimps, namely Group 4 does not seem to correlate well with rhesus Group 2, which on the same grounds was supposed to be the expression of the 4b specificity in rhesus monkeys. Chimp Group 3 which is

Table 1.

INCIDENCE OF RhL-A REACTIVITIES ON RHESUS, SPECIOSA AND CHIMPANZEE LYMPHOCYTES

RhL-A	Rhesus cells (%)	Speciosa cells (%)	chimp cells (%)
1	45	0	0
2	22	12	76
3	63	42	5
4	58	58	0
5	23	18	50
6	37	66	16
7	59	8	53
8	47	42	39
9	26	0	0
10	26	0	26
11	46	36	13
12	43	32	0
13	11	0	61
14	8	8	100

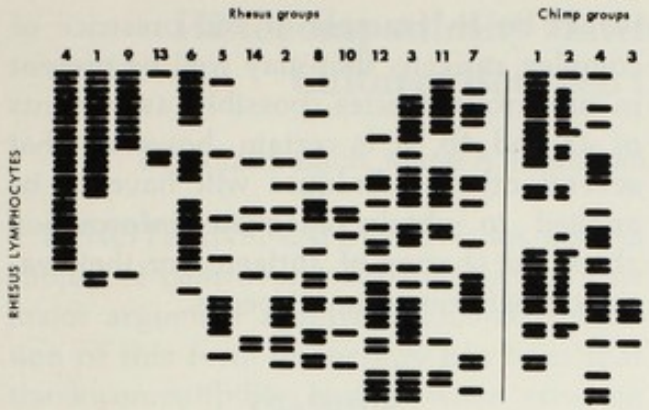

Fig. 2. Comparison of reactivity patterns of chimp sera and rhesus sera with 41 rhesus cells (cytotoxicity). Chimp groups that showed no reactivity have been left out. Longer symbols, all sera of a group reacting positively; shorter symbols, part of the sera of a group reacting positively.

related to the human 7c specificity does not show a clear-cut correlation with any of the rhesus groups. Cross-absorptions and further analysis of the RhL-A and ChL-A systems may throw more light on the as yet unexplained complexity of the 4a and 4b systems.

Cross-typing of Speciosa Cells With Rhesus Sera

As can be seen in Fig. 3, most of the rhesus leukocyte specificities seem to be represented on speciosa cells with the exception of groups 10, 1, 9, and 13. Again, a contrasting distribution can be seen between the 4a like specificities 4 and 6 and the other groups.

The frequency of these rhesus groups in speciosa monkeys can also be seen in Table 1; for most groups the frequencies in speciosa monkeys are not significantly different from those in rhesus monkeys. Incidentally, 3 isoantisera produced in two speciosa monkeys showed an identical pattern when tested with speciosa cells. These two monkeys as well as their immunizing donor did not react with any rhesus group.

In an attempt to raise speciosa isoantisera for tissue typing of rhesus monkeys, two speciosa monkeys that had been typed with rhesus typing sera were immunized with a speciosa donor differing for one rhesus group only (Group 12). As hoped for, these monkeys produced a serum that showed group 12 specificity when tested on rhesus cells (Table 2). Similar results have been obtained with chimpanzee isoantisera; several reagents of low specificity in chimpanzees showed a high degree of HL-A specificity when used for human tissue typing. It is tempting to explain this phenomenon by assuming that these isoantisera contain several antibodies and that only those antigens that are shared between two species are identified in cross-species typing. Further application of this principle may have practical implications for the production of typing reagents in general.

*Van Rood's original nomenclature; it cross-reacts with HL-A7, AA, and FJH.

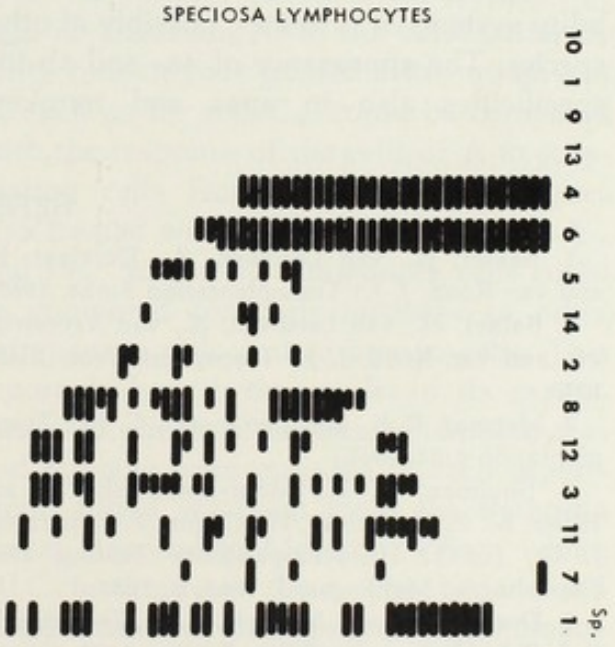

Fig. 3. Reactivity of rhesus sera with 50 speciosa cells (cytotoxicity). Numbers stand for rhesus leukocyte groups. Sp. 1 represents reactivity of 3 speciosa sera, derived from two different donors. Longer symbols, all sera of a group reacting positively; shorter symbols, part of the sera of a group reacting positively.

Table 2.

PRODUCTION OF TYPING REAGENTS FOR RHESUS MONKEYS BY ISO-IMMUNIZATION OF SPECIOSA MONKEYS

		RhL-A specificities
Speciosa recipients	1	4 6
	2	4 6 7
Immunizing Speciosa donor		4 6 **12***

* sera had RhL-A 12 specificity in Rhesus monkeys

Looking at the presented, preliminary results of cross-species typing, it seems likely that some of the observed cross-reactivities may provide interesting information concerning the basic structure and the phylogeny of the major histocompatibility systems of primates, possibly of other species. The appearance of 4a- and 4b-like specificities also in apes and monkeys would be an example of the presence of complex antigens that may well be present in numerous species, possibly as variants of 4a and 4b. It is certain, however, that several other techniques will have to be applied to obtain adequate information about the sharing of antigens (or their variants) between animal species.

SUMMARY

Isoantisera defining eight antigenic determinants in chimpanzees were used also for the typing of rhesus monkeys. A similar approach was used in typing chimpanzees and speciosa monkeys with a panel of rhesus isoantisera defining 14 rhesus leukocyte specificities. Several similarities as well as differences were found that may lead to a better understanding of the antigenic systems of primate species including man.

Current cross-species studies, which include cross-species absorptions, should provide useful information also regarding the phylogeny of the major histocompatibility systems of primates.

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Histocompatibility Matching. VII. Mixed Leukocyte Cultures Between Chimpanzee and Man

By Fritz H. Bach, Marie A. Engstrom, Marilyn L. Bach, and Kenneth W. Sell

XENOTRANSPLANATION has been a subject of controversy for some years. One major argument against serious consideration of this form of therapy has been that the incompatibility barrier when crossing species would be greatly increased. With our increasing understanding of the different types of immunological reactions, the possibility exists that the major problem in xenotransplantation is the presence of preformed antibodies, a humoral response, which attack the xenogeneic donor kidney. This still leaves open the question of how severe the cellular immune response (thought by many to be the main reaction leading to classical allograft rejection) might be against a xenografted organ. A few isolated instances of moderately prolonged survival of chimpanzee kidneys in humans lends support to the concept that, in certain cases, such a transplant might be considered. The choice of the chimpanzee as a donor species can be based on anthropological considerations that have been strengthened by the findings that man and chimpanzee lymphocytes share many of the antigens that are grouped within HL-A, the major histocompatibility locus in man.[1-3]

Our purpose in this study was to investigate the incompatibility between chimpanzee and man as reflected in the mixed leukocyte culture (MLC) test.[4,5] This test has been shown by a number of studies to be a quantitatively meaningful measure of histocompatibility in man.[6-8] It seems reasonable to extrapolate, given appropriate controls, the findings obtained in MLC to xenogeneic testing, although no firm evidence for this exists.

The basic protocol used was to study chimpanzee and human cells in all possible combinations in the one-way mixed leukocyte culture test.[9] As such, the response of cells of a human, A, to the stimulation by other human cells treated with mitomycin C, such as B_m and C_m, could be compared with the response of the cells of A to stimulating cells from different chimpanzees also treated with mitomycin C, such as V_m and W_m. Similarly, chimpanzee cells could be compared for their response to other chimpanzee cells and to human cells. This report deals with the results of six experiments in which a number of chimpanzees and humans were included in each experiment. Blood was sent from the Bethesda Naval Hospital to Madison, Wisc., where MLC testing was done. The heparinized blood from the chimpanzees and humans was allowed to sediment in Bethesda and the leukocyte-rich plasma from each sample was mixed with an approximately equal volume of Medium 199 buffered with Hepes for shipment at 4°C to Madison. After arrival in Madison, usually within

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10 hr, the cells were resuspended in fresh medium and MLC tests were performed as extensively detailed elsewhere.[9,10]

Whereas the degree of stimulation in each experiment varied somewhat (in the same general range that we find in mixed cultures done in man) significant stimulation was obtained in every experiment allowing a comparison of the response of man to man, man to chimp, chimp to man, and chimp to chimp. Selected parts of three experiments are given in Table 1. The letters A through G refer to humans; the letters S through Z to chimpanzees. The mean values given for each mixture are the average of quadruplicate cultures plus or minus the standard deviation of those values.

The most striking observation is that human cells respond to chimpanzee cells in a manner which is grossly similar, in quantitative terms, to their response to other human cells. For instance in Experiment 2, the human responding cells, C and D, respond to the same general extent to each other and to the cells of E, another human,

Table 1.

MLC in Man and Chimpanzee

Experiment I			
AA_m*	584 ± 181**cpm	SS_m	1526 ± 433 cpm
AB_m	5518 ± 2513	ST_m	8841 ± 1609
	11435 ± 2997		14099 ± 3480
AS_m	25643 ± 5176	SA_m	8299 ± 1974
	33753 ± 6028		19313 ± 7641
AT_m	1892 ± 513	SB_m	3296 ± 716
	4862 ± 2105		4554 ± 1268
Experiment II			
CC_m	435 ± 28	DD_m	386 ± 17
CD_m	4998 ± 1288	DC_m	2237 ± 781
CE_m	15060 ± 3800	DE_m	8061 ± 1033
CU_m	9152 ± 1807	DU_m	4661 ± 997
CV_m	23521 ± 2029	DV_m	18455 ± 2525
CW_m	11281 ± 2741	DW_m	9399 ± 2032
UU_m	1251 ± 403	VV_m	1160 ± 44
UV_m	79882 ± 4595	VU_m	24980 ± 2552
UW_m	64010 ± 3728	VW_m	18258 ± 4458
UC_m	4082 ± 677	VC_m	6083 ± 1230
UD_m	17993 ± 5782	VD_m	12264 ± 2779
UE_m	15364 ± 1145	VE_m	19591 ± 3409
Experiment III			
FF_m	588 ± 185	XX_m	603 ± 82
FG_m	12298 ± 1392	XX_m	24185 ± 1019
FX_m	1768 ± 246	XZ_m	7389 ± 1318
FY_m	7035 ± 454	XF_m	7653 ± 238
FZ_m	12113 ± 2193	XG_m	30789 ± 547

* A - G are humans; S - Z are chimpanzees.

** ± standard deviation of the mean.

as they do to the cells of U, V, and W, three chimpanzees. It is noteworthy that there is wide variability in the extent to which human cells respond to different chimpanzee stimulating cells; similarly, there is variability in the extent to which chimpanzee cells respond to human cells. This has been previously noted for variations in the strength of the HL-A alleles in allogeneic human mixtures.[11]

In Experiment 1 allogeneic and xenogeneic responses were tested at two concentrations of stimulating cells, the second twice the first. In the past we have reported that under such conditions, if one is testing in the right range of cell concentrations, an approximate doubling of the counts per minute (cpm) of tritiated thymidine incorporated is observed. In these experiments, this was shown to be true not only in human-human mixtures but also in mixtures where human cells respond to chimpanzee-stimulating cells, where chimpanzee cells react to human-stimulating cells, and in the allogeneic chimpanzee mixtures. Although not a critical argument, this finding gives us some confidence that the use of chimpanzee cells both for responding cells and stimulating cells at the same concentrations as human cells were reasonable concentrations with which to work.

We have tried to summarize all of the data obtained in this study in two ways to allow further comparison. In the first case, we have expressed stimulation in the four types of mixtures, chimpanzee responding to chimpanzee, human responding to human, chimpanzee responding to human, and human responding to chimpanzee, as the ratio of cpm incorporated in the allogeneic or xenogeneic mixture to the cpm in the control isogeneic mixture. In the second case, we have expressed the actual cpm incorporated in certain of the mixtures as ratios of the cpm in other mixtures.

A total of six experiments were done. In

every experiment all chimpanzees and humans were tested in every possible combination. In one experiment, six chimpanzees and four humans were tested; in a second experiment, three chimpanzees and three human; in a third experiment, four chimpanzees and four humans; in a fourth experiment, two chimpanzees and three humans; in a fifth experiment, two chimpanzees and two humans, and, in a sixth experiment, one chimpanzee and two humans.

The overall results can be expressed in the two ways outlined above. First, the cpm present in the allogeneic or xenogeneic mixture are expressed as a multiple of those present in the isogeneic mixture. The average ratio for chimpanzee responding to chimpanzee was 12.74 (range 1.5–35); the average ratio for human responding to human was 11.41 (range 6.5–16.5); the average ratio for chimpanzee responding to human was 7.6 (range 2.4–14.4); and the average ratio for human responding to chimpanzee was 16.74 (range 3.2–43.9). It seems proper to compare, for instance, the response of human cells to other human cells with the response of those human cells to chimpanzee cells. As such, it would appear that there is no significant difference in the responses, although there is a tendency for a slightly higher response of human cells to chimpanzee cells than of human cells to other human cells. This is fully consistent with the data presented in Table 1. It should be noted, however, that there is also a tendency for a higher response of chimpanzee cells to other chimpanzee cells than to human cells. This could thus indicate that chimpanzee cells are simply more stimulatory. Certainly, no gross differences exist; there is wide overlap in the responses in the allogeneic and xenogeneic mixtures.

If the data are expressed as a ratio of the average cpm present in the different combinations, similar conclusions may be reached. The ratio of the average cpm present in chimpanzees-chimpanzees mixtures over the average cpm incorporated in mixtures in which chimpanzee cells respond to human stimulating cells is 1.347 with a range from 0.456 to 3.22 in the different experiments. The ratio of the average cpm in mixtures involving humans responding to humans over the average cpm in humans responding to chimpanzees is 0.962 with a range from 0.369 to 1.924. These results are thus consistent with those presented above. i.e., humans appear to respond somewhat more to chimpanzees than to other humans, and chimpanzees appear to respond more to other chimpanzees than to humans. Again, there is substantial overlap.

It is difficult to draw any firm conclusions regarding the in vivo prognosis for xenotransplantation from such studies. Whereas there is evidence that the MLC test will meaningfully quantitate differences at HL-A, the evidence that this is prognostic for transplantation in man, other than in the case of HL-A-identical siblings, is only minimal. As such, this must be regarded as a guideline study. Further complicating the interpretation of these data is the question of whether it is proper to compare the response of, let us say human cells, to human and chimpanzee-stimulating cells using similar numbers of cells as stimulating cells. The fact that approximately twice as many cpm are incorporated if the number of stimulating cells is doubled, as well as the similar number of cpm present in the isogeneic mixtures of man (average 1433 cpm) and isogeneic mixtures of chimpanzees (average 1182 cpm) provide some basis for making these comparisons. Whereas there is good evidence in allogeneic systems that the MLC test is a measure of delayed type hypersensitivity reaction, it is not clear whether xenogeneic mixtures are equally a reflection of delayed type hypersensitivity.

Nonetheless it would not be unreason-

able to suggest, on the basis that chimpanzees do not stimulate human responding cells to a much greater extent than human stimulating cells, that one might hope to find some chimpanzees who would, if only the thymic arm of the immune response had to be considered, be reasonable donors.

ACKNOWLEDGMENT

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Principles of Blood Grouping in Apes and Monkeys: Human, Simian, and Cross-immune Types

By A. S. Wiener, J. Moor-Jankowski, and W. W. Socha

IT IS A PARTICULAR PLEASURE AND SATISFACTION to participate in this symposium, since it makes us feel no longer isolated in our interest in antigenic similarities and differences, homologues and analogues, of blood group specificities in man, apes and monkeys. The earliest significant investigation on the blood groups of apes and monkeys was by Landsteiner and Miller,[1] who introduced the use of eluates as testing reagents for A-B-O blood grouping and demonstrated unequivocally the sharing of this human blood group system by chimpanzee, orangutan, and gibbon. Landsteiner and Miller did not succeed, however, in elucidating the A-B-O blood groups in gorilllas or in monkeys, because the red cells of those primates do not react with A-B-H reagents. This problem was solved a decade later by one of us (A. S. W.) by demonstrating that monkeys are A-B-H secretors so that their A-B-O blood groups can be determined by tests on saliva and serum. At about the same time parallel investigations on M-N-like blood factors present in apes and monkeys led Landsteiner and Wiener to the discovery of the Rh-factor in man. This, in turn, led to an enormous expansion in human blood grouping and the development of new techniques which have been adapted to our studies in nonhuman primates.

In 1962 Wiener was joined by Moor-Jankowski,[2] who had earlier applied to the study of primate serum proteins the same principles that had led to the discovery of the Rh factor. A long-term plan of investigation was established, the first stage of which was the study, during the years 1962–1966, of the human blood groups and blood factors of the A-B-O, M-N, Rh-Hr, I-i, and Lewis systems in a large number of primate animals, from a variety of species.

HUMAN-TYPE BLOOD GROUPS

The work on the A-B-O groups in apes was greatly facilitated by our introduction, in 1963, of anti-**A** and anti-**B** reagents produced by absorption with chimpanzee group O red cells, instead of the laboriously prepared and less dependable eluates. New testing methods for other factors were also introduced, which entailed comparative titrations; so too was use of appropriately diluted antisera, to avoid interference from nonspecific heteroagglutinins. This refined absorption and titration-dilution technique is applicable to the blood group systems A-B-O, M-N and Rh-Hr and I-i but not to other human-type blood group systems that are detected with reagents of generally lower titer. We have, therefore, after a number of inconclusive pilot studies, excluded such blood factors from our panel of tests, with the exception of the Lewis system which can be determined by inhibi-

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*In this article and the next, to avoid ambiguity, symbols for blood factors (serological specificities) and their corresponding antibodies are printed in bold face type, symbols for genes and genotypes in italics, and symbols for agglutinogens and phenotypes in regular type.

Table 1. Current Status of A-B-O Blood Groups in Primates*

Species	Blood Groups Encountered	Antigens on Red Cells	Subgroups† of A	Antigens Present in Saliva
Man	O, A, B, AB	A, B, H	A_1, A_2, A_3, etc., and intermediates	A, B, H (Sec. and nS)‡
Chimpanzee				
Pan troglodytes	O, A	A, H	A_1^{Ch}, A_2^{Ch}	A, H
Pan paniscus	O, A	A, H	A_1	A, H
Orangutans				
Pongo pygmaeus	A, B, AB	A, B	A_1	A, B, H (Sec. and nS)
Gibbon				
Hylobates	A, B, AB	A, B, H	A_1, A_2	A, B, H
Siamans				
Symphalangus syndactylus	B	B, H	—	B, H
Gorilla				
Gorilla g. gorilla	B	Absent	—	B, H
Baboons§				
Papio	O, A, B, AB	Absent	—	A, B, H
Drills				
Mandrillus leucophaeus	A	Absent	—	A, H
Geladas				
Theropithecus gelada	'O'	Absent	—	H
Celebes black apes				
Cynopithecus niger	O (?), A, B	Absent	—	A, B, H
Patas monkeys				
Erythrocebus patas	A	Absent	—	A, H
Vervet monkeys				
Cercopithecus pygerythrus	A, B	Absent	—	A, B, H
Rhesus monkey				
Macaca mulatta	B	Absent	—	B, H
Crab-eating macaques§				
Macaca irus	O, A, B, AB	Absent	—	A, B, H
Pig-tailed macaques				
Macaca nemestrina	O, B	Absent	—	B, H
Stump-tail macaques				
Macaca speciosa	B	Absent	—	B, H
Spider monkeys				
Ateles	'O', A, B	B-like	—	A, B, H
Capuchins				
Cebus albifrons	'O', B	B-like	—	B, H
Squirrel monkeys				
Saimiri sciurea	'O', A	B-like	—	A, H
Marmosets				
Various species	A	B-like	—	A, H-like

*All data based on our own findings except the presence of group O in dwarf chimpanzee by Schmitt.

†Subgroups of A not determinable in tests on saliva.

‡All animals are secretors (Sec) except where indicated (nS).

§Group O is uncommon among baboons.

§Group O is uncommon among baboons and crab-eating macaques.

tion tests on saliva. Thus, we do not consider reliable reports by others of the demonstration of blood factors such as **Lu**, **Fy**, **Jk**, etc., on the red cells of primate animals using routine methods, especially since none of these reports made any men-

tion of titration or dilution experiments, nor of the blind technique of reading the reactions, used routinely for all our own tests and which is indispensable, especially for weakly reacting reagents. The scope of the information accumulated by our team on A-B-H blood factors in primates is illustrated by the summary table (Table 1).

The importance of the A-B-O blood groups for transplantation in primate animals has been established. In apes and in man, where the reciprocal antibodies in the plasma are reactive with the red cells as well as with the antigens in secretions, an immediate nonfunctioning of an A-B incompatible chimpanzee kidney transplanted to man was observed by Reemtsma. Even though in baboon the A-B-H group specific substances are not demonstrable on the red cells but only in secretions and tissues, it was shown by Hitchcock and Starzl that baboon kidneys transplanted to man had a better clinical course when matched with the recipient for A-B-O. In homotransplantation of baboon kidneys Murphy has demonstrated consistently better survival of kidneys matched for the A-B-O blood groups.

SIMIAN-TYPE BLOOD GROUPS

The next step of our planned investigations concerned the search for blood groups and factors peculiar to apes and monkeys. There had been three previous investigations along these lines, all dealing with saline-reactive agglutinating reagents. In 1932, Fischer and Klinkhart described individual differences in rhesus monkey *(Macaca mulatta)* red cells demonstrable with the serum of a rhesus immunized with cynomolgus monkey *(Macaca fascicularis)* red cells. In 1936, three chimpanzees immunized with A-B-O compatible human red cells by Landsteiner and Levine produced antisera that detected individual differences in chimpanzee red cells. In 1961, Owen and Anderson, using rabbit antisera prepared against rhesus red cells, demonstrated five blood factors in rhesus monkeys, designated as A, B, C, D, and E, of which A and B were allelic and C and E closely associated. None of these three studies was continued further, and the antisera, with the possible exception of those of Owen and Anderson, are no longer available.

Several reports, including that of Owen and Anderson emphasized the difficulty of producing antisera in nonhuman primates by isoimmunization. Nevertheless, in our investigations, with the exception of the pilot study on Celebes black apes *(Cynopithecus niger)*, we deliberately chose isoimmunization in order to avoid nonspecific heteroagglutinins present in the raw antisera produced in rabbits and other standard experimental animals. Our immunization schedule took into an account early observations of one of us (J. M.-J.) in serum protein work which indicated that prolongation of the course of immunization beyond a year or more may be required to produce results. Moreover, our concept of a special immunological relationship among closely related species led us to include cross-immunization as an essential part of our program. The initial isoimmunization and cross-immunization experiments were carried out by us during 1963–1969. They resulted in new insight into the principles of blood group specificities in monkeys, apes, and man, and their mutual relationships which, in turn, led to the formulation of the new concept of human-type, simian-type and cross-immune type primate blood group specificities and of homologues and analogues of human blood groups in apes and monkeys. The human-type blood groups are those determined by reagents originally prepared for testing human blood, though the reagents generally require further processing to remove nonspecific heteroagglutinins. These reagents, therefore, define in simians the homologues of the human blood group factors, including the A-B-O and H, M-N, and Rh-Hr

reactions. The existence of the second category, that of simian-type blood factors, was previously unrecognized as such; it includes those factors of simian blood detected with reagents produced by immunization with red cells of apes and monkeys.

The following simian-type blood group specificities have thus far been identified in our studies.

Chimpanzees—V-A-B system: **V**, $\mathbf{A^c}$, $\mathbf{B^c}$, C-E-F system: $\mathbf{C^c}$, $\mathbf{c^c}$, $\mathbf{E^c}$, $\mathbf{F^c}$ Others: $\mathbf{G^c}$, $\mathbf{H^c}$, $\mathbf{I^c}$, $\mathbf{J^c}$, $\mathbf{K^c}$, $\mathbf{L^c}$, $\mathbf{M^c}$, **Lindsay, Mandy**

Gibbons—$\mathbf{A^g}$, $\mathbf{B^g}$, $\mathbf{C^g}$, and five others presently under study

Baboons—$\mathbf{A^p}$, $\mathbf{B^p}$, $\mathbf{C^p}$, $\mathbf{G^p}$, and more than six others presently under study

Rhesus monkeys—$\mathbf{A^{rh}}$, $\mathbf{B^{rh}}$, $\mathbf{C^{rh}}$, $\mathbf{D^{rh}}$, and more than six others presently under study

Cynomolgus monkeys—four blood factors, presently under study

Celebes black apes—$\mathbf{A^{ba}}$

Some of the simian-type blood factors studied by us have already been shown to be analogues of the human M-N-S and Rh-Hr factors. The cross-immune blood factors, on the other hand, are those detected by antisera produced by cross-immunization between closely related species rather than within the same species. The cross-immune antisera thus necessarily define simian-type blood factors shared by closely related species. An independent parallel study in 1964 by cross-immunization of chimpanzees with human blood carried out by Zmijewski and Metzgar resulted in defining of two chimpanzee blood factors.

The use in our investigations of all the techniques that are accepted as standard for human blood typing, has provided insight when classifying the specificities into separate blood group systems. The methods included saline agglutination, antiglobulin, ficinated rell cell method, and the antiglobulin test using ficinated red cells. Thus, in early stages of our investigations on the V-A-B system found by us in chimpanzees, we thought it to be the analogue of the human M-N-S system because of parallel reactions obtained in tests on chimpanzee red cells using anti-$\mathbf{N^v}$ lectin (*Vicia graminea*) and using chimpanzee antisera produced by cross-immunization with human red cells. The hypothesis was later confirmed, when it was found that V-A-B antigens were destroyed by treatment of the red cells with the proteolytic enzyme ficin, as are the antigens of the M-N-S system in man. On the other hand, the C-E-F system of chimpanzees was believed to be the analogue of the human Rh-Hr system because the reagents reacted best with ficinated red cells, as in Rh-Hr reactions in man. This hypothesis was later confirmed when human anti-$\mathbf{Rh_o}$ sera, fractionated by absorption with particular chimpanzee red cells, proved to give reactions on chimpanzee red cells parallel with the chimpanzee isoimmune serum anti-c^c.

Along the same lines, in gibbons, baboons, and rhesus monkeys, particular isoimmune sera are reactive by the saline agglutination and antiglobulin methods but not for ficinated red cells, while other antisera are reactive by all methods. Again, we presume that we are dealing with analogues of the human M-N-S and Rh-Hr systems, respectively, so that further experiments have been planned to test this concept.

The next step in our investigation will be to define homologues and analogues among blood group factors and systems shared by some of the monkeys but not present in man. Extension of these studies will provide additional insight into the phylogenetic relationships among primate species.

The general concept of our investigative approach has provided us with a new additional category of reagents, namely, isoimmune sera prepared in one species and used for typing animals from other closely

related species of primates. Here again, we have drawn upon the experience acquired 10 yr ago from studies on serum proteins. In our more recent blood typing experience, we have found, for example, that isoimmune sera produced in baboons can be used not only for typing baboon red cells but also for typing blood from some species of macaques, and, similarly, that certain isoimmune sera produced in rhesus monkeys define individual differences also in irus monkeys and vice-versa.

A further logical step is to test human red cells with reagents produced for simian-type blood factors in order to detect homologues of simian specificities in man. Experiments along those lines, which have so far yielded only inconclusive results, are being continued.

The Primate Blood Group Reference Laboratory in New York is available to all investigators. At the Rijswijk Workshop all chimpanzees were tested by us for the blood factors of human type **A**, **A_1**, **B**, and H and simian types **V**, **A^c**, **B^c**, **C^c**, **c^c**, **E^c**, **F^c**, **G^c**, **H^c**, and **Lindsay**, and rhesus monkeys for **D^{rh}**, **E^{rh}** (three different sera), and **F^{rh}**. We expect to continue testing the antisera of Stone, Duggleby, and Sullivan which, so far, have not given conclusive results in our hands.

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Methodology of Primate Blood Grouping

By W. W. Socha, A. S. Wiener, E. B. Gordon, and J. Moor-Jankowski

IT WILL BE ASSUMED that the readers are familiar with the principles of human blood grouping. Therefore, in this presentation, only aspects peculiar to blood typing of simians will be discussed.

Tests on nonhuman primates can be classified in two main categories: tests for human-type blood factors and tests for simian-type blood factors. Tests for human type blood factors, as the term implies, are carried out with reagents originally prepared for typing human blood, and therefore detect homologues of the human blood groups occurring in apes and monkeys. On the other hand, tests for simian-type blood factors are carried with reagents prepared by hetero- and isoimmunization with red cells of apes and monkeys. Some of the simian-type blood factors detected by these reagents have been shown to be analogues of human blood groups.

DETERMINATION OF HUMAN-TYPE BLOOD GROUPS

A-B-O Typing of Apes: With exception of gorillas, this is done with the same techniques as used for testing human blood. However, prior to their use, the anti-**A** and anti-**B** sera* have to be absorbed with group O red cells of chimpanzees to remove the nonspecific heteroagglutinins reactive for ape red cells. In tests for agglutinogen A, lectins from lima beans *(Phaseolus vulgaris)* as well as anti-**A** snail agglutinins *(Helix pomatia)* may be used without prior absorption, since they do not contain heteroagglutinins. The snail agglutinins are very potent and can be highly diluted for testing.

All group A bloods from apes are further tested with anti-$\mathbf{A_1}$ reagents. Anti-$\mathbf{A_1}$ lectin *(Dolichos biflorus)* has the advantage that it can be used without prior absorption. All A-B-O blood grouping results have to be confirmed by reverse tests of the animal's serum against human red cells of groups A_1, A_2, and B, and O (used as control) after the serum has been absorbed with human group O cells to remove nonspecific heteroagglutinins. It is our practice to test the red cells of all nonhuman primates also with anti-**H** lectin *(Ulex europeus)*; these tests are done as in human blood grouping, since there are no nonspecific heteroagglutinins to contend with.

Gorillas and Old World Monkeys: Red cells of gorillas as well as of Old World monkeys cannot be used for A-B-O grouping because they do not react with anti-**A**, anti-**B**, or anti-**H** reagents. However, all the gorillas and monkeys tested by us have proved to be secretors of the A-B-H substances in their saliva. The saliva is tested by inhibition technique, as in man, and reverse grouping tests for anti-**A** and anti-**B** are done on the sera of the animals. For the inhibition tests, the anti-**A**, anti-**B** and anti-**H** reagents are diluted depending on their titers to yield reagents with four to eight agglutinating units. The indicator cells for anti-**A** are human A_2 red cells; human B cells are used for anti-**B**, and human O cells for anti-**H**. The reagents do not have to be absorbed with chimpanzee group O red cells before use, because the indicator cells are of human origin, so that nonspecific heteroagglutinins are not in-

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volved. We do not depend on a one-tube tests, but test the saliva in five fourfold dilutions; thus, at the same time we determine the inhibition titer. Subgroups cannot be determined for group A baboons, because, as in man, the inhibition tests on saliva do not clearly distinguish subgroups.

M-N Types: For these tests, anti-**M** and anti-**N** reagents prepared from rabbit antisera are used as in routine human blood typing. With these reagents interference from heteroagglutinins are largely avoided by dilution, so that, with some exceptions, they can be used directly and by the same technique as for man. We also use anti-$\mathbf{N^v}$ lectin, *(Vicia graminea)*, which detects an N-like specificity of human and ape red cells. In cases of weak reactions when there may be uncertainty whether the factor in question, for example **M**, is really present on the ape red cells, the reagent is absorbed with human M cells. This should eliminate the reactivity also for ape cells if the weak reactions with the unabsorbed anti-**M** reagent were actually due to an M specificity of ape red cells. The anti-**M** and anti-**N** antibodies can be fractionated by absorption with red cells of apes and monkeys, and the results obtained in this way are of considerable interest not only for immunologists but also for taxonomists.

Rh-Hr Types: For these tests, Rh-Hr antisera of human origin are used. The same techniques are applied as for testing human red cells and, in our hands, the ficinated red cell technique gives dependable results with the least effort. Further tests are necessary to determine whether the reagents contain nonspecific heteroagglutinins for ape blood; no simple method has been devised to remove them without weakening the type-specific antibodies. However, when the reagents give negative reactions in direct tests on ape blood, the specificity being tested for is obviously absent from the tested cells. If, on the other hand, positive reactions are obtained, further tests are required to determine whether these are type-specific or due to other antibodies in the reagent. One of the methods used is to titrate the positively-reacting simian red cells in parallel with positive human red cells. Since the reagents used by us are of high titer, similarity of titers may be considered evidence that the reactions are indeed type-specific. A more definitive proof is to absorb the reagent, anti-$\mathbf{Rh_o}$, for example, with human Rh_o positive red cells; if this removes reactions also for the ape red cells, the reaction of the unabsorbed serum is considered type-specific. Like anti-**M** and anti-**N** sera, human anti-$\mathbf{Rh_o}$ sera can also be fractionated by absorption with ape red cells.

Other Human-Type Blood Factors: The reagents for testing most other human blood groups systems are generally of low titer. Therefore, the results are poorly reproducible even in man, and particularly unsuitable for testing simian red cells, because of interference of nonspecific heteroagglutinins. Therefore, in our investigations we have limited our red cell tests to the A-B-O, M-N, Rh-Hr and I-i systems. The reports in the literature that we have read claiming to have demonstrated specificities such as Kell, Kidd, Duffy, etc., on the red cells of nonhuman primates are mostly based on simple direct tests or tests with eluates, and significantly make no mention of confirmatory tests by titration and absorption, or the use of the blind technique, and are therefore discounted by us.

SIMIAN-TYPE BLOOD GROUPS

Production of Reagents: The simian-type reagents have mostly been produced by isoimmunization thus avoiding the interference of heteroagglutinins. In most of our experiments, the isoantisera have been produced by mixing 1 ml of packed washed red cells with an equal volume of complete Freund's adjuvant and administering the mixture into multiple sites by deep intra-

Table 1. Comparison of Results of Titration by the Saline Agglutination Method Antiglobulin Method, Ficinated Red Cell Method, and Dextran Method

Method of titration	Titration of isoimmune rhesus monkey serum no. 216									Titration of isoimmune rhesus monkey serum no. 230									Control negatively reactive red cells
	Undil.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Undil.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
Saline agglutination	+	tr	tr	-	-	-				tr	tr	-	-	-					-
Antiglobulin	++±	++	++	++	++	++	++	++±	±	++	++	++	++	++	++	++±	++±	+	-
Ficin	+	++	++	++	++	++	+	tr	-	-	-	±	±	±	±	±	±	-	-
Dextran	++	++	++	++±	++±	+	-	-	-	+	tr	-	-	-	-	-	-	-	-

muscular injection. The injections are repeated 6 wk later, using red cells mixed with incomplete Freund's adjuvant. Animals with a good antibody response are subjected to plasmapheresis while the titers are at their peak.

The Tests: The methods of testing and titration of the antisera obtained are the same as in human blood typing: the saline agglutination method, the antiglobulin method, the ficinated red cell method, and at times, the ficinated red cell antiglobulin method. Theoretically, the antiglobulin serum used for the tests should be prepared by immunization with the serum or globulin of the species being studied. We have indeed used such antiglobulin sera, but find that antihuman globulin serum gives virtually the same results. However, the antihuman as well as the antimonkey globulin sera must be first absorbed with pooled washed simian red cells.

Other workers have tested rhesus monkey red cells by the dextran method, originally developed for tests on mice, for which species the methods used for man are inapplicable. Since our objective has been to approximate conditions in man, we found it inappropriate to use a method developed for mice, especially since the techniques for human blood typing give clear reproducible results with monkey blood. Moreover, in our hands, the dextran method does not always give clear-cut and reproducible reactions.

The reason why we use all three methods, saline agglutination, antiglobulin, and ficin is that different antibodies react by different techniques. In Table 1 are shown, for example, comparative titrations against rhesus monkey red cells using the saline agglutination, antiglobulin, ficinated red cell and dextran methods. As can be seen, the antibodies in question are of the "univalent" or "incomplete" variety, since no clumping occurred by the saline method using untreated red cells. The presence of high-titered antibodies is nevertheless demonstrated by the antiglobulin test. By the ficin method the first serum gave good reactions but of lower titer than by antiglobulin, while the second serum reacted much more weakly and with a prozone. The results by the dextran method were the least distinct.

As another example, Table 2 compares the reactions of a chimpanzee isoimmune serum with the red cells of five chimpanzees, using three different methods of titration. No reactions were obtained by the saline agglutination method, showing that no γM antibodies were present. Strong reactions were obtained, however, by the antiglobulin and ficin methods, indicating the presence of γG antibodies. By the antiglobulin method red cells of chimpanzee 168

Table 2. Comparison of Titrations of Serum of Isoimmunized Chimpanzee No. 4 (John) by Three Different Methods

Test red cells of chimpanzee			Titers by methods of		
Number	Name	Simian-type blood group	Saline agglutination	Anti-globulin	Ficinated red cells
No. 1	Buddha	V.A cef	0	24	1
No. 168	Walter	V.B CeF	0	214	214
No. 64	Duane	v.B cef	0	12	0
No. 225	Andy	V.AB cef	0	32	0
No. 4	John	v.B CEf	0	0	0

reacted in very high titer, red cells from three other chimpanzees reacted in considerably lower titer, while the fifth failed to react. On the other hand, titrations by the ficin method gave clear reactions and high titers only with the red cells of chimpanzee 168. Thus, the serum used contained antibodies of at least two distinct specificities, one of a very high titer for the factor F^c of the chimpanzee C-E-F system, and the second weaker and reacting only by antiglobulin and not by the ficinated red cell method, and, therefore, presumably detecting a specificity belonging to the V-A-B chimpanzee blood group system. Of course, the two antibodies in this antiserum might have proved separable by absorption with appropriate chimpanzee red cells, but by our use of more than one method more information could be obtained with less effort.

For the above reason, the use of three different techniques has been routine in our laboratory. In macaques, for example, we have been able to classify specificities according to whether or not the antibodies reacted by the saline agglutination method, and there could be further fundamental classification of specificities: (1) those specificities reactive by the antiglobulin method but not by the ficin technique and therefore belonging to systems analogous to the human M-N-S and the V-A-B system of chimpanzees; (2) specificities reactive by the ficin as well as the antiglobulin technique, and therefore presumably belonging to blood group systems analogous to the human Rh-Hr system and the chimpanzee C-E-F system.

One pitfall is the occurrence of a prozone in titrations by the ficin method of freshly obtained isoimmune rhesus monkey sera, not observed by us in other species. Obviously, if only one-tube tests were done with such antisera, false negative reactions could result. If the serum is inactivated, however, by heating for half an hour at 56°C, the prozone disappears (Table 3), showing that it was due to interference from complement present in the fresh serum, and not to so-called "optimal proportions." The presence of complement is

Table 3. Elimination of Prozone by Inactivation of Isoimmune Rhesus Monkey Serum No. 228

Method of titration	Condition of serum	Reactions with serum dilutions									Negative control red cells
		Undil.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
Saline agglutination	Fresh	-	-	-							-
Antiglobulin	Fresh	+++	+++	+++	+++	+++	++	++	++	tr	-
Ficin	Fresh	-	-	-	tr	+	++	+	tr	-	-
Ficin	Inactivated	+++	++	++	++	++	++	tr	-	-	-

Table 4. Cross Reactions of Two Anti-A^P Isoimmune Baboon Sera, with Red Cells of Macques and Gelada Monkeys (Saline Agglutination Method).

Antiserum	Reactions with red cells of															
	Papio cynocephalus				Macaca mulatta				Macaca fascicularis				Theropithecus gelada *			
	Nos. 2	9	11	13	Nos. 208	222	228	230	Nos. 60	160	168	98	Nos. 5	6		
Anti-A^P (B-2)	-	++	++	-	+++	++	+++	+++	++	-	++	-	-	-		
Anti-A^P (B-11)	-	++	++	-	+++	-	+++	-	-	-	-	-	-	-		

*While geladas gave negative reactions for A^P, they were both positive with other baboon isoimmune sera.

sometimes apparent when hemolysis occurs in first few tubes of the titration. Instead of inactivating the serum, the titrations can be converted to the antiglobulin method, by washing the red cells in each tube and then adding antiglobulin serum, whereupon the prozones disappear. Evidently, there are no prozones when the antiglobulin method is used, whether the red cells are ficinated or tested untreated.

Isoimmune sera can be used not only for blood typing within the same species, but also in closely related species; e.g., isoimmune rhesus sera can be used for typing stumptailed macaques, and vice versa. It should be noted that two isoantisera that show parallel polymorphism within their own species, can differ in their reactions with red cells from animals of another species. As shown in Table 4, two anti-A^P isoimmune baboon sera tested against red cells from *Macaca mulatta, M. fascicularis,* and *Theropithecus gelada* gave differing reactions proving that the two antisera differed in specificity even though they gave parallel reactions in tests on red cells from baboons.

A different kind of reagent is produced by immunization with blood from closely related species. For example, chimpanzees cross-immunized with human red cell produced antibodies not only reactive for human red cells but also defining individual differences in chimpanzees. Since the use of these cross-immune antisera involve no special techniques of testing, they will not be further discussed here.

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Immunogenetic Studies of Rhesus Monkeys. III. Statistical Analysis of Relationships Between Antigenic (Blood Group) Factors of Rhesus Monkeys

By C. R. Duggleby, W. F. Duggleby, and W. H. Stone

MANY LABORATORIES use primates of diverse or unknown origins in immunogenetic studies. This paper will illustrate a basic problem in the statistical analysis of associations between blood group antigens of such primate populations.

Eleven rhesus monkeys *(Macaca mulatta)* at the Wisconsin Regional Primate Research Center were isoimmunized with erythrocytes. The resulting raw sera were subjected to absorption analysis to provide reagents of serologic unity.[2] Five hundred and twenty-three lab-reared and 461 feral animals (of diverse origins) were tested with the 12 reagents produced. The test results on 332 complete families were analyzed to ascertain the genetic unity of the reagents. In addition to this family study, 2×2 contingency tests were conducted for the purpose of discovering the genetic relationships of the antigenic factors defined by the 12 reagents. Yates' correction factor and Fisher's exact test were used where appropriate.[1,2]

The 12 reagents detected 11 antigenic factors, belonging to six blood group systems. The G system consists of four alleles, $G,^1$ $G,^2$ $G,^3$ and $G.^4$ $G,^1$ $G,^2$ and G^3 are linear subtypes. G^4 is an autosomal codominant allele of $G,^1$ $G,^2$ and G^3 (Table 1.) One reagent is a dosage reagent that detects only G^4 homozygotes.[2] The H system consists of two codominant alleles, H^1 and H^2. At least two codominant alleles, I^1 and I^2 were found to be part of the I system. $J,^1$ $K,^1$ and L^1 were factors that appeared to be unrelated to any other factors in this study.[1]

In the course of this investigation, highly significant statistical associations were observed between seven pairs of blood group factors known by family studies not to be alleles, subtypes or members of the same phenogroup.[1] For example, the χ_1^2 for the 2×2 test for reagents J-1 and K-1 is 27.0, $p < 0.0001$. On the other hand, the χ_1^2 for the 2×2 test for reagents I-1 and I-2 is not significant, $\chi_1^2 = 2.68$, $p = 0.10$, although family data indicate they are codominant alleles.[1]

Penrose's sib-pair method[4] was used to learn the basis of the $\chi_1^2 = 27.0$ for reagents J-1 and K-1, when compared in a 2×2 contingency test. This method uses the phenotypes of full sibs to determine linkage. Sibs are scored on the basis of whether they are alike or unlike for each of the two traits. Table 2 shows how the data are recorded. When no linkage exists, the four entries will show a random distribution. When linkage does exist, cells *a* and *d* will be disproportionately large, sibs tending to be alike in both traits or unlike in both. When this method was applied to J_1 and K_1 (as well as numerous other pairs of antigenic factors), far too many pairs

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Table 1.

GENETIC SYSTEMS OF RHESUS BLOOD GROUPS

System	No. Reagents	Detectable Antigenic factors	No. Phenotypes	No. Genotypes
G	5	G_1, G_2, G_3, G_4	7	10
H	2	H_1, H_2	3	3
I	2	I_1, I_2	4	6
J	1	J_1	2	3
K	1	K_1	2	3
L	1	L_1	2	3

were alike in both traits, but only a small proportion unlike in both (see Table 2 for representative data).

At least one situation could produce these results. If a series of populations is amalgamated into one population, the proportion of homozygotes in this resulting population will be greater than that in the F_1 from the amalgamated, randomly-mating population by a factor equal to the variance in the gene frequencies among the unamalgamated populations (Wahlund's effect).[3]

If the Primate Center feral population were an amalgam of this type, in Penrose's sib-pair method too many F_1 sibs would be alike for both antigenic factors, since more parents than expected would be homozygous. In the limiting case, where all parents are homozygous, although not necessarily for the same alleles, all full sibs will be alike. This seems a plausible explanation for the results of Penrose's sib-pair method (Table 2). Furthermore, if the feral population were an amalgam of a number of populations, linkage disequilibrium might occur. This should be reduced by 50% each generation with random mating and independent assortment. The 2 × 2 contingency test for reagents J-1 and K-1, based on the feral population, gives a χ_1^2 of 27.0. However, when a 2 × 2 test is run, based only on the lab-reared population, the χ_1^2 is reduced to 8.5. This is what is expected if the feral parental population were an amalgam with extreme linkage disequilibrium regarding J^1 and K^1.

The higher proportion of homozygotes in the feral population (amalgamated) might reduce the χ^2 below the level of significance (taken here as 0.01) for a 2 × 2 test involving allelic genes, such as I^1 and I^2 appear to be. The χ_1^2 for the 2 × 2 contingency test for I-1 and I-2 was not significant ($\chi_1^2 = 2.68$, $p = 0.10$) when based on the feral population. However, if a 2 × 2 test is run for these two reagents based on the lab-reared population, the χ^2 *is* significant ($\chi_1^2 = 13.8$, $p < 0.001$).

Table 3 presents the χ^2's with probabilities less than 0.01 from 2 × 2 contingency tests on the feral and lab-reared populations. Only those associations which can not be ascribed to allelism, a subtype association or a phenogroup association have been included. Note that most of the significant χ^2's are reduced from the parental generation to the lab-reared (or offspring) generation. At the suggestion of Dr. James Crow (personal communication) the χ^2's for each population have been summed. This is perhaps more informative than the examination of the change in χ^2's between generations for each pair of antigenic factors separately, since they are not independent events. The sum of the χ^2's for

PENROSE'S SIB-PAIR METHOD FOR DETERMINING LINKAGE

		Phenotype A: Sibs like	Phenotype A: Sibs unlike
Phenotype B	Sibs like	a	b
	Sibs unlike	c	d

		I_2 phenotype: Sibs like	I_2 phenotype: Sibs unlike
I_1 phenotype	Sibs like	18	1
	Sibs unlike	7	0

		K_1 phenotype: Sibs like	K_1 phenotype: Sibs unlike
J_1 phenotype	Sibs like	18	3
	Sibs unlike	2	3

Table 3.

SIGNIFICANT χ^2'S ON GENETICALLY UNRELATED ANTIGENIC FACTORS

Factors	461 feral animals		526 lab-reared animals	
	χ^2	Probability	χ^2	Probability
H_2 vs J_1	9.52	$p<.005$	1.36	$p = .25$
I_1 vs J_1	12.79	$p<.005$	20.64	$p<.001$
I_2 vs J_1	9.31	$p<.005$	1.02	$.50>p>.25$
G_1 vs J_1	7.70	$p=.005$	11.11	$p<.005$
K_1 vs J_1	27.01	$p<.0001$	8.46	$p<.005$
G_1 vs I_1	6.75.	$p=.01$	1.99	$.25>p>.10$
I_1 vs K_1	9.92	$p<.005$	0.81	$.50>p>.25$

the feral parental generation is 83.00, that for the lab-reared offspring generation is 45.39. This shows a considerable decrease, as would be expected if the factors were not associated. Furthermore, this evidence is somewhat conservative, since the number of feral animals (461) is smaller than the number of lab-reared animals (526). The χ^2 should therefore be larger for the larger sample if the association were not a spurious one.

Without family studies, these spurious χ^2's might have been accepted as indicating true genetic associations between factors. While the precise cause of the spurious χ^2's derived from 2×2 tables could not be determined with certainty, the population structure of the primate center rhesus colony (an amalgam of populations), the resulting linkage disequilibrium and the breeding structure (few males fathering most of the F_1 generation) all clearly contribute to the apparent associations. Thus, statistical analysis of relationships between antigenic factors seems to have some pitfalls when the research animals used are of diverse or unknown origin or matings are highly nonrandom.

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Immunogenetic Studies of Rhesus Monkeys. IV. Serologic and Genetic Tests With Reagents From Wisconsin and The Netherlands

By P. T. Sullivan, C. Blystad, and W. H. Stone

DUGGLEBY, STONE, AND BLYSTAD[1,2] described one heteroimmune and 11 isoimmune rhesus erythrocyte typing reagents (agglutinins) developed at the Laboratory of Genetics, Madison, Wis., in cooperation with the Wisconsin Regional Primate Research Center. These reagents were processed to satisfy the serologic criterion of unity as described by Stone,[3] i.e., each reactive cell, when used for absorption, should remove the reactivity for all other reactive cells. A colony of 984 rhesus monkeys comprised of 461 feral and 523 lab-reared animals was typed with these reagents. On the basis of the reactions of the feral animals, 2 × 2 contingency tables for all pairwise combinations of reagents were analyzed by a computer. In addition, pedigree analysis was done to show that the detected specificities were inherited as simple Mendelian codominant alleles. Three hundred thirty-two complete families (mother-father-child) were tested.

The analysis showed that these reagents belonged to six independent genetic systems which were designated with the letters G, H, I, J, K, and L. The nomenclature of the individual reagents and their distribution in the systems are given in Table 1. Subsequent immunizations and analyses have produced additional reagents.

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Fifteen reagents from the Wisconsin laboratory were used to type 231 rhesus at the Rijswijk Radiobiological Institute, The Netherlands. The results confirmed the analyses of Duggleby et al. In addition, it has been possible to compare the reactivity patterns of the Wisconsin reagents to the reactivity patterns of sera used in the Rijswijk laboratory. These included 65 sera used in the dextran/BSA techniques to test 154 unrelated monkeys and 54 sera tested in the saline and, occasionally, antiglobulin technique against a panel of 41 unrelated animals.

MATERIALS AND METHODS

Source of Madison Reagents: A panel of 50 Rhesus monkeys at the Wisconsin Regional Primate Research Center was available for our use as donors and recipients in isoimmunizations and as a source of erythrocytes for the analysis and absorption of raw antisera. A goat anti-rhesus γ-globulin was prepared and used for the antiglobulin test as described previously.

Source of Rijswijk Antisera: The Rijswijk isoantisera were produced by a varied program of immunizations including skingrafts and injections of whole blood as described by Hirose and Balner.[4]

Antiglobulin (Coombs) Test, Immunizations, Tests for Serologic and Genetic Unity: The procedures were the same as described previously.[1,2]

Tests for Association of Factors: One hundred fifty-four unrelated animals from the colony at Rijswijk were typed with the 15 Wisconsin reagents. The same animals were tested in the dextran/BSA technique by Hardonk. These results were compared in 2 × 2 contingency tests analyzed by a computer. In addition, family data were studied. Thirty-two females were mated repeatedly to five breeding males, each female being mated with the same male each time, to

Table 1. The Blood Group Systems of Rhesus Monkeys

System	Number of Reagents	Detectable Blood Factors	Number of Phenotypes	Number of Genotypes	Kinds of Genotypes	Kinds of Phenotypes
G	6	G_1, G_2, G_3, G_4	9	10	G^1/G^1, G^1/G^2, G^1/G^3, G^1/G^4	G_1, G_1 G_4, G_1 G_3
					G^2/G^2, G^2/G^3, G^2/G^4	G_2, G_2 G_4, G_2 G_3
					G^3/G^3, G^3/G^4	G_3, G_3 G_4
					G^4/G^4	G_4
H	2	H_1, H_2	3	3	H^1/H^1, H^1/H^2	H_1, H_1 H_2
					H^2/H^2	H_2
I	2	I_1, I_2	4	6	I^1/I^1, I^1/I^2, I^1/I^i	I_1, I_1 I_2
					I^2/I^2, I^2/I^i	I_2
					I^i/I^i	I_i
J	1	J_1	2	3	J^1/J^1, J^1/Ji	J_1
					Ji/Ji	J_i
K	1	K_1	2	3	K^1/K^1, K^1/K^k	K_1
					K^k/K^k	K^k
L	1	L_1	2	3	L^1/L^1, L^1/L^l	L_1
					L^l/L^l	L_l

From reference 2.

generate families including up to five full sibs. A total of 77 offspring were tested.

RESULTS

The G-system: The reactivity pattern of reagents of the G-system is given in Table 2. No cell negative for all reagents has been found. Thus the alleles detected, G^1, G^2, G^3 and G^4 form a closed allelic system.

A new reagent (to be reported more extensively later) which detects the G^3 allele in the presence of G^1 or G^2 has been developed in our Wisconsin laboratory. This reagent reacted negatively with all animals tested at Rijswijk. Thus the G-system in this population exhibits only three alleles G^1, G^2, and G^4. This is not a surprising finding since G^3 is a low frequency allele. As this population was obviously not mating at random, genetic analysis was conducted on the basis of Mendelian expected ratios. The large numbers of offspring (19 and 16, respectively) from the two phenotypically G_1 males, allowed the unequivocal assignment of the G^1/G^2 genotype to both males. Two other males were G^2/G^2 homozygotes, and the fifth male was a G^4/G^4 homozygote. Eight matings involving 23 offspring included individuals whose genotype was still indeterminate; G^1/G^1 or G^2, and could not be analyzed further.

Table 2. Reaction Patterns of the G Blood Group of Rhesus Monkeys

Phenotype of Cells	Reagents G-1	G-2	G-3	G-4	G-4D	Number of Rijswijk Animals Feral	Lab-reared	Total	Proposed Genotype
G_1	+	+	+	—	—	48	32	80	G^1/G^1, G^1/G^2 or G^1/G^3
G_1 G_4	+	+	+	+	—	12	5	17	G^1/G^4
G_2	—	+	+	—	—	56	10	66	G^2/G^2 or G^2/G^3
G_2 G_4	—	+	+	+	—	31	23	54	G^2/G^4
G_3	—	—	+	—	—	0	0	0	G^3/G^3
G_3 G_4	—	—	+	+	—	0	0	0	G^3/G^4
G_4	—	—	—	+	+	7	7	14	G^4/G^4
G_{null}	—	—	—	—	—	0	0	0	G^n/G^n
						154	77	231	

Table 3. Reaction Patterns of the H Blood Group System of Rhesus Monkeys

Phenotype of Cells	Reagents H-1	Reagents H-2	Proposed Gentoypes
H_1	+	—	H^1/H^1
$H_1\ H_2$	+	+	H^1/H^2
H_2	—	+	H^2/H^2
H_{null}	—	—	H^n/H^n

As expected, two matings of type $G^4/G^4 \times G^4/G^4$ produced five offspring, all G^4/G^4. Four matings of type $G^4/G^4 \times G^2/G^2$ produced 11 offspring, all G^2/G^4. Five matings of types $G^4/G^4 \times G^2/G^4$ and $G^4/G^4 \times G^2/G^1$ produced four G^2 and six non-G^2 offspring ($\chi_1^2 = 0.4$). Ten matings of types $G^2/G^2 \times G^1/G^2$ and $G^2/G^2 \times G^1/G^4$ produced 13 G^1 and ten non-G^1 offspring ($\chi_1^2 = 0.29$).

Using the saline technique to test 41 unrelated monkeys, four unabsorbed Rijswijk sera were found exhibiting the G-4 specificity. After testing 151 unrelated animals using the dextran/BSA technique, one of these Rijswijk sera, Hm, showed a significant positive correlation with G-4 (probability of independence < 0.001) when analyzed by a 2 x 2 contingency test. Another serum produced by the same serum donor gave parallel results. This result is compatible with the observation that our Madison G-4 reagent is an unabsorbed antiserum. Furthermore the specificity has repeatedly been found at Wisconsin in antisera taken shortly after birth from multiparous rhesus females.

The H-System: The reactivity pattern of the reagents of the H-system is given in Table 3. These reagents were found to react best in the antiglobulin test and they have been routinely employed with this method. No cells negative for both reagents have been found. Thus, the alleles H^1 and H^2 form a closed allelic system. Since H-1 is not presently available, only H-2 was used in the typing at Rijswijk. The results of these tests are presented in Table 5.

Using the saline technique on tests with 41 unrelated monkeys, eight unabsorbed Rijswijk sera from five donors exhibited weak H-2 specificity. A subsequent test of these sera with 20 random monkeys using the Madison antiglobulin reagent enhanced the H-2 specificity. These same sera, when tested using the dextran/BSA technique against 151 random monkeys, gave a significant positive correlation with the Madison H-2 reagent. These results are compatible with hypothesis that the antiserum behaves like an incomplete antibody.

The I-System: The reactivity pattern of the two reagents of the I-system is given in Table 4. All four possible phenotypic classes were found: ++, +—, —+ and ——. As in the results of Duggelby, Stone, and Blystad, the 2 × 2 contingency test, based on unrelated animals did not give a significant χ_1^2 for independence. A $\chi_1^2 = 2.13$ ($0.20 > p > 0.10$) was calculated based on the testing of 151 Rijswijk monkeys. However, a test cross-mating $I^1/I^2 \times I^i/I^i$ produced five offspring: two I_1 and

Table 4. Reaction Patterns of the I Blood Group System of Rhesus Monkeys

Phenotype of Cells	Reagents I-1	Reagents I-2	Number of Rijswijk Animals: Feral	Lab-reared	Total	Proposed Genotypes
I_1	+	—	58	41	99	I^1/I^1, I^1/I^i
$I_1\ I_2$	+	+	15	2	17	I^1/I^2
I_2	—	+	25	3	28	I^2/I^2, I^2/I^i
I^i	—	—	56	31	87	I^i/I^i
			154	77	231	

three I_2. These results corroborate the finding of Duggleby et al. in 19 similar test crosses suggesting allelism of the genes controlling I_1 and I_2. No I_1I_2 or I_i offspring have been produced in these matings.

The J-, K-, and L-Systems: The results of the Rijswijk typing with reagents J-1, K-1, and L-1 are presented in Table 5. The results from family data are not inconsistent with the hypothesis of Duggleby et al. that each reagent detects an independent Mendelian codominant allele. However, since all five Rijswijk breeding males were J_1K_k, not all desirable test crosses occured in this population.

Using the panel of 41 unrelated monkeys, two Rijswijk sera were found that exhibited the K-1 specificity. One of these was tested in the dextran/BSA against 151 unrelated animals, and analysis showed a significant positive correlation (probability of independence < 0.001). Two other Rijswijk sera gave parallel results in the dextran/BSA technique. This result agrees with the observation that the anti K-1 specificity occurs frequently in raw antisera produced at Wisconsin.

New Madison Reagents: Five new monospecific reagents have been developed in the Madison laboratory (unpublished). Due to the low frequency of the factors detected and the small size of the mating population at Rijswijk, data available to date are insufficient to establish the mode of inheritance of these factors. Further results wil be published later.

DISCUSSION

Throughout our studies we have stressed the genetic and serologic criteria of unity. All sera employed as typing reagents were tested for monospecificity by absorption with a number of reactive cells, each of which must have removed reactivity for all positive cells. Conclusive testing of genetic hypotheses demands family data, although computer analysis of population data can often provide important working hypotheses.

The results of these comparison studies demonstrate that certain strong antigenic factors are common to different laboratory colonies of rhesus monkeys. Duggleby et al. found that the anti-D^{rh} serum reported by Moor-Jankowski et al.[5,6] contained the specificities anti K-1 and anti H-2. This relatively frequent occurrence of anti-G-4, anti-K-1, and anti-H-2 is understandable when the frequency of these factors is considered (each approximately 10–20%). In any sizeable program of immunizations one is quite likely to find a positive donor and a negative recipient. The reactions of these sera in the dextran/BSA technique attest to

Table 5. Reaction Patterns of H-2, J, K, and L Blood Group Reagents of the Rhesus Monkey

System	Phenotype of Cells	Reaction With Reagent	Number of Rijswijk Animals			Proposed Genotype
			Feral	Lab-reared	Total	
H	H-2(+)	+	19	3	22	H^2/H^2 or H^2/H^1
	H-2(—)	—	135	74	209	H^1/H^1
J	J^1	+	117	67	184	J^1/J^1 or J^1/J^i
	J^i	—	37	10	47	J^i/J^i
K	K^1	+	35	16	51	K^1/K^1 or K^1/K^k
	K^k	—	119	61	180	K^k/K^k
L	L^1	+	111	62	173	L^1/L^1 or L^1/L^l
	L^l	—	43	15	58	L^l/L^l

their validity as specific agglutinins. The response of the various anti-H-2 sera in the antiglobulin technique indicates that this specificity is an incomplete antibody as previously reported. The blood group factors described in this work provide useful genetic markers for many other studies in a species increasingly important in medical research.

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New Data on H-2 Locus: Their Implications for Interpretation of Major Histocompatibility Loci in Different Species, Including Man

By P. Démant and P. Iványi

IN THE PAST DECADE, a single major histocompatibility system (MHS) has been found in every mammalian and avian species studied.[1] The two best analyzed systems, H-2 in the mouse and HL-A in man, have been of the greatest importance for the concept of MHS. As the essential features of H-2-HL-A analogy have recently been reviewed,[2,3] this paper will be restricted to the analysis of some selected aspects of H-2 relevant to the understanding of MHS problems in general and HL-A problems in particular. As the similarity between H-2 and HL-A does not extend to terminology, the term H-2 allele is the equivalent of HL-A haplotype, and H-2 specificity is equivalent to the HL-A antigen. While some HL-A antigens are allelic, the H-2 specificities may be mutually exclusive.

A number of new H-2 specificities have been described since the last publication of the complete H-2 chart:[4] 15[5], 20, 21, 24;[6] 24;[7] 34;[8] 35, 36, 41, 42, 43;[9] 37, 38, 39;[10] and 46, 47.[11] The H-2 specificities restricted to wild mice are listed separately. The well-known nonrandomness of the distribution of H-2 specificities among the panel of inbred mouse strains[12,13] was further emphasized by addition of these new specificities, and more detailed serological studies have resulted in a description of two complexes of closely related specificities (H-2.1 complex[6] and H-2.3 complex[9]) and of two series of mutually exclusive private specificities.[5]

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The private specificities are restricted to a single H-2 allele and its crossover derivatives. They are localized in the K or D ends of the H-2 gene map. Because no H-2 allele can have more than two private specificities, one at its K end and one at its D end, two mutually exclusive series of H-2 specificities exist: the H-2K series wtih specifities 15,16,17,19,20,21,23,31,33, and the H-2D series with specificities 2,4,9,30,32. Specificity 18 has yet to be localized by crossover. Several alleles have no defined specificity of H-2D or H-2K series. Some private specificities are the strongest H-2 specificities of all, while the strength of others is intermediate or weak. The two series of H-2 specificities appear to be similar to the first (LA) and second (Seven or Four) series of allelic HL-A antigens.[14-16] Antisera against private specificities are monospecific only with alleles included in the H-2 chart. Their complexity is revealed when tested with alleles not included in the H-2 chart.[52]

The H-2.1[6] and H-2.3[9] complexes each contain a group of specificities that have a number of similar properties. The specificities of the H-2.1 complex (1,5,11,23,24,25) are all present in alleles a,h,k,m, which have the H-2K private specificity 23. These alleles are named 1-complete because they uniformly give the strongest reactions and most effective absorption with anti-1 antibodies; 1-negative alleles do not react with anti-1 sera. The variety of anti-1 antibodies is revealed in their reactions with the large group of 1-intermediate alleles. These alleles react only with some anti-1 sera; the

reactions are of variable strength and reproducibility, although always weaker than with 1-complete alleles. The absorption capacity of 1-intermediate alleles ranges from complete absorption to no absorption with different anti-1 sera, and the allelic pattern is different for each antiserum. This makes the resolution of anti-1 sera difficult, and we have to name the involved specificities by a group designation H-2.1. Anti-1 antibodies can be produced using 1-intermediate alleles as donors and recipients. The important anti-1 antisera feature that ties them into a common family is that although they may have been made against different 1-intermediate alleles, they are invariably absorbed more efficiently by 1-complete alleles than by 1-intermediate alleles, including the donor's allele. The specificities of the H-2.3 complex (4,10,13,35,36,41,42, 43) are all present in alleles a,d,i,u, which also have the H-2D private specificity 4 (3-complete alleles, probably all with an identical D end) and in reactivity differ from 3-intermediate alleles. The properties of anti-3 antibodies resemble those of anti-1's except that the antisera can be more easily resolved into distinct specificities and the difference between absorption capacity of 3- complete and 3-intermediate alleles is smaller.

The unusual properties of the H-2.1 (H-2.3) family may be explained by the assumption that the relevant part of the H-2 end-product acquires different, slightly modified forms in different 1-intermediate (3-intermediate) alleles. Gene duplications with subsequent unequal crossing over and mutation have been suggested as the genetic mechanisms generating this variability of H-2.1 (H-2.3) antigenic sites.[6,17]

Several H-2 specificities (7,8,34,37,38,39, 46,47) belong neither to the H-2.1 or H-2.3 complex nor to the mutually exclusive series of private antigens. These may form inclusion systems, but their relationship to each other and to other H-2 specificities cannot be ascertained before more crossover data are available.

The problem of the H-2 gene map has been reviewed by Shreffler.[17,18] In the initial period of analysis, it was possible to construct a consistent multipoint gene map of H-2 specificities, but as more alleles were analyzed and using also Ss-Slp markers, there was an increase in the number of exceptions to the rules of gene mapping necessary to keep the map's linear structure. From the beginning, it has been clear that the best defined specificities never map into the central regions (C,V,E,A) and antigenic products of allelic forms of the postulated central regions have never been detected. Together with the anomalous behavior of H-2.1, H-2.3, and H-2.5 in the recombinant alleles, these facts have led to doubts about the existence of such central regions and the serological individuality of their antigenic products. We have tested the existence of central regions by the transplantation method,[19] and no clear-cut skin graft rejections could be attributed to incompatibility at the postulated central regions. This result was in sharp contrast to incompatibilities at peripheral regions K and D; these resulted invariably in graft rejection no later than 20 days. The transplantation analysis data and the proposed new concepts of H-2 chart[15] are in accordance with the postulated new model of H-2,[17] assuming two principal H-2 regions (subloci), K and D, derived by duplication of a single ancestral gene, and subsequent divergence by unequal crossing over and mutation. According to this theory, the Ss-Slp region is a chance inclusion in H-2.

An important implication of this model is the control of some specificities by two different regions of H-2 (cross-reactions between the subloci). This assumption helps to accommodate specificities formerly placed in the central regions but exhibiting anomalous transmission in recombinants, if they are considered to be serological and

genetic entities. It is based on the existence of antisera made in recipients with a recombinant allele, immunized with donors carrying one original allele, and reacting with the second original allele. Theoretically, such reactions should not occur, barring the unequal crossover. The first such antiserum was the anti-3 produced in H-2^o recipients[20] and reacting with both original alleles, H-2^k and H-2^d. The anti-35 and anti-36 sera also belong to this class.[9,42] They can be made in H-2^h recipients and will react with both original alleles of H-2^h, H-2^a, and H-2^b. All three specificities (3, 35, and 36) belong to the H-2.3 complex and suggest that there exists a group of specificities with a similar distribution but which can react with products of both H-2K and H-2D regions. This situation is perhaps analogous to the anti-4^a and anti-4^b antisera, the reactions of which appear to be associated with antigens of both the first and second HL-A subloci.[21]

The presently available data on H-2 recombinants are compatible with the existence of allelic substitution in two distinct chromosomal regions, H-2K and H-2D, with an intercalated Ss-Slp determinant. The antigenic products of both regions are very complex and may comprise one private specificity and a number of specificities shared with other alleles at the same or at the other region. The extent of these regions, whether they can be subdivided by recombination, and how the determinants for the individual specificities they control are organized remains to be seen. Convincing evidence of an intra-region recombination is still lacking. The complex mechanisms and interactions postulated for the shaping of the serotype of a particular HL-A allele may well exist in the H-2 system and would make detection of an intraregion crossover rather difficult.[22] The existence of regions determining products of minor antigenic strength cannot be excluded, and it must be kept in mind that our models are based on a small sample of recombinants. Nevertheless, similarity with the postulated two-component model of HL-A[14-16] (cross-reacting alleles at two subloci) cannot escape attention.

It is recognized that transplantation reactions in diffierent allelic combinations of donor and recipient vary in strength. The weakness of specificity 32 is particularly well known. It has been hypothesized that the strength of the allograft reaction is related to the number of specificities against which it is directed,[23,24] although some data did not fit this hypothesis.[25] An analysis of H-2 recombinants in the mixed lymphocyte reaction has revealed the great strength of H-2K incompatibility,[26,27] and it has been shown that H-2K incompatibility is stronger in the graft-versus-host reaction[28] and that H-2D incompatibility is more susceptible to ALS immunosuppression.[29] The difference between the intensity of the reaction against H-2K and H-2D incompatibility is approximately twofold. It appears, therefore, that the H-2 region at which incompatibility occurs may be more critical for the intensity of the subsequent immune reaction than the number of target specificities. It might be of considerable clinical interest if the same rule could be shown to apply to HL-A.

The overall analogy in histocompatibility genetics should be stressed. The most remarkable and best documented point is that of the 15–30 segregating H loci that can be demonstrated in the different species, only one in every species is a major locus. (For details of MHS definition, see Ref. 1). Very little is known about the weak non-MHS loci in species other than the mouse, although the MHS chart already exists in rats and is being worked out for other species[1,31,32] with the possible exception of extremely cross-reacting chicken MHS antigens.

Besides the antigenic characteristics, interest is focusing on MHS associations with

other genes and traits. Most data stem from the mouse and man. No clear analogy could be found yet between the genes loosely linked with MHS in these two species (T and W-st, fused in mice,[2] and Hp[33] and PGM_3[34] in man), but this may be due to insufficient data.[46] H-2 is closely linked with TL,[45] Ss-Slp,[18] Ir-1,[44] Ryv-1,[35] Hom-1,[43] and H-Y rejection genes.[36,37] It seems of extreme importance that the analogue of the Ir-1 locus in guinea pigs is linked with MHS,[39] as this is the first indication that associations and linkage of MHS are not due to chace. We do not know whether the rat Ss protein[38] is linked with H-1. No direct evidence for such associations exists in man except for circumstantial evidence[1,21] supported by some finding about the association of HL-A with disease of a possibly immunologic nature,[47-51] thus implying involvement of a type of MHS-influenced immune process known in the mouse.[36,37,40,41]

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Antigenic Determinants on Immunoglobulins of Nonhuman Primates

By Erna van Loghem and S. D. Litwin

SEROLOGIC INVESTIGATIONS have shown cross-reactivity between man and nonhuman primates. These investigations have considerably contributed to the knowledge of origin and evolution of cellular and humoral elements like red cells, white cells and serum proteins. Extensive research has also been carried out on the evolution and phylogeny of immunoglobulins, which provide the antibody reagents for these investigations. In normal serum, immunoglobulins occur in multiple forms; they can be distinguished in different classes and subclasses. The molecules are similarly constructed and consist of two pairs of polypeptides. The genes coding for these polypeptides evolved through a process of gene doubling and multiple duplication.[1] Humoral antibodies are only found in vertebrates and probably arose at the time of the emergence of placoderm-derived fishes.[2,3]

Once duplication took place, mutation led to the modification of the genes, thus developing specialized functions. At present at least three genes for light chains and ten genes for heavy chains are known in man. The polypeptides produced by these genes can be distinguished from each other by specific antisera. There are also regions on the various polypeptides that were not changed during evolution from a common ancestor. This follows from the finding of homologies in amino acid sequences.

Studies on immunoglobulin determinants in various species will reveal deviation on one hand and relationship of structural and functional properties of immunoglobulin genes on the other. In Table 1, determinants are classified according to limitation of their specificity. Determinants common to all immunoglobulins but not occurring in other proteins represent basic sequences of all classes and subclasses. Nonmarkers are determinants that occur on the molecules of several subclasses but as a genetic marker in only one of them. It is highly probable that nonmarker antigens reflect basic sequences present in original genes and that mutation in one of the duplicated genes altered the DNA region coding for the antigen into a marker, thus creating polymorphism for this gene. One allele carries the new marker while the other conserves the original nonmarker antigen. It is clear that reagents detecting nonmarkers can only be used as typing reagents for isolated monoclonal proteins. The specificity of class and subclass determinants is more restricted; reagents for these determinants do not detect genetic polymorphism. The specificity of genetic markers is most limited in all, since they appear on the alleles of one particular subclass only. Genetic markers originate from mutation of existing genes. The difference may relate to the change of only one single aminoacid. The mutation may change the structure or function very little so that the new mutated gene will persist next to the original gene performing the same function. The markers are characteristic flags by which allelic genes of a particular subclass are recognizable and can be studied in whole serum. It was the purpose of our study to investigate the occurrence of various established human immunoglobulin determinants in nonhuman primates.

From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, and The New York Hospital, Cornell Medical Center, New York, N.Y.

MATERIALS AND METHODS

The reagents used are listed in Table 2. Human anti-Rh sera and isolated proteins were used as antigens. Antisera were obtained by immunizing experimental animals (chimpanzees, baboons, monkeys, and rabbits). Human antisera were obtained from normal healthy donors with naturally occurring antibodies and from patients with immunoglobulin deficiencies and transfusion reactions. The nonhuman primates tested were apes, Old World monkeys, New World monkeys and prosimians (kindly provided by Dr. J. Mortelmans, Dr. C. Zmijewski, Dr. O. Mühlbock, and LEMSIP). Antigen-antibody systems were used

Table 1. Immunoglobulin Determinants

Common to all subclasses
Nonmarkers
Class or subclass specificities
Genetic markers (Gm, Am, Inv)

Table 2. Antigenic Determinants Investigated and Reagents Used for Typing

Subclass	Determinant	Antiserum	Antigen
IgG1	Gm(a)	human	anti-Rh 3069
	Gm(a)	monkey	anti-Rh 3069
	Gm(z)	rabbit	anti-Rh 3069
	Gm(z)	human	anti-Rh 3069
	Gm(f)	human	anti-Rh Korv
	Gm(x)	human	anti-Rh 2880
IgG1-IgG2-IgG3	non-b^0	chimpanzee	anti-Rh 2290
IgG2-IgG3	non-g	monkey	anti-Rh Scher
IgG2	$\gamma 2$	rabbit	IgG2 protein Bl, Gm (n—)
	Gm(n)	rabbit	IgG2 protein Ja, Gm (n+)
IgG3	$\gamma 3$	rabbit	anti-Rh 2127
	$\gamma 3$	baboon	anti-Rh 2127
	$\gamma 3$	monkey	anti-Rh Perd
	Gm(b^0)	human	anti-Rh 2127
	Gm(b^1)	human	anti-Rh 2127
	Gm(b^3)	human	anti-Rh 2127
	Gm(b^5)	human	anti-Rh 2127
	Gm(g)	rabbit	anti-Rh Perd
	Gm(s)	human	anti-Rh Scher
	Gm(t)	human	anti-Rh Bor
	Gm(c^3)	human	anti-Rh Bor
	Gm(c^5)	human	anti-Rh Bor
	Pa	baboon	anti-Rh 2127
	Bo	chimpanzee	anti-Rh 2036
IgG4	$\gamma 4$	human	anti-Rh v E1
	$\gamma 4$	rabbit	anti-Rh v E1
	$\gamma 4$	monkey	anti-Rh v E1
	$\gamma 4$	baboon	anti-Rh v E1
	$\gamma 4$	rabbit antichimp	anti-Rh v E1
	$\gamma 4$	rabbit antibaboon	anti-Rh v E1
IgA	α	human	IgA1 protein 3011
	α	rabbit	IgA1 protein 3011
	α	monkey	IgA1 protein 3011
	α	rabbit anti-*Macaca*	IgA1 protein 3011
IgA2	Am(1)	human	IgA2 protein Her
K chains	Inv(1)	human	anti-Rh 2290
	Inv(a)	human	anti-Rh 2290

In some cases, reagents, were provided by the following investigators: Dr. S. D. Lawler, Dr. K. L. G. Goldsmith, Dr. C. Zmijewski, Dr. B. G. Grobbelaar, Dr. H. H. Fudenberg, Dr. D. Wiebecke, Dr. J. B. Natvig, Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), Dr. M. C. Botha, and Dr. C. Ropartz.

Table 3. Human γ1 Determinants in Nonhuman Primates

Species	Number	Human Gm(a)*	Monkey Gm(a)	Rabbit Gm(z)	Human Gm(z)
G. gorilla	14	+	+	+	—
P. paniscus	3	+	+	+	+
P. troglodytes	91	+	—	+	+
P. pygmaeus	25	—	—	+	—
H. lar	14	+	—	+	—
Papio	10	—	—	+	—
T. gelada	9	—	—	+	—
Macaca	60	—	—	—	—
C. fulginosus	14	—	—	—	—
E. patas	7	—	—	—	—
C. aethiops	20	—	—	—	—
Cebus a.	3	—	—	—	—
Tupaia	6	—	—	—	—
Loris	3	—	—	—	—

*Source of antiserum.

in the agglutination-inhibition test and applied for the determination of genetic markers and nonmarkers and for subclass specific determinants.[4] Altogether, 34 different specificities could be detected by this system.

RESULTS

The results include those of previous investigations.[5,6] Three different *Papio* species and five different *Macaca* species are noted under one heading for reasons of simplification. Determinants present in at least one species are shown in Tables 3–7. The results of typing for human γ 1 determinants are shown in Table 3. The dwarf chimpanzee *(P. paniscus)* shows the closest similarity to man since it reacted with four systems determining human Gm(za) proteins. Gm(z) as detected by rabbit antiserum is the only γ 1 determinant found in some Old World monkey species. In New World monkeys and prosimians, no γ 1 determinants are found. Polymorphism for any of the γ 1 markers occurs in none of the species.

The results of typing for human γ 2 determinants are shown in Table 4. The only genetic marker of γ 2 polypeptides known, Gm(n), was not present in any of the species tested, but γ 2 specific determinants, as detected by a rabbit antiserum, were found in all species except for *Loris*. Non-b^0 and non-g are included in Table 4. Although they are genetic markers of human IgG3 proteins, they are indicative of other human subclasses since non-b^0 is present in all IgG1 and IgG2 proteins and non-g in all IgG2 proteins. Non-g was only found in chimpanzees, but non-b^0 is evidently of old origin. Most interesting is the finding that there is polymorphism for this determinant in chimpanzees, baboons, and cercocebus.

The results of typing for human γ 3 determinants are shown in Table 5. Subclass specific determinants as detected by rabbit and baboon but not by monkey antisera, are found only in some species of the great apes. Several determinants that are genetic markers in man are frequently found in nonhuman primates. When they occur, there is polymorphism in at least one of the species investigated. The combination of Gm(s), (b^0), and (c^5), which occurs in many species of Old World monkeys, is poly morphic in chimpanzees, where the gene producing non-b^0 behaves as an antithetical allele.[7] Of these three determinants, only Gm(b^0) is polymorphic in orangutans. It always occurs with Gm(b^3) and Gm(b^5).

Table 4. Human γ2 Determinants in Nonhuman Primates

Species	Number	Rabbit γ2*	Chimp Non-b°	Monkey Non-g
G. gorilla	14	+	+	—
P. paniscus	3	+	+	—
P. troglodytes	91	+	41+	+
P. pygmaeus	25	+	—	—
H. lar	14	+	—	—
Papio	10	+	+	—
T. gelada	9	+	7+	—
Macaca	60	+	+	—
C. fulginosus	14	+	5+	—
E. patas	7	+	+	—
C. aethiops	20	+	+	—
Cebus a.	3	+	—	—
Tupaia	6	+	—	—
Loris	3	—	—	—

*Source of antiserum.

The occurrence of Gm(b^5) is similar to that of Gm(b^0) except for chimpanzees which are all Gm(b^5) positive. The interesting finding of Gm(c^3) with only one of several anti-Gm(c^3) specific human antisera has been discussed before.[5,6]

The last determinant shown in Table 5, for which no Gm symbol has yet been assigned, is preliminary noted as Bo (Bonnie). This determinant seems to be of very ancient origin, because only the prosimians did not carry this determinant. We included Bo because it most probably is specific for human IgG3 proteins. In several *Macaca* species, Bo is the only polymorphic immunoglobulin marker. Chimpanzees also show polymorphism for Bo. No relation could be found with other polymorphic Gm factors. The Gm factors (g), (b^1), (t), and (Pa)[8] were not found in any of the species tested.

The results of typing for human γ 4 determinants are shown in Table 6. Naturally occurring anti-γ 4 specific antibodies found in man react with gorillas and with both species of chimpanzees. Immunization of baboons, monkeys, and rabbits with human IgG4 proteins resulted in the production of antisera reacting with both chimpanzee species only. Rabbits immunized with chimpanzee or baboon globulins produced antisera of identical IgG4 specificity in man but of different specifrcity in nonhuman primates. The antiserum anti-chimpanzee reacted with all great apes, while the antiserum antibaboon reacted with all Old and New World monkeys. Both antisera failed to react with prosimians.

The results of typing for human α determinants are shown in Table 7. The four antisera noted in this table react specifically with an α determinant occurring in IgA1 and IgA2 proteins. Therefore the antisera could be used with red cells coated with IgA1 or IgA2 proteins, as both systems give identical results. The determinant detected by the human anti-α antiserum was present in all nonhuman primates tested but not in other mammalians such as horse, sheep, rabbit, and guinea pig. The monkey and rabbit anti-α antisera were raised by immunization with an IgA1 protein different from the one used as antigen in the test system. All great apes reacted with the monkey antiserum and only some of them with the rabbit antiserum. Another rabbit antiserum raised by immunization with an immunoglobulin preparation from *Macaca* cynomolgous and absorbed with IgA-deficient human serum detected in human IgA1 and IgA2 proteins an α determinant that also appears in great apes and Old World monkeys but now in New World monkeys and prosimians. The Am factor, the only genetic marker of human IgA2 proteins known, and the genetic markers Inv(l) and Inv(a) of human κ chains were not found.

DISCUSSION

The serologic cross-reactivity between man and nonhuman primates is to a certain degree dependent on the species used as antibody producer. The experimental animal providing the antiserum must be sufficiently phylogenetically remote to recognize the injected proteins as foreign antigens. The more phylogenetically, the

Table 5. Human γ3 Determinants in Nonhuman Primates

Species	Number	Baboon,* Rabbit γ3	Human Gm(s)	Human Gm(b^0)	Human Gm(c^5)	Human Gm(b^5)	Human Gm(b^3)	Human Gm(c^3)	Chimp Bo
G. gorilla	14	+	+	+	—	+	+	—	+
P. paniscus	3	+	+	+	—	+	—	—	+
P. troglodytes	91	+	78+	78+	78+	+	12+	85+	48+
P. pygmaeus	25	—	+	17+	+	17+	17+	—	+
H. lar	14	—	+	+	+	+	—	—	+
Papio	10	—	+	+	+	+	—	—	+
T. gelada	9	—	+	+	+	+	—	—	+
Macaca	60	—	+	+	+	+	—	—	17+
C. fulginosus	14	—	+	+	+	—	—	—	+
E. patas	7	—	—	—	—	—	—	—	+
C. aethiops	20	—	—	—	—	—	—	—	+
Cebus a.	3	—	—	—	—	—	—	—	+
Tupaia	6	—	—	—	—	—	—	—	—
Loris	3	—	—	—	—	—	—	—	—

*Source of antiserum.

more differences may be expected. The antisera will then be polyspecific, resulting in quantitative cross-reaction.

Qualitative molecular evolution will only be known when total amino-acid sequences of monoclonal proteins can be determined. Until then, monospecific determinants may give information, although so-called monospecific antisera may still contain several antibodies reacting with distinctive determinants simultaneously present on the same antigen. The exact correlation of chemical structure, location, and specificity of only a few determinants is known. The genetic markers tested in this study are probably all correlated with a difference of not more than one or two amino acids.

The presence of human γ 1 and γ 3 genetic markers in apes and Old World monkeys indicates the existence of corresponding subclass genes in these species. As long as no monoclonal proteins have been

Table 6. Human γ4 Determinants in Nonhuman Primates

Species	Number	Human γ4*	Baboon, Monkey, Rabbit γ4	Rabbit (Antichimp) γ4	Rabbit (Antibaboon) γ4
G. gorilla	14	+	—	+	—
P. paniscus	3	+	+	+	—
P. troglodytes	91	+	+	+	—
P. pygmaeus	25	—	—	+	—
H. lar	14	—	—	+	—
Papio	10	—	—	—	+
T. gelada	9	—	—	—	+
Macaca	60	—	—	—	+
C. fulginosus	14	—	—	—	+
E. patas	7	—	—	—	+
C. aethiops	20	—	—	—	+
Cebus a.	3	—	—	—	+
Tupaia	6	—	—	—	—
Loris	3	—	—	—	—

*Source of antiserum.

found in nonhuman primates, it is questionable that this molecular distribution also exists in these species. Whenever possible, more than one test system was used for subclass specific determinants, because it is known that more than one specificity can be confined to a certain subclass. There is indeed a difference in reaction of anti-γ 3 reagents. Rabbit and baboon antisera detect an antigen common to man and some species of the great apes while the monkey antiserum reacts with human IgG3 proteins only. The anti-γ 4 antisera produced in rabbits, baboons, and monkeys by immunization with human IgG4 proteins show identical specificity when testing nonhuman primates, but rabbit antisera produced by immunization with chimpanzee and baboon immunoglobulins, although having identical specificity when testing human proteins, show differences when testing nonhuman primates. The human anti-α serum obtained from an IgA-deficient patient and the monkey and rabbit antisera produced by immunization with human IgA1 proteins each show a different pattern. It is striking that the rabbit antihuman IgA1 shows the least cross-reactivity. However, this does not mean that rabbits possess IgA heavy chains similar to lower nonhuman primates, because immunization with *Macaca* immunoglobulins resulted in specific antisera detecting an α determinant common to man, apes, and Old World monkeys.

Interesting data were obtained from typing for nonmarkers. The only reagents available for typing nonmarkers were anti-non-b^0 from chimpanzee and anti-non-g from monkey. The nonmarker antigens occur in more than one subclass in man. This may be the same for those species in which these determinants are present without polymorphism, but the finding of polymorphism for non-b^0 in *Pan troglodytes*, *Theropithecus gelada*, and *Cercocebus fulginosus* indicates the occurrence of this determinant in one subclass only. However, this does not prove that the species showing polymorphism possess only a locus corresponding with human IgG3 and no loci corresponding with human IgG1 and IgG2, because the nonmarker may be absent from the proteins coded by the latter loci or located in such a way that serologic expression is impossible. The data obtained suggest that α determinants evolved before any of the other determinants investigated. Thereafter, γ 2 determinants appeared, followed by γ 3, γ 4, γ 1 determinants. The species *Pan paniscus* shows the closest re-

Table 7. Human α Determinants in Nonhuman Primates

Species	Number	Human α*	Monkey α	Rabbit α	Rabbit (anti-*Macaca*) α
G. gorilla	14	+	+	+	+
P. paniscus	3	+	+	+	+
P. troglodytes	91	+	+	+	+
P. pygmaeus	25	+	+	—	+
H. lar	14	+	+	—	+
Papio	10	+	—	—	+
T. gelada	9	+	—	—	+
Macaca	60	+	—	—	+
C. fulginosus	14	+	—	—	+
E. patas	7	+	—	—	+
C. aethiops	20	+	—	—	+
Cebus a.	3	+	—	—	—
Tupaia	6	+	—	—	—
Loris	3	+	—	—	—

*Source of antiserum.

lationship with man, in agreement with the findings of Moor-Jankowski et al.[10]

SUMMARY

Two hundred seventy-nine samples from 20 species of apes, Old World monkeys, New World monkeys, and prosimians were tested for 34 different immunoglobulin specificities. Twenty-three specificities were found to be present in one or more nonhuman species. Determinants corresponding with human determinants appeared in the following order: α, γ 2, (γ 3, γ 4), and γ 1. Polymorphism was found in chimpanzees, orangutans, *Theropithecus*, *Macaca*, and *Cerocebus*. The use of nonhuman primate species for the study of immunoglobulins is interesting from two points of view: as subjects to investigate phylogenetic relationship to man and to select animals as antibody producers and antigen suppliers.

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Red Cell Enzyme Polymorphisms in Rhesus Monkeys and Chimpanzees

By P. Meera Khan, H. van Someren, W. W. de Jong, and M. Vervloet

A QUANTITATIVE ASSESSMENT of the proportion of cells of donor origin in male recipients of bone marrow from female donors has been made either by demonstrating leukocytes with female drumsticks or by analyzing the chromosomes of cells from marrow, peripheral blood, and lymph nodes. In such a procedure, sex becomes a limiting factor in choosing the donors and the origin of circulating erythrocytes in chimeras will still be obscure. The use of different homozygotes (for red cell enzyme variants detectable on zymograms) as partners in donor-recipient pairs may abolish these limitations (Epstein, R. B.: personal communication). Apart from this, the zymogram analysis is easier, cheaper, and quicker than chromosome analysis in assessing the proportion of cells of donor origin in experimental radiation chimeras that survive bone marrow transplantation.

In a group of 50 randomly chosen outbred dogs, we have detected two homozygotes for an electrophoretic variant allele for the red cell indophenol oxidase (IPO).[1] Thus we got interested in a search for red cell enzyme polymorphisms also in other species which are currently used for experimental transplantation.

In the present study we have screened 254 Rhesus monkeys *(Macaca mulatta)* and 30 presumably unrelated adult chimpanzees *(Pan satyrus)* for the electrophoretic behavior of different genetically determined red cell enzyme systems. It was found that two of 11 loci maintained a high degree of polymorphism in rhesus monkeys.

MATERIALS AND METHODS

The macaques included 164 random adults of both sexes and 90 children: 67 of these children fell into 29 families raised by 5 male and 29 female parents. The chimpanzees and rhesus monkeys are maintained at the Primate Center T.N.O., Rijswijk.

Erythrocytes from heparinized blood samples were washed and lysed,[2] using "lysis buffer,"[3] and the following enzymes, excepting PHI (see legend to Fig. 2), were assayed on Cellogel as in Ref. 3: glucose-6-phosphate dehydrogenase (G6PD), NADP-dependant isocritrate dehydrogenase (NADP-IDH), indophenol oxidase (IPO), lactate dehydrogenase A (LDH A), lactate dehydrongenase B (LDH B), NAD-dependant malate dehydrogenase (NAD-MDH), 6-phosphogluconate dehydrogenase (6PGD), 3-phosphoglycerate kinase (PGK), phosphoglucomutase$_1$ (PGM_1), phosphoglucomutase$_2$ (PGM_2), and phosphohexose isomerase (PHI).

RESULTS

In the present series of *M. mulatta*, the loci for 6PGD and PHI exhibited a high degree of polymorphism (Table 1). Two individuals out of 164 macaques were found to be heterozygous for a PGM_1 variant (Fig. 3). The rest of the enzymes studied did not show any variation.

Zymogram patterns of various 6PGD, PHI, and PGM phenotypes found in the rhesus monkeys are seen in Figs. 1, 2 and 3, respectively. The population data is presented in Table 1.

Among the 30 chimpanzees studied, one homozygote and one heterozygote for a fast-moving 6PGD variant and only one presumed homozygote for a PGM_1 variant were encountered. The rest of the enzymes exhibited no variation.

DISCUSSION

Ritter et al. have studied 425 individuals belonging to 36 different species of primates which included 16 rhesus monkeys and three chimpanzees[7] and have reported transspecific variability of a series of red cell enzymes.[7-9] Barnicot and Cohen[10] have also reported somewhat similar data obtained from 218 individuals belonging to

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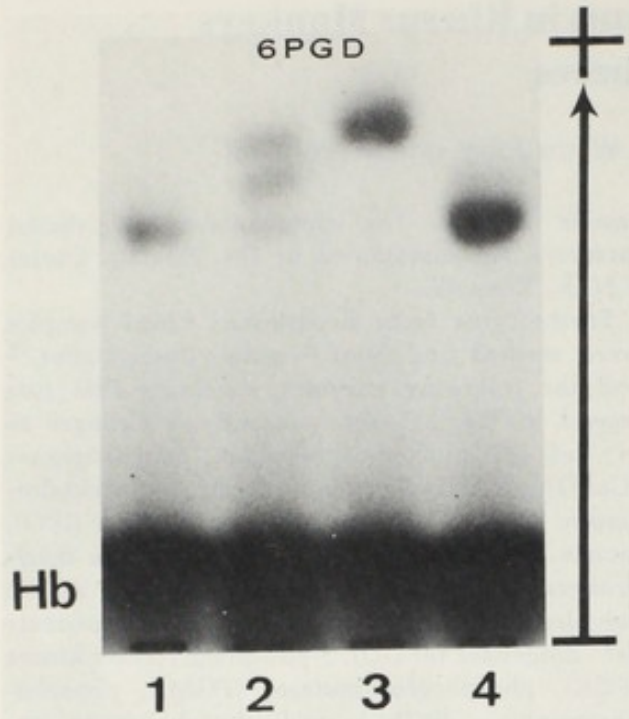

Fig. 1. Electropherogram of red cell phosphogluconate dehydrogenase of *Macaca mulatta* showing the patterns of the usual phenotype of PGD A (channels 1 and 4), the fast variant PGD F (channel 3), and the heterozygote PGD AF (channel 2). Note the hemoglobin (Hb) bands close to the origin (bottom).

22 different species of primates which included neither rhesus monkeys nor chimpanzees.

Tariverdian et al.[8] have noticed three electrophoretic phenotypes (A, AF, and F) of 6PGD determined by a pair of codominant alleles (*PGD*^{a} and *PGD*^{f}) at an autosomal locus in the macaques. These phenotypes were found to be indistinguishable between *M. mulatta* and *M. irus*. As this variation appears to be similar to that found in the present series of *M. mulatta*, we used the same nomenclature (Table 1).

Barnicot and Cohen[10] did not include the PHI system in their study, while Tariverdian et al.[9] observed no PHI variation in their macaques. Figure 2 shows the electrophoretic patterns of PHI in the macaques. The single band moving towards the cathode in channels 3 and 4 is the usual homozygous pattern (PHI 1). We found one instance in which the cathodal band and the band migrating with hemoglobin, seen in channels 1 and 2 of Fig. 2, were absent and the remaining three main anodal bands

Table 1. Distribution of 6PGD and PHI Variants in 164 Macaques*

	Percentages of Phenotypes			Allele Frequencies	
6PGD	PGD A	PGD AF	PGD F	*PGD*^{a}	*PGD*^{f}
Observed	64.63	29.27	6.10	79.27	20.73
Expected	62.84	32.86	4.30		

$\chi^2 = 1.19658$ with two degrees of freedom
p is > 0.5 and < 0.7.

	Percentages of Phenotypes			Allele Frequencies	
PHI	PHI 1	PHI 2-1	PHI 2	*PHI*^{1}	*PHI*^{2}
Observed	84.76	14.63	0.61	92.07	7.93
Expected	84.77	14.60	0.63		

P.S. The observed and the expected values are embarassingly fitting.

*These data suggest that various phenotypes of G6PD as well as PHI are quite in equilibrium and in agreement with the hypothesis that these phenotypes were determined by a pair of codominant alleles at an autosomal locus in each case.

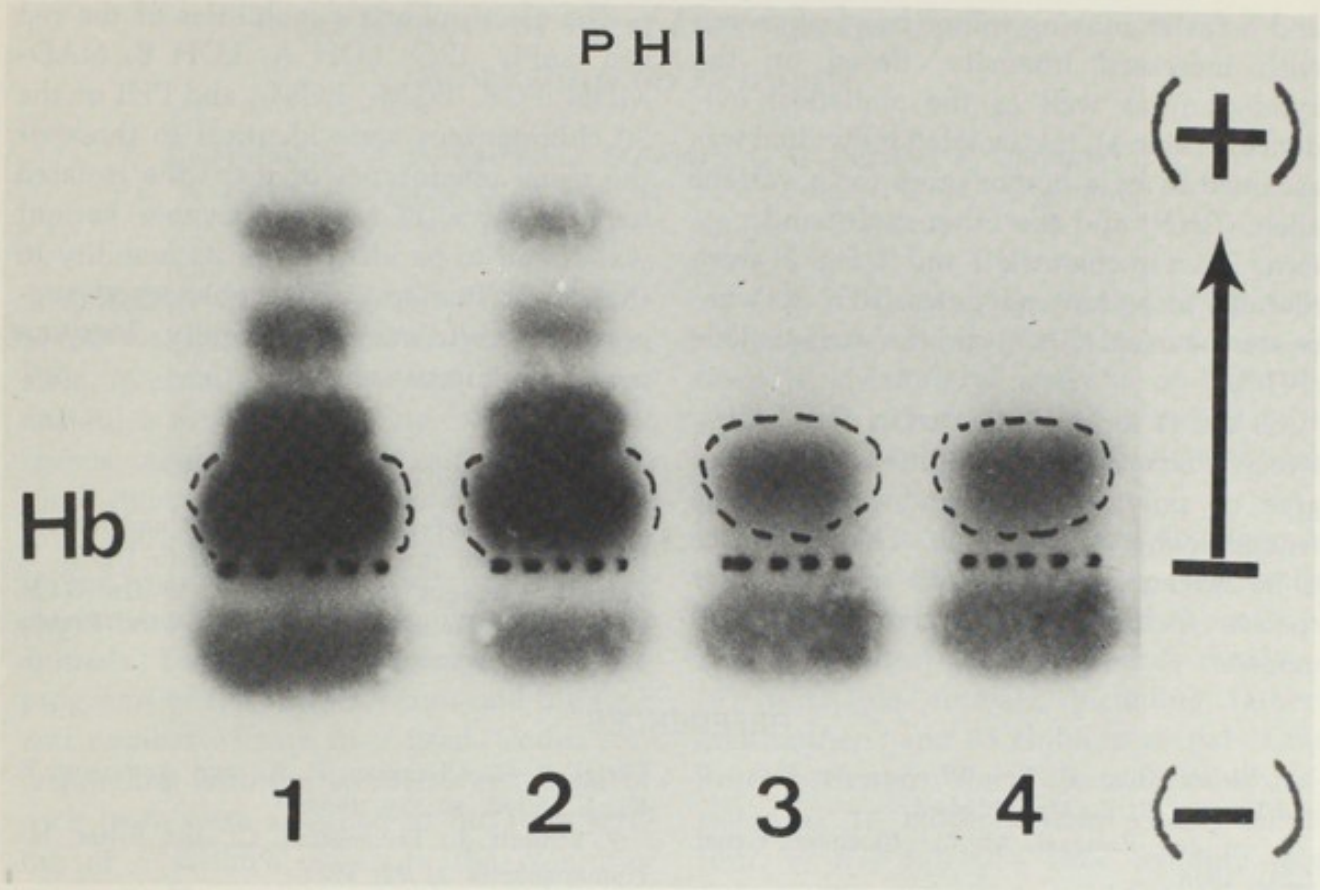

Fig. 2. Zymograms of phosphohexose isomerase in rhesus monkeys showing the PHI 1 (channels 3 and 4) and PHI 2-1 (channels 1 and 2) patterns. The broken ovals indicate the position of hemoglobin (Hb) and the dotted lines below Hb represent the origin. Cellogel electrophoresis was done in buffer system III for 3 hr following the general procedure described by Meera Khan.[3] Staining mixture consists of 2 mg of fructose-6-phosphate (F6P) in 1.0 ml of A_1 and 0.2 ml each of D_3, G_1, G_2, and B_1 along with 10 μl of F_1 (Ref. 3 for the description of these symbols and the details on reagents).

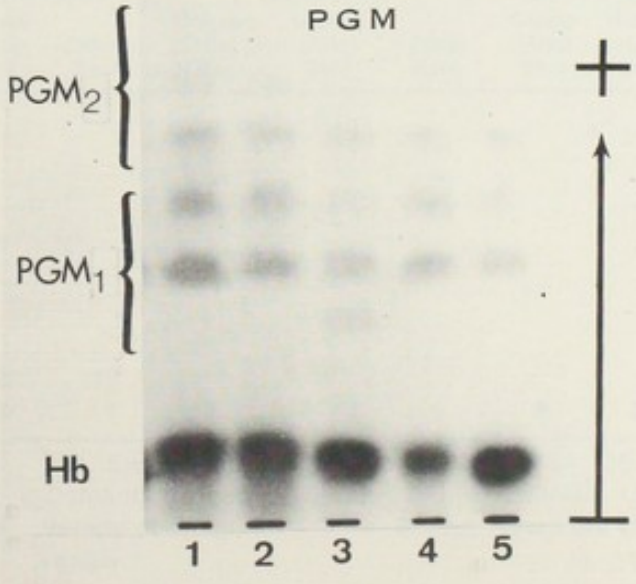

Fig. 3. Phosphoglucomutase isozymes in macaques. The pattern of the Rhesus variant PGM_1 2-1 is seen in channel 3. The fast component of the variant is probably overlapping the slow component of PGM_1 1.

and a faster moving minor band appeared with increased intensity. Based on the zymogram as well as the statistical evidence, (Table 1), this isolated individual was assumed to be a homozygote for a variant allele *(PHI^2)* and the other multiband patterns (seen in channels 1 and 2, Fig. 2) were thought to be heterozygotes (PHI 2-1) between the usual *(PHI^1)* and the variant allele *(PHI^2)*.

Of the 11 loci investigated in *M. mulatta*, two *(PGD* and *PHI)* exhibited a high degree of polymorphism and one *(PGM_1)* tended to be polymorphic. This incidence is in agreement with those seen in other species including man,[4] fruit fly,[5] and mouse.[6]

The electrophoretic mobilities of the red cell G6PD, IPO, LDH A, LDH B, NAD-MDH, PGK, PGM_1, PGM_2, and PHI of the 30 chimpanzees were identical to those of the usual phenotypes of man: the isolated instance of a PGM_1 homozygote variant was found to be identical in its mobility to that of the human PGM_1 2 phenotype, suggesting evolutionary proximity between man and chimpanzee.

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Joint Report of First Histocompatibility Workshop on Primates

By H. Balner, A. D. Barnes, J. D'Amaro, C. C. Darrow, P. Démant, B. W. Gabb, A. Piazza, R. S. Metzgar, H. F. Seigler, G. N. Rogentine, J. J. van Rood, F. Ward, and R. L. Zweerus

THE PRINCIPAL AIM OF THE WORKSHOP was a better definition of and, if possible, international agreement about, nomenclature for the leukocyte isoantigens of rhesus monkeys and chimpanzees. At the same time, a number of related investigations were performed such as cross-species typing (mainly of chimpanzee cells with human antisera) and red cell typing of the same animals. Table 1 lists the principal typing programs of the various teams and the type and number of sera they used. Codes corresponding with the senior investigators of each team were assigned to facilitate tracing of a serum's origin in the computer printout. These codes are also used in the tables showing the groups of highly correlated sera. Although the emphasis was on defining isoantigens by means of isoantisera of a species, the close phylogenetic relation between chimpanzees and man allowed a meaningful analysis of data obtained for chimp cells when tested with both chimp-antihuman and human-antihuman antisera.

In the course of 9 workdays, 202 animals were typed; 60 chimpanzees (the entire Rijswijk colony except a recently born child and its mother) and 142 rhesus monkeys (87 unrelated animals, including fathers and mothers, and 55 children reared at the Primate Center. The Rhesus families consisted of 16 mothers with usually three, four, or five full sibs sired by only four fathers. The animals were bled in the early morning. At about 10 a.m., suspensions of separated lymphocytes were available. By general agreement, separation of lympho-

Table 1. Teams Participating in Workshop and Their Principal Typing Programs

		Rhesus Monkeys				Chimpanzees				
		Leukocytes		Erythrocytes		Leukocytes			Erythrocytes	
Code	Team	Rhesus (Allo) Sera	Other Sera	Rhesus (Allo) Sera	Other Sera	Chimp (Allo) Sera	Human (Allo) Sera	Other Sera	Chimp (Allo) Sera	Human (Allo) Sera
RA,	New York						51			
VR,	Leiden						19			
JO,	Marburg					14		29		
ME,	Duke		4			22	59	44		
BA,	Rijswijk	136	4	75		129				
BR,	Birmingham	41	5				3			
RO,	Bethesda	109								
DE,	Prague		30							
SU,	Madison			12	1					
SO,	New York			5					8	6

RA = Rapaport, New York University. VR = van Rood, Leiden University. JO = Johannsen, Behringwerke, Germany. ME = Metzgar, Duke University. BA = Balner, Primate Center TNO, Holland. BR = Barnes, Queen Elizabeth Hospitals, England. RO = Rogentine, NIH, USA. DE = Démant, Prague University. SU = Sullivan, University of Wisconsin. SO = Socha, New York University.

Table 2

RhL-A 8

freq. pos.	serum number and name							
.379	215	BA 902.1	-					
.414	216	BA 1285.1	.930	-				
.391	260	BA 1157.2	.879	.810	-			
.416	271	RO 966-E*	.893	.920	.814	-		
.253	259	BA 1171.3	.835	.777	.815	.766	-	
.552	110	RO 631-F	.701	.663	.560	.706	.588	-
			215	216	260	271	259	110

RhL-A 9

freq. pos.	serum number and name									
.356	16	BA 42A	-							
.345	14	BA 41E	.975	-						
.379	210	BA 954.1	.929	.906	-					
.390	269	RO 403-F*	.920	.894	.836	-				
.405	47	RO 446-F	.877	.853	.826	.972	-			
.402	208	RO 309-F*	.858	.835	.832	.893	.828	-		
.402	209	RO 405-F*	.837	.814	.810	.761	.723	.739	-	
.414	158	BR 2167-1805	.775	.748	.802	.84	.770	.745	.801	-
			16	14	210	269	47	208	209	158

RhL-A 10

freq. pos.	serum number and name					
.287	233	RO 303-F*	-			
.345	234	BA 1121 XI	.875	-		
.322	235	BA 1262.1	.759	.743	-	
.299	236	BA CF.2	.862	.741	.679	-
			233	234	235	236

*Tested by Balner et al.; one-stage cytotoxicity.

cytes on Ficoll-Isopaque gradients was centralized to save blood and enable the teams to work with identical suspensions.

Special forms were provided for serum identification and data scoring. Scores ranged from 0 to 5 (0 was negative, and 5 was the highest positive reaction), and the teams were informed that the computer would regard 3 and higher scores as positive and 2 and lower as negative. The percentage of killed cells for the scoring of 2 or 3, a negative or positive reaction, was determined individually by each team. The data were recorded on scoring forms and transferred to punch cards usually the same evening.

On the day before the symposium, parts of the computer printout were given to the team leaders (2 × 2 tables for the reactivity patterns of their own sera compared with all other sera for significantly positive or negative correlations). This enabled each team to group its own sera according to similar reactivity patterns and to prepare dot charts for workshop data analysis.

Papers (not incorporating workshop results) were presented on the first day of the symposium, and on the second day workshop results were analyzed. Conclusions and decisions about nomenclature were sometimes reached by a majority vote but usually by unanimous agreement. Our confidence in the conclusions reached was increased by the active participation of a number of HL-A experts: Albert, Bodner, Kissmeyer-Nielsen, Payne, Svejgaard, Zmijewski, and Trnka.

A Library of Computer Programs for genetic analyses written by Piazza was used

Table 3

RhW-2

freq. pos.	serum number and name					
.256	225	BA EB	-			
.299	226	BA EC	.833	-		
.221	228	BA CB	.844	.748	-	
.402	227	BA BC	.653	.591	.643	-
			225	226	228	227

RhW-12

freq. pos.	serum number and name				
.299	140	RO B2621	-		
.333	133	RO 129	.817	-	
.349	24	BA 61A	.793	.716	-
			140	133	24

RhW-13

freq. pos.	serum number and name							
.161	212	BA UU.1	-					
.208	262	BO 3-MRI26*	.837	-				
.126	213	BA BP.2	.775	.701	-			
.218	214	BA 1229.2	.753	.672	.712	-		
.250	164	BR 2169-2201	.603	.496	.461	.617	-	
.236	165	BR 2169-0502	.533	.457	.482	.569	.736	-
.222	166	BR 2169-2004	.655	.581	.505	.599	.694	.804	-
			212	262	213	214	164	165	166

RhW-14

freq. pos.	serum number and name					
.126	223	BA V.2	-			
.218	281	BA V.4	.813	-		
.164	224	BA N.1	.801	.634	-	
.138	31	BA 107A	.751	.605	.757	-
			223	281	224	31

*Tested by Balner et al.; one-stage cytotoxicity.

to analyze the results of the workshop.[1] Piazza, Gabb, D'Amaro, and Zweerus were chiefly resposible for the data analysis.

Rhesus Monkeys

Tables 2 to 4 show the groups of antisera assigned an RhL-A or RhW (workshop) designation. The usual method of association analysis was employed: sera were selected on the basis of significant positive correlations(r), taking into account the strength of the antisera and possible inclusions. Eleven such groups of sera were found, and by general agreement they were accepted as defining an antigenic determinant.

Because of previously published data concerning most of the sera constituting nine of the groups, agreement was reached to assign the names indicated in Tables 2–4. Following the procedure used at the human histocompatability workshop in 1970, groups were divided into RhL-A and RhW designations. RhL-A designations were accepted for three of the groups on the basis of available information of family and population studies.[2,3] Groups RhW 7 and RhW 16 (Table 4) probably identify a common determinant, while the sera of group RhW 7 define the narrower specificity. The sera of groups RhW 11 and RhW 15 show a similar relationship. The newly assigned specificities and the corresponding group designations used before by different investigators are shown in Table 5, and gene frequencies for each group, calculated from a sample of 87 unrelated animals, are indicated. Family data for the newly assigned specificities are being investigated and will be published in due course.

Chimpanzees

While the proposition to regard RhL-A as the major histocompatibility system of rhesus monkeys was unanimously accepted, there was controversy about whether or not the existence of one major histocompatibility system of chimpanzees could be accepted on the basis of the current knowledge of chimpanzee isoantigens. The proponents of accepting a ChL-A locus argued that most of the chimp specificities described in the literature were defined by groups of isoantisera prepared independently in different laboratories, that many of the reagents were operationally monospecific, and that a previous analysis of an admittedly small population (sample size, 193) allowed the assumption of at least one series of allelic specificities.[4] All this, plus the striking similarity between several chimp specificities and antigens of both

Table 4

RhW - 7

freq. pos.	serum number and name					
.448	252	BA 549.1	-			
.448	248	BA 1174.3	.652	-		
.540	250	BA 905.1	.547	.522	-	
.726	60	RO 700-F	.504	.558	.552	-
			252	248	250	60

RhW - 16
(broad RhW - 7)

freq. pos.	serum number and name					
.786	44	RO 463 - E	-			
.845	45	RO 989 - E	.739	-		
.833	58	RO 632 - F	.778	.780	-	
.726	60	RO 700 - F	.720	.623	.656	-
.839	112	RO 636 - F	.819	.636	.691	.623	-
			44	45	58	60	112

RhW - 11

freq. pos.	serum number and name					
.540	244	BA 1233.1	-			
.414	245	BA 1240.1	.728	-		
.540	247	BA BU.1	.630	.588	-	
.325	263	BO 4-MRI30*	.650	.788	.684	-
			244	245	247	263

*Tested by Balner et al.; one-stage cytotoxicity.

RhW - 15
(broad RhW - 11)

freq. pos.	serum number and name					
.631	89	RO 683-2	-			
.702	90	RO 640-F	.797	-		
.691	91	RO 649-F	.662	.747	-	
.690	242	BA 951.1	.635	.715	.634	-
			89	90	91	242

Table 5. Leukocyte Specificities of Rhesus Monkeys: Relation Between Workshop and Previous Nomenclature

Workshop Nomenclature	Original Nomenclature of Groups at			Gene Frequencies and Standard Errors
	Rijswijk	Bethesda	Birmingham	
RhL-A 6	RhL-A 6	Group 2	No previous	0.219 (0.033)
RhL-A 9	RhL-A 9	Group 1	designation	0.197 (0.032)
RhL-A 10	RhL-A 10	Group 5 (approximately)	of groups available	0.149 (0.028)
RhW 2	RhL-A 2			0.149 (0.028)
RhW 12	RhL-A 12	(new)		0.169 (0.029)
RhW 13	RhL-A 13			0.090 (0.022)
RhW 14	RhL-A 14			0.065 (0.019)
RhW 7	RhL-A 7			0.284 (0.037)
RhW 16 (broad 7)		Group 3		0.545 (0.047)
RhW 11	RhL-A 11			0.257 (0.035)
RhW 15 (broad 11)		Group 8		0.442 (0.044)

For identification of sera defining these groups and their associations, see Tables 2–4. Rijswijk, Bethesda, and Birmingham sera are indicated with prescripts Ba, RO, and BR, respectively.

Gene frequencies for workshop groups were calculated from sample of 87 unrelated animals.

segregant series of HL-A, was put forward as argument in favor of accepting a ChL-A system at this stage. A formal proof of the existence of one major histocompatibility system by family analyses or population studies on a large sample of chimpanzees may not be possible for many years to come. Nonetheless, most of the discussion participants were in favor of delaying the decision about the existence of one major histocompatibility system of chimpanzees, and only ChW designations were assigned. To emphasize the phylogenetic closeness between man and chimpanzees, the groups of antisera defining chimp specificities shown to be the unequivocal analogues of established human antigens were given HL-A related designations (ChW 4 for 4a, ChW 6 for 4b, ChW11 for HL-A 11). Tables 6–9 show the groups of antisera which, on the basis of positive correlations (r), were assigned ChW designations. Analysis of chimpanzee population data was similar to the rhesus analysis. As can be seen in Tables 6–9, chimp-antihuman sera and human isoantisera were also accepted as defining chimpanzee groups. Sera included in the groups have a probability less than or equal to 0.001 in Fisher's exact test for 2 × 2 tables.

ChW groups that defined specificities not firmly related to any of the known human specificities were assigned sequence numbers above 100. Although some of the groups were clearly associated with other groups (ChW 106 was a short ChW 105,

Table 6

ChW = 4

(related to 4a)

freq. pos.	serum number and name	source of serum									
.845	43 ME Kong 0270	C-C									
.759	65 ME Peck 0968	C-C,H	.76								
.862	64 ME Peck 0369	C-C,H	*	.71							
.828	49 ME Peck 0370	C-C,H	.69	.81	.88						
.817	25 ME Sny 1070	H-H	.89	.86	.70	.83					
.743	209 ME Rogan 0370	C-C,H	.48	.61	*	.55	.51				
.770	61 BA Ci 24	C-C	*	.57	.62	.63	.48	.57			
.750	170 BA Ci 26	C-C	.65	.81	.59	.70	.76	.82	.69		
.759	62 ME Alice 0371	C-C	.54	.62	.59	.70	.63	.61	.47	.72	
.737	58 ME Larr 1068	C-C,H	.62	.77	.68	.77	.72	.57	.53	.68	.77
			43	65	64	49	25	200	161	170	82 58

* $p > 0.001$.

ChW = 6

(related to 4b)

freq. pos.	serum number and name	source of serum								
.621	46 ME Wand 0270	C-C,H								
.574	141 BA Re 2	C-C	.61							
.623	142 BA Ma 3	C-C	.78	.70						
.574	139 BA An 8	C-C	.64	.46	.63					
.557	167 BA Re 53	C-C	.72	.77	.74	.63				
.531	237 BA-C-An 12	C-C	.78	.71	.67	.75	.71			
.408	236 BA-C-An 8	C-C	.58	.70	.51	.53	.66	.70		
.404	230 BA-C-Re 2	C-C	.61	.63	.55	.63	.63	.69	.79	
.404	232 BA-C-Ma 3	C-C	.61	.63	.55	.63	.63	.72	.83	.93
			46	141	142	139	167	237	236	230 232

MS=Metzger et al. Ba=Balner et al. VB=van Rood et al. C-C=chimp-antichimp. C-H=chimp-antihuman. C-C,H=chimp-antichimp and human. *complement fixation technique was used. In all other cases, cytotoxicity was used.

Table 7

ChW - 110
(short ChW - 6)

freq.-pos.	serum number and name		source of serum						
.255	35	ME Jac 1-69	H-H						
.367	39	ME Ra 1-67	H-H	.79					
.397	61	ME Laur 0868	C-C,H	.75	.82				
.421	50	ME Jenda W	C-C,H	.75	.67	.78			
.448	79	ME Jenda 0369	C-C,H	.72	.72	.83	.96		
.379	51	ME Jenda 0866	C-C,H	.71	.78	.89	.82	.87	
				35	39	61	50	79	51

ChW - 107
(short ChW - 6)

BA-C-Re 2

BA-C-Ma 3	+	-
+	22	1
-	1	33

r = 0.93

frequency positives
Re 2 .404
Ma 3 .404

ChW - 11
(related to HL-A 11)

freq.-pos.	serum number and name		source of serum			
.410	130	BA Ti 5	C-C			
.388	221	BA-C-4268L	C-C	.87		
.311	126	BA Do 20	C-C	.66	.70	
				130	221	126

ChW - 108
(short ChW - 11)

freq.-pos.	serum number and name		source of serum			
.333	244	BA-C-Do 13	C-C			
.281	226	BA-C-Kn 1	C-C	.88		
.316	249	BA-C-Qu 2	C-C	.88	.92	
				244	226	249

ChW 107 a short ChW 6, and ChW 108 a short ChW 11) most of the participants preferred to assign separate symbols rather than designations that might reveal a correlation between the groups. Several participants disagreed with this policy and would have preferred a nomenclature making associations recognizable. The relation between the newly assigned ChW specifr-cities and the group designations previously used in publications of the Duke and Rijswijk/Leiden investigators are shown in Table 10.

Red Cell Typing

Although red cell typing was not one of the original aims of the workshop, the willingness of the principal primate red cell experts to participate was greatly appreciated. Since the Rijswijk chimpanzees and rhesus monkeys used during the workshop can be regarded as a reference panel for white cell specificities, maximal knowledge of their red cell phenotypes was considered valuable extra information. The 60 chimpanzees were typed for the antigens of the human ABO system and for the simian VAB and CEF systems established previously by Wiener and Moor-Jankowski.[5,6] The distribution of specificities was

Table 8

ChW - 101
(related to HL-A 1)

freq.-pos.	serum number and name		source of serum				
.350	16	ME Rag 4-71	H-H				
.383	194	*ME Dunc.0370	C-C	.96			
.283	34	ME Lew	H-H	.85	.83		
.300	VR	VR 3271	H-H	.78	.83	.75	
				16	194	34	VR

*Tested by Balmer et al.

ChW - 102

freq.-pos.	serum number and name		source of serum				
.410	129	BA Abe 24	C-C				
.551	231	BA-C-Qu 0	C-C	.75			
.475	158	BA Abe 23	C-C	.74	.65		
.567	179	ME Lucy 0270	C-C,H	.65	.75	.55	
				129	231	158	179

ChW - 103

freq.-pos.	serum number and name		source of serum			
.333	26	ME Dalp 1	H-H			
.311	146	BA Ge 14	C-C	.77		
.311	148	BA Jac 4	C-C	.51	.77	
				26	146	148

ChW - 104

freq.-pos.	serum number and name		source of serum				
.655	59	ME Larr 1168	C C,H				
.607	124	BA Jac 7	C C	.61			
.508	125	BA Ol 1	C C	.78	.68		
.534	44	ME Elga 0270	C C,H	.74	.55	.69	
				59	124	125	44

Table 9

ChW - 105
(related to 7c)

freq. pos.	serum number and name	source of serum					
.295	136 BA IS 4	C-C					
.310	69 ME ALS 0770	C-C,H	.88				
.328	70 ME ALS 0870	C-C,H	.84	.96			
.379	55 ME Mart 0968	C-C	.82	.78	.74		
.328	138 BA Ka 5	C-C	.77	.64	.61	.59	
			136	69	70	55	138

ChW - 106
(short ChW 105)

freq. pos.	serum number and name	source of serum				
.190	93 ME Mart 0871	C-C				
.193	224 BA-C-Is 4	C-C	.71			
.193	225 BA-C-Jo 1	C-C	.71	.89		
.193	228 BA-C-Ka 5	C-C	.71	1.00	.89	
			93	224	225	228

ChW - 109

freq. pos.	serum number and name	source of serum			
.183	20 ME MHP 1	H-H			
.241	48 ME Halp 0270	C-C,H	.70		
.207	54 ME Halp 0269	C-C,H	.67	.71	
			20	48	54

ChW-111

freq. pos.	serum number and name	source of serum			
.175	250 BA-C-Si 5	C-C			
.158	243 BA-C-Di 3	C-C	.81		
.293	87 ME Lucy 0671	C-C,H	.77	.61	
			250	243	.87

not found to be significantly different from that described for other chimpanzee colonies. The rhesus monkeys were typed by three groups of investigators (New York, Madison, and Rijswijk). Each team used its own reagents and techniques. It was decided to postpone the assignment of a uniform international nomenclature until further comparisons had been made, but interesting correlations were found between the reactivity patterns of antisera used by the three teams of investigators (Table 11).

At this time, a comparison between the reactivity patterns obtained with red cell

Table 10. Leukocyte Specificities of Chimpanzees: Relation Between Workshop and Previous Nomenclature

Workshop Nomenclature	Original Rijswijk/ Leiden Nomenclature	Original Duke Nomenclature	Similarities With Human Antigens	
			Firm	Vague
ChW 4	1	I, II	4a	HL-A1
ChW 6	4	III	4b	
ChW 11	8		HL-A11	
ChW 101		VIII		
ChW 102	2	VI*		
ChW 103	5			
ChW 104	6	I		
ChW 105	3	IV		7c AA
ChW 106	3 (short)			7c AA
ChW 107	4 (short)			4b
ChW 108	8 (short)		HL-A11	
ChW 109				
ChW 110		VII		4b
ChW 111	7 (short)			

*Duke VI completely includes ChW 102.

typing reagents and patterns obtained with leukocyte typing reagents has not been made. Time limitation prevented us from running this particular computer program (for chimpanzee and rhesus cells). Similarity of reactivity patterns would imply

Table 11. Associations Between Reactivity Patterns of Rhesus Erythrocyte Isoantisera (Positive Correlations > 0.70)

Sample size 120

	$*_{K-1}$	D^{Rh}	E^{Rh}-212	$*_{20}$	$*_{G-2}$
D^{Rh}-6	.791				
E^{Rh}-212	.941	.82			
E^{Rh}-218	.941	.82	1.00		
F^{Rh}-3				.73	
$*_{G-3}$					1.00

Sample size 81

	$*_{H-2}$	G^5	1121^7	1121^{10}	1146^7	1146^{10}	1159^1
G^5	.93						
1121^7	(.67)	.73					
1121^{10}	.72	.78	(.68)				
1146^7	.88	.78	.75	.71			
1146^{10}	.83	.78	.85	.71	.89		
1164^3	.86	.92	(.68)	.72	.71	.71	
C''							.86

Rh superscripts indicate New York antisera. Numeral superscripts indicate Rijswijk antisera. Asterisks indicate Madison reagents.

that leukocyte specificities might be recognizable on red cells and vice versa. If significant correlations are indeed found, the results will be published.

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Index