

INVESTIGATIONS OF THE GENETIC ASPECTS OF
THE MIXED LYMPHOCYTE CULTURE REACTION IN
SOUTHERN AFRICA

THESIS

submitted to the University of Cape Town

by

Ernette D. du Toit, M.B.,Ch.B., (Cape Town)

in part fulfilment of the requirements for the
degree of DOCTOR OF MEDICINE

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"Blood is thicker than water"

(= the tie of relationship)

c.1180 HEINRICH DER GLICHESSERE

In: *Reinhart Fuchs*. "Ouch hoerich sagen, daz sippebluot von wassere niht verdirbet" (The Oxford dictionary of English proverbs, compiled by William George Smith. Oxford University Press, 1935 reprint 1936, p.69).

Included in a pocket on the back page are reprints of the following articles:-

1. Eijsvoogel, V.P., Van Rood, J.J., Du Toit, E.D. and Schellekens, P.Th.A. (1972a): Position of a locus determining mixed lymphocyte reaction distinct from the known HL-A loci. Eur. J. Immunol. 2, 413.
2. Eijsvoogel, V.P., Du Bois, M.J.G.J., Melief, C.J.M., De Groot-Kooy, M.L., Koning, C., Van Rood, J.J., Van Leeuwen, A., Du Toit, E.D., and Schellekens, P.Th.A., (1972b): Position of a locus determining mixed lymphocyte reaction (MLR), distinct from the known HL-A loci, and its relation to cell-mediated lympholysis (CML). In: Histocompatibility Testing, 1972. Eds. Dausset, J. and Colombani, J. Pp 501-508. Munksgaard, Copenhagen.
3. Eijsvoogel, V.P., Koning, L., De Groot-Kooy, L., Huismans, L., Van Rood, J.J., Van Leeuwen, A. and Du Toit, E.D. (1972c): Mixed lymphocyte culture and HL-A. Transplant. Proc. IV, 199.

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TERMINOLOGY

The terminology which has been adopted includes the following terms :-

1. *Transplantation antigens* may be defined as antigens on cells and tissues which after grafting may induce in the recipient an immune response resulting in rejection phenomena. Snell (1948) defined histocompatibility genes as genes whose products are cell surface antigens which determine the acceptance or rejection of transplants. He therefore reserved the designation histocompatibility (H) for those regions (loci) whose antigens are involved in graft rejection.
2. Since there is more than one locus controlling histocompatibility on a limited area of a particular chromosome, Bach (1973) introduced the term *major histocompatibility complex (MHC)* to refer to the entire segment which determines not only the serologically defined loci, but also other components possibly involved in graft rejection.
3. *Tissue typing* is the term in general use for the serological test used to determine which H antigens an individual carries. It is a necessary preliminary to the matching of graft tissue for a particular recipient.

4. The term *HLA*:

- (a) When used alone in this Thesis, *HLA* implies only the A and B loci. This is because typing sera for the third or C locus were not commonly available until recently, and typing was not done on any of the subjects included in this study.
- (b) In accordance with the recommendations of the WHO Nomenclature Committee which met in Aarhus, Denmark, after the Sixth Histocompatibility Conference in July, 1975, the hyphen has been dropped from the term *HLA*. In this Thesis it is therefore not used except in Chapter One, where the history of the term *HLA* is given.
- (c) The new equivalent designations as recommended by the WHO Nomenclature Committee in 1975 have been applied to the antigens for HLA-A and B loci. The provisionally identified specificities, as before, carry the additional letter W, which is inserted between the locus letter(s) and the allele and specificity number. Table A gives a list of synonyms for the various antigens of the HLA-A, B, C and D series currently recognized (WHO, 1975).

TABLE A

LISTING OF CURRENTLY RECOGNIZED HLA SPECIFICITIES

(From WHO, 1975)

New Locus A	Previous First, LA Locus	New Locus B	Previous Second 'Four'	New Locus C	Previous Third	New Locus D	Previous LD, MLC-I
HLA-A1	HL-A1	HLA-B5	HL-A5	HLA-CW1	T1	HLA-DW1	LD 101
HLA-A2	HL-A2	HLA-B7	HL-A7	HLA-CW2	T2	HLA-DW2	LD 102
HLA-A3	HL-A3	HLA-B8	HL-A8	HLA-CW3	T3	HLA-DW3	LD 103
HLA-A9	HL-A9	HLA-B12	HL-A12	HLA-CW4	T4	HLA-DW4	LD 104
HLA-A10	HL-A10	HLA-B13	HL-A13	HLA-CW5	T5	HLA-DW5	LD 105
HLA-A11	HL-A11	HLA-B14	W14			HLA-DW6	LD 106
HLA-A28	W28	HLA-B18	W18			HLA-DW7	
HLA-A29	W29	HLA-B27	W27			HLA-DW8	
HLA-AW23	W23	HLA-BW15	W15				
HLA-AW24	W24	HLA-BW16	W16				
HLA-AW25	W25	HLA-BW17	W17				
HLA-AW26	W26	HLA-BW21	W21				
HLA-AW30	W30	HLA-BW22	W22				
HLA-AW31	W31	HLA-BW35	W5				
HLA-AW32	W32	HLA-BW37	TY				
HLA-AW33	W19.6, Fe55	HLA-BW38	W16.1				
HLA-AW34	Malay 2	HLA-BW39	W16.2				
HLA-AW36	Mo*	HLA-BW40	W10				
HLA-AW43	BK	HLA-BW41	Sabell				
		HLA-BW42	MWA				

STANDARD ABBREVIATIONS

CML	Cell-mediated lympholysis
cpm	Counts per minute
GvH	Graft-versus-host
HLA	Human leukocyte, locus A
H-2	Histocompatibility 2
LD	Lymphocyte defined
m	Mitomycin-C
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
PHA	Phytohaemagglutinin
RR	Relative response
SD	Serologically defined
se	Standard error
SR	Stimulation ratio

ABSTRACT

The genetic relationship between the serologically detectable antigens of the HLA-A and B loci and the mixed lymphocyte culture (MLC) reaction is of utmost importance in the understanding of histocompatibility. From the literature it appeared that neither the HLA-A, B genotype nor the MLC reaction is the complete answer to donor-recipient selection in organ transplantation. This study was therefore initiated in June 1971 in an attempt to clarify the problem. It is inherent in a project of this nature that much time is spent in collecting samples from donors in diverse areas. Most experiments had to be repeated two or three times, therefore some of the problems we set out to resolve were clarified by other workers before we were able to complete our investigations.

The Thesis is presented in four main parts : Introduction; Materials and Methods; Results; and Discussion. In the Introduction (*Part I*) a review of the human and mouse histocompatibility systems and their clinical significance is given to provide a background to the study (Chapter One). The findings in man and the mouse are reported, as the histocompatibility antigen system of the mouse has been studied in greater detail than that of any other experimental animal and as a close analogy between these two species has been demonstrated. This review includes concepts as accepted at the beginning of 1977. Following this first Chapter, the motivation for the study is dealt with in Chapter Two.

The Materials and Methods section (*Part II*), describes the groups chosen for investigation (Chapter Three); and the methods employed (Chapter Four).

Chapter Three starts with a brief explanatory note on the concepts of consanguinity and genetic non-relatedness. The reason for this is that the groups studied included unrelated individuals at the one extreme and the offspring of consanguineous unions at the other. The San (Bushmen), a small group living in geographical isolation, were selected for study as a group intermediate between these two extremes. A short description of the San and Indians (Muslims) is also included for those readers who are unfamiliar with these two ethnic groups.

The methods (Chapter Four) are reported in detail. They constitute a new field of research at this University. The setting up of the MLC test during field work is particularly difficult; the problems encountered are therefore discussed and a list of materials used is given in the Appendix. This may help future research teams.

Part III has been devoted to the results and this is by far the longest section of the Thesis. In Chapter Five the format for presenting the results and the different approaches used in expressing MLC reactivity are described. The results are reported in detail from Chapter Six to Chapter Ten. A chapter is devoted to each of the major groups and an interpretation of the data is given at the end of each chapter. This interpretation is considered necessary as some findings were observed that are unrelated to the initial aims and were

sufficiently significant to require recording. The expression of MLC reactivity remains a universal problem for those workers who employ this technique. In Chapter Eleven, the correlation between the different parameters used in this study - the mean cpm, stimulation ratio (SR) and relative response (RR) as well as the morphology - are compared.

Part IV, Chapter Twelve, of the Thesis has been reserved for a general discussion of the results. Original evidence was obtained for the control of strong MLC reactivity at a single locus (or genetic region) HLA-D, closely linked to HLA-B, and located outside the HLA-A, B segment of the major histocompatibility complex (MHC). Data in support of this were obtained from unrelated HLA serologically non-identical MLC negative combinations and families in which recombinational events had occurred. The HLA-D region appears to control an allelic system in which the specificity of the determinants is to some extent related to the specificities of the determinants of the HLA-B locus. The identification of an MLC homozygous cell derived from incest, allowed the demonstration of the one-way reaction, which proved that the MLC reaction is solely controlled by one chromosome. It also made the definition of an HLA-D specificity DW3 in unrelated individuals possible. The results of this 'MLC typing' experiment suggested that the DW3 determinant (as it was recognized by the Sixth International Histocompatibility Workshop homozygous typing cells, of which the above-mentioned MLC homozygous cell was one), could be more complex. The weak cell proliferations responsible for 'typing responses' in HLA-D typing experiments between unrelated individuals, were also observed with the homozygous

'typing cells' within the inbred family. The weak responses characteristic of 'typing responses' cannot therefore be completely explained by weak disparities at other HLA loci or by cross-reactions between similar HLA-D specificities, and have still to be satisfactorily explained.

Tissue typing has proved to be a tremendous success in living related donor situations, but far less useful when applied to cadaver donors. However, from all the data available it appears that the HLA-A and B loci and the MLC reaction do play a role in histocompatibility, but the relative importance of the HLA-A, B and D loci is not yet clearly understood. The conclusion that can be drawn from the study of the genetic control of immune response and clinical transplantation, emphasizes, however, the significance of the HLA-D segment of the MHC.

PART I

INTRODUCTION

CHAPTER ONE - A REVIEW OF THE LITERATURE OF THE GENETICS OF THE
HUMAN AND MOUSE HISTOCOMPATIBILITY SYSTEMS AND THEIR
CLINICAL SIGNIFICANCE

I SEROLOGICALLY RECOGNIZED ANTIGENS FOR HISTOCOMPATIBILITY

(i) Leukocyte Antigens in the Mouse

H-2 the Major Histocompatibility Antigen System
in the Mouse

The MHC in the Mouse

Non-H-2 Systems

(ii) Leukocyte Antigens in Man

HLA the Major Histocompatibility Antigen System in Man

The MHC in Man

Clinical Significance of Leukocyte Matching in Man

Conclusion

II DETERMINANTS WHICH CONTROL CELLULAR IMMUNE REACTIONS WHICH
CANNOT AS YET BE RECOGNIZED SEROLOGICALLY *IN VITRO*

Mixed Lymphocyte Culture (MLC) Reactions

(i) MLC Studies in the Mouse

(ii) MLC Studies in Man

Cell-mediated Lympholysis (CML) Test

(i) CML Studies in the Mouse

(ii) CML Studies in Man

Graft-versus-Host (GvH) Reactions

(i) GvH Reactions in the Mouse

(ii) GvH Reactions in Man

I SEROLOGICALLY RECOGNIZED ANTIGENS FOR HISTOCOMPATIBILITY

In 1936 Gorer working with inbred strains of mice, discovered four blood group antigens and showed (Gorer, 1937) that one of them, antigen II, was also present in fixed tissues and had a major influence in determining susceptibility or resistance to tumour transplants as well as on red cell agglutination. Following the suggestion of Snell (1948), the designation antigen II was changed to H-2.

It was Medawar in 1946, who found that antigens associated with transplantation reactions in the rabbit were present on leukocytes, and interest was thus aroused in the study of these antigens as a possible means of tissue identification and matching. He showed second-set skin graft rejection when the recipients were pre-immunized with leukocytes from the donor. The same phenomenon was shown in man when injections of donor leukocytes into a recipient resulted in accelerated rejection of a skin graft from the same donor (Friedman et al., 1961). In addition, in man cytotoxic antibodies developed against the lymphocytes of the donor after skin grafting (Walford et al., 1964). These two facts provided evidence that human leukocytes carried human transplantation antigens.

(i) Leukocyte Antigens in the Mouse

Since experimental work on the histocompatibility antigens of laboratory animals suggests general principles which may be applicable to man, it is important to look at these briefly in order to clarify the findings in man.

In species other than mouse and man it has also been found that a single histocompatibility locus predominates as the major locus (Table I.1). However, the histocompatibility antigens of the mouse have been studied in greater detail than those of any other experimental animal, and from this work it has become evident that there is a close analogy between man and mouse.

TABLE I.1. LIST OF MAJOR HISTOCOMPATIBILITY LOCI IN OTHER SPECIES

The AgB or H-I locus in rats	(7,9)
The B locus in chickens	(8)
The SL-A locus in swine	(11)
The DL-A locus in dogs	(10)
The RhL-A locus in Rhesus monkeys	(2)
The ChL-A locus in chimpanzees	(1)
The RL-A locus in rabbits	(3,6)
The H-2 locus in mice	(4,5)

1. Balner et al. (1967)	7. Palm (1964)
2. Balner et al. (1971)	8. Schierman & Nordskog (1964)
3. Black (1967)	9. Silvers et al. (1967)
4. Gorer (1937)	10. Templeton & Thomas (1971)
5. Gorer (1938)	11. Vaiman et al. (1970)
6. Matej (1970)	

H-2 The Major Histocompatibility Antigen System in the Mouse

In the mouse there are many different histocompatibility antigen systems determined by independent genetic loci. The H-2 (histocompatibility 2) system is the major serologically detectable histocompatibility system (Snell and Stimpfling, 1966; Iványi, 1970; Klein and Shreffler, 1971). The development by Snell (1958) of numerous H-2 congenic strains (strains genetically identical except for a chromosomal segment containing at least one distinctive gene) established the genetic polymorphism of the system and the major role of H-2 in transplantation.

The murine H-2 antigens are cell surface components determined by the H-2 loci. The H-2 system is now thought to consist of two closely linked loci, H-2K and H-2D (Fig. I.1) within the MHC and controlled by genes on the 17th chromosome. The H-2 genes on an individual chromosome are known as a *haplotype*.

The one locus is probably derived from the other by duplication. Originally the antigens must have been essentially identical, and chemically they are still very similar (Démant et al., 1971; Klein and Shreffler, 1971; Cullen et al., 1972; Démant et al., 1973; Snell and Cherry, 1974). Biochemical analysis of purified H-2 allo-antigens has revealed only two classes of glycoproteins, one carrying H-2K specificities, the other carrying H-2D specificities (Nathenson, 1970). Similar results were obtained by Neauport-Sautes et al. (1973) in 'capping' studies on intact lymphocytes with fluorescent anti-H-2 sera. Each locus has numerous alleles and the antigens determined by each allele evoke, and react with, a number of antibodies (Snell et al., 1973). Forty-one haplotypes have been detected so far in laboratory strains as well as in wild mice.

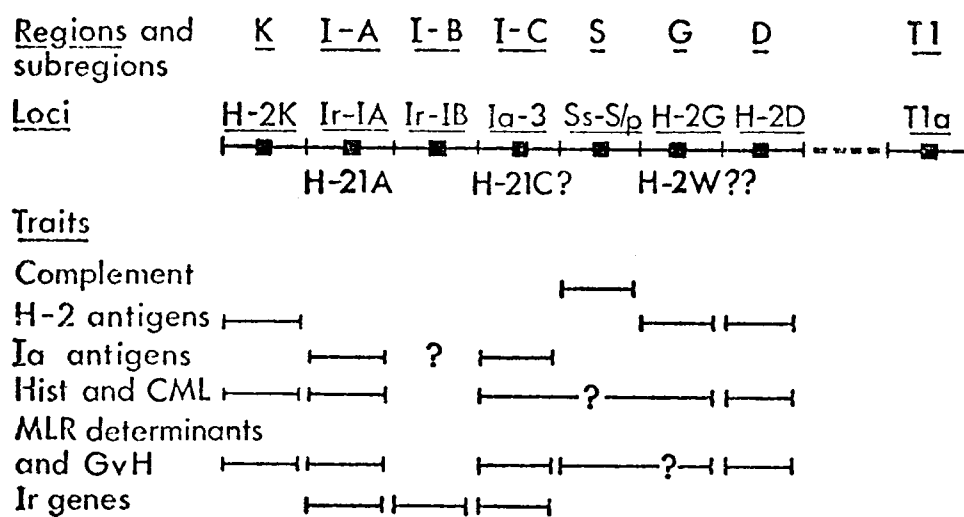


Figure I.1. A schematic diagram of the major histocompatibility complex (H-2) in the mouse and associated traits. Some of the presently recognized regions and sub-regions and the marker-loci are listed. The bars indicate associated genetic traits. The question marks indicate that no definite position for a locus can be determined.

There are 'private specificities' which are uniquely determined by either the H-2K or the H-2D gene. Thus each H-2 haplotype has two private specificities, one determined by the K gene, and one by the D gene (Snell et al., 1971a, b). In addition there are 'public specificities' which map as though they belong in either the K or D genes or both (Klein and Shreffler, 1971; Stimpfling, 1971).

The H-2 antigens are found on a variety of cells, including erythrocytes, leukocytes, epithelial cells and parenchymatous cells of most organs. H-2 typing is usually carried out by serological tests on either erythrocytes or leukocytes. Mouse erythrocytes will agglutinate in the presence of the appropriate H-2 antibodies if special diluent media are used. Testing of leukocytes is usually carried out by means of cytotoxic tests in which lymphocytes, antibody and complement are allowed to react together.

It should be noted that it is still not clear whether the genes controlling the H-2D and H-2K specificities are on a single locus or region composed of several loci carrying identical genes, or whether the genes are complex regions composed of several loci, each responsible for the control of a part of the final number of different antigenic specificities produced by the region.

The knowledge of the H-2 structure is largely based on recombinants within the H-2 region. At least thirty-five recombinants have occurred in crosses specifically designed for H-2 analysis (Shreffler, 1970). The recombination fraction between the D and K subdivision of the mouse H-2 system is approximately 0.5 - 1% (Shreffler, 1967; Festenstein, 1975). These H-2 recombinations have permitted subdivision of the chromosomal segment between H-2K and H-2D and in sorting out various traits to various regions of the complex. This chromosome segment between and

including H-2K and H-2D is termed the major histocompatibility complex (MHC) or H-2 complex on the 17th mouse chromosome.

The MHC in the Mouse (Fig. 1.1)

According to the data available at present, the MHC in the mouse is thought to include at least six distinct genes, defining six regions or subregions, K, I-A, I-B, I-C, S and D. Recently, another region, the G region has been added, between the S and D regions (David et al., 1975; Klein et al., 1975). Although the Tl region has not been formally included as part of the MHC, it will be discussed because it has an important relationship with H-2 as it controls similar traits and is very closely linked.

These various genetic regions or subregions within the MHC of the mouse and the marker loci, as depicted in Fig. 1.1, will now be discussed as the information derived from them is applicable to man.

The K and D regions: From the present concept of the genetic control of H-2 antigenic specificities it can be assumed that there are two regions, H-2K and H-2D. Most of the traits ascribed to the K and D regions are probably controlled by single H-2K and H-2D genes, whose products are probably cell-membrane proteins and are the classic H-2 antigens. These H-2 antigens detected by serological tests, are also referred to as serologically defined (SD) antigens and are situated at the left and right ends of the MHC respectively.

The strong graft rejections associated with the MHC of the mouse have always been attributed to the serologically detected

antigens. However, this view has been questioned by some authors (Amos, 1971; Bailey et al., 1971), and the evidence of Boyse et al. (1972) who defined the H(TIa) (see T1 region) and of Klein et al. (1974a) defining H-2I (see I region) indicates that graft rejection can be mediated by non-H-2K, non-H-2D genes, which are associated with the MHC but whose products are not serologically defined. Shreffler and David (1975) in a review concluded that it seems very likely that the H-2K and H-2D gene products as detected serologically are also histocompatibility antigens, but conclusive proof is still lacking.

The K and D regions also play some role in the control of the mixed lymphocyte culture reaction, cell-mediated lympholysis and the graft-versus-host reaction that will be discussed in more detail in Part II of this Chapter.

The I region: There has been much confusion about the genetic interpretation of the I (immune response) region and although varying numbers of subregions have been suggested by different groups (Sachs et al., 1975; Dorf and Benacerraf, 1975), there is presently only good evidence for two subregions I-A and I-C and possibly a third, the I-B region as depicted in Fig. 1.1 (Klein and Hauptfeld, 1976; Shreffler et al., 1976). The experimental evidence for these subregions will now be discussed.

The I region in the central segment of the mouse MHC is of particular interest because during the last few years it has become apparent that it plays a major role in numerous immunological phenomena including genetic control of immune responsiveness, mixed lymphocyte and graft-versus-host reactions, graft rejection, and cellular interaction in the immune system (Shreffler and David, 1975). Much effort has been put into the identification of the I region gene products as understanding of the nature and inter-relationships of these reactions depends to a large extent on

knowledge of the molecular entities involved.

The immune response-1 (Ir-1) gene, was first described by McDevitt and Sela (1967). It controls the immune responsiveness to a series of branched synthetic polypeptide antigens, and is situated between the H-2K and Ss-Slp loci (McDevitt et al., 1972).

Experiments by Lieberman et al. (1972) on a recombinant strain led to the division of the I region into two subregions : I-A (formerly called Ir-1 and I-B (formerly called Ir-IgG). From further studies it now seems likely that there must be at least three distinct Ir genes in the I region, dividing it into the I-A, I-B and I-C subregions (Snell et al., 1976). However, it must be emphasized that the definition of the I-B subregion, by its responses to the Ir-IgG (Lieberman et al., 1972), Ir-LDH_B (Melchers et al., 1973) and Ir-Nase (Lozner et al., 1974) hinges upon a single H-2 recombinant B10.A (4R), which behaves strangely in every respect (Klein and Hauptfeld, 1976). Therefore most authors in this field have reserved judgement about the existence of the I-B subregion.

When several pairs of recombinant strains differing only in the I and S regions became available, a number of laboratories produced a variety of different antisera that reacted with cell-membrane allo-antigens, genetically controlled by the I region - now referred to as *Ia for immune associated* (David et al., 1973; Götze et al., 1973; Hauptfeld et al., 1973; Hämmerling et al., 1974; Shreffler et al., 1974). Simultaneously Sachs and Cone (1973) were able to identify Ia antigens with conventional anti-H-2 sera by making them Ia-specific after absorption with H-positive/Ia negative cells from syngeneic animals. All Ia specificities seem to fall into the I-A subregion except for three different Ia specificities from eight different recombinants which defined the new I-C subregion. From recent experimental data presented by Shreffler et al. (1976) it appears that no known Ia determinants

are controlled by the I-B subregion. These Ia antigens have a different tissue distribution from the H-2K and H-2D antigens, being present mainly on B cells, but also on some T lymphocytes, sperm macrophages and epidermal cells. They are heterogeneous in function and some may also be lymphocyte activating determinants (Lads) (Festenstein, 1975).

Shreffler and David (1975) emphasized that the Ir genes that map in I-A and I-B subregions are not necessarily the same genes that control other traits mapped in the same subregions. Some of these other reactions, like the mixed lymphocyte and graft-versus-host reaction, where the I region appears to be implicated will be discussed in Section II of this Chapter. As shown in Fig. I.1, the existence of a strong histocompatibility locus within the I region, designated H-2I (now called H-2IA or H-2A) has also been postulated (Klein et al., 1974a) and at least one, but more likely, two weak loci (H-2IC and H-2W) (Klein and Hauptfeld, 1976).

The S region: The Ss (serum serological) and Slp (sex-limited protein) genes in this region are located between the H-2K and H-2D loci. These determine quantitative and allotypic variations in serum β globulin (Shreffler and Owen, 1963; Passmore and Shreffler, 1970; Démant et al., 1973). Studies by Démant et al. (1973) have shown that genes in the Ss-Slp region are also involved in the control of the complement level. The S region thus far appears not to be involved in control of cell-membrane allo-antigens, histocompatibility factors or immune responses.

The G region: The best-established locus between the S and D regions of the mouse MHC is the H-2G locus. This locus is defined by specificity H-2.7 (David et al., 1975; Klein et al., 1975). H-2.7 is unique among H-2 specificities in that it is much more abundant on red cells than on lymphocytes.

Evidence concerning the association of any histocompatibility effect with the G region is ambiguous. Loci determining a weak mixed lymphocyte response may also be located in the G region. It is interesting to note that in the HLA as well as the H-2 system there is a third locus with some resemblance to the major cell-membrane allo-antigen determining loci.

The Tl region: This region is marked by the Tla locus (thymus and leukaemia antigens) which controls allo-antigens expressed on thymocytes of some strains and on cells of some leukaemias (Old et al., 1963). It maps on the right side of the H-2D region and is linked to this region (Boyse et al., 1964) so that Tla incompatibility frequently accompanies H-2 incompatibility. Boyse et al. (1972) in skin-graft experiments between two pairs of congenic strains, differing only in the Tl region demonstrated the presence of a gene or genes, denoted H(Tla), determining antigens in the Tl region. It was subsequently confirmed by Démant and Graff (1973).

Non-H-2 Systems

Very little is known about the loci controlling weak transplantation antigens in species other than the mouse. In general the H-2 immunity is much easier to induce and detect than non-H-2 immunity. Also, the H-2 immunity is usually much stronger than the non-H-2 immunity, as manifested by the speed of onset and vigour of the response. Non-H-2 differences may, however, be the explanation for the rejection of skin grafts exchanged between mice strains which have the same H-2 genotype. Through the use of congenic strains it has been reported that there are ten non-H-2 autosomal H-loci and there are sixteen others under investigation (Graff and Bailey, 1973). At each of these non-H-2 loci, varying numbers of alleles have been found. Whereas serological techniques

can be applied in H-2 typing, histogenetic techniques have been necessary for typing non-H-2 alleles. When donor and host strains differ by H-2 plus non-H-2 loci the humoral response is quite strong. The response is usually somewhat weaker when the donor and host differ only at the H-2 locus (Snell, 1970). The production of antibodies to non-H-2 antigens has been reported only sporadically (Winn et al., 1958). In recent investigations non-H-2 antibodies in low titres have been demonstrated by haemagglutination (Cantrell and Hildemann, 1973).

(ii) Leukocyte Antigens in Man

As mentioned before, transplantation antigens seem to be present on leukocytes, and this observation led to the study of these cells and the major human transplantation system, the HLA system (HLA - Human Leukocyte, locus A). It was found that the HLA-A and B antigens are not only present on leukocytes and platelets, but on all nucleated cells and that they are determined by genes situated on a pair of autosomal chromosomes, C6 which carries the MHC in man (Westerveld et al., 1973).

Leukocyte and platelet antigens which are independent of the HLA system are known, but these systems do not seem to be involved in transplantation. Most of the emphasis in this study is placed on the HLA system and its antigens, which are the serologically defined (SD) antigens and the lymphocyte defined (LD) determinants which lead to lymphocyte activation either *in vitro* (MLC test) or *in vivo*.

HLA The Major Histocompatibility Antigen System in Man

In 1954 Dausset investigated sixty cases with leuko-agglutinating antibodies and found that 90% of these had received multiple transfusions. He concluded that transfusions might play

an important role in the development of these antibodies. This has been confirmed by many workers, and it is now generally accepted that leukocyte antibodies may be formed after multiple transfusions as an immunological response directed against foreign iso-antigens on the leukocytes in the transfused blood. Dausset suggested that these antisera could serve for serotyping of H antigens in humans.

In 1958 Payne and Rolfs as well as Van Rood et al. (1958) independently showed that iso-antibodies may also be formed against leukocytes during pregnancy. At least 10-40% (depending on technique used) of women with multiple pregnancies form antibodies against leukocytes. These antibodies are found to be directed against antigens present on leukocytes of the husband and are formed by the mother as a response to foreign foetal antigens. Leukocyte antibodies may also develop after experimental and clinical grafting of skin and organs.

The first leukocyte antigen detected by allo-immune human sera was described by Dausset (1958). He found that sera from seven out of twenty-seven polytransfused patients showed more or less the same agglutination pattern when tested against a panel of leukocytes. Leukocytes from the seven patients were not agglutinated by their own serum nor the serum from the other six patients in this group, and Dausset concluded that they lacked the same antigen which he called MAC. He also reported that monozygotic twins showed identical agglutination patterns when examined with different leukocyte antisera, but dizygotic twins did not. Leukocyte antigens therefore seemed to be genetically determined, and this was proved by family studies (Payne and Rolfs, 1958; Van Rood et al., 1959).

The first leukocyte group system was described by Van Rood in 1962, when he introduced computer techniques in this field. On the basis of the chi-square values, a computer programme helped to select sera with highly similar reaction patterns. Three sera were found to give agglutination patterns which were significantly similar but not identical. From this it was concluded that the strong association between these three sera was caused by the sera sharing one or more antibody component. These antibodies were called anti-4a. In addition, five other sera with similar but contrasting distribution to the three 4a antisera were found, thus suggesting that the antigens 4a and 4b possessed by the individual reacting with the respective antisera might be determined by alleles. Family studies (Van Rood and Van Leeuwen, 1963) confirmed this and showed that the 4a and 4b genes were inherited as simple Mendelian autosomal co-dominant alleles. The system was called Leukocyte Group Four.

In the years 1964-1967 this approach helped to define a number of leukocyte antigens in different laboratories (Table I.2) (Kissmeyer-Nielsen and Thorsby, 1970). Dausset et al. (1965) described ten leukocyte antigens (1-10). On the basis of significant positive and negative correlations between the respective sera and defined antigens, it was concluded that the ten antigens described belong to one complex genetic system which was named Hu-1.

In 1965 it was demonstrated at a serological workshop in Leyden that some of the antigens identified by different workers were closely related but none of the sera gave completely identical results (Histocompatibility Testing, 1965). At a later workshop arranged in Torino in 1967, it was found that many antisera gave identical results against the cell panel. In addition, results obtained at the workshop and data presented by three groups of

TABLE 1.2

HL-A NOMENCLATURE AS SUGGESTED BY THE NOMENCLATURE COMMITTEE IN 1968 AND PREVIOUS DESIGNATIONS^a

(From Kissmeyer-Nielsen and Thorsby, 1970)

New HL-A nomenclature ^b	Amos	Batchelor	Cepellini	Dausset	Kissmeyer- Nielsen	Payne/ Bodmer	Van Rood	Shulman	Terasaki	Walford
HL-A1 ^c	19	1	To-8	11	LA1	LA1	LA1	-	1	Lc-1
HL-A2 ^c	1	5	To-9	1 or Mac	LA2	LA2	8a	PIGrLy ^{B1}	2	Lc-2
HL-A3 ^c	4	-	To-10	12	LA3	LA3	LA3	H111	8	Lc-3
HL-A4										
HL-A5 ^d	45	25	To-5	5	-	-	Da5	-	6	-
HL-A6										
HL-A7 ^d	2	-	To-20	10	-	4d	7c	-	5	Lc-8
HL-A8 ^d	41	2	To-7	8	-	7d	7d	-	11	Lc-7

a A dash (-) indicates that no symbol has been allocated within the nomenclature concerned.

b HL-A4 has been reserved for one of the higher frequency 4a factors and HL-A6 for a 4b factor.

c LA series.

d Four series.

workers (Ceppellini et al., 1967; Dausset et al., 1967; Van Rood et al., 1967), showed that most of the leukocyte antigens are determined by closely linked genes at one chromosomal region. Following the recommendation of the WHO Nomenclature Committee in 1968, this was called the HLA locus (Human Leukocyte locus A). It is probably the major histocompatibility system in man which is comparable to the H-2 system in the mouse.

Great confusion has been caused by the different nomenclatures used by the various workers in the field. At the Torino Workshop in 1967, the Nomenclature Committee was appointed, and in 1968 they put forward the view that the factors typed for should be indicated by a number following the symbol for the system, e.g. HL-A1, HL-2, etc.

Table I.2 shows the correlation between this nomenclature and the previous 'local' designations at the time. HL-A1, HL-A2 and HL-A3 belong to the LA series of antigens first described by Payne et al. (1964). In 1969 Dausset et al. named this series the First series of HL-A antigens. Many more alleles have since been found to belong to this series of the HL-A system. In 1973 Botha and his co-workers reported a new antigen within this complex, BK (now named HLA-AW43). This antigen appears to be unique among Southern African and hybrid populations partly derived from them. It has been demonstrated in our laboratory in Khoikhoi Nama, Central !Kung (San), Cape Coloureds, two Negroid populations, Xhosa and Dama, and two unrelated South African Caucasoid individuals (Botha et al., 1973, 1975a, b; Nurse et al., 1975). So far it has not been reported in any other population group in the world.

HL-A5, HL-A7 and HL-A8 belong to the Four series of antigens described by Van Rood et al. (1967). This series was called the 4A/4B series and the 4/6/7 series by Ceppellini et al. (1967).

The antigens 7c and 7d of Van Rood's original Seven series of antigens are closely related to HL-A7 and HL-A8. Several more alleles have been found to belong to the Four series, called the Second series of HL-A antigens by Dausset et al. (1969).

The MHC in Man (Fig. 1.2)

Almost two years after the commencement of this study, the term major histocompatibility complex (MHC) was introduced by Bach (1973) to include the genetic information carried by the entire chromosomal region responsible for histocompatibility. He also suggested the terms serologically defined (SD) and lymphocyte defined (LD) on the basis of the current methods of detection of these genes. More recently serological techniques have been described by Van Leeuwen et al. (1973) and others, which appear to define the LD antigens. For many years prior to this the HLA system, consisting of the LA (or locus A) and the Four (or locus B) series of antigens, and the H-2 system, were the only known instances of the histocompatibility systems in man and the mouse.

The MHC of man, which has its analogue H-2 in the mouse and also in many other species, is now known to contain many genes controlling cell surface determinants, immune response differences, some components of the complement system (in man C3 pro-activator (Bf), C2 and probably C4) and perhaps other related functions connected with the general cell-cell recognition (Bodmer, 1975).

As a result of the increasing number of loci being defined in the region, the WHO Nomenclature Committee which met in Aarhus, Denmark, in June 1975, after the Sixth Histocompatibility Workshop, recommended the following :

- i) HLA (the hyphen previously used in the designation of the HLA system has been dropped in order to minimize the number of symbols required to define loci and specificities of this system) should be the name given to the whole MHC region on chromosome 6.
- ii) The individual loci within it will be called :-
 - Locus A (formerly LA or first or SD-I locus)
 - Locus B (formerly the FOUR or second or SD-2 locus)
 - Locus C (formerly the AJ or third or SD-3 locus)
 - Locus D (formerly MLC-I or Major MLC locus or LD-I).
- iii) The probable order of these four loci is:
D, B, C and A as shown in Fig. 1.2.
- iv) New loci of the HLA region will be named accordingly as they become clearly identified (Bodmer, 1975).

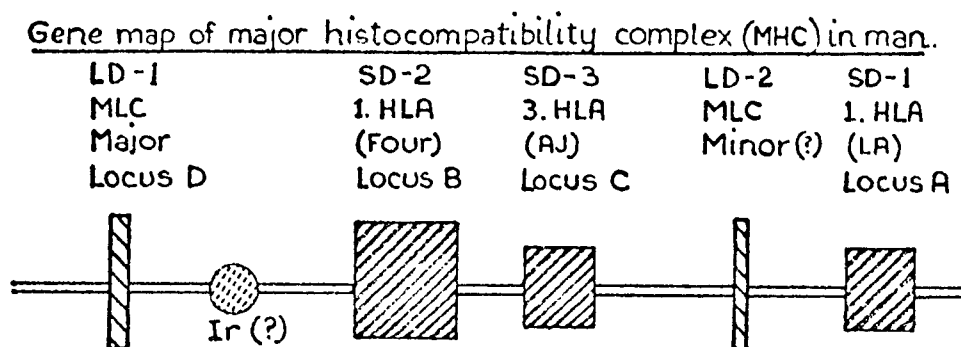


Figure 1.2. A schematic concept of the HLA region. The probable order of the loci is given. The Ir genes and the ones predisposing for certain diseases are thought to lie in the region between the D locus (Major MLC) and the B locus but its exact position is still not clear.

HLA-A, B, and C loci: The antigens at these three loci are serologically identifiable and are also known as SD antigens. Two segregant series of HLA antigens - those of locus A and locus B - have been known to exist in man for some time, and therefore most of the earlier work on the HLA system includes only the antigens of the A and B loci. The possibility of a third segregant series of HLA antigens on the AJ or SD-3 or the C locus, closely associated with the B locus, was suggested as early as 1970 by Sandberg et al., and later confirmed by Svejgaard et al. (1972). As a result of the Sixth Workshop in Aarhus, locus C is now serologically well defined.

Each chromosome thus carries information for one A, one B, one C and one D alternative allelic antigen. Such a set of antigens jointly transmitted by the same parental chromosome is called a *haplotype*, and each nucleated cell definitely carries the information for four antigens belonging to the A and B loci,

and probably also two for the C and two for the D loci. Family studies have proved that the A and B antigens are determined by alleles and inherited as co-dominant traits, i.e. both antigenic specificities belonging to one series are expressed in the heterozygote (Svejgaard et al., 1970). When less than four antigens are recognized in an individual, this is due to homozygosity and/or the presence of antigens which cannot be recognized by the available antisera.

Recent genetic data from the Sixth Histocompatibility Workshop, on the C locus specificities, suggest that the same may be true of them. Table A shows the nineteen antigens of the A locus, twenty of the B locus, and five of the C locus which were recognized by the 1975 Workshop sera (WHO, 1975). In the majority of families the HLA (loci A, B and C) haplotypes are handed on unaltered to offspring, but several cases of *crossing-over* between the A and B loci series have been described (Kissmeyer-Nielsen et al., 1969; Dausset et al., 1970; Bodmer et al., 1970; Mayr and Mickerts, 1971; Eijsvoogel et al., 1972c). In 1971 Svejgaard et al. collected data from 309 families which yielded information on linkage between the A and B segregant series of the HLA transplantation system and calculated a recombination fraction of 0.8%. Recombinations may account for a number of unexpected HLA-A, B typings when studying families.

Recently a few instances of recombination of the C locus with the A and B loci have been reported. In eight of these recombinations, the C locus antigen followed the antigen of the B locus during the crossing-over event, and in two the C and A series antigens stayed together (Belvedere et al., 1975; Staub-Nielsen et al., 1975). These data and the observation that in chromosomes derived by crossing-over between the A and B loci antigens, the C locus antigens usually segregate with the B

locus, suggest that the B and C loci are close to each other. As previously stated, recombination is a relatively rare event, but when considered at a population level, it could account for all possible haplotypic combinations between the alleles of the A and B loci (Ceppellini and Van Rood, 1974).

Despite the fact that crossing-over is known to take place between various HLA loci, some HLA alleles can be found together on the same haplotype with a much higher or lower frequency than expected from the gene frequency of the alleles in any given population. This phenomenon is called *linkage disequilibrium* and is frequently noticed within the HLA system. This genetic association or linkage disequilibrium is usually expressed as a delta value (Δ) that is defined as the difference between the observed haplotype frequency and that expected from the product of the gene frequencies (Mattiuz et al., 1970). The delta values for the different combinations of alleles vary considerably from population to population. For instance, in our laboratory among South African Caucasoids, the highest delta value is found in the HLA-A1, B8 haplotype; in the Xhosa (a Southern African Negroid population) in the HLA-AW30, BW42 haplotype; and in the Cape Coloureds the HLA-AW24, BW40 haplotype. The reason for these associations is unknown.

Using twenty antigens at the A locus and twenty-one antigens at the B locus (one blank allele is counted at both the A and B loci) we calculated that $20 \times 21 = 420$ haplotypes may exist, the majority of which have actually been observed. It can thus be calculated with the formula:

$$\text{Genotypes} = \frac{h(h+1)}{2}, \text{ where } h = \text{number of haplotypes,}$$

that 88,410 genotypes and 48,510 phenotypes can be found. The frequencies range from 1% for the most common (e.g. A1,

B8/A3,B7 in Caucasoids) to extremely rare haplotypes with frequencies of 0.01%. These high frequencies illustrate the problem in selecting unrelated individuals, who are identical, for clinical transplantation. This has become an even greater problem as the number of antigens and the loci involved are now known to be much larger. Among siblings however, the likelihood of finding an HLA-A, B identical is 25% because only four different genotypes can be found, excluding the rare occurrence of crossing-over.

The study of families with recombinants has provided important information about the relative importance of the particular parts of the MHC with regard to the outcome of transplants.

MLC determinants and the D locus: The mixed lymphocyte culture (MLC) reaction is measured by the proliferative response when the lymphocytes of two unrelated individuals who are HLA-A, B non-identical are cultured together. In 1972 in studies with Eijsvoogel we showed that there are probably two MLC loci in man (Fig. 1.2). These loci carry determinants identified by the mixed lymphocyte culture (MLC) test (Eijsvoogel et al., 1972a, b). Although the term *locus* will be used for both the *Major* MLC locus (or HLA-D locus) and the '*minor*' MLC locus, it must be emphasized that it is still not clear whether this corresponds to one structural gene locus or a cluster of very closely linked loci.

The Major MLC locus, LD-I, now called the D locus, is a strong locus which maps outside the HLA-A, B and C regions of the MHC, and the '*minor*' MLC locus or LD-2 is a much weaker locus, closely linked to the A locus. In man the D locus is closely linked to, but outside, the B Locus, whereas in the

mouse the strong MLC locus (or loci) in the I region is also close to H-2K, the analogue of HLA-B in man but probably inside the H-2K and H-2D regions.

Following the discovery that the determinants of the MLC reaction were controlled by one or more loci that were genetically separable from the A and B loci although closely linked to them, a number of laboratories clearly established that it was possible to use MLC reactions to a set of specially selected lymphocytes to define MLC determinants, i.e. to do 'MLC typing' (see Methods). Family and population data presented at the Sixth International Histocompatibility Workshop also suggested that there are six well-defined D locus determinants, and possibly two others not so well defined which behave as if controlled by a series of alleles at this locus (Table A). It was also clear that new information will probably lead to 'splitting' of these MLC determinants and possibly of the D locus itself as in the case of the serologically defined specificities of the A, B and C loci. The strong linkage disequilibrium between the D and B loci alleles was confirmed (Bodmer, 1975).

Ir region: The immune response genes (Ir genes) and those predisposing to certain diseases are thought to lie in the region between the B and D loci in man, as shown in Fig. 1.2. Nearly all associations so far observed between disease and HLA primarily concern B or D series determinants, whereas the association with A and C series antigens is secondary. This Ir region was first described in the mouse where it lies between the H-2K and H-2D loci.

At the Sixth International Workshop on Histocompatibility Testing in Aarhus, a number of laboratories using different serological techniques described Ia-type specificities in man. These Ia antigens differ from the HLA-A, B and C antigens in having a tissue distribution that is relatively specific, being present mainly on B-lymphocytes and rarely on T-lymphocytes. Initial studies on Ia specificities suggest that this is a complex and polymorphic system, and their significance for clinical transplantation and disease association may be of utmost importance.

Clinical Significance of Leukocyte Matching in Man

With the advent of *human organ and skin transplantation*, much interest was aroused in the further study of HLA-A, B typing and its effect on the clinical outcome of transplantation.

In 1970 Kissmeyer-Nielsen and co-workers said:

"The HLA system seems to be the only genetic system (besides the ABO system) which has major influence on human transplantations ... other alleles determining antigens with decisive influence on human transplantations must - if they exist be closely linked with the LA and FOUR series ... This important statement was based on the following observations :

1. Prolonged survival of skin grafts between HLA identical *siblings*;
2. uniformly good results of kidney transplantation between such siblings; and
3. lack of stimulation when mixed lymphocyte cultures are performed between LA- and FOUR-identical siblings."

(Kissmeyer-Nielsen et al., 1970,

p. 105)

Reports from some other centres also gave encouraging results with regard to HLA-A, B matching and transplantation of kidneys or skin in siblings and to a lesser extent in transplant programmes using unrelated donors (Batchelor et al., 1971; Morris et al., 1971).

However, in America, Terasaki and co-workers did not find a good correlation, and they cautioned transplanters to view their results with reservation.

"In order for decisive correlation to be obtained with any system of matching, it would be expected that incompatibility would consistently cause failure, and that uniform compatibilities would assure success. Obviously, neither premise has been completely fulfilled. Successful transplantation has often been achieved, despite frank antigenic mismatches. There have even been numerous instances of proven or probably multiple incompatibilities with an excellent result.... In addition it is necessary to explain failures despite apparently good HLA matches. The presence of such cases in every large series means that other factors may significantly effect the results after renal transplantation. These could include other antigens within or outside the HLA system, surgical technical considerations, and original host disease to mention only three. Consequently, while good HLA matching should be a desirable condition in performing organ transplantation, the HLA system may only be the tip of an enormous and as yet poorly understood biological iceberg."

(Halgrimson et al., 1971, p. 144)

It became evident to many that the clinical outcome of renal transplants from live donors to related family recipients correlated well with the closeness of the HLA-A, B match, but the value of HLA matching in unrelated transplants was not clear. Oliver and Van Rood independently showed

that the B locus incompatibilities of the HLA system have a more important role in graft rejection than those of the A locus, especially in individuals with circulating anti-HLA antibodies (pre-immunized individuals) (Oliver et al., 1972; Van Rood et al., 1973). In the mouse the H-2K end of the H-2 system had also been found to play a dominant role in skin-graft rejection (Démant and Nouza, 1971; Klein, 1972).

In spite of the evidence for the important role HLA-A and HLA-B antigens played in vital organ graft survival, especially in related transplants, it became clear that HLA matching was not the full answer to histocompatibility because even HLA identical sibling transplants still require immunosuppression.

Conclusion

1. Because other loci within the MHC must also determine strong histocompatibility antigens and because of close linkage, these loci will almost always be identical in HLA identical siblings.
2. Loci outside the MHC may influence graft survival which may explain rejection between HLA identical siblings.

II DETERMINANTS WHICH CONTROL CELLULAR IMMUNE REACTIONS WHICH CANNOT AS YET BE RECOGNIZED SEROLOGICALLY *IN VITRO*

These determinants should not be called antigens as long as it has not been conclusively shown that they can elicit a specific immune response either *in vivo* or *in vitro*. For many years the HLA-A and HLA-B, and the H-2 antigens were the only known products of the major histocompatibility complex in man and the mouse. It was only after the description of the mixed lymphocyte culture (MLC) test by Hirschhorn et al. (1963) and Bain et al. (1963, 1964) that several investigators tried to demonstrate a relationship between the mixed lymphocyte reaction (MLR) phenomenon and histocompatibility.

The first indication of association between MLR and the MHC in the mouse was reported by Dutton (1966), using congenic strains. In man, it was shown that stimulation in the MLR was primarily due to the antigens of the A and B loci of the HLA system (Amos and Bach, 1968). These findings suggested that the *in vitro* parallel of graft rejection was the genetically controlled MLR phenomenon, caused by differences of the major histocompatibility chromosomal region. Since MLC activation in mice is dependent on differences in the MHC, it was assumed that the classical H-2 antigen differences were responsible for the stimulation.

However, Rychlíková et al. (1970, 1971) demonstrated that the major H-2-associated MLR stimulation was due to differences controlled by the K-end of the mouse MHC. Further analysis of intra-H-2 recombinants showed that K-end differences stimulated almost as strongly as complete MHC differences, but D-end differences stimulated weakly or not at all. However, at about the same time Yunis and Amos and others suggested that the determinants of the MLC reaction in man were controlled

by one or more locus genetically separable from the A and B loci, although closely linked to them (Yunis and Amos, 1971; Yunis et al., 1971). These developments stimulated additional research, including this study, to localize the MLC determinants more precisely in man.

It has now been determined, by our studies with Eijsvoegel, in collaboration with others, that the strongest MLC response in man is caused by a separate locus (now called the D locus) distinct from the A and B loci (Eijsvoegel et al., 1972a, b,c), and a weaker response by the 'minor' MLC locus (see gene map of MHC in man, Fig. 1.2) (Eijsvoegel et al., 1972a, b; Mempel et al., 1973a). The experimental evidence for this will be presented in more detail later in this thesis. From the discussion in the section on MLC studies in the mouse, it will become clear that new recombinant congenic strains of mice were of particular importance for the genetic resolution of the MLC determinants in the mouse.

Several authors have recently suggested that the allograft response can be divided into at least two phases: an initial *sensitizing phase* of allograft reaction, when competent (T) cells react to foreign histocompatibility antigens on the transplanted tissue by proliferation and differentiation; and a late *destructive phase* in which the graft is rejected. The *in vitro* parallel of the former is the MLC reaction and of the latter the cell-mediated lympholysis (CML) test. The CML test was first described by Lightbody et al. (1971) and based on observations by Hirschhorn et al. (1965) and Solliday and Bach (1970).

In the CML test normal lymphocytes (e.g. A) are sensitized (because of differences at the MLC loci) by Mitomycin-C-treated stimulating cells (e.g. B_m) in a one-way MLC. After 6-10 days *effector* cells are produced, which can specifically kill freshly prepared ⁵¹Cr-labelled, tumour or phytohaemagglutinin (PHA) blast (*target cells*) syngeneic to the MLC stimulating cell B, provided there are HLA-A and B incompatibilities between them. The amount of ⁵¹Cr released from the cells, expressed as a per cent of maximal release, is a measure of the amount of cytotoxicity which has taken place (Häyry and Defendi, 1970; Svedmyr and Hodes, 1970; Häyry et al., 1972).

The results obtained using the MLC and CML tests have given further information regarding the genetic structure of the MHC in man and the mouse. Although there are minor differences between the findings in the two species, the same general principles are applicable to both. These findings will be discussed in this Chapter.

The so-called graft-versus-host (GvH) reaction occurs when competent lymphoid cells are transferred from a donor to a recipient who is unable to reject them. These grafted cells survive and have time to recognize the host antigens and react immunologically against them. Instead of the normal transplantation reaction of host-versus-graft, the reverse takes place, the so-called GvH reaction.

In young mice there can be inhibition of growth (runting), splenic enlargement and haemolytic anaemia (due to the production of antibodies to red cells). In the human, anaemia, fever, weight loss, diarrhoea, rash and splenomegaly are seen. The stronger the transplantation antigen differences, the more severe the reaction will be. The regions on the MHC of man and the mouse that appear to be responsible

for the GvH reaction will be discussed later in this Section.

Mixed Lymphocyte Culture (MLC) Reactions

The MLC test is one measure of MHC disparity (Widmer et al., 1973a). When lymphocytes from two unrelated mice or individuals are mixed *in vitro*, a proliferative response is usually observed (see Methods for details of test).

(i) MLC Studies in the Mouse

In the mouse three separate genetic systems, alleles of which segregate independently from one another, have been shown to cause stimulation in culture if different in two animals. The most important is in the I region of the MHC. The second region which is *not* linked to H-2 and the MHC, the Mls (originally M) locus, has been demonstrated by Festenstein et al., 1971, 1972, 1974) (see below for further details on the Mls locus). The third region which carries the theta (θ) antigen, an antigenic marker on thymus-dependent lymphocytes of the mouse, also appears capable of introducing MLC activation (Peck et al., 1973).

The evidence for the existence of MLC loci separate from the HLA-A and HLA-B loci in man led to a search for similar separability of the mouse MLR determinants from the determinants of known H-2 antigens by Bach et al. (1972a, b) and Meo et al. (1973). Their results suggested that the major (but not only) MLR stimulation by the MHC was controlled by the I region of the complex. (In the studies of Rychlíková et al. (1970, 1971) previously mentioned, the

K-end differences had also included I region differences). More recently Shreffler and David (1975) concluded from studies of mice with a recombinant haplotype derived from a cross-over within the I region, that the most likely location for the strong MLR determinant(s) is the I-A subregion and that the H-2K locus serves as a serological marker for this strong MLC locus.

Attention must once more be drawn to the analogy with the D locus in man, but there may be one important difference: unlike in man, the strong MLC locus in the mouse may be located inside the H-2K and H-2D region. Current genetic data indicate that the K region, the D region, a site on one or the other side (or both sides) of the S region, and possibly, a discrete site (weak) the G region, positioned between the Ss and H-2D loci of the MHC play relatively minor roles in MLR stimulation, although quite frequently differences in these regions yield statistically significant stimulation (Shreffler and David, 1975). The information concerning the MLR in the mouse, is summarized in Fig. 1.1 in the column marked MLR determinants and GvH.

It should be stressed that there is no evidence either for the strong I region-associated or the weak K, D and S region-associated MLR stimulation, whether one is dealing with a single locus or several loci.

Similar to the superiority of K-end incompatibility over the D-end incompatibility in the MHC of the mouse is the superiority of incompatibility at the chromosomal segment bearing the B locus over the incompatibility at the chromosomal segment bearing the A locus in man (Eijssvoogel et al., 1972a, b).

A single genetic region known as the *Mls* (*mouse minor MLC-stimulation*) locus, independent of the H-2 and other histocompatibility loci, has been demonstrated in the mouse (Festenstein, 1966, 1970, 1973, 1974, 1975; Festenstein et al., 1972). This locus is polymorphic and was detected and characterized because of its strong lymphocyte activating properties. It has four co-dominant determinants M^1 to M^4 . At present these Mls locus determinants are not serologically detectable. The Mls locus behaves as a single gene complex, or linkage group, and it is not yet known whether it is the first detectable marker of an undiscovered linkage group. The tissue distribution of Mls locus gene products has not been established, but present evidence is that they are present on lymphocytes but not on erythrocytes (Festenstein and Démant, 1973). Studies by these authors have also shown that there are no gross allogeneic effects of the Mls locus on skin graft, heart-muscle graft, spleen-cell transplantation, allogeneic bone-marrow transplantation, parabiosis and tooth-germ transplantation.

(ii) MLC Studies in Man

The first indication that MLC activation is induced by genetically determined factors in humans was the lack of MLC reactivity between lymphocytes of monozygotic twins found by Bain and Hirschhorn (Bain et al., 1963; Bain and Lowenstein, 1964; Hirschhorn et al., 1963).

The position of the genes responsible for eliciting MLC reactivity was thought to be in the same chromosomal region as the A and B loci when Amos and Bach (1968) and later others (Sørensen and Kissmeyer-Nielsen, 1969; Schellekens et al., 1970) showed that lymphocytes from HLA

identical siblings scarcely ever show MLC stimulation. In 1971 Yunis et al. first proposed that the MLR stimulation in man is mediated by a genetic determinant closely linked to the A and B loci, but distinct from them, and separable by recombination. Findings by several workers have since built up evidence to support this:-

1. Cases of MLC stimulation between HLA-A and B *identical siblings* have been reported (Amos and Bach, 1968; Eijsvoogel et al., 1970, 1972a; Yunis and Amos, 1971; Mempel et al., 1972). The frequency of such anomalous responses is about one per cent.
2. Cases of non-stimulation in MLC between HLA-A and B *non-identical siblings* were found by us (Eijsvoogel et al., 1972a, b) and others (Dupont et al., 1971; Mempel et al., 1972).

In line with these findings are the following observations:

Only a few cases of non-stimulation between HLA-A and B *non-identical unrelated individuals* have been reported (Pentycross et al., 1972; Mempel et al., 1973a, b).

There is a relatively low frequency (10%) of non-stimulation between HLA-A and B *identical unrelated individuals* (Eijsvoogel et al., 1970; Sørensen and Staub-Nielsen, 1970; Sengar et al., 1971; Mempel et al., 1973b).

In 1971 Yunis and Amos reported a family in which recombination between the A and B loci and an MLC locus appeared highly probable (Yunis and Amos, 1971). However, the proof that MLC reactivity is determined by a region or locus distinct from the known HLA-A and B loci, outside the A—B interval on the side of the B locus, was obtained in family KA we studied together with Eijsvoogel and co-workers which will be presented in Chapter Seven in more detail.

In studies performed by us (Eijsvoogel et al., 1972a, b) a weak but not completely negative response was observed in four out of twelve sibling combinations differing only for an HLA determinant. If these reactions are also caused by structures other than the A locus, a second or 'minor' MLC locus (loci) situated between the A and B loci may exist as outlined in Fig. 1.2. The genes of this 'minor' MLC locus might either stay together with the corresponding gene of the D locus (and of the B locus) depending on the point of crossing-over in the chromosomes, resulting in an absence of stimulation; or stay with the corresponding gene of the A locus, thus resulting in weak stimulation when tested with siblings different for the A locus determinant only. Further evidence for such a 'minor' MLC locus has come from more recent studies by Mempel et al. (1973a) and Thorsby et al. (1973).

Linkage disequilibrium as previously mentioned is the tendency for alleles at different, usually closely linked loci, not to be associated at random in a population. It is in general the main cause of the association between functions controlled by linked genes in a population. This is the probable basis for most of the known associations between HLA and disease which have been interpreted as due to linkage disequilibrium between alleles of the B locus

and alleles at an immune response locus linked to it (Bodmer, 1975). Definite evidence of linkage is given by the occurrence of recombination.

Until recently only rough estimates of the recombination frequency between the B and D loci in man have been made by Ceppellini (1.0%) (1971) and Thorsby et al. (0.4%) (1975). These estimates were based on the number of HLA-A and B identical siblings stimulating each other in the MLC test. In the literature, thirteen possible cases of recombinations between the D locus and the B locus have been reported (Amos and Bach, 1968; Bach et al., 1969; Plate et al., 1970; Yunis et al., 1971; Dupont et al., 1972, 1974a; Eijssvoogel et al., 1972c; Mempel et al., 1972; Sasportes et al., 1972; Thorsby et al., 1973; Keuning et al., 1975). Keuning and co-workers (1975) attempted to calculate the recombination fraction in a study of thirty-nine families with 167 children where two maternal recombinations between the B and D loci were found in two different families. They calculated the recombination frequency between the B and D loci to be 0.0074 which does not differ too much from that between the A and B loci of the HLA system.

With the discovery of an independent strong MLC locus, or the D locus as it is now called, closely linked to the B locus, and with the previously mentioned difficulty in demonstrating a strong correlation between kidney-graft survival and HLA-A and B matching, it was suggested by some authors that the MLC locus (loci) alone or in conjunction with the B locus could play a role in the rejection process.

Since there is a good correlation between HLA identity and MLC non-stimulation among *sibling pairs* and there is also a well-established correlation between serological identity and kidney-graft survival in this group, it is apparent that in HLA identical sib combinations, no conclusions will emerge about the relative importance of MLC identity as such, for organ-graft survival.

The general impression gained from *skin-graft* experiments by several workers (Koch et al., 1972; Van Rood et al., 1973; Sasportes et al., 1973; Ward and Seigler, 1973) is that MLC incompatibility shortens the skin-graft survival time in both HLA identical and non-identical *unrelated* donor-recipient combinations. It should, however, be pointed out that the presence or absence of MLC reactivity is not necessarily the only variable between these different donor-recipient pairs. The particular HLA antigen(s) for which the donor-recipient combinations differ may also vary from one pair to the other. With reference to this, it should also be noted that Van Rood et al. (1973) showed that in a group of HLA identical unrelated donor-recipient pairs, the average MLC reactivity was lower than between the HLA non-identical group. This is almost certainly a result of the linkage disequilibrium between HLA and MLC loci described by several workers (Sørensen and Staub-Nielsen, 1970; Eijsvoegel et al., 1970; Bach et al., 1970). Skin grafting in HLA recombinant families has generally failed to show that D locus disparity alone can cause skin-graft rejection (Ward and Seigler, 1973; Yunis et al., 1973). From these data it is obvious that it is still very difficult to draw conclusions about the impact on skin-graft survival of HLA-A and B incompatibility and MLC incompatibility separately.

Cochrum et al. (1973) reported a study comparing MLC reactivity and graft survival in fifty-nine *cadaveric* kidney transplants. All combinations were divided into groups of high and low MLC stimulation (stimulation ratio < 8 or stimulation ratio > 8), and they found that high MLC reactivity correlated with relatively poor graft survival, and low MLC reactivity with relatively good graft survival. Before concluding that the histo-incompatibility in the first group may be attributed to MLC disparity, it must be emphasized that the HLA-A and B disparities could not be controlled in this experiment and that the kind of HLA-A and B incompatibilities in the different groups may not be comparable. Because of the linkage disequilibrium between several of the more common determinants of the HLA-B and HLA-D loci, the selection of a group of donors and recipients on the basis of less disparity at HLA-D is likely to result in the inadvertent selection of certain HLA-B antigens that are found in linkage disequilibrium with certain HLA-D determinants. The group with low MLC reactivity may therefore have less disparity of the HLA-B locus antigens as well. The relative importance of the serologically defined HLA antigens versus the MLC determinants may not be perceived until carefully designed experiments, in which the MLC determinants are the only variable, can be analyzed. Another variable in the assessment of the influence of MLC and HLA-A and B on histocompatibility that has emerged from the various studies is the effect of *immunization* before grafting either by blood or tissue of the organ donor or of other individuals. This can affect the prognosis of the transplant. Some individuals will form antibodies against leukocytes, but others will fail to do so.

Hamburger et al. (1971) and Van Hooff et al. (1972) published data showing that matching for MLC in *non-immunized*

recipients improved kidney-graft survival. Because of the previously mentioned linkage disequilibrium between MLC and HLA, matching for MLC will usually imply HLA-A and B identity as well, and thus improved graft survival is seen by many workers in the HLA-A and B identical groups.

If an individual is *pre-immunized* as demonstrated by the presence of circulating antibodies, matching for HLA antigens, especially the B locus antigens, becomes of prime and overriding importance in skin and kidney allograft survival (Van Hooff et al., 1972; Oliver et al., 1972; Van Rood et al., 1973, 1977). This is also in accordance with the data obtained in the mouse, where the H-2K region seems to be more immunogenic in skin-graft rejection than the H-2D region (Klein, 1966; Démant et al., 1971).

The explanation put forward by Van Rood in 1974 for these *in vivo* findings was that allograft rejection occurs as a two-phase mechanism in which there is an interaction of the MLC determinants and the HLA determinants, especially the B locus determinants. The two phases are :-

The recognition phase: In a *non-immunized* recipient, or in the recipient who has had blood transfusions but failed to form cytotoxic leukocyte antibodies, the immune mechanism of the recipient will recognize the foreign graft through differences in the MLC determinants. Thus matching of MLC determinants in this situation will improve graft survival.

The rejection phase: In the *immunized* recipient, i.e. an individual in whom the immune mechanism has been activated through encountering an incompatible MLC

determinant, the second phase of allograft response, the rejection phase is set in motion through killer cells and / or cytotoxic antibodies directed at the target tissue. In such recipients the destruction of the grafted organ takes place with the B locus determinants of the graft as the main target. The exact role of the A locus determinants is not yet clear.

The *in vitro* findings by Eijsvoogel et al. (1973a) employing the cell-mediated lympholysis (CML) test is in accordance with this hypothesis (see below for further details).

From all the data available thus far it appears that both the HLA-A and B loci and the MLC reaction do play a role in allograft rejection, but because of the limited data in which either HLA-A and B or MLC determinants are the only variable, a conclusive answer cannot yet be reached concerning their relative importance in histocompatibility. Furthermore, the lack of complete correlation could indicate that MHC loci other than those determining the HLA-A, B, C and the MLC reactivity may be of greater importance for histocompatibility in man.

The Cell-mediated Lympholysis (CML) Test

Hirschhorn et al. (1965) were the first to demonstrate that the entire *in vivo* cycle, from the sensitization of human lymphocytes, to the development of cytotoxicity towards allogeneic fibroblasts could be obtained *in vitro*. A similar target cell destruction is observed when using the cells from the same human lymphoid cell line as stimulators in an MLC and also as target cells in a subsequent cytotoxicity assay

(Hardy et al., 1970). Experiments by Solliday and Bach (1970) showed that although this cytotoxic effect is maximal when tested on target cells identical with the stimulating cells, it may, to a limited degree, show cross-reactivity when tested on target cells of lymphoid cell lines other than the one used as the initial stimulator.

Subsequently Lightbody et al. (1971) showed that blast cells, obtained through PHA stimulation *in vitro* of normal peripheral blood lymphocytes, are sensitive *targets* for lysis by *effector* lymphocytes, which have been activated as *responder* cells in a unilateral MLC. This cytotoxic effect could be shown to be specific in that PHA transformed target cells, which are syngeneic to the responder cells, are not (or only very weakly) lysed, while target cells syngeneic to the stimulator cells in the MLC are lysed to the highest degree. This model, which was called cell-mediated lympholysis (CML), allows further evaluation of the function of the serologically determined antigens and MLC determinants, respectively.

(i) CML Studies in the Mouse

The CML method has been applied to the mouse for only a relatively short period, so new evidence is still being accumulated. However, in a number of studies that have employed recombinants in combinations that can test one region at a time (Abbasi et al., 1973; Alter et al., 1973; Nabholz et al., 1974; Schendel et al., 1973; Sørensen and Hawkes, 1973; Widmer et al., 1973b) there has been agreement that the *killing phase* of the reaction is directed against products of either the K or the D regions, with little difference in the 'strength' of the two regions. There is still no definite evidence whether this effector phase in CML is directed towards the H-2K or H-2D

specificities or antigens determined by very closely linked loci present on the stimulator cell and absent on the responder cell. An exception to the above-mentioned findings has been noted by Widmer et al. (1973c). They found reciprocal MLC stimulation, skin-graft rejection and CML in the C57BL/6-H(z1) combination, although no serological differences have to date been detected between these two strains.

There is even more controversy about the regions in which incompatibilities are required for *induction* of killer cells. In general the positive CML required a positive MLR in the first phase of the test (Alter et al., 1973; Abbasi et al., 1973; Sørensen and Hawkes, 1973). These findings are consistent with the studies of Eijssvoegel et al. (1973b) on the HLA system (see (ii) for further discussion). However, a number of exceptions have recently been observed by different laboratories (Klein et al., 1974b; Nabholz et al., 1975; Wagner et al., 1975). These workers showed that killing can occur with MLC only, in the absence of K and D region differences. Furthermore, K and D region differences alone in the absence of any I region differences can lead to graft rejection and CML (Klein et al., 1974b). In one particular instance, involving strain B10.A(5R), another paradoxical pattern of reactivity was observed (reviewed by Démant, 1973). In the combination B10.A(5R) responder/B10.D2 target, involving strong MLR and K region differences, CML was not reported, whereas in the reciprocal combination which also gave a strong MLR, CML was observed. One possible explanation for the discrepancies could be an insensitivity of *in vitro* assays. More recently, Festenstein and Démant (1975) have presented evidence for the existence of a mouse CML-stimulating locus (ECS), mapping in the I-B and I-C subregions of the H-2 complex. In contrast to these findings, Abbasi and Festenstein (1973) have

shown that incompatibility at the non-H-2-Mls locus does not result in a positive CML, although it causes a rather strong MLR. The exact roles of the K, D and I regions in CML thus still remain unresolved in the mouse.

(ii) CML Studies in Man

In 1972 Miggiano et al. reported CML studies in families and demonstrated a greater destruction of specific target cells derived from siblings differing for two haplotypes with the responder, than of specific target cells differing only for one. Preliminary evidence was also provided that the serologically defined antigens might determine the specificity of this cell-mediated cytotoxicity.

To evaluate the genetic aspects of CML further, we, together with Eijsvoegel and his co-workers, performed some preliminary experiments on a family that had previously demonstrated recombinational events within the MHC. The tentative conclusion reached seemed to be that the sensitization for CML takes place only when stimulating and responding cells differ for MLR (D locus) determinants, but the actual destruction is not directed against the D locus determinants, but rather against the serologically defined HLA-A and B antigens (Eijsvoegel et al., 1972b) or antigens determined by very closely linked loci (Eijsvoegel et al., 1973a).

These conclusions are supported by the findings of Bonnard et al. (1973) and Mawas et al. (1973) in man, and by other workers in mice. However, exceptions have recently been reported in man, showing that MHC gene products, in addition to the classical A and B loci antigenic specificities, can act as targets for destruction (Mawas et al., 1975; Kristensen and

Grunnet, 1975). Similar exceptions have also been observed in mice as discussed in the previous section. Evidence supporting the concept of a separate CML locus inside the HLA system, and in marked linkage disequilibrium with the HLA-B antigens as first suggested in mice by Festenstein et al. (1974), has emerged from the data presented by Mawas et al. (1974, 1975) and Goulmy et al. (1975). More recently, it has been suggested that the HLA-D determinants are not essential for the generation of cytotoxic cells (Long et al., 1975). It should be emphasized that for the time being this evidence can only be considered as suggestive.

By virtue of the presence of children with recombination between the A and B loci of HLA in families, Eijsvoogel et al. (1973a) were able to evaluate the relative importance of the A and B loci in the development of cytotoxicity in the CML test. They found the limited number of B locus antigens (B8,B5 and BW35) that they could test for, to induce strong cytotoxicity in the CML test. The four A locus antigens (A1, A3, A11 and A28) in this experiment elicited no, or only weak, cytotoxicity. In contrast to their findings Trinchieri et al. (1973) in their studies of unrelated individuals also found killing but to a minor extent, against the antigens of the A locus.

Because of the possible discrepancy between these results with regard to the part played by the A locus antigens in the CML test, Eijsvoogel et al. (1973b) performed CML studies on ten unrelated individuals who differed for only one A locus antigen. From this study and the previous studies on the recombinant siblings, they concluded that although some antigens of the A locus (A1, A3, A11 and A28) are weak antigens for CML, other antigens, e.g. A2, can cause pronounced cytotoxicity. This therefore suggests that in the CML test some antigens of

a particular locus may be more immunogenic than others. It should be noted that Curtoni et al. (1973) also found the antigen A2 to be stronger *in vivo*, using experimental skin-graft survival time and the normal lymphocyte transfer (NLT) reaction.

Eijsvoogel et al. (1973b) investigated the question of whether the MLR-stimulating determinant and the antigen(s) at which subsequent destruction is directed must be present on the same (stimulator) cell. In a *genetic three-cell experiment* using a cell that on its own cannot stimulate the responder by virtue of MLC 'identity' (recombination) and an *artificial three-cell experiment* where, because of heat treatment, which abolishes the MLC-activating capacity but maintains HLA antigens, Schellekens and Eijsvoogel (1970) showed that one cell type induced the activation, while the other presented the specificity for CML. These findings have subsequently been confirmed by Mawas et al. (1973) and by Bonnard et al. (1973) in man, and similar observations have been made by Schendel et al. (1973) in mice.

Very recently Eijsvoogel et al. (1976) have published data which seem to indicate that the cells proliferating in response to an allogeneic (HLA-D) stimulus in the MLC belong to a different T cell subpopulation from the cells that become specifically cytotoxic towards HLA-B and A determinants or determinants coded for by closely linked loci. This is in agreement with the findings of Bach et al. (1973) in mice and those of Zier and Bach (1976) in man.

At present it is impossible to come to a final conclusion about a possible correlation between CML *in vitro* and human histocompatibility *in vivo*. There is, however, some circumstantial evidence that either MLC or HLA-A and B matching (and

particularly for antigens of the B locus) on its own improves graft survival. Retrospective analysis of kidney-graft survival has suggested that matching for MLC or the B antigens improves the outcome (Cochrum et al., 1973). A retrospective analysis of cadaver transplants (Oliver et al., 1972; Van Hooff et al., 1972) showed a significant correlation between matching for B locus antigens and kidney-graft survival. In the study by Van Hooff et al. (1972) this was shown only in those recipients with preformed leukocyte antibodies. Both these authors suggest that this improvement might be a reflection of a CML-like mechanism *in vivo*.

Graft-versus-Host (GvH) Reactions

A graft-versus-host (GvH) reaction can only develop when the *graft* is *not* rejected in the presence of antigenic differences between the recipient and the donor. In man and the mouse and in some other species like the dog, rat and chicken, which have been studied, it has been demonstrated that incompatibilities in the MHC region play the most important role in GvH reactions (Bach and Bach, 1974). The greater the antigenic differences between host and donor, the higher the frequency and severity of the GvH reaction.

(i) GvH Reactions in the Mouse

Early on in studies of the GvH reaction in the mouse, it was recognized that incompatibilities in the MHC play the most prominent role (for a review see Klein, 1975). In a recent publication, Klein (1976) suggested that the GvH reactions can be divided into two phases, proliferative and effector. The *proliferative* phase involves stimulation of donor lymphocytes by cell-membrane antigens of the host, usually followed by non-specific proliferation of the host

lymphocytes. These proliferations lead to an enlargement of lymphoid organs, primarily spleen and lymph nodes, and this enlargement can then be used as an indicator of the proliferative phase of GvH (Simonsen et al., 1958).

The *effector* phase is characterized by the generation of donor-derived effector lymphocytes that can attack and destroy tissues of the host. The attack on the host tissues may be expressed by a variety of symptoms, the most conspicuous of which are loss of body weight, general runting, and, in extreme cases, death of the host. The two phases have their corresponding *in vitro* correlates in the MLC reaction (proliferative phase) and CML (the effector phase).

Studies by Klein and Park (1973) and by Oppøltova and Démant (1973) established that not all the H-2 regions of the MHC played an equal role in the *proliferative* phase of GvH. Antigens controlled by the I region, more specifically the I-A subregion, induce much stronger GvH in newborn mice hosts than antigens controlled by other regions and subregions of the MHC. Subsequently, Klein (1976) demonstrated, in adult mouse hosts, that the strongest proliferative GvH was obtained when the donor cells reacted against the entire MHC of the host. The response to I region differences was weaker than the anti-H-2 response but, at the same time, was considerably stronger than the response to any other single H-2 region difference. However, these other H-2 region differences did lead to significant GvH stimulation. Thus, although antigens controlled by the I-A subregion play a predominant role in the proliferative phase of GvH, antigens controlled by the H-2K and H-2D loci in the K and D regions respectively, are also capable of inducing significant GvH. The K and D regions lead to a stimulation comparable to the stimulation by the I-B + I-C subregions. S region differences probably produce no significant effect.

From these studies and from those by Rodey et al. (1974) it can be concluded that both MLC reactivity and the GvH reaction in the mouse are primarily controlled by the same genetic loci in or close to the I region. These findings thus support the notion that the MLR is indeed an *in vitro* correlate of the proliferative phase of GvH.

Klein (1976) performed experiments to assay the genetic requirements of the *effector* phase of GvH by injecting parental spleen cells intravenously into young adult F_1 hybrids irradiated twenty-four hours before injection. They tentatively concluded from these experiments that both K and I region differences lead to fatal GvH, although the latter may do so less effectively than the former.

These *in vivo* results again closely parallel those obtained using the *in vitro* CML assay for effector lymphocytes, thus suggesting that the CML assay is indeed a correlate of the effector phase of GvH.

(ii) GvH Reactions in Man

This reaction is of utmost importance in allogeneic bone-marrow grafts because the success of bone-marrow transplantation depends on the removal of two immunological barriers, the host-versus-graft reaction which is similar to that encountered in all other organ or tissue grafts, and the GvH reaction which is caused by the fact that the bone-marrow graft contains cells or can produce cells which under certain conditions are capable of setting up an immunological reaction against the recipient.

Three groups of patients have so far been treated with allogeneic bone-marrow with some success :-

Patients with *severe combined immunodeficiency (SCID)* disease who apparently lack the bone-marrow stem cells which can develop into mature T and B lymphocytes;

patients suffering from *aplastic anaemia*; or

patients suffering from *leukaemia*.

Allogeneic bone-marrow transplantation in the treatment of children with *SCID* has been very informative about the role of histocompatibility in transplantation. Successful transplantation and immunological reconstitution in this disease have depended largely on the use of HLA- and MLC identical *sibling* donors (Buckley, 1971; Dupont et al., 1974b). Bone marrow from a family donor mismatched by a single haplotype has invariably resulted in fatal GvH disease (Van Bekkum, 1972; Park et al., 1973). One patient with *SCID* has been successfully reconstituted with bone marrow from an MLC-matched sibling who was, however, HLA-A mismatched, as a result of recombination within the HLA chromosomal region (Gatti et al., 1968). The HLA recombination had presumably led to HLA-B, D identity between donor and recipient but non-identity for the HLA-A determinant. Engraftment and immunological reconstitution have also been achieved in a second patient with *SCID* in which an HLA-A, B non-identical but MLC-matched maternal uncle was used as a bone-marrow donor (Koch et al., 1973). Retrospective analysis of this family, however, suggests there was no recombination, as first suspected. Although the father and maternal grandfather were both HLA-A, B heterozygous, they both appear to have been HLA-D homozygous. Thus the patient and maternal uncle were MLC non-

responsive because they each carried the same two HLA-D specificities, although on different and unrelated haplotypes (Dupont et al., 1976). This is an informative case because it suggests that matching for bone-marrow transplantation could be achieved for non-sibling donors by matching for HLA-D.

L'Esperance et al. (1975) used bone marrow from an *unrelated* donor MLC identical but incompatible for one HLA-A antigen, in repeated graftings of a child with SCID. Chimaerism was achieved with complete immunological reconstitution. A second patient with SCID has been grafted with an unrelated, HLA-D identical, but one HLA-A antigen non-identical donor. There was some evidence of immunological reconstitution, when the patient died on day 30 from cytomegalovirus pneumonia (Horowitz et al., 1975).

The final outcome of these attempts at immunological reconstitution by bone-marrow transplantation with unrelated MLC-matched bone marrow cannot yet be evaluated. It seems likely, however, that non-immunized recipients (at least in SCID patients), with common HLA-D determinants in the MHC, can be matched with unrelated individuals for the purpose of bone-marrow transplantation.

Clinical bone-marrow transplantation in patients with *aplastic anaemia* or *leukaemia* is more complex than bone-marrow transplantation in children with SCID. Firstly, these patients are immunocompetent and require immunosuppressive drugs before transplantation. Secondly, they have frequently received multiple transfusions of blood products, which, in the dog, have been shown to increase the chance of even a well-matched graft being rejected (Storb et al., 1971). In spite of pre-graft immunosuppression, graft rejection has been reported in up to

25% of transplants between HLA-A, B identical MLR non-reactive sibling pairs (Storb et al., 1974).

The incidence of GvH after bone-marrow transplantation from HLA-A, B identical MLC negative *siblings* is much greater in patients with aplastic anaemia or leukaemia than in patients with SCID. Following bone-marrow transplantation the incidence of clinically significant GvH reaction exceeds 50% in most series (Thomas et al., 1975; U.C.L.A. Bone Marrow Transplant Team, 1976) and may be as high as 80% in patients with acute myelogenous leukaemia (Thomas et al., 1973). The reason for increased intensity of GvH reaction in these patients is unknown. Important differences between patients with aplastic anaemia or leukaemia and infants with SCID may include age factors, differences engendered by the primary disease process, pre-sensitization caused by blood transfusions and complications introduced by immunosuppressive or antileukaemia therapy.

Donor selection for marrow transplantation in patients suffering from acute leukaemia has been restricted with rare exceptions to HLA identical, MLC non-reactive siblings. Gale et al. (1975) reported on bone-marrow transplantation in a young male with acute myelogenous leukaemia, in which case marrow from a sero-identical, but MLR positive, female sibling donor was used. There was evidence of engraftment, but leukaemia recurred along with predominantly host markers, and the patient died on day 38. Although the family MLC experiment was interpreted as representing an HLA recombination between HLA-B and HLA-D, formal proof of recombination is not available. Because of the early recurrence of leukaemia and the apparent loss of the graft, the potential for GvH reaction in this MLR positive situation could not be adequately evaluated.

In *summary*, the mechanism of GvH is still unknown. There is a tendency to equate MLR and GvH reactions in the mouse. In man, however, it is obvious that the GvH reaction can occur between HLA identical, MLR negative sibs. Because almost all information has been collected from bone-marrow graft exchanges between HLA identical siblings, no precise information is available whether it is the MLC locus itself or a locus nearby that determines the onset of GvH. In this connection it should be noted that non-HLA circulating antibodies have been found after GvH reaction between HLA identical MLC negative sibs (Jeannet et al., 1973; Gluckman, 1974). In one patient with aplastic anaemia the MLC and CML tests became positive with the donor's lymphocytes, suggesting that immunization against non-MHC antigens can be responsible for unexpected MLC and CML reactions. Thus even identity for the MLC region may perhaps not be an absolute prerequisite in the selection of donor-recipient combinations for bone-marrow allografting.

CHAPTER TWO - MOTIVATION FOR THIS STUDY

I THE ROLE OF HLA-A AND B ANTIGENS IN HISTOCOMPATIBILITY

II THE MIXED LYMPHOCYTE CULTURE (MLC) REACTION

THE OBJECTIVE AND MOTIVATION

From a review of the literature it appeared that neither the HLA-A, B genotype nor the MLC reaction is the complete answer to donor-recipient selection in organ transplantation. Hence, the main aim of this project was to study the genetic relationship between the serologically detectable products of the HLA-A and HLA-B loci on the one hand and MLC reactivity on the other in relation to histocompatibility.

I THE ROLE OF HLA-A AND B ANTIGENS IN HISTOCOMPATIBILITY

Investigation of the role of HLA-A and HLA-B antigens in histocompatibility has shown that:-

Organ-graft survival is significantly better when donor and recipient are HLA-A and B identical siblings than when donor and recipient are siblings not identical for HLA-A and B.

When donor and recipient are unrelated to each other no such clear correlation between HLA-A and B matching and organ-graft survival could be noticed.

An explanation for this discrepancy may be that serological typing has often been incomplete because of the lack of a complete range of antisera. This would frequently not affect the assessment of HLA identity in families, but could influence the possibilities of true matching among unrelated individuals. Further possible explanations for these findings must, however, be considered:

(a) Serological typing of the products of the A and B loci does not really recognize (all) the antigens of the major histocompatibility locus :

It could be that the antigens presently recognized are still heterogeneous, or the antisera polyspecific, containing more than one, or a cross-reacting, antibody. This would not affect the outcome of typing results in families because there are only a limited number of antigens present within a family, but it could influence the HLA-A and B matching between unrelated individuals.

Serological typing does not recognize products of other loci in the same chromosomal region, but recognition of the segregation of the particular chromosome within families is sufficient for the selection of siblings identical for all the other loci of that chromosomal region. This of course is not the case when unrelated people have to be matched.

Serological typing does not recognize genotypes; therefore if genic interaction occurs in these systems it generally cannot be assessed between unrelated cadaver donors because haplotyping is often not possible.

(b) True identity for the major histocompatibility complex (MHC) between unrelated people does not result in a similar histocompatibility as seen between HLA identical siblings, due to the cumulative effect of minor histocompatibility loci.

Assuming that most immunogenetic systems are extensively polymorphic one would expect that approximately 25% of the loci that are different in unrelated individuals will be identical in siblings. However, in view of the data presented by Bildsøe et al. (1970) one would expect such an effect to influence skin-graft survival rather than organ transplantation. Bildsøe performed kidney, heart and skin-graft experiments in rats, from donors belonging to segregating F_2 hybrid populations to recipients of one of the parental strains. The presence or absence of a major locus incompatibility was determined by serological typing and MLC, showing a 100% correlation. Skin-graft rejection was invariably rapid in both the major locus identical and non-identical combinations. Kidney and heart transplants were rejected in an acute or sub-acute manner in those combinations which were incompatible for the major locus, with one single exception. Similar grafts were still surviving up to 300 days, if no major locus incompatibility existed. These findings provide further confirmation of the one-locus hypothesis for organ transplantation and the cumulative effects of multiple minor loci in skin-graft rejection.

II THE MIXED LYMPHOCYTE CULTURE (MLC) REACTION

The data available at the time the present study was initiated were the following:

HLA identical sibling pairs give MLC negative reactions, while HLA non-identical sibling pairs show MLC positive reactions. This implies that disparities at the MHC chromosomal region are responsible for the MLC reactivity. However, it had also been demonstrated that the vast majority of serologically identical unrelated pairs show positive MLC reactions.

These findings reveal a striking similarity between MLC compatibility and the serological matching for HLA-A and B (see above).

For this reason it seemed highly relevant to study further the genetic aspects of MLC in an effort to provide knowledge of, and insight into, the requirements for histocompatibility.

Regarding the underlying mechanism of MLC reactivity, two different hypotheses could be postulated :

1. MLC reactivity is the result of an overall recognition phenomenon of the whole MHC, detecting incompatibilities for any product of this region.
2. MLC reactivity is caused by incompatibilities for one or more loci, separate from HLA-A and B, but still belonging to the MHC.

1. If this hypothesis were right, then the same situations, as mentioned under (a) as possible explanations for the findings among HLA sero-identical individuals, would also result in positive MLC reactions, and only true identity of the HLA-A and B products would allow negative MLC reactions. These latter identical combinations could then be expected to be more histocompatible than the first group.

The influence of minor locus incompatibilities on organ-graft survival could then eventually also be evaluated by comparing graft survival in HLA-A and B identical, MLC-negative, unrelated donor-recipient combinations with that between HLA identical siblings.

2. If MLC reactivity were controlled by only one or more loci, belonging to the MHC but separate from HLA-A and B, then MLC studies would sooner or later reveal combinations which were HLA-A and/or B different but MLC-negative. Such combinations could possibly be found in families in which recombinational events had occurred. In unrelated combinations such evidence for separate MLC locus or loci might also be obtained, but this would probably imply the testing of a large number of HLA-A and B different combinations, since there is no alternative way of pre-selection, and MLC polymorphism is well known to be extensive.

However, if one or more separate MLC loci could be demonstrated to exist, this would certainly also have important implications for histocompatibility; it would be highly relevant then to investigate whether the products of the genes of such a locus or loci, either alone or in combination or by interaction with products of HLA-A and B (and possibly other loci), are the major histocompatibility antigens.

Once evidence for one or more separate MLC loci has been obtained, it would be an indication that the degree of polymorphism and the frequency of some of the alleles should be analyzed. Some token of this could be obtained by determining the frequency with which unrelated parents share one MLC allele. Such a shared allele can only be demonstrated in family studies, since one may then expect to find children MLC identical to one or to the other parent. Furthermore, MLC homozygous children can also be expected.

This then poses the important question whether indeed in an MLC between a homozygous and a heterozygous cell only

the homozygous cell will react to the one foreign allele of the heterozygote, while the heterozygote will not react at all since the homozygote has nothing foreign, to respond to.

If, however, genic interaction were to take place and thus influence the final products, such natural one-way reactions would not occur. Such an effect of genic interaction on the final MLC products would have enormous implications in other respects as well, since it would make any form of typing extremely difficult. The question of whether genic interaction does affect the final products can be answered by analyzing homozygous-heterozygous MLC reactions applying procedures like Mitomycin treatment or X-irradiation of one of the two cell suspensions, thus blocking its proliferative potential but maintaining its stimulating properties.

The best chance of finding MLC homozygous individuals lies in the studying of families where either incest, or at least a close family relationship between the parents exists, thus increasing the chance of a common MLC allele shared by the parents.

The above-mentioned considerations formed the motivation for studying the MLC reactivity:

1. in unrelated individuals selected on the basis of HLA-A and B typing;
2. in randomly chosen families with non-consanguineous parents;
3. in families selected for varying degrees of consanguinity between the parents.

PART II

MATERIALS AND METHODS

CHAPTER THREE - THE GROUPS STUDIED

SELECTION OF ETHNIC GROUPS

Consanguinity and Inbreeding
Consanguinity and Genetic Non-relatedness

FAMILIES AND GROUPS STUDIED

- I UNRELATED INDIVIDUALS
- II RANDOM NON-CONSANGUINEOUS FAMILIES
- III SAN (BUSHMEN) FAMILIES

The San (Bushmen)

Language
Geographical position of the group studied
The family unit

- IV CONSANGUINEOUS FAMILIES

- i) First-degree Consanguineous Families
- ii) Fourth-degree Consanguineous Indian Families

The Indians (Muslims)

Arrival of the Indians
Indian (Muslims) in the Cape Peninsula
Language and religion
The Indian family
Family planning

RECOGNITION OF MLC DETERMINANTS (MLC TYPING) BY MEANS OF MLC HOMOZYGOUS CELLS

SELECTION OF ETHNIC GROUPS

The population of South Africa and South West Africa is characterized by its ethnic diversity. In the course of many decades in the country's history, different ethnic groups have settled as separate peoples in certain areas of the land. The respective groups all retained their own identities and are still distinguishable in the present population. There has been little intermarriage and because each was made up of a relatively small number, often geographically isolated, a certain degree of inbreeding resulted. Thus the chance of unrelated individuals inheriting a common HLA-A, B, C and D haplotype is greater. Gene drift and founder effects also have a much larger effect on breeding groups of restricted size. Thus if gene drift, small population, linkage disequilibrium, natural selection, and a degree of consanguinity are operating, the chance of compatibility between individuals is greatly increased. There are a number of distinct population groups in South Africa and South West Africa - the Indians, San (Bushmen), Nama, Coloureds, Whites and Negro (with their many linguistic and geographical subdivisions). These separate population groups are distinguished from one another by their different languages, physical appearance and cultures, and to a large extent also by the areas in which they have settled and now live.

Of these population groups, two were selected for inclusion in this study - the *Indian (Muslims)* and the *San (Bushmen)*. There is a relatively large group of Muslim Indians in Cape Town, among whom first-cousin marriages are known to be common, therefore a search for consanguineous families among this group was undertaken (see page 82 for further details on the Indians). The decision to include the San (Bushmen) as one of the populations to be studied for the

Fifth International Histocompatibility Workshop provided us with a unique opportunity to study the San (see page 70 for a detailed account of the San).

Consanguinity and Inbreeding

If two individuals have at least one ancestor in common they are said to be consanguineous. This common ancestor must not be too distant, or else the concept becomes meaningless, and also consanguinity would then not be easily detectable. On the basis of the theory of evolution, all individuals of a species are to some extent consanguineous, because all are descended from a remote common ancestor. Thus consanguinity, and the degree of inbreeding, must be defined as being applicable only to unions established after some evolutionary time-point at which, from a practical point of view, everyone is considered to be unrelated (Cavalli-Sforza and Bodmer, 1971).

The offspring of consanguineous parents is by definition inbred, i.e. possesses genes identical by descent. Consanguineous relations are defined in civil and canon law by the number of 'steps' within a pedigree that lie between the two related individuals. First-degree consanguinity therefore is a union between parent and offspring, second-degree is that between siblings or grandparent and grandchild. Other examples of consanguineous relations are those between uncle or aunt and nephew and niece (third-degree), between first cousins (fourth-degree) and between second cousins (sixth-degree) (Stern, 1960).

In genetic terms the degree of inbreeding is much more rigorously defined. The co-efficients of inbreeding for individuals and populations, designated F by Wright, and α by Bernstein, are equivalent. It may be defined as the probability that an individual is homozygous at a given locus for

two genes 'identical' insofar as they are both derived from a single gene present in a common ancestor. Marriages between relatives, or consanguineous marriages, are important genetically since the inbred individual may carry a double dose of such a gene.

The frequency with which consanguineous unions occur in different populations depends partly on the population structure and also on social customs. Some ancient societies, for example, the ancient Egyptians and the Incas, favoured marriages of brothers and sisters of the reigning dynasty because they considered 'royal blood' only worthy of mixing with other 'royal blood'. A few societies today still prefer marriages between relatives to marriage between unrelated persons, but the customs or laws of most societies consider incest taboo. The exact degree of relationship at which a mating is considered incestuous may vary slightly from one society to another, but in general, all societies prohibit parent-offspring and brother-sister matings.

Different societies vary in their judgement on marriages between uncle-niece or nephew-aunt and sometimes on even more distant degrees of consanguinity. In some states in America, for example, the union of second cousins is prohibited. The Roman Catholic Church does not permit first-cousin marriages without special dispensation (see Appendix for Form B-I 31, which gives details of "Prohibited Degrees of Relationships under the South African Law"). These restrictions probably result from observation of the more frequent occurrence of recessively inherited diseases in the progeny of consanguineous marriages (Cavalli-Sforza and Bodmer, 1971; Stern, 1960).

Consanguinity and Genetic Non-relatedness

Curt Stern (1960) has pointed out that a sharp distinction cannot be drawn between consanguinity and genetic non-relatedness since there must be many people descended from common ancestors who are unaware of the fact. In most geographical regions, man does not reproduce within pedigrees completely isolated from one another, but rather in a complex network of relationships that joins all or most strains together in a single reproductive unit. This is true even when different races inhabit the same territory or where society and customs favour a separateness of population layers or castes. In the course of generations such barriers against intermarriage prove ineffective, particularly as both intra- and extra-marital unions lead to an interchange of genes between acknowledged groups. Therefore when the pedigrees of any group of apparently unrelated people living in the same territory are carefully traced, many will be shown to possess common ancestors within the last few centuries. The San (Bushmen) families in this study comprise a *small* group of unrelated individuals of this kind and in addition to being one race, they have also been geographically isolated. It follows that, in such a group with only a limited number of genes, if two 'unrelated' people marry, they contract in reality a distinctly consanguineous marriage.

FAMILIES AND GROUPS STUDIED

A short description of the families and unrelated groups investigated is given in this section.

I UNRELATED INDIVIDUALS

The following groups were selected from a large number of healthy people typed in our laboratory as part of family studies or population genetic analysis from 1973 - 1976.

i) Unrelated individuals different for HLA-A and B

Bilateral and unilateral MLC tests were performed on 318 and 513 pairs respectively who carried different A and B locus antigens. This group was compiled from the parental pairs who were unrelated, unrelated controls and groups of unrelated individuals (Table VI.1).

ii) Unrelated individuals identical for HLA-A and B

Bilateral MLC tests were performed on seven unrelated individuals with HLA-A and B phenotype A1,3;B7,8 as well as one with phenotype A1,3;B7,W35 and a control X with the phenotype A9,28;B14,W17 (Table VI.2).

II RANDOM NON-CONSANGUINEOUS FAMILIES

Random families, in which the parents had varying degrees of HLA-A and B disparity, were selected from family MLC studies performed in our laboratory. The detailed results on six families which illustrate various aspects of the genetic relationship between HLA-A, HLA-B and MLC, have been included.

1. FAMILY BEU: Sibling II-6 suffered from aplastic anaemia and therefore the HLA-A and B genotypes of her parents and siblings were determined as shown in Fig. 7.1. The discovery of a recombinant locus A and locus B haplotype in sibling II-3 prompted further MLC studies between the available siblings. The patient died before a suitable donor could be found in this family.
2. FAMILY KA: We studied the MLC reactivity in five families with serologically demonstrable recombination between the HLA-A and HLA-B loci. In all cases, the limited function of the A locus with

regard to the MLC reactivity was demonstrable. For a detailed report on these families see Eijssvoegel et al. (1972c).

In contrast with all the other HLA identical siblings in the families mentioned above, who showed no MLC reactivity at all, an unexpected pattern was found in family KA (Table VII.7). Bilateral and unilateral MLC tests were performed on family KA on two occasions. In each experiment, control MLC tests were carried out with the lymphocytes of a healthy, unrelated individual X.

3. FAMILY DEW: This family could be studied because sib II-2 (Fig. 7.2) was a candidate for renal transplantation who developed a strong lymphocytotoxic antibody positive with all individuals with the exception of those phenotypically identical to her for HLA-A and B. Renal transplantation from a related donor, HLA-A and B identical, MLC non-reactive and negative in the cytotoxic cross-match was then considered. This idea was abandoned when familial glomerulonephritis was found to be present in several members.

The MLC studies were continued, however, because of interest roused by the HLA-A and B genotypes in the family.

4. FAMILY VDW: The HLA-A and B genotypes were determined and MLC tests performed on the parents, child II-1 and her brother II-2, with a view to transplantation of a kidney to II-1 (Fig. 7.3). The patient received a kidney from her brother, and is still well with good renal function three years later.

5. FAMILY DEL: HLA typing for the A and B loci as well as mixed lymphocyte cultures was done in this family in order to find a matched kidney donor for the patient, sib II-5 (Fig. 7.5). A renal transplant was not performed because she was found to be psychiatrically unsuitable.
6. FAMILY GIA: The GIA family was investigated with a view to finding a possible HLA-A, B and MLC compatible donor for sibling II-1 who required bone-marrow transplantation (Fig. 7.6). Unfortunately she died before the transplant could be performed.

MLC experiments and HLA-A and B typings were done on an additional twenty-one families (Table VII.28), with unrelated parents, in an effort to find how many of these parental pairs shared one MLC allele. These families were all Caucasoids and unlike the San where there are difficulties in determining non-consanguinity, this group was selected from an outbred population.

III SAN (BUSHMEN) FAMILIES

An expedition was undertaken to study the San in Tsumkwe in South West Africa as part of the Fifth International Histocompatibility Workshop held in Evian, France. I accompanied Dr M.C. Botha of our laboratory, Professor J.J. van Rood of Leyden, Dr. V.P. Eijsvoegel of Amsterdam and Professor Trefor Jenkins of Johannesburg into the field in June 1971. For this Workshop HLA-A and B typing of various populations was undertaken to establish the gene frequencies for these HLA antigens among different populations. This provided Dr Eijsvoegel of Amsterdam and myself with an ideal opportunity to do MLC studies on the following seven San families:

1. SAN FAMILY I: The parents and five siblings as shown in Fig. 8.1 were HLA-typed, and MLC studies were performed on the whole family with the exception of sib II-3.
2. SAN FAMILY II (Bigamous): The HLA antigens were defined in the father, his one wife, I-2A and their six children as shown in Fig. 8.2. MLC studies were also done in all possible combinations on this group, with the exception of sib II-4 because of insufficient lymphocytes. The other wife, I-2B and her children were not available for study.
3. SAN FAMILY III (Bigamous): The HLA serology for the A and B loci of the father, both spouses and their children was determined, but because of the limited technical facilities available for doing large MLC tests, the one wife I-2B was omitted from the MLC study (Fig. 8.3).
4. SAN FAMILY IV: The parents and three of the five siblings in this San family were studied (Fig. 8.4). Siblings II-4 and II-5 were not available for testing.
5. SAN FAMILY V: All five members of this family were HLA-A and B typed and MLC tested as shown in Fig. 8.5.
6. SAN FAMILY VI: MLC studies were performed on the parents and four children (Fig. 8.6). The HLA serology was unsatisfactory because of technical problems, but could not be repeated as the results were only known on our return from the expedition.
7. SAN FAMILY VII: The HLA serology of the whole family was determined, but the father I-1 had to be omitted from the MLC test because there were insufficient cells from him (Fig. 8.7).

The San (Bushmen)

At the anthropological conference organized by Prof. Philip V. Tobias and Prof. T. Jenkins and sponsored by the South African Institute for Medical Research and the Royal Society of South Africa, which was held in Johannesburg in June 1971, the participants agreed to use the words SAN and KHOIKHOI as the designation for the Bushmen and Hottentot people respectively (Jenkins and Tobias, 1977). San is the name by which the 'Bushmen' were known to the Khoikhoi, and the name Khoikhoi which the 'Hottentots' used to refer to themselves means 'men of men'. The word Khoisan (made up of the words Khoikhoi 'Hottentots' and San 'Bushmen') is sometimes used to refer to these two groups collectively.

The San of the present day are descended from people of the later Stone Age who were living in South Africa as long ago as 10,000 years. There has, however, been a great deal of mixture between them and later arrivals in Southern Africa.

In 1956 Tobias estimated that there were 55,000 San still surviving, but two more recent estimates, of Lee (1965) and Silberbauer (1965) put this figure somewhat lower. The number of San was estimated to be 13,300 of the total population of 588,300 in South West Africa in 1966 (Jenkins, 1972). The San, the earliest known inhabitants of Southern Africa, were distributed over most of this area, as is shown on the map (Fig. 3.1) based on the occurrence of their rock art and other remnants. However, the present-day San are virtually confined to Botswana, South West Africa and Angola.

Traditionally the San were primitive hunter-gatherers who lived in small independent family units and spoke non-Bantu click languages (Fig. 3.2). They existed by hunting with



Figure 3.1. A map of Africa showing the suggested past and the present distribution of the San peoples (after Tobias 1964).

bow and arrow, collecting edible wild plants, catching fish and using only natural or the most primitive shelters against the wind (Fig. 3.3). The most obvious physical features (Figs 3.4 - 3.8) of the San are their short stature, with small hands and feet, slender build, light yellowish-brown skin colour, flat face with slanting eyes and prominent epicanthic and other folds, tightly spiralled tufts of hair ('peppercorns') and a tendency to accumulate a great deal of fat on the buttocks (steatopygia) (Tobias, 1964; Jenkins, 1972).

Language: Recently the San have been divided into the following groups according to the language they speak :

- i) *Tshu-kwe*, a group of five languages, related to the Nama language that is spoken by the most dominant of the original Hottentot groups of South West Africa.
- ii) *Bush* languages, which are not related to Nama. There are three distinct forms of Bush, designated A, B and C and they are not necessarily related (Westphal, 1963). The B form is spoken by a relatively small community and Bush C by fewer than one hundred individuals. Bush A is important because of the number of people who still speak it. There are three dialectic forms, now generally known as northern, central and southern !Kung.

This study concerned itself with the *central division* of the !Kung-speaking people who live at *Tsumkwe*.

Geographical position of the group studied: There are about 17,000 !Kung of the central dialect group, and they occupy an area of thousands of square miles extending west and also east from Grootfontein in South West Africa to



Fig. 3.2
A young San
hunter.



Fig. 3.3 Young girl standing in front of primitive hut.



Fig. 3.4 San family in front of hut with a few amenities of Western civilization such as metal bucket, cup and cast-off clothes.



Fig. 3.5 Mother and child at Tsumkwe. The peppercorn hair is well demonstrated in her child.



Fig. 3.6 A young San woman from the Tsumkwe settlement. Note the high cheek-bones, flat face with broad flat nose, small ears, slender build and yellowish-brown skin colour.



Fig. 3.7 An old San woman, showing the slanting eyes with prominent epicanthic and other eye folds.



Fig. 3.8 A San child being breast-fed.

Western Ngami in Botswana (300 miles) and north-south from the Okavango River down to Gobabis in South West Africa (400 miles) (Fig. 3.9). On the periphery of this area, thousands of these !Kung work as servants for White, Herero and Kavango farmers. Many are seasonally independent hunter-gatherers, others have been permanently dependent settlers for the last three generations. An isolated group of 1,000 - 2,000 completely independent hunter-gatherers occupies the central part of this geographical area. They depend on the many permanent surface water-sources present in the Nyae-Nyae plains and at Tsumkwe, and are isolated from the peripheral area by a broad belt (40-80 miles) of arid, waterless country.

In recent years efforts have been made, with a view to the provision of medical, educational and other services, to concentrate the San at various points. Thus a settlement was started at Tsumkwe, where there are about 300-500 San at any one time. This settlement provided the material for our study.

The San at Tsumkwe live mainly by hunting and gathering although rations of food are provided for the very young, elderly and ill. A substantial number of males are employed by the Administration of this area as labourers on the roads and waterholes, and to erect fences, and attempts are being made to teach them agriculture and husbandry. There is also a school for the children, as well as a modern hospital clinic which functions daily. In addition to the San at Tsumkwe there are isolated groups scattered throughout the semidesert central area at waterholes, who still lead a largely traditional, independent existence. However, most of these !Kung have acquired a few of the amenities of Western civilization such as steel knives, plastic containers, metal cooking utensils and cast-off clothes and shoes. Most of them speak only their own dialect, are illiterate and very unsophisticated.

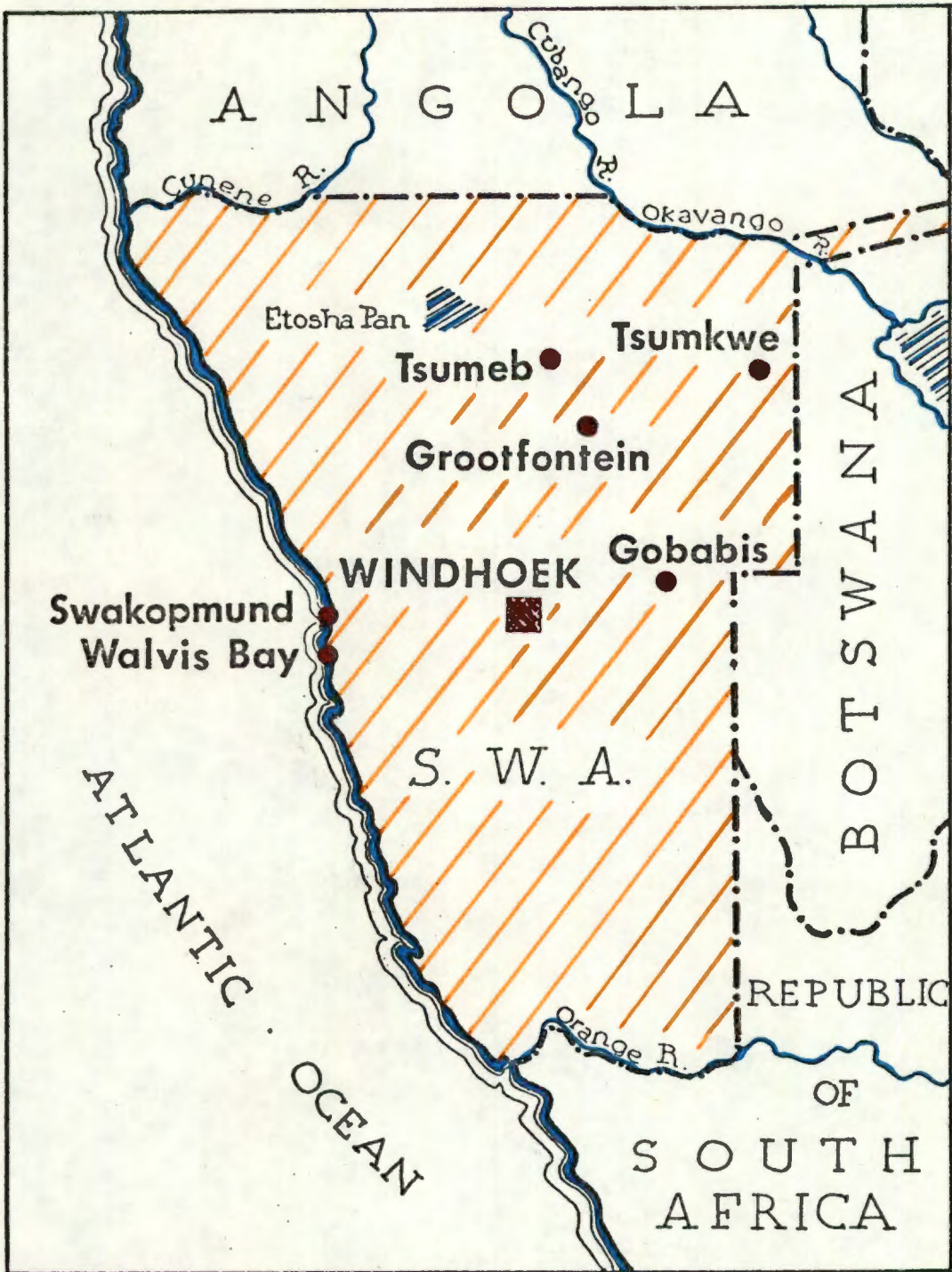


Figure 3.9. Map of South West Africa. Tsumkwe, a tiny village, is shown on the N.E. border close to Botswana. Grootfontein, the nearest large town, is 290 km away.

The family unit: The unit is the family with matriarchal dominance and even in the main settlement at Tsumkwe there is little social organization to leadership. Bigamy is accepted among the San, and where this occurs both wives and their children are equal members of the family unit. Two such families are included in this study. Divorce, remarriage and adultery do occur but are not prevalent (Botha et al., 1973).

One of the problems encountered in studying the San was the difficulty in ensuring that we were dealing with families and their biological relationship. Kinship terms are much wider and have a much less specific meaning than in Western societies. For example, a man may refer to another individual as 'my brother' whereas they may not share a single parent. This is because people with the same names are regarded as brothers. Among the !Kung there are approximately fifty names for males and fifty for females. Foster children who are accepted into a family are thereafter consistently referred to as 'our child' and could therefore inadvertently be included in a sibship study. Language differences, lack of expert interpreters and this different concept of kinship introduced problems in establishing family relationships in this study. However, in Tsumkwe itself a missionary resident in the area for ten years, who knew the people and their language, provided invaluable assistance.

Since 1953 the !Kung in the Tsumkwe area are protected and now live in virtual isolation. They marry strictly within their own community and have an average of three living children per couple. Their average life expectancy is estimated to be about forty-five years.

The San were selected for this study because of their small numbers and geographical isolation, important factors in producing genetic relatedness.

IV CONSANGUINEOUS FAMILIES

We decided that the most obvious way of finding individuals truly homozygous at the MLC locus (or loci) was to look for the HLA-A, B homozygous offspring of consanguineous parents, as they may be expected to be homozygous by descent for the whole MHC and therefore also for the MLC genes.

Twenty-five per cent of father-daughter offspring on average may be expected to be HLA-homozygous whereas only one in sixteen of first-cousin marriages may be. Furthermore even if the latter are selected as being homozygous for the A and B antigens, there is a considerable chance that one of the haplotypes has been introduced by another ancestor, especially if the haplotype in question is common in the population. For example, if only the first cousins and their children have been typed there is only a 50% chance that the haplotypes of an HLA-A1,B8 homozygous child are identical by descent. In addition the possibility exists that a crossing-over event may have taken place between the B and D loci in one of the six meiotic divisions which have occurred from the common ancestor to the first-cousin offspring.

i) First-degree Consanguineous Families

For the reasons detailed above, we first set out to find first-degree consanguineous offspring. Seven such families were traced in the Cape Town area, but only two families were available for study.

1. FAMILY COO: This is a Caucasoid family, consisting of a father and mother with sixteen siblings (Fig. 9.1). The father mated with his eldest daughter and the two offspring from this union are thus consanguineous in the first degree. This is common knowledge in the family and among their neighbours, and the father, mother and daughter by whom he had the children all admitted this. The family lives under very poor socio-economic circumstances, and the father and several of the sons drink alcohol to excess.

The HLA types of the parents and twelve of the children in generation II and the two children from the consanguineous union, generation III, were determined. MLC studies were done on the parents, sib II-1 and her two children III-1, III-2 and sibs II-13 and II-16.

2. FAMILY MEI: This Cape Coloured family in which the father mated with his daughter and produced a child came to my attention because of a paternity dispute (Fig. 9.2). The brother II-2 was not included in the MLC experiment.

ii) Fourth-degree Consanguineous Indian Families

Consanguineous marriages are still reasonably common in the Indian Muslim community in Cape Town, and a search for such families was accordingly undertaken. The task was hampered by the reluctance of many individuals to participate in the study.

Three Indian Muslim families where the parents were related were studied :

1. FAMILY FEG: This is a Cape Indian Muslim family in which the parents are first cousins (II-1 and II-2), being the offspring of two brothers I-1 and I-2 (Fig. 9.3). The father, II-1, immigrated to South Africa from Rander in India as a young adult, and the mother, II-2, at the age of two years. Both parents and seven of the ten siblings were tested. There is a pair of monozygotic twins among the siblings.

2. FAMILY HOO: This Cape Indian Muslim family was also studied because of consanguinity in the parents III-1 and III-2 whose fathers II-2 and II-3 were brothers as shown in Fig. 9.4. The mothers of III-1 and III-2 were also related, i.e. I-2 the mother of III-2 was the maternal great-aunt of III-1.

Although the father III-1 was born in South Africa, his father came from Surat in India (near Bombay), his wife III-2 was born in India and immigrated to South Africa as a young girl. Father III-1, mother III-2 and their twelve children were tested.

3. FAMILY CAD: The third Cape Indian Muslim family tested consisted of parents II-1 and II-2, who are first cousins, and their two children (Fig. 9.5).

The Indians (Muslims)

The Indian (Muslim) families presented in this study are part of a group of $\pm 11,500$ Indians in the Cape Peninsula area of whom $\pm 65\%$ are Muslims. According to a report of the Department of Indian Affairs in 1973, there are a total of 620,417 Indians in South Africa, of whom 21,617 are in the

Cape Province, 80,556 in the Transvaal, 3,441 in the Black Homelands and the majority, 514,803, are in Natal. A more recent estimate of the number of Indians in South Africa is 750,000 in 1975.

The arrival of the Indians:

In 1859 Natal passed legislation for the importation of labourers from India. This was because the pioneering European sugar planters in Natal experienced considerable difficulties with the local Zulu labourers, whom they found to be unstable and not in possession of the necessary agricultural skills. Experience in other sugar-producing territories indicated that Indian labour was far superior.

After lengthy negotiations and petitions from the colonists to the Court of Directors of the British East India Company, to the Secretary of State for the Colonies and to the Government of India, legislation was passed and two ships, the *Belvedere* at Calcutta and the *Truro* at Madras, were chartered to bring the first shipload of 340 Indian immigrant labourers to Natal. In November 1860 the ships landed in Natal with 318 persons of whom 16 were Muslims.

The Indians who came to Natal under contract were imported primarily to serve on the sugar plantations. They were indentured to their employers for three years; hence the term '*indentured*' labourers. After expiry of this first term of indenture, the labourers could enter a new contract of service

or on payment of £2.10 for each year unfulfilled in the new contract buy their freedom. They were then free to seek employment elsewhere in the colony and follow whatever vocation they chose. Many of these 'free' Indians were quickly absorbed in other parts of the colony including the Cape at higher rates of pay. They were employed as artisans, cooks, house-servants, tailors, laundrymen or in other occupations. There was an interval of eight years from 1866 to 1874 when no 'indentured' labourers were imported, because of agitation of the White colonists, with whom they were competing in trade, and because of the world depression. The importation of Indian labour was resumed in 1874, and continued until 1911, when the Government of India finally stopped it. In all, 152,184 persons were introduced as indentured labourers between 1860 and 1911. There were a few Christians and a small number of Muslims among these 'indentured' labourers, but the majority were Hindus belonging to different caste systems and spoke Tamil and Hindi.

Following on the indentured labourers, many Muslim and a small number of Hindu traders came to the new colony from the province of Gujarat in British India, speaking Gujarati and Urdu. They are referred to in official records as 'Arab Traders' to distinguish them from the class of labourers imported under indenture. Almost at the same time *other groups of adventurers* with a variety of skills - goldsmiths, silversmiths, and workers in metals and precious stones - followed. These two classes are described as '*free-passenger Indians*' in official records. There are no statistics to indicate the numbers of these free-passenger Indians as they were not bound to be registered with the Protector of Indian Immigrants (South African Encyclopaedia, 1972, "Indians"). The majority of the Indians who immigrated to the Cape and the Transvaal were of this 'free-passenger' type who came after

the indentured labourers (Brand, 1966).

Indians (Muslims) in the Cape Peninsula: Of the total of 620,412 Indians in South Africa, only 16 per cent (99,266) are Muslims, according to the 1970 census. This study has been undertaken in the Cape Peninsula where there are $\pm$ 11,500 Indians, and 65 per cent (7,475) of these are Muslims. Most of these Muslims came from scattered areas in North India, others came from Cochin in the south and from Hyderabad, now the capital of the Central Province, Andhra Pradesh.

In a survey done by Brand (1966) it appeared that most of the Indians in the Cape Peninsula were born in India, and migrated directly to the Cape. Many of the men, however, married only after arriving at the Cape. This explains why he observed a higher proportion of Cape Town-born women. The majority of the Indian Muslims in the Cape are Gujarati-speaking or Urdu-speaking. They identify themselves with their religio-linguistic group, and in some instances with their broader caste group, rather than with the Indian population as a whole. Brand (1966) writes :-

"It has often been noted by students of immigrant communities across the world that there are numerous forces operating toward facilitating assimilation of the immigrant community into the host society on one hand, and toward the building up of a resistance to such assimilation on the other. In South Africa these factors have been extremely complex in character owing to the heterogeneity of the population, and to historical developments. In spite of indications of increasing assimilation, interest has constantly been aroused by the way the Indians in South Africa have succeeded in maintaining their ethnic identity."

(Brand, 1966, p.1)

Language and religion: In order of magnitude the linguistic groups among the South African Indians are the following: Tamil 38%, Hindi 25%, Gujarati and Urdu 25%, Telugu 12%. On the basis of religious affiliation, the South African Indians can be classified roughly as follows: 72% Hindu, 16% Muslims, 12% Christian (according to the 1970 census). The Hindu and Muslim sections have retained their forms of worship, festivals and other customs almost intact, with the exception of their caste system which they forfeited when they left their country. In some parts of the country they are even more pious than the original groups in India.

The Indian family: The Indian family differs in some important aspects from Negro and White families in South Africa even when these groups live under similar economic conditions in the same urban environment. The most obvious differences are:

The greater emphasis laid on arranged marriages - they traditionally marry within their family group and first-cousin marriages are common among the Muslims. (It is, however, laid down in the Holy Qur'an, Chapter iv, Verse 23 p. 186 (see Appendix) that unions between parent-child, brother-sister, uncle-niece and aunt-nephew, and other close relatives are prohibited.)

A higher proportion of Indian households are occupied by more than one related family - few live completely on their own, as the lonely and old are usually cared for by a young household.

Indian society is strongly patrilineal, with the oldest living patriarch at the apex of the structure where he exercises a very strong influence. This structure is known as

'kool' or 'kootem' in Hindi or Gujarati and as 'koodoobhia' or 'koodoomor' among Tamil- and Telugu-speaking Indians (Indian South Africans, 1964).

The reasons why Muslim Indians favour parallel-cousin marriages are:-

A persistence of Hindu social attitudes in a group only historically recently Islamicized.

In the small communities in India where they originated there was a tendency to concentrate wealth in families and not to disperse it by out-marriages.

The families are usually fairly large and it is not unusual to find as many as ten to twelve children in a family. The reason is partly socio-economic - a lack of birth control and the fact that children are a form of wealth and insurance for old age. Traditionally parents stay with their children, and are not sent to old-age homes. Kinship is regarded as of primary importance and kinsmen are expected to render financial assistance in times of need. This help is usually not given from a sense of duty but from a sense of family. To help a kinsman is not only obligatory, it is a privilege, because of the urge to maintain relationships. It is an old Muslim practice to share everything within the family.

Family planning: There appears to have been little family planning among the older generation of Indian Muslims in South Africa, although all forms of birth control are sanctioned. In the year 1107 Imaam Ghazzali wrote his masterpiece "Ihya - Uloom - Iddien" (the revivification of the science of religion) in which he makes it clear that birth control is permissible, but he does not agree to its practice because of a fear of not earning enough to feed and rear children. In June 1953 a council of learned men met in Cairo

to discuss the problem of birth control in order to give a directive to all Muslims on the question. The whole council agreed that birth control is a decision to be left to individual practice, for reasons of necessity concerning the individual: that it is not permissible for all (i.e. for the whole nation); and that it is not right that there should be state legislation on birth control (Dien, 1968).

The three Indian Muslim families studied belong to this group of people who have retained their identity and customs in the multiracial society of South Africa. They were uniquely appropriate for this study as their social customs still favour first-cousin marriages.

In *summary*, there were available three categories of family suitable for studying the MLR. In the first place, there were two families, one Caucasoid and one Coloured, in which there were offspring who were the products of first-degree consanguineous matings; in the second, the San families came from a population in which consanguinity was inevitable for almost any family, though its degree could not be assessed accurately; and finally there were families in which the offspring were known to have resulted from fourth-degree consanguineous marriages and which came from a population in which this type of marriage is preferentially practised.

THE RECOGNITION OF MLC DETERMINANTS (MLC TYPING) BY MEANS OF MLC HOMOZYGOUS CELLS

We used the MLC homozygous cells from sib III-1 from family C00 (Pedigree 9.1) in unilateral MLC tests with a group selected from a large panel of HLA-A, B typed unrelated individuals whose genotypes had been established by typing their families (Table X.1).

The test group consisted of the following :-

- a) Cells from sib III-1 (C00) homozygous for HLA-A1,B8 and MLC determinants by descent.
- b) Four unrelated individuals Nos. 2-5 homozygous for the serological determinants HLA-A1,B8.
- c) In addition there were two unrelated individuals Nos. 6 and 7 who were HLA haplo-identical with the previous five individuals in this group.
- d) The control X carried four other HLA-A and B antigens.

CHAPTER FOUR - METHODS

I THE MIXED LYMPHOCYTE CULTURE (MLC) TEST

Preparation of Lymphocyte Suspension

Mitomycin-C Treatment of Cells

Lymphocyte Culture

Determination of Thymidine-2-¹⁴C Incorporation

Harvesting procedure

Counting radioactivity

Morphological Interpretation

MLC Typing

II HLA TYPING

a) San

b) Other Families and Individuals

III RED CELL ANTIGEN TYPING

IV OTHER TECHNIQUES OCCASIONALLY EMPLOYED

V SETTING UP A LABORATORY DURING FIELD WORK ON THE SAN (BUSHMEN) AT TSUMKWE

Collection of Blood Samples

Problems Encountered

I THE MIXED LYMPHOCYTE CULTURE (MLC) TEST

Bilateral and unilateral MLC tests were performed employing the technique developed by Schellekens and Eijsvoegel (1968) and Du Bois et al. (1973) with slight modifications.

The amount of blood required varies depending on the size of the family or group on which the MLC is performed. We found the number of cells required for a particular MLC test could be calculated on a lymphocyte yield of approximately 1×10^6 per 1 ml of whole blood. Using this method, we had less than 5% granulocytes and monocytes, and an erythrocyte - lymphocyte ratio of $< 1:1$, in keeping with the findings of Schellekens and Eijsvoegel (1968).

The technique used for the MLC test is described in full, in order to illustrate the problems of setting it up for field work. (See Appendix for list of materials and equipment taken on the expedition to Tsumkwe.) The technique used for the collection of blood in the field at Tsumkwe varied from the standard method, and will be described.

Preparation of Lymphocyte Suspension

Venous blood was defibrinated by shaking for ten minutes in a sterile conical glass flask with glass beads (one bead for each 1 ml of blood). Aliquots of defibrinated blood were mixed with equal amounts of Eagle's minimum essential medium for suspension cultures (MEM-S) (Eagle, 1959) supplied by Grand Island Biology Co., New York, U.S.A., buffered with 0.025 M Tris. This medium was used throughout the MLC test.

The lymphocytes were isolated using the equilibrium density centrifugation method described by Böyum (1968). This separation fluid consists of ten parts of 33.9% sodium metrizoate solution (Nyegaard & Co., AS. Oslo, Norway) mixed with twenty-four parts of 9% Ficoll (Pharmacia, Uppsala, Sweden), giving a final density of 1.077. Eight ml of the diluted blood was then layered on top of 4 ml of the Ficoll-sodium metrizoate mixture in a Falcon tissue culture tube (16 x 125 mm, Cat. No. 3033). This gradient was centrifuged at room temperature for twenty minutes at 1,000 g (as measured at the interface). After centrifugation the lymphocytes form a layer at the interface, and most of the red cells and granulocytes sediment to the bottom. The supernatant medium plus the serum were aspirated with a Pasteur pipette by means of a vacuum pump and discarded. Then the lymphocyte layer, together with approximately 1 cm of fluid above and below it, was removed with a Pasteur pipette. The cells were washed twice by resuspension in Eagle's MEM-S and centrifuged for ten minutes at 400 g at room temperature. After the removal of the second washing fluid by means of a Pasteur pipette and suction pump, the sediment was resuspended in a known volume of MEM-S + 20% heat-inactivated AB serum (stored at -70°C).

A leukocyte count was performed on an electronic cell counter (Coulter Electronics Model F) by taking 0.05 ml of cell suspension with a sterile volumetric pipette into 25 ml of Isoton (Coulter). Finally the cell concentration was adjusted to 3×10^5 cells per ml by the addition of MEM-S supplemented with 20% heat-inactivated human AB serum, penicillin 100 U/ml and streptomycin 100 $\mu\text{g/ml}$. The penicillin and streptomycin were always freshly prepared for the experiments except at Tsumkwe where stock solutions were made and refrigerated at -18°C until used.

Mitomycin-C Treatment of Cells

A normal MLC is a two-way reaction: both lymphocyte populations can stimulate each other to transform and synthesize DNA. As DNA synthesis is the measure of the reactivity, it can be made *unilateral* by inhibiting the DNA synthesis of one of the two lymphocyte suspensions.

Mitomycin-C (Bach and Voynow, 1966) was used for this purpose in these experiments, X-rays (Kasakura and Lowenstein, 1965) and N-mustard derivatives (Ceppellini et al., 1965) have also been used. After such treatment the cells retain their powers of stimulating the non-treated population to produce DNA, and this is usually called a *one-way or unidirectional* reaction.

The Mitomycin-C treated cells were prepared by transferring a known volume of cells from the original concentrated suspension to a 50 ml Falcon tube (Cat. No. 2070). The volume of cell suspension must not be more than 1 ml, to prevent diluting the Mitomycin-C, but the number of cells is not important. This cell suspension was then diluted by the addition of 4 ml of MEM-S, and finally 0.16 ml of Mitomycin-C solution was added to bring it to a final concentration of 20 µg/ml. After mixing, the cells were incubated at 37°C for thirty minutes in a waterbath. Subsequently the cells were washed twice in 20 ml of MEM-S. The centrifugation was performed for ten minutes at 400 g at room temperature. The supernatant medium was removed, and if necessary clumps were removed by microfiltration. The cells were resuspended in a calculated volume of MEM-S + 20% heat-inactivated AB serum, penicillin 100 U/ml and streptomycin 100 µg/ml to a concentration of 3×10^5 cells per ml.

Lymphocyte Culture

The lymphocytes were cultured in 11 x 70 mm polystyrene tubes with caps and spherical internal bottoms (Nunc, Roskilde, Denmark, Cat. No. 1090). The lymphocytes of both individuals were always present in equal numbers of 1.5×10^5 cells in the reaction mixture giving a total of 3×10^5 cells in a final volume of 1 ml in each culture tube. *Each combination was performed in triplicate or quadruplicate.* This fourth tube was included initially for a morphological preparation, in order to exclude toxicity in case of a negative thymidine-2-C¹⁴ incorporation and also to compare the morphological results with those of the thymidine incorporation.

1. Two-way or bilateral cultures

Aliquots of 0.5 ml per culture tube of cell suspension from both donors were distributed by means of an automatic pipette with disposable tips (Biopette Becton, Dickenson & Company, Cat. No. 0010-30) resulting in a 1:1 ratio in a final volume of 1 ml.

2. One-way or unilateral (Mitomycin-C treated cultures)

Then 0.5 ml of Mitomycin-C treated stimulatory 1.5×10^5 cells (designated, e.g. A_m: B_m, etc.) + 0.5 ml non-Mitomycin-C treated responding cells 1.5×10^5 (designated, e.g. A, B, etc.) were mixed in the culture tubes.

3. Controls

Phytohaemagglutinin (PHA, Burrough Wellcome, Cat. No. NR-10) and negative controls were always included for each suspension (see Table IV.1 for example of protocol for MLC tests). The PHA stimulation was carried out by the

TABLE IV.1. EXAMPLE OF PROTOCOL FOR MLC TEST IN THE COMBINATION A + B

BILATERAL MLC TEST	UNILATERAL MLC TEST
A + B <u>Control</u> A (PHA) - Maximal stimulation and cell viability A Background control	$A_m + B$ (unilateral in both directions) $A + B_m$ <u>Control</u> A (PHA) - Maximal stimulation and cell viability $A + A_m$ Background control and Mitomycin-C inhibition

m = Mitomycin-C treated

If one of these combinations has been omitted, it is always mentioned in the discussion of the final data.

addition of 0.05 ml of PHA to each tube by means of a disposable tuberculin syringe, the final concentration of PHA then being 40 $\mu\text{g/ml}$ of tissue culture medium. Each of the PHA - and negative control tubes contained 3×10^5 cells in 1 ml of culture medium from the same cell donor.

All the tubes were then capped and placed in an incubator at 37°C . The PHA-stimulated cultures were harvested after seventy-two hours, and all other cultures were terminated after 144 hours.

Determination of Thymidine-2- ^{14}C Incorporation

Twenty-four hours before harvesting the cells, 0.075 μCi thymidine-2- ^{14}C in 0.05 ml culture medium, of a specific activity of 59 mC/mM (Amersham, England) was added and the tubes were well shaken. (The specific activity was hotter than required, but the only one locally available.)

Harvesting procedure : After twenty-four hours the contents of each tube were transferred to a vertically placed 20-ml syringe without plunger, with the centrally placed needle aperture downwards, which was connected to a Millipore Swinnex - 13 mm filter unit (Millipore Corporation, Bedford, Mass., U.S.A.) containing a glass-fibre filter (Cat. No. AP2001300). The needle-side of the filter-holder was connected to a vacuum line (see Fig. 4.1 for a sketch of this hand-made apparatus consisting of twelve of the above-described syringes and filter-holders). (This unit was kindly donated to us by Dr V.P. Eijssvoogel of the Central Laboratory of the Netherlands, Red Cross Blood Transfusion, Amsterdam, Netherlands.) A vacuum was then applied to this and the culture medium containing the

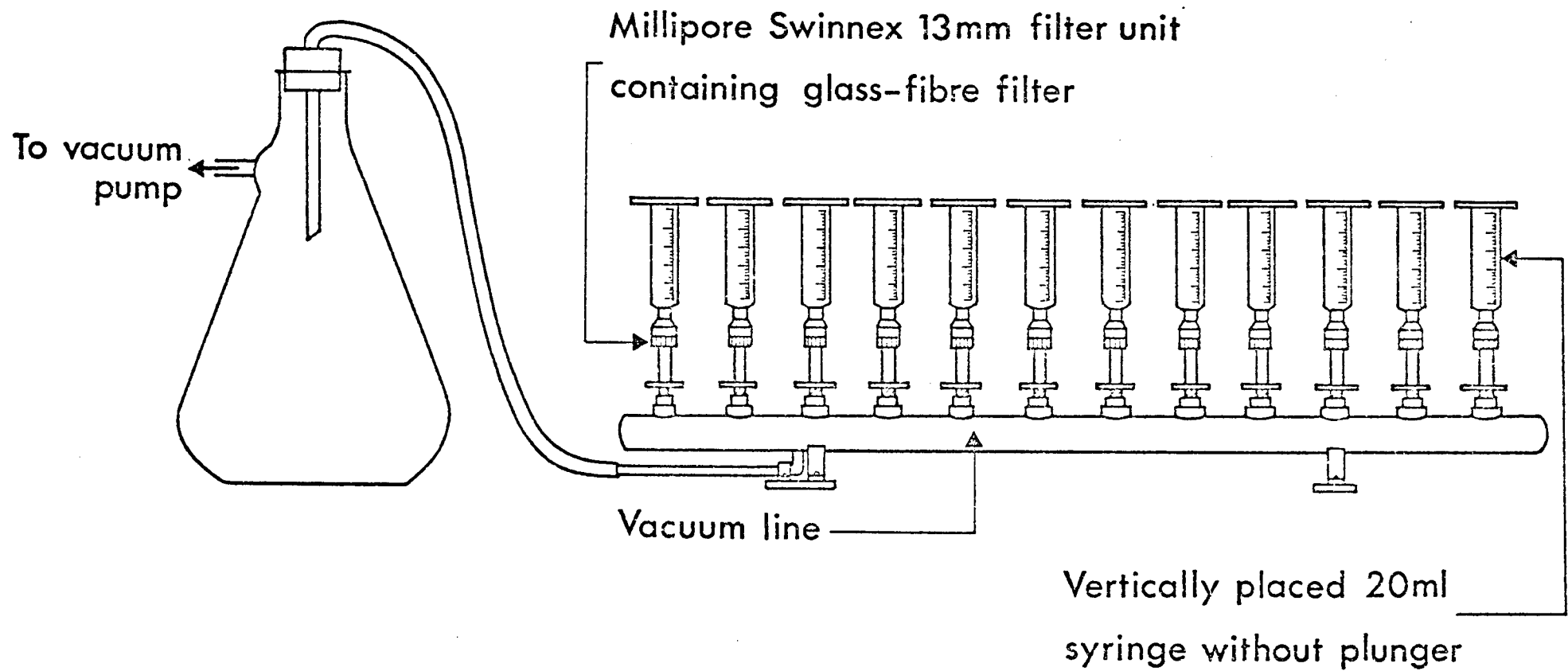


Figure 4.1. Sketch of apparatus used for the harvesting of mixed lymphocyte culture (MLC) tests.

non-incorporated radioactivity sucked through the filter, while the lymphocytes remained on top of it. Subsequently, the culture tube was rinsed with 2 ml of saline which was also transferred to the syringe. Finally the lymphocytes were washed with 20 ml distilled water (this will induce lysis of the remaining red cells on the filter) in the same manner as before, whereafter the filter was removed from the filter-holder, placed in a counting vial and dried overnight at 37°C in an incubator.

Counting radioactivity : After the addition of 2 ml of scintillation fluid (11.25 g PPO, 1 g POPOP, and 2.5 litre toluene) to each counting vial, the radioactivity was determined by means of a Packard TRI-CARB Liquid Scintillation Spectrometer, Model 3385 (efficiency 90-95%) for ten minutes.

The arithmetic mean and the standard error (se) of each triplicate set of tubes were calculated and expressed as counts per minute (cpm).

Morphological Interpretation

When the lymphocytes obtained from human blood are kept in culture without any apparent stimulus a small number of them spontaneously transform into 'blast' cells. But, in the presence of foreign cells, this transformation into 'blast' cells is greatly increased. It is difficult to quantitate this morphological phenomenon of the lymphocytes *in vitro* in a meaningful way. We used the percentage of 'blast' cells after 144 hours of incubation as an indication of the responsiveness of the lymphocytes.

±	denotes < 5% blast formation
+	denotes > 5% < 10% blast formation
++	denotes > 10% < 15% blast formation
+++	denotes > 15% < 30% blast formation
++++	denotes > 30% blast formation

Macrophages were not included in these counts.

In the *negative* control cultures, blastoid transformation was seen in less than 2% of cells examined and in the majority, transformation was less than 0.5%. In the *positive* control cultures, where cells from single donors were stimulated with PHA, the transformation was much more noticeable, often 95% or more of the cells in culture after seventy-two hours. Blastoid transformation usually varied from 5-15% of the cells in the mixed cultures but was as a rule absent when lymphocytes of HLA identical siblings were grown together. There are, of course, various inherent sources of error in this method, such as the problems of a visual counting technique, and as many as 10,000 cells had sometimes to be examined to achieve a significant result, especially because of the tendency for blast cells to be distributed at the edge of the smear.

Phase-contrast microscopy was used to study the morphology of the MLC cultures. This method was chosen because of its simplicity and ease compared with the acetic orceine staining technique, especially in the field studies on the San group. In addition it was an insurance against failure of any sort of assessment of the MLC tests if the final discs for the liquid scintillator counting were lost or damaged during transport to the laboratory. The extra cells cultured for morphological examination were prepared by discarding the culture media and gentle agitation of the cells on a Whirlmix. The contents of the tube was then transferred to a clear slide, sealed with a coverslip and examined immediately under a phase-contrast

microscope. It must be emphasized that lymphocytes and the large 'blast'-like cells derived from them by transformation are both actively motile cell types. Their appearance in the living state is very different from that observed in conventional fixed-stained slides, which do not show the dynamic interrelationships between cells in culture by means of intercellular processes termed uropods (Greek: 'oura', tail and 'podos', foot, stalk) (McFarland et al., 1966). The motile lymphocytes in suspension cultures are easily recognized by their irregular 'pear-shaped' configuration.

In the wet preparation the cell has an overall diameter of 16 μ and the nucleus is central with prominent nucleoli. 'Blast' cells in mitosis are readily recognized and some workers rely on the percentage of mitotic figures in a preparation to indicate the extent of the *in vitro* response. In highly active cultures mitoses were frequent and the lymphoblasts were usually present in large aggregates often around a central macrophage.

MLC Typing

MLC typing cells are cells from individuals who carry the same information for the MLC stimulating determinant on both chromosomes, i.e. individuals who are known to be homozygous for the HLA-D locus determinant usually on the basis of family studies. Although HLA-D homozygous individuals are found in the outbred population, especially in families where the parents share one common HLA-A, B haplotype the best source is the homozygous offspring of parents who are related.

For MLC typing the one-way test is used (in which the stimulator cells are inactivated by X-rays or Mitomycin-C)

so that it can act as a stimulus only for the responder cells that are not inactivated. The basic concept of this test is that if a test cell does not show an MLC response to a given homozygous typing cell, then it presumably has the determinant carried by the typing cell, e.g. -

1. If X is the cell to be typed (responder cell) and a/a is the homozygous typing cell (stimulator cell);

if the MLC test is *negative*, the MLC type of X must be a/a, a/b, a/c, etc;

if the MLC test is *positive*, the MLC type of X must be b/c, etc.

The MLC test can also be reversed if the initial test with the homozygous cell a/a as stimulator was negative, e.g. -

2. If X_m versus a/a is *negative*, then X must be homozygous for the typing cell a/a.

The reactions of a panel of cells to a series of typing cells can be analyzed according to the same principles used for the analysis of serological reactions, to establish specificities defined by similar reactions to a set of typing cells. For the Sixth International Histocompatibility Workshop, fifteen laboratories collaborated and each used sixty-two carefully selected homozygous typing cells to type a panel of not more than 100 responders.

From the analysis of these data it was concluded that: six and possibly eight determinants could be defined at the D locus. These determinants behaved as if controlled by a series of alleles at the D locus. It was also clear that, as in the case of the serologically defined specificities of the A, B and C loci, new information will probably lead to 'splitting' of these MLC determinants and possibly of the D locus itself.

The strong linkage disequilibrium between the D and B loci alleles was also confirmed (Bodmer, 1975).

II HLA TYPING

The HLA antigens of the A and B loci were determined by the two-stage microlymphocytotoxicity technique recommended by the National Institute of Health (N.I.H.), U.S.A..

a) San

Blood was collected in the Hyland transport set (for full description see page 109 and Fig. 4.2) and typed with the Fifth Histocompatibility Workshop collection of anti-sera. In addition to performing the lymphocytotoxicity testing, all samples were tested by the N.I.H. standard micro platelet complement fixation technique and the micro-EDTA agglutination test (Van Rood et al., 1970). The results of these tests showed poor correlation with the cytotoxicity results (except for sera recognizing the well-defined antigens) and have therefore not been included in this study. Further details of the serological studies on the San have been previously reported by us (Botha et al., 1973).

b) Other Families and Individuals

Fresh blood samples were collected in Thromboliquine (NV Organon Oss, Holland) and purified by Ficoll-Isopaque flotation as described by Böyum (1968). These cells were tested with a battery of 140 local and other sera.

III RED CELL ANTIGEN TYPING

The antigens listed in Table IV.2 were tested in all the families. The erythrocyte genetic markers were not done on the HLA identical unrelated groups as they were not relevant to the study.

At the onset of the study the Henshaw and the Lewis (Le^a) antigens were also included, but as they are not done as a matter of routine in our laboratory, they were later omitted except in the two San families IV and VI where additional markers were sought to solve the question of disputed parentage. As indicated in Table IV.2 commercially obtained antisera were always used according to the instructions of the manufacturers and standard methods were used for the locally prepared antisera. A clotted sample was taken and saline suspensions made from the cells at the bottom of the clotted sample tube, care being taken to remove all clots.

IV OTHER TECHNIQUES OCCASIONALLY EMPLOYED

Since the serum protein and red cell enzyme determinations were kindly performed in Professor T. Jenkins' Laboratory at the South African Institute for Medical Research in Johannesburg, the methods are not described here in detail. They are reported in his Thesis 'Genetic Polymorphisms of Man in Southern Africa' (1972). A list of the serum proteins and red cell enzymes tested for and a brief description of the techniques employed are given in Table IV.3. These serum protein and red cell enzyme tests were used initially on the three Indian families but were then omitted as none of them showed any peculiarity. They were, however, again used on San families IV and VI as they provided additional markers to elucidate the problem of disputed parentage.

TABLE IV.2. ANTIBODIES AND TECHNIQUES USED FOR RED CELL

ANTIGEN TYPING

Antigen	Technique Employed	Source of Antibody
AB	Saline, tube	Local
A ₁	Saline, tube Lectin	Extracted from Dolichos biflorus commercial
Rh(C), (c̄), (D), (e)	Saline, tube	Commercial
E	Saline, tube	Local
M, N	Saline, tube	Commercial
S, s̄	Coombs	Commercial
Kell (K)	Coombs	Local
Cellano (k̄)	Coombs	Commercial
P ₁	Saline, tube	Commercial
Duffy (Fy ^a)	Coombs	Local
Henshaw (He)	Saline, tube	Produced locally in a rabbit
Lewis (Le ^a)	Coombs	Local

TABLE IV.3. TECHNIQUE USED FOR SERUM PROTEIN AND RED CELL
ENZYME DETERMINATIONS

Serum Proteins	Technique Employed
a) Haptoglobins (Hp)	Starch gel electrophoresis method of Smithies (1955) using the discontinuous buffer system of Poulik (1957) and stained with benzidine.
b) Transferrins (Tf)	Starch gel electrophoresis method of Smithies (1955) and discontinuous buffer system of Poulik (1957), then same run as for Hp identification but stained with Amido Black 10B.
Red Cell Enzymes	
<u>Peptidase</u>	
A	Horizontal starch gel electrophoresis, then staining technique of Lewis and Harris (1967).
B	
C	
D	
<u>Adenosine deaminase(ADA)</u>	Method of Spencer et al. (1968).
<u>Glucose-6-phosphate dehydrogenase (G-6-PD)</u>	Phosphate buffer system of Mathai et al. (1966). The one half of the starch gel was stained for G-6-PD activity by a mixture consisting of 75 ml warm (45°C) Tris-HCl buffer (0.05 M) to which was added immediately prior to pouring on the gel: 9.9 mg G6P, 4.2 mg NADP, 10 mg NBT (or 50 mg INT or 4 mg MTT and 2.5 mg PMS). Incubation for 45 mins in the dark at 37°C is usually sufficient to demonstrate the zones of G-6-PD activity.
<u>6-phosphogluconate dehydrogenase (6-PGD)</u>	The same phosphate buffer system and starch gel preparation as for G-6-PD was employed, one half being stained for G-6-PD and the other for 6-PGD. Staining was carried out using 12 mg

Technique Employed

	6-PG, 1.1 mg NADP, 7 mg NBT (or 25 mg INT or 1.2 mg MTT) 0.5 mg PMS and 0.2 ml $MgCl_2$ (5.1 g/l) are dissolved in 6 ml of Tris-HCl buffer (0.05 M, pH) and mixed with 6 ml molten agar before it was poured over the gel. Bands of enzyme activity develop at 37°C in the dark in about 30 minutes.
<u>Phosphoglucomutase (PGM₁)</u> <u>(PGM₂)</u>	Horizontal starch gel electrophoresis using the Tris-maleate buffer, pH 7.4 (Hopkinson and Harris, 1965; 1966; Harris et al. 1968). The gel was stained with 50 ml Tris-HCl buffer (0.05 M) and 40 ml $MgCl_2$ (0.025 M) warmed to 37°C and mixed with 10 mg NADP, 50 mg G-1-P, 10 mg MTT (or 20 mg NBT or 250 mg INT, 20 λ G-6-PD (activity = 140 units per ml) and 10 mg PMS. This mixture was poured over the gels and incubated in the dark at 37°C for 1 - 3 hours.
<u>Adenylate Kinase (AK)</u>	Horizontal starch gel electrophoresis using the discontinuous histidine-citrate buffer system pH 7.0 (Fildes and Harris, 1966). The gel is stained with an agar overlay consisting of 11 ml of 2% molten agar mixed with 12 ml of Tris-HCl buffer (0.5 M, pH 8.0), 36.6 mg glucose, 74.1 mg $MgCl_2 \cdot 6 H_2O$, 9.7 mg ADP, 4.6 mg NADP 20 mg NBT, 2.1 mg PMS, 5 λ G-6-PD suspension i.e. approximately 0.04 μ /ml and hexokinase 1.4 λ . The zones of AK activity develop after incubation in the dark at 37°C for 30-60 minutes.
<u>Acid phosphatase (AP)</u>	Horizontal starch gel electrophoresis in the buffer system of Karp & Sutton (1967). The gel was stained according to the method of Hopkinson et al.(1963), modified according to Jenkins (1972).

V SETTING UP A LABORATORY DURING FIELD WORK ON THE SAN
(BUSHMEN) AT TSUMKWE

Setting up the MLC test during field work is a formidable task, and this would not have been possible without the meticulous attention to detail by Dr. V.P. Eijsvoogel of Amsterdam in planning it. We were also fortunate that Dr M.C. Botha was able to visit Tsumkwe prior to our departure to assess the available facilities, which were :-

- a) A medical clinic where we were able to use four relatively dust-free rooms (Fig. 4.2)
 - i) one for bleeding and the separation of blood
 - ii) one for the MLC tests
 - iii) one for Prof. T. Jenkins of Johannesburg to do anthropological measurements and separate blood samples for red cell and serum enzyme studies
 - iv) one for Prof. J.J. van Rood, Dr J. van der Does and Miss A. van Leeuwen to do the micro-EDTA agglutination tests
- b) An electrical generator and adequate power plugs
- c) Refrigerator facilities
 - i) two refrigerators (+4°C)
 - ii) a deep freeze cabinet (-18°C)
- d) Adequate bench space $\pm$ 3 sq. metres in each room, plus tables for equipment.
- e) An interpreter: Family relationships between individuals were of utmost importance, particularly for the MLC studies. The resident missionary, the Rev. F. Weich, was not only able to interpret for us, but he also knew the family relationships. Despite this, problems arose as shown in San family IV where some members were



Fig. 4.2 The modern hospital clinic at Tsumkwe where field laboratory tests were done.



Fig. 4.3 The village of Samengaigai near Tsumkwe showing the typical countryside of the area during the autumn season.

not biologically related.

- f) Seasons: The study was planned for autumn to avoid the heat of summer and the rainy season when the San move away from Tsumkwe to better hunting grounds at distant waterholes (Fig. 4.3).

Collection of Blood Samples

The blood samples were collected using the Hyland Trans-Type Lymphocyte Transport-Storage System (Hyland, California, U.S.A.) (see Fig. 4.4 for a sketch of this set). A special 'Transport Storage Medium' was also provided in which lymphocytes could be preserved at room temperature, for up to 14 days to facilitate shipment of the cells to the laboratory for lymphocytotoxicity testing.

Using a sterile taking-set, blood was drawn into a 125-ml Hyland vacuum bottle containing 15-20 small glass beads. The blood was then defibrinated by gentle rotation ($\pm$ 5-10 minutes). A further 10 cc of EDTA blood (for platelet complement fixation and micro-leuko-agglutination tests) and 5 cc of clotted blood (for red cell genotyping and serum enzyme studies) were then collected with the same taking-set in separate vacutainers. After defibrinating the blood in the Hyland collecting bottle, the remaining vacuum was released and 5-10 cc of blood was withdrawn with a syringe and needle for MLC testing.

Subsequently an equal volume of sterile erythrocyte sedimenting agent was added to the remaining defibrinated blood in the Hyland collecting bottle, mixed, and allowed to stand at room temperature for 30 to 40 minutes to allow sedimentation of the red cells. When sedimentation of the red cells was apparent, the long needle of the 'Trans-Type Lymphocyte Separation Set'

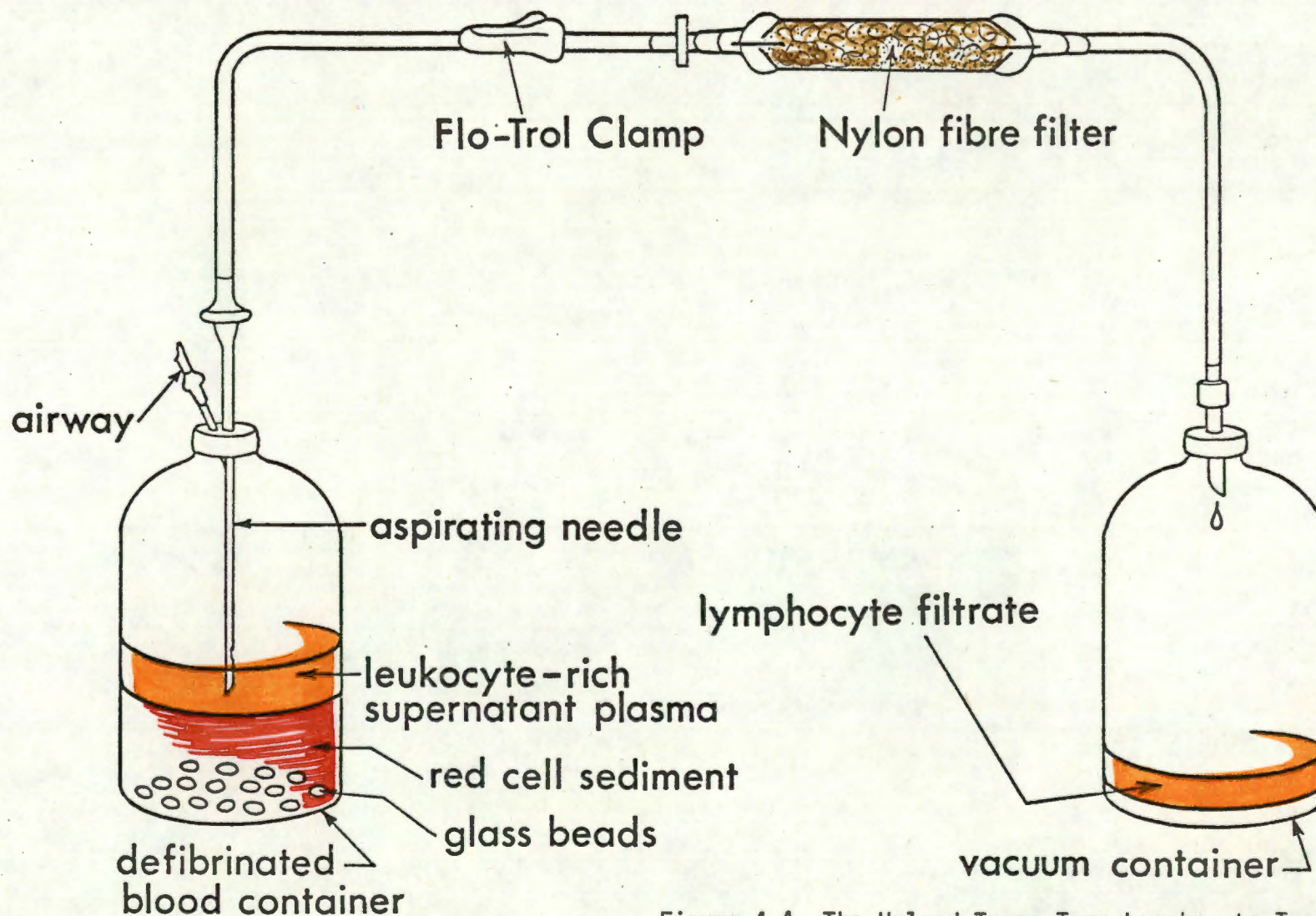


Figure 4.4. The Hyland Trans-Type Lymphocyte Transport-Storage System, used for separating lymphocytes.

was inserted into the leukocyte-rich supernatant. The 'FLO-TROL Clamp' of the tubing was then released and the supernatant drawn off through the nylon fibre filter at a slow rate into a sterile, evacuated 125-ml bottle as illustrated in Fig. 4.4. When this filtration procedure was completed, the clamp was closed and the line disconnected from the bottle into which the supernatant had passed. An equal volume of 'Transport Storage Medium' was then added to this bottle.

The cells for lymphocytotoxic testing were divided into two. One of these was sent to Leiden, Holland, and the other to Cape Town. The division was made as an insurance against loss or delay during air transport.

Problems Encountered

The logistic problems of a study so far from the laboratory were enormous, and this limited the extent of the investigation. Over 300 samples were taken and transported by air to Leiden and when possible to Cape Town, but only 100 samples were suitable for HLA-A and B typing. Blood was collected in Hyland transport sets (as previously described) which had been tried and found to be satisfactory both in Leiden and Cape Town.

Laboratory work was often hampered by the delay in gathering an entire family together at a specific time. The San are traditionally nomadic people, who may suddenly decide to go hunting for the day or even for a few weeks. Thus in some families the MLC studies were done on members of the same family on different occasions, and in others the studies were incomplete. Some of the families lived in isolated settlements some distance away from Tsumkwe and this meant long journeys

through rough country to bring them to the clinic for bleeding. In a few instances bleeding was performed in the 'bush', but this was extremely difficult. The time factor was also a problem as the expedition was planned for only ten days. One day was needed to unpack and install various items, and another to pack, leaving eight days for setting up MLC tests.

Performing an MLC test with 600 tubes per day needs two people for 6 - 8 hours, e.g. :

- 1) A family of two parents, four children, and two unrelated controls means a total of twenty-eight possible combinations. If only a bilateral MLC with PHA and negative controls are performed in quadruplicate, 176 tubes are needed. If both unilateral and bilateral MLCs are done in all possible combinations, 432 tubes are required.
- 2) A family of two parents and six children with two unrelated controls brings the number to 260 (bilateral MLC) and 440 (unilateral MLC).

As PHA controls had to be harvested on the third day, and the rest on the sixth day, from the third day harvesting had to be included into the daily scheme, and this took another 4 - 6 hours. White cell counts had to be done by a visual method and this also takes longer than by the electronic cell-counting method. The cultures that had not yet been harvested on the day of departure from Tsumkwe had to be carried in an insulated box to keep them as close to 37°C as possible and transported by air to Cape Town where further incubation and final harvesting were performed. The filters of the completed cultures were carried home and the radio-activity counted at the laboratory.

Although the expedition was in autumn, it was often very hot, and the room temperature was well above the

required 20 - 22⁰C. This may have contributed to the death of some of the cells. The transport of the equipment to and from Tsumkwe also presented a major problem. A large proportion of materials not available in South Africa at the time were flown from the Netherlands to Windhoek.

PART III

RESULTS

CHAPTER FIVE - PRESENTATION OF RESULTS

Presentation Format

Expression of MLC Reactivity

Presentation Format

The results on each of the families and the HLA-A and B identical unrelated groups studied are presented separately as follows :-

i) A diagrammatic representation of the pedigree of each family is given. The symbols and numbering are those conventionally used in genetics (Fig. 5.1): females are denoted by circles, males by squares, consanguineous matings by double and non-consanguineous by single lines between symbols, the generations by Roman and the individuals in a generation by Arabic numerals. Sibships are given in order of birth. The numbers under each member of the family (as for example in the pedigree of family BEU, Fig. 7.1) represent the HLA-A and B antigens. The letters a,b,c and d denote the HLA haplotypes. Additional symbols used for special purposes are explained in the legend of the pedigree in which they occur.

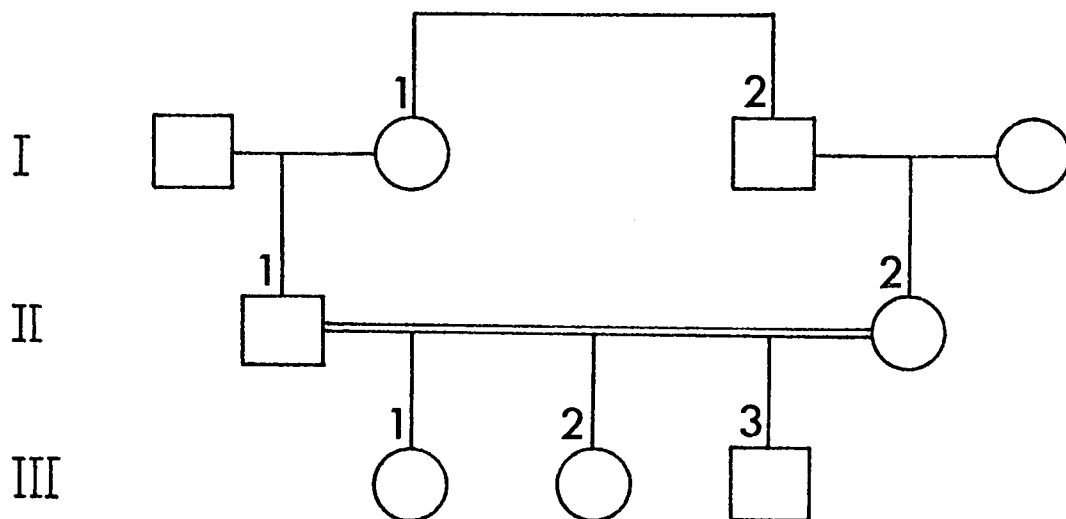


Figure 5.1. A sample pedigree

ii) A Table with the HLA-A and B genotypes and haplotypes (e.g. Table VII.1) of the individuals who were available for HLA typing in each family and unrelated group is given with a comment on the results.

iii) A Table with erythrocyte genetic markers (e.g. Table VII.2) of all the available members of the families is given with a comment on these data. In the first three families studied, families BEG, HOO and CAD (Chapter Nine), additional erythrocyte genetic markers, i.e. Henshaw and Lewis, were tested for, as well as serum proteins and red cell enzymes. None of these showed any peculiarity and as they were not essential for assessment of paternity and were not routine tests in the laboratory when this study was commenced, they were not done on the rest of the families with the exception of San families IV and VI where the question of mixed parentage arose. The erythrocyte genetic markers were not done on the HLA-A and B identical unrelated groups as they were not relevant to the study.

iv) The Tables with the results of the bilateral and/or unilateral MLC experiments performed are given, e.g. Table VII.3. The left-hand column is the identification of the persons according to the pedigree in the families or the code number of individuals in the unrelated groups. The HLA haplotypes represented by the letters a, b, c and d are given in the next column, sometimes with the A and B antigens if relevant to the discussion. Phenotypes only, are given for unrelated individuals where family studies have not been done to establish the genotypes or haplotypes.

All cultures were performed in triplicate and for each combination the mean and standard error (se) of the three results

expressed in counts per minute (cpm) are given. The values enclosed in the rectangles are those discussed in the text. The results of the negative controls for each cell in both the unilateral and bilateral MLC tests are indicated by a diagonal stippled line running through them. In each experiment, control MLC tests were carried out with the lymphocytes from at least one healthy, unrelated individual (X), and sometimes a second unrelated individual (Y). The ability of each cell to respond to phytohaemagglutinin (PHA) is also recorded except in San family II, where there were too few cells available. In the unilateral MLC tests, the Mitomycin-C treated stimulator cells are marked with the subscript m, always listed in the left-hand vertical column, whereas the responder cells are listed horizontally at the top of the Table.

Expression of MLC Reactivity

The majority of the MLC test results here are expressed as a mean of the triplicate in *cpm* with the variation within the triplicate expressed as a standard error (se). It should, however, be emphasized that like most investigators we have found a considerable variation in the degree of reactivity in MLC tests performed on different days. The cause of this variation is not clear. It may be the result of minor technical variations, but this seems unlikely, as a strict protocol is always adhered to. It may, however, be an additional effect caused by a change in the pool of circulating lymphocytes (Eijsvoegel, 1974). As a result of the difficulty in finding an expression of MLC reactivity which gives comparable

values for the same MLC combinations performed on different days, and which also distinguishes clearly between D locus identical sibs and combinations differing for one or two alleles at the D locus, several other approaches have been suggested.

We have used two of these: the *stimulation ratio (SR)*, and the *relative response (RR)* in expressing the MLC reactivity in some of the experiments. The stimulation ratio was calculated by dividing the mean cpm by the cpm of the corresponding autologous control mixture. The definition of SR is:

$$SR = \frac{\text{mean-cpm}}{\text{autologous control-cpm}}$$

and it indicates how many times the counts were higher than the control counts. As recommended in the Joint Report from an MLC Workshop (1974), the SR is expressed as a decimal to prevent confusing SRs with RRs.

The relative response (RR) for a given responder was calculated as follows:

$$RR = \frac{\text{mean-cpm} - \text{autologous control-cpm}}{\text{reference-cpm} - \text{autologous control-cpm}} \times 100$$

The mean cpm for a given responder are corrected by subtracting the autologous control cpm. The highest test cpm observed with the corresponding responder cells that day was used as the reference value because this should reflect the general responsiveness of the cell. The response against each stimulator is then expressed as a percentage of the reference cpm minus the autologous control cpm without decimals.

Initially, *morphological* assessment of the MLC cultures was done on all the families in an effort to find another parameter by which to judge whether stimulation between individuals was present and to what extent, and to see how these values compared with the thymide incorporation results. Later in the study these were omitted as little additional information could be gained from them. The morphology results are given in the same Tables as the thymidine incorporation results in the bilateral MLC tests, and in separate Tables in the unilateral cultures.

CHAPTER SIX - THE UNRELATED INDIVIDUALS

- (i) Unrelated Individuals Different for HLA-A and B
- (ii) Unrelated Individuals Identical for HLA-A and B

Interpretation of Results

Two groups of unrelated individuals, selected on the basis of HLA-A and B typing, were studied.

(i) Unrelated Individuals Different for HLA-A and B

As part of this study MLC tests were done on unrelated HLA-A and B different combinations, employing the bilateral MLC technique in 318 cases, and the unilateral technique in 513.

The results of all the tests on these HLA-A and B different unrelated individuals are summarized in Table VI.1. Included is a small number of unrelated persons with varying degrees of HLA disparity as well as some who are HLA-A and B identical. None of the 318 *bilateral* MLC combinations was negative, and only one of the 513 *unilateral* tests was negative among the individuals differing for two A locus and two B locus antigens (Group a, Table VI.1). One of the thirty-eight *bilateral* MLC tests (Groups b and d), where the MLC pairs shared one B locus antigen and differed for one or two A locus antigens, was MLC negative and eleven of the seventy *unilateral* MLC combinations in the same group, were negative. In one pair who shared the same HLA-B antigen but differed for the two HLA-A antigens, reciprocal non-stimulation was observed in the unilateral MLC test (Group c). Among the HLA-A and B identical combinations (Group e) there were four *bilaterally* negative pairs and thirteen *unilaterally* negative pairs.

(ii) Unrelated Individuals Identical for HLA-A and B

This group comprised seven unrelated individuals with the HLA-A and B phenotype A1,3;B7,8 one with the phenotype A1,3;B7,W35 and a control X, with the phenotype A9,28;B14,W17.

TABLE VI.1. MLC REACTIVITY IN HLA-A AND B TYPED UNRELATED INDIVIDUALS

Group	No. of HLA antigens different in unrelated pairs					
	A locus antigens	B locus antigens	No. of bilateral MLC combinations	No. of unilateral MLC combinations	No. of bilateral MLC negative tests	No. of unilateral MLC negative tests
a	2	2	318	513	0	1
b	2	1	4	13	0	3
c	2	0	-	28	-	2
d	1	1	34	57	1	8
e	0	0	42	26	4	13

The results of *bilateral* MLC tests carried out among these are shown in Table VI.2. This experiment was performed at the onset of this study when the importance of doing uni-directional MLC tests was not fully understood and was not routinely done.

The three parameters included in these results - the stimulation ratio (SR), mean cpm $\pm$ se and the morphological assessment - are not always in full agreement. The SR, which shows how many times the counts were higher than the control counts, is dependent on the control values. This was clearly illustrated in this MLC test by the low SR seen in all the combinations where the high control value of individual R0 (Table VI.2) formed part of the calculation. The morphological results in this experiment are also at variance with the thymidine incorporation results in a number of instances. Hence only the actual mean cpm's were taken into consideration for the assessment of this MLC.

Thus from the results of the mean cpm values in Table VI.2, it is clear that there were four combinations resulting in low MLC stimulation in this group, i.e. TA vs R0, V0 vs R0, V0 vs TA and L0 vs BA. Cells R0, TA and V0 are probably genotypically identical or nearly identical for HLA-A, B and D, and L0 and BA probably represent another such semi-identical group, different from the first.

The two remaining cells VT and DL with the phenotype A1,3;B7,8 stimulate each other and the previously mentioned negative groups strongly, and must therefore carry different D locus determinants which are also non-identical with the D locus determinants of the former negative groups. Of the two remaining HLA-A and B non-identical cells, neither WA (HLA-A1,3;B7,W35) nor the control X (HLA-A9,28;B14,W17) were

TABLE VI.2

BILATERAL MLC TEST BETWEEN SEVEN UNRELATED INDIVIDUALS WITH HLA PHENOTYPE A1,3;B7,8 AS WELL AS ONE INDIVIDUAL WITH THE PHENOTYPE A1,3;B7,W35 AND A CONTROL X WHO CARRIED OTHER HLA ANTIGENS

(Each entrance contains from top to bottom: SR, mean cpm $\pm$ se.
The morphological assessment is given in the right upper triangle)

Subject	HLA phenotype	VT	RO	DL	TA	BA	VO	LO	WA	X	PHA
		A1,3;B7,8	A1,3;B7,8	A1,3;B7,8	A1,3;B7,8	A1,3;B7,8	A1,3;B7,8	A1,3;B7,8	A1,3;B7,W35	A9,28;B14,W17	
VT	A1,3;B7,8	89 ± 13	++	+	++	$\pm$	+	+	$\pm$	++	+++
RO	A1,3;B7,8	8.9 7,085 ± 259	1,494 ± 235	+	$\pm$	+	$\pm$	+	++	++	+++
DL	A1,3;B7,8	48.6 5,931 ± 234	9.2 7,603 ± 686	155 ± 16	+(+)	+	+	$\pm$	++	++	++
TA	A1,3;B7,8	59.9 8,872 ± 483	2.3 1,964 ± 295	37.7 6,841 ± 85	207 ± 34	+	-	+	++	++	+++
BA	A1,3;B7,8	48.2 3,810 ± 276	8.6 6,751 $\pm 1,033$	39.2 4,392 $\pm 1,019$	44.1 6,099 ± 530	69 ± 16	+	-	+(+)	++	++
VO	A1,3;B7,8	37.4 5,425 ± 162	2.2 1,931 ± 104	25.3 4,511 ± 466	3.8 789 ± 197	27.7 3,751 ± 777	119 ± 22	$\pm$	++	++	+++
LO	A1,3;B7,8	40.0 3,442 ± 486	7.4 5,861 ± 598	32.7 3,894 ± 335	26.9 3,909 ± 190	20.6 1,573 ± 193	29.5 4,201 ± 590	83 ± 21	+(+)	+(+)	+++
WA	A1,3;B7,W35	11.9 -3,035 ± 215	8.8 8,489 ± 452	23.3 6,727 ± 675	35.7 11,232 ± 637	24.2 5,938 ± 581	17.7 5,537 ± 277	26.1 6,592 ± 305	251 ± 36	++	+++
X	A9,28;B14,W17	52.2 8,876 ± 49	13.3 11,698 ± 630	53.4 10,847 ± 445	55.0 12,603 ± 730	57.7 9,241 ± 344	22.2 5,039 ± 389	40.2 6,722 ± 611	45.9 15,438 $\pm 1,585$	422 ± 113	+++
PHA		20,243 $\pm 2,095$	10,281 ± 460	11,339 ± 593	15,863 ± 564	5,553 ± 623	15,355 ± 758	20,325 ± 86	7,851 ± 661	11,908 ± 444	

PHA = Phytohaemagglutinin.

--- = Stippled line through negative control.

Unusual MLC reactions are outlined within the Table.

shown to be MLC (or locus D) identical with the other cells or with each other.

Interpretation of Results

Among 318 bilateral and 513 unilateral unrelated combinations that differed for their HLA-A and B locus alleles, there was only one pair that showed lack of MLC response in one direction but with positive response in the opposite direction (Group a, Table VI.1). One of the thirty-eight bilateral combinations that shared one B locus antigen but differed for one or two A locus antigens, was MLC negative. Among the six individuals who are HLA-B identical but HLA-A non-identical one pair showed reciprocal non-stimulation.

However, four of the forty-two (Group e, Table VI.1) and four of the twenty-one (Table VI.2) HLA-A and B identical unrelated pairs were MLC negative or weakly positive in the bilateral MLC experiments. This 12,6% non-stimulation observed in these two combined groups, is in keeping with the 10% incidence of MLC identity reported among HLA sero-identical unrelated combinations in the literature (Mempel et al., 1973b; Eijssvoegel et al., 1970, 1971).

An increased number of these HLA heterozygous unrelated combinations showed lack of MLR in one direction, but a positive response in the opposite direction. A possible explanation for this observation may be that some of these HLA-A, B heterozygous individuals are homozygous for their MLC determinants (HLA-D homozygous) as recently observed by Mempel et al. (1973b) and Dupont et al. (1974a).

Out of the group of seven unrelated persons with the HLA phenotype A1,3;B7,8 there were two non-stimulatory groups, the one consisting of three individuals TA, RO and VO and the other of two LO and BA. There was clear stimulation between members belonging to the two different non-stimulatory groups. For example, TA who does not react against RO and VO shows strong stimulation with LO and BA. This provides evidence of the existence of at least two different MLC specificities which could be demonstrated among these seven individuals.

CHAPTER SEVEN - RANDOM NON-CONSANGUINEOUS FAMILIES

1. Family BEU
2. Family KA
3. Family DEW
4. Family VDW
5. Family DEL
6. Family GIA

Interpretation of Results

The MLC experiments on *families BEU and KA*, each containing one sibling in whom recombination between the genes determining the antigens of the HLA-A and HLA-B loci had occurred, are presented. Following on that the results on *family DEW*, where the parents shared one HLA-A, B haplotype, and three other *families (VDW, DEL and GIA)* with varying degrees of HLA-A and B disparity, are reported in detail.

Family MLC tests were performed on *twenty-one families* where the parents were unrelated and the results are summarized in Table VII.28. A number of these families were tested during assessment of the relatives for bone-marrow and renal transplantation. In twelve of these twenty-one families the parents shared a B locus determinant. Out of the twelve families, five (i.e. 2, 10, 12, 13 and 21) had parents who were HLA haplo-identical (the same A and B locus determinant on one chromosome). Furthermore in three of the twelve families (i.e. families 3, 4 and 8) one of the parents was homozygous for a B locus determinant as well as sharing this determinant with the other parent. None of these parents who were B locus homozygous was homozygous for a D locus determinant.

In the remaining nine families the parents did not share a B locus determinant. There were ten HLA sero-identical parent-child combinations, of which three were MLC negative. Of the eighty-three offspring tested, twenty-one were HLA-B homozygous but only one child appeared to be MLC homozygous according to the family MLC testing (see Table VII.29 for protocol of a 'family MLC test').

1. Family BEU

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 7.1, and presented in tabular form in Table VII.1. Table VII.2 shows the erythrocyte genetic markers, and Tables VII.3, VII.4, VII.5 and VII.6 give the results of the MLC tests.

The HLA types of the parents and eleven of the thirteen children are reported here. Four well-defined HLA-A and B antigens were present in each of the parents, and these segregated regularly into four haplotypes in all the children except sib II-3, who gave evidence of recombination within the paternally derived haplotypes A3,BW17/AW33,B7, resulting in the recombinant haplotype A3,B7.

The parents had two HLA antigens in common, i.e. AW33 and BW17, but in both instances these antigens were on separate chromosomes. Three of the children inherited haplotypes *ac* and were homozygous for BW17. Four sibs inherited haplotypes *bc*, two *ad* and sib II-5 alone the haplotypes *bd*.

The results of the erythrocyte genotyping on the parents and five of the children are presented. The erythrocyte genotyping was not done on sib II-1. From these results there is nothing to suggest that sibling II-3 does not properly belong to this family.

The available members of this family were initially tested in a bilateral MLC test to find a possible MLC negative bone-marrow donor for sib II-6 (the patient). When it was evident that no HLA identical sibling donor existed among these siblings, it became of utmost importance to do unilateral MLC

tests to determine whether sib II-3 (the only possible donor, because sib II-12 was too young) did indeed have a recombinant paternal HLA haplotype as suggested by the serological typing results.

The bilateral MLC result (Table VII.3) between sib II-6 (the patient) and the recombinant II-3, who differed from II-6 only with respect to the B locus antigen, BW17, was strongly positive. The two HLA-A and B identical siblings II-7 and II-8 did not stimulate each other in this MLC experiment and the controls with X and Y were good.

In the bilateral and unidirectional MLC tests presented in Tables VII.4 and VII.5 sib II-3 (recombinant) and II-5 who were HLA identical except for the A locus antigen A3 in the recombinant, were non-stimulatory in both directions, suggesting that the MLC stimulation was not associated with a difference at the A locus. The lymphocytes from the unrelated control X failed to stimulate sibs II-3 and II-5 (Table VII.5), therefore we felt it necessary to repeat the test to confirm these findings.

The unilateral MLC experiment was repeated on the father and six of the children after the death of sib II-6. The lymphocytes of sib II-12 who carried the same A and B antigens as the deceased (II-6) were included. These results are presented in Table VII.6 as relative responses only, to illustrate the findings clearly.

Sib II-3 (recombinant) again did not stimulate or respond to sib II-5, with whom she was serologically identical except for the A locus antigen A3. This confirms their D locus identity. The lymphocytes of sib II-3 (recombinant) and sib II-12 showed reciprocal stimulation although they were serologically identical except for two B locus antigens. They

shared the AW33,B27 maternal haplotype but differed in so far as II-3 had the recombinant haplotype HLA-A3,B/ derived from the paternal haplotype HLA-A3,BW17/AW33,B7 and II-12 the paternal haplotype HLA-A3,BW17. The strong stimulation observed between sibs II-3 (recombinant) and sib II-12 confirms that they must differ for a D locus determinant, which must have resulted from the crossing-over event of the B locus antigen B7 in sib II-3. This confirms that the D locus determinant is in close association with the B locus. The HLA-A and B identical siblings II-1 and II-7 also gave a clearly negative result in this MLC experiment.

Both parents carried the B locus antigen BW17, resulting in two of their offspring, sibs II-1 and II-7, being homozygous for this antigen. The maternal and paternal chromosomes must, however, have different D locus determinants accompanying the BW17 antigen because sibs II-1 and II-7 stimulate the father in the unilateral MLC as reported in Table VII.6.

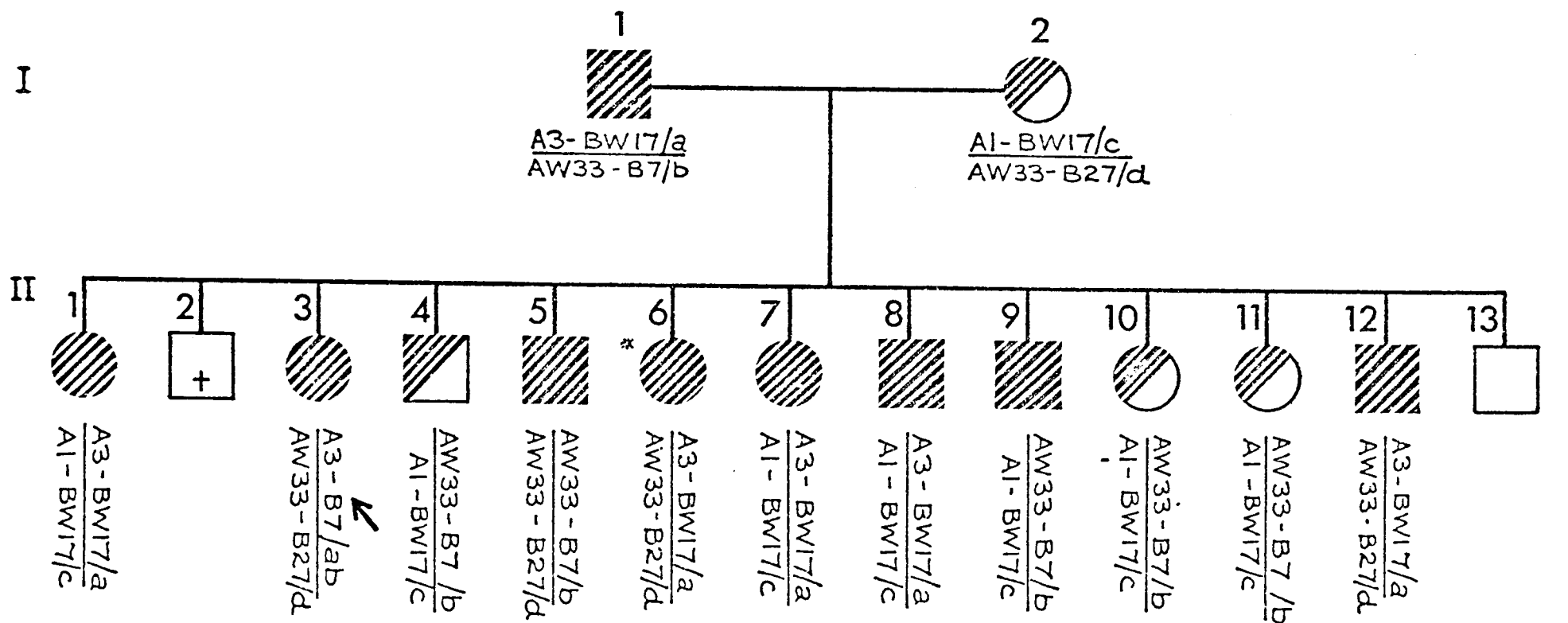


Figure 7.1. Pedigree of family BEU with HLA-A and B genotypes and haplotypes.

The paternal HLA haplotypes are designated ab, and the maternal cd.

↙ marks the recombinant haplotype

* denotes the patient for bone-marrow transplant

+ indicates deceased sibling

	MALE	FEMALE
HLA & MLC tested		
Only HLA tested		
Not tested		

TABLE VII.1

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FAMILY BEU

(See Pedigree Fig. 7.1)

HLA Genotypes	Haplotypes	Subject
A3-BW17 AW33-B7	a b	I-1 Father
A1-BW17 AW33-B27	c d	I-2 Mother
A3-BW17/AW33-B27	a/d	II-6 Sib (patient)
A3-BW17/AW33-B27	a/d	II-12 Sib
A3-BW17/A1-BW17	a/c	II-1 Sib
A3-BW17/A1-BW17	a/c	II-7 Sib
A3-BW17/A1-BW17	a/c	II-8 Sib
A3-B7/AW33-B27	a,b/d	↗ II-3 Sib
AW33-B7/A1-BW17	b/c	II-4 Sib
AW33-B7/A1-BW17	b/c	II-9 Sib
AW33-B7/A1-BW17	b/c	II-10 Sib
AW33-B7/A1-BW17	b/c	II-11 Sib
AW33-B7/AW33-B27	b/d	II-5 Sib

↗ The arrow identifies the sibling with the recombinant HLA-A,B haplotype.

TABLE VII.2

ERYTHROCYTE GENETIC MARKERS ON FAMILY BEU

(See Pedigree Fig. 7.1)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		P_1	<u>Duffy</u> Fy^a
				K	$\bar{k}$		
F I-1	A_1	R_1/R_1	$M\bar{s}/M\bar{s}$	-	+	-	+
M I-2	A_1	R_0/r	$MS/M\bar{s}$	-	+	+	+
↗ Sib II-3	A_1	R_1/r	$M\bar{s}/M\bar{s}$	-	+	+	+
Sib II-4	A_1	R_1/r	$M\bar{s}/M\bar{s}$	-	+	+	-
Sib II-5	A_1	R_1/R_0	$M\bar{s}/M\bar{s}$	-	+	+	+
Sib II-6 (patient)	O	R_1/r	$MS/M\bar{s}$	-	+	+	-
Sib II-7	A_1	R_1/r	$M\bar{s}/M\bar{s}$	-	+	+	+
Sib II-8	O	R_1/r	$MS/M\bar{s}$	-	+	+	+
Sib II-9	A_1	R_1/R_0	$MS/M\bar{s}$	-	+	+	+
Sib II-10	A_1	R_1/R_0	$M\bar{s}/M\bar{s}$	-	+	+	+
Sib II-11	A_1	R_1/R_0	$MS/M\bar{s}$	-	+	+	+
Sib II-12	A_1	R_1/R_0	$M\bar{s}/M\bar{s}$	-	+	+	+

↗ Sibling with the recombinant HLA haplotype.

TABLE VII.3

BILATERAL MLC TESTS ON FAMILY BEU

(Each entrance contains from top to bottom: SR, mean cpm $\pm$ se)

Subject		 11-3	11-6 (patient)	11-7	11-8	X	Y
	HLA genotypes and haplotypes	A3,B7/ab AW33,B27/d	A3,BW17/a AW33,B27/d	A3,BW17/a A1,BW17/c	A3,BW17/a A1,BW17/c	HLA phenotype A2,28;BW35,12	HLA phenotype A1,29;BW21,TT
 Sib 11-3	A3,B7/ab AW33,B27/d	119 ± 5					
Sib 11-6 (patient)	A3,BW17/a AW33,B27/d	40.1 3,214 ± 274	42 ± 5				
Sib 11-7	A3,BW17/a A1,BW17/c	84.5 11,938 ± 915	121.2 12,365 ± 237	163 ± 19			
Sib 11-8	A3,BW17/a A1,BW17/c	159.9 17,111 $\pm 1,149$	136.5 13,112 ± 321	1.4 181 ± 13	96 ± 15		
X	HLA phenotype A2,28;BW35,12	44.5 14,389 $\pm 1,403$	47.2 13,475 ± 761	45.4 15,696 ± 828	37.8 11,801 ± 884	528 ± 98	
Y	A1,29;BW21,TT	90.0 17,017 ± 873	120.8 18,243 ± 810	102.4 21,624 $\pm 1,506$	121.7 21,671 $\pm 2,058$	64.2 25,317 $\pm 3,143$	260 ± 65
PHA		16,749 ± 262	17,467 ± 355	25,346 $\pm 1,782$	21,700 $\pm 1,519$	39,287 $\pm 1,349$	27,697 $\pm 3,842$

X & Y = Healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative control.

 The arrow identifies the sibling with the recombinant haplotype.

The negative reaction is outlined within the Table.

TABLE VII.4

BILATERAL MLC TESTS ON FAMILY BEU

(Each entrance contains from top to bottom: SR, mean cpm $\pm$ se)

Subject		 11-3	11-5	X
	HLA genotypes and haplotypes	$\frac{A3,B7/ab}{AW33,B27/d}$	$\frac{AW33,B7/b}{AW33,B27/d}$	HLA phenotype A1,9;B7,8
 Sib 11-3	$\frac{A3,B7/ab}{AW33,B27/d}$	102 ± 2		
Sib 11-5	$\frac{AW33,B7/b}{AW33,B27/d}$	1.7 192 ± 15	115 ± 39	
X	HLA phenotype A1,9;B7,8	99.8 11,384 ± 754	27.8 3,369 ± 296	127 ± 19
PHA		17,288 ± 399	21,751 ± 564	20,994 ± 282

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin.

 = The arrow identifies the sibling with the recombinant haplotype.

--- = Stippled line through negative controls.

The negative reactions are outlined within the Table.

TABLE VII.5

UNILATERAL MLC TESTS ON FAMILY BEU

(Each entrance contains from top to bottom: SR, mean $\text{cpm} \pm \text{se}$)

Subject	↗ 11-3	11-5	X
HLA genotypes and haplotypes	A3,B7/ab AW33,B27/d	AW33,B7/b AW33,B27/d	HLA phenotype A1,9;B7,8
↗ Sib 11-3 _m A3,B7/ab AW33,B27/d	74 ±13	1.3 96 ±17	40.7 4,763 ±1,161
Sib 11-5 _m AW33,B7/b AW33,B27/d	1.0 77 ±11	70 ±24	25.4 2,979 ±215
X _m HLA phenotype A1,9;B7,8	4.5 340 ±92	1.6 113 ±22	117 ±44
PHA	17,288 ±399	21,751 ±564	20,994 ±252

X = Healthy unrelated individual.

m = Mitomycin-C treated.

PHA = Phytohaemagglutinin.

↗ = The arrow identifies the sibling with the recombinant haplotype.

--- = Stippled line through negative controls.

The negative reactions are outlined within the Table.

TABLE VII.6

UNILATERAL MLC TESTS ON FAMILY BEU
(Results expressed as a relative response)

Subject		F 1-1	↗ 11-3	11-5	11-12	11-1	11-7	11-9	X	Y
	HLA genotypes and haplotypes	A3,BW17/a AW33,B7/b	A3,B7/ab AW33,B27/d	AW33,B7/b AW33,B27/d	A3,BW17/a AW33,B27/d	A3,BW17/a A1,BW17/c	A3,BW17/a A1,BW17/c	AW33,B7/b A1,BW17/c	HLA phenotype A2,-;BW35,-	HLA phenotype A2,29;B12,W15
F 1-1 _m	A3,BW17/a AW33,B7/b	0	55	56	63	79	100	95	82	100
↗ Sib 11-3 _m	A3,B7/ab AW33,B27/d	nd	0	0	42	90	41	58	100	77
Sib 11-5 _m	AW33,B7/b AW33,B27/d	nd	4	0	47	100	97	86	65	80
Sib 11-12 _m	A3,BW17/a AW33,B27/d	56	56	50	0	20	50	93	91	72
Sib 11-1 _m	A3,BW17/a A1,BW17/c	59	52	82	nd	0	0	nd	63	57
Sib 11-7 _m	A3,BW17/a A1,BW17/c	73	93	48	63	1	0	67	52	90
Sib 11-9 _m	AW33,B7/b A1,BW17/c	58	100	78	100	77	40	0	71	84
X _m	HLA phenotype A2,-;BW35,-	81	75	68	89	91	84	100	0	0
Y _m	A2,29;B12,W15	100	72	100	77	84	96	96	0	0

X & Y = Two healthy unrelated individuals.

nd = Not determined.

m = Mitomycin-C treated.

--- = Stippled line through negative controls.

↗ = The arrow identifies the sibling with the recombinant haplotype.

The unusual reactions are outlined within the Table.

2. Family KA

The HLA-A and B genotypes and haplotypes of each member of this family are presented in tabular form in Table VII.7. Child 10 represents the recombinant of the HLA-A and HLA-B loci. Tables VII.8, VII.9 and VII.10 give the results of the bilateral and unilateral MLC tests.

A detailed account of the findings in this family and four others, each containing one sibling in which recombination between the genes determining the antigens of the A locus and the B locus had occurred, has been previously reported by us (Eijsvoegel et al., 1972a, b and c). The very weak functional activity of the A locus antigens with regard to MLC reactivity was confirmed. It should be pointed out, however, that of the twelve MLC reactions performed in these experiments between siblings differing only for an HLA-A antigen, four displayed weak but not completely negative reactions. Normal positive MLC reactions were always found in the tests involving differences for the antigens of the B locus only.

In *family KA* the children 1 and 2, who differed from the recombinant, child 10, for the A locus antigen (A2 versus A11) only, showed virtually no stimulation or response in this combination, while the children 8 and 9 who differed only for a determinant of the B locus (BW35 versus B8), demonstrated consistent stimulation and response when tested with the recombinant (Table VII.8).

In contrast to all the other HLA identical siblings in the previously mentioned four families, who showed no MLC reactivity at all, family KA showed further anomalies in the

relationship between HLA-A and B and MLR (Table VII.9). The HLA identical sibling combination 1 + 2 with the haplotypes *bc*, were MLC negative. The combination of siblings 4 + 5, with the haplotypes *bd*, was also negative, but the combinations 4 + 3 and 5 + 3 were positive in the bilateral and unilateral MLC tests notwithstanding the fact that sibling 3 was HLA-A and B identical to siblings 4 and 5. Further analysis showed that sibling 3 was MLR negative with another group of two HLA identical siblings 1 and 2 (*bc*) who differed from the former group for one HLA-A, B haplotype (*c vs d*). This indicates that child 3 belongs to the MLC identical group of children 1 and 2 and not to the MLC identical group, made up of the children 4 and 5.

Finally sibling 10 in whom the recombination between HLA-A and B was demonstrable, neither stimulated nor responded to sibling 3, notwithstanding a difference for three HLA-A and B antigens, although all the MLC tests performed with siblings 4 and 5 were clearly positive as shown in Table VII.10.

The other HLA-A, B identical sibling combinations 6 + 7, with the haplotypes *ad*, and siblings 8 + 9, with the haplotypes *ac*, were MLC identical (Table VII.9).

TABLE VII.7

HLA-A AND B TYPING FAMILY KA

(From Eijssvoegel, V.P., Van Rood, J.J.,
Du Toit, E.D. and Schellekens, P.Th.A.,
Eur. J. Immunol., 1972a, 2:413)

Parents	HLA Genotypes	Haplotypes
Father	A2 -B8	a
	A11-BW35	b
Mother	A3 -B7	c
	A1 -B8	d
Children	Haplotypes	
1.	A11-BW35/A3-B7	b/c
2.	A11-BW35/A3-B7	b/c
3.	A11-BW35/A1-B8	b/d
4.	A11-BW35/A1-B8	b/d
5.	A11-BW35/A1-B8	b/d
6.	A2-B8 /A1-B8	a/d
7.	A2-B8 /A1-B8	a/d
8.	A2-B8 /A3-B7	a/c
9.	A2-B8 /A3-B7	a/c
↗10.	A2-BW35/A3-B7	ab/c

↗ The arrow identifies the child with the recombinant HLA-A,B haplotype.

The new equivalent designations for the HLA-A and B loci antigens have been assigned to the 'old' antigens, as laid down by the WHO Nomenclature Committee in Aarhus, Denmark in July 1975.

TABLE VII.8

BILATERAL AND UNILATERAL MLCs INVOLVING SIB 10, THE RECOMBINANT
BETWEEN HLA-A AND HLA-B IN FAMILY KA

(From Eijsvoegel, V.P., Koning, L., de Groot-Kooy, L., Huismans, L., Van Rood, J.J., Van Leeuwen, A. and Du Toit, E.D. Transplant. Proc. 1972c, IV : 199)

HLA Differences	Sib No.	cpm	se
HLA-A only	10 + 1	62	14
	10 _m + 1	55	19
ab/c + bc	10 + 1 _m	53	27
	10 + 2	63	9
	10 _m + 2	71	13
	10 + 2 _m	142	21
HLA-B only	10 + 8	3035	307
	10 _m + 8	2540	328
ab/c + ac	10 + 8 _m	1223	123
	10 + 9	1069	75
	10 _m + 9	1041	215
	10 + 9 _m	690	32
Controls	10 + X _m	2905	649
	1 + X _m	2519	300
	2 + X _m	2010	273
	10 _m + X	3966	241
	1 _m + X	3095	252
	2 _m + X	4451	130
	10	20	4
	1	21	8
	2	34	17
	8	125	4
	9	26	5

* m = Mitomycin-C treated.

TABLE VII.9

MLCs BETWEEN SIBLINGS OF FAMILY KA

(From Eljsoogel, V.P., Van Rood, J.J., Du Toit, E.D. and Schellekens, P.Th.A. Eur. J. Immunol. 1972a, 2 : 413)

	Sib	cpm	se		Sib	cpm	se
HLA Identicals				1-haplotype differences			
ad/ad	6 + 7	117	37	b/c + b/d	1 + 4	1959	152
					1 _m + 4	1578	182
					1 + 4 _m	1689	188
a/c + a/c	8 + 9	167	43		2 + 4	1655	354
					2 _m + 4	1550	108
					2 + 4 _m	1817	84
b/c + b/c	1 + 2	33	4		1 + 5	2108	486
					1 _m + 5	1772	211
					1 + 5 _m	1489	109
b/d + b/d	4 + 5	37	5		2 + 5	3078	212
	4 _m + 5	72	10		2 _m + 5	2078	125
	4 + 5 _m	135	25		2 + 5 _m	1318	117
	4 + 3	2124	384		1 + 3	122	34
	4 _m + 3	1061	99		1 _m + 3	37	20
	4 + 3 _m	2605	193		1 + 3 _m	344	53
	5 + 3	3982	574		2 + 3	105	39
	5 _m + 3	1787	258		2 _m + 3	58	15
	5 + 3 _m	1903	260		2 + 3 _m	338	32

m = Mitomycin-C treated.

The more commonly used term "haplotype" has been used instead of "allelic".

TABLE VII.10

BILATERAL AND UNILATERAL MLC INVOLVING
SIBLINGS 3, 4, 5 AND 10 OF FAMILY KA

(From Eijssvoogel, V.P., Van Rood, J.J., Du Toit, E.D.
 and Schellekens, P.Th.A. Eur. J. Immunol. 1972a, 2 :
 413)

HLA Genotype Combination	Sib	cpm	se
ab/c + bd	10 + 3	353	92
	10 _m + 3	233	72
	10 + 3 _m	192	25
	10 + 4	2452	55
	10 _m + 4	2048	140
	10 + 4 _m	nd	
	10 + 5	2394	239
	10 _m + 5	1770	158
	10 + 5 _m	1466	105

m = Mitomycin-C treated.

nd = Not determined.

3. Family DEW

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 7.2, and presented in tabular form in Table VII.11. Table VII.12 shows the erythrocyte genetic markers, and Tables VII.13, VII.14 and VII.15, give the results of the MLC tests.

In both generations of this family the parents (unrelated individuals) shared the common haplotype HLA-A1,B8. The mother of the *first set of siblings*, I-2 is deceased and therefore her HLA genotype for loci A and B was postulated from the serological typings in the children. From the segregation of the antigens in the children it appeared that the father I-1 and the mother I-2 shared a common haplotype, HLA-A1,B8. This haplotype is designated α' in the father, and α'' in the mother. The second haplotype in the father, I-1, HLA-A11,B7, is marked α in order to prevent confusion with II-1, and the second haplotype of the mother, I-2, as d . Siblings II-2, II-4 and II-5 are all homozygous for HLA-A1,B8. The siblings II-7 and II-11 are HLA-A and B identical heterozygotes and II-9 and II-12 are haplo-identical.

The mother of the *second generation family* (II-2), is one of the homozygous HLA-A1,B8 siblings of the first set. Her husband, an unrelated individual, also has the haplotype HLA-A1,B8 on one chromosome designated α in Table VII.11 and a second haplotype HLA-A2, B12 on the b chromosome. From the serological results sibs III-1, III-3 and III-4 and father II-1 are identical for their HLA-A and B antigens. The mother and child III-2 are both homozygous HLA-A1,B8. The inheritance of HLA haplotypes has been derived from the MLC data and will be discussed with the results of the MLC tests.

The inheritance of the erythrocyte genetic markers does not reveal any evidence, in either generation of this family, of any parentage other than that stated. The first generation and the sibship engendered by it could unfortunately not be tested in the MLC test. The two parents and their four children in the second nuclear family were tested in bidirectional and unidirectional MLC tests in all possible combinations on three different occasions with two unrelated controls X and Y as well as autologous and PHA controls. As there was little variation from day to day the results of only one set of experiments are presented in Tables VII.13, VII.14 and VII.15.

Three of the four children III-1, III-3, III-4 and the father, II-1 are HLA-A and B identical heterozygotes. These three children must have inherited the *b* haplotype HLA-A2,B12 from their father and could have inherited either one of the mother's HLA-A1,B8 haplotypes. Since there was non-stimulation between two of the HLA-A and B identical father-child combinations, i.e. father II-1 versus children III-3 and III-4 in the bidirectional and unidirectional thymidine incorporation results and morphology, it must be concluded that these children inherited the maternal HLA-A1,B8 haplotype (designated α' in Table VII.11) which carries a D locus determinant identical with the determinant on the father's HLA-A1,B8 haplotype (designated α in Table VII.11). The third child III-1 carried the same A and B antigens as the father but was positive with father in the bidirectional and unidirectional MLC tests according to the thymidine and morphology results. Therefore this child must have inherited the HLA-A1,B8 haplotype from her mother (designated α'' in Table VII.11), which carries a different D locus determinant to the HLA-A1,B8(α) haplotype of the father.

From these results it was concluded that the mother, although HLA-A1,B8 homozygous, carried different D locus specificities on her two chromosomes; one identical to the D locus specificity carried on the paternal chromosome α ; and the other, different. This resulted in the paternal haplotype α (HLA-A1,B8) and the maternal haplotype α' (HLA-A1,B8) being identical for the HLA loci A, B and D and the paternal haplotype α , and the maternal haplotype α'' non-identical for the D locus. The mother II-2 and child III-2 who were HLA-A and B identical and both homozygous HLA-A1,B8 were non-stimulatory in the bilateral and unilateral MLC tests, both from the thymidine and morphological results. Child III-2 must have inherited from her father haplotype α , which has the same HLA-A, B and D determinants as the mother's haplotype α' , and from the mother the haplotype α'' , which has a D locus determinant different from that accompanying haplotypes α and α' . This resulted in child III-2 being identical to her mother for the HLA-A and B antigens as well as the D locus specificities.

When the mean *cpm* value was taken to analyse these results, we found a range of 23-107 (mean 59) among combinations with expected unidirectional non-stimulation, 464-3284 (mean 1288) for the haplo-identicals and 622-7228 (mean 2822) for the unrelated individuals.

The mean *stimulation ratio* (SR) of all combinations with expected unidirectional non-stimulation in this family was 2.1 (range 1.0-4.6) when calculated from the SR values presented in Table VII.14. In the haplo-identical combinations it was 62.3 (range 14.0-158.3), and with the unrelated individuals 78.9 (range 24.3-277.5).

The range of *relative responses* (RR) (Table VII.14), in the unidirectional non-stimulatory combinations was from 0-4 (mean 1); in the haplo-identical from 10-100 (mean 48); and with the two unrelated individuals it was 14-100 (mean 72).

Thus analysing the results in this family using either the mean cpm, stimulation ratios or relative responses shows a clear dichotomy between the combinations with expected non-stimulation and the haplo-identical as well as the unrelateds. None of these three parameters were, however, able to separate the haplo-identicals from the unrelated individuals.

The results of all these tests therefore confirm that the mother II-2 and child III-2 are homozygous for HLA-A and B antigens but heterozygous for the locus D determinants. Sib III-1 who is HLA-A and B identical to the father, II-1, and sibs III-3 and III-4, reacts like a haplo-identical in culture with them, because of the different D locus determinant carried by III-1.

HLA-A and B identity between parent-child and siblings naturally does not necessarily predict MLC non-reaction. It is important to exclude this sort of situation by family analysis before recombination between the D locus and the A and B loci is suggested in HLA-A and B identical sibling combinations who stimulate each other in culture.

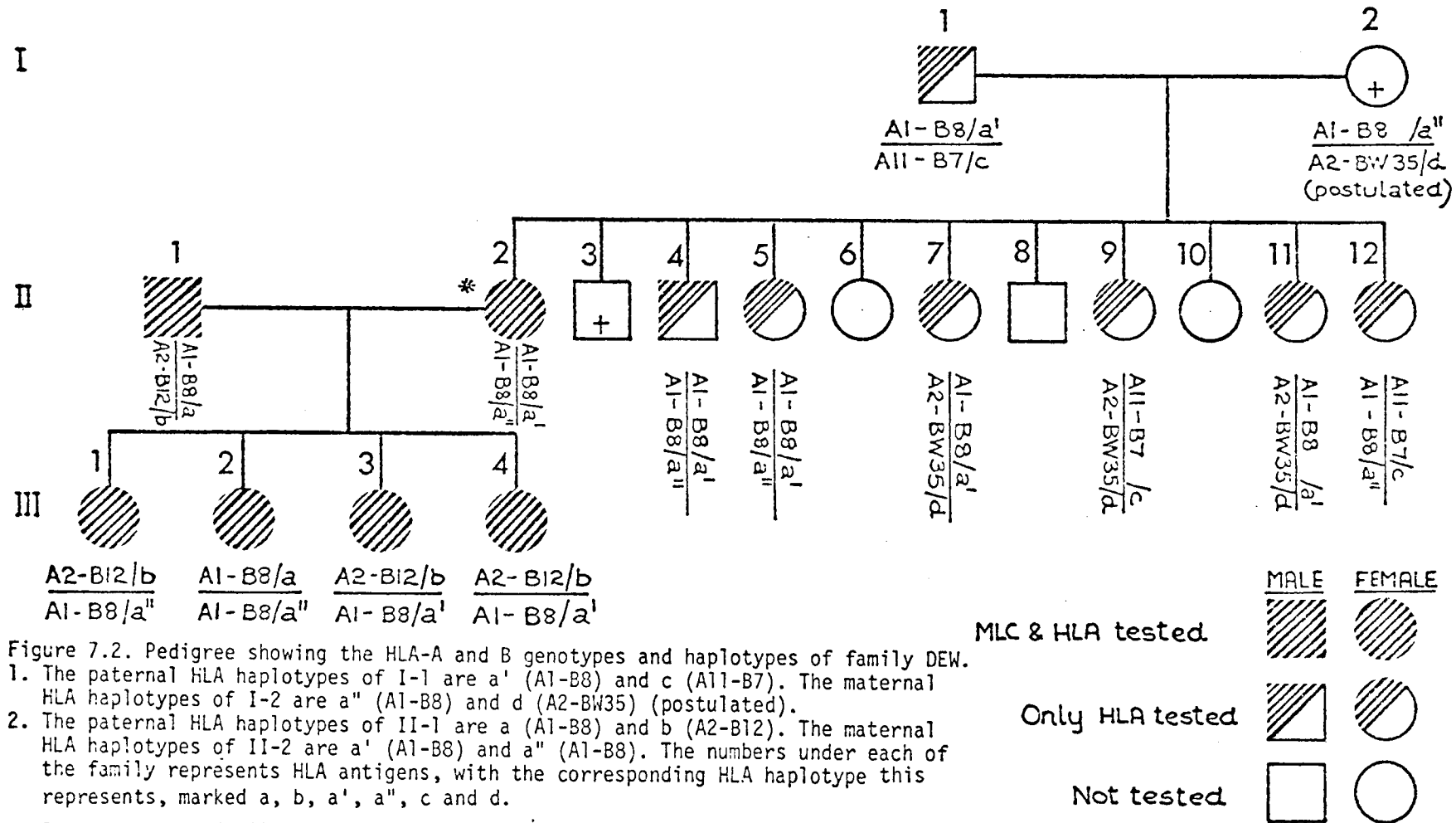


Figure 7.2. Pedigree showing the HLA-A and B genotypes and haplotypes of family DEW.

1. The paternal HLA haplotypes of I-1 are a' (A1-B8) and c (A11-B7). The maternal HLA haplotypes of I-2 are a'' (A1-B8) and d (A2-BW35) (postulated).
2. The paternal HLA haplotypes of II-1 are a (A1-B8) and b (A2-B12). The maternal HLA haplotypes of II-2 are a' (A1-B8) and a'' (A1-B8). The numbers under each of the family represents HLA antigens, with the corresponding HLA haplotype this represents, marked a, b, a', a'', c and d.

+ Denotes mother (I-2) and sib (II-3) who are deceased.

* Denotes the candidate for renal transplantation.

TABLE VII.11

FAMILY DEWHLA-A AND B GENOTYPES AND HAPLOTYPES OF PARENTS AND FIRST SIBSHIP
(See Pedigree Fig.7.2)

HLA Genotype	Haplotype	Subject
A1-B8 A11-B7	a' c	I-1 Father
A1-B8 A2-BW35	a'' d	I-2 Mother (deceased)
A1-B8/A1-B8	a'/a''	II-2 Sib
A1-B8/A1-B8	a'/a''	II-4 Sib
A1-B8/A1-B8	a'/a''	II-5 Sib
A11-B7/A2-BW35	c/d	II-9 Sib
A1-B8/A2-BW35	a'/d	II-7 Sib
A1-B8/A2-BW35	a'/d	II-11 Sib
A11-B7/A1-B8	c/a''	II-12 Sib

HLA-A AND B GENOTYPES AND HAPLOTYPES OF PARENTS AND SECOND SIBSHIP

HLA Genotype	Haplotype	Subject
A1-B8 A2-B12	a b	II-1 Father
A1-B8 A1-B8	a' a''	II-2 Mother
A1-B8/A1-B8	a/a''	III-2 Sib
A2-B12/A1-B8	b/a''	III-1 Sib
A2-B12/A1-B8	b/a'	III-3 Sib
A2-B12/A1-B8	b/a'	III-4 Sib

TABLE VII.12

FAMILY DEWERYTHROCYTE GENETIC MARKERS OF PARENTS AND FIRST SIBSHIP

(See Pedigree Fig. 7.2)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P_I</u>	<u>Duffy</u> Fy(a)
				K	k		
F I-1	B	R ₁ /r	MS/Ms	+	+	-	+
M I-2 (postulated)		R ₁ /r	Ms/Ns				
Sib II-2 (patient)	B	R ₁ /r	Ms/Ms	-	+	-	+
Sib II-4	B	r/r	Ms/Ms	+	+	-	-
Sib II-5	B	R ₁ /r	Ms/Ns	-	+	+	-
Sib II-7	B	r/r	Ms/Ns	+	+	-	+
Sib II-9	B	R ₁ /R ₁	MS/Ms	-	+	-	+
Sib II-11	B	R ₁ /R ₁	Ms/Ms	-	+	-	+
Sib II-12	B	R ₁ /R ₁	MS/Ns	-	+	-	-

ERYTHROCYTE GENETIC MARKERS OF PARENTS AND SECOND SIBSHIP

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P_I</u>	<u>Duffy</u> Fy(a)
				K	k		
F II-1	O	R ₁ /r	Ms/Ns	-	+	+	-
M II-2 (patient)	B	R ₁ /r	MS/Ns	-	+	-	+
Sib III-1	O	R ₁ /r	Ms/Ns	-	+	+	-
Sib III-2	B	r/r	Ns/Ns	-	+	+	-
Sib III-3	B	R ₁ /r	Ms/Ns	-	+	+	+
Sib III-4	O	r/r	Ms/Ns	-	+	-	-

TABLE VII.13

FAMILY DEW
BILATERAL MLC TESTS OF PARENTS AND SECOND SIBSHIP

(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		II-1	II-2	III-2	III-1	III-3	III-4	X	Y	PHA
	HLA haplotype	ab	a'a''	aa''	ba''	ba'	ba'	HLA phenotype A2,3;BW40,W17	HLA phenotype A1,11;B5,W35	
F II-1	ab	98 ± 37	++	++	++	-	-	++	++	+++
M II-2	a'a''	10,984 $\pm 1,973$	151 ± 48	-	++	+	+	++	++	+++
Sib III-2	aa''	8,948 ± 769	399 ± 142	52 ± 2	++	++	++	++	++	++++
Sib III-1	ba''	13,343 ± 346	7,540 $\pm 2,025$	5,477 ± 845	95 ± 23	+	+	++	++	+++
Sib III-3	ba'	489 ± 192	13,549 ± 673	9,433 ± 385	11,033 $\pm 1,288$	85 ± 58	-	++	++	++++
Sib III-4	ba'	334 ± 11	12,073 $\pm 1,139$	13,577 $\pm 2,554$	8,626 ± 553	152 ± 49	79 ± 10	+	++	+++
X	HLA phenotype A2,3;BW40,W17	24,967 $\pm 1,046$	18,354 ± 356	24,873 $\pm 2,129$	24,706 ± 248	15,128 $\pm 1,614$	11,703 ± 366	39 ± 5	++	+++
Y	A1,11;B5,W35	7,502 $\pm 1,421$	8,795 ± 344	18,027 $\pm 2,931$	16,870 $\pm 2,080$	11,869 ± 413	11,319 $\pm 1,206$	7,237 ± 734	49 ± 7	+++
PHA		34,037 $\pm 1,619$	21,778 ± 916	26,706 ± 52	41,217 $\pm 1,535$	22,992 $\pm 1,227$	26,245 $\pm 1,968$	45,746 $\pm 1,820$	25,660 ± 485	

X & Y = Healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative control.

Unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the positive reactions.

TABLE VII.14

FAMILY DEW
UNILATERAL MLC TESTS OF PARENTS AND SECOND SIBSHIP

(Each entrance contains from top to bottom: SR, RR and mean cpm $\pm$ se.
The highest test counts observed with the corresponding responder cells that day, which
is the basis for the calculation of the relative response are marked with an asterisk.)

Subject		II-1	II-2	III-2	III-1	III-3	III-4	X	Y
HLA haplotype		ab	a'a''	aa''	ba''	ba'	ba'	HLA phenotype A2,3;BW40,W17	HLA phenotype A1,11;B5,W35
F II-1 _m	ab	37.7 100 23 $\pm$ 8	37.7 100 *1,132 $\pm$ 46	99.7 79 2,594 $\pm$ 915	106.9 38 1,283 $\pm$ 171	1.2 0 27 $\pm$ 5	2.9 2 105 $\pm$ 13	94.8 87 5,215 $\pm$ 1,182	82.2 97 3,370 $\pm$ 274
M II-2 _m	a'a''	89.8 28 2,067 $\pm$ 186	30 $\pm$ 5	1.0 0 28 $\pm$ 8	158.3 56 1,900 $\pm$ 107	58.1 94 1,220 $\pm$ 9	80.6 88 2,904 $\pm$ 52	81.6 74 4,488 $\pm$ 1,322	71.7 85 2,943 $\pm$ 339
Sib III-2 _m	aa''	98.7 31 2,271 $\pm$ 650	2.7 4 82 $\pm$ 24	26 $\pm$ 11	54.5 19 654 $\pm$ 195	33.1 53 697 $\pm$ 138	91.2 100 *3,284 $\pm$ 709	78.2 71 4,305 $\pm$ 678	67.2 73 2,759 $\pm$ 235
Sib III-1 _m	ba''	34.7 10 800 $\pm$ 96	28.0 73 840 $\pm$ 32	27.6 21 720 $\pm$ 148	12 $\pm$ 1	24.3 38 512 $\pm$ 101	14.0 14 504 $\pm$ 63	60.2 55 3,312 $\pm$ 749	73.3 87 3,026 $\pm$ 113
Sib III-3 _m	ba'	1.7 0 39 $\pm$ 0	20.5 53 617 $\pm$ 84	17.8 13 464 $\pm$ 163	83.5 29 1,002 $\pm$ 99	21 $\pm$ 7	1.7 0 64 $\pm$ 8	38.0 34 2,091 $\pm$ 172	47.2 55 1,937 $\pm$ 234
Sib III-4 _m	ba'	4.6 1 107 $\pm$ 35	28.6 75 858 $\pm$ 123	40.2 31 1,046 $\pm$ 422	81.3 29 976 $\pm$ 253	1.1 0 23 $\pm$ 6	36 $\pm$ 2	108.5 100 *5,969 $\pm$ 471	84.0 100 *3,447 $\pm$ 207
X _m	HLA phenotype A2,3;BW40,W17	314.2 100 *7,228 $\pm$ 497	31.2 82 936 $\pm$ 192	125.5 100 *3,265 $\pm$ 289	277.5 100 *3,331 $\pm$ 199	61.6 100 *1,294 $\pm$ 233	72.0 78 2,598 $\pm$ 30	55 $\pm$ 23	72.1 85 2,957 $\pm$ 226
Y _m	A1,11;B5,W35	47.2 14 1,037 $\pm$ 187	29.7 78 892 $\pm$ 54	43.3 33 1,127 $\pm$ 173	186.0 66 2,232 $\pm$ 173	29.6 47 622 $\pm$ 82	37.5 40 1,351 $\pm$ 175	24.3 25 1,615 $\pm$ 137	41 $\pm$ 0
PHA		34,037 $\pm$ 1,619	21,778 $\pm$ 916	26,706 $\pm$ 52	41,217 $\pm$ 1,535	22,992 $\pm$ 1,227	26,245 $\pm$ 1,968	45,746 $\pm$ 1,820	25,660 $\pm$ 485

X & Y = Healthy unrelated individuals.
PHA = Phytohaemagglutinin.
m = Mitomycin-C treated.
--- = Stippled line through negative controls.

Unusual reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the positive reactions.

TABLE VII.15

FAMILY DEW
RESULTS ON MORPHOLOGY OF THE UNILATERAL MLC (TABLE VII.14) OF PARENTS AND SECOND SIBSHIP

Subject		II-1	II-2	III-2	III-1	III-3	III-4	X	Y
	HLA haplo-type	ab	a'a''	aa''	ba''	ba'	ba'	HLA phenotype A2,3;BW40,W17	HLA phenotype A1,11;B5,W35
F II-1 _m	ab	-	+	+	+	-	-	+	+
M II-2 _m	a'a''	+	-	-	+	+	+	++	++
Sib III-2 _m	aa''	+	-	-	(+)	+	+	+	++
Sib III-1 _m	ba''	(+)	+	+	-	(+)	(+)	++	+
Sib III-3 _m	ba'	-	+	+	(+)	-	-	+	+
Sib III-4 _m	ba'	-	+	(+)	+	-	-	+	+
X _m	HLA phenotype A2,3;BW40,W17	+	+	++	++	++	+	-	++
Y _m	A1,11;B5,W35	++	+	+	++	++	+	++	-

X & Y = Healthy unrelated individuals.

m = Mitomycin-C treated.

--- = Stippled line through negative controls.

The unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the positive.

4. Family VDW

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 7.3, and presented in tabular form in Table VII.16. Table VII.17 shows the erythrocyte genetic markers, and Tables VII.18, VII.19 and VII.20 give the results of the MLC tests.

The family was characterized serologically for HLA. The parents shared an HLA antigen A3, as well as antigen BW35; in the mother they were on the same chromosome, but in the father these two antigens appeared to be on different chromosomes as judged by the inheritance of the antigens in the children. The two siblings were haplo-identical as they both inherited the *d* haplotype from their mother. In addition sib II-1 was homozygous for BW35, a B locus antigen situated in close proximity to the D locus. Sib II-2 was homozygous for HLA-A3.

There is no evidence from erythrocyte genetic markers of parentage other than as stated.

Table VII.18 records the results of the bilateral MLC test between the father and the two children with unrelated controls X and Y. The results of two unilateral experiments on the whole family with three unrelated controls are presented in Tables VII.19 and VII.20. The SR was calculated in the bilateral experiment (Table VII.18) and the SR and the RR were calculated in the two unilateral cultures in an attempt to clarify the picture (Tables VII.19 and VII.20).

The mean cpm's as well as the SR between father I-1 and sib II-1 as well as between sibs II-1 (patient) and sib

II-2 were considerably lower than in the other combinations in the bilateral MLC test as shown in Table VII.18. This diminished reactivity between sibs II-1 and II-2 was confirmed in a repeat bilateral culture, the results of which are not included. In this same experiment the reaction between sib II-2 and the unrelated X was also much weaker than one would expect if they carried different D locus determinants (as usually observed between unrelates).

In order to investigate these reactions two unilateral MLC tests were performed and these are recorded in Tables VII.19 and VII.20. They were performed on two occasions on the parents, sib II-1 (patient), sib II-2 with X (the same unrelated control used in the repeat bilateral experiment where diminished reactivity was observed) and two other unrelated controls Y and Z.

The weakly positive or negative MLC reactions between sibs II-1 (patient) and II-2 were confirmed in both experiments. Sib II-1 did not respond to sib II-2 and in the reverse situation the response of sib II-2 to sib II-1 was also negative or perhaps very weakly positive. The explanation for this is not clear. It cannot be because the paternal *b* chromosome (HLA-A28,BW35) and the maternal *d* chromosome (HLA-A3,BW35) carry identical D locus determinants in this family, since sib II-1 who carries these two chromosomes, stimulated both parents strongly. Thus homozygosity of the HLA-B locus in sib II-1 did not result in D locus homozygosity. Furthermore, homozygosity of the father, I-1, at the D locus can also not explain these results, because there would then be weak or non-stimulation of father for both children and not only for II-2. Another explanation that can be put forward is that the accompanying MLC determinants of the paternal haplotype *a*, and the maternal haplotype *c*, are the same. The

lack of MLC reaction between sib II-2 and the mother I-1 could be explained on these lines, but not the negative MLC results between sibs II-2 and II-1, who carry the paternal haplotype *b*.

It should also be noted that the MLC reactions between sib II-2 and the mother are only weakly positive or negative. The response of sib II-2 to father is weak but definitely positive on both occasions and in the reverse situation he stimulates father strongly.

The reactions between the unrelated individual X with the HLA genotype A3,BW35/A10,BW35 and sib II-2 were consistently weak or negative. The observation that X is homozygous for the B locus determinant BW35 does not seem to have any direct bearing on the non-stimulation of X and sib II-2 because the MLC response of the mother who carries the same chromosome A3,BW35 that sib II-2 inherited, responds to X. In addition, sib II-1 who also inherited this maternal chromosome responds strongly to X. Furthermore, the MLC reactivity between sib II-2 and Y (another unrelated), with totally different A and B locus antigens, was very much reduced. Finally it is important to note that sib II-2 is able to respond and stimulate some individuals normally as for example the reactions II-2 versus Z and also father's response to II-2. The explanation for the negative and weak MLC reactions between sib II-2 and the mother, sib II-1 (patient), X and Y cannot easily be found on the conventional lines of D locus determinants being shared because all these individuals, who responded and stimulated sib II-2 weakly reacted strongly to each other. Figure 7.4 is a schematic drawing and summarizes these findings. The possible explanations for this observation will be discussed at the end of this Chapter.

A comparison of the *mean cpm*, *SR* and *RR* is shown in Table VII.21, between those individuals who appeared to be identical, haplo-identical and non-identical (unrelated) in the two MLC experiments recorded in Tables VII.19 and VII.20. There is a difference between the identicals and haplo-identicals according to all three parameters, but not between the haplo-identical and unrelated individuals.

The MLC results of II-2_m versus Y (Tables VII.19 and VII.20) fall between those of the identicals and haplo-identicals when judged by the mean cpm values, but the *SR* and *RR* values suggest haplo-identity. The MLC reaction between I-1_m versus II-2 is also difficult to interpret, as the results are again between those of the identicals and haplo-identicals in these cultures.

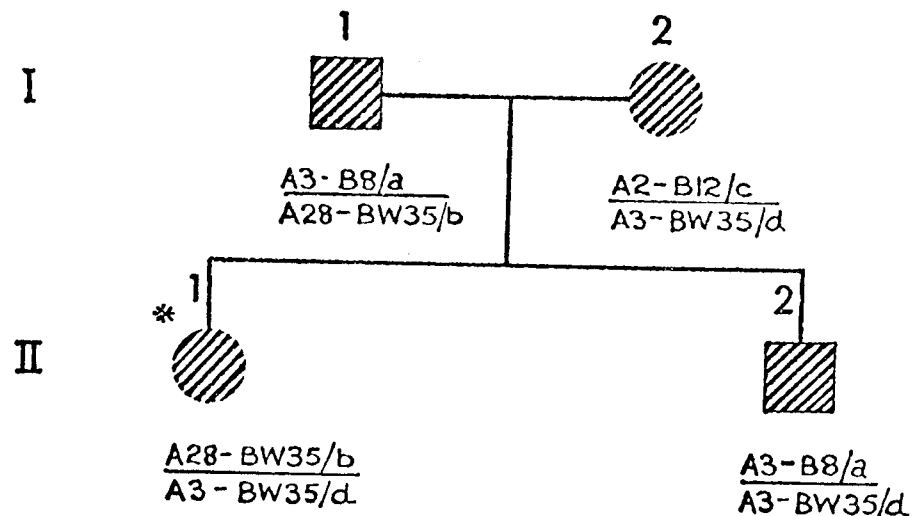


Figure 7.3. Pedigree of family VDW showing HLA-A and B genotypes and haplotypes. The father's HLA haplotypes are designated ab and the mother's cd. Both parents, the proband and her brother were tested by HLA and MLC studies.

* Renal transplant recipient.

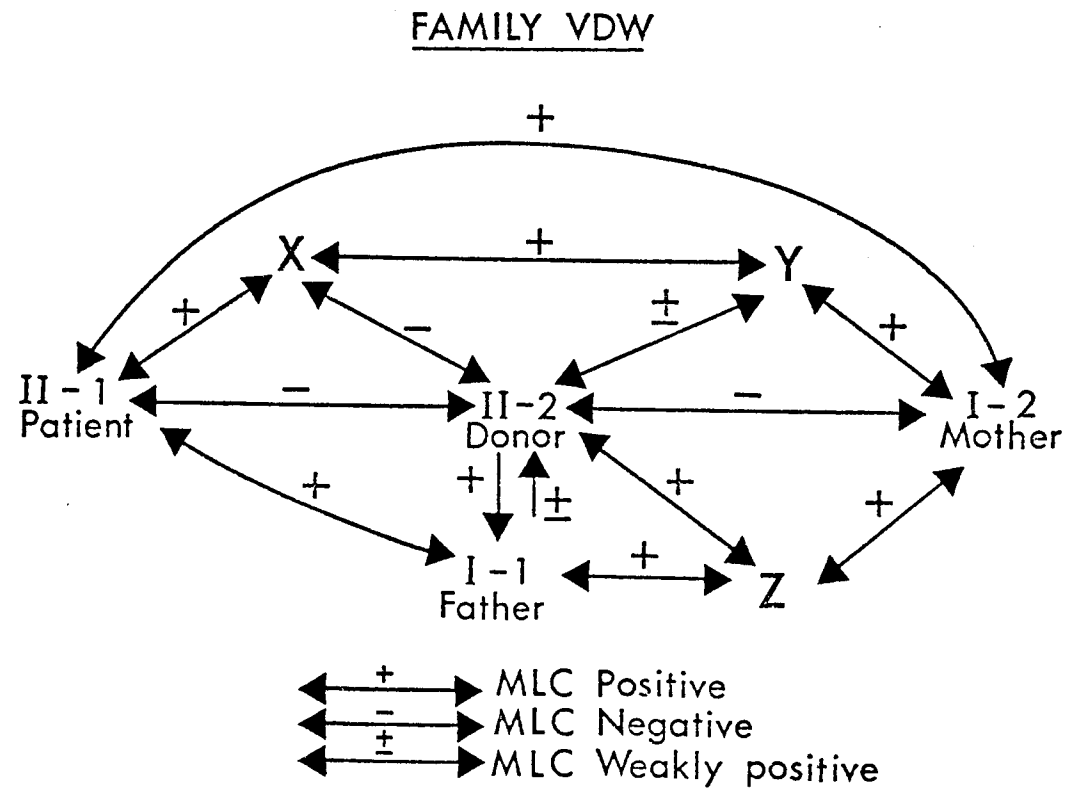


Figure 7.4. A schematic drawing of the MLC reactions between sib II-2 (donor) and sib II-1 (patient), father (I-1), mother (I-2), X, Y and Z (unrelated individuals).

TABLE VII.16

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FAMILY VDW

(See Pedigree Fig. 7.3)

HLA Genotype	Haplotype	Subject
A3-B8	a	I-1 Father
A28-BW35	b	
A2-B12	c	I-2 Mother
A3-BW35	d	
A28-BW35/A3-BW35	b/d	II-1 Sib (patient)
A3-B8/A3-BW35	a/d	II-2 Sib (donor)

TABLE VII.17

ERYTHROCYTE GENETIC MARKERS FAMILY VDW

(See Pedigree Fig. 7.3)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u> K $\bar{k}$	P_1	<u>Duffy</u> Fy(a)
F I-1	0	R ₂ /r	MS/MS	- +	-	+
M I-2	0	R ₁ /r	MS/N $\bar{s}$	- +	-	-
Sib II-1 (patient)	0	R ₁ /r	MS/N $\bar{s}$	- +	-	+
Sib II-2 (donor)	0	r/r	MS/N $\bar{s}$	- +	-	+

TABLE VII.18

BILATERAL MLC TESTS ON FAMILY VDW

(Each entrance contains from top to bottom: SR, mean cpm $\pm$ se)

Subject		I-1	II-1	II-2	X	Y
	HLA genotypes and haplotypes	A3,B8/a A28,BW35/b	A28,BW35/b A3,BW35/d	A3,B8/a A3,BW35/d	HLA phenotype A1,3;B12,W35	HLA phenotype A3,28;BW21,W35
F I-1	A3,B8/a A28,BW35/b	211 ± 15				
Sib II-1 (patient)	A28,BW35/b A3,BW35/d	16.3 2,073 $\pm 1,711$	44 ± 20			
Sib II-2 (donor)	A3,B8/a A3,BW35/d	139.7 26,125 $\pm 1,041$	21.2 2,212 ± 339	164 ± 38		
X	HLA phenotype A1,3;B12,W35	127.6 26,422 $\pm 1,635$	171.9 21,325 ± 796	193.4 35,601 $\pm 2,055$	204 ± 23	
Y	A3,28;BW21,W35	101.0 27,682 $\pm 2,217$	125.9 23,928 $\pm 2,026$	28.6 7,160 ± 93	82.9 22,384 $\pm 2,062$	337 ± 87
PHA		20,518 ± 829	19,666 ± 859	36,902 $\pm 1,158$	26,327 ± 573	30 592 ± 360

X & Y = Two healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

Unusual MLC reactions are outlined within the Table.

TABLE VII.19

UNILATERAL MLC TESTS ON FAMILY VDW.

(Each entrance contains from top to bottom: SR, RR and mean cpm $\pm$ se. The highest test counts observed with the corresponding responder cells that day, which is the basis for the calculation of the RR, are marked with an asterisk)

Subject		FI-1	MI-2	II-1 (patient)	II-2 (donor)	X	Y	Z
	HLA genotypes and haplotypes	A3,B8/a A28,BW35/b	A2,B12/c A3,BW35/d	A28,BW35/b A3,BW35/d	A3,B8/a A3,BW35/d	A3,BW35 A10,BW35	HLA phenotype A9,W33;B14,27	HLA phenotype A1,2;BW21,TT
F I-1 _m	A3,B8/a A28,BW35/b	169 ±63	73.6 100 *23,498 ±2,631	18.5 32 4,101 ±856	16.7 15 2,944 ±173	32.9 66 12,659 ±1,001	205.3 93 12,998 ±2,310	58.1 84 19,829 ±2,205
M I-2 _m	A2,B12/c A3,BW35/d	131.7 81 22,265 ±3,931	319 ±110	36.7 65 8,117 ±328	4.4 3 784 ±207	47.7 97 18,341 ±1,594	168.3 76 10,607 ±126	63.6 92 21,690 ±2,439
Sib II-1 _m (patient)	A28,BW35/b A3,BW35/d	71.8 44 12,137 ±246	55.6 75 17,743 ±1,575	221 ±18	12.0 10 2,121 ±96	49.0 100 *18,838 ±1,882	200.9 91 12,657 ±1,576	48.7 70 16,636 ±1,454
Sib II-2 _m (donor)	A3,B8/a A3,BW35/d	74.8 45 12,658 ±779	5.8 6 1,861 ±801	2.5 2 564 ±79	176 ±55	4.0 6 1,554 ±568	47.7 21 3,008 ±612	68.5 100 *23,383 ±671
X _m	A3,BW35 A10,BW35	139.2 85 23,529 ±1,753	66.8 90 21,316 ±1,848	55.8 100 *12,339 ±919	11.2 9 1,927 ±136	384 ±11	215.9 98 13,607 ±2,133	54.1 78 18,473 ±723
Y _m	HLA phenotype A9,W33;B14,27	75.4 46 12,744 ±1,031	45.4 61 14,509 ±1,432	39.2 69 8,672 ±1,457	12.6 11 2,220 ±639	31.4 63 12,077 ±2,341	63 ±32	57.9 84 19,756 ±1,547
Z _m	A1,2;BW21,TT	161.7 100 *27,339 ±290	64.5 87 20,607 ±1,552	47.9 85 10,590 ±407	102.9 100 *18,123 ±1,599	36.6 74 14,083 ±1,423	220.2 100 *13,878 ±1,754	341 ±151
PHA		16,474 ±718	7,161 ±169	11,972 ±430	7,090 ±597	7,712 ±604	8,514 ±1,000	23,398 ±1,124

X,Y & Z = Three healthy unrelated individuals.
 PHA = Phytohaemagglutinin.
 m = Mitomycin-C treated.
 --- = Stippled line through negative controls.

The unusual MLC reactions are outlined within the Table, the solid lines for the negative reactions and interrupted lines for the weakly positive reactions.

TABLE VII.20

UNILATERAL MLC TESTS ON FAMILY VDW

(Each entrance contains from top to bottom: SR, RR and mean cpm $\pm$ se. The highest test counts observed with the corresponding responder cells that day, which is the basis for the calculation of the RR are marked with an asterisk)

Subject		F1-1	M1-2	11-1 (patient)	11-2 (donor)	X	Y	Z
	HLA genotypes and haplotypes	A3,B8/a A28,BW35/b	A2,B12/c A3,BW35/d	A28,BW35/b A3,BW35/d	A3,B8/a A3,BW35/d	A3,BW35 A10,BW35	HLA phenotype A9,W33;B14,27	HLA phenotype A1,2;BW21,TT
F 1-1 _m	A3,B8/a A28,BW35/b	128 ±19	102.7 95 23,727 ±1,561	16.1 38 4,528 ±7	18.0 14 3,426 ±588	75.1 60 13,231 ±558	140.9 88 12,681 ±1,655	76.4 84 19,193 ±839
M 1-2 _m	A2,B12/c A3,BW35/d	138.2 80 17,693 ±15	231 ±68	30.1 74 8,465 ±793	2.9 1 558 ±103	94.8 76 16,700 ±568	134.9 84 12,145 ±493	89.8 100 *22,545 ±920
Sib 11-1 _m (patient)	A28,BW35/b A3,BW35/d	92.9 53 11,897 ±248	65.1 60 15,055 ±911	281 ±35	9.3 7 1,780 ±269	124.0 100 *21,829 ±2,144	145.5 91 13,097 ±256	68.0 75 17,075 ±630
Sib 11-2 _m (donor)	A3,B8/a A3,BW35/d	93.8 54 12,018 ±792	5.6 4 1,295 ±90	2.0 2 578 ±69	190 ±18	3.7 2 653 ±51	40.0 24 3,601 ±905	80.9 99 20,322 ±2,820
X _m	A3,BW35 A10,BW35	163.5 94 20,939 ±3,076	107.4 100 *24,810 ±1,952	39.9 100 *11,219 ±793	5.1 3 983 ±33	176 ±15	158.6 100 *14,276 ±1,124	71.3 79 17,900 ±2,535
Y _m	HLA phenotype A9,W33;B14,27	167.3 97 21,418 ±2,224	91.3 84 21,104 ±635	35.5 88 9,980 ±2,093	14.3 11 2,731 ±299	82.3 66 14,493 ±679	90 ±34	76.6 85 19,251 ±2,142
Z _m	A1,2;BW21,TT	172.4 100 *22,073 ±1,741	90.9 84 21,017 ±290	39.6 99 11,134 ±692	116.3 100 *22,101 ±1,014	77.0 61 13,555 ±500	153.0 96 13,775 ±977	251
PHA		12,730 ±90	4,933 ±245	7,401 ±599	6,852 ±352	6,701 ±548	7,664 ±34	15,049 ±1,164

- X, Y & Z = Three healthy unrelated individuals.
 PHA = Phytohaemagglutinin.
 m = Mitomycin-C treated.
 --- = Stippled line through negative controls.

The unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the weakly positive reactions.

TABLE VII.21

THIS TABLE SUMMARIZES THE RESULTS OF THE MEAN CPM, SR AND RR IN THE TWO UNILATERAL MLC EXPERIMENTS IN TABLE VII.19 (EXP. I) AND TABLE VII.20 (EXP. II) BETWEEN MLC IDENTICAL, HAPLO-IDENTICAL AND NON-IDENTICAL (UNRELATED) INDIVIDUALS IN FAMILY VDW

	M E A N CPM		M E A N SR		M E A N RR	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
IDENTICAL	1575 (range 564-2220)	1225 (range 558-2731)	7.5 (range 2.5-12.6)	6.1 (range 2.0-14.3)	6 (range 2-11)	4 (range 1-11)
HAPLO-IDENTICAL	10951 (range 4101-17743)	10392 (range 4528-15055)	51.4 (range 18.5-74.8)	52.6 (range 16.1-93.8)	52 (range 32-75)	48 (range 14-74)
UNRELATED	16965 (range 8672-27339)	17242 (range 9980-22545)	91.5 (range 31.4-220.2)	104.1 (range 35.5-172.4)	84 (range 46-100)	87 (range 60-100)

5. Family DEL

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 7.5, and presented in tabular form in Table VII.22. Table VII.23 shows the erythrocyte genetic markers, and Table VII.24 gives the results of the MLC tests.

The parental HLA genotypes and haplotypes were postulated from the serological results in the eight children, who were all available for testing. There were three groups of HLA-A and B identical siblings in this family as shown in Table VII.22. Siblings II-1, II-4 and II-7 who were HLA-A and B identical each showed four clearly defined antigens. In the other five siblings only three antigens could be defined, but because the four possible parental haplotypes were all present, the genotypes and haplotypes for the father and mother could be postulated. The father appears to have had four different HLA-A and B antigens, but the mother must have been homozygous for the HLA antigen B12. The father also carried the antigens B12 and A2 present in the mother, but on different chromosomes.

From the results of tests for erythrocyte genetic markers it appears likely that these were true sibs, although the parents could not be tested.

The eight siblings and two unrelated controls X and Y were tested in the bilateral MLC test in all possible combinations. The HLA-A and B genotypes and haplotypes are both listed in Table VII.24 as they appear to be relevant. The results of the thymidine incorporation and SR in sibs II-1, II-4 and II-7 who were HLA identical for loci A and B, with

haplotypes *ac*, were negative. The same was true for sibs II-3 versus II-5 with haplotypes *ad*, and sibs II-6 versus II-8 with haplotypes *bc*, confirming the three groups of HLA-A and B identical siblings to be non-stimulatory in the MLC test. All the siblings have the B locus antigen B12, either from the paternal *b* haplotype or from the maternal *c* or *d* haplotypes. Three siblings, II-6, II-8 and II-2, were homozygous for B12. Sibs II-6 and II-8 inherited the paternal haplotype *b* and maternal haplotype *c* and sib II-2 inherited haplotypes *bd*. However, from the thymidine incorporation results and SRs all these combinations are strongly positive, therefore the two maternal chromosomes probably carry different D locus determinants although B12 homozygous. Furthermore, the D locus determinant on the father's *b* chromosome which carries the same antigen B12 is also different from either of the mother's D locus determinants.

The value of calculating SRs to find non-stimulatory combinations is demonstrated in this family where some of the autologous control values are rather high. This may or may not be reflected in the mean cpm, as for example in combinations II-1 versus II-4 and II-3 versus II-5. The SR indicates how many times the mean cpm were higher than the control counts. The mean SR for the HLA-A and B identical sibs in this culture was 1.2 and the range 0.4 - 1.9 whereas the mean cpm for these sibs was 341 and the range 162-646. The mean SR for the haplo-identical siblings was 52.1 (range 13.1-90.6) and 121.8 (range 43.8-259.6) with the unrelated controls X and Y.

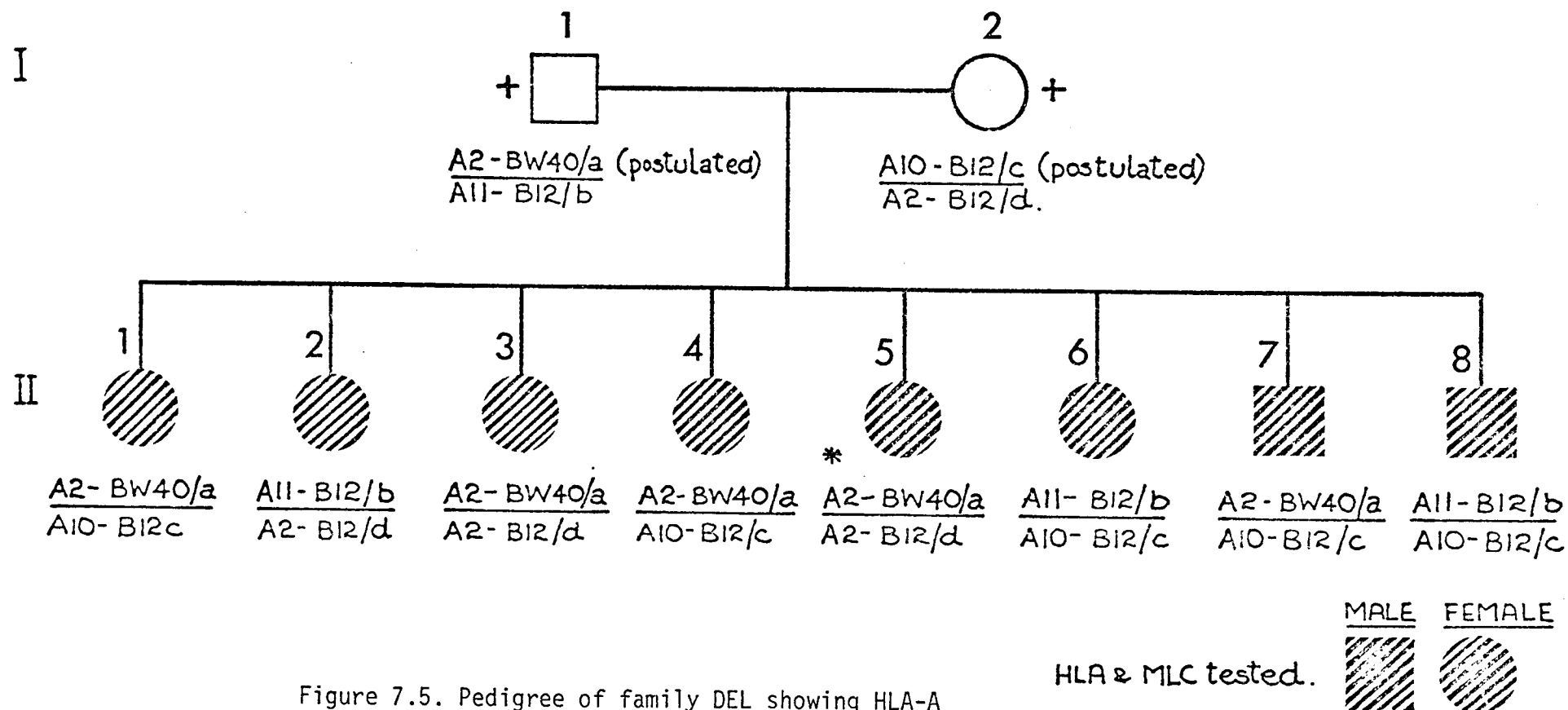


Figure 7.5. Pedigree of family DEL showing HLA-A and B genotypes and haplotypes. The paternal HLA haplotypes are ab and the maternal cd.

* denotes candidate for renal transplantation.

+ indicates deceased parents.

TABLE VII.22

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FAMILY DEL

(See Pedigree Fig. 7.5)

HLA Genotype	Haplotype	Subject
* A2-BW40 A11-B12	a b	I-1 Father
* A10-B12 A2-B12	c d	I-2 Mother
A2-BW40/A10-B12	a/c	II-1 Sib
A2-BW40/A10-B12	a/c	II-4 Sib
A2-BW40/A10-B12	a/c	II-7 Sib
A2-BW40/A2-B12	a/d	II-3 Sib
A2-BW40/A2-B12	a/d	II-5 Sib (patient)
A11-B12/A10-B12	b/c	II-6 Sib
A11-B12/A10-B12	b/c	II-8 Sib
A11-B12/A2-B12	b/d	II-2 Sib

* These HLA-A and B genotypes and haplotypes were postulated, the parents are deceased.

TABLE VII.23

ERYTHROCYTE GENETIC MARKERS ON FAMILY DEL

(See Pedigree Fig. 7.5)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P₁</u>	<u>Duffy</u>
				K	$\bar{k}$		Fy(a)
Parents	I-1) I-2)	deceased					
Sib II-1	A ₁	R ₁ /R ₀	M $\bar{s}$ /N $\bar{s}$	-	+	-	+
Sib II-2	A ₁	R ₁ /R ₀	M $\bar{s}$ /N $\bar{s}$	+	+	-	+
Sib II-3	A ₁	R ₁ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	-	+
Sib II-4	A ₁	R ₁ /R ₀	M $\bar{s}$ /N $\bar{s}$	+	+	-	+
Sib II-5 (patient)	A ₁	R ₀ /R ₀	M $\bar{s}$ /N $\bar{s}$	-	+	+	+
Sib II-6	A ₁	R ₁ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	-	+
Sib II-7	A ₁	R ₁ /R ₀	M $\bar{s}$ /N $\bar{s}$	-	+	-	+
Sib II-8	A ₁	R ₁ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	+	+

TABLE VII.24

BILATERAL MLC TESTS ON FAMILY DEL

(Each entrance contains from top to bottom: SR, mean cpm $\pm$ se)

Subject		11-1	11-4	11-7	11-3	11-5 (patient)	11-6	11-8	11-2	X	Y
	HLA haplotype	A2-BW40/a A10-B12/c	A2-BW40/a A10-B12/c	A2-BW40/a A10-B12/c	A2-BW40/a A2-B12/d	A2-BW40/a A2-B12/d	A11-B12/b A10-B12/c	A11-B12/b A10-B12/c	A11-B12/b A2-B12/d	HLA phenotype A1,3;B7,W22	HLA phenotype A3,9;BW35,W15
Sib 11-1	A2-BW40/a A10-B12/c	397 ± 100									
Sib 11-4	A2-BW40/a A10-B12/c	1.9 646 ± 135	270 ± 47								
Sib 11-7	A2-BW40/a A10-B12/c	1.5 199 ± 71	1.1 257 ± 52	199 ± 9							
Sib 11-3	A2-BW40/a A2-B12/d	25.7 14,149 ± 762	25.3 12,317 ± 362	25.3 12,653 $\pm 1,268$	702 ± 158						
Sib 11-5 (patient)	A2-BW40/a A2-B12/d	61.8 14,350 $\pm 1,130$	68.8 11,628 ± 740	90.6 12,051 ± 276	1.1 442 ± 15	68 ± 18					
Sib 11-6	A11-B12/b A10-B12/c	21.3 10,136 $\pm 1,685$	37.7 15,551 $\pm 1,928$	23.9 9,016 ± 726	24.1 15,168 $\pm 1,214$	40.0 12,452 $\pm 1,168$	554 ± 186				
Sib 11-8	A11-B12/b A10-B12/c	20.5 5,513 $\pm 1,440$	30.4 5,695 ± 642	28.0 4,270 ± 555	30.0 12,114 $\pm 2,408$	110.3 9,490 $\pm 1,282$	0.4 162 ± 40	105 ± 19			
Sib 11-2	A11-B12/b A2-B12/d	37.6 14,220 $\pm 1,096$	56.7 17,813 ± 819	66.1 18,445 ± 382	13.1 6,981 ± 578	46.3 9,867 ± 203	31.3 14,522 $\pm 1,759$	49.6 11,519 ± 439	359 ± 52		
X	HLA phenotype A1,3;B7,W22	66.3 15,994 $\pm 1,387$	69.3 12,266 ± 754	136.6 19,410 ± 995	53.3 22,944 $\pm 1,420$	219.0 16,645 $\pm 1,111$	43.8 13,988 $\pm 1,080$	167.9 15,954 ± 522	67.7 15,032 $\pm 2,511$	85 ± 25	
Y	A3,9;BW35,W15	88.5 21,239 $\pm 2,126$	114.2 20,228 $\pm 4,313$	151.6 21,380 $\pm 1,225$	62.8 24,708 $\pm 1,630$	259.6 19,733 $\pm 1,125$	57.4 18,336 $\pm 1,751$	179.6 16,886 ± 599	76.4 16,885 ± 954	251.8 21,159 $\pm 2,867$	84 ± 17
PHA		28.155 $\pm 1,236$	28.160 $\pm 1,986$	26.895 $\pm 1,708$	14.610 $\pm 1,253$	20.357 $\pm 1,890$	24.656 $\pm 1,638$	20.566 $\pm 1,250$	29.314 $\pm 1,051$	35.497 $\pm 2,543$	32.732 $\pm 4,869$

X & Y = Healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

The negative MLC reactions are outlined within the Table.

6. Family GIA

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 7.6, and presented in tabular form in Table VII.25. Table VII.26 shows the erythrocyte genetic markers and Table VII.27 gives the results of the MLC tests.

The HLA genotypes for loci A and B were determined in the father and children and postulated for the deceased mother I-2. The four possible parental haplotypes were present in the eight children. As shown in Table VII.25, five of the children were HLA-A and B identical, with parental haplotypes *bc* and two were HLA-A and B identical with haplotypes *bd*. Child II-2 was the only one with haplotypes *ac*. The HLA genotype of III-1, child of II-1 (patient) could not be determined because his father was not typed.

From the erythrocyte genetic marker results they appeared to be true siblings. The father and seven of his children and the grandchild III-1 were tested for the MLC reaction. Child II-6 was the only one not included in this culture. The bilateral MLC tests between the four siblings II-1, II-4, II-7 and II-8 who all have the same HLA haplotypes *bc* were negative according to the thymidine incorporation data, or some were weakly positive from the morphological results. The SRs in this group were low, the mean being 1.5 falling into the accepted value for MLC negative HLA-A and B identical sibling combinations in this laboratory. The other two HLA-A and B identical siblings, II-3 and II-5 were also MLC negative with an SR of 1.2.

There was a big range in the mean cpm value as well as in the SRs among the haplo-identical pairs in this family. The range of cpm's was from 1,053-26,516 and the range of SRs 10,1-199,5 in the haplo-identical pairs. The mean cpm value for the haplo-identical combinations was 10,902 and the mean SR 62.6. However, the control values with X and Y were also higher than usually observed by us. The mean cpm value with the two unrelated controls was 26,026 and mean SR 147.1.

It is of interest that the reaction between I-1 and III-1, his grandchild, was like a haplo-identical combination. Child III-1 had the HLA-A and B phenotype A9,W32;B7,12, but because his mother II-1 had the haplotype AW32,B12, his probable genotype was A9,B7/AW32,B12. The haplotype AW32,B12 in II-1 is maternally derived and was probably transmitted to child III-1. The A9,B7 was likely to have been transmitted to child III-1 by his father, who was not typed. There appear to be two possible explanations why I-1 and III-1 should react like haplo-identicals:

1. The 'foreign' haplotype A9,B7 may carry the same D locus determinant as the haplotype A1,B7 present in I-1. It is noteworthy that the MLC reaction of III-1 versus II-2, the only sibling who had the haplotype A1,B7 from the father, was also somewhat reduced.
2. The second explanation is that child III-1 inherited a recombinant HLA locus B and D haplotype from his mother II-1, which would make him MLC haplo-identical to I-1. It would have been interesting to test III-1 against I-2 to determine whether they reacted like haplo-identicals in MLC. There is a good correlation between the results of the thymidine incorporation and morphology in this family.

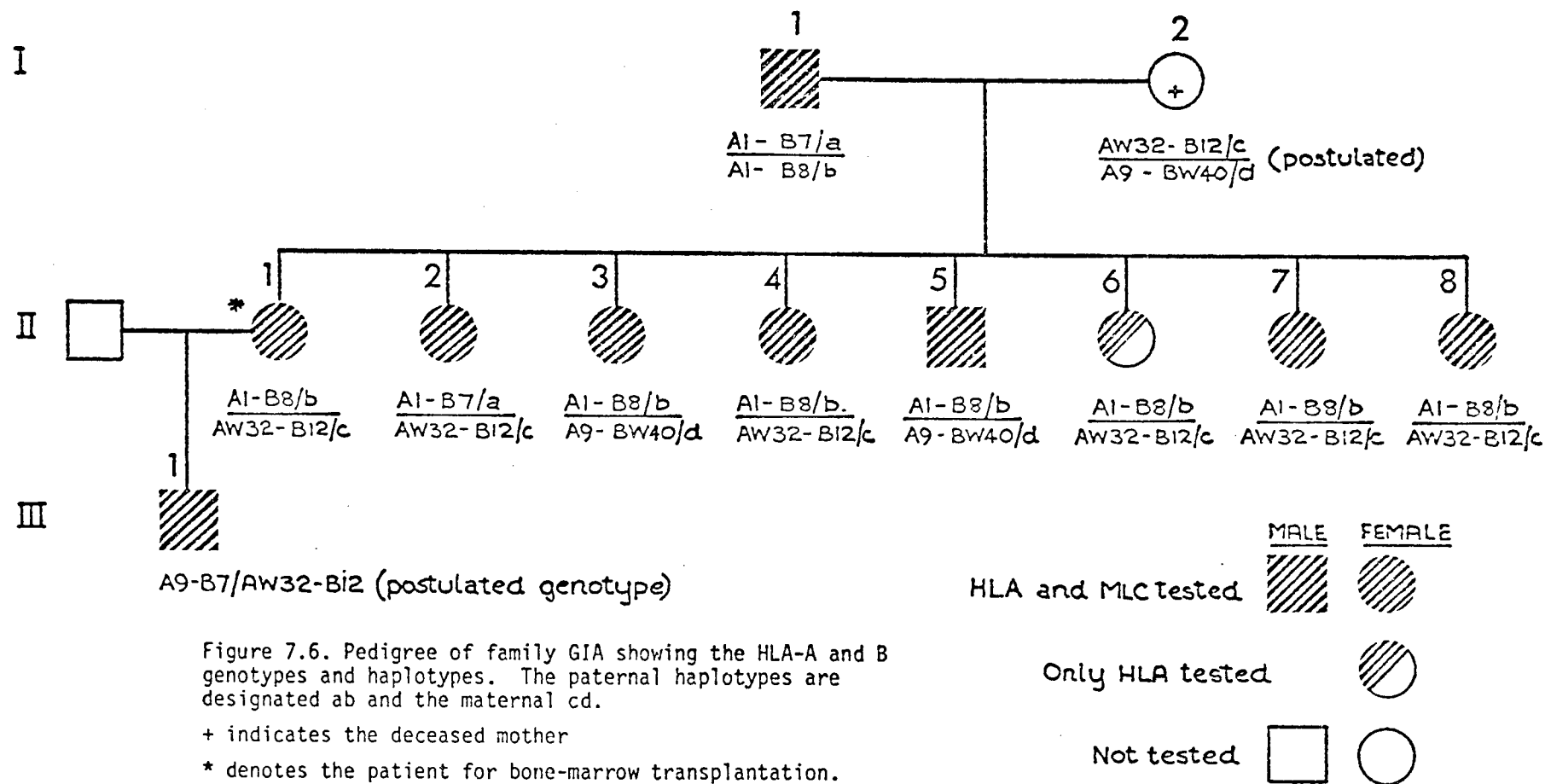


Figure 7.6. Pedigree of family GIA showing the HLA-A and B genotypes and haplotypes. The paternal haplotypes are designated ab and the maternal cd.

+ indicates the deceased mother

* denotes the patient for bone-marrow transplantation.

TABLE VII.25

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FAMILY GIA

(See Pedigree Fig. 7.6)

HLA Genotype	Haplotype	Subject
A1-B7 A1-B8	a b	I-1 Father
*AW32-B12 A9-BW40	c d	I-2 Mother
A1-B8/AW32-B12	b/c	II-1 Sib (patient)
A1-B8/AW32-B12	b/c	II-4 Sib
A1-B8/AW32-B12	b/c	II-6 Sib
A1-B8/AW32-B12	b/c	II-7 Sib
A1-B8/AW32-B12	b/c	II-8 Sib
A1-B7/AW32-B12	a/c	II-2 Sib
A1-B8/A9-BW40	b/d	II-3 Sib
A1-B8/A9-BW40	b/d	II-5 Sib
HLA Phenotype		
A9,W32;B7,12	** -	III-1 child of II-1

* The HLA-A and B genotype of deceased mother I-2 has been postulated.

** The HLA haplotype is not shown, as the father was not typed.

TABLE VII.26

ERYTHROCYTE GENETIC DATA FAMILY GIA

(See Pedigree Fig. 7.6)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P₁</u>	<u>Duffy</u> Fy(a)
				K	k		
F 1-1	A ₁	R ₁ /r	MS/Ms	-	+	+	+
M 1-2 (postulated)		R ₁ /R ₂	Ms/Ns				
Sib 11-1 (patient)	A ₁	R ₁ /r	Ms/Ns	-	+	+	-
Sib 11-2	A ₁	R ₁ /R ₁	Ms/Ns	-	+	+	-
Sib 11-3	A ₁	R ₂ /r	Ms/Ns	-	+	+	+
Sib 11-4	A ₁	R ₁ /R ₂	MS/MS	-	+	+	+
Sib 11-5	O	R ₁ /r	MS/MS	-	+	-	+
Sib 11-6	A ₁	R ₁ /R ₁	MS/MS	-	+	+	+
Sib 11-7	O	R ₂ /r	MS/MS	-	+	+	+
Sib 11-8	O	R ₁ /R ₁	MS/MS	-	+	+	-
Sib 111-1	O	r/r	MS/MS	-	+	-	+

TABLE VII.27

BILATERAL MLC TESTS ON FAMILY GIA

(Each entrance contains from top to bottom: SR and mean cpm $\pm$ se with morphology results in the right upper triangle)

Subject		I-1	II-1 (patient)	II-4	II-7	II-8	II-2	II-3	II-5	III-1	X	Y	PHA
	HLA genotype and haplotype	A1-B7/a A1-B8/b	A1-B8/b AW32-B12/c	A1-B8/b AW32-B12/c	A1-B8/b AW32-B12/c	A1-B8/b AW32-B12/c	A1-B7/a AW32-B12/c	A1-B8/b A9-BW40/d	A1-B8/b A9-BW40/d	HLA phenotype A9,W32;B7,12	A2,3;BW40,W17	A2,28;BW35,12	
F I-1	A1-B7/a A1-B8/b	63 ± 9	(+)	(+)	(+)	+	+	++	+	+	++	++	+++
Sib II-1 (patient)	A1-B8/b AW32-B12/c	21.5 3,037 ± 721	220 ± 77	$\pm$	$\pm$	-	+	+	+	$\pm$	++	++	+++
Sib II-4	A1-B8/b AW32-B12/c	18.2 1,061 ± 413	1.7 237 ± 18	53 ± 4	-	-	+	++	++	+	++	++	++++
Sib II-7	A1-B8/b AW32-B12/c	10.5 1,053 ± 224	1.3 245 ± 22	1.3 124 ± 96	138 ± 12	$\pm$	++	++	++	+	++	+	++++
Sib II-8	A1-B8/b AW32-B12/c	16.2 2,692 ± 175	2.7 668 ± 242	0.6 100 ± 26	1.5 300 ± 66	261 ± 64	++	++	++	+	++	+	++
Sib II-2	A1-B7/a AW32-B12/c	39.1 8,691 $\pm 1,295$	13.3 4,005 $\pm 1,664$	21.7 4,710 ± 870	32.5 8,429 ± 918	40.6 13,043 $\pm 1,764$	381 ± 30	++	++	+	++	++	+++
Sib II-3	A1-B8/b A9-BW40/d	199.5 20,552 $\pm 1,878$	68.4 12,459 ± 785	178.4 17,488 ± 977	136.6 19,268 ± 918	98.7 19,941 $\pm 1,766$	58.9 14,437 $\pm 1,461$	144 ± 57	-	+	++	+	++
Sib II-5	A1-B8/b A9-BW40/d	104.2 21,792 ± 877	56.3 16,218 $\pm 3,372$	125.3 25,561 ± 738	93.1 23,011 $\pm 2,079$	86.0 26,516 $\pm 1,317$	68.4 25,182 $\pm 2,013$	1.2 299 ± 159	356 ± 146	++	+++	++	++
Sib III-1	HLA phenotype A9,W32;B7,12	22.5 1,559 ± 122	10.1 1,495 ± 108	75.7 4,849 ± 406	40.6 4,351 ± 240	42.8 7,201 $\pm 1,203$	15.6 3,573 ± 615	90.3 9,938 $\pm 1,153$	69.1 14,946 ± 322	76 ± 8	++	+	+++
X	A2,3;BW40,W17	231.4 28,004 ± 933	92.0 18,308 ± 762	339.1 39,345 $\pm 1,493$	243.6 38,490 $\pm 3,710$	149.0 32,780 $\pm 2,358$	134.5 37,676 $\pm 1,455$	338.4 54,482 $\pm 8,206$	247.1 66,001 $\pm 3,363$	179.3 22,762 $\pm 2,626$	179 ± 17	++	+++
Y	A2,28;BW35,12	96.7 13,064 $\pm 1,710$	50.5 10,766 ± 897	115.9 15,069 $\pm 1,913$	67.5 11,619 $\pm 1,998$	55.2 12,932 $\pm 1,091$	20.4 6,007 ± 742	107.8 18,867 $\pm 1,277$	86.2 24,227 $\pm 5,986$	50.7 7,159 ± 519	191.3 36,923 $\pm 3,600$	207 ± 41	+++
PHA		32,418 $\pm 1,962$	46,029 ± 958	35,897 $\pm 2,172$	32,218 $\pm 2,599$	39,704 $\pm 3,283$	20,783 ± 982	17,880 ± 466	24,542 ± 953	29,105 $\pm 1,737$	45,023 $\pm 3,617$	41,266 $\pm 5,479$	

X & Y = Two healthy unrelated individuals.
 PHA = Phytohaemagglutinin.
 --- = Stippled line through negative controls.

The unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the weak reactions.

Interpretation of Results

In *family BEU* sib II-3 had a recombinant paternally-derived HLA-A and B haplotype, and MLC studies showed that the MLC locus determinant went with the B locus antigen during the crossing-over event. The parents both carried the B locus antigen BW17 but differed for their accompanying MLC determinants. The HLA-A and B identical sibling combination II-7 (*ac*) versus II-8 (*ac*) and II-1 (*ac*) versus II-7 (*ac*) showed reciprocal non-stimulation in the MLC studies.

Sibling 10 in *family KA* had a recombinant paternally-derived HLA-A and B haplotype. The results confirm the very weak or non-stimulatory effect of antigens of the A locus. Further anomalies in the MLC tests, however, indicate that the antigens of the B locus are not fully responsible for MLC activation either. Among the group of three HLA-A, B identical siblings (3, 4 and 5), one (3) proved to be MLC positive with the other two (4 and 5), but MLC negative with another group of two HLA-A, B identical siblings (1 and 2) who differed for one haplotype from the former group. Furthermore, the child (3) who showed this unexpected MLC reaction pattern also proved to be MLC negative with sibling 10 who had the serologically demonstrable recombination between the A and B loci alleles, which resulted in a difference for three HLA antigens.

These findings were interpreted as strong evidence for the occurrence of a recombination between the serologically determined HLA loci and an MLC determining locus (or loci) which can thus be localized outside the previously recognized HLA region, close to the B locus.

In the *family DEW* the mother II-2 and daughter III-2 were homozygous for HLA-A and B but heterozygous for the D locus. The father's one MHC chromosome carried A, B and D loci identical to one of the mother's chromosomes. Identity for HLA-A and B in parent-child and sibling combinations does not necessarily predict identity for the D locus determinants. This sort of MLC study should be done in families before recombination between the B and D loci is suggested in HLA-A and B identical parent-child or sibling combinations who stimulate each other.

The parents of *family VDW* shared a B locus antigen BW35 which resulted in sib II-1 being homozygous for this antigen, but from the family MLC test the accompanying D locus determinant appeared to be different. There appeared to be reciprocal non-stimulation in combinations II-2 versus II-1 and II-2 versus I-2. Sib II-2 responded to and stimulated unrelated individuals X and Y weakly, or not at all. The father stimulated sib II-2 only weakly but responded strongly to stimulation by II-2.

The lack (or reduction) of response and stimulation by sib II-2 to his mother (I-2), sister (II-1), and two unrelated individuals, X and Y, as well as the fact that he did not respond to his father (or only weakly), cannot be explained by a recombinational event of the D locus determinants in either the paternal or maternal chromosomes inherited by sib II-2, nor can it be explained by simple sharing of a D locus determinant, because I-2, II-1, X and Y all stimulated each other as illustrated diagrammatically in Fig. 7.4. The inclusion phenomenon (Van den Tweel et al., 1973; Dupont et al., 1973), analogous with the cross-reactivity patterns

described in HLA serology also fails to explain these results, since inclusion of the D locus determinant(s) could only explain unilateral non-stimulation by sib II-2, but not the mutually negative MLRs between II-2 and the afore-mentioned cells.

Sib II-2 was capable of normal stimulation and response in MLC as was seen in the reactions between II-2 and Z (Tables VII.19 and VII.20). However, in the MLC between sib II-2 and father, the response of II-2 to father was weak but there was no reduction in the stimulatory capacity of II-2 towards father's cells.

A possible explanation for these latter observations is that there may be separate response and stimulator genes responsible for the MLC reaction in man (Yunis and Amos, 1971). They named these factors or genes MLR-S and MLR-R. They also pointed out that if this MLR-S locus is a separate locus, mutation or deletion of it should be possible. Yunis and Amos (1971) suggested that such response genes could be similar to the immune response (Ir) genes that are postulated to exist within the MHC. The genetic control of MLR would then be determined by genes coding for stimulating determinants (HLA-D) and genes coding for responsiveness to specific HLA-D determinants. If this hypothesis is indeed true, it may well be that sib II-2 lacked the appropriate genes necessary for response to a *specific* HLA-D determinant. This hypothesis is consistent with the findings in mice that unidirectional MLR can occur in genetic constitution based on differences in the MHC alone (e.g. B10.A(4R) responds to B10.A(2R), whereas B10.A(2R) fails to respond to B10.A(4R) (Bach et al., 1972a). The two recombinant strains have the

same parental strains and differ only for two of the I sub-regions. The strong MLC reaction between sib II-2 and Z proves that the lymphocytes of the former were capable of stimulating and responding, and excluded a deletion of the MLR-S and MLR-R genes in sib II-2.

The patient, sib II-1, received a kidney from her brother sib II-2 and had an uneventful clinical course in the post-operative period with minimal signs of rejection. It is now three years since the transplant, and she is well and has good renal function. The kidney graft was performed because these siblings showed a negative or weak response in the MLC tests, in spite of being only HLA haplo-identical. This shows the importance of not ignoring anomalous weak MLC reactions within a family, and possibly also between unrelated individuals, when selecting for organ transplantation.

The mother of *family DEL* was homozygous for the B locus antigen B12, and father had this same antigen on one chromosome, but the accompanying D locus determinants are different on all three chromosomes. The three groups of siblings identical for HLA-A and B are MLC negative. The renal transplant between the patient and the HLA-A, B and D identical sibling was not done for medical reasons.

The two groups of HLA-A and B identical siblings in *family GIA* are MLC negative. There is a distinct possibility that grandfather I-1 and grandchild III-1 may carry the same D locus determinants, derived from different ancestors, on one chromosome. The prospective bone-marrow transplant recipient died before she could receive a transplant.

By performing MLC experiments between family members, it is easy to demonstrate the frequency with which unrelated parents share one MLC allele. Table VII.29 shows the protocol for such *family MLC experiments*.

Children who are MLC identical with one or the other parent, as for example sibs 2 and 4 in Table VII.29 inherit one MLC allele from that parent and the second allele, the shared MLC allele, from the other parent. If the two parents carry identical MLC alleles, it implies that MLC homozygous children may be found like, e.g. sib 1 in Table VII.29.

More than half the parents in the *randomly selected unrelated families* shown in Table VII.28 shared an HLA-B antigen, and five sets of parents were HLA-A, B haplo-identical. Among these there were ten pairs of sero-identical parent-child combinations but only three of them were MLC negative, indicating that the MLC allele on the two serologically identical haplotypes of the parents of these three children were identical. In another family where the parents were HLA-A9,B12 haplo-identical, their only child, who inherited both these parental haplotypes, was MLC homozygous which means that these parents must also have shared one MLC allele.

TABLE VII.28

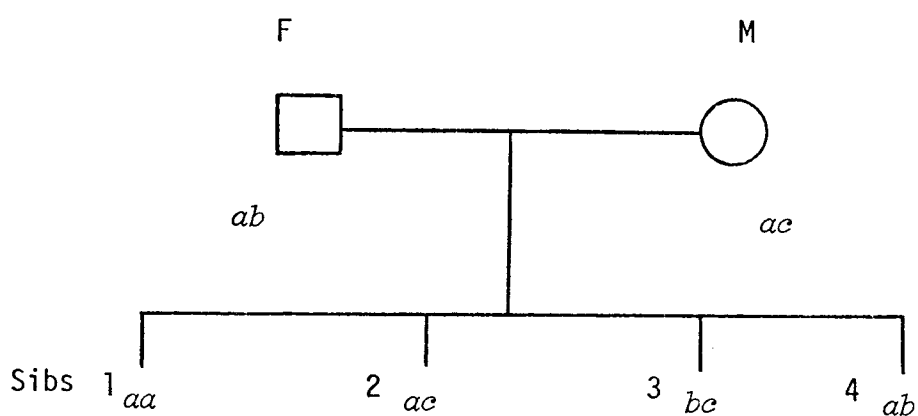
A SUMMARY OF THE HLA-A AND B TYPINGS OF THE PARENTS, IN TWENTY-ONE RANDOM NON-CONSANGUINEOUS FAMILIES, ON WHOM 'FAMILY MLC TESTS' WERE PERFORMED. THE NUMBER OF D LOCUS HOMOZYGOUS OFFSPRING IN THE LAST COLUMN WAS DEDUCED FROM THE MLC RESULTS

Family Number	HLA-A and B genotypes of parents	No. of children tested in each family	No. of sero-identical parent-child combinations	No. of children homozygous for B Locus D Locus	
1	F A2,B7/A1,BW40 M A9,B7/A2,B5	2	0	2	0
2*	F A9,BW17/AW43,BW17 M A9,BW17/A9,BW17	5	5	5	0
3	F AW30,B8/A10,B12 M A1,B12/A2,B12	3	0	0	0
4	F A2,BW40/A11,B12 M A10,B12/A2,B12	8	0	3	0
5	F A1,B7/A1,B8 M AW32,B12/A9,BW40	8	0	0	0
6	F A3,BW15/A11,- M A2,BW35/A3,BW17	5	0	0	0
7	F A3,B8/A28,BW35 M A2,B12/A3,BW35	2	0	1	0
8	F A2,BW40/A11,B12 M A10,B12/A2,B12	8	0	2	0
9	F A3,BW17/AW33,B7 M A1,BW17/AW33,B27	9	0	2	0
10*	F A2,BW35/A11,BW15 M A9,B5/A2,BW35	2	0	2	0
11	F A1,B27/A2,BW15 M A1,B7/A1,BW37	2	0	0	0
12*	F A1,B8/A29,B12 M A29,B12/A2,B7	1	0	1	1
13*	F A2,B12/-,- (postulated) M A2,B12/A2,BW15	3	1	2	0
14	F A11,BW15/AW30,B7 M A1,BW17/A2,BW22	5	0	0	0
15	F A28,B14/AW43,BW21 M A29,B18/A28,-	2	0	0	0
16	F A2,BW40/AW30,BW42 M AW43,B12/AW31,BW17	3	0	0	0
17	F A2,-/A3,BW35 M A3,B7/A2,BW40	2	0	0	0
18	F A2,BW15/A28,BW35 M A1,B8/A3,BW35	2	0	0	0
19	F AW30,B7/A28,BW35 M AW30,-/A10,BW17	3	0	0	0
20	F A2,BW22/A28,B8 M A2,BW35/A3,BW15	4	0	0	0
21*	F A1,B8/A2,B12 M A1,B8/A1,B8	4	4	1	0
TOTAL	21 families	83 children	10	21	1

* Denotes the families with HLA haplo-identical parents.

TABLE VII.29. PROTOCOL FOR TESTING SHARING OF MLC ALLELES
BY THE FATHER AND THE MOTHER

If the letters a , b and c denote the MLC alleles and the father and mother share one allele a , the pedigree can be written as follows :



The MLC results should be:

F	+	M	= MLC positive in both directions
F	+	Sib 4	= MLC negative in both directions
M	+	Sib 2	= MLC negative in both directions
aa_m	+	ab	= MLC negative
aa_m	+	ac	= MLC negative

but

ab_m	+	aa	= MLC positive
ac_m	+	aa	= MLC positive

The subscript m = Mitomycin-C treated

CHAPTER EIGHT - SAN (BUSHMEN) FAMILIES

1. San family I
2. San family II (Bigamous)
3. San family III (Bigamous)
4. San family IV
5. San family V
6. San family VI
7. San family VII

Interpretation of Results

The results of HLA-A, B and MLC experiments on seven San families tested during field work are given. This group provided an ideal opportunity to study the genetic relationship between HLA-A and B and the MLC reaction in a small isolated community where apparently unrelated parents may possess the same genes. The explanation for this could be that in such a small group there are only a limited number of genes. Furthermore, many of them may have common ancestors.

Due to an error, the radio-active thymidine, used in the MLC tests on the San, was of very low specific activity, and in order to obtain results comparable to the normal, the tests had to be counted for much longer periods. Therefore in some instances the background counts of the bottle, disc and scintillating fluid without cells were greater than the actual test counts, and when the background counts were subtracted from the test cpm, this gave a negative value. Such counts have a negative sign preceding the mean cpm values.

1. San family I

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 8.1, and presented in tabular form in Table VIII.1. Table VIII.2 shows the erythrocyte genetic markers, and Table VIII.3 gives the results of the MLC tests.

Three different HLA antigens could be identified in each individual in generations I and II. From the segregation of the antigens in the children it could be postulated that the father I-1 and the five siblings were all homozygous for

HLA-BW17. The absence of the maternal haplotype *c* from the siblings makes it impossible to say whether the mother I-2 was also homozygous for HLA-BW17 or whether she had an unidentified B locus antigen in that position.

No specimens were available from siblings II-4 and II-5 for testing for erythrocyte markers. There was no evidence of other parentage than that stated among the three siblings tested.

Blood from the parents and four of the siblings II-1, II-2, II-4 and II-5, was available for MLC testing. The parents and siblings II-1, II-4 and II-5 were tested in all possible combinations on one occasion. Controls with unrelated individuals were omitted in the first test because of a shortage of cells from the family. Siblings II-2 and II-4 were tested separately on a second occasion and unfortunately the other members of the family were then not available because of the nomadic habits of the San. Although the father was homozygous for the HLA antigen BW17, and the mother also had this antigen BW17 on one chromosome, the MLC determinants which go with the father's HLA haplotype A3,BW17(*a*) and the mother's haplotype AW30,BW17(*d*) must be different on account of the positive MLC results between the father and the three siblings who are also homozygous for BW17, by virtue of the fact that they had the paternal haplotype *a* and maternal *d* haplotype (Table VIII.3, 1st Culture).

It was clear from both the morphology and thymidine incorporation that sibs II-4(*ad*) versus II-5(*ad*) (1st Culture), and II-2(*bd*) versus II-4(*ad*) (2nd Culture), were negative with good controls. Unfortunately II-2(*bd*)

versus II-5 (*ad*) could not be tested. The negative reaction between sibs II-4 and II-5 confirmed their MLC as well as HLA-A and B identity. However, the unexpected negative result between II-2 (*bd*) and II-4 (*ad*) as well as the positive MLC results between the HLA-A and B identical sibs II-1 (*ad*) versus II-4 (*ad*) and II-1 (*ad*) versus II-5 (*ad*) warrants further discussion.

A recombination between the paternal *a* and *b* chromosomes may have occurred between the HLA locus B and the Major MLC locus (locus D) in sib II-2, who had the HLA haplotypes *bd*, or the father who was homozygous for HLA-BW17 may also have been homozygous for the accompanying D locus determinant, thereby producing the negative MLC result between sibs II-2 and II-4, i.e. *bd* versus *ad*. Alternatively this could be the result of a recombination between the paternal chromosomes *a* and *b* between HLA-B and HLA-D in sib II-4. This same phenomenon may have taken place in sib II-5. This would explain the positive reaction between sibs II-5 versus II-1 and II-4 versus II-1 as well as the negative MLC results between II-4 and II-5. Unfortunately II-2 versus II-1 and II-2 versus II-5 were not tested. The positive MLC results between sibs II-1 versus II-4 and II-1 versus II-5 could also be due to a segregation of the paternal or maternal D and B loci in child II-1. From these results it is impossible to deduce whether these results were due to a recombination of paternal *ab* or maternal *cd* chromosomes.

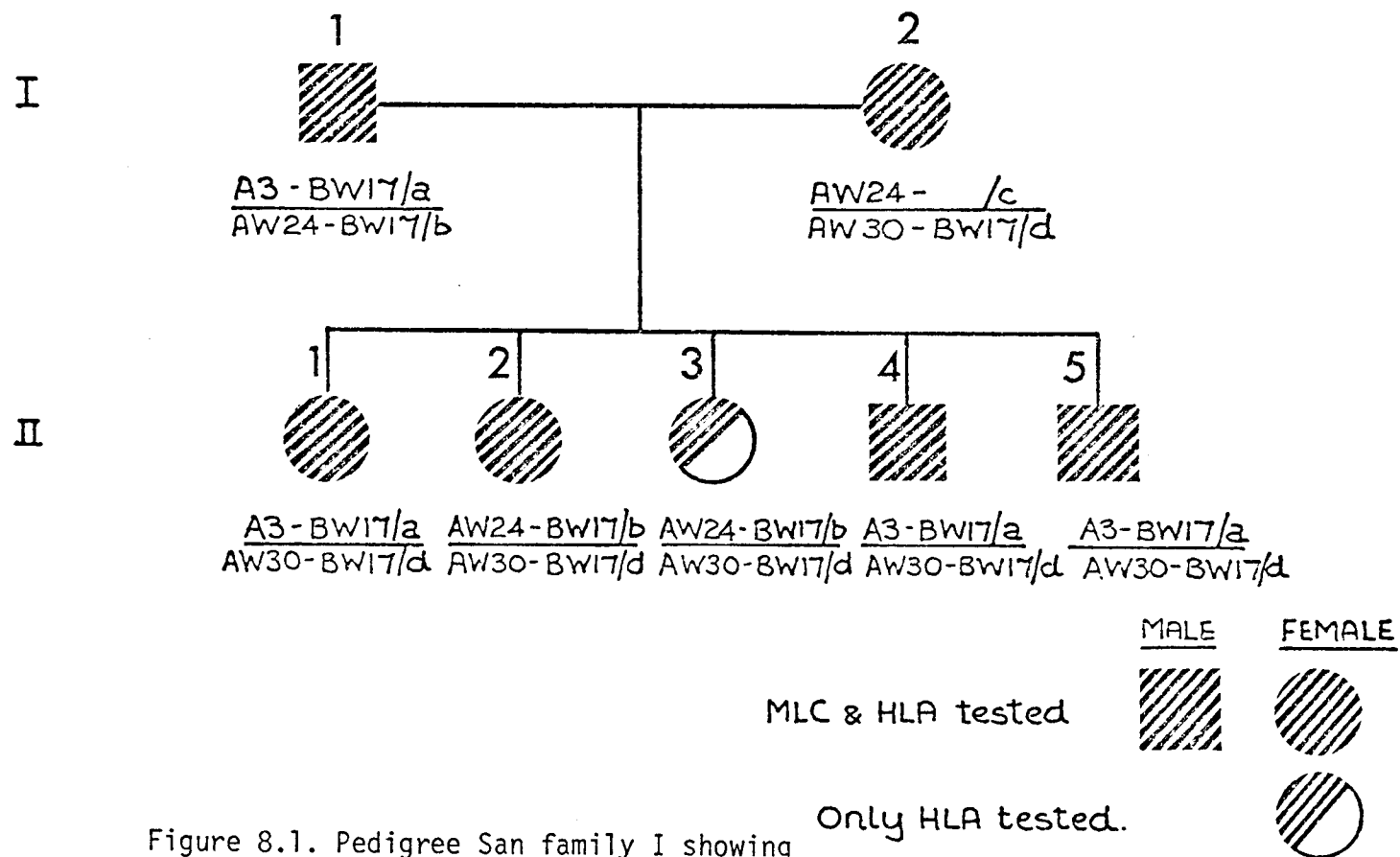


Figure 8.1. Pedigree San family I showing HLA-A and B genotypes and haplotypes. The paternal haplotypes are designated ab and the maternal cd.

TABLE VIII.1

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY I
(See Pedigree Fig. 8.1)

HLA Genotype	Haplotype	Subject
A3-BW17 AW24-BW17	a b	I-1 Father
AW24- AW30-BW17	c d	I-2 Mother
AW24-BW17/AW30-BW17	b/d	II-2 Sib
AW24-BW17/AW30-BW17	b/d	II-3 Sib
A3-BW17/AW30-BW17	a/d	II-1 Sib
A3-BW17/AW30-BW17	a/d	II-4 Sib
A3-BW17/AW30-BW17	a/d	II-5 Sib

TABLE VIII.2

ERYTHROCYTE GENETIC MARKERS SAN FAMILY I
(See Pedigree Fig. 8.1)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u> K $\bar{k}$	P_1	<u>Duffy</u> Fy(a)
F I-1	0	R_0/R_0	$NS/N\bar{s}$	- +	+	+
M I-2	0	R_0/R_0	$N\bar{s}/N\bar{s}$	- +	+	+
Sib II-1	0	R_0/R_0	$N\bar{s}/N\bar{s}$	- +	+	+
Sib II-2	0	R_0/R_0	$NS/N\bar{s}$	- +	+	+
Sib II-3	0	R_0/R_0	$NS/N\bar{s}$	- +	+	-
Sib II-4	not tested					
Sib II-5	not tested					

TABLE VIII.3

BILATERAL MLC TESTS ON SAN FAMILY I

(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

1ST CULTURE							
Subject		I-1	I-2	II-1	II-4	II-5	PHA
	HLA haplotype	A3-BW17/a AW24-BW17/b	AW24- /c AW30-BW17/d	A3-BW17/a AW30-BW17/d	A3-BW17/a AW30-BW17/d	A3-BW17/a AW30-BW17/d	
F I-1	A3-BW17/a AW24-BW17/b	49 ±3	++	++	++	++	+++
M I-2	AW24- /c AW30-BW17/d	3,335 ±1,107	60 ±35	++	++	++	++
Sib II-1	A3-BW17/a AW30-BW17/d	2,189 ±824	1,932 ±515	28 ±1	++	++	+++
Sib II-4	A3-BW17/a AW30-BW17/d	4,007 ±1,591	2,726 ±1,325	2,889 ±1,235	129 ±28	-	++
Sib II-5	A3-BW17/a AW30-BW17/d	442 ±69	2,067 ±416	901 ±363	49 ±26	28 ±0	
PHA		2,019 ±54	2,444 ±97	3,523 ±168	11,381 ±631	9,072 ±345	

2ND CULTURE						
Subject		II-2	II-4	X	Y	PHA
	HLA haplotype	AW24-BW17/b AW30-BW17/d	A3-BW17/a AW30-BW17/d	nd	nd	
Sib II-2	AW24-BW17/b AW30-BW17/d	24 ±4	-	++	++	+++
Sib II-4	A3-BW17/a AW30-BW17/d	91 ±19	35 ±7	++	++	++
X	nd	799 ±145	837 ±53	61 ±32	++	+++
Y	nd	1,084 ±278	1,674 ±31	911 ±337	50 ±48	+++
PHA		4,849 ±588	3,567 ±765	3,114 ±100	4,040 ±333	

X & Y = Two healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

nd = Not determined.

The negative MLC reactions are outlined within the Table.

2. San family II (bigamous)

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 8.2, and presented in tabular form in Table VIII.4. Table VIII.5 shows the erythrocyte genetic markers, and Tables VIII.6 and VIII.7 give the results of the MLC tests.

The father and his wife I-2A and their six children were typed for the HLA-A and B antigens. Only three antigens were identified in the father and four in the mother. The father and mother shared one HLA antigen, AW26. In the two siblings II-1 and II-4 with haplotypes *ac*, the B locus antigen on the paternal α chromosome again evaded identification. Three sibs, II-2, II-3 and II-6, inherited haplotypes *bc*, and one sib, II-5, haplotypes *bd*.

The results of the tests for erythrocyte markers did not suggest parentage other than that stated.

The PHA controls were omitted because of a scarcity of cells. The *bilateral* results of both the morphology and thymidine incorporation of the combinations II-2 versus II-3, II-3 versus II-6 and II-2 versus II-6 with the identical HLA haplotypes *bc* were negative with good controls. The thymidine incorporation data of II-1 versus II-3 and II-1 versus II-6, i.e. *ac* + *bc* were negative or weakly positive, especially as the control with Y was relatively low. The morphology results of II-1 versus II-2, II-3 and II-6 with haplotypes *bc* were negative or weakly positive.

From the *unilateral* MLC data in Table VIII.7 it has not been conclusively shown that sib II-1 with haplotype *ac* also

belonged to the previous group of MLC negative individuals with the HLA haplotype *bc*. The Mitomycin-C data in this experiment were particularly difficult to interpret.

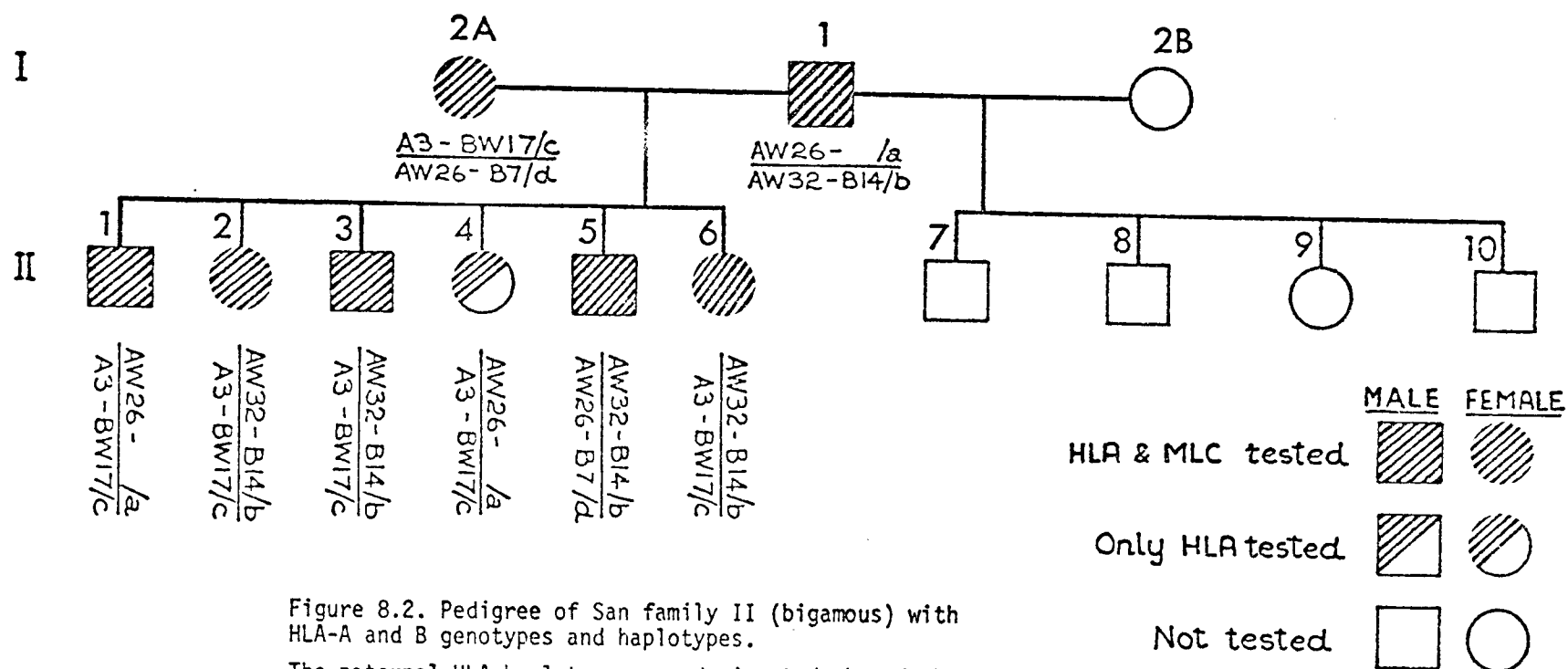


Figure 8.2. Pedigree of San family II (bigamous) with HLA-A and B genotypes and haplotypes.

The paternal HLA haplotypes are designated ab and the maternal (I-2A) cd.

TABLE VIII.4

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY II (BIGAMOUS)
 (See Pedigree Fig. 8.2)

HLA Genotype	Haplotype	Subject
AW26- AW32-B14	a b	I-1 Father
A3-BW17 AW26-B7	c d	I-2A Mother
AW26- /A3-BW17	a/c	II-1 Sib
AW26- /A3-BW17	a/c	II-4 Sib
AW32-B14/A3-BW17	b/c	II-2 Sib
AW32-B14/A3-BW17	b/c	II-3 Sib
AW32-B14/A3-BW17	b/c	II-6 Sib
AW32-B14/AW26-B7	b/d	II-5 Sib

TABLE VIII.5

ERYTHROCYTE GENETIC MARKERS SAN FAMILY II (BIGAMOUS)

(see Pedigree Fig. 8.2)

Subject		<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		P_1	<u>Duffy</u> Fy(a)
					K	$\bar{k}$		
F	I-1	O	R_0/R_0	$M\bar{S}/N\bar{S}$	-	+	+	-
M	I-2A	A_1	R_0/R_0	$M\bar{S}/N\bar{S}$	-	+	+	-
Sib	II-1	A_1	R_0/R_0	$N\bar{S}/N\bar{S}$	-	+	-	-
Sib	II-2	A_1	R_0/R_0	$M\bar{S}/N\bar{S}$	-	+	-	-
Sib	II-3	O	R_0/R_0	$M\bar{S}/N\bar{S}$	-	+	-	-
Sib	II-4	A_1	R_0/R_0	$M\bar{S}/N\bar{S}$	-	+	-	-
Sib	II-5	O	R_0/R_0	$N\bar{S}/N\bar{S}$	-	+	+	-
Sib	II-6	A_1	R_0/R_0	$N\bar{S}/N\bar{S}$	-	+	-	-

TABLE VIII.6

BILATERAL MLC TESTS ON SAN FAMILY II (BIGAMOUS)

(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		I-1	I-2A	II-1	II-2	II-3	II-6	II-5	X	Y
	HLA haplo-type	ab	cd	ac	bc	bc	bc	bd	nd	nd
F I-1	ab	63	weakly positive	+	?	++	++	+	+++	+++
M I-2A	cd	488 ± 220	-10	+	+	++	++	+	+++	++
Sib II-1	ac	153 ± 26	265 ± 72	-27	[$\pm$ $\pm$ $\pm$]			++	+++	+++
Sib II-2	bc	157 ± 35	804 ± 612	147 ± 43	50	[- -]		++	+++	++
Sib II-3	bc	787 ± 144	428 ± 63	42 ± 32	-5 ± 19	38	-	++	+++	++
Sib II-6	bc	153 ± 67	140 ± 46	49 ± 28	-11 ± 5	4 ± 7	-1	+	+++	+++
Sib II-5	bd	199 ± 19	55 ± 294	588 ± 198	265 ± 78	199 ± 63	465 ± 93	-34	+++	+++
X	nd	4,415 $\pm 2,917$	3,247 $\pm 2,690$	1,143 ± 635	850 ± 137	6,330 $\pm 1,080$	3,044 ± 244	4,090 $\pm 2,550$	98	+++
Y	nd	2,629 ± 578	1,827 ± 922	349 ± 230	2,046 ± 656	1,278 ± 344	1,549 ± 491	4,075 $\pm 1,614$	6,657 ± 648	2
PHA		1,436 ± 148	1,039 ± 31	1,338 ± 138	1,609 ± 587	4,941 ± 161	3,853 ± 65	4,515 ± 482	3,209 ± 78	2,544 ± 65

X & Y = Two healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

- = A negative sign in front of the cpm value denotes a count less than the background.

nd = Not determined.

Unusual MLC reactions are outlined within the Table, solid lines for negative reaction and interrupted lines for the doubtful reactions.

TABLE VIII.7

UNILATERAL MLC TESTS ON SAN FAMILY II (BIGAMOUS)
(expressed in cpm $\pm$ se)

Subject		I-1	I-2A	II-1	II-2	II-3	II-6	II-5	X	Y
	HLA haplo-type	ab	cd	ac	bc	bc	bc	bd	nd	nd
F I-1 _m	ab	nd	93 ± 11	224 ± 107	731 ± 467	833 ± 364	129 ± 50	1,006 ± 354	3,956 $\pm 1,785$	1,746 ± 633
M I-2A _m	cd	577 ± 293	nd	268 ± 152	438 ± 94	455 ± 168	132 ± 18	113 ± 48	2,007 $\pm 1,273$	1,351 ± 475
Sib II-1 _m	ac	463 ± 252	147 ± 81	nd	135 ± 93	51 ± 14	30 ± 23	132 ± 62	3,054 $\pm 1,177$	1,209 ± 530
Sib II-2 _m	bc	99 ± 25	116 ± 24	273 ± 101	nd	86 ± 26	-6 ± 7	722 ± 515	2,722 $\pm 1,915$	1,703 ± 613
Sib II-3 _m	bc	214 ± 102	122 ± 11	231 ± 105	112 ± 119	nd	-7 ± 18	167 ± 86	466 ± 157	701 ± 368
Sib II-6 _m	bc	32 ± 20	63 ± 33	40 ± 27	54 ± 7	47 ± 12	nd	87 ± 30	316 ± 46	915 ± 372
Sib II-5 _m	bd	244 ± 80	54 ± 29	1,171 ± 275	137 ± 82	798 ± 447	474 ± 192	nd	1,383 ± 885	2,902 $\pm 1,407$
X _m	nd	304 ± 87	862 ± 295	2,191 ± 544	3,568 $\pm 2,714$	2,808 ± 239	1,997 ± 799	215 ± 54	nd	2,077 $\pm 1,108$
Y _m	nd	891 ± 599	1,466 ± 339	2,303 ± 942	1,269 ± 171	3,054 ± 234	3,604 ± 363	1,097 ± 461	5,170 $\pm 2,009$	nd

X & Y = Two healthy unrelated individuals.

m = Mitomycin-C treated.

nd = Not determined (autologous controls were unfortunately omitted).

- = A negative sign in front of the cpm value denotes a count less than the background.

--- = Stippled line through negative controls.

Unusual MLC reactions are outlined within the Table, solid lines for negative reactions and interrupted lines for the doubtful reactions.

3. San family III (bigamous)

The pedigree of this family together with HLA-A and B genotypes and haplotypes, is shown in Fig. 8.3, and presented in tabular form in Table VIII.8, Table VIII.9 shows the erythrocyte genetic markers, and Table VIII.10 gives the results of the MLC tests.

The HLA genotypes for loci A and B of the father, his two wives and their respective children were serologically characterized. Four distinct antigens were demonstrated in the mother I-2B, but only two in mother I-2A, and three in the father, who must have been homozygous for HLA-B8 in order to have passed on this antigen to sibs II-1 and II-4 who inherited the paternal *b* haplotype as well as to sibs II-2 and II-3 who inherited the *a* haplotype. The four different haplotypes from the father I-1 and mother I-2A, segregate normally in their four children. The two children belonging to mother I-2B were HLA-A and B identical and two of the parental haplotypes were present in them.

The erythrocyte genetic markers again segregated normally in the two families.

The HLA serology of the father, both spouses and their children were determined, but because of the limited technical facilities available to do large MLC tests, the one wife I-2B was omitted from the MLC study. MLC tests were carried out on the father I-1, the mother I-2A and their four offspring in all possible combinations. The two full siblings belonging to mother I-2B were also tested in the same MLC test with the above-mentioned, as well as two controls X and Y.

The father I-1 was homozygous for the HLA antigen B8, but carried different D locus determinants on the two MHC chromosomes as shown by the positive MLC tests with II-1 versus II-2 and II-3 versus II-4 where the sibling pairs were identical for the maternal haplotypes but inherited either the *a* or *b* haplotypes from the father.

The results of the thymidine incorporation as well as the morphology between mother I-2A and sib II-4, i.e. *bd* versus *cd* were negative with good controls. This suggested that the maternal *c* chromosome and the paternal *b* chromosome may share a common D locus determinant thus resulting in mother I-2A and child II-4 being identical for their D loci although only HLA haplo-identical. This would also explain the negative or weakly positive thymidine incorporation and morphological results for the MLC tests between father I-1 (haplotypes *ab*) and sib II-2 (haplotypes *ac*) who would also be identical for their D locus determinants, but only HLA-A and B haplo-identical. If as postulated the father I-1 and mother I-2A shared a common D locus determinant on one of their two chromosomes, this would have resulted in a much weaker stimulation when their cells were cultured together than normally seen with HLA-A, B and D non-identical unrelated parents. The degree of stimulation in the MLC test between father I-1 and mother I-2A was low according to the mean cpm and morphology, thus supporting the above postulate.

The HLA-A and B identical sibs II-5 and II-6 (haplotypes *ae*) failed to stimulate each other in the bidirectional MLC tests as assessed by both the thymidine incorporation and the morphology results.

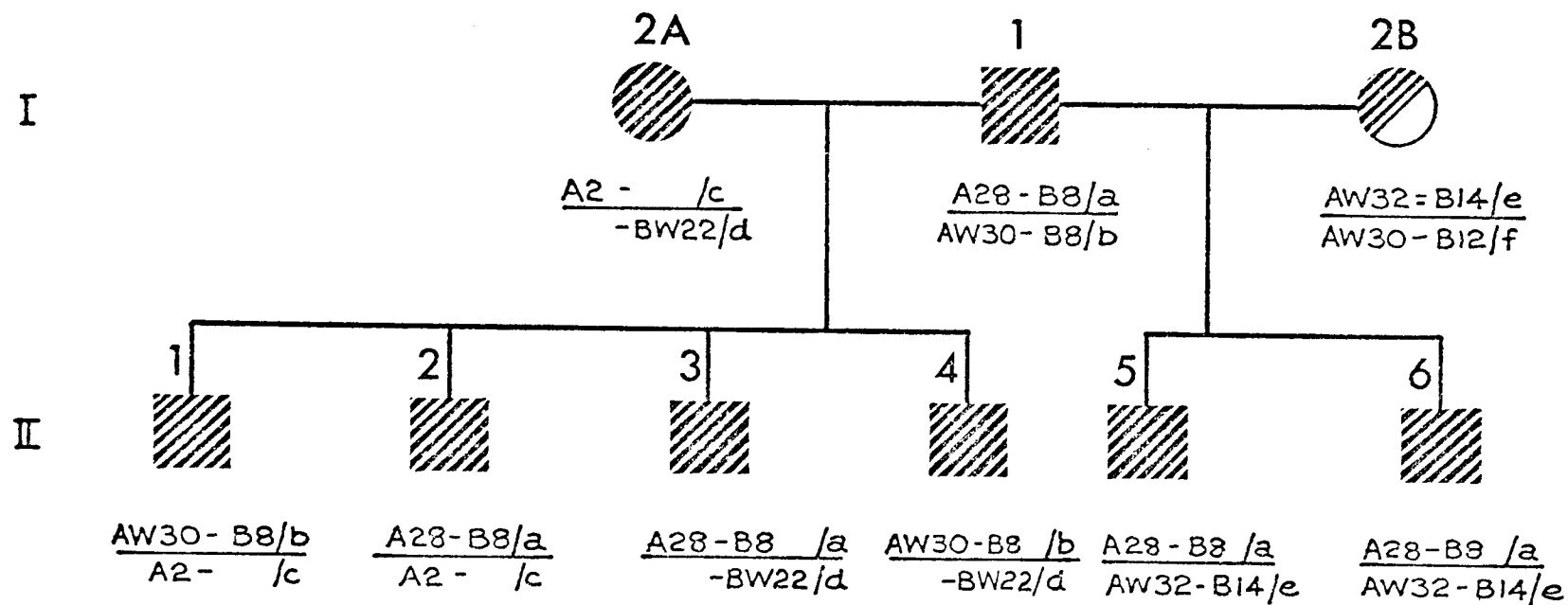


Figure 8.3. Pedigree San family III (bigamous) with HLA-A and B genotypes and haplotypes. The father's HLA haplotypes are designated ab, mother I-2A's cd, and mother I-2B's ef.

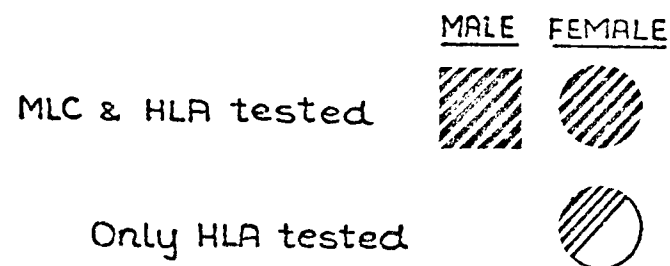


TABLE VIII.8

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY III (BIGAMOUS)

(See Pedigree Fig. 8.3)

HLA Genotype	Haplotype	Subject
A28-B8 AW30-B8	a b	I-1 Father
A2- -BW22	c d	I-2A Mother
AW32-B14 AW30-B12	e f	I-2B Mother
Children from mother I-2A		
AW30-B8/A2-	b/c	II-1 Sib
A28-B8/A2-	a/c	II-2 Sib
A28-B8/-BW22	a/d	II-3 Sib
AW30-B8/-BW22	b/d	II-4 Sib
Children from mother I-2B		
A28-B8/AW32-B14	a/e	II-5 Sib
A28-B8/AW32-B14	a/e	II-6 Sib

TABLE VIII.9

ERYTHROCYTE GENETIC MARKERS SAN FAMILY III (BIGAMOUS)

(See Pedigree Fig. 8.3)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P₁</u>	<u>Duffy</u> Fy(a)
				K	k		
F I-1	O	R ₀ /R ₀	M ⁻ S/M ⁻ S	-	+	+	-
M I-2A	A ₁	R ₀ /R ₀	M ⁻ S/N ⁻ S	-	+	+	+
Sib II-1	O	R ₀ /R ₀	M ⁻ S/M ⁻ S	-	+	+	-
Sib II-2	A ₁	R ₀ /R ₀	M ⁻ S/M ⁻ S	-	+	+	-
Sib II-3	O	R ₀ /R ₀	M ⁻ S/N ⁻ S	-	+	+	+
Sib II-4	O	R ₀ /R ₀	M ⁻ S/M ⁻ S	-	+	+	-
F I-1	O	R ₀ /R ₀	M ⁻ S/M ⁻ S	-	+	+	-
M I-2B	A ₁	R ₀ /R ₀	N ⁻ S/N ⁻ S	-	+	+	-
Sib II-5	A ₁	R ₀ /R ₀	M ⁻ S/N ⁻ S	-	+	+	-
Sib II-6	O	R ₀ /R ₀	M ⁻ S/N ⁻ S	-	+	+	+

TABLE VIII.10

BILATERAL MLC TESTS ON SAN FAMILY III (BIGAMOUS)
(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		I-1	I-2A	II-1	II-2	II-3	II-4	II-5	II-6	X	Y	PHA
	HLA haplotype	A28-B8/a AW30-B8/b	A2- /c -BW22/d	AW30-B8/b A2- /c	A28-B8/a A2- /c	A28-B8/a -BW22/d	AW30-B8/b -BW22/d	A28-B8/a AW32-B14/e	A28-B8/a AW32-B14/e	nd	nd	
F I-1	A28-B8/a AW30-B8/b	22 ± 35	$\pm$	++	$\pm$	++	++	++	+++	++	+++	++
M I-2A	A2- /c -BW22/d	72 ± 61	-1 ± 5	+	+	+	-	+	+	+	+	++
Sib II-1	AW30-B8/b A2- /c	336 ± 143	48 ± 17	7 ± 19	++	+	$\pm$	++	+++	+++	++	++
Sib II-2	A28-B8/a A2- /c	18 ± 11	323 ± 111	1,321 ± 738	35 ± 36	$\pm$	$\pm$	++	++	++	++	+++
Sib II-3	A28-B8/a -BW22/d	348 ± 201	140 ± 34	1,716 ± 520	129 ± 72	58 ± 8	-7	++	++	++	++	++
Sib II-4	AW30-B8/b -BW22/d	333 ± 136	-3 ± 7	248 ± 14	581 ± 281	277 ± 97	7 ± 1	++	++	++	++	++
Sib II-5	A28-B8/a AW32-B14/e	2,435 ± 821	442 ± 121	4,813 $\pm 1,652$	3,854 $\pm 1,318$	1,872 ± 426	1,105 ± 371	-4 ± 19	-	++	++	+++
Sib II-6	A28-B8/a AW32-B14/e	1,700 ± 659	569 ± 274	3,549 ± 702	1,855 ± 978	2,189 $\pm 1,035$	1,099 ± 600	18 ± 17	-14 ± 53	+	++	+++
X	nd	1,837 ± 912	1,020 ± 502	411 ± 66	2,974 $\pm 1,731$	1,917 $\pm 1,342$	1,776 ± 871	2,533 $\pm 1,323$	1,171 ± 77	20 ± 15	++	++
Y	nd	1,167 $\pm 2,480$	578 ± 292	3,473 ± 137	3,820 $\pm 1,227$	2,352 $\pm 1,273$	993 ± 450	6,082 ± 991	3,508 $\pm 1,738$	4,504 $\pm 1,727$	2,000 ± 3	+++
PHA		489 ± 119	1,699 ± 782	705 ± 136	1,223 ± 128	1,419 $\pm 1,078$	1,236 ± 840	4,897 ± 352	2,294 $\pm 1,537$	2,955 $\pm 2,084$	3,867 $\pm 2,693$	

X & Y = Two healthy unrelated individuals.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

- = A negative sign in front of the cpm value denotes a count less than the background.

nd = Not determined.

The unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the weakly positive reactions.

4. San family IV

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 8.4 and presented in tabular form in Table VIII.11. Table VIII.12 shows the erythrocyte and serum protein genetic markers, and Table VIII.13 gives the results of the MLC tests.

The results of the HLA serology should be viewed with reservation as they are unclear because of a high background staining in all the tests. The antigens assigned to the parents and the three siblings who were typed were not clearly defined.

The erythrocyte genetic markers, serum proteins and red cell enzymes are all included in the results because they provided additional evidence that parentage in this family was not as stated. The father I-1, was tested three times with anti- $\bar{s}$ and was negative each time. The three sibs could therefore be excluded on the MNS $\bar{s}$ system. In addition sib II-1 was excluded on haptoglobin and transferrin and sib II-2 on ABO and PGM $_1$.

Bilateral cultures were performed in all possible combinations on the parents and sibs II-1, II-2 and II-3 with two unrelated controls X and Y. The morphology was omitted because of a lack of lymphocytes. The results of the thymidine incorporation of the tests between sibs II-2 and II-3 who appeared to be HLA-A and B identical were somewhat lower than the other combinations, but not what one would expect in a truly negative culture. These results, taken in conjunction with the poor HLA serology and exclusion of paternity of all three siblings on the results of the erythrocyte genetic markers, serum proteins and red cell enzymes, confirmed that parentage of these children was not as stated.

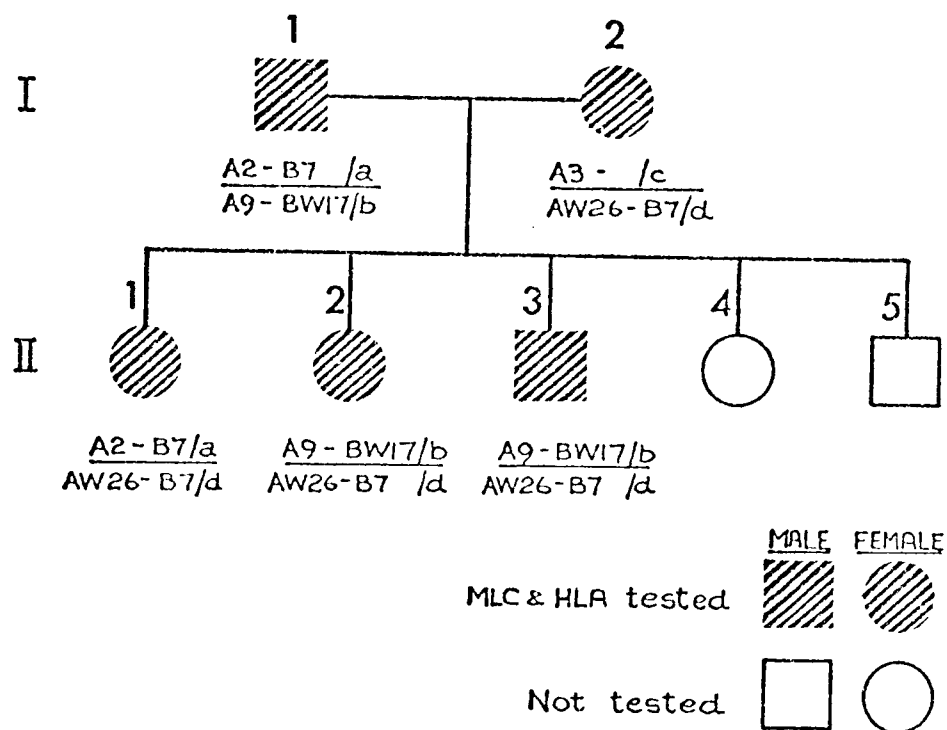


Figure 8.4. Pedigree of San family IV with HLA-A and B genotypes and haplotypes. The paternal HLA haplotypes are designated ab, and the maternal cd.

TABLE VIII.11

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY IV

(See Pedigree Fig. 8.4)

HLA Genotype	Haplotype	Subject
A2-B7	a	I-1 Father
A9-BW17	b	
A3-	c	I-2 Mother
AW26-B7	d	
A2-B7/AW26-B7	a/d	II-1 Sib
A9-BW17/AW26-B7	b/d	II-2 Sib
A9-BW17/AW26-B7	b/d	II-3 Sib

TABLE VIII.12

GENETIC DATA ON SAN FAMILY IV

(See Pedigree Fig. 8.4)

Subject	ABO	Rhesus probable genotype	MNSs	He	Kell		P ₁	Duffy Fy(a)	Serum Proteins		Red Cell Enzymes										
					K	k			Hp	Tf	Peptidases				ADA	G6PD	6PGD	PGM ₁	PGM ₂	AK	AP
											A	B	C	D							
F I-1	O	R ₀ /R ₀	MS/NS	+	-	+	+	-	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	RB
M I-2	O	R ₀ /R ₀	M ^s /M ^s	-	-	+	+	+	2-1	CD	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	nd	AB
Sib II-1	O	R ₀ /R ₀	M ^s /N ^s	-	-	+	+	+	<u>2-2</u>	<u>D</u>	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	B
Sib II-2	A _{bantu}	R ₀ /R ₀	M ^s /N ^s	-	-	+	+	+	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	<u>2-2</u>	1-1	2-1	RB
Sib II-3	O	R ₀ /R ₀	M ^s /N ^s	-	-	+	+	+	2-1	C	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	RA
Sib II-4	Not tested																				
Sib II-5	Not tested																				

nd = Not determined.
The exclusions are underlined.

TABLE VIII.13

BILATERAL MLC TESTS ON SAN FAMILY IV

(expressed in cpm $\pm$ se)

Subject		I-1	I-2	II-1	II-2	II-3	X	Y
	Haplo- type	ab	cd	ad	bd	bd	nd	nd
F I-1	ab	329 ± 193						
M I-2	cd	5,077 ± 28	1,387 ± 487					
Sib II-1	ad	1,187 ± 46	6,049 $\pm 1,398$	4,130 ± 877				
Sib II-2	bd	3,701 ± 117	7,078 ± 243	4,489 $\pm 1,817$	961 ± 558			
Sib II-3	bd	2,602 $\pm 1,858$	4,566 $\pm 1,144$	4,892 ± 221	1,220 ± 203	124 ± 31		
X	nd	709 ± 49	6,001 $\pm 1,194$	8,660 ± 505	7,022 ± 54	5,271 $\pm 1,747$	2,441 ± 114	
Y	nd	1,933 $\pm 1,287$	3,110 $\pm 1,782$	7,329 $\pm 1,604$	3,381 $\pm 1,381$	7,835 ± 102	2,115 ± 171	1,253 $\pm 1,008$
PHA		2,733 $\pm 1,968$	4,006 $\pm 1,642$	3,645 $\pm 2,212$	6,062 $\pm 1,387$	6,201 ± 831	7,264 ± 130	9,255 ± 449

X = Two healthy unrelated individuals

PHA = Phytohaemagglutinin

--- = Stippled line through negative controls nd = Not determined

The unusual MLC reaction is enclosed in a smaller rectangle within the table.

5. San family V

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 8.5, and presented in tabular form in Table VIII.14. Table VIII.15 shows the erythrocyte genetic markers, and Table VIII.16 gives the results of the MLC tests. Only three well-established HLA antigens were identified in the father I-1 and four in the mother I-2. The parents shared one HLA antigen, i.e. HLA-A2. The three siblings were HLA-A, B identical as all three inherited HLA haplotypes *a* and *d* from their parents.

Blood from only the father and one sibling II-2 was available for erythrocyte genotyping, and therefore no conclusions could be drawn about parentage from the red cell genotyping.

The MLC tests were performed on the cells from the two parents and their three HLA-A and B identical siblings in all possible combinations. Both from the thymidine incorporation data and the morphology, sibs II-1 versus II-2 and II-2 versus II-3 appear to be negative. One would therefore expect sibs II-1 versus II-3 also to be negative, which seems dubious both for the thymidine incorporation and the morphology. The morphology seems to indicate negative or weak reactions of the mother I-2 with sibs II-1, II-2 and II-3 with good controls with X and Y. From the thymidine incorporation data, however, the control with Y is negative and weak with X, so without further tests between the mother and the three sibs, little can be said, although these counts do not exclude negative or weak reactivity.

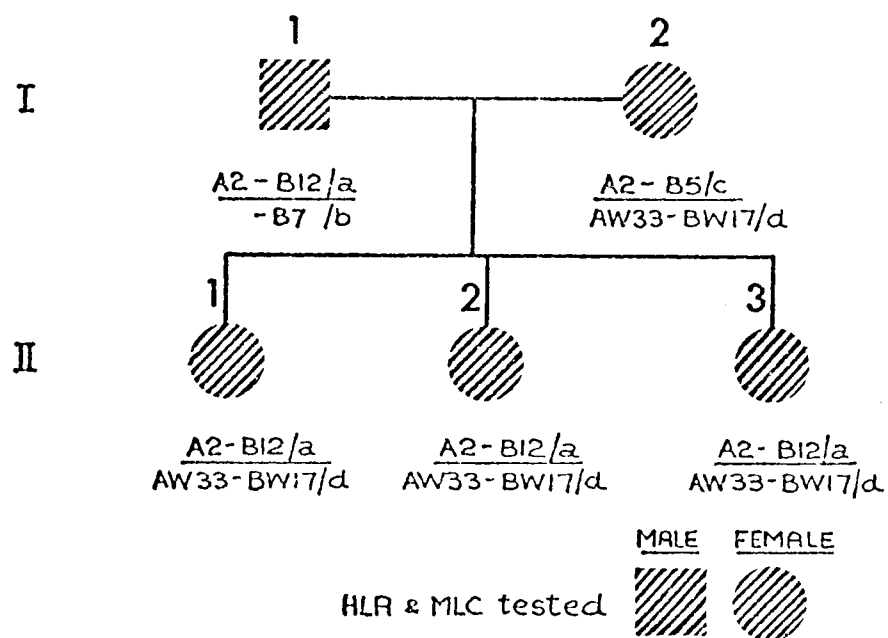


Figure 8.5. Pedigree San family V with HLA-A and B genotypes and haplotypes. The paternal HLA haplotypes are designated ab and the maternal cd.

TABLE VIII.14

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY V
(See Pedigree Fig. 8.5)

HLA Genotype	Haplotype	Subject
A2-B12 -B7	a b	I-1 Father
A2-B5 AW33-BW17	c d	I-2 Mother
A2-B12/AW33-BW17	a/d	II-1 Sib
A2-B12/AW33-BW17	a/d	II-2 Sib
A2-B12/AW33-BW17	a/d	II-3 Sib

TABLE VIII.15

ERYTHROCYTE GENETIC MARKERS SAN FAMILY V
(See Pedigree Fig. 8.5)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u> K $\bar{k}$	P_1	<u>Duffy</u> Fy(a)
F I-1	O	R_0/R_0	$M\bar{s}/M\bar{s}$	- +	+	-
M I-2	not tested					
Sib II-1	not tested					
Sib II-2	O	R_0/R_0	$M\bar{s}/N\bar{s}$	- +	+	-
Sib II-3	not tested					

TABLE VIII.16

BILATERAL MLC TESTS ON SAN FAMILY V

(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		I-1	I-2	II-1	II-2	II-3	X	Y	PHA
	HLA haplotype	A2-B12/a -B7/b	A2-B5/c AW33-BW17/d	A2-B12/a AW33-BW17/d	A2-B12/a AW33-BW17/d	A2-B12/a AW33-BW17/d	nd	nd	
F I-1	A2-B12/a -B7/b	124 ± 5	++	++	++	++	++	++	++
M I-2	A2-B5/c AW33-BW17/d	282 ± 146	9 ± 5	$\pm$	$\pm$	$\pm$	++	+	++
Sib II-1	A2-B12/a AW33-BW17/d	360 ± 81	59 ± 21	37 ± 12	-	(+)	++	(+)	+++
Sib II-2	A2-B12/a AW33-BW17/d	366 ± 50	168 ± 138	48 ± 61	50 ± 14	-	++	++	+++
Sib II-3	A2-B12/a AW33-BW17/d	218 ± 40	57 ± 38	230 ± 188	58 ± 18	60 ± 53	++	(+)	++
X	nd	649 ± 38	151 ± 28	498 ± 60	580 ± 19	837 ± 178	61 ± 32	++	++
Y	nd	377 ± 59	22 ± 45	256 ± 62	348 ± 10	374 ± 47	911 ± 237	50 ± 48	++
PHA		2,644 ± 231	768 ± 240	1,062 ± 271	2,149 ± 653	4,368 ± 412	3,114 ± 100	4,040 ± 333	

X & Y = Two healthy unrelated individuals.
 PHA = Phytohaemagglutinin.
 --- = Stippled line through negative controls.
 nd = Not determined.

The unusual MLC reactions are outlined within the Table,
 solid lines for the negative reactions and interrupted
 lines for the doubtful reactions.

6. San family VI

The pedigree only is given in Fig. 8.6. The HLA serology could not be done on this family as the cells were all dead on arrival in the laboratory. Table VIII.17 shows the erythrocyte and serum protein genetic markers, and Table VIII.18 gives the results of the MLC tests.

The red cell genotypes, serum proteins and red cell enzyme results have been fully tested in this family in an effort to elucidate the genetic relatedness of this family. This was of particular importance because HLA typing results were not available.

The ABO grouping on the father, I-1, presented some difficulties. In the laboratory he was classified as either an A_{intermediate} or A₁. The classification as A_{intermediate} depends on the grade of agglutination by *Dolichos biflorus* extract; father, I-1 gave a ++ reaction, where with an A₁ individual one would expect a ++++ reaction. The father's reaction to *Ulex europeus* extract was also weak but present. The father is thus probably A_{intermediate} and though this would not be enough for a court of law it ought to incline more to paternal exclusion than inclusion. Furthermore, II-1, II-2 and II-4 would appear to be excluded on P₁ but there were difficulties with the anti-P₁ in use at that time, and the reactions of II-1 and II-2 were not strong. On the other hand the Rhesus and AK results are against it, especially as the R₁ allele is not common in the San but, if the community is inbred, even this evidence is less than convincing.

The cells from the whole family were cultured in all possible combinations except for father I-1 and mother I-2

versus sib II-2 due to insufficient lymphocytes from II-2. The morphological MLC result indicates a negative or weakly positive reaction between sibs II-2 and II-3 with good controls. The thymidine incorporation data are not contradictory but the controls are bad, thus allowing no definite conclusion. The weak but probably still positive reaction in the thymidine incorporation result of father I-1 versus sib II-3 cannot be confirmed in the morphology because this preparation could not be interpreted. Further information could not be obtained from I-1 versus II-2 because this combination was not tested. The thymidine incorporation data of II-2 versus II-4 and II-3 versus II-4 were both negative, but the control with X was also negative and the morphology positive. Therefore, in the absence of further tests it cannot be concluded whether sibs II-2, II-3 and II-4 had the same MLC determinants.

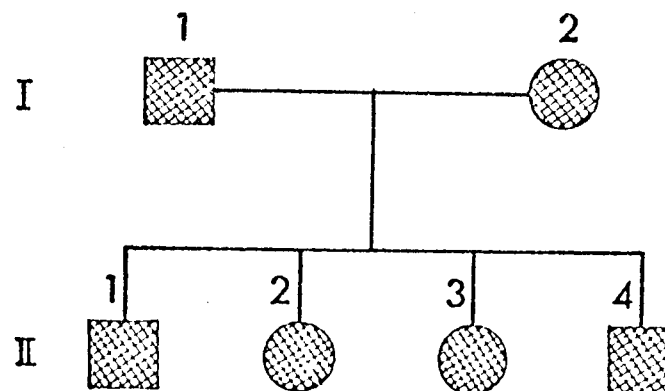


Figure 8.6. Pedigree San family VI
(MLC tested only).

TABLE VIII.17

GENETIC DATA ON SAN FAMILY VI
(See Pedigree Fig. 8.6)

Subject	ABO	Rhesus probable genotype	MNSs	He	Kell		P ₁	Duffy	Serum Proteins		Red Cell Enzymes											
					K	k			Fy(a)	Hp	Tf	Peptidases				ADA	G6PD	6PGD	PGM ₁	PGM ₂	AK	AP
												A	B	C	D							
F I-1	A _{Int}	R ₁ /R ₀	N ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	-	+	2-1	C	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	2-1	B	
M I-2	A ₂	R ₀ /R ₀	M ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	-	+	0-0	CD	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	RB	
Sib II-1	A ₁	R ₁ /R ₀	M ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	±	+	2-1	CD	1-1	1-1	1-1	1-1	1-1	B	A	nd	nd	2-1	RB	
Sib II-2	A _{bantu}	R ₀ /R ₀	M ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	±	+	2-1	CD	1-1	1-1	1-1	1-1	1-1	B	A	nd	nd	1-1	B	
Sib II-3	A ₁	R ₁ /R ₀	N ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	-	+	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	nd	nd	2-1	B	
Sib II-4	A ₁	R ₀ /R ₀	N ⁻ S ⁻ /N ⁻ S ⁻	-	-	+	+	+	0-0	C	1-1	1-1	1-1	1-1	1-1	B	A	nd	nd	nd	RB	

nd = Not determined.

TABLE VIII.18

BILATERAL MLC TESTS ON SAN FAMILY VI

(expressed in cpm $\pm$ se with morphology results in the upper right triangle)

Subject		I-1	I-2	II-1	II-2	II-3	II-4	X	PHA
	HLA typing not done	nd	nd	nd	nd	nd	nd	nd	
F I-1	nd	5 ± 29	++	++	nd	?	++	++	++
M I-2	nd	890 ± 542	58 ± 24	++	nd	+	++	++	+++
Sib II-1	nd	1,388 ± 727	2,191 $\pm 1,373$	6 ± 0	++	++	++	++	++
Sib II-2	nd	nd	nd	569 ± 98	78 ± 0	±	++	++	++
Sib II-3	nd	173 ± 57	306 ± 17	259 ± 69	65 ± 70	2 ± 24	++	(+)	++
Sib II-4	nd	852 ± 234	578 ± 67	608 ± 90	5 ± 13	-43 ± 29	58 ± 26	+	++
X	nd	535 ± 33	794 ± 104	1,077 ± 174	-82 ± 13	-13 ± 29	-10 ± 5	13 ± 8	++
PHA		213 ± 41	1,518 ± 506	702 ± 49	1,313 ± 333	967 ± 252	707 ± 30	797 ± 62	

nd = Not determined.

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin

The unusual MLC reactions are outlined within the Table, solid lines for possible negative reactions and interrupted lines for doubtful reactions.

--- = Stippled line through negative controls

- = A negative sign in front of the cpm value denotes a count less than the background.

7. San family VII

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 8.7, and presented in tabular form in Table VIII.19. Table VIII.20 shows the erythrocyte genetic markers, and Table VIII.21 gives the results of the MLC tests.

The HLA serology of the parents and their four children was done and four antigens were identified in each individual. The parental haplotypes segregated normally in the children. Siblings II-1 and II-2 were HLA-A and B identical and the other two siblings were non-identical.

Unfortunately no blood was available from the parents for red cell genotyping, but from the erythrocyte genetic marker tests, the four children appeared to be true siblings.

Mixed lymphocyte culture tests were performed on the mother and the four children with the control X. The father was not available for this test. It was apparent both from the morphology and thymidine incorporation data that the HLA-A and B identical sibs II-1 versus II-2 were non-stimulatory with good controls. It is noteworthy that tests of the mother I-2 versus II-2 and II-3 were morphologically weakly positive, and the thymidine incorporation results were relatively low but definitely positive. This seems to indicate a good correlation between these two parameters.

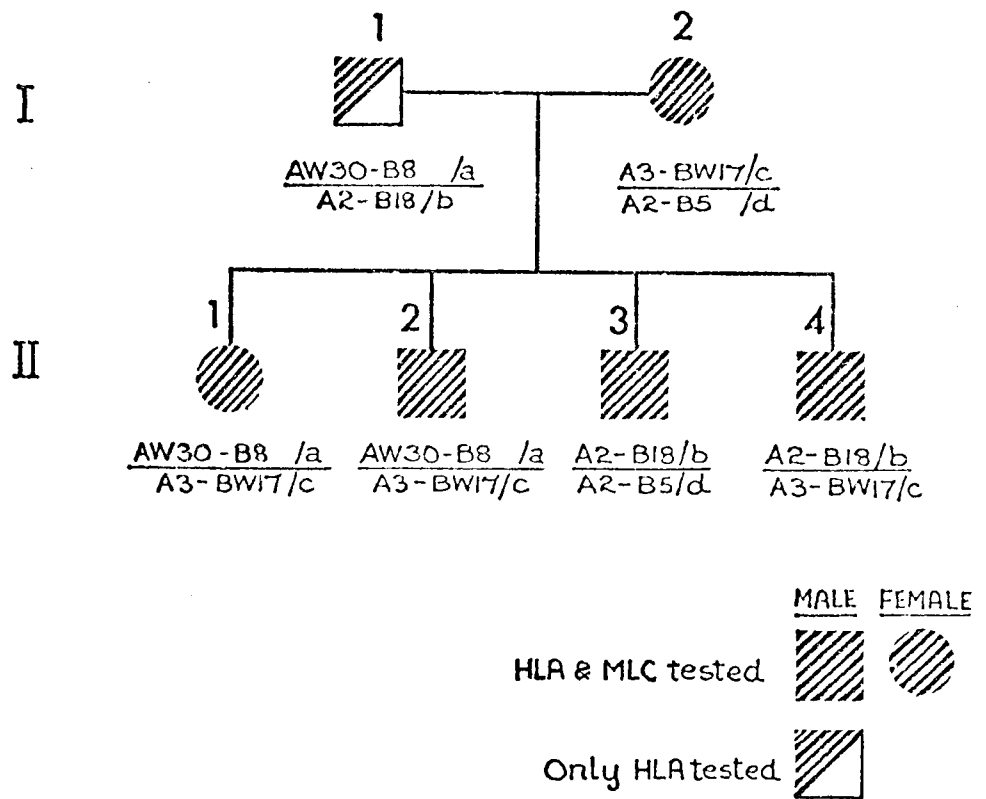


Figure 8.7. Pedigree of San family VII showing HLA-A and B genotypes and haplotypes. The paternal HLA haplotypes are designated ab and the maternal cd.

TABLE VIII.19

HLA-A AND B GENOTYPES AND HAPLOTYPES OF SAN FAMILY VII

(See Pedigree Fig. 8.7)

HLA Genotype	Haplotype	Subject
AW30-B8 A2-B18	a b	I-1 Father
A3-BW17 A2-B5	c d	I-2 Mother
AW30-B8/A3-BW17	a/c	II-1 Sib
AW30-B8/A3-BW17	a/c	II-2 Sib
A2-B18/A2-B5	b/d	II-3 Sib
A2-B18/A3-BW17	b/c	II-4 Sib

TABLE VIII.20

ERYTHROCYTE GENETIC MARKERS SAN FAMILY VII

(See Pedigree Fig. 8.7)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u> K $\bar{k}$	P_1	<u>Duffy</u> Fy(a)
Parents not tested						
Sib II-1	O	R_0/R_0	$M\bar{S}/M\bar{S}$	- +	+	-
Sib II-2	O	R_0/R_0	$M\bar{S}/N\bar{S}$	- +	+	-
Sib II-3	O	R_0/R_0	$M\bar{S}/N\bar{S}$	- +	+	-
Sib II-4	O	R_0/R_0	$M\bar{S}/N\bar{S}$	- +	+	-

TABLE VIII.21

BILATERAL MLC TESTS ON SAN FAMILY VII
(expressed in cpm $\pm$ se with morphology results)

Subject		I-2	II-1	II-2	II-3	II-4	X	PHA
	HLA haplo-type	$\frac{A3-BW17/c}{A2-B5/d}$	$\frac{AW30-B8/a}{A3-BW17/c}$	$\frac{AW30-B8/a}{A3-BW17/c}$	$\frac{A2-B18/b}{A2-B5/d}$	$\frac{A2-B18/b}{A3-BW17/c}$	nd	
M I-2	$\frac{A3-BW17/c}{A2-B5/d}$	91 ± 98	++	$\boxed{\pm}$	$\boxed{\pm}$	++	++	+
Sib II-1	$\frac{AW30-B8/a}{A3-BW17/c}$	1,063 ± 366	9 ± 65	$\boxed{-}$	++	++	++	++
Sib II-2	$\frac{AW30-B8/a}{A3-BW17/c}$	$\boxed{380}$ ± 57	$\boxed{-56}$ ± 24	62 ± 24	++	++	++	++
Sib II-3	$\frac{A2-B18/b}{A2-B5/d}$	691 ± 464	1,253 ± 109	4,855 ± 623	22 ± 64	++	++	++
Sib II-4	$\frac{A2-B18/b}{A3-BW17/c}$	2,007 ± 409	1,265 ± 431	2,507 $\pm 1,482$	3,097 ± 578	6 ± 23	++	++
X	nd	2,157 ± 681	4,169 $\pm 1,590$	4,055 $\pm 1,764$	1,714 ± 610	2,195 $\pm 1,115$	181 ± 29	++
PHA		72 ± 29	848 ± 76	1,030 ± 276	602 ± 147	1,401 ± 276	934 ± 141	

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

- = A negative sign before the cpm value denotes counts less than background.

nd = Not determined.

The negative MLC reactions are outlined within the Table with solid lines and the doubtful reactions with interrupted lines.

Interpretation of Results

The gene frequencies for some of the HLA-A and B antigens in the San varied considerably compared with other Southern African populations, e.g. Cape Coloureds and Xhosas. We have reported these findings in detail (Botha et al., 1973, 1975a and b; Nurse et al., 1975).

In *San family I* the father is homozygous for the HLA antigen BW17, and possibly for the D locus determinants, while the mother's D locus determinant accompanying BW17 is different from the father's D locus determinant(s) accompanying the antigen BW17 present on both his MHC chromosomes. There is a distinct possibility of recombination of either the paternal or maternal chromosomes between HLA-B and D in two of the offspring in this family. The existence of a 'hot spot' giving rise to recurrent recombinations has been suggested by Dupont et al. (1974a). They reported on the first family in which recombination on approximately the same location of the chromosome has been observed, resulting in two siblings inheriting the two possible recombinant chromosomes.

San family II has three HLA-A and B identical siblings who appear to be MLC negative. There is a possibility of a recombination of the B and D loci in sib II-1, but the results are inconclusive.

In *San family III* the father is homozygous for HLA-B, but heterozygous for HLA-D. In spite of being HLA serologically non-identical, the paternal *b* and the maternal *c* chromosomes appeared to have identical D locus determinants. This resulted in sib II-2 and the father I-1 being MLC negative, although they were only HLA-A, B haplo-identical. If the

paternal *b* and maternal *c* chromosomes carried the same D locus determinants, sib II-1 with the HLA haplotype *bc* could be homozygous for the D locus although heterozygous for HLA-A and B. Unfortunately unilateral MLC tests were not done in this family and therefore homozygosity of sib II-1 for the D locus determinant could not be proved. The negative MLC reactions in the presence of B locus differences suggest that the D locus determinants are not always associated with the same B locus antigens or with the same HLA haplotype. This also supports the view that MLC activation is brought about by D rather than HLA-A and B differences.

San family IV is not a true biological family, since all three siblings are excluded on the results of the erythrocyte genetic markers, serum protein and red cell enzymes. The HLA serology was poor, and the MLC data confirm the two apparently HLA-A and B identical siblings as MLC non-identical. This family illustrates the problems encountered in establishing true family relationships.

In *San family V* two of the three HLA-A and B identical siblings were also MLC negative. The rest of the MLC data were, however, inconclusive. The HLA serological typing could not be done in *San family VI*. The erythrocyte genetic data show no definite exclusions, while the MLC data were inconclusive and of little value in view of the absence of HLA serological results. The two HLA-A and B identical siblings of *San family VII* were MLC negative.

CHAPTER NINE - CONSANGUINEOUS FAMILIES

First-degree Consanguineous Families

1. Family C00
2. Family MEI

Fourth-degree Consanguineous Indian Families

1. Family BEG
2. Family H00
3. Family CAD

Interpretation of Results

First-degree Consanguineous Families

In our search for first-degree consanguineous offspring we found seven cases with a total of nine offspring in Cape Town. In four of these families the offspring were infants and therefore could not be studied. The remaining three families consisted of: one family with two HLA heterozygous idiot children (having IQs < 20) (data not presented); family C00 (see pedigree, Fig. 9.1) with one HLA heterozygote (III-2) who was feeble-minded (IQ 60) and the HLA homozygote (III-1) with an IQ of 80; and the third family MEI (see pedigree, Fig. 9.2) with one HLA heterozygous offspring aged two years who was mentally retarded.

During our search for offspring of incestuous matings we gained the impression that the incidence was considerably higher than expected among the lower socio-economic groups in Cape Town, but difficult to trace because of the possible legal complications for the families.

1. Family C00

The pedigree of this family, together with HLA genotypes and haplotypes, is shown in Fig. 9.1, and presented in tabular form in Table IX.1. Table IX.2 shows the erythrocyte genetic markers, and Tables IX.3, IX.4 and IX.5 give the results of the MLC tests.

Four well-established HLA-A and B antigens could be identified in each individual tested in generations I and II. The four different HLA haplotypes *ab* and *cd* of the parents I-1 and I-2 segregate normally in their children. The haplotypes of the dizygotic twins II-14 and II-15 are *ac* and *bc*,

respectively. The first-degree consanguineous union between I-1 and his daughter II-1 produced two offspring; one homozygous for the HLA haplotype A1-B8, presumably originating from the father I-1, and a second child with the haplotypes HLA-A1-B8/AW26-B18, both of which again originated from I-1.

The erythrocyte genetic markers give no evidence of mixed parentage among siblings in generation II and do not exclude parentage of I-1 and II-1 for sibs III-1 and III-2.

MLC tests were performed in all possible combinations on three occasions on the members of this family who were willing to donate blood for this purpose. The conclusions that could be reached from the results of the three experiments were the same; therefore data are presented on only one of these experiments.

In the *bilateral* MLC test the combination I-1 versus II-16 was omitted because the tube with blood from II-16 broke during centrifugation, resulting in a shortage of lymphocytes from this individual. The father I-1 and his first-degree consanguineous child III-2 who were HLA-A and B identical were also MLC negative, as assessed by both thymidine incorporation and morphological assessment. It should also be noted that the two HLA-A and B identical sibs II-1 and II-13 with the haplotypes *ae* were mutually non-stimulatory according to the results of thymidine incorporation and morphology. The negative and PHA controls in these cultures were good.

The *unilateral* MLC tests again showed the HLA-A and B identical combinations II-1 versus II-13 and I-1 versus III-2 to be non-stimulatory in both directions.

Surprisingly however, the homozygous cells of III-1 (*aa*) stimulated the heterozygous *ab* cells of I-1, III-2 and *ac* cells of II-1 and II-13, although in some instances more weakly than in the opposite direction. The possible explanations of this phenomenon will be discussed later. The results of thymidine incorporation were again in agreement with the morphological assessment. The controls in this experiment were good.

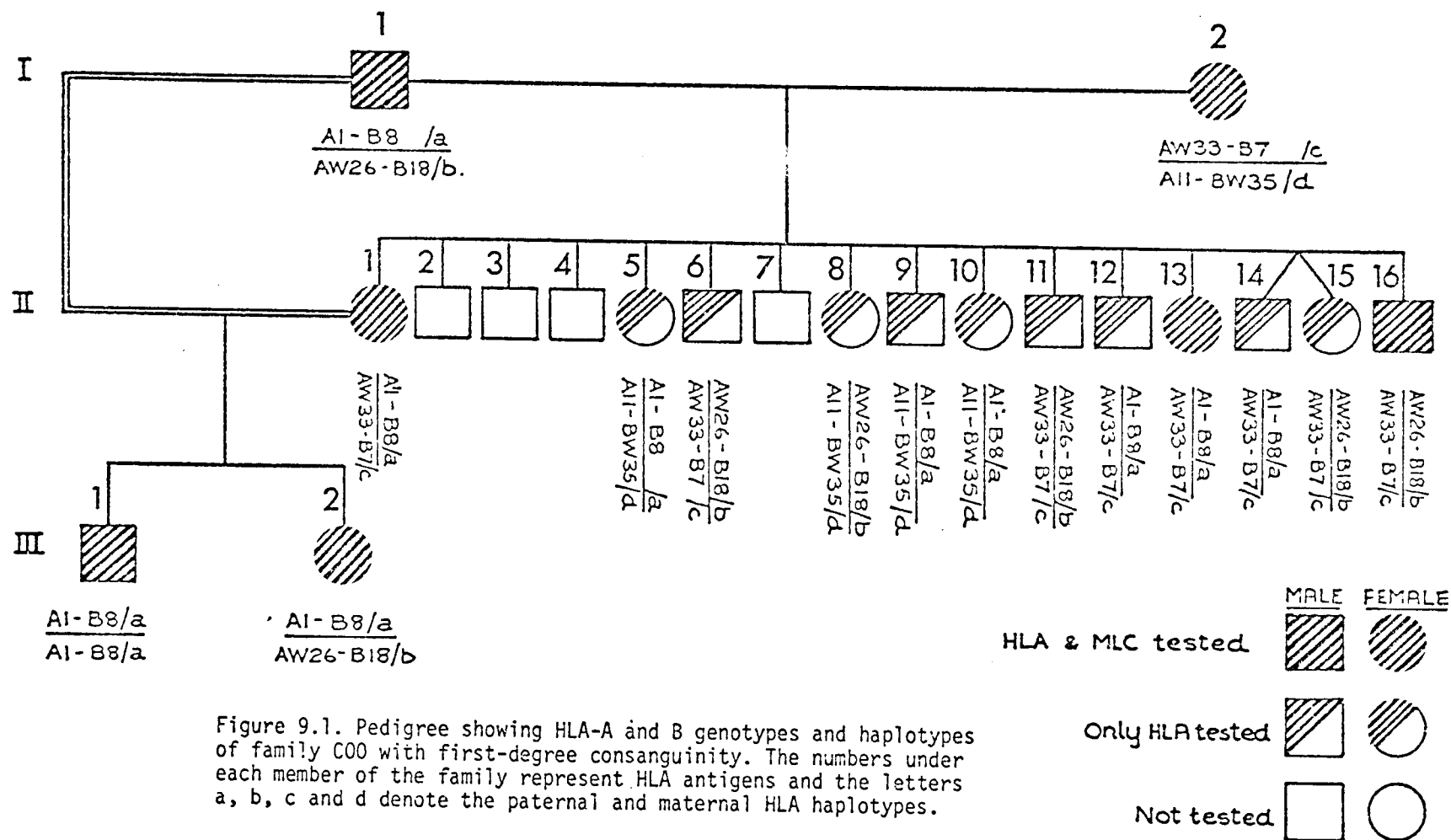


Figure 9.1. Pedigree showing HLA-A and B genotypes and haplotypes of family C00 with first-degree consanguinity. The numbers under each member of the family represent HLA antigens and the letters a, b, c and d denote the paternal and maternal HLA haplotypes.

TABLE IX.1

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FIRST-DEGREE
CONSANGUINEOUS FAMILY COO

(See Pedigree Fig. 9.1)

HLA Genotype	Haplotype	Subject
A1-B8 AW26-B18	a b	I-1 Father
AW33-B7 A11-BW35	c d	I-2 Mother
A1-B8/AW33-B7	a/c	II-1 Sib (mother)
A1-B8/AW33-B7	a/c	II-12 Sib
A1-B8/AW33-B7	a/c	II-13 Sib
A1-B8/AW33-B7	a/c	II-14 Sib *
A1-B8/A11-BW35	a/d	II-5 Sib
A1-B8/A11-BW35	a/d	II-9 Sib
A1-B8/A11-BW35	a/d	II-10 Sib
AW26-B18/AW33-B7	b/c	II-6 Sib
AW26-B18/AW33-B7	b/c	II-11 Sib
AW26-B18/AW33-B7	b/c	II-15 Sib *
AW26-B18/AW33-B7	b/c	II-16 Sib
AW26-B18/A11-BW35	b/d	II-8 Sib
		<u>Children First-Degree Consanguinity</u>
A1-B8/A1-B8	a/a	III-1 Sib
A1-B8/AW26-B18	a/b	III-2 Sib

* dizygotic twins.

TABLE IX.2

ERYTHROCYTE GENETIC MARKERS FIRST-DEGREE CONSANGUINEOUS FAMILY C00

(See Pedigree Fig. 9.1)

Subject	ABO	Rhesus probable genotype	MNSs	Kell		P ₁	Duffy Fy(a)
				K	k		
F I-1	A ₁	R ₁ /r	N $\bar{s}$ /N $\bar{s}$	-	+	+	-
M I-2	B	R ₁ /R ₁	MS/N $\bar{s}$	-	+	-	-
Sib II-1 (mother)	A ₁ B	R ₁ /r	MS/N $\bar{s}$	-	+	-	-
Sib II-6	A ₁ B	R ₁ /r	MS/N $\bar{s}$	-	+	+	-
Sib II-8	A ₁ B	R ₁ /r	MS/N $\bar{s}$	-	+	-	-
Sib II-9	A ₁ B	R ₁ /r	MS/N $\bar{s}$	-	+	-	-
Sib II-12	A ₁ B	R ₁ /r	N $\bar{s}$ /N $\bar{s}$	-	+	-	-
Sib II-13	B	R ₁ /r	N $\bar{s}$ /N $\bar{s}$	-	+	-	-
*Sib II-14	B	R ₁ /r	N $\bar{s}$ /N $\bar{s}$	-	+	+	-
*Sib II-15	B	R ₁ /r	N $\bar{s}$ /N $\bar{s}$	-	+	-	-
Sib II-16	A ₁ B	R ₁ /r	MS/N $\bar{s}$	-	+	-	-
Children First-Degree Consanguinity							
Sib III-1	A ₁	R ₁ /r	MS/N $\bar{s}$	-	+	-	-
Sib III-2	A ₁	R ₁ /r	MS/N $\bar{s}$	-	+	-	-

* dizygotic twins.

TABLE IX.3

BILATERAL MLC TESTS ON FIRST-DEGREE CONSANGUINEOUS FAMILY COO

(expressed in cpm $\pm$ se and with morphology results in the right upper triangle)

Subject		I-1	I-2	II-1	II-13	II-16	III-1	III-2	X	PHA
	HLA haplo-type	ab	cd	ac	ac	bc	aa	ab	HLA phenotype A2,3;B5,14	
F I-1	ab	89 ± 12	+++	++	++	nd	++	-	++	++
M I-2	cd	9,245 ± 513	87 ± 12	+	++	++	+++	++	++	+++
Sib II-1 (mother)	ac	2,478 ± 794	1,722 ± 281	118 ± 38	-	++	+	+	++	+++
Sib II-13	ac	7,313 ± 188	5,648 ± 29	201 ± 121	46 ± 2	+++	++	++	++	++
Sib II-16	bc	nd	4,706 ± 430	4,699 ± 545	5,042 ± 127	58 ± 7	++	++	++	+++
Sib III-1	aa	2,924 ± 684	4,694 ± 408	2,610 ± 279	2,776 ± 462	6,921 ± 521	139 ± 35	++	+++	+++
Sib III-2	ab	188 ± 11	9,339 ± 869	4,400 ± 345	7,643 ± 460	4,260 ± 528	5,113 ± 130	136 ± 19	++	++
X	HLA phenotype A2,3;B5,14	7,791 ± 698	9,096 ± 817	2,666 ± 570	9,728 ± 557	7,945 ± 395	6,685 ± 464	9,386 ± 573	110 ± 12	++
PHA		4,759 ± 447	17,030 ± 562	15,982 ± 854	19,384 ± 709	21,413 $\pm 1,626$	18,108 ± 220	17,489 ± 718		

nd = Not determined.

PHA = Phytohaemagglutinin.

X = Healthy unrelated individual.

--- = Stippled line through negative controls.

The negative reactions are outlined within the Table.

TABLE IX.4

UNILATERAL MLC TESTS ON FAMILY C00
(expressed in cpm $\pm$ se)

Subject		I-1	I-2	II-1	II-13	II-16	III-1	III-2	X
	HLA haplo-type	ab	cd	ac	ac	bc	aa	ab	HLA phenotype A2,3;B5,14
F I-1 _m	ab	62 ± 16	4,165 ± 733	1,554 ± 160	3,161 ± 165	1,576 ± 215	1,758 ± 238	94 ± 12	3,070 ± 247
M I-2 _m	cd	1,674 ± 223	36 ± 2	421 ± 83	1,380 ± 378	1,486 ± 82	1,264 ± 232	1,885 ± 304	2,614 ± 173
Sib II-1 _m	ac	809 ± 130	867 ± 164	45 ± 9	37 ± 3	438 ± 81	426 ± 81	1,040 ± 128	2,420 ± 21
Sib II-13 _m	ac	213 ± 60	726 ± 100	30 ± 5	30 ± 3	329 ± 24	198 ± 26	498 ± 79	2,460 ± 129
Sib II-16 _m	bc	312 ± 34	518 ± 124	326 ± 98	553 ± 93	34 ± 2	478 ± 39	261 ± 14	1,353 ± 58
Sib III-1 _m	aa	627 ± 47	2,795 ± 228	487 ± 37	243 ± 148	1,515 ± 37	39 ± 3	557 ± 104	1,294 ± 98
Sib III-2 _m	ab	66 ± 20	2,731 ± 36	794 ± 73	1,604 ± 97	668 ± 17	852 ± 160	90 ± 30	1,526 ± 228
X _m	HLA phenotype A2,3;B5,14	1,627 ± 213	2,223 ± 335	2,296 ± 131	2,997 ± 112	1,271 ± 423	1,088 ± 116	1,553 ± 211	27 ± 1

X = Healthy unrelated individual.

m = Mitomycin-C treated.

--- = Stippled line through negative controls

The unusual reactions are outlined within the Table.

TABLE IX.5
RESULTS ON MORPHOLOGY OF UNILATERAL MLC ON FAMILY C00
(Table IX.4)

Subject		I-1	I-2	II-1	II-13	II-16	III-1	III-2	X
	HLA haplo-type	ab	cd	ac	ac	bc	aa	ab	HLA phenotype A2,3;B5,14
F I-1 _m	ab	-	++	+	++	+	+	-	+
M I-2 _m	cd	+	-	(+)	+	+	+	+	+
Sib II-1 _m	ac	+	+	-	-	+	+	+	+
Sib II-13 _m	ac	+	+	-	-	+	+	+	+
Sib II-16 _m	bc	+	+	+	+	-	+	+	+
Sib III-1 _m	aa	(+)	++	(+)	(+)	+	-	(+)	++
Sib III-2 _m	ab	-	++	+	+	+	+	-	+
X _m	HLA pheno-type A2,3;B5,14	+	++	+	++	+	++	+	-

X = Healthy unrelated individual

m = Mitomycin-C treated

--- = Stippled line through negative controls

The unusual reactions are outlined within the Table.

2. Family MEI

The pedigree of this family, together with HLA-A and B genotypes and haplotypes, is shown in Fig. 9.2, and presented in tabular form in Table IX.6. Table IX.7 shows the erythrocyte genetic markers, and Tables IX.8, IX.9 and IX.10 give the results of the MLC tests.

The inheritance of the HLA antigens did not show any deviation from normal. The HLA-A and B genotype of the offspring III-1, of the alleged consanguineous union between I-1 and II-1, did not help to prove or disprove this, but it seems very likely that they could be the parents particularly as maternity of I-2 for III-1 could be excluded from the results in the Rhesus system (Table IX.7). In addition, parentage of I-1 and II-1 for III-1 seems highly likely from the probable Rhesus genotype results.

The mixed lymphocyte culture reactions reported here were unfortunately only performed on one occasion because of lack of further co-operation by the family involved.

The *bidirectional* stimulation between I-2 and II-1 was somewhat greater than one would expect between mother and daughter who were identical for one HLA haplotype. This may be because of the fact that the negative control for the mother I-2 was also higher than usual.

In the *unidirectional* MLC test, cells from III-1 responded weakly to the cells from I-2 and II-1, but adequately to I-1 and X. It should be noted that II-1 and III-1 were identical for their B locus antigens and may therefore also be identical for the D locus; the same was

not, however, true for III-1 and I-2. In the reverse situation the response of I-2 and II-1 to III-1 was as one would expect in the one-haplotype-identical situation. The response of the father I-1 to II-1 and III-1 who were haplo-identical with him was rather lower than expected; it is, however, noteworthy that his response to the two unrelated HLA-A and B non-identical individuals I-2 and X and to PHA was also weak.

The SR and RR were calculated in this culture in an effort to find whether there were meaningful differences between the various reactions. There was a fair correlation between the results of the mean cpm and RR. The two reactions which appeared to be negative from the cpm results, i.e. the response of III-1 to I-2 and II-1, gave low RR values, and the weakly positive responses of I-1 to II-1 and III-1, as well as those of I-2 and II-1 to III-1, gave intermediate RR values. The SR values were less reliable, e.g. the SRs of 2.3 and 3.4 obtained with I-1 responding to II-1 and III-1 would indicate a negative MLC response which is doubtful from the cpm and RR values.

The results of the morphological assessment are in agreement with the results of the thymidine incorporation in both the bilateral and unilateral MLC tests. Without further testing no conclusions about the possible sharing of D locus determinants could be made in this family.

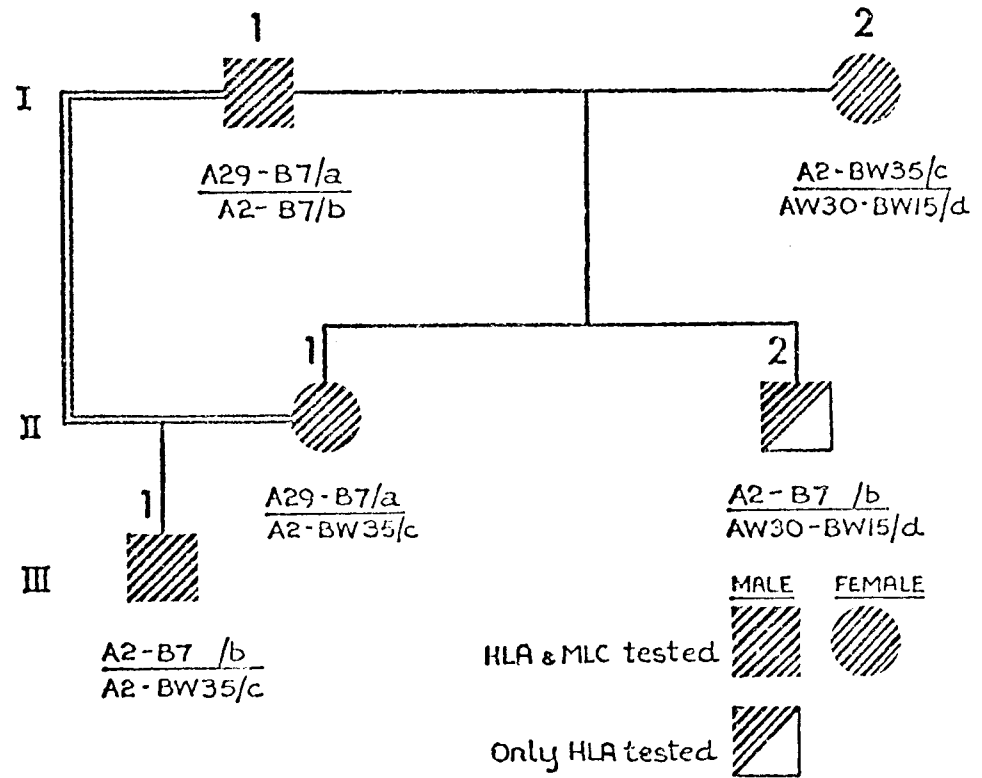


Figure 9.2. Pedigree of the family MEI with first-degree consanguinity showing the HLA-A and B genotypes and haplotypes. The letters ab denote the paternal and cd the maternal haplotypes.

TABLE IX.6

HLA-A AND B GENOTYPES AND HAPLOTYPES OF FIRST-DEGREE
CONSANGUINEOUS FAMILY MEI

(See Pedigree Fig. 9.2)

HLA Genotype	Haplotype	Subject
A29-B7	a	I-1 Father
A2-B7	b	
A2-BW35	c	I-2 Mother
AW30-BW15	d	
A29-B7/A2-BW35	a/c	II-1 Sib
A2-B7/AW30-BW15	b/d	II-2 Sib
		<u>Child First-Degree</u> <u>Consanguinity</u>
A2-B7/A2-BW35	b/c	III-1 Sib

TABLE IX.7

ERYTHROCYTE GENETIC MARKERS FIRST-DEGREE CONSANGUINEOUS FAMILY MEI

(See Pedigree Fig. 9.2)

Subject	<u>ABO</u>	<u>Rhesus</u> probable genotype	<u>MNSs</u>	<u>Kell</u>		<u>P_I</u>	<u>Duffy</u> Fy(a)
				K	$\bar{k}$		
F I-1	O	R ₀ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	-	+
M I-2	B	R ₁ /R ₂	M $\bar{s}$ /N $\bar{s}$	-	+	+	+
Sib II-1 (mother)	B	R ₁ /R ₀	M $\bar{s}$ /N $\bar{s}$	-	+	-	+
Sib II-2	O	R ₂ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	-	-
Child First-degree Consanguinity							
Sib III-1	B	R ₀ /R ₀	M $\bar{s}$ /M $\bar{s}$	-	+	-	+

TABLE IX.8

BILATERAL MLC TESTS ON FIRST-DEGREE CONSANGUINEOUS FAMILY MEI
(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		I-1	I-2	II-1	III-1	X	PHA
	HLA haplo-type	A29-B7/a A2-B7/b	A2-BW35/c AW30-BW15/d	A29-B7/a A2-BW35/c	A2-B7/b A2-BW35/c	HLA phenotype A2,3;BW40,W17	
F I-1	A29-B7/a A3-B7/b	184 ± 58	+	+	(+)	+	++
M I-2	A2-BW35/c AW30-BW15/d	5,564 ± 426	434 ± 36	+	(+)	+	++
Sib II-1	A29-B7/a A2-BW35/c	3,274 ± 280	15,533 $\pm 1,395$	166 ± 34	(+)	+	++
Sib III-1	A2-B7/b A2-BW35/c	3,430 ± 635	2,638 ± 215	2,752 ± 452	105 ± 12	+	++
X	HLA phenotype A2,3;BW40,W17	7,329 ± 553	6,397 ± 441	8,625 ± 232	7,349 ± 213	116 ± 32	++
PHA		11,296 ± 784	18,397 ± 106	18,837 ± 793	25,619 ± 901	25,086 ± 332	

X = Healthy unrelated individual
 PHA = Phytohaemagglutinin
 --- = Stippled line through negative controls

The unusual reaction is outlined within the Table

TABLE IX.9

UNILATERAL MLC TESTS ON FIRST-DEGREE CONSANGUINEOUS FAMILY MEI

(Each entrance contains from top to bottom: SR, RR and mean cpm $\pm$ se)

Subject		I-1	I-2	II-1	III-1	X
	HLA genotype and haplotype	A29-B7/a A2-B7/b	A2-BW35/c AW30-BW15/d	A29-B7 /a A2-BW35/c	A2-B7/b A2-BW35/c	HLA phenotype A2,3;BW40,W17
F I-1 _m	A29-B7/a A2-B7/b	192 ±4	23.8 99 13,855 ±1,320	29.2 29 3,621 ±163	48.5 100 *3,012 ±612	40.4 71 8,206 ±553
M I-2 _m	A2-BW35/c AW30-BW15/d	7.7 51 1,497 ±307	582 ±171	50.7 52 6,295 ±1,100	3.5 1 222 ±28	44.7 79 9,087 ±718
Sib II-1 _m	A29-B7 /a A2-BW35/c	2.3 9 443 ±60	9.1 33 5,307 ±11,074	124 ±4	4.7 7 297 ±19	56.0 100 *11,348 ±343
Sib III-1 _m	A2-B7/b A2-BW35/c	3.4 18 665 ±127	4.5 14 2,646 ±1,037	11.8 12 1,468 ±659	62 ±5	42.7 72 8,670 ±1,291
X _m	HLA phenotype A2,3;BW40,W17	14.1 100 *2,713 ±545	25.5 100 *14,898 ±290	96.0 100 *11,916 ±1,383	36.0 73 2,233 ±46	203 ±24
PHA		11,296 ±784	18,397 ±106	18,837 ±793	25,619 ±901	25,086 ±332

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin.

m = Mitomycin-C treated.

--- = Stippled line through negative controls.

The unusual reactions are outlined within the Table.

The asterisk marks the highest test cpm observed with the corresponding responder cells used for calculating the RR.

TABLE IX.10

RESULTS ON MORPHOLOGY OF MLC TESTS

(Table IX.9)

Subject		I-1	I-2	II-1	III-1	X
	HLA genotype and haplotype	$\frac{A29-B7/a}{A2-B7/b}$	$\frac{A2-BW35/c}{AW30-BW15/d}$	$\frac{A29-B7/a}{A2-BW35/c}$	$\frac{A2-B7/b}{A2-BW35/c}$	HLA phenotype A2,3;BW40,W17
F I-1 _m	$\frac{A29-B7/a}{A2-B7/b}$	-----				+
M I-2 _m	$\frac{A2-BW35/c}{AW30-BW15/d}$					+
Sib II-1 _m	$\frac{A29-B7/a}{A2-BW35/c}$					+
Sib III-1 _m	$\frac{A2-B7/b}{A2-BW35/c}$					+
X _m	HLA phenotype A2,3;BW40,W17	+	+	+	+	
PHA		++	++	++	++	

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin.

m = Mitomycin-C treated.

--- = Stippled line through negative controls.

The unusual reactions are outlined within the Table.

Fourth-degree Consanguineous Indian Families

In the three first-cousin marriages presented, none of the parents shared HLA-A, B haplotypes and therefore none of their offspring were HLA-A, B homozygotes. The parents in one family (BEG) carried an identical HLA-B determinant.

Subsequent to the completion of this study we have made a public appeal through the news media for offspring of first-cousin marriages and we have collected a further eighteen Caucasoid families with thirty-four offspring. Three of these offspring are homozygous for the serologically determined HLA antigens and family MLC experiments are in progress to determine whether they are also MLC homozygous.

1. Indian family BEG

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 9.3, and presented in tabular form in Table IX.11. Table IX.12 shows the erythrocyte genetic markers, and Table IX.13 gives the results of the MLC tests.

Although the parents in this family were first-cousins, they did not share an HLA haplotype. The seven siblings who were HLA-A and B typed can be divided into two groups. One inherited the haplotypes *ad*, and the other *ae* from their parents. Among the *ad* group there was a set of twins, sibs III-7 and III-8, who were probably monozygotic.

There was no evidence from the erythrocyte genetic markers to suggest that the parentage was other than as stated. The results of the red cell genotypes, serum proteins and red cell enzymes, once again confirmed that sibs III-7 and III-8 were probably monozygotic twins.

Mixed lymphocyte cultures were performed on three occasions on the parents and on seven of their available children. The results of the three experiments led to the same conclusions and therefore the data on only one are presented here. The two clusters of HLA-A and B identical siblings, i.e. III-4, III-5, III-7 and III-8 with haplotypes *ad* and III-2, III-9 and III-10 with haplotypes *ae* were MLC negative. It is of special interest that the set of apparently monozygotic twins do not stimulate each other to any significantly lesser extent than the other HLA-A and B identical sibs in the same HLA cluster or the other HLA cluster. The negative and positive controls in this experiment were generally good. In general, morphological assessment of this MLC tallied well with the results of thymidine incorporation in the family, confirming the two HLA-A and B identical sib clusters to be non-stimulatory.

Results of the bilateral MLC test between individuals with one and two haplotype differences (Table IX.14) confirm that differences of two haplotypes are accompanied by a stimulation twice as high as with one haplotype difference only.

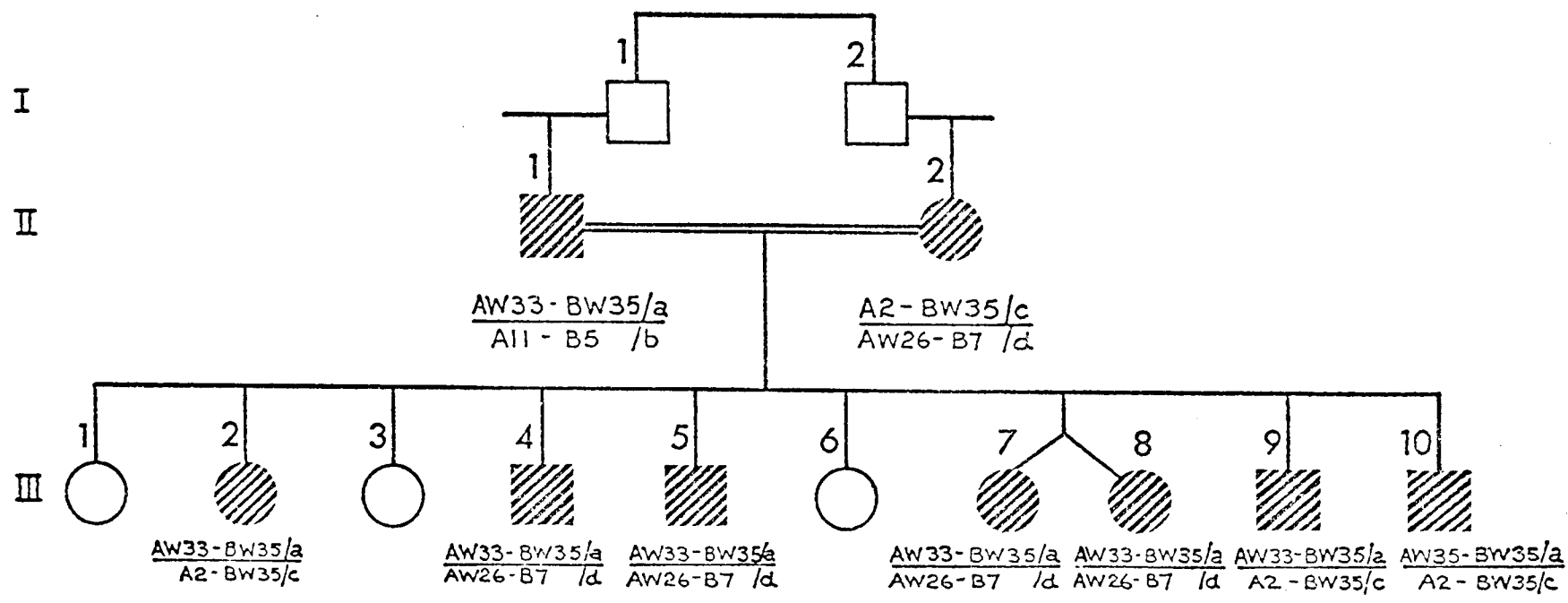


Figure 9.3. Pedigree of Indian family BEG with HLA-A and B genotypes and haplotypes. The paternal HLA haplotypes are designated ab and the maternal cd.

	<u>MALE</u>	<u>FEMALE</u>
HLA & MLC tested		
Not tested		

TABLE IX.11

HLA-A AND B GENOTYPES AND HAPLOTYPES OF INDIAN FAMILY BEG
WITH FOURTH-DEGREE CONSANGUINITY

(See Pedigree Fig. 9.3)

HLA Genotype	Haplotype	Subject
AW33-BW35	a	II-1 Father
A11-B5	b	
A2-BW35	c	II-2 Mother
AW26-B7	d	
AW33-BW35/AW26-B7	a/d	III-4 Sib
AW33-BW35/AW26-B7	a/d	III-5 Sib
AW33-BW35/AW26-B7	a/d	III-7 Sib *
AW33-BW35/AW26-B7	a/d	III-8 Sib *
AW33-BW35/A2-BW35	a/c	III-2 Sib
AW33-BW35/A2-BW35	a/c	III-9 Sib
AW33-BW35/A2-BW35	a/c	III-10 Sib

* Probably monozygotic twins.

TABLE IX.12

GENETIC DATA ON INDIAN FAMILY BEG WITH FOURTH-DEGREE CONSANGUINITY
(See Pedigree Fig. 9.3)

Subject	ASO	Rhesus probable genotype	MNS $\bar{s}$	He	Kell		P $_1$	Duffy Fy(a)	Lewis Le(a)	Serum Proteins		Peptidases				Red Cell Enzymes						
					K	$\bar{k}$				Hp	Tf	A	B	C	D	ADA	G6PD	6PGD	PGM $_1$	PGM $_2$	AK	AP
F II-1	O	R $_1$ /R $_2$	MS/N $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	2-1	B	A	2-1	1-1	1-1	AB
M II-2	B	R $_1$ /R $_2$	M $\bar{s}$ /M $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	AB
Sib III-2	O	R $_2$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	2-1	B	A	2-1	1-1	1-1	B
Sib III-4	B	R $_2$ /R $_2$	M $\bar{s}$ /N $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	AB
Sib III-5	O	R $_2$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	AB
Sib III-7*	O	R $_1$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	A
Sib III-8*	O	R $_1$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	1-1	C	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	A
Sib III-9	B	R $_1$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	2-1	B	A	2-1	1-1	1-1	AB
Sib III-10	B	R $_1$ /R $_2$	MS/M $\bar{s}$	-	-	+	+	+	-	2-1	C	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	B

* Probably monozygotic twins.

3. Indian family CAD

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 9.5, and presented in tabular form in Table IX.18. Table IX.19 shows the erythrocyte and serum protein genetic markers, and Table IX.20 gives the results of the MLC tests.

Once again the fourth-degree consanguineous parents did not have an HLA haplotype in common. All four antigens were identified in the parents and they segregated normally in the two siblings who shared one common haplotype from the father.

No evidence of other than the accepted parentage could be demonstrated from determinations of the erythrocyte and serum protein genetic markers. The MLC results in this family did not reveal anything of particular interest.

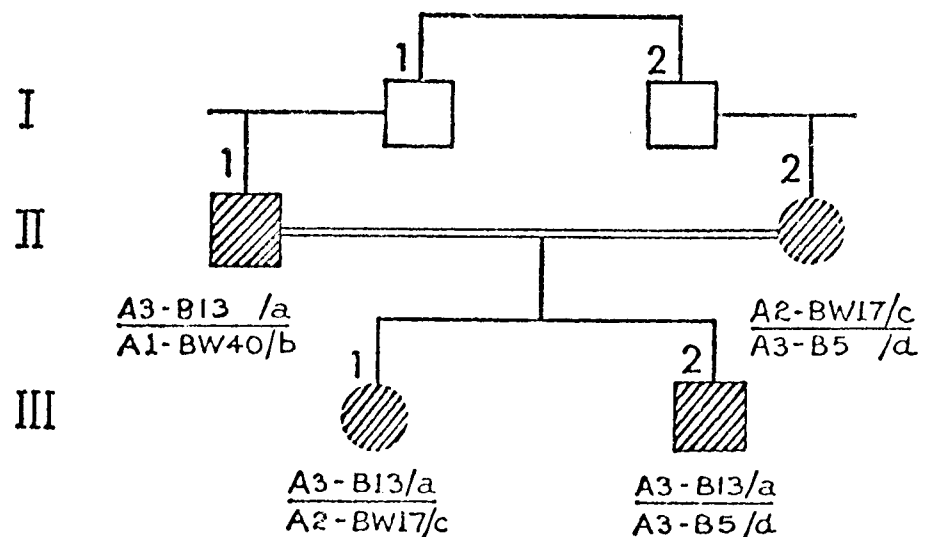


Figure 9.5. Pedigree of Indian family CAD showing HLA-A and B genotypes and haplotypes. Paternal HLA haplotypes are designated ab and maternal cd.

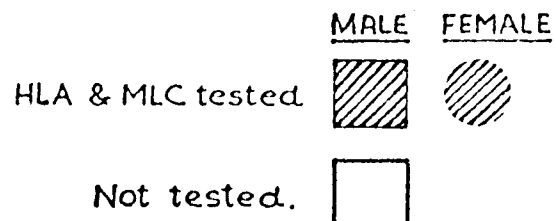


TABLE IX.13

BILATERAL MLC EXPERIMENT ON INDIAN FAMILY BEG WITH FOURTH-DEGREE CONSANGUINITY
(expressed in cpm $\pm$ se with morphology results in the right upper triangle)

Subject		II-1	II-2	III-4	III-5	III-7*	III-8*	III-2	III-9	III-10	X
	HLA haplotype	ab	cd	ad	ad	ad	ad	ac	ac	ac	HLA phenotype A9,11;B12,W40
F I-1	ab	23 ± 3	++	++	+++	++	++	++	++	++	
M II-2	cd	14,503 ± 607	69 ± 6	+++	+++	++	++	++	++	++	
Sib III-4	ad	7,050 ± 847	14,032 ± 834	58 ± 3	-	-	-	++	++	++	
Sib III-5	ad	4,679 ± 407	14,987 ± 392	99 ± 41	57 ± 4	-	-	++	++	++	
* Sib III-7	ad	4,812 ± 459	12,401 ± 920	74 ± 12	56 ± 4	28 ± 2	-	++	++	++	
* Sib III-8	ad	5,290 ± 647	12,964 $\pm 1,982$	123 ± 39	80 ± 11	35 ± 3	263 ± 25	+	++	++	
Sib III-2	ac	4,744 ± 498	10,528 $\pm 1,097$	6,147 ± 441	5,591 ± 197	4,049 ± 612	3,390 ± 556	42 ± 1	-	-	
Sib III-9	ac	9,015 ± 826	16,717 $\pm 1,071$	3,350 ± 560	7,678 $\pm 1,638$	10,348 ± 695	6,373 ± 752	47 ± 7	60 ± 2	-	
Sib III-10	ac	4,857 ± 668	12,948 ± 351	5,566 ± 745	5,960 ± 138	4,555 ± 709	5,630 ± 526	24 ± 1	81 ± 3	44 ± 8	
X	HLA phenotype A9,11;B12,W40	20,656 $\pm 1,387$	27,525 $\pm 1,547$	15,817 $\pm 1,595$	19,063 $\pm 2,113$	20,008 $\pm 2,581$	20,702 ± 975	13,700 $\pm 1,434$	21,840 $\pm 1,416$	14,814 ± 979	172 ± 17
PHA		33,566 $\pm 2,972$	42,201 $\pm 1,770$	38,466 ± 90	40,740 $\pm 1,113$	42,061 $\pm 1,841$	39,578 ± 555	24,372 $\pm 1,118$	33,194 $\pm 1,041$	33,116 $\pm 1,646$	

- X = Healthy unrelated individual.
 PHA = Phytohaemagglutinin.
 --- = Stippled line through negative controls.
 * = Probably monozygotic twins.

The negative MLC reactions are outlined within the Table.

TABLE IX.14

RESULTS BILATERAL MLC TEST ON INDIAN FAMILY BEG (WITH FOURTH-DEGREE
CONSANGUINITY) BETWEEN INDIVIDUALS WITH ONE AND TWO HAPLOTYPE
DIFFERENCES

(expressed in average cpm)

Combinations	No. Tested	Average cpm
Two haplotype differences	5	15,812
One haplotype difference	26	7,833

2. Indian family H00

The pedigree of this family, together with HLA-A and B genotypes and haplotypes is shown in Fig. 9.4, and presented in tabular form in Table IX.15. Table IX.16 shows the erythrocyte genetic markers, and Table IX.17 gives the results of the MLC tests.

The parents, who are first-cousins, do not share common HLA haplotypes. All four of the paternal HLA antigens have been identified, but only three of the mother's. The haplotypes segregated normally in the siblings, among whom there were four groups of HLA-A and B identical siblings representing the four possible combinations in this family.

No peculiarities could be demonstrated among the erythrocyte genetic markers.

MLC determinations were performed on two separate occasions on the whole family consisting of a father, mother and twelve children. The results obtained in the one experiment only are presented as there was little variation between the two tests. There are four clusters of HLA-A and B identical siblings that were of interest in this family :-

siblings IV-1, IV-5, IV-11 with haplotypes *ac*
 siblings IV-2, IV-4, IV-8, IV-10 with haplotypes *ad*
 siblings IV-6, IV-7, IV-12 with haplotypes *bd*
 siblings IV-3, IV-9 with haplotypes *bc*.

The autologous control values for some of the siblings in this culture are much higher than usual, i.e. siblings IV-1, IV-2, IV-4, IV-5 and IV-8. If these high background counts are subtracted from the mean cpm the sibs in the four HLA-A and B identical groups appear to be truly MLC negative. The controls with X and PHA are good except for the PHA value of III-2 which is rather low. The results of the morphology for the HLA-A and B identical siblings confirmed their MLC identity with the exception of IV-1 versus IV-5 in the *ae* group and IV-2 versus IV-4 in the *ad* group, which were weakly positive.

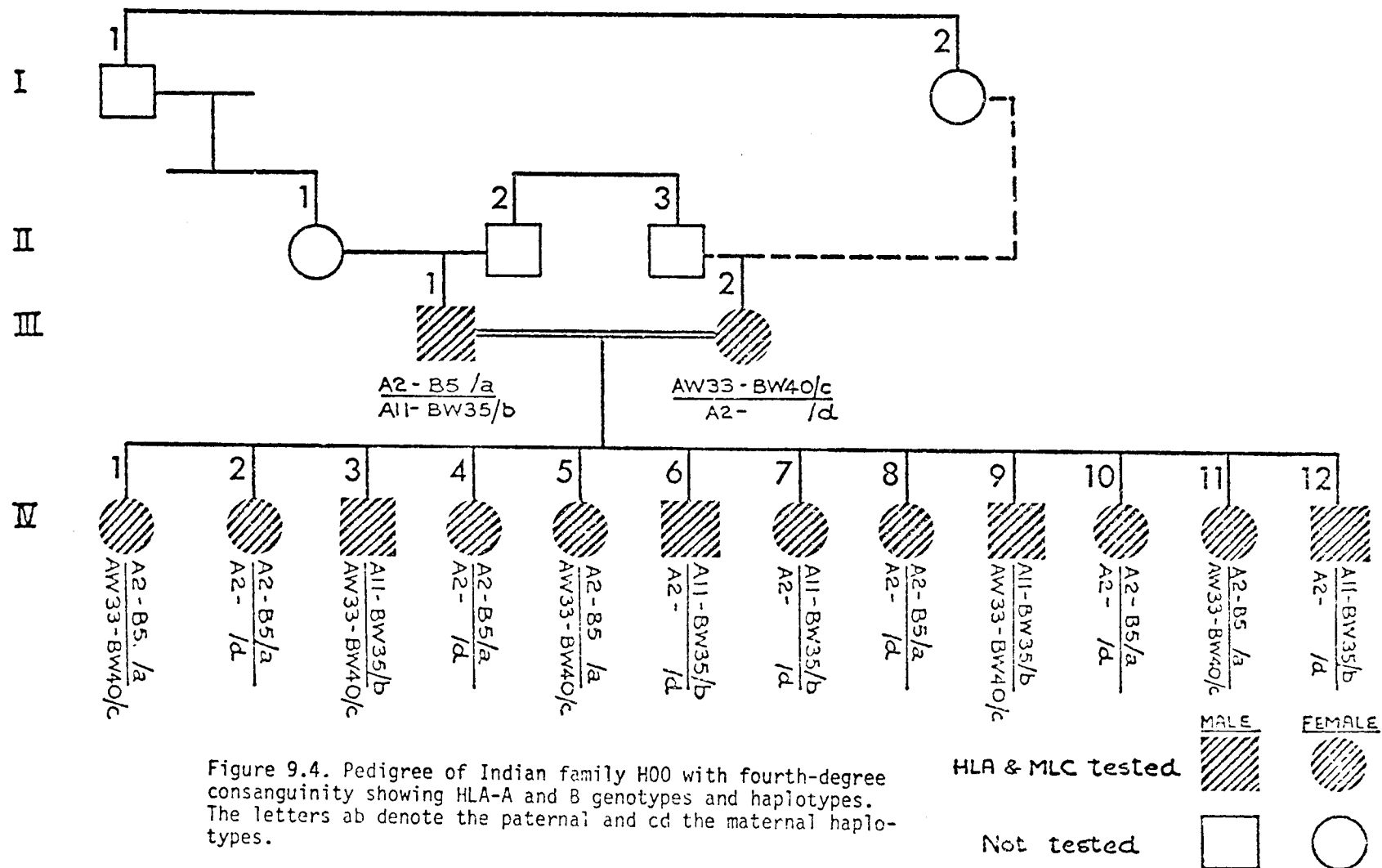


TABLE IX.15

HLA-A AND B GENOTYPES AND HAPLOTYPES OF INDIAN FAMILY H00
WITH FOURTH-DEGREE CONSANGUINITY

(See Pedigree Fig. 9.4)

HLA Genotype	Haplotype	Subject
A2-B5 A11-BW35	a b	III-1 Father
AW33-BW40 A2-	c d	III-2 Mother
A2-B5/AW33-BW40	a/c	IV-1 Sib
A2-B5/AW33-BW40	a/c	IV-5 Sib
A2-B5/AW33-BW40	a/c	IV-11 Sib
A2-B5/A2-	a/d	IV-2 Sib
A2-B5/A2-	a/d	IV-4 Sib
A2-B5/A2-	a/d	IV-8 Sib
A2-B5/A2-	a/d	IV-10 Sib
A11-BW35/A2-	b/d	IV-6 Sib
A11-BW35/A2-	b/d	IV-7 Sib
A11-BW35/A2-	b/d	IV-12 Sib
A11-BW35/AW33-BW40	b/c	IV-3 Sib
A11-BW35/AW33-BW40	b/c	IV-9 Sib

TABLE IX.16

GENETIC DATA ON INDIAN FAMILY HOO WITH FOURTH-DEGREE CONSANGUINITY
(See Pedigree Fig. 9.4)

Subject	ABO	Rhesus probable genotype	MNSs	He	Kell		P ₁	Duffy Fy(a)	Lewis Le(a)	Red Cell Enzymes										
					K	k̄				Peptidases				ADA	G6PD	6PGD	PGM ₁	PGM ₂	AK	AP
										A	B	C	D							
F III-1	A ₂	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	AB
M III-2	A ₂	R ₁ /R ₁	M $\bar{S}$ /N $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	A3
Sib IV-1	A ₂	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	AB
Sib IV-2	O	R ₁ /R ₁	M $\bar{S}$ /N $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	AB
Sib IV-4	O	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	-	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	AB
Sib IV-5	A ₂	R ₁ /R ₁	M $\bar{S}$ /M $\bar{S}$	-	-	+	-	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	B
Sib IV-6	A ₂	R ₁ /R ₁	M $\bar{S}$ /M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	AB
Sib IV-7	A ₂	R ₁ /R ₁	M $\bar{S}$ /M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	B
Sib IV-8	A ₂	R ₁ /R ₁	M $\bar{S}$ /N $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	AB
Sib IV-9	A ₂	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	A3
Sib IV-10	A ₂	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	AB
Sib IV-11	A ₂	R ₁ /R ₁	MS/N $\bar{S}$	-	-	+	+	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	A
Sib IV-12	A ₂	R ₁ /R ₁	M $\bar{S}$ /M $\bar{S}$	-	-	+	-	+	-	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	1-1	B

TABLE IX.17
BILATERAL MLC TEST ON INDIAN FAMILY HOO WITH FOURTH-DEGREE CONSANGUINITY

(expressed in cpm $\pm$ se with morphology results
in the right upper triangle)

Subject		F III-1	M III-2	IV-1	IV-5	IV-11	IV-2	IV-4	IV-8	IV-10	IV-6	IV-7	IV-12	IV-3	IV-9	X	PHA
	HLA haplotype	ab	cd	ac	ac	ac	ad	ad	ad	ad	bd	bd	bd	bc	bc	HLA phenotype A2,3; BW40, W17	
F III-1	ab	257 +31	++	++	++	+	++	++	++	++	++	+	+	++	+	++	+++
M III-2	cd	5,872 +178	250 +38	++	++	++	++	++	++	++	++	+	+	++	+	++	++
Sib IV-1	ac	4,002 +742	4,434 +1,175	404 +29	(+)	-	++	+	++	+	++	+	++	nd	++	++	++
Sib IV-5	ac	7,250 +25	10,703 +749	715 +107	354 +29	-	+++	++	++	++	+++	+	++	+++	++	++	+++
Sib IV-11	ac	5,185 +529	5,957 +661	338 +80	394 +104	84 +16	+	++	++	+	+++	+	++	++	+	+++	+++
Sib IV-2	ad	5,542 +372	6,063 +733	2,725 +191	6,058 +269	5,475 +743	1,473 +72	(+)	-	-	++	+	++	+	+	+++	++
Sib IV-4	ad	6,275 +607	7,169 +381	5,233 +389	5,535 +678	5,131 +672	1,462 +125	649 +38	-	-	+	+	+	++	+	++	+++
Sib IV-8	ad	10,437 +912	10,495 +442	10,984 +874	1,184 +250	6,147 +1,076	361 +50	555 +208	1,112 +74	-	++	+	+	++	++	+++	+++
Sib IV-10	ad	4,192 +471	3,320 +272	7,726 +273	6,669 +348	2,603 +446	546 +137	630 +94	353 +49	275 +13	+	++	++	++	+	+++	+++
Sib IV-6	bd	6,737 +659	6,399 +848	7,705 +491	18,023 +269	9,113 +300	4,881 +823	4,907 +873	11,379 +687	3,167 +488	243 +52	-	-	+++	+	+++	+++
Sib IV-7	bd	2,503 +316	6,018 +37	11,534 +268	10,887 +1,001	4,025 +277	5,605 +641	6,997 +808	7,686 +205	2,004 +51	163 +20	76 +3	-	++	-	++	++
Sib IV-12	bd	4,122 +102	6,393 +532	6,945 +444	14,072 +253	7,479 +149	5,439 +538	5,974 +312	11,744 +599	4,162 +653	391 +108	168 +5	211 +22	+++	++	+++	+++
Sib IV-3	bc	10,308 +343	9,591 +242	nd	14,449 +937	14,007 +1,914	16,970 +1,349	15,095 +716	15,301 +187	15,115 +687	17,009 +627	14,070 +1,481	11,000 +1,420	85 +6	-	+++	+++
Sib IV-9	bc	3,067 +206	3,669 +236	2,744 +161	7,219 +764	2,711 +150	5,210 +36	5,296 +343	10,827 +878	2,767 +527	3,732 +490	3,175 +749	2,867 +436	327 +44	105 +44	++	+++
X	HLA phenotype A2,3; BW40, W17	19,206 +1,655	19,510 +552	27,604 +1,006	22,657 +836	20,219 +491	26,126 +2,307	19,984 +808	18,774 +263	17,350 +773	17,975 +550	15,908 +625	18,504 +456	16,948 +825	19,105 +712	343 +58	
PHA		12,829 +377	4,320 +97	9,672 +282	18,341 +406	18,510 +683	15,930 +177	13,933 +349	15,030 +1,252	14,908 +197	17,839 +1,035	17,027 +578	11,434 +929	9,004 +549	20,173 +1,734		

The unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the high control values.

nd = Not determined
X = Healthy unrelated individual
PHA = Phytohemagglutinin
--- = Stippled line through negative controls

TABLE IX.18

HLA-A AND B GENOTYPES AND HAPLOTYPES OF INDIAN FAMILY CAD
WITH FOURTH-DEGREE CONSANGUINITY

(See Pedigree Fig. 9.5)

HLA Genotype	Haplotype	Subject
A3-B13 A1-BW40	a b	II-1 Father
A2-BW17 A3-B5	c d	II-2 Mother
A3-B13/A2-BW17	a/c	III-1 Sib
A3-B13/A3-B5	a/d	III-2 Sib

TABLE IX.19

GENETIC DATA ON INDIAN FAMILY CAD WITH FOURTH-DEGREE CONSANGUINITY

(See Pedigree Fig. 9.5)

Subject	ABO	Rhesus probable genotype	MNSs	He	Kell		P ₁	Duffy Fy(a)	Lewis Le(a)	Serum Proteins		Peptidases				Red Cell Enzymes						
					K	k				Hp	Tf	A	B	C	D	ADA	G6PD	GPGD	PGM ₁	PGM ₂	AK	AP
F II-1	B	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	2-1	c	1-1	1-1	1-1	1-1	1-1	B	A	1-1	1-1	2-1	B
M II-2	B	R ₁ /r	M $\bar{S}$ /M $\bar{S}$	-	-	+	+	+	-	2-2	c	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	2-1	B
Sib III-1	B	R ₁ /r	MS/M $\bar{S}$	-	-	+	+	+	-	2-2	c	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	2-1	B
Sib III-2	O	R ₁ /R ₁	MS/M $\bar{S}$	-	-	+	+	+	-	2-2	c	1-1	1-1	1-1	1-1	1-1	B	A	2-1	1-1	1-1	B

TABLE IX.20

BILATERAL MLC TESTS ON INDIAN FAMILY CAD WITH FOURTH-DEGREE CONSANGUINITY
(expressed in cpm $\pm$ se)

Subject		F II-1	M II-2	III-1	III-2	X
	HLA haplo-type	ab	cd	ac	ad	HLA phenotype A9,11;B12,W40
F II-1	ab	512 ± 26	485 ± 97	624 ± 49	679 ± 141	608 ± 104
M II-2	cd	8,799 ± 693				
Sib III-1	ac	6,013 ± 251	3,517 ± 149	3,845 ± 135	13,354 ± 430	9,333 ± 769
Sib III-2	ad	10,642 ± 821	10,121 ± 243			
X	HLA phenotype A9,11;B12,W40	10,118 ± 816	9,719 ± 150	9,865 ± 446	13,354 ± 430	608 ± 104
PHA		10,425 ± 895	8,029 ± 689	13,619 $\pm 1,194$	11,108 ± 751	

X = Healthy unrelated individual.
 PHA = Phytohaemagglutinin.
 --- = Stippled line through negative controls.

Interpretation of Results

In the *first-degree consanguineous family COO*, the parents did not share an HLA haplotype and the three groups of siblings identical for HLA-A and B were MLC negative. The father and the daughter with whom he mated shared an HLA haplotype and produced one offspring homozygous for the MHC chromosome and one heterozygote. A weak response was observed when the homozygote was used to stimulate the heterozygotes in this family, but proof that sib III-1 is truly MLC homozygous could be obtained from the fact that the HLA sero-identical father-child combination (I-1 versus III-2) was mutually non-responsive.

In *family MEI*, in which first-degree consanguinity was also present, the offspring from the consanguineous union was not homozygous, and the MLC results were inconclusive.

In none of the *fourth-degree consanguineous families* was there sharing of HLA-A, B haplotypes between the parents. Unfortunately unilateral MLC experiments were not done on these families because of difficulties encountered in persuading them to donate further blood samples. Therefore no final conclusion could be drawn with regard to the sharing of MLC determinants.

In *family BEG* there were two groups of siblings identical for HLA-A and B and MLC negative. Furthermore individuals with differences of two haplotypes stimulated twice as much as those with only one haplotype difference.

Family HOO included four groups of HLA-A and B identical siblings who were MLC negative, while *family CAD* was not informative, since the two siblings were HLA-A and B non-identical and MLC positive.

CHAPTER TEN - THE RECOGNITION OF MLC DETERMINANTS
(MLC TYPING) BY MEANS OF MLC HOMOZYGOUS CELLS

Interpretation of Results

Unilateral MLC tests were carried out on a group consisting of cell III-1 (C00) from the first-degree consanguineous family (see Pedigree, Fig. 9.1) known to be homozygous for HLA-A1,B8 and the MLC locus determinants as well as any other unidentified determinants on the MHC chromosomal region; four unrelated individuals, Nos. 2, 3, 4 and 5 with the genotype HLA-A1,B8/A1,B8; two unrelated individuals Nos. 6 and 7 with the genotypes A1,B8/A3,B7 and A1,B8/A3,BW35 respectively; and X, the control with four totally different HLA-A and B antigens (Table X.1).

The rationale behind this test was to use the lymphocytes from III-1 (C00) which were known to be MLC homozygous as: i) stimulating cells; and ii) as responder cells. A negative or weak response of lymphocytes from any individual A, stimulated by these cells is taken to indicate that one or both of the MLC determinants of the responding cells A are identical with the MLC determinant carried by cell III-1 (C00) (= 'typing cell'). If the MLC test is again negative when the 'typing cell' acts as a responder cell towards A (now the stimulator cells), A must also be homozygous for the same MLC determinants carried by the 'typing cell'.

The MLC reactivity of each combination is given as an SR, RR and mean cpm $\pm$ standard error in order to compare their value in judging identical, one-haplotype and two-haplotype different individuals.

This group was MLC tested in all possible combinations on two different occasions and the results of the first experiment were confirmed. 'Typing cell' III-1 (C00) elicited no response from Nos. 2 and 4 and a very weak response from No. 5 (negative in the repeat test). Therefore *one* or *both* of the D locus (or Major MLC locus) determinants of Nos. 2, 4

TABLE X.1

UNILATERAL MLC TEST BETWEEN THE MLC HOMOZYGOTE 111-1 (C00), 4 UNRELATED HLA-A1,B8 HOMOZYGOTES AND 2 UNRELATED INDIVIDUALS HLA HAPLO-IDENTICAL WITH THE PREVIOUS 5 INDIVIDUALS
(Each entrance contains from top to bottom: SA, RR and mean cpm $\pm$ se)

Subject	111-1 C00 homozygous	2	3	4	5	6	7	X
HLA genotype or haplotype	A1,B8/A1,B8	A1,B8/A1,B8	A1,B8/A1,B8	A1,B8/A1,B8	A1,B8/A1,B8	A1,B8/A3,B7	A1,B8/A3,BW35	HLA phenotype A2,W33;B12,W17
111-1 C00 homozygous	A1,B8/A1,B8	1.2 0 79 ± 19	26.7 26 4,648 $\pm 1,211$	1.1 0 150 ± 25	3.7 4 1,586 ± 512	65.1 37 7,164 ± 462	6.7 13 3,332 ± 551	178.3 66 12,162 ± 548
2 _m	A1,B8/A1,B8	8.4 14 3,261 ± 503	37.4 37 6,517 ± 120	1.6 0 214 ± 2	4.7 6 1,982 ± 614	69.8 40 7,681 $\pm 2,174$	8.9 17 4,385 ± 497	136.6 50 9,295 ± 943
3 _m	A1,B8/A1,B8	16.2 30 6,292 $\pm 1,346$	62.0 16 4,036 ± 798	44.0 22 5,553 ± 685	20.1 31 8,404 ± 482	72.2 41 7,949 $\pm 1,132$	5.0 9 2,462 ± 116	225.6 83 15,342 $\pm 2,805$
4 _m	A1,B8/A1,B8	19.5 36 7,570 $\pm 1,162$	134.6 37 8,750 ± 555	25.5 25 4,440 ± 220	32.1 50 13,429 $\pm 2,767$	44.4 25 4,892 $\pm 1,034$	19.3 33 9,759 ± 209	81.6 30 5,550 ± 924
5 _m	A1,B8/A1,B8	5.7 9 2,232 ± 779	17.8 4 1,159 ± 355	17.6 16 3,071 ± 631	13.4 6 1,691 ± 851	51.3 29 5,653 ± 140	16.8 36 8,310 ± 673	100.3 37 6,826 $\pm 1,153$
6 _m	A1,B8/A3,B7	46.4 90 18,004 $\pm 3,565$	225.5 62 14,662 $\pm 1,385$	65.7 66 11,437 $\pm 2,282$	158.7 81 20,004 $\pm 1,425$	4.9 6 2,062 ± 701	45.0 100 *22,178 $\pm 3,407$	269.2 100 *18,311 ± 819
7 _m	A1,B8/A3,BW35	40.7 79 15,823 $\pm 1,910$	300.8 83 19,558 $\pm 1,901$	98.4 99 17,122 $\pm 2,168$	194.9 100 *24,565 $\pm 2,590$	62.1 100 *25,963 $\pm 1,154$	160.0 93 17,608 $\pm 1,488$	203.5 75 13,838 $\pm 1,307$
X _m	HLA phenotype A2,W33;B12,W17	51.1 100 *19,854 ± 909	361.7 100 *23,513 $\pm 1,141$	99.0 100 *17,226 $\pm 2,525$	162.2 82 20,284 ± 764	41.2 65 17,235 $\pm 2,334$	170.9 100 *18,803 ± 743	34.7 76 17,087 $\pm 1,492$
PHA		32,553 ± 951	51,159 $\pm 6,170$	36,263 $\pm 2,533$	25,117 $\pm 2,016$	30,078 $\pm 1,269$	41,567 $\pm 3,467$	41,127 $\pm 2,466$

X = Healthy unrelated individual.

PHA = Phytohaemagglutinin.

--- = Stippled line through negative controls.

m = Mitomycin-C treated.

* = Marks the highest test cpm observed with the corresponding responder cells used for calculating the relative response.

Unusual MLC reactions are outlined within the Table, solid lines for the negative reactions and interrupted lines for the weakly positive reactions.

and 5 must be identical with that of III-1 (C00). There is an excellent correlation between the SR and RR values and the mean cpm values in these combinations.

In the reverse situation the response of III-1 (C00) to No. 5 was weakly positive. No. 2 stimulated III-1 (C00) moderately and No. 4 stimulated III-1 (C00) quite strongly. From this it would seem that Nos. 2 and 5 probably differ from III-1 (C00) for one or two 'minor' MLC determinant(s) only, and No. 4 probably carries a different D locus determinant on one chromosome.

It is of interest to compare the reactions of Nos. 2, 4 and 5 who appeared to be typed by the 'typing cell' III-1 (C00). No. 2 did not stimulate No. 4 but a strong MLC response was observed when No. 4 stimulated No. 2. This could be because No. 2 had *both* the D locus determinants of III-1 (C00) and No. 4 had *one* in common with III-1 (C00) and 2. No. 2 elicited no, or only a weak, typing response from No. 5 (the repeat test was negative), and No. 5 did not stimulate (or only weakly) the 'typing cell' III-1 (C00) as well as Nos. 2 and 4. Nos. 2 and 5 could therefore be identical for their D locus determinants but may differ from each other and III-1 (C00) for some 'minor' MLC loci. This would make the 'typing cell' III-1 (C00) and No. 2 and 5 identical for their D locus determinants, and because III-1 (C00) was homozygous for the D locus determinant, Nos. 2 and 5 were possibly also homozygous for the same D locus determinant. No. 2 did, however, elicit some response from III-1 (C00) which suggested either that the 'minor' MLC determinants of III-1 (C00) may be included in those of No. 2 or an absence of such determinants (0 = null-determinants, Table X.2); but then No. 2 should also have stimulated No. 5 if No. 5 had exactly the same MLC determinants as III-1 (C00). No. 5 may, however, have been only slightly

TABLE X.2

SCHEMATIC CONCEPT OF MLC DETERMINANTS OF SOME OF THE CELLS TESTED
(Table X.1)

POSSIBLE MLC - LOCUS GENOTYPES				
Cell No.	Major MLC locus (D locus)	'Minor' MLC locus	Major MLC locus (D locus)	'Minor' MLC locus
III-1 (C00)	C00	0	C00	0
No. 2	C00	a	C00	b
No. 5	C00	0	C00	b
No. 4	C00	-	4	-
No. 3	3	-	3	-
No. 7	3	-	7	-

0 = null - determinant

different from III-1 (C00) (Table X.2), e.g. one 'minor' MLC determinant b ; hence the weak stimulation of No. 5 versus III-1 (C00). No. 2 may differ from III-1 (C00) for 'minor' MLC determinants, i.e. a and b, and therefore may differ from No. 5 for the 'minor' MLC determinant a only. No. 3 did not stimulate No. 7 (or only weakly); hence this cell could have been homozygous for one of the D locus determinants present on the lymphocytes of No. 7. In the reverse situation No. 7 was strongly stimulatory towards No.3, and No.7 must therefore have carried one D locus determinant different from those of No. 3.

The weak response of No. 5 to No. 6 observed in this culture could not be reproduced in the second test; this was therefore probably a false negative reaction. The three parameters SR, RR and mean cpm's in this experiment again correlated well in the clear-cut negative MLC tests, but in the weakly positive reactions, the mean cpm values and the RR values correlated more closely with each other than with the SR.

Interpretation of Results

We tested the MLC homozygous cells from sib III-1 from family C00, in a unilateral MLC experiment against a panel of unrelated individuals, of whom some were HLA serologically identical with the 'typing cell' III-1 (C00) and others only HLA haplo-identical.

The 'typing cell' III-1 (C00) elicited a 'typing response' from Nos. 2, 4 and 5 from which can be deduced that Nos. 2, 4 and 5 carry the same D locus determinant as the 'typing cell' on *one* or *both* MHC chromosomes (Table X.2). In the reverse situation the response of the 'typing cell' was weak when stimulated by Nos. 2 and 5; therefore they may be homozygous for the D locus determinant of the 'typing cell' III-1 (C00). No. 2 may have carried two, and No. 5 one, 'minor' MLC determinant different from III-1 (C00) as shown in Table X.2. No. 2 and No. 5 may also differ from each other for one 'minor' MLC determinant, and No. 4 probably carried the D locus determinant of III-1 (C00) on one chromosome.

Since the completion of this study, cell III-1 (C00) was included as one of the sixty-two homozygous 'typing cells' used in the collaborative studies for the Sixth International Workshop on Histocompatibility Testing held in Aarhus, Denmark, in 1975. This cell (Workshop number 16005) was shown to define the new D locus specificity DW3, mostly found in individuals carrying the B8 antigen.

Recently completed family MLC experiments confirmed Nos. 2 and 5 to be homozygous for their D locus determinants. Since these two cells appeared to carry III-1 (C00)-'like' D locus determinants, and since we know that III-1 (C00) defined the

DW3 specificity, it seems likely that Nos. 2 and 5 are two additional DW3 typing cells. Thus, reference typing cells can be obtained by reciprocal non-stimulation with lymphocytes previously shown to be homozygous, as well as by family MLC testing. When used as stimulators, in population studies presently being performed in our laboratory, Nos. 2 and 5 behaved operationally like DW3 homozygous cells. However, the results in this experiment also indicate the complexity of the genetic constitution with regard to the determinants involved in MLC. The D locus determinants as they were recognized during the Sixth International Histocompatibility Workshop may be poorly defined and may in fact be broad determinants which include several other (still unrecognized) specificities.

In a recent review Dupont and co-workers (Dupont et al., 1976) discuss the various possible explanations for narrow specificities, of which III-1 (C00) as presented in Table X.2 may be an example, and broader specificities, e.g. Nos. 2 and 5. They suggested that broad and narrow D specificities may represent different allelic products sharing a common cross-reacting determinant; they may also represent the complex products of sub-loci or pseudo-alleles within the D locus; or disparities at other genetic determinants.

Whatever may be the explanation for the complexity observed, it can be concluded from the data that Nos. 2 and 5 carry MLC determinants which are 'like' those possessed by the 'typing cell' III-1 (C00) only with reference to the D locus determinant. The determinants represented by the three different MLC homozygous cells III-1 (C00), 2 and 5 that appear to belong to the same group HLA-DW3, may represent *cross-reacting specificities of the same allelic system* or they may represent the *products of sub-loci in strong positive linkage disequilibrium*.

CHAPTER ELEVEN - PROBLEMS ENCOUNTERED IN EXPRESSION
OF MLC REACTIVITY

Empirically the measurement of proliferation in MLC can be ranked in different groups based on antigenic differences between responder and stimulator. A clear separation exists between HLA-A, B identical sibling combinations on the one hand, and family members or unrelated combinations that differ for one or more HLA-D specificities or haplotypes, on the other. The mean for one haplotype response is approximately half the mean for two haplotype responses. The data presented on family BEG in Table IX.14 (Chapter Nine) illustrate this point. A considerable overlap between the one haplotype- and two haplotype-different groups has been demonstrated in several studies (Albertini and Bach, 1968; Schellekens and Eijssvoegel, 1970; Sørensen and Kissmeyer-Nielsen, 1969), as well as in our data.

In the course of this study an attempt was made to analyse the results of some of the MLC tests using the three different approaches, i.e. *mean counts per minute (cpm)*, *stimulation ratio (SR)* and the *relative response (RR)*, in order to find which of these parameters could distinguish clearly between D locus-identical combinations and those differing for one or two haplotypes. In addition, the results of the *morphology* were also recorded in a number of tests.

In Table XI.7 the MLC results of *two of the families (DEW and VDW)* presented in Chapter Seven are compared when expressed as mean cpm, SRs and RRs. In each family the mean value for the combinations that appeared to be identical, haplo-identical or non-identical was calculated using the three previously mentioned parameters.

TABLE XI.1

THIS TABLE SHOWS THE MLC RESULTS OF FAMILY DEW AND FAMILY VDW (TWO EXPERIMENTS ON DIFFERENT DAYS) EXPRESSED AS MEAN CPM, SR AND RR VALUES. A COMPARISON USING THE MEANS OF THESE INDICES IN IDENTICAL, HAPLO-IDENTICAL AND NON-IDENTICAL COMBINATIONS IS MADE, THE LEVELS OF SIGNIFICANCE BEING SHOWN.

		FAMILY DEW			FAMILY VDW				
		Number of combinations	Mean Values	Standard Deviation	Number of combinations	Exp. 1 Mean Values	Standard Deviation	Exp. 2 Mean Values	Standard Deviation
Mean cpm	Identical	8	59 xxx	35	7	1576 xxx	654	1225 xxx	799
	Haplo-identical	22	1243 xxx	784	5	10951 xx	5133	10393 xxx	4023
	Non-identical	26	2823	1639	28	16966	4880	17474	4392
Stimulation Ratio (SR)	Identical	8	2.11 xxx	1.23	7	7.50 xxx	4.28	6.13 xxx	4.31
	Haplo-identical	22	59.50 N.S.	37.99	5	51.48 N.S.	23.87	59.60 x	35.61
	Non-identical	26	86.12	70.99	28	91.58	61.63	104.08	40.10
Relative Response (RR)	Identical	8	0.88 xxx	1.46	7	6.71 xxx	3.45	4.29 xxx	3.55
	Haplo-identical	22	48.36 xxx	30.25	5	52.20 xxx	17.40	55.80 xxx	13.01
	Non-identical	26	72.04	26.12	28	84.64	14.60	87.71	12.01

SIGNIFICANCE LEVELS FOR THE DIFFERENCE OF THE MEANS OF THE POPULATIONS ARE INDICATED AS: xxx = .005
 xx = .01
 x = .025
 N.S. = Not significant

THE NULL HYPOTHESIS THAT THERE IS NO TRUE DIFFERENCE BETWEEN THE MEANS WAS TESTED UNDER THE ASSUMPTIONS BOTH OF NORMALITY AND NON-NORMALITY. NO DIFFERENCE IN THE SIGNIFICANCE LEVELS WAS OBSERVED.

The mean cpm takes account of raw data only. The SR (see page 119) as an index is particularly sensitive to the magnitude of the background. This is verified by noting that no clear distinction could be made between haplo-identical and non-identical combinations in Table XI.1. The third index, the RR in which the results are stabilized by subtracting the autologous control and expressing each reaction as a percentage of the response to a stimulator that definitely carries different D locus determinants, appears to distinguish more clearly between identical, haplo-identical and non-identical individuals. The variance is noted to be lower the more identical the combinations are. The higher variance in haplo-identical and non-identical combinations is presumably an indication of their heterogeneity.

The MLC results of the *two groups of unrelated individuals (Groups 1 and 2)*, are expressed as mean cpm's, SRs and RRs in *Table XI.2*. A comparison using these three parameters in those individuals who had previously been shown by HLA serology and MLC testing to be identical and non-identical was made, the level of significance being shown. Since all the individuals were unrelated, haplo-identical combinations could not be selected. As shown in Table XI.2 all three indices provided clear distinction between combinations.

The *morphological* assessment correlated quite well with the results of the thymidine incorporation, especially in the strongly positive MLC combinations and the clearly negative tests as shown for example in the data on families COO, MEI and BEG. However, in some instances, like family

TABLE XI.2

THIS TABLE GIVES THE MLC RESULTS OF THE TWO GROUPS (1 & 2) OF IDENTICAL AND NON-IDENTICAL UNRELATED INDIVIDUALS, EXPRESSED AS MEAN CPM, SR AND RR VALUES. A COMPARISON USING THE MEANS OF THESE INDICES IN IDENTICAL AND NON-IDENTICAL COMBINATIONS IS MADE, THE LEVELS OF SIGNIFICANCE BEING SHOWN.

		GROUP 1			GROUP 2		
		Number of combinations	Mean	Standard deviation	Number of combinations	Mean	Standard deviation
Mean cpm	Identical	12	404 xxx	500	10	1500 xxx	1097
	Non-identical	43	7854	3852	45	12324	6582
Stimulation Ratio (SR)	Identical	12	2.36 xxx	2.22	10	6.26 xxx	5.51
	Non-identical	43	73.24	59.39	45	95.67	85.87
Relative Response (RR)	Identical	12	1.67 xxx	3.34	10	5.20 xxx	4.61
	Non-identical	43	56.07	27.73	45	58.09	30.38

SIGNIFICANCE LEVELS FOR THE DIFFERENCE OF THE MEANS OF THE POPULATIONS ARE INDICATED AS: xxx = .005

H00 and the San families V, VI, and VII, the results of the morphology were inconclusive or contradicted those of the thymidine incorporation.

The morphological assessment is a time-consuming and exacting technique, greatly dependent on technical variations and individual interpretation. It cannot be recommended as a routine measure of MLC reactivity, except as a check on toxicity and in exceptional circumstances (such as the field study on the San) to guard against a possible mishap with tests that cannot be repeated.

In *summary*, since the discrimination between weak responses and the group of responses representing one-haplotype-different sibling or parent-child combinations is relatively poorly defined, the limits of a 'typing response' cannot be clearly delineated and remain a major problem in MLC interpretation. In order to minimize technical variations in experimental results, many attempts have been made to normalize the data. As discussed previously, responder function can be normalized by using relative responses. Stimulator function is now normalized by using the methods of Ryder and Thomsen (Thomsen et al., 1975; Ryder et al., 1975).

PART IV

DISCUSSION

CHAPTER TWELVE - THE MLR AND HISTOCOMPATIBILITY

The object of this study was to investigate the genetic relationship between the serologically detectable products of the HLA-A and HLA-B loci on the one hand, and MLC reactivity on the other in an effort to provide knowledge and insight into the requirements for histocompatibility.

I Thus in order to elucidate the question posed in Chapter Two, II.2 - whether MLC reactivity is controlled by one or more loci belonging to the MHC, but separate from the serologically determined HLA-A and B antigens - we studied the following two groups :-

- (i) unrelated combinations with varying degrees of HLA-A and B disparity
- (ii) families in which recombinational events had occurred

(i) *Unrelated Combinations with Varying Degrees of HLA-A and B Disparity (Chapter Six)*

The fact that non-stimulation between HLA-A and B mismatched unrelated individuals could be found, indicates that the serologically determined HLA antigens are not solely responsible for MLC reactivity. Since we found only a few such non-stimulatory pairs in a large group, and since this observation was noted in many thousands of MLC tests performed all over the world (Mempel et al., 1973b; Pentycross et al., 1972; Van den Tweel et al., 1973), it must be assumed that there is a fairly high degree of polymorphism in the MLC system.

The observation that selection for HLA-A and B identity among unrelated persons increased the frequency of non-stimulating pairs to about 10%, indicates that HLA-A antigens and/or the HLA-B antigens must have some relation to the

specificity of the MLC-controlling determinants. However, it also demonstrated that the HLA-A and B antigens cannot be the major genetic factors controlling MLC. This concept is further supported by the observation of some MLC identical combinations in our experiments and in those of others (Mempel et al., 1973b), among the individuals who share HLA-B specificities but who carry different HLA-A specificities. This would give support to the suggestion that the MLC specificities are closely linked with the B locus.

Evidence suggestive of at least two defined MLC determinants was found among the HLA-A1,3;B7,8 identical group.

(ii) *Families in which recombinational events had occurred*
(Chapter Seven)

As pointed out in Chapter Two, II.2, the discovery of combinations which are HLA-A and/or B different but MLC negative, especially in families where a recombinational event has taken place, would be further proof of the existence of a separate MLC-controlling entity. The possibility that the MLC reaction might be controlled by a separate gene closely linked to the serologically detectable HLA antigens was suggested by Yunis and Amos (1971).

In our experiments performed in collaboration with Eijsvoegel et al. (1972c), five families were studied, each containing a sibling in which recombination between the genes determining the antigens of the A and B loci had occurred. These results, as well as those of family BEU (see pg 130 confirmed the findings of Yunis et al. (1971), Dupont et al., (1971) and Lebrun et al. (1971), that the antigens of the A

locus have a very weak or non-stimulatory effect on the MLC test. On the other hand, pronounced MLC activity was found in tests involving differences for the determinants of the B locus only.

Further findings in family KA demonstrated a separate inheritance of the antigens of the B locus and the MLC determinants, probably by recombination, which implied independence of the MLC reaction from both the HLA-A and HLA-B loci. Consequently this separate MLC locus (or loci) can now be mapped not in between, but outside the previously recognized HLA region, close to the B locus. It should be pointed out, however, in the light of the earlier mentioned findings of occasional very weak stimulation between siblings differing only for an A locus antigen, that this may reflect A-locus-associated regions governing weak MLC reactivity. Further evidence for a 'minor' MLC locus has also been provided by Thorsby et al. (1973), but final proof for the existence of such a locus has not been presented.

These findings, together with the findings of HLA serologically non-identical unrelated MLC negative combinations, supported the hypothesis of a separate region or locus for the genetic control of the MLC reaction.

This concept of a separate MLC locus (or loci) explains firstly the frequent findings of positive MLC tests between carefully serotyped HLA-A and B identical unrelated individuals, if it is assumed that the MLC locus (or loci) displays a polymorphism comparable with that of the HLA-A and B loci. Secondly the weak or negative MLC reactions observed between approximately 10% of HLA sero-identical unrelated individuals suggest a

genetic linkage disequilibrium between the serologically defined HLA antigens (and particularly the HLA-B locus) and the determinants controlling strong MLC reactions.

II Once evidence for a separate MLC locus (or loci) had been obtained, family MLC studies were done on randomly chosen families with non-consanguineous parents (Chapter Seven), the San (Chapter Eight) and families selected for varying degrees of consanguinity between the parents (Chapter Nine).

The tests were performed according to the protocol set out on pg 184 in order to find the following :-

- (i) the frequency with which unrelated parents shared one MLC allele
- (ii) the identification of MLC homozygous individuals

(i) *Sharing of MLC Alleles*

Twenty-one randomly chosen families with eighty-three offspring in which the non-consanguineous parental pairs had varying degrees of HLA-A and B disparity, were tested. In only two of the families tested (Table VII.28), family 21 (= family DEW) and family 12, was there evidence of sharing of MLC alleles between the parents.

In *family DEW*, three of the children, sibs III-2, III-3 and III-4 were MLC negative with their respective HLA sero-identical parents. However, sib III-1, although also HLA-A,

B identical with the father, was MLC positive. The detailed family MLC experiment (Table VII.14), illustrates the point that identity for the serologically determined HLA antigens in parent-sib or sib-sib combinations does not necessarily result in MLC identity even when the parents share one MLC allele.

The only offspring in *family 12* was homozygous for the HLA-A, B haplotype shared by the parents. In the unilateral MLC experiments this child did not stimulate either of the parents, but in the reverse situation stimulation was observed, suggesting homozygosity of the MLC alleles in the child.

More recently Mempel et al. (1973b), Dupont et al. (1973), Lebrun et al. (1973) and others described several families, selected only on the basis of the parents sharing one of the more common HLA-A, B haplotypes, e.g. HLA-A1,B8;A3,B7 or A2,B12. The results proved that the MLC determinants on the shared parental haplotype were also identical. The incidence of such MLC haplo-identity between unrelated parents who are HLA-A, B haplo-identical was so much greater than in a randomly chosen family study such as ours, that this tends to confirm the linkage disequilibrium. Furthermore it indicates a relatively restricted polymorphism of the MLC determinants.

The MLC tests in *family GIA* (Table VII.27) suggested that the grandfather I-1 and his grandchild III-1 who shared the HLA determinant B7 on one haplotype, but differed for the A antigen on the same haplotype, were MLC haplo-identical. A common genetic source could not be traced for the two haplotypes in question. In 1973, Dupont et al. showed that the B locus antigen B7 is very frequently associated with a specific and relatively

common MLC determinant in the Danish population and called this determinant LD7a (now called DW2). They also mention that when DW2 is present in a single dose in related individuals, they react as haplo-identical siblings in the MLC tests. Finally they observed that the association between HLA-B7 and the MLC locus determinant DW2 may be even stronger than the association between HLA-A3 and B7. Recently, additional family studies have demonstrated that the association between MLC determinants and the HLA-A, B antigens is strongest between MLC- and the HLA-B determinants.

However, it must be emphasized that in spite of the observed genetic linkage disequilibrium between certain common HLA-A, and/or B antigens and the MLC determinants as well as the relatively restricted MLC polymorphism, this favours MLC identity between unrelated HLA-A and B identical individuals in a normal outbred population to a very limited extent.

(ii) *The Identification of MLC Homozygous Individuals*

Family MLC tests also constituted the basis for finding individuals who are MLC homozygous. The one-way MLC test was employed using Mitomycin-C treatment of cells as described in the Methods in Chapter Four. The basic concept is that, provided genic interaction does not occur as mentioned in Chapter Two, II.2, MLC homozygosity will result in reactions which are positive when the heterozygote stimulates the homozygote, but which are negative when the heterozygous cells are used as the responders. When testing a family for a possible homozygous cell, the most informative combinations to study are those of the HLA sero-identical parent-child. If this combination is mutually non-responsive, the HLA homozygous child must be MLC homozygous assuming that a recombination has not

occurred.

(a) *The San* at Tsumkwe (Chapter Eight), a small population group, living in the same geographically isolated region where genetic relatedness could be expected, was therefore included in our attempt to find MLC homozygous individuals.

Out of the five informative families (family IV was excluded on paternity testing, and family VI could not be HLA-A and B typed) that were HLA serologically typed and MLC tested, none of the parental pairs were HLA-A, B haplo-identical. In only one family, (family I), did the parents carry an identical B locus determinant, HLA-BW17. Furthermore, the father who was homozygous for HLA-BW17 also appeared to be MLC homozygous. In San family III the parents appeared to be MLC haplo-identical although HLA-A, B non-identical. This resulted in MLC identity between sib II-2 and the father, although they were HLA-A, B haplo-identical and sib II-1 was possibly MLC homozygous in spite of being HLA-A, B heterozygous. Unfortunately proof for this was not obtained because further unilateral MLC tests could not be performed.

However, evidence in support of the findings in this family comes from a recent report of a family study by Suciu-Foca and Dausset (1975), where negative MLC reactions were observed in the presence of a B locus incompatibility. The observation in families like these, that an HLA-A, B heterozygous cell can be MLC homozygous (demonstrating that one MLC determinant may occur together with more than one HLA-B determinant), further suggests that the polymorphism of the MLC system is relatively restricted. It also supports the concept that MLC activation is controlled by a separate gene(s).

Finally it should be mentioned that in two of the San families (I and II), the possibility of a recombination of the HLA-B and MLC locus (or loci) of one of the parental chromosomes was considered.

Without the opportunity to repeat and extend the experiments in this small but apparently interesting group, a formal conclusion about the genetic aspects of HLA-A and B and MLC and the degree of inbreeding among the San at Tsumkwe could not be drawn.

(b) Among families in which the parents were *consanguineous* (Chapter Nine) only family C00 proved to be of interest. The first-degree consanguineous union between father and daughter produced an HLA-A1,B8 homozygous child (III-1). This child should be genotypically homozygous for the whole MHC, including the MLC alleles, by descent (except for the remote possibility that a mutation has taken place). The introduction of the HLA-A1,B8 haplotype from outside was excluded by erythrocyte genetic studies. Indirect proof that the two identical serological haplotypes inherited by sib III-1 must also carry identical MLC determinants was obtained from the fact that the HLA sero-identical father-child combination (I-1 versus III-2) showed reciprocal non-stimulation.

It should, however, be noted that a weak response was observed in the unilateral MLC experiments when the homozygote, III-1, stimulated parental cells and cells from other family members heterozygous for the HLA haplotype of the homozygote. This was the first case of a homozygous cell derived from incest, where a weak but definite stimulation of parental cells was

reported (Du Toit, 1973). Others have observed this same phenomenon with homozygous cells from first-cousin marriages and from families in the outbred population (Eijssvoegel et al., 1971; Jorgensen et al., 1973; Thorsby and Piazza, 1975). Some of the explanations put forward for this weak response will be discussed later in this Section.

III MLC Typing

Mempel et al. (1973b) were the first to suggest that it was possible to use the MLC reactions to individuals known to be MLC homozygous to recognize the same MLC determinants in any unrelated individual, i.e. to do 'MLC typing' (for detailed explanation see Chapter Four, pg. 100).

The locus controlling strong MLC reactions was named HLA-D, following nomenclature established by the Sixth International Histocompatibility Workshop, 1975, and the HLA-D homozygous reference cell used for the assignment of a particular HLA-D specificity to a given individual was called a 'typing cell' (Thorsby and Piazza, 1975).

One of the most important difficulties encountered in MLC typing is that sharing of HLA-D determinants between homozygous stimulating and heterozygous responding cells seldom results in a completely negative MLC reaction, as for instance seen between HLA identical sibling combinations. Therefore a 'typing response' is rarely a true negative response. It is of particular interest to note that, even with an incestuous family such as family C00 where the MLC homozygous child should be genotypically homozygous by descent, the typing responses are of the same level of magnitude as the typing responses between

unrelated individuals. Therefore the weak responses characteristic of typing responses cannot be completely explained by differences at other loci within the MHC, or cross-reactions between similar HLA-D determinants.

Several explanations can be given for this phenomenon. It has been suggested that some kind of blastogenic factor released from inactivated homozygous stimulating cells upon recognition of the foreign MLC determinant(s) on the non-inactivated heterozygous responding cells, can induce mitotic activity of these cells. This has been called 'back stimulation' (Thorsby and Piazza, 1975).

Another possibility is allelic interaction, which implies that an allelic gene has to modify the phenotypic expression of a corresponding gene and even abolish it completely. If such an effect existed, one would expect it to be of equal strength on the two haplotypes in the parent-child HLA haplo-identity situation and not a modification of their relative identity (see Dupont et al., 1976, for review).

From the MLC typing experiment described in Chapter Ten, it is clear that the DW3 determinant, as it was recognized by the Sixth International Histocompatibility Workshop homozygous typing cells, of which III-1 (C00) (Workshop number 16005) was one, could be much more complex. This should therefore be taken into account when explaining the results with D locus homozygous typing cells. At present it is not clear whether these findings should be attributed to cross-reacting specificities of the same allelic system; to the involvement of products of subloci that exist in the population in strong positive linkage disequilibrium; or to disparities at other genetic determinants.

IV Histocompatibility

The ABO blood group system is the first genetic system that provides strong transplantation antigens, therefore it is of fundamental clinical importance and must be taken into account in all transplantations, except, perhaps, when the recipient suffers from severe combined immunodeficiency.

The importance of the major histocompatibility region in man (HLA) in skin and organ graft survival, in eliciting GvH and in blood component therapy is clearly established, but the significance of the individual components has been difficult to determine. HLA identity is of primary importance within families for skin transplantation and in kidney transplantation; the best graft survival is obtained with grafts obtained from HLA identical sibling donors. HLA identical sibs are probably identical not only for HLA-A, B and C, but also for HLA-D and most likely for the chromosomal region between these loci. This is in contrast to the situation when unrelated cadaver kidneys are used. Even when they are closely matched for the HLA-A, B and C antigens, as is currently done, this does not give a much higher chance that donor and recipient are identical for other loci within the complex. From all the transplantation data available in the world, it appears that HLA sero-identity alone is an inadequate predictor of skin or kidney allograft behaviour between unrelated individuals.

Nevertheless, cadaver kidneys are the most frequently used source of kidneys for transplantation, and, to overcome the problem of matching for the extremely polymorphic HLA system, national and regional organizations have been created to promote organ exchange. Because of the limited data in which

either the serological HLA antigens or the MLC determinants are the only variable, a conclusive answer cannot yet be reached concerning their relative importance in histocompatibility.

Matching for HLA-D, or some other closely associated determinants, could have a great influence on the prognosis of kidney grafts when unrelated donors are used (Van Rood et al., 1977). It has been shown that in the absence of strong MLC reactivity, very few cytotoxic lymphocytes develop (Eijssvoegel et al., 1973a), and it is possible that, in the absence of MLC activation, matching for the serologically determined HLA antigens may be of less clinical importance. At present, matching for D locus determinants by homozygous typing cells is impossible since the length required for an MLC test is about one week. It would be of great practical value if rapid methods for detection of D locus determinants and for Ia antigens, closely associated with D, were available. In this respect the use of a very recently described test, the Primed Lymphocyte-Typing (PLT) test, which may allow MLC typing within twenty-four hours, might be very useful (Bach et al., 1975; Sheehy et al., 1975). The detection of products of other loci in the MHC might possibly be of equal importance for the prognosis of graft survival.

Thus, the relative importance of the different loci of the MHC in clinical transplantation is yet poorly understood.

APPENDIX

B-I 31

Vir Afrikaans sien keersy.

DECLARATION FOR THE PURPOSE OF A MARRIAGE.

N.B.—Print clearly

PARTICULARS OF APPLICANT

1. Identity number_____
2. Surname_____
3. First names in full_____
4. Date of birth_____ 5. Country of birth_____
6. Marital status: State bachelor, spinster, widower, widow or divorcee, as the case may be.

7. Population group_____ 8. Sex_____
9. Permanent residential address_____

PARTICULARS OF PROSPECTIVE *HUSBAND/WIFE

10. Identity number_____
11. Surname (present legitimate surname in the case of a woman)_____
12. Maiden name_____
13. First names in full_____

I *declare under oath/solemnly declare that the particulars given above are to the best of my knowledge and belief true and correct and—

- (i) that we are not within the prohibited degrees of relationship (see footnote);
- (ii) that there is no lawful impediment to our marriage; and
- †(iii) that I have the written consent of *my parents/guardian/Commissioner of Child Welfare/the Minister of the Interior/a competent court, as the case may be.

19_____

Signature

* Delete whichever is not applicable.

† Delete the whole of the paragraph if applicant is a major.

The deponent has acknowledged that he/she knows and understands the contents of this affidavit which was sworn to/affirmed and signed before me at _____ on this the _____ day of _____ 19_____

Signature (Commissioner of Oaths)

Capacity

(Full names in block letters)

(Area for which appointed)

Footnote:

PROHIBITED DEGREES OF RELATIONSHIP

1. Attention is drawn to the fact that it is lawful—
 - (a) for a man to marry the sister or any female relative of his deceased or divorced wife, except an ancestor or descendant of such wife; and
 - (b) for a woman to marry the brother or any other male relative of her deceased or divorced husband, except an ancestor or descendant of such husband.
2. As a guide it should be mentioned that it is not lawful for a man to marry any person mentioned in column (1) or for a woman to marry any person mentioned in column (2):—

Column (1)	Column (2)	Column (1) (continued)	Column (2) (continued)
Mother.	Father.	Wife's father's mother.	Husband's father's father.
Daughter.	Son.	Wife's mother's mother.	Husband's mother's father.
Father's mother.	Father's father.	Wife's son's daughter.	Husband's son's son.
Mother's mother.	Mother's father.	Wife's daughter's daughter.	Husband's daughter's son.
Son's daughter.	Son's son.	Son's son's wife.	Son's daughter's husband.
Daughter's daughter.	Daughter's son.	Daughter's son's wife.	Daughter's daughter's husband.
Sister.	Brother.	Father's sister.	Father's brother.
Wife's mother.	Husband's father.	Mother's sister.	Mother's brother.
Wife's daughter.	Husband's son.	Brother's daughter.	Brother's son.
Father's wife.	Mother's husband.	Brother's daughter's daughter.	Brother's son's son.
Son's wife.	Daughter's husband.	Brother's son's daughter.	Brother's daughter's son.
Father's father's wife.	Father's mother's husband.	Sister's daughter.	Sister's son.
Mother's father's wife.	Mother's mother's husband.	Sister's daughter's daughter.	Sister's son's son.
		Sister's son's daughter.	Sister's daughter's son.

24. Also (prohibited are)
 Women already married,
 Except those
 Whom your right hands possess : ⁵³⁷
 Thus hath God ordained
 (Prohibitions) against you :
 Except for these, all others
 Are lawful, provided
 Ye seek (them in marriage)
 With gifts from your property,—
 Desiring chastity, not lust. ⁵³⁸
 Seeing that ye derive
 Benefit from them, give them
 Their dowers (at least) ⁵³⁹
 As prescribed ; but if,
 After a dower is prescribed, ye
 agree
 Mutually (to vary it),
 There is no blame on you,
 And God is All-knowing,
 All-wise.

٢٤- وَالْمُحْصَنَاتُ مِنَ النِّسَاءِ
 إِلَّا مَا مَلَكَتْ أَيْمَانُكُمْ
 كَتَبَ اللَّهُ عَلَيْكُمْ
 وَأُحِلَّ لَكُمْ مِمَّا وَرَاءَ ذَلِكَ
 أَنْ تَبْتَغُوا بِأَمْوَالِكُمْ
 مُحْصَنِينَ غَيْرَ مُسْفِهِينَ
 فَمَا اسْتَمْتَعْتُمْ بِهِ مِنْهُنَّ
 فَأْتُوهُنَّ أَجُورَهُنَّ فَرِيضَةً
 وَلَا جُنَاحَ عَلَيْكُمْ
 فِيمَا تَرَاضَيْتُمْ بِهِ مِنْ بَعْدِ الْفَرِيضَةِ
 إِنَّ اللَّهَ كَانَ عَلِيمًا حَكِيمًا

25. If any of you have not
 The means wherewith
 To wed free believing women,
 They may wed believing
 Girls from among those
 Whom your right hands possess : ⁵⁴⁰
 And God hath full knowledge
 About your Faith.
 Ye are one from another :
 Wed them with the leave
 Of their owners, and give them
 Their dowers, according to what

٢٥- وَمَنْ لَمْ يَسْتَطِعْ مِنْكُمْ
 طَوْلًا أَنْ يَنْكَحَ الْمُحْصَنَاتِ الْمُؤْمِنَاتِ
 فَمِنْ مَا مَلَكَتْ أَيْمَانُكُمْ
 مِنَ فَتَيَاتِكُمُ الْمُؤْمِنَاتِ
 وَاللَّهُ أَعْلَمُ بِإِيمَانِكُمْ بَعْضُكُمْ مِنْ بَعْضٍ
 فَانْكِحُوهُنَّ بِإِذْنِ أَهْلِهِنَّ
 وَأَتُوهُنَّ أَجُورَهُنَّ بِالْمَعْرُوفِ

537. Whom your right hands possess : i.e., captives in a Jihād, or war under the orders of the righteous Imām against those who persecute Faith. In such cases formal hostility dissolves civil ties.

538. After defining the prohibited degrees, the verse proceeds to say that women other than those specified may be sought in marriage, but even so, not from motives of lust, but in order to promote chastity between the sexes. Marriage in the original Arabic is here described by a word which suggests a fortress (ḥiṣn) : marriage is, therefore, the fortress of chastity.

539. As the woman in marriage surrenders her person, so the man also must surrender (besides some part of his independence) at least some of his property according to his means. And this gives rise to the law of Dower. A minimum dower is prescribed, but it is not necessary to stick to the minimum, and in the new relationship created, the parties are recommended to act towards each other with the greatest confidence and liberality.

540. That is, captives taken in a Jihād : see note 537 above. "Your right hands" does not mean necessarily that she has been assigned to you, or is your property. All captures in war belong to the community ; they are "yours" in that sense. If you seek such a person in marriage, do it from no base motives. Safeguard your faith, and see that she too does believe. In that case, after all, she is of the human brotherhood, and her condition is accidental and redeemable. If the slave bore a child to her master, she would become free. The slave condition is now out of date, in the true spirit of Islam. But there are other conditions in which a woman's (or man's) freedom is restricted, and the principle would apply there also.

Is reasonable : they should be
Chaste, not lustful, nor taking
Paramours : when they
Are taken in wedlock,
If they fall into shame,
Their punishment is half
That for free women.
This (permission) is for those
Among you who fear sin ;
But it is better for you
That ye practise self-restraint.
And God is Oft-forgiving,
Most Merciful.

SECTION 5.

26. God doth wish
To make clear to you
And to show you
The ordinances of those
Before you ; and (He
Doth wish to) turn to you
(In Mercy) : and God
Is All-knowing, All-wise.

27. God doth wish
To turn to you,
But the wish of those
Who follow their lusts
Is that ye should turn
Away (from Him),—
Far, far away.

28. God doth wish
To lighten your (difficulties) :
For man was created
Weak (in flesh).

29. O ye who believe ! ⁵⁴¹
Eat not up your property

مُتَّصِنَاتٍ غَيْرِ مُسْتَفْعَاتٍ
وَلَا مُتَّخِذَاتٍ أَخْدَانٍ
فَإِذَا أَحْصَيْنَ فَإِنَّ اتَيْنَ بِثُلَاثَةِ
فَعَلَيْهِنَّ نِصْفُ مَا عَلَى الْمُحْصَنَاتِ مِنَ
الْعَذَابِ ذَلِكَ لِمَنْ خَشِيَ الْعَنَتَ مِنْكُمْ
وَأَنْ تَصْبِرُوا سَخِيرٌ لَكُمْ
وَاللَّهُ غَفُورٌ رَحِيمٌ ۝

٢٦- يُرِيدُ اللَّهُ لِيُبينَ لَكُمْ
وَيَهْدِيَكُمْ سُنَنَ الَّذِينَ مِنْ قَبْلِكُمْ
وَيَتُوبَ عَلَيْكُمْ
وَاللَّهُ عَلِيمٌ حَكِيمٌ ۝

٢٧- وَاللَّهُ يُرِيدُ أَنْ يَتُوبَ عَلَيْكُمْ
وَيُزِيلَ الَّذِينَ
يَتَّبِعُونَ الشَّهَوَاتِ
أَنْ تُبْسِلُوا مِثْلَ هَذِهِمُ ۝

٢٨- يُرِيدُ اللَّهُ أَنْ يُخَفِّفَ عَنْكُمْ
وُخْلُقَ الْإِنْسَانَ ضَعِيفًا ۝

٢٩- يَا أَيُّهَا الَّذِينَ آمَنُوا لَا تَأْكُلُوا أَمْوَالَكُمْ

541. Let me paraphrase this verse, for there is profound meaning in it. (1) All your property you hold in trust, whether it is in your name, or belongs to the community, or to people over whom you have control. To waste is wrong. (2) In ii. 1-3 the same phrase occurred, to caution us against greed. Here it occurs, to encourage us to increase property by economic use (traffic and trade), recalling Christ's parable of the Talents (Matt. xxv. 14-30), where the servants who had increased their master's wealth were promoted and the servant who had hoarded was cast into darkness. (3) We are warned that our waste may mean our own destruction ("nor kill or destroy people's lives. We must commit no violence. This is the opposite of "trade and traffic by mutual good-will." (4) Our violence to our own brethren is particularly preposterous, seeing that God has loved and showered His mercies on us and all His creatures.

MATERIALS SHIPPED BY AIR TO WINDHOEK

<u>Quantity</u>	<u>Article</u>
150	30 ml plastic syringes
200	needles
2500	tissue culture tubes (Falcon, Cat. No. 3033)
750	50 ml tubes (Falcon, Cat. No. 2070)
1450	Pasteur pipettes
7200	culture tubes (Nunc, Cat. No. 1090)
7200	caps for tubes (Nunc, Cat. No. 1095)
25	leukocyte pipettes
15	counting chambers (Bürker Türk)
200 ml	Türk solution
300	1 ml disposable pipettes (Falcon, Cat. No. 7506)
500	5 ml disposable pipettes (Falcon, Cat. No. 7529)
400	10 ml disposable pipettes (Falcon, Cat. No. 7551)
60	1 ml luer syringe (Johnson)
100	luer needle 0.9 x 50 mm (Johnson)
80	carton boxes (empty)
6	test tube racks (8 holes)
6	racks (Tomado)
18	glass pencils
200	protocol papers
20	printed papers
1	plastic tray 20 x 20 x 7 cm

<u>Quantity</u>	<u>Article</u>
2	biopettes (B.D. 00.10.29)
2000	biotips 1 ml (B.D. 00.10.30)
4	pairs of forceps
6300	filters 13 mm (Millipore)
1000	slides (Menzel) 76 x 26 mm
2000	coverglasses (Menzel) 26 x 21 mm
3	pipette balloons
20	pipette nipples
2	filtration apparatus
50	syringes (30 ml)
50	filter-holders
5	leukocyte pipette tubes
25	pieces of short tubing
10	plastic racks (66 holes)
1	roll autoclave tape
10	plastic bags (empty)
4	sterile pairs of forceps
25	tissue culture flasks 250 ml (Falcon, Cat. No. 3024)
110	Linbro disposable trays (54 holes)
20	portfolios
2	incubators ($37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) one extra in case of breakdown
6	centrifuges
1	waterbath (37°C)
3	vacuum flasks (1 of 1 litre, 2 of 5 litres) and rubber connexions
3	microscopes
3	electric cords with an appropriate variety of plugs and adaptors
	insulation tape
1	pipette shaker for leukocyte counting pipettes, should hold 6-8 pipettes

<u>Quantity</u>	<u>Article</u>
1	cell counter
1	whirlmix (with adjustable speed)
15 L	saline
60 L	distilled water

Refrigerator space for :-

AB serum	11 bottles	(refrigerator essential)
MEM-S medium	100 bottles	(refrigerator desirable)
Ficoll-Isopaque	10 bottles	(refrigerator desirable)

(Once MEM-S and Ficoll-Isopaque bottles opened, refrigeration essential).

One test tube rack with PHA, Mitomycin-C, etc. (refrigerator essential).

One plastic box with thymidine-2-C¹⁴ (refrigerator essential).

Deep freeze space for :-

5 tubes (Falcon, Cat. No. 3033) penicillin and streptomycin stock solution.

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V.P. Eijssvoegel⁺, J.J. van Rood^o, E.D. du Toit* and P.Th.A. Schellekens⁺

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology of the University of Amsterdam⁺, the Department of Immuno-Haematology of the University Hospital, Leyden^o and the Provincial Blood Grouping Laboratory, Cape Town*

Position of a locus determining mixed lymphocyte reaction distinct from the known HL-A loci*

Bilateral and unilateral mixed lymphocyte culture (MLC) tests were performed in five families, each containing one sibling in which recombination between the genes determining the antigens of the LA and Four segregant series of HL-A had occurred. The results confirm the very weak or non-stimulatory effect of antigens of the LA segregant series, bringing to seven the total number of alleles for which this has been shown so far. Further anomalies in one of the families, however, indicate that the antigens of the Four segregant series are not fully responsible for MLC activation either. Of a group of three HL-A identical siblings, one proved to be MLC-positive with the other two, but MLC-negative with another group of two HL-A identical siblings, which group displayed a one-allelic difference for HL-A with the former group. Furthermore, the child showing this unexpected MLC reaction pattern proved also to be MLC-negative with the earlier mentioned sibling who had the serologically demonstrable recombination between the LA and Four series, notwithstanding a difference for three HL-A antigens.

These findings are interpreted as strong evidence for the occurrence of a recombination between the HL-A loci and a MLC determining locus, which can thus be localized outside the previously recognized HL-A region close to the Four locus. The relationships between HL-A, MLC, skin graft rejection and organ transplantation are re-examined in the light of this concept.

1. Introduction

Studies in sibling combinations have shown that mixed lymphocyte culture (MLC) reactivity is closely associated with the HL-A loci [1-3].

Recent investigations [4-7] of the MLC reactions of a total of five individuals with a serologically demonstrable recombination between the LA (or first) and Four (or second) segregant series of HL-A with their respective siblings, indicated that a difference only for the LA series, at least for the antigens studied (HL-A1, HL-A2, HL-A3, A028, W 28), did not elicit a MLC response. A difference only in antigens of the Four series (HL-A7, W27, HL-A12, W15, A041) was always accompanied by pronounced MLC reactivity. This parallels

the situation in the mouse, in which the predominant role of the K-region of the H2-locus in MLC reactivity has been described [8].

Among unrelated individuals, however, such an association between HL-A and MLC reactivity is considerably less evident [9-14], notwithstanding the fact that a few non-related HL-A-identical individuals have been found to be MLC-identical as well [9-11, 15].

Serological heterogeneity of the HL-A antigens has long been held responsible for this discrepancy. The fact, however, that the actual gene products eliciting MLC reactivity have never been conclusively identified as being the HL-A antigenic determinants as such, allows the assumption that MLC reactivity may not be controlled by the genes of the Four locus either, but by a separate polymorphic genetic system, closely linked to HL-A. Evidence for this latter concept, which was initially raised as a possibility by Amos and Bach [1], Plate et al. [16], Kissmeyer-Nielsen et al. [17] and ourselves [9, 10], and recently put forward in an elegant hypothesis by Yunis and Amos [13] and discussed in detail by Ceppellini [18], might be obtained from the demonstration of recombination between the HL-A loci and a MLC-controlling genetic entity.

* A brief account of some of this work was presented at the Symposium on Cellular Aspects of Transplantation Immunology, February 1972, Paris.

Correspondence: Vincent P. Eijssvoegel, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam-W., The Netherlands

Abbreviations: MLC: Mixed lymphocyte culture

The few reported positive MLC reactions between HL-A identical siblings [1, 4, 16, 19] may well be the result of such crossing-over, although other explanations, such as preimmunization [10] could not always be excluded. Stronger evidence for such crossing-over might be derived from the demonstration of an absence of MLC reactivity of such a recombinant, when tested with siblings differing for one of the HL-A haplotypes.

So far, one family has been reported in the literature by Yunis and Amos [13] in which recombination between HL-A and a MLC-locus has been made highly probable. These data formed the basis on which these authors formulated their hypothesis.

The present MLC studies involve five families each containing a sibling with serologically demonstrable recombination between the LA and Four segregant series.

In addition to a confirmation in all cases studied of the limited function of the LA locus in regard to MLC reactivity, strong evidence was obtained for the occurrence of a crossing-over between the Four locus and a MLC-locus in a sibling of one of these families.

2. Material and methods

2.1. HL-A typing

Lymphocytes were tested using a modified Kissmeyer-Nielsen lymphocytotoxicity test [20, 21] and a microagglutination technique [21]. A complement fixation test on platelets [22] was also performed. Altogether, a panel of 120 well-established antisera was used. All families were typed twice with the exception of family MEL. The five families studied were selected from a series of 276 consecutively typed families, containing 12 possible recombinants*. Selection was based on the following criteria: family size, availability for further experiments and conclusiveness of the serological findings on which the assumption of recombination was based.

2.2. Mixed lymphocyte culture

Bilateral and unilateral MLC tests were performed in a modification of a previously described technique [23]**. Lymphocytes were separated from defibrinated blood by Ficoll-Iso-

* Van Rood, J.J. and van Leeuwen, A., unpublished data.

** Du Bois, M.J.G.J., Huismans, L., Schellekens, P.Th.A. and Eijvoogel, V.P., submitted for publication.

paque gradient centrifugation [24]. After washing and electronic cell counting, the lymphocytes were resuspended in a concentration of 3×10^5 per ml in Minimum Essential Medium for spinner cultures (MEM-S, Gibco, Grand Island, N.Y. U.S.A.) buffered with Tris (0.025 M) and supplemented with 20 % fresh heat-inactivated human AB serum, penicillin (100 I.U. per ml), and streptomycin (100 µg per ml). For unilateral MLC tests mitomycin-C treatment was applied [25]. The total volume per culture tube was 1 ml and the two suspensions were always mixed in a 1 : 1 ratio.

The cells were cultured in polystyrene vials (Nunc, Roskilde, Denmark, cat. no. 1090) at 37 °C. To determine the reactivity, [^{3} H]thymidine (0.075 µCi, specific activity 6.25 mCi/mM, C.E.N., Mol, Belgium) was added 120 h after the initiation of the culture. The cells were harvested after a further 24 h of incubation, collected and washed on glass fiber filters (Millipore, Bedford, Mass., U.S.A.), which were then dried. Counting was performed during 10 min in a Packard liquid scintillation counter, using 2 ml of scintillation fluid. All cultures were performed in triplicate and family KA was studied at two different occasions with virtually similar results. The arithmetical mean and the standard error of each set of triplicate cultures were determined and expressed as cpm. In each experiment control MLC's were carried out with the lymphocytes of a healthy, non-related individual (X).

3. Results

3.1. General remarks

The HL-A phenotypes and haplotypes of each member of the five families are shown in Table 1. The last child of each family represents the recombinant of the LA and Four segregant series.

The results of the bilateral and unilateral MLC between the recombinants and their respective siblings together with the controls are given in Table 2.

3.2. Family MEL

As the father of this family had died, his HL-A genotype had to be deduced from the findings in the other family members. The assumption of recombination is based, in this case, on the absence of the antigen W28 in child #9 rather than on the presence of an antigen not occurring in the mother.

Table 1. HL-A typing and haplotypes of five families

Family MEL			Family HOP			Family HA			Family STE			Family KA		
P	(3-W5/W28- 5) ^a	ab	P	2-8/9-5	ab	M	2- 12/11-W5	ab	P	11-5/ 9- 8	ab	P	2- 8/11-W5	ab
M	3- 7/W28-W5	cd	M	1-5/1-8	cd	P	3-? / 1- 8	cd	M	2-7/W28-W5	cd	M	3- 7/ 1- 8	cd
Child			Child			Child			Child			Child		
1.	W28- 5/ 3- 7	bc	1.	2-8/1-8	ad	1.	11-W5/ 3-?	bc	1.	11-5/ 2- 7	ac	1.	11-W5/ 3- 7	bc
2.	W28- 5/ 3- 7	bc	2.	9-5/1-5	bc	2.	2- 12/ 3-?	ac	2.	11-5/ 2- 7	ac	2.	11-W5/ 3- 7	bc
3.	3-W5/W28-W5	ad	3.	9-5/1-8	bd	3.	1- W5/ 1- 8	bd	3.	11-5/W28-W5	ad	3.	11-W5/ 1- 8	bd
4.	3-W5/W28-W5	ad	4.	9-5/1-8	bd	4.	11-W5/ 1- 8	bd	4.	9-8/W28-W5	bd	4.	11-W5/ 1- 8	bd
5.	3-W5/W28-W5	ad	5.	2-8/1-5	ac	5.	11-W5/ 1- 8	bd				5.	11-W5/ 1- 8	bd
6.	3-W5/ 3- 7	ac				6.	2- 12/ 1- 8	ad				6.	2- 8/ 1- 8	ad
7.	3-W5/ 3- 7	ac										7.	2- 8/ 1- 8	ad
8.	W28- 5/W28-W5	bd										8.	2- 8/ 3- 7	ac
												9.	2- 8/ 3- 7	ac
9.	3- 5/ 3- 7	a/b c	6.	2-5/1-5	a/b c	7.	2-W5/ 3-?	a/b c	5.	11-8/ 2- 7	a/b c	10.	2-W5/ 3- 7	ad

a) Deducted from the findings in the other family members. W28 = Ba*; W5 = R*.

Table 2. Bilateral and unilateral MLC involving recombinants between LA and Four segregant series of HL-A in five families

HL-A difference	Family MEL			Family HOP			Family HA			Family STE			Family KA		
	sib #	cpm	s.e.a)	sib #	cpm	s.e.	sib #	cpm	s.e.	sib #	cpm	s.e.	sib #	cpm	s.e.
<i>First series only:</i>															
<i>a/b c + bc</i>	9 +1	243	23	6 +2	176	64	7 +1	406	110				10 +1	62	14
	9 _m +1 _b)	80	15	6 _m +2	244	78	7 _m +1	157	52				10 _m +1	55	19
	9 +1 _m	216	11	6 +2 _m	96	23	7 +1 _m	140	29				10 +1 _m	53	27
	9 +2	174	23										10 +2	63	9
	9 _m +2	88	37										10 _m +2	71	13
	9 +2 _m	69	3										10 +2 _m	142	21
<i>Second series only:</i>															
<i>a/b c + ac</i>	9 +7	4 710	444	6 +5	4 651	155	7 +2	2 880	169	5 +1	1 314	63	10 +8	3 035	307
	9 _m +7	2 797	166	6 _m +5	3 136	76	7 _m +2	2 629	140	5 _m +1	1 009	35	10 _m +8	2 540	328
	9 +7 _m	2 731	883	6 +5 _m	2 532	359	7 +2 _m	3 060	318	5 +1 _m	1 335	332	10 +8 _m	1 223	123
	9 +6	4 137	147							5 +2	1 128	308	10 +9	1 069	75
	9 _m +6	3 190	559							5 _m +2	1 011	77	10 _m +9	1 041	215
	9 +6 _m	2 964	281							5 +2 _m	909	74	10 +9 _m	690	32
<i>Controls:</i>															
	9 +X _m	2 806	360	6 +X _m	3 968	320	7 +X _m	3 130	271				10 +X _m	2 905	649
	1 +X _m	1 985	412	2 +X _m	4 564	708	1 +X _m	2 913	292				1 +X _m	2 519	300
	2 +X _m	2 745	283	6 _m +X	2 544	18	7 _m +X	3 043	90				2 +X _m	2 010	273
	9 _m +X	3 448	374	2 _m +X	3 023	470	1 _m +X	2 199	214				10 _m +X	3 966	241
	1 _m +X	3 266	443										1 _m +X	3 095	252
	2 _m +X	5 448	452										2 _m +X	4 451	130
	9	95	5	6	15	1	7	41	6	5	21	1	10	20	4
	1	46	12	2	34	5	1	95	43	1	20	4	1	21	8
	2	155	37	5	52	13	2	95	27	2	28	10	2	34	17
	6	308	157										8	125	4
	7	89	1										9	26	5

a) s.e. = standard error; b) m = mitomycin-C-treated.

The children # 1 and # 2, differing with the recombinant # 9 only for a LA series determinant (W28) are neither stimulated by, nor stimulate, the recombinant. The children # 6 and # 7 who differ with the recombinant only for a Four series determinant (HL-A5 versus W5) both stimulate and are stimulated at levels similar to the reactions with the control X.

3.3. Family HOP

The child # 2 differs with the recombinant # 6 for a LA series determinant (HL-A2 versus HL-A9) only, which results in marginal reactivity when compared with the control (less than 10 %). Child # 5, differing with the recombinant for a Four series determinant (HL-A5 versus HL-A8) only, stimulates and is stimulated at normal levels.

3.4. Family HA

Child # 1 differs with the recombinant # 7 for a LA series determinant (HL-A2 versus HL-A11) only, which results in very weak to marginal stimulation (~ 5 % of the control). Child # 2 differs for an antigenic determinant of the Four series (W5 versus HL-A12) only, which results in normal stimulation in the bilateral and both unilateral tests.

3.5. Family STE

As the bc genotype was not represented in this family, only a difference for a Four series determinant (HL-A8 versus HL-A5) could be evaluated involving the children # 1 and # 2 with the recombinant # 5. Normal bilateral and unilateral reactivity was found in both cases.

3.6. Family KA

The children # 1 and # 2, differing with the recombinant # 10 for a LA series determinant (HL-A2 versus HL-A11)

only, showed virtually no stimulatory capacity or response in this combination, while the children # 8 and # 9, who differ only for a determinant of the Four series (W5 versus HL-A8), demonstrated consistent stimulation and response, when tested with the recombinant.

In contrast to all the other HL-A identical siblings in the families described above, which showed no MLC-reactivity at all, an unexpected pattern was found in family KA (Table 3).

The combination of the children # 1 and # 2, both of the genotype bc was indeed MLC-negative. Of the genotype bd, the combination of the children # 4 + # 5 was also negative, but the combination # 4 + # 3 and # 5 + # 3 were evidently positive in the bilateral and unilateral MLC's notwithstanding the fact that child # 3 carried the same antigens of the Four series (W5 and HL-A8) as the children # 4 and # 5. Further analysis showed that child # 3 was MLC-negative with the children # 1 and # 2, who carried the antigen HL-A7, thus belonging to the genotype bc. This indicates that child # 3 belongs to the MLC-identical group of children # 1 and # 2 and not to the MLC-identical group, consisting of the children # 4 and # 5. Finally, child # 10 with the genotype a/b,c neither stimulated nor responded to sibling # 3, notwithstanding a difference for three HL-A antigens, although all the reactions performed with the siblings # 4 and # 5 were clearly positive. The other HL-A identical siblings combinations (children # 6 + # 7 of the genotype ad and children # 8 + # 9 of the genotype ac) were MLC-identical.

4. Discussion

The reliability of the serological data is essential for the interpretation of our MLC findings. With some restriction for family MEL (see section 3.2.) and apart from the unlikely

Table 3. Bilateral and unilateral MLC involving the siblings 1, 2, 3, 4, 5, 6, 7, 8 and 9 of family KA

HL-A genotype combination	sib	cpm	s.e.
bc + bc	1 + 2	33	4
	4 + 5	37	5
	4 _m + 5 ^{a)}	72	10
	4 + 5 _m	135	25
	4 + 3	2 124	384
	4 _m + 3	1 061	99
	4 + 3 _m	2 605	193
	5 + 3	3 982	574
	5 _m + 3	1 787	258
	5 + 3 _m	1 903	260
bc + bd	1 + 4	1 969	152
	1 _m + 4	1 578	182
	1 + 4 _m	1 689	188
	2 + 4	1 655	354
	2 _m + 4	1 550	108
	2 + 4 _m	1 817	84
	1 + 5	2 108	486
	1 _m + 5	1 772	211
	1 + 5 _m	1 489	109
	2 + 5	3 078	212
	2 _m + 5	2 078	125
	2 + 5 _m	1 318	117
	1 + 3	122	34
	1 _m + 3	37	20
	1 + 3 _m	344	53
	2 + 3	105	39
a/b c + bd	2 _m + 3	58	15
	2 + 3 _m	338	32
	10 + 3	353	92
	10 _m + 3	233	72
	10 + 3 _m	192	25
	10 + 4	2 452	55
	10 _m + 4	2 048	140
	10 + 4 _m	n.d. b)	
	10 + 5	2 394	239
	10 _m + 5	1 770	158
ad + ad	10 + 5 _m	1 466	105
	6 + 7	117	37
ac + ac	8 + 9	167	43

a) m = mitomycin-C-treated.

b) n.d. = not determined.

occurrence of illegitimacy, the presence of a child with recombination in each of these families seems rather conclusive.

The six bilateral and twelve unilateral MLC-tests between the HL-A recombinant children and their respective siblings, who differ only for an antigenic determinant of the LA segregant series, show no or marginal reactivity. On the other hand, normal, positive MLC reactions are found in the eight bilateral and sixteen unilateral tests involving differences for determinants of the Four segregant series only. So far, these data confirm and extend the earlier mentioned findings from Yunis et al. [4], Dupont et al. [5], Lebrun et al. [6] and Gatti et al. [7], bringing to seven the total number of antigens from the LA segregant series, shown not to elicit pronounced MLC reactivity (HL-A1, HL-A2, HL-A3, A028, W28, HL-A9, HL-A11). From gene frequency data, it can be calculated that these together comprise 93 % of the genes of the LA segregant series of the Caucasian population [26]. Although it is still possible that the products of the remaining 7 % of the genes elicit normal MLC reactions, the very limited function

of this locus in regard to the MLC is obvious and it seems justified to postulate that normal MLC reactivity is independent of this genetic system.

The further findings in family KA reveal that child # 3 displays an unexpected MLC reaction pattern in the two positive bilateral and the four positive unilateral reactions with its HL-A identical siblings # 4 and # 5. In previous studies [10, 27] we have provided evidence that the exceptional finding of MLC reactivity between HL-A identical siblings might be explained by preimmunization. Such immunization could have been caused by pregnancy, bloodtransfusions, skin grafting or cross-reactivity of bacterial sensitization.

In our tests, however, immunity due to skin grafting as demonstrable in the MLC persisted for only 2 - 3 weeks. It seems, therefore, extremely unlikely that immunization could explain the positive reactions between child # 3 and siblings # 4 and # 5, especially since similar results were obtained with an interval of 8 weeks.

The additional finding that child # 3 was MLC-negative with both the siblings # 1 and # 2, who differed for one haplotype with # 3, indicates that the positive reactions in the combinations # 3 + # 4 and # 3 + # 5 will rather have to be explained by a separate inheritance of the HL-A antigens and the MLC determinants, probably by recombination, which implies that MLC is independent from both the LA and Four locus.

Final evidence for the importance of this MLC locus emerges from the fact that the MLC reactions between the siblings # 3 and # 10 are virtually negative, regardless of a difference for three HL-A antigens. Consequently, this separate MLC locus can now be mapped not in between but outside the previously recognizable HL-A region, close to the Four locus. Localization between the LA and Four locus would require the assumption of a double recombination to explain the above results. This seems rather unlikely as the impression emerges from the overall data regarding MLC-positive HL-A identical siblings that the crossing-over frequency between HL-A and MLC is of about the same order of magnitude as that between the LA and Four locus.

The concept of a separate MLC locus explains, firstly, the frequent finding [9-14] of MLC reactivity between carefully serotyped, HL-A identical, unrelated individuals, if it is assumed that the MLC locus displays a polymorphism comparable to that of HL-A. The findings [9-11, 15] of the few HL-A identical, MLC-negative, unrelated individuals suggest a degree of linkage disequilibrium similar to that found between the LA and Four segregant series of HL-A [13].

The existence of a separate MLC locus could also explain why we have never been able to induce normal lymphocyte reactivity with allogeneic platelets, which are known to carry HL-A antigens. It may well be that these cells lack the MLC determinants, which may also be absent from the lymphocyte extracts, which proved to be non-stimulatory for many investigators [28-30], although the extraction procedure could be expected to preserve at least some of the HL-A antigens. In this respect it might be noted that recent reports [31]* describe lymphocyte transformation in rats and humans elicited by allogeneic epidermal cells. Epidermal cells of mice are reported to carry the theta-antigen [32].

* Leguit, P. and Eijssvoegel, V.P. (unpublished observations).

Our previously reported finding [10] that the lymphocytes of a patient with thymic dysplasia, although carrying HL-A antigens, did not stimulate the cells of any of the four unrelated non-HL-A-identical normal individuals tested in the MLC, may be relevant to this and suggest a possible relationship between the thymus and those cells which bear lymphocyte-stimulating determinants.

It should be pointed out, however, that four of the twelve MLC reactions reported here between siblings differing only for a LA series determinant displayed marginal but not completely negative reactions (see Table 2). This may indicate that, besides the above-described locus controlling, pronounced MLC reactivity, other systems exist which can elicit very weak MLC reactivity. The fact that such weak stimulation occurred in some, but not all, of the recombinants may suggest that at least one such locus is situated between the Four and LA series, close to the latter. This might well parallel the findings of Klein et al. [33] and Festenstein [34] of occasional stimulation by D-end differences in the mouse and fits with the duplication model as postulated by Shreffler et al. [35] for the H2 region in the mouse. As it has been described [14, 18] that HL-A identical, unrelated individuals, which are MLC-positive, show on the average a weaker stimulation than do HL-A non-identical pairs, this may suggest that these different MLC loci can exert cumulative effects.

In the light of the present findings it seems difficult to explain the inhibitory effect of antibody directed against antigens of the LA or Four segregant series on MLC-reactivity [36, 37] by a masking of the specific stimulating determinants. The fact, however, that HL-A antibody also inhibits the responding cells, either in the MLC or in antigen-stimulated lymphocyte transformation, together with the finding that xenogeneic anti-L-chain serum [38] and anti-lymphocyte serum [39, 40] causes a similar inhibition, indicates that the binding of antibody to the lymphocyte membrane *per se* probably interferes with either its stimulating or responding capacity, possibly by hampering lymphocyte-lymphocyte or lymphocyte-macrophage interaction.

The question arises whether graft rejection is primarily determined by incompatibilities of the HL-A loci or of this major MLC locus. If we accept the latter to be the case, it becomes very easy to explain why the improvement of kidney graft survival by HL-A typing has been so much more significant with sibling donors than with unrelated donors.

It might be argued that the studies we performed earlier in collaboration with others [10, 41] do not seem to indicate a predominant role of this MLC locus or of the HL-A locus in skin graft rejection. In these experiments, involving five HL-A identical, unrelated individuals, experimental skin grafts from four of these were transplanted to the fifth. Two of the donors were MLC-negative with the recipient and also among each other; the other two showed a clearly positive reaction with the recipient as well as among each other. The grafts from both groups were equally rejected in twelve days and the control graft in eleven days. Although these studies concerned only one recipient and more such experiments will have to be performed before definite conclusions can be drawn, it should be considered that studies in the mouse [42, 43] have shown that multiple minor locus incompatibilities can exert cumulative effects on skin graft rejection and finally mimic a major locus difference in skin graft survival time. The existence of a similar effect of minor loci on skin graft

survival in man seems very well feasible, when the range in the data reported by Ceppellini [44] is considered. This author reported the survival times in days, together with the standard deviation of skin grafts exchanged between HL-A identical siblings to be 20.4 s.d. 4.23 ($n = 12$) and between unrelated, non-selected individuals 12.11 s.d. 1.55 ($n = 206$).

Organ graft rejection, on the other hand, might well be dependent on one single (histocompatibility) locus only. Simonsen [45] formulated his one-locus hypothesis for organ graft rejection on the basis of the human kidney transplantation data, although these were heavily "contaminated" by immunosuppression. To prove this hypothesis, Bildsoe et al. [46] performed kidney, heart and skin graft experiments in rats, from donors belonging to segregating F_2 hybrid populations to recipients of one of the parental strains. The presence or absence of a major locus incompatibility was determined by serological typing and MLC, showing a 100% correlation. Both in the major locus identical and non-identical combinations, skin graft rejection was invariably rapid. Kidney and heart transplants were rejected in an acute or subacute manner in those combinations, which were incompatible for the major locus, with one single exception. Similar grafts were still surviving up to 300 days, if no major locus incompatibility existed. These findings were demonstrated in two different strain combinations, thus confirming the one-locus hypothesis for organ graft rejection, contrasting in this respect with the rejection of skin grafts.

If we assume, on the basis of the human kidney transplant data, that the HL-A locus is not of sole importance for organ transplantation, neither the above-mentioned skin graft experiments between unrelated individuals nor the one-locus hypothesis seems to be in conflict with the concept that this MLC locus is either on its own, or in combination with HL-A or any other closely linked locus, controlling organ graft rejection.

The point could be raised whether the predominant role of the passenger lymphocyte in the sensitization of the recipient by organ transplantation [47-51] may be associated with the correlation between the one-locus dependency of MLC reactivity and organ graft rejection.

Further experiments with animals, in which recombination has occurred between the serologically demonstrable, so-called major histocompatibility locus and the (major) MLC locus, will have to be performed to elucidate the exact role of each of these loci in organ graft rejection.

After this study was completed, evidence for a separate MLC locus was also reported by Sasportes et al. [52].

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Mixed Lymphocyte Culture and HL-A

By V. P. Eijssvoegel, L. Koning, L. de Groot-Kooy, L. Huismans,
J. J. van Rood, A. van Leeuwen, and E. D. duToit

THE CORRELATION between HL-A and mixed lymphocyte culture (MLC) is well-established among siblings.¹⁻³ Recent studies^{4,6} involving four individuals with serological evidence of recombination between the LA (or first) and Four (or second) segregant series described that only the differences in determinants of the Four segregant series were accompanied by MLC reactivity. This parallels the situation in the mouse, in which the predominant role of the K region of the H-2 locus in MLC reactivity has been described.⁷ Among unrelated individuals, however, the correlation between HL-A and MLC is considerably less evident.⁸⁻¹² Although the serological heterogeneity of the HL-A antigens has long been held responsible for this discrepancy, the fact that the actual products eliciting MLC reactivity have never been conclusively identified as being the HL-A antigenic determinants as such, allows the assumption that MLC reactivity may be controlled by a polymorphism of a separate genetic system, closely linked to HL-A. For this latter concept (initially raised as a possibility by Amos and Bach,¹ recently worked out in an elegant hypothesis by Yunis and Amos,¹¹ and discussed

in detail by Ceppellini,²⁹) evidence may be obtained from the demonstration of recombination between the HL-A loci and a MLC-controlling genetic entity.

The few reported positive MLC reactions between HL-A-identical siblings^{1,4,13,14} may well be the result of such crossing over, although other explanations, such as preimmunization,⁸ could not always be excluded. Stronger evidence for recombination is provided by the finding of the absence of MLC reactivity among siblings who share only one haplotype. MLC data of one family indicating this situation were, among other things, the basis on which Yunis and Amos¹¹ formulated their hypothesis.

We studied the MLC reactivity in five families with serologically demonstrable recombination between the LA and Four segregant series. In all cases, the limited function of the LA locus in regard to MLC reactivity was demonstrable. One of these families also showed further anomalies, from which the occurrence of crossing over between the Four locus and an MLC locus can be concluded.

MATERIALS AND METHODS

HL-A Typing

Lymphocytes were tested using a modified Kissmeyer-Nielsen lymphocytotoxicity test,^{15,16} and a microagglutination technique.¹⁶ Also, a complement fixation test on platelets¹⁷ was performed. Altogether, a panel of 120 well-established antisera was used.

Mixed Lymphocyte Culture

Bilateral and unilateral MLC tests were performed in a modification of a previously described technique.^{18,19} All cultures were performed in triplicate, and family KA was studied on two

From the Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service, Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Department of Immunohematology of the University Hospital, Leyden, Netherlands, and the Provincial Blood Grouping Laboratory, Cape Town, South Africa.

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Table 1. HL-A Typing and Haplotypes of Five Families

Family MEL			Family HOP			Family HA			Family STE			Family KA		
P*†	(3-W5/W28- 5)	ab	P 2-8/9-5	ab		M 2- 12/11-W5	ab		P 11-5/ 9- 8	ab		P 2- 8/11-W5	ab	
M	3- 7/W28-W5	cd	M 1-5/1-8	cd		P 3-? / 1- 8	cd		M 2-7/W28-W5	cd		M 3- 7/ 1- 8	cd	
Child			Child			Child			Child			Child		
1	W28- 5/ 3- 7	bc	1 2-8/1-8	ad		1 11-W5 / 3-?	bc		1 11-5/ 2- 7	ac		1 11-W5/ 3- 7	bc	
2	W28- 5/ 3- 7	bc	2 9-5/1-5	bc		2 2- 12/ 3-?	ac		2 11-5/ 2- 7	ac		2 11-W5/ 3- 7	bc	
3	3-W5/W28-W5	ad	3 9-5/1-8	bd		3 11-W5 / 1- 8	bd		3 11-5/W28-W5	ad		3 11-W5/ 1- 8	bd	
4	3-W5/W28-W5	ad	4 9-5/1-8	bd		4 11-W5 / 1- 8	bd		4 9-8/W28-W5	bd		4 11-W5/ 1- 8	bd	
5	3-W5/W28-W5	ad	5 2-8/1-5	ac		5 11-W5 / 1- 8	bd					5 11-W5/ 1- 8	bd	
6	3-W5/ 3- 7	ac				6 2- 12/ 1- 8	ad					6 2- 8/ 1- 8	ad	
7	3-W5/ 3- 7	ac										7 2- 8/ 1- 8	ad	
8	W28- 5/W28-W5	bd										8 2- 8/ 3- 7	ac	
												9 2- 8/ 3- 7	ac	
9	3- 5/ 3- 7	a/b c	6 2-5/1-5	a/b c		7 2-W5 / 3-?	a/b c		5 11-8/ 2- 7	a/b c		10 2-W5/ 3- 7	a/b c	

* W28, Ba*; W5, R*.

† Deducted from the findings in the other family members.

different occasions with virtually similar results. The geometrical mean and the standard error of each set of triplicate cultures were determined and expressed as cpm. In each experiment control, MLC's were carried out with the lymphocytes of a healthy, nonrelated individual (X).

RESULTS

The HL-A phenotypes and haplotypes of each member of the five families are shown in Table 1. The last child of each family represents the recombinant of the LA and Four segregant series. The results of the bilateral and unilateral MLC's between the recombinants and their respective siblings, together with the controls, are given in Table 2.

The six bilateral and 12 unilateral MLC tests between the HL-A recombinant children and their respective siblings; who differ only for an antigenic determinant of the LA segregant series, show no or marginal reactivity. On the other hand, normal positive MLC reactions are found in the eight bilateral and 16 unilateral tests involving differences for determinants of the Four segregant series only.

In contrast to all the other HL-A-identical siblings in the described families, who showed no MLC reactivity at all, in family KA an unexpected pattern was found (Table 3). The combination of children 1 and 2, both of the genotype bc, was indeed MLC negative. Of the genotype bd, the combination of children 4 + 5 was also negative, but the combination 4 + 3 and 5 + 3 were evidently positive in the bilateral and unilateral MLC's, notwithstanding the fact that child 3 carried the same antigens of the Four series (W5 and HL-A8) as children 4 and 5. Further analysis showed that child 3 was MLC-negative with children 1 and 2, who carried the antigen HL-A7, thus belonging to the genotype bc. This indicates that child 3 belongs to the MLC-identical group of children 1 and 2 and not to the MLC-

identical group, consisting of children 4 and 5. Unfortunately, child 10 could not be included in the investigations involving siblings 3, 4, and 5. The other HL-A-identical siblings combinations (children 6 + 7 of the genotype ad and children 8 + 9 of the genotype ac) were indeed MLC identical.

DISCUSSION

First, these data confirm and extend the earlier mentioned findings from Yunis,⁴ DuPont,⁵ and Lebrun,⁶ bringing the total number of antigens from the LA segregant series, shown not to be involved in MLC reactivity, to seven (HL-A1, HL-A2, HL-A3, A028, W28, HL-A9, and HL-A11). From gene-frequency data, it can be calculated that together, these comprise 98% of the genes of the LA segregant series of the White population.²⁰ Although it is still possible that the products of the remaining 2% of the genes do elicit MLC reaction, the very limited function of this locus in regard to the MLC is obvious, and it seems justified to postulate that MLC reactivity is independent of this genetic system. The further findings in family KA demonstrate a separate inheritance of the antigens of the Four segregant series and the MLC determinants, probably by recombination, which implies that another locus, apart from HLA, controls MLC.

If it is assumed that this MLC locus displays a polymorphism comparable to that of HL-A, the frequent finding⁸⁻¹² of MLC reactivity between carefully serotyped HL-A-identical unrelated individuals might be explained. The few HL-A-identical individuals described to be MLC negative as well^{8,9,21} suggest a degree of linkage disequilibrium similar to that found between the LA and Four loci of HL-A.¹¹

The question could be raised whether, rather than the HL-A locus alone, the MLC locus or any other closely linked locus may,

Table 2. Bilateral and Unilateral MLC's Involving Recombinants Between LA and Four-Segregant Series of HL-A in Five Families

HL-A difference	Family MEL			Family HOP			Family HA			Family STE			Family KA		
	Sib No.	cpm	SE	Sib No.	cpm	SE	Sib No.	cpm	SE	Sib No.	cpm	SE	Sib No.	cpm	SE
First Series Only															
a/b c + bc	9 +1	243	23	6 +2	176	64	7 +1	406	110				10 +1	62	14
	9 _m +1*	80	15	6 _m +2	244	78	7 _m +1	157	52				10 _m +1	55	19
	9 +1 _m	216	11	6 +2 _m	96	23	7 +1 _m	140	29				10 +1 _m	53	27
	9 +2	174	23										10 +2	63	9
	9 _m +2	88	37										10 _m +2	71	13
	9 +2 _m	69	3										10 +2 _m	142	21
Second Series Only															
a/b c + ac	9 +7	4.710	444	6 +5	4.651	155	7 +2	2.880	169	5 +1	1.314	63	10 +8	3.035	307
	9 _m +7	2.797	166	6 _m +5	3.136	76	7 _m +2	2.629	140	5 _m +1	1.009	35	10 _m +8	2.540	328
	9 +7 _m	2.731	883	6 +5 _m	2.532	359	7 +2 _m	3.060	318	5 +1 _m	1.335	332	10 +8 _m	1.223	123
	9 +6	4.137	147							5 +2	1.128	308	10 +9	1.069	75
	9 _m +6	3.190	559							5 _m +2	1.011	77	10 _m +9	1.041	215
	9 +6 _m	2.964	281							5 +2 _m	909	74	10 +9 _m	690	32
Controls	9 +X _m	2.806	360	6 +X _m	3.968	320	7 +X _m	3.130	271				10 +X _m	2.905	649
	1 +X _m	1.985	412	2 +X _m	4.564	708	1 +X _m	2.913	292				1 +X _m	2.519	300
	2 +X _m	2.745	283	6 _m +X	2.544	18	7 _m +X	3.043	90				2 +X _m	2.010	273
	9 _m +X	3.448	374	2 _m +X	3.023	470	1 _m +X	2.199	214				10 _m +X	3.966	241
	1 _m +X	3.266	443										1 _m +X	3.095	252
	2 _m +X	5.448	452										2 _m +X	4.451	130
	9	95	5	6	15	1	7	41	6	5	21	1	10	20	4
	1	46	12	2	34	5	1	95	43	1	20	4	1	21	8
	2	155	37	5	52	13	2	95	27	2	28	10	2	34	17
	6	308	157										8	125	4
	7	89	1										9	26	5

*m, Mitomycin-C treated.

Table 3. Bilateral and Unilateral MLC's Involving Siblings

HL-A Genotype Combination	Sibling	cpm	SE
bc + bc	1 +2	33	4
	4 +5	37	5
bd + bd	4 _m +5	72	10
	4 +5 _m	135	25
	4 +3	2.124	384
	4 _m +3	1.061	99
	4 +3 _m	2.605	193
	5 +3	3.982	574
	5 _m +3	1.787	258
	5 +3 _m	1.903	260
	1 +4	1.969	152
	1 _m +4	1.578	182
bc + bd	1 +4 _m	1.689	188
	2 +4	1.655	354
	2 _m +4	1.550	108
	2 +4 _m	1.817	84
	1 +5	2.108	486
	1 _m +5	1.772	211
	1 +5 _m	1.489	109
	2 +5	3.078	212
	2 _m +5	2.078	125
	2 +5 _m	1.318	117
	1 +3	122	34
	1 _m +3	37	20
	1 +3 _m	344	53
	2 +3	105	39
	2 _m +3	58	15
	2 +3 _m	338	32
ad + ad	6 +7	117	37
ac + ac	8 +9	167	43

*m, Mitomycin C treated.

either on its own or in combination, be of major importance for histocompatibility. The more limited improvement by HL-A typing of kidney graft survival between

unrelated individuals when compared with siblings, would favor such concept.

In earlier collaborative studies²² we could not demonstrate a predominant role of either the HL-A locus or the MLC locus on skin graft survival between unrelated individuals. However, the antigens eliciting skin graft rejection may well have to be distinguished from those involved in organ graft rejection. Minor locus incompatibilities were shown to have a cumulative effect on skin graft rejection in mice^{23,24} finally even mimicking a major locus difference. The range in the data of skin graft survival times between HL-A-identical siblings^{25,26} seems not to exclude a similar phenomenon in humans.

On the other hand, heart and kidney graft survival in rats was shown to be mainly dependent on one (major) histocompatibility locus.²⁷ This seems to confirm the one-locus hypothesis for kidney graft rejection in humans, as postulated by Simonsen.²⁸

If we assume on the basis of the human kidney transplant data that the HL-A locus is not of sole importance for organ graft rejection, neither the above-mentioned skin graft experiments between unrelated individuals nor the one-locus hypothesis seems to be in conflict with the concept that the MLC locus, or any other closely linked locus, is either on its own, or in combination with each other and/or with HL-A, controlling organ graft rejection.

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Position of a Locus Determining Mixed Lymphocyte Reaction (MLR), Distinct from the Known HL-A Loci, and Its Relation to Cell – Mediated Lympholysis (CML)

V. P. EIJSVOOGEL, M. J. G. J. DU BOIS, C. J. M. MELIEF,
M. L. DE GROOT-KOORY, C. KONING, J. J. VAN ROOD,
A. VAN LEEUWEN, E. DU TOIT, & P. TH. A. SCHELLEKENS

*Central Laboratory of the Netherlands Red Cross Blood Transfusion Service
and the Laboratory for Experimental and Clinical Immunology of the
University of Amsterdam; the Department of Immuno Haematology
of the University Hospital Leyden and the Provincial Blood Grouping
Laboratory, Cape Town.*

An association between HL-A dissimilarity and mixed lymphocyte reactivity was demonstrated in sibling combinations (Amos & Bach 1968, Schellekens *et al.* 1970, Freiesleben Sørensen 1970). Since the HL-A region can be divided into the LA (or first) locus and the FOUR (or second) locus, the question could be raised whether the products of both contribute to the MLR, or whether one of these two, or even a closely linked locus, is responsible for the MLR.

MLR: Determined by LA or FOUR Locus?

Recent publications (Yunis *et al.* 1971, Dupont *et al.* 1971, Lebrun *et al.* 1971, Gatti *et al.* 1971) of the mixed lymphocyte reactions of a total of five individuals with a serologically demonstrable recombination between the LA and FOUR segregant series of HL-A, with their respective siblings, indicated that a difference only for the LA series, at least for the antigens studied (HL-A1, HL-A2, HL-A3, AO28 (W32), W28) did not elicit a MLR, while a difference only in the antigens of the FOUR series (HL-A7, W27, HL-A12, W15, AO41) was always accompanied by pronounced mixed lymphocyte reactivity.

We studied five such families, each containing a sibling in which recombination between the genes determining the antigens

of the LA and FOUR segregant series had occurred, with virtually similar results (Eijsvoogel *et al.* 1972). The six bilateral and twelve unilateral MLR-tests between the HL-A recombinant children and their respective siblings, who differ only for an antigenic determinant of the LA segregant series, showed either no or very weak reactivity. On the other hand, pronounced MLR's were found in the eight bilateral and sixteen unilateral tests involving differences for determinants of the FOUR segregant series only.

These data confirm and extend the earlier mentioned reports (Yunis *et al.* 1971, Dupont *et al.* 1971, Lebrun *et al.* 1971, Gatti *et al.* 1971) bringing the total number of antigens from the LA segregant series which have been shown not to elicit pronounced mixed lymphocyte reactivity, to seven (including HL-A9 and HL-A11). From gene frequency data it can be calculated that together these comprise more than 90% of the genes of the LA segregant series in the Caucasian population (e.g. Van Rood *et al.* 1971). Although it is still possible that the products of the remaining genes do elicit normal MLR, the very limited function of this locus in regard to the MLR is obvious, and it seems justified to postulate that normal MLR is independent of this genetic system. However, the very weak reactivity, found between some recombinants and their

respective siblings differing only for the LA series (less than 10% of normal control values) was definitely above non-stimulated control levels and was found consistently in particular combinations, which suggest some weak MLR stimulating property determined either by the LA locus or by a closely linked locus.

MLR: FOUR Locus or a Closely Linked Locus?

The association between HL-A and MLR is considerably less evident among unrelated individuals (Eijssvoogel *et al.* 1970, Eijssvoogel *et al.* 1971, Bach *et al.* 1971, Johnston & Bashir 1971, Yunis & Amos 1971, Freiesleben Sørensen & Staub Nielsen 1970), notwithstanding the fact that a few non-related HL-A identical individuals have been found to be MLR-identical as well (Eijssvoogel *et al.* 1970, Eijssvoogel *et al.* 1971, Bach *et al.* 1971, Festenstein 1971).

Serological heterogeneity of the HL-A antigens is generally blamed for this discrepancy. The fact, however, that the actual gene products eliciting MLR have never been conclusively identified as being HL-A antigenic determinants as such, allows the assumption that the normal MLR may not be controlled by the genes of the FOUR locus either, but rather by a separate polymorphic genetic system, closely linked to HL-A. Evidence for this latter concept, which was raised initially as a possibility by several authors (Amos & Bach 1968, Plate *et al.* 1970, Kissmeyer-Nielsen *et al.* 1970, Eijssvoogel *et al.* 1970, Eijssvoogel *et al.* 1971) and recently put forward in an elegant hypothesis by Yunis & Amos (1971) and discussed in detail by Ceppellini (1971), might be obtained from the demonstration of recombination between the HL-A loci and a MLR controlling genetic entity. The few reported positive MLR's between HL-A identical siblings (Amos & Bach 1968, Yunis *et al.* 1971, Plate *et al.* 1970, Bach *et al.* 1969) may well be the result of such crossing over, although other explanations, such as preimmunization (Eijssvoogel *et al.* 1971) could not always be excluded. Stronger evidence for such crossing over might be derived from the demonstration of an absence

TABLE I
HL-A typing of Family KA

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

W5 = R*

of mixed lymphocyte reactivity between such a recombinant and its siblings, which differ for one of the HL-A haplotypes. So far, one family has been reported in the literature by Yunis & Amos (1971) in which recombination between HL-A and a MLC locus has been made highly probable. These data formed among other things, the basis on which these authors formulated their hypothesis. Recently, Sasportes *et al.* (1972) and Mempel *et al.* (1972) each reported a similar family.

One of the earlier mentioned families (Table I), which we studied with respect to presence of a serologically demonstrable recombination between the LA and FOUR locus, showed further anomalies in the association between HL-A and MLR. Of a group of three HL-A identical siblings (nos. 3, 4 and 5), one (no. 3) proved to be consistently MLR positive with the other two (nos. 4 and 5), but MLR negative with another group of two HL-A identical siblings (nos. 1 and 2), which displayed a one-allelic difference for HL-A with the former group (Table II). Furthermore, the child (no. 3), who showed this unexpected MLR pattern reacted marginally with the earlier discussed sibling (no. 10) in which the recombination between the LA and FOUR series was demonstrable. The latter lack of pronounced

TABLE II
MLR's in family KA (expressed in cpm, $\pm$ s.e.)

sib no.		1.	2.	3.	4.	5.	8.	9.	10.	X
	geno type	bc	bc	bd	bd	bd	ac	ac	a/bc	
1.	bc	21 ± 4								2,519 (X_m)
2.	bc	33 ± 4	34 ± 17							5,024 ± 270
3.	bd	122 ± 34	150 ± 39	52 ± 9						5,173 ± 567
4.	bd	1,959 ± 125	1,655 ± 354	2,124 ± 384	46 ± 3					4,341 ± 339
5.	bd	2,108 ± 485	3,078 ± 212	3,982 ± 574	37 ± 5	20 ± 4				5,443 ± 290
8.	ac						125 ± 4			5,470 ± 225
9.	ac						167 ± 43	26 ± 5		2,233 ± 407
10.	a/bc	62 ± 14	63 ± 2	353 ± 92	2,452 ± 55	2,394 ± 239	3,935 ± 307	1,069 ± 75	20 ± 4	4,355 ± 209
1_m	bc			37 ± 20	1,578 ± 182	1,772 ± 211			53 ± 27	3,095 ± 252
2_m	bc			58 ± 15	1,550 ± 108	2,078 ± 125			142 ± 21	4,451 ± 130
3_m	bd	344 ± 53	338 ± 32		2,605 ± 193	1,903 ± 260			192 ± 25	2,966 ± 160
4_m	bd	1,689 ± 188	1,817 ± 84	1,061 ± 99		72 ± 10				2,595 ± 275
5_m	bd	1,489 ± 109	1,318 ± 117	1,787 ± 258	135 ± 25				1,466 ± 105	2,603 ± 71
8_m	ac								1,223 ± 123	3,102 ± 207
9_m	ac								690 ± 32	1,639 ± 176
10_m	a/bc	55 ± 19	71 ± 13	233 ± 72	2,048 ± 140	1,770 ± 158	2,540 ± 328	1,041 ± 215		3,954 ± 241
X_m		2,519 ± 300	2,010 ± 273	2,015 ± 212	2,377 ± 44	2,892 ± 163	1,248 ± 327	1,026 ± 14	2,905 ± 649	

mixed lymphocyte reactivity occurred in spite of a difference for three well defined HL-A antigens.

These findings demonstrate a separate inheritance of the HL-A antigens and the determinants eliciting pronounced MLR, probably by recombination, which implies independence of this MLR from both the LA and FOUR loci. Consequently, this separate MLR locus can now be mapped not in between but outside the previously recogniz-

able HL-A region, close to the FOUR locus. Localization between the LA and FOUR locus would require the assumption of a double recombination to explain the above results. This seems rather unlikely as the impression emerges from the overall data regarding MLR positive HL-A identical siblings that the crossing over frequency between HL-A and MLR is of about the same order of magnitude as that between the LA and FOUR locus.

In the light of our earlier mentioned findings of occasional very weak stimulation between siblings differing only for determinants of the LA series, it could be postulated that this may reflect the existence of a second (minor) MLR locus, situated between the LA and FOUR locus. Depending on the localization of the crossing over in the chromosome, the gene of this locus might either stay together with the corresponding gene of the FOUR locus (and of the major MLR locus), resulting in an absence of stimulation in the above mentioned situation, or stay with the corresponding gene of the LA locus, thus resulting in weak stimulation when tested with siblings serologically differing for LA determinants only. However, as the evaluation of weak MLR's is less reliable with the presently available techniques, further extension of such experiments with more sensitive methods will be required before any conclusions with regard to a second, minor MLR locus can be drawn. The concept of a separate (major) MLR locus explains firstly the frequent finding (Eijssvoogel *et al.* 1970, Eijssvoogel *et al.* 1971, Bach *et al.* 1971, Johnston & Bashir 1971, Yunis & Amos 1971, Freiesleben Sørensen & Staub Nielsen 1970) of mixed lymphocyte reactivity between carefully serotyped HL-A identical unrelated individuals if it is assumed that the MLR locus displays a polymorphism comparable to that of HL-A. The finding (Eijssvoogel *et al.* 1970, Eijssvoogel *et al.* 1971, Bach *et al.* 1971, Festenstein 1971) of the few HL-A identical MLR negative unrelated individuals suggests a degree of linkage disequilibrium similar to that found between the LA and FOUR segregant series of HL-A (Yunis & Amos 1971).

The existence of a separate MLR locus might also explain why we have never been able to induce normal lymphocyte reactivity with allogeneic platelets, which are known to carry HL-A antigens. It may well be that these cells lack the MLR determinants, which may also be absent from the lymphocyte extracts and which proved to be non-stimulatory in the hands of many investigators (Kasakura & Lowenstein 1965, Ceppellini *et al.* 1965, Hardy & Ling 1969), although the extraction procedure could be

expected to preserve at least some of the HL-A antigens. In this respect it might be noted that recent reports (Main *et al.* 1971, Leguit & Eijssvoogel) describe lymphocyte transformation in rats and humans elicited by allogeneic epidermal cells. Epidermal cells of mice are reported to carry the theta-antigen (Scheid *et al.* 1972). Our previously reported finding (Eijssvoogel *et al.* 1971) that the lymphocytes of a patient with thymic dysplasia (Nezeloff syndrome), although carrying HL-A antigens, did not stimulate the cells of any of the four unrelated non-HL-A-identical normal individuals tested in the MLR, may be relevant to this and may suggest a possible relationship between the thymus and those cells which bear lymphocyte stimulating determinants. Recent experiments by MacLaurin (1972) in rats have indeed demonstrated the thymus-dependence of MLR stimulating lymphocytes.

In the light of the present findings it seems hard to explain the inhibitory effect of antibody directed against antigens of the LA or FOUR segregant series on mixed lymphocyte reactivity (Ceppellini *et al.* 1971, Grumet & Leventhal 1970) by a masking of the specific stimulating determinants. The fact, however, that HL-A antibody also inhibits the responding cells, either in the MLR or in antigen-stimulated lymphocyte transformation, together with the finding that xenogeneic anti L-chain serum (Greaves *et al.* 1969) and anti-lymphocyte serum (Eijssvoogel *et al.* 1969, Revillard *et al.* 1970) causes a similar inhibition, indicates that probably the binding of antibody to the lymphocyte membrane per se interferes with either its stimulating or responding capacity possibly by hampering lymphocyte-lymphocyte or lymphocyte-macrophage interaction.

CML: HL-A Locus or MLR Locus, or both?

Hirschhorn *et al.* (1965) were the first to demonstrate that human lymphocytes stimulated in the MLR, display cytotoxicity towards allogeneic fibroblasts. When using the cells from the same human lymphoid cell line as both the stimulating cells and the target cells, a similar cytotoxic effect could be demonstrated (Hardy *et al.* 1970).

Although this cytotoxic effect is maximal when tested towards the stimulating cell type, it may, to a more limited degree, show cross reactivity with other lymphocyte lines (Solliday & Bach 1970). Häyry & Defendi (1970) demonstrated that target cell destruction by MLR activated mouse lymphocytes did not affect cells, which were syngeneic with the responding cell population, thus reflecting an immunospecific destruction.

Lightbody, Ceppellini and their colleagues (1971) and later Lightbody & Bach (1972) reported specific destruction of human PHA stimulated lymphocytes by MLR activated cells. According to Miggiano *et al.* (1972) the destruction of cells from the stimulating donor is greater when the responding cells are derived from a family member differing for two alleles of HL-A with the stimulator than when the donor differs for only one allele.

To further evaluate the genetic aspects of this cell-mediated lympholysis (CML) we performed some preliminary experiments with this model in the family KA mentioned earlier. The technique used was based on the same principle as described by Lightbody *et al.* (1971): stimulator and responder lymphocytes are cultured in unilateral MLR for 6 days. Target cells of the stimulator, of the responder and of others are collected on the same day and cultured for three days

without stimulant, whereafter PHA is added for another 3 days. These PHA blasts are then labelled with ^{51}Cr and mixed with the MLR in a ratio of 50 responding cells to 1 target cell. Destruction is allowed to take place during 4 hours at 37°C. After centrifugation half of the supernatant (I) is separated from the sediment and the rest of the supernatant (II), and the destruction is calculated by dividing the radioactivity in I by half of the sum of the activity in I and II. Similarly, the spontaneous ^{51}Cr -release of the target cells without responding cells is determined as well as the maximal ^{51}Cr -release by freeze-thawing of the cells. The final results of a particular experiment are obtained by subtracting the spontaneous release from the experimental release and expressing this difference as a percentage of the maximal release minus spontaneous release. The results of our preliminary experiments are given in Table III and can be summarized as follows:

- if the responding cells are cultured with stimulator cells which differ both for MLR and HL-A determinants (MLR positive), then the stimulating cells are destroyed;
- if the responding cells are cultured with stimulator cells which differ only for MLR determinants (MLR positive), the stimulating cells are not destroyed;

TABLE III

CML's in family KA (expressed in percentage destruction; see text)

Responding cells		Stimulating cells in MLR		Result MLR	Target cells				
sib no.	geno type	sib no.	geno type		no. 1 (bc)	no. 2 (bc)	no. 3 (bd)	no. 4 (bd)	no. 5 (bd)
5	bd	1	bc	+	<i>21,1</i>	<i>17,5</i>	1,2*	-0,8	0,2
1	bc	5	bd	+	2,9	1,3	<i>11,3**</i>	<i>10,1</i>	<i>12,2</i>
4	bd	3	bd	+	3,9*	2,0*	3,7*	3,0	2,9
3	bd	4	bd	+	1,7	-0,5	0,6	-1,9*	-0,2*
1	bc	3	bd	-	3,84	0,69	4,8	1,0	6,0
3	bd	1	bc	-	4,8	3,3	1,9	-1,5	2,3

Values in italics are considered to reflect specific destruction.

* = no destruction regardless common MLR determinants of stimulating and target cells.

** = destruction in the absence of common MLR determinants of stimulating and target cells.

- if the responding cells are cultured with stimulator cells which differ only for HL-A determinants (MLR negative), the stimulating cells are not destroyed;
- if the responding cells are cultured with stimulator cells which differ both for MLR and HL-A determinants (MLR positive) and are then tested with target cells which have only the MLR determinants in common with the stimulator cells, but the HL-A determinants of the responding cells, no destruction is found;
- if the responding cells are cultured with stimulator cells which differ both for MLR and HL-A determinants (MLR positive) and are then tested with target cells which have only the HL-A determinants in common with the stimulator cell, but the MLR determinants of the responding cell, destruction can be demonstrated.

A tentative conclusion seems to be that the sensitization for CML takes place only when stimulating and responding cells differ for MLR determinants, but that the actual destruction is not directed against MLR determinants, but rather against HL-A antigens. Although further extension of such experiments is required before any definite conclusion can be established, the question arises whether similar differences in sensitization and effector mechanisms between MLR and HL-A determinants may affect *in vivo* histocompatibility.

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