Serological Identification and Characterization of New HLA Class I and Non-HLA Antigens

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ABSTRACT

Human leukocyte antigens (HLA) are polymorphic major histocompatibility complex (MHC) cell surface glycoproteins that function in immune regulation and response, and enable immunologic discrimination between self and non-self. Knowledge regarding the immunological role of individual HLA antigens and their association with disease susceptibility is dependent upon the ability to define each antigen and its variant(s). While determination of HLA class II (HLA-DR, -DQ, and -DP) antigens has gradually advanced to molecular biology methodology in recent years, much of the identification of class I (HLA-A, -B, and -C) antigens still largely relies on serological techniques using allo- or monoclonal antisera.

This report embarks on an investigation of the ethnic diversity, using alloantisera, of three groups of HLA class I antigens, namely A2, A11, and C-locus antigens, and to define antigens that might be restricted to certain ethnic populations. As transplantation therapy has been widely accepted as the choice of treatment for patients with severe haematological disorders, such as acute leukemia, it is essential to match MHC antigens of unrelated donor and recipient pairs to avoid graft rejection or graft-versus-host disease and to reduce administration of immune suppressants.

Results obtained from this study indicate that HLA-A2 consists of at least four variants as measured by isoelectric focusing assay. However, only three serological variants are demonstrated by alloantisera. Results further confirmed that two variants of HLA-A11 exist, as measured by serology and isoelectric focusing. The more

frequent variant, A11.1, is found in donors of all ethnic backgrounds. The less frequent variant, A11.2, is found only in Oriental populations. This study also reveals two "new" serologically defined HLA-C locus antigens: HLA-Cw Hunt and HLA-Cw Grace. These two "new" antigens are found in Black, Caucasian, East Indian and Oriental ethnic groups. Identification of these two new C-locus antigens reveals that approximately 11% of the population was falsely typed and possess only one or no C-locus antigen. This study also identifies for the first time an alloantiserum that defines an ethnic-specific human minor histocompatibility antigen, hmHΦ, found only in the Oriental population with a frequency of approximately 5.5%. It was not found in 1,000 Caucasian, 200 East Indian, and 60 Black blood donors.

The data obtained from this investigation may be valuable in determining the degree of compatibility to forecast the outcome of an organ transplantation or to evaluate linkage in susceptibility to a disease. In addition, the establishment of the Oriental-restricted hmHΦ antigen may be utilized in anthropological population surveys and for study of the role of minor histocompatibility antigens in transplantation.

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LIST OF ABBREVIATIONS

HLA	human leukocyte antigen(s)
MHC	major histocompatibility complex
IHW	International Histocompatibility Workshop
WHO	World Health Organization
NIH	National Institute of Health
KD	kilo-dalton
kb	kilo-base
CD	cell differentiation antigen(s)
LCT	lymphocytotoxicity
MLC	mixed-lymphocyte culture
MLR	mixed-lymphocyte reaction
PLT	primed lymphocyte test
PCR	polymerase chain reaction
SSOP	sequence-specific oligonucleotide probe
SSP	sequence-specific primer
DNA	deoxyribonucleic acid
SBT	sequence based typing
1D-IEF	one-dimensional isoelectric focusing
EBV	Epstein-Barr virus
mH	minor histocompatibility antigen(s)

PBL	peripheral blood lymphocyte
ACD	acid citrate dextrose
RPMI	Roswell Park Memorial Institute
CFDA	carboxylfluorescein diacetate
RT	room temperature
RAM Ig	rabbit anti-mouse immunoglobulin
EDTA	ethylene diaminetetra acetic acid
SDS	sodium dodecyl sulfate
hmHΦ	human minor histocompatibility antigen Φ
H-Y	Y-chromosome associated minor histocompatibility antigen
MoAb	monoclonal antibody

1. INTRODUCTION

1.1 General Introduction

1.1.1 MHC

The major histocompatibility complex (MHC) was discovered originally as a genetic locus controlling rapid rejection of tissue grafts expressing surface antigens. In humans, these transplantation antigens are referred to as the human leukocyte antigens (HLA) while in mice they are termed H-2 antigens. The discovery of MHC restriction and the molecular identification of MHC genes and their products has subsequently led to a unified theory of the principal physiological function of MHC molecules to act as guidance molecules for immune responses (1, 2). The genes found within the HLA system are involved in the recognition of foreign antigen in association with HLA gene products that is referred to as restricted recognition. Moreover, it is a process that has evolved in a manner that enables the immune system in mammals to respond effectively to foreign antigens while at the same time to recognize but not respond to self antigens (2, 3, 4, 5).

The human MHC is located on the short arm of chromosome 6 at 6p21.3, and the mouse MHC on chromosome 17. In both species, it consists of three major linked gene clusters. The class I (HLA-A, -B and -C) and class II (HLA-DR, -DQ and -DP) regions encode highly polymorphic cell surface molecules involved in immune recognition of antigens. The class III region contains genes for complement proteins C2, factor B and C4 as well as genes for the steroid 21-hydroxylase (6). In addition, in the class III region, many novel genes have been located between the Hsp70-Hom and G7a genes and between the B144 and TNF-A genes (7).

1.1.2 Genetic Organization of MHC

The overall size of the human MHC is about 4,000 kb. A significant advance in the identification of genes in the MHC is the discovery of a large number of novel genes in the class I region. Currently, there are about 18 highly related class I genes which include those encoding the classical transplantation antigens (HLA-A, -B, and -C), class I related (or non-class I) genes (i.e. HLA-E, -F, -G, and -H) (7), pseudogenes, and truncated genes or gene fragments (8). The class II genes are arranged in a block consisting of five major subregions. These subregions are named: DR, DQ, DP, DN, and DO. Each subregion of DR, DQ, and DP contains at least one alpha/beta pair of genes. The genes responsible for complement proteins C2, factor B, and C4 are located in class III region (**Figure 1**) (7).

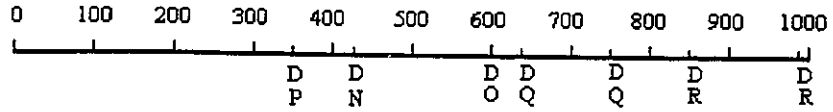
All three MHC regions evidently have undergone expansion and contraction throughout evolution. Some of the duplicated genes have lost their functions and have become pseudogenes. Others are clearly expressed as protein products and some remain of undefined status (9, 10).

The class I related genes have been sequenced and shown to encode intact HLA class I proteins distinct from the classic HLA-A, -B, and -C antigens. Understanding of the functional role of this group of antigens remains incomplete (11, 12).

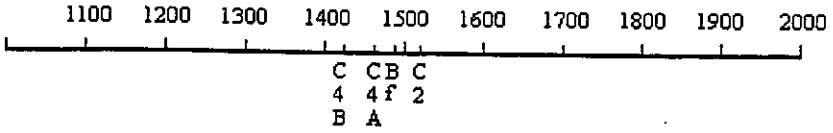
The classical class I genes encode HLA-A, -B, and -C antigens. Polymorphism of this group of antigens is believed to play an important role in establishing the diversity of immune responsiveness within populations and constitutes the major basis of rejection in tissue transplantation (13, 14).

Figure 1. Gene map of the MHC. The MHC gene complex contains a large number of individual genes expanding approximately 4,000 kb on the short arm of chromosome 6. Three major sets of molecules are encoded within this region: class I, II, and III antigens. A total of 18 class I genes have been reported to date. The class II (HLA-D) region is subdivided into DP, DN, DO, DQ, and DQ regions. The class III region has many novel genes, genes for complement components (C4, C2, and Bf), and genes for tumour necrosis factor (TNF) and heat-shock proteins (Hsp-70).

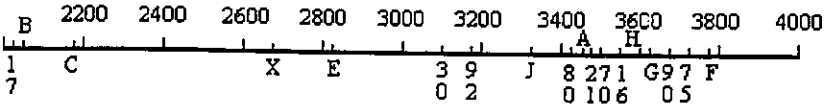
Class II region



Class III region



Class I region

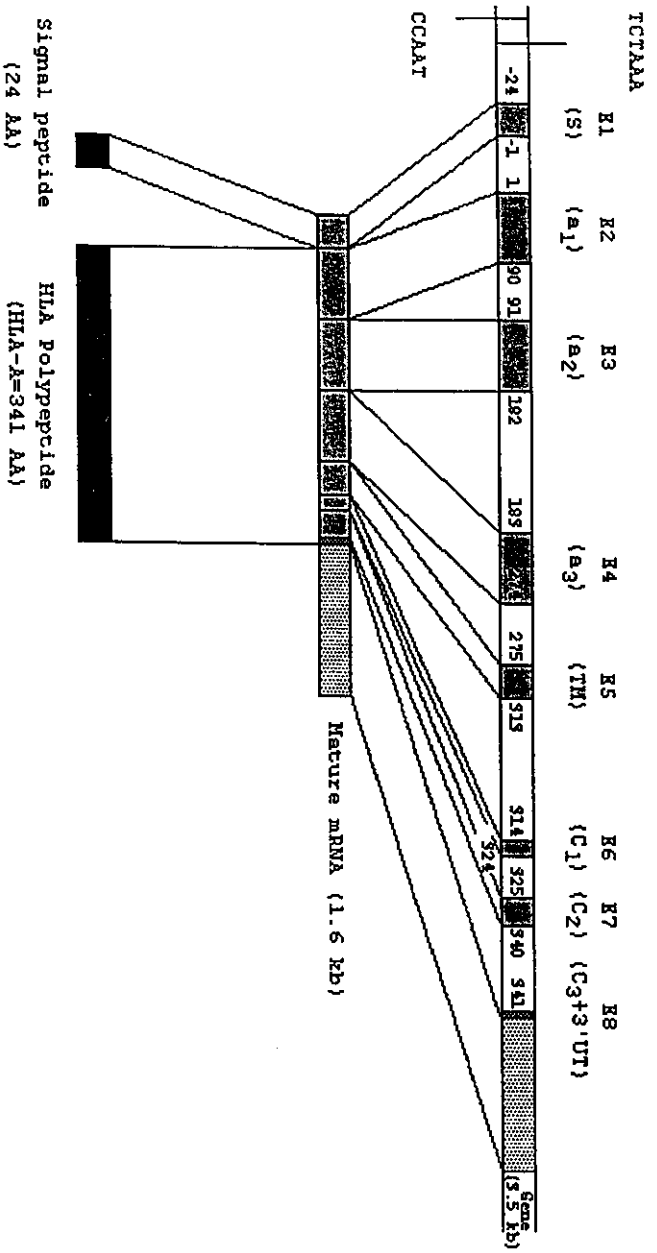


1.1.3 Structure of Class I and Class II Genes

1.1.3.1 Class I Genes

The typical class I HLA gene has eight exons (**Figure 2**). The first exon encodes a 5' untranslated sequence of approximately 18 nucleotides, followed by the codons for a hydrophobic leader peptide responsible for the insertion of the heavy chain through the surface membrane. The peptide is about 24 amino acids long, although for some heavy chains it has been reported much longer. The second and third exons contain about 270 nucleotides each and encode the polymorphic alpha 1 and alpha 2 domains respectively. The fourth exon consists of a conserved nucleotide sequence encoding the alpha 3 domain. This exon has considerable homology to immunoglobulin constant-region exons. The alpha 3 domain appears to be the part of the heavy chain that binds the beta-2-microglobulin to form the class I molecular complex. The beta-2-microglobulin gene contains an exon of structure similar to the immunoglobulin constant region exons (15). The fifth exon contains about 120 nucleotides and encodes the transmembrane segment of hydrophobic and basic amino acids which may interact with the phosphates of membrane phospholipids. The sixth and seventh exons are small coding regions, of about 11 and 15 codons respectively, which encode the cytoplasmic tail. This part of the molecule contains phosphorylation sites which appear to be transmission of activation signals mediated by class I molecules (16). There is considerable variability in the amino acid composition of the cytoplasmic tail of HLA molecules of different specificities. This could mean differences in signal transmission or anchoring capabilities of the molecule. The last exon, the eighth, is the longest, containing over 400 residues, and generally encodes the 3' untranslated region. In

Figure 2. Class I gene (HLA-A) as present on the chromosome 6. The class I gene is segmented into protein-coding regions (exons; filled) and non-coding regions (introns; blank). E1 = exon for signal peptide (s); E2, E3, and E4 = exons for alpha 1, alpha 2, and alpha 3 domains; E5 = exon for the transmembrane region (TM); E6 and E7 = exons for the cytoplasmic region and for the untranslated segment (3' UT). TCTAAA and CCAAT are conserved promoter sequences. The numbers -24 to 341 represent amino acid residues of the class I protein molecule. A 1.6 kb mature mRNA is translated into a class I polypeptide.


TCTAAA
CCAAT
E1
E2
E3
E4
E5
E6
E7
E8
(S)
(a1)
(a2)
(a3)
(TM)
(C1)
(C2)
(C3+3'UT)
-24
-1
1
90
91
192
193
275
313
314
325
340
341
Gene (3.5 kb)
Mature mRNA (1.6 kb)
Signal peptide (24 AA)
HLA Polypepcide (HLA-A=341 AA)

some cases, the translation termination codon is already present at the end of the seventh exon (17).

1.1.3.2 Class II Genes

The HLA-D region is approximately 900 kb long and can be represented schematically as shown in **Figure 3**. The A genes encode the alpha chains of polypeptide and the B genes encode the beta chains of polypeptide of each HLA-D dimer. The structure of each class of genes resembles that of the HLA class I genes depicted in **Figure 2** with the following different features:

a. exon 4 of the DR A gene encodes membrane portion, cytoplasmic portion and proximal cytoplasmic portion of the DR alpha peptide chain.

b. exon 5 of the DR A gene encodes distal 3' untranslated portion of the DR A chain.

c. exon 6 of the DR A gene is absent.

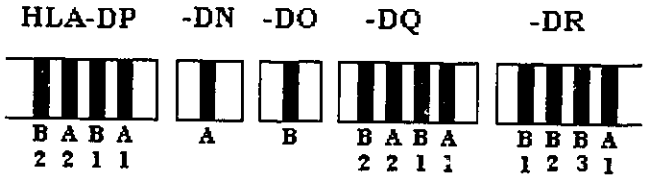
d. exon 4 of the DR B gene encodes proximal 3' untranslated region of the DR B chain.

e. exon 5 of the DR B gene encodes middle cytoplasmic portion of the DR beta peptide chain.

f. exon 6 of the DR B gene encodes distal cytoplasmic portion of the DR B beta chain.

The allelic polymorphism of the HLA-D genes is found in exon 2 where highly variable regions alternate with highly conserved regions. The genes of DR B1, DR B3, DR B4, DR B5, DQ A1, DQ B1, DP A1, and DP B1 contribute to most of the HLA-D polymorphism seen today (18).

Figure 3. Schematic presentation of HLA class II genes. The HLA-D region consists of HLA-DP, -DN, -DO, -DQ, and -DR five major loci. The A genes encode the alpha chains and the B genes the beta chains of each HLA-D heterodimer. The structure of each gene resembles that of the HLA class I genes.


HLA-DP
-DN
-DO
-DQ
-DR
B A B A
2 2 1 1
A
B
B A B A
2 2 1 1
B B B A
1 2 3 1

1.1.4 General Function of MHC Products

1.1.4.1 Class I

The great majority of functional studies of class I gene products deals with MHC restriction of virus-specific CD8+ cytotoxic T cells. Studies indicate that the primary role of class I molecules is in mediating T cell immune surveillance of virally-infected or neoplastically-transformed cells. Class I molecules primarily present endogenous peptides, those synthesized within the cell (e.g. peptides derived from viral proteins or cytosolic "self" antigens). HLA class I molecules must bind peptide for stability and efficient expression at the cell surface (19). Recent characterization of class I-bound peptides has revealed that the size of peptides is from 9-11 residues, with nonamers binding with the highest affinity. Peptides bound by any one MHC allele share an allele-specific consensus motif (20). A hydrophobic residue at the -COOH end appears to be a critical anchor position (21).

The role of class I alloantigens in transplantation immunity mediated by alloantibodies and cytotoxic CD8+ T lymphocytes is also well known. These findings clearly implicate the important role of class I molecules in interactions of the immune response. CD8+ cytotoxic T lymphocytes must carry out their effector functions by recognizing antigen determinants, in the form of peptides, in association with class I antigens (19). Essentially, it is this central character in the immune regulation stage performed by the class I molecules that provides a self-defense mechanism of our body.

1.1.4.2 Class II

The functional role of class II molecules is also in immune response and immune regulation. CD4+ T lymphocytes can recognize antigen only presented in the context

with "self" or compatible MHC-encoded class I or class II molecules expressed by antigen-presenting cells. This MHC restriction or self-recognition has been demonstrated in various animal studies and the portions of the MHC molecules which are recognized, together with antigen, are often referred to as restriction elements. Antigen determinants, in the form of peptides, from foreign organisms are displayed in the antigen-presenting cell membrane in association with class II molecules. This complex is recognized by CD4+ T cells with a specific receptor (22). Therefore, with their normal functions the class II molecules safeguard our immune system from engaging in self-destructive activity. Similar to class I molecules, HLA class II molecules must bind peptides for stability and efficient expression at the cell surface (19). Class II molecules present exogenous as well as endogenous peptides, for example, peptides derived from extracellular microbes or "self" antigens which may be internalized. The peptide-binding groove of class II molecules binds larger peptides of 10-14 residues. Heterogeneity at the -COOH terminus suggests that the groove may be open at this end (23).

1.1.4.3 Class III

Unlike class I and class II gene products, some class III molecules are actively secreted. C2 and Factor B are serine proteases which, on activation, associate with C4 and C3 respectively and then form the C3 convertase of the classical and alternative pathways of complement activation. Activation of the complement system is a potent mechanism for initiating and amplifying inflammation. Activation products of complement proteins stimulate chemotaxis and activation of leukocytes. Thus, class III gene products are constituents of natural immune surveillance of the host. Ironically,

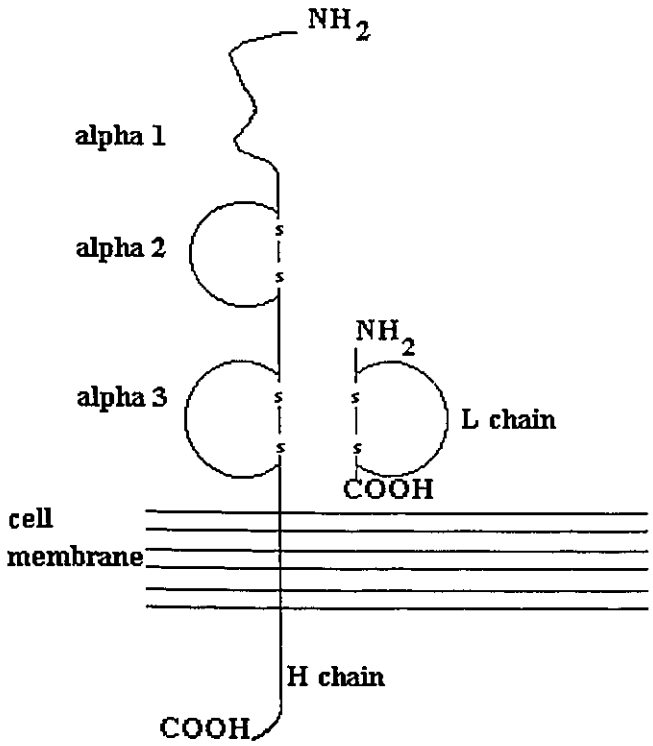
Jan Klein (1) does not consider complement components to be members of the MHC family. Instead, he regards a set of 16 low molecular mass proteins (LMP) discovered by Monaco and McDevitt during a routine examination of class I and class II gene expression on BALB.B (H-2b) mouse cells as the class III products (24). Class III products, whether complement components or low molecular mass proteins, will not be considered further as that is beyond the scope of this thesis.

1.1.5 Structure of Class I and Class II Molecules

1.1.5.1 Structure of Class I Molecules

Each class I molecule is a tertiary complex comprised of a heavy alpha chain polypeptide and a light chain polypeptide called beta-2-microglobulin. The alpha chain is encoded for by the class I genes on chromosome 6, whereas beta-2-microglobulin is encoded for by a gene located on chromosome 15 (25). The class I antigen molecules are therefore heterodimers (**Figure 4**). The heavy chain is a glycoprotein that has a molecular weight of between 40 and 45 KD, whereas beta-2-microglobulin has a molecular weight of approximately 13 KD. The heavy chain of class I molecules is arranged into five distinct domains. Three of them (alpha 1, alpha 2, and alpha 3), each consisting of 90 amino acid residues in length, are found externally on the cell. The alpha 2 and alpha 3 domains have disulphide linkages that are arranged in a manner analogous to that of the immunoglobulin molecules. The fourth domain, composed of about 40 amino acid residues, is embedded by its hydrophobic residues within the membrane. A fifth domain composed of 30 amino acid residues projects into the cytoplasm. These domains are folded in such a manner that alpha 1 and 2 are linked together by noncovalent bonds, whereas the alpha 3 domain is linked to the beta-

Figure 4. Schematic structure of the HLA class I molecule. HLA class I antigens consist of two polypeptide chains: the heavy (H) chain alpha and the light (L) chain beta-2-microglobulin. The H chain consists of separate domains; the alpha 1, alpha 2, alpha 3, transmembranal region, and cytoplasmic tail. The light chain is non-covalently associated with the alpha 3 domain.

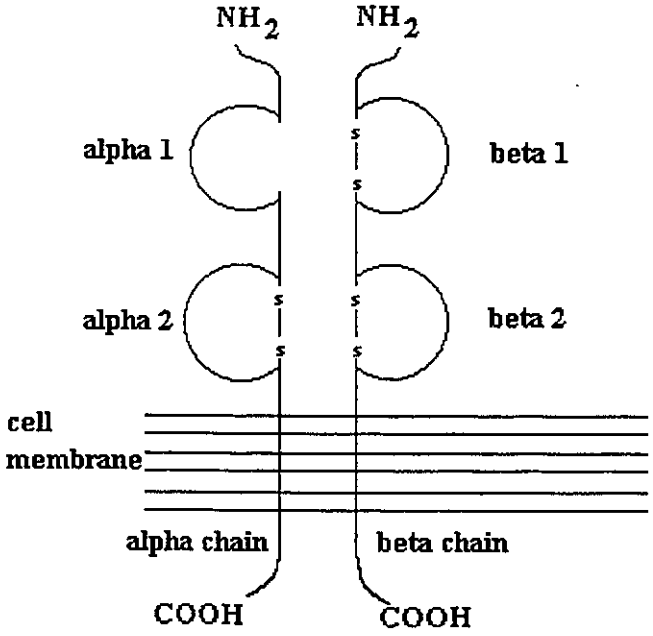

NH2
alpha 1
alpha 2
S
S
NH2
alpha 3
S
S
S
S
L chain
COOH
cell
membrane
H chain
COOH

2-microglobulin by a similar type of bonding (26). The function of beta-2-microglobulin is thought to involve the expression and transport of HLA class I to the cell surface (1). Despite high interlocus homology, the HLA-A, -B, and -C antigens retain unique features. Furthermore, there are locus-specific amino acids, e.g., Met-138 and Met-189 for HLA-A; Arg-239 for HLA-B; Val-52, Glu-183, and Glu-268 for HLA-C (27). Polymorphism of the HLA-A, -B, and -C antigen specificities are represented by amino acid sequences in alpha 1 and alpha 2 domains. The alpha 3 domain is for the most part uniform. The most significant feature of the HLA class I antigens is that the allelic differences are concentrated in only a few regions on the molecules. The highest degree of polymorphism is found in the alpha 1 domain involving amino acid residues 9, 24, 41, 43, 45, and 62-80. In the alpha 2 domain, the polymorphic positions are in the regions of 95-116, 147-157, 161-163, and 177-194 residues (28). Individual antigens of the same locus may vary by up to 50 amino acids. Two closely related antigens (e.g. A2 and A28) have been shown to have a 10 amino acid variation (29).

1.1.5.2 Structure of Class II Molecules

The class II molecules are also heterodimers (**Figure 5**). They are composed of two peptide chains, an alpha chain weighing 30 to 33 KD and a beta chain weighing 27 to 29 KD. Each class II polypeptide chain is made up of four domains. The alpha 1 and alpha 2, and the beta 1 and beta 2 are each 90 amino acid residues in length. The third domain for each chain is the transmembrane region which contains about 30 amino acid residues. Similar to the class I molecules, it is embedded by its hydrophobic residues within the membrane. The last domain, containing only 15 amino

Figure 5. Schematic structure of the HLA class II molecule. HLA class II antigens consist of two polypeptide chains: the alpha chain and the beta chain. The alpha chains consist of alpha 1, alpha 2, tranamembranal and cytoplasmic regions. The beta chains consist of beta 1, beta 2, transmembranal and cytoplasmic domains.


NH2
NH2
alpha 1
beta 1
alpha 2
beta 2
cell
membrane
alpha chain
beta chain
COOH
COOH

acid residues, constitutes the cytoplasmic tail. In a fashion similar to that found in immunoglobulin molecules, the alpha 2 domain, proximate to the cell surface, is stabilized by an intrachain disulphide bridge between residues 107 and 163. Both beta 1 and beta 2 domains have stabilizing intrachain disulphide bridges, producing loops between positions 15 and 79, and 117 and 173 respectively (26, 30, 31). The alpha 1 and alpha 2 domains of the DR antigen contain an asparagine-linked carbohydrate moiety at positions 78 and 118, whereas the beta chain has a glycan linked at position 19 (32). The N-terminal domain (alpha 1 or beta 1) is highly polymorphic, whereas the second domain (alpha 2 or beta 2) is more conserved and has structural homology to immunoglobulin constant regions, as has been observed with the alpha 3 domain and beta-2-microglobulin of the class I molecules. It is the diversification of amino acid sequences in alpha 1 and beta 1 domains that reflects the heterogeneity of HLA-DR, -DQ and -DP antigenic specificities (1).

The amino acid sequences of different alpha chains of DR, DQ, and DP exhibit a high degree of homology. Any two sequences compared display a 50-60% identity. The differences occur along the whole length of the peptide chains, although the membrane-integrated sections may exhibit as much as 80% homology, while the short cytoplasmic portions vary considerably. The alpha 1 domains have less homology than the alpha 2 domains. DR alpha chains are relatively nonpolymorphic and DP alpha chains have a low degree of polymorphism. However, DQ alpha chains exhibit a high degree of amino acid sequence variability which clusters in the N-terminal alpha 1 domain (32).

The beta chains of DR, DQ, and DP molecules show a 60-70% homology and

appear to be somewhat more closely related than the corresponding alpha chain (33). The distribution of amino acid residues which differ between chains encoded by these three subregions is not limited to a particular region, although the N-terminal beta 1 domains are slightly more divergent than the beta 2 domains next to the membrane. Analogous to alpha chains, the transmembrane portions of beta chains show considerable homology, whereas the cytoplasmic portions vary considerably in amino acid sequences and length. The beta chains encoded by the DR, DQ, and DP subregions all display a considerable degree of biochemical polymorphism. The amino acid sequence variability is largely confined to the N-terminal beta 1 domains, where 15-20% of the residues may differ between the nonallelic beta chains, especially those encoded by DR and DQ (34).

1.1.6 Distribution of Class I and Class II Antigens

The biosynthesis and subsequent expression of class I antigens on the cell surface is a complex process controlled by regulatory mechanisms at the transcriptional level and post-translational processes involving the association with beta-2-microglobulin, glycosylation, and membrane insertion. Nascent HLA peptides are first transported and inserted into the membrane of the endoplasmic reticulum where within minutes they become associated with beta-2-microglobulin, which plays a central role in the intracellular transport. After core and terminal N-glycosylation, class I molecules are expressed at the cell surface 30 to 45 minutes later (35). One variation of the pathways of class I and class II membrane transport is that class I molecules are transported from Golgi directly to the cell membrane, while class II molecules are transported to endosomes and then to the cell membrane (36).

Native class I HLA complex molecules are found on the surfaces of most cells in amounts from approximately 10^3 to 10^6 molecules per cell (1). Until recently the tissue distribution of class I HLA antigens was believed ubiquitous. Only erythrocytes have been considered to lack class I HLA antigens (in certain individuals, weak expression of A28, B7, and B17 has been observed). Platelets express class I antigens, but it has been suggested that this occurs via a passive absorption of soluble class I molecules from the plasma (37). The expression of class II HLA antigens was initially thought to be limited to bone marrow-derived cells. They were first identified as expressed on peripheral blood B lymphocytes and could not be found on differentiated plasma cells. Class II molecules can also be found on antigen-processing cells such as Langerhans' cells, dendritic cells, monocytes and macrophages (38). Erythroid and granulocyte precursor cells often transiently express DR and DP, but not DQ antigens (39). The majority of resting T lymphocytes are class II antigen negative. However, following activation in MLC or by antigen or mitogen, T lymphocytes synthesize and express DR and DQ antigens on their cell surface (1, 2).

1.1.7 General Characteristics of HLA Antigens

An exceptional level of amino acid diversity is the one characteristic of HLA alloantigens that distinguishes them from most other proteins in nature. It is believed that the HLA system is derived from a common ancestral gene which has undergone duplication and mutation over the centuries, leading to the almost individual polymorphism seen today (9, 10). In addition to the extensive polymorphism, the second major characteristic of the HLA antigens is the high degree of crossreactivity. This crossreactivity can be due to structural similarities between two or more antigenic

determinants (epitopes). An antibody against an epitope on one HLA molecule may recognize a structurally similar epitope of another HLA molecule, although it may bind that epitope with a lower affinity (40). The third outstanding feature of the HLA antigens is the linkage disequilibrium phenomenon. The HLA antigens are codominantly inherited. The close linkage of the genes, however, dictates a more complicated inheritance, namely the "en bloc" inheritance of an antigen together with its neighbouring antigens. En bloc, the antigens of linked genes are collectively named "haplotype", an expression to describe the genetic constellation. Within families one would expect that products of closely-linked genes would be inherited en bloc, as a haplotype. Therefore, on a population level, certain combinations of alleles (haplotypes) are found more frequently than would be expected from the products of their allele frequencies alone. The driving force for this phenomenon is not completely understood. Haplotype frequencies can be derived from family studies or be calculated from extensive population data (41). The fourth unique feature of the HLA antigens is the occurrence of predominance of certain alleles or variants in specific populations (42). In other words, the HLA alleles are distributed unevenly among ethnic groups. Studies of DR haplotypes in American Africans have shown multiple differences from Caucasian haplotypes. In American Africans, 50% express one DR1 variant and the remaining 50% express the second DR1 variant. Only one of these variants is predominant in the Caucasian population (10). Further examples of note are the association of DR5 and DQ haplotypes in Caucasians and Blacks (10), and the distribution of A28 alleles in Caucasians (43). In the Caucasian population, DR5 is almost always observed with DQ3; however, 40% of DR5 American Blacks are DQ1

(10). For HLA-A28 (which includes A*6801, A*6802, and 68.3 alleles), it was found that the HLA-A*6801 allele was prevalent in northern European populations, in contrast to A*6802 which was prevalent in the peoples of Mediterranean basin. In North America, A*6801 and 68.3 have a combined frequency of 88.2% in Caucasians, and A*6802 was the most frequent type among Blacks (60%) (43).

1.1.8 Clinical Importance of HLA Antigens

1.1.8.1 Transplantation

HLA molecules are extremely important in tissue and organ transplantation (13). Disparity in small regions of HLA proteins can be responsible for triggering a T-or B-lymphocyte response (14). Peptides that trigger CD4+ T lymphocyte responses, critical for anti-HLA immunoglobulin (IgG) and delayed-type hypersensitivity reactions, are now being defined (44). The outcome of bone marrow transplantation between unrelated individuals is critically dependent on the degree of HLA matching between the donor and recipient. Very subtle HLA disparities can have drastic effects on success of engraftment, as evidenced by the observation that a single amino acid difference in HLA class I can cause graft rejection (45).

There is considerable controversy about the relevance of HLA-DR and -DQ antibodies in renal transplantation. The precise definition of the specificities of the class II antibody causing a positive crossmatch is important. There are a number of published studies in which the specificity of the antibody has been examined and the general consensus view is that DR antibodies are detrimental to renal allografts (46, 47). However, a successful transplantation of a patient with a positive B lymphocyte crossmatch due to DQ antibody has been reported (48). In general, the matching of

class I and class II alleles and variants improves the acceptance and maintenance of organ allogenic transplants and lessens the requirement for the use of immunosuppressive drugs (49).

1.1.8.2 Disease Association

The discovery of associations of HLA with certain diseases has been a significant breakthrough in understanding the genetics of these diseases. It is not surprising that the discoveries made concerning the genetics of HLA class I and HLA class II molecules would stimulate research in the area of a disease linkage with the HLA system (50). The model that had been emerging from these investigations was one emphasizing the essential role of HLA in immune responses. Any enhancement or deficiency of response involving the roles of class I and class II antigens could have critical consequences in terms of the types of immune responses mounted by an individual.

Involvement of HLA antigens and involvement of genes closely linked with the HLA complex were proposed as the mechanisms for the association of HLA and diseases (1, 2, 6). Hypotheses proposing HLA antigens act as receptors for pathogenic organisms (51, 52) or display molecular mimicry of infectious agents (53, 54) were proposed as the mechanisms for the association of HLA with disease. HLA antigen has also been hypothesized to be not directly involved in the causation of disease. Instead, HLA is thought to be a marker for disease susceptibility genes that are linked very closely with the HLA system. Immune response genes and metabolic genes are the suggested prime candidates (1). Correlation between HLA and autoimmune disease has been suggested by researchers (1, 55, 56). Examples of the linkage between HLA

and diseases are: B27 and ankylosing spondylitis; DR2 and narcolepsy; HLA haplotype A1, B8, DR3 and susceptibility to juvenile-onset diabetes; and, HLA-C alleles and Psoriasis vulgaris (50).

The pathogenesis of ankylosing spondylitis and seronegative spondylarthropathies, such as Reiter's syndrome and reactive arthritis involving HLA-B27 molecules, was further elucidated by the demonstration of HLA-B27-restricted bacterial-specific cytotoxic CD8+ T lymphocyte clones derived from the synovial fluid of patients with ankylosing spondylitis or reactive arthritis (57). Pockets in the antigen-binding cleft of a B27 molecule which displays a specific sequence motif were revealed to bind four side-chains and the amino and carboxyl termini of nonameric endogenous self-peptides (58, 59).

Study of HLA may also be used as a tool for the investigation of human diversity and epidemiology (60). HLA allele confers resistance to severe malaria in West African populations, as illustrated using HLA-B53 peptide. It was found that among malaria-immune Africans, HLA-B53-restricted cytotoxic T-lymphocytes recognized a conserved nonamer peptide from liver-stage-specific-antigen-1 of *Plasmodium falciparum*. This observation suggested a possible molecular basis for the role of HLA in disease susceptibility, and supports the possibility of utilizing liver-stage-specific-antigen-1 of *Plasmodium falciparum* as a malaria vaccine component (60).

1.1.9 Methods for HLA Antigen Detection

1.1.9.1 Serology

The polymorphisms of HLA gene products were originally investigated by serological procedures. Leukoagglutination was initially utilized and later the

complement-mediated lymphocytotoxicity (LCT) test. These procedures use alloantisera obtained from women with a history of pregnancy or individuals who received a blood transfusion. Recently, murine monoclonal antibodies have become available as HLA typing reagents. Although devised as early as the 1970's, the LCT test remains a widely accepted methodology for tissue typing laboratories for HLA class I antigen identification (1).

1.1.9.2 Cellular Typing

The mixed-lymphocyte reaction (MLR) or mixed-lymphocyte culture (MLC) is an *in vitro* assay that measures primarily HLA-D locus specificities. In one-way MLR assays, leukocytes from one individual (the responder) are cultured with mitomycin C-treated or irradiated leukocytes from another individual (the stimulator). If the stimulator cells (mostly B lymphocytes and monocytes) express incompatible HLA antigens, especially class II antigens controlled by the HLA-D region, responder T lymphocytes will undergo MLR proliferation. On the other hand, no MLR proliferation is observed if the stimulator is compatible (1).

A second cellular assay to define HLA polymorphisms is the primed lymphocyte test (PLT) which utilizes MLR-activated T cells as responders against a second (or different) stimulator cells. A panel of primed T lymphocytes with different specificities is usually tested for ability to undergo secondary proliferation with cells from an unknown donor. In this test, a response of cell proliferation indicates that the donor cells express the cellular determinant defined by the specific PLT cells (61).

1.1.9.3 Genomic Typing Methods

The introduction of PCR (polymerase chain reaction) method into the HLA field

has greatly facilitated the investigation of polymorphisms in HLA genes at the nucleotide level, particularly in HLA class II genes. Several methodologies are currently in use. HLA-DR oligotyping is the "classical" approach which involves amplification of specific HLA genes (in exon 2 of HLA class II sub-region of the MHC) using the polymerase chain reaction, immobilization of DNA on nylon membranes (dot blots), and sequence-specific oligonucleotide probe (SSOP) hybridization. The template DNA is usually derived from blood cells, but cells discarded after serological testing, cultured cells, buccal mucosa, and hair follicles have also been used (62).

Sequence-specific amplification using sequence-specific primer (PCR-SSP) is an another way to HLA-type a person. The principle of this technique is that each group of alleles or an individual allele making up a serological specificity is amplified by a primer pair exactly matched to that group. By keeping the PCR conditions stringent, primer pairs will not non-specifically amplify other closely-related alleles. Identification of the alleles is based on the presence or absence of amplified products observed after agarose gel electrophoresis (63). Sequence-based typing (SBT) was developed as an alternative molecular approach to the analysis of HLA class I and class II polymorphism. HLA class I SBT is based on direct sequencing of PCR-amplified HLA-A, -B, and -C cDNAs. The SBT strategy allows direct determination of the sequences of all polymorphic HLA genes in any individual without prior knowledge of an HLA type as defined by other methods, such as serology, SSOP, or SSP (64).

1.1.10 Detection of HLA Class I Variants

The detection of variants (subgroups or splits) of HLA-A, -B, and -C alleles is

most often achieved by using sera which are specific for the antigens. For instance, HLA-A9 antigen was first discovered in an LCT test using alloantisera. Soon it was realized that this antigen could be serologically further subgrouped, using specific antibodies, to HLA-A23, HLA-A24, and HLA-A24.3, each representing a variant of the A9 allele. Other examples of such demarcation include: HLA-B54, -B55, and -B56 of HLA-B22; and HLA-A29, -A30, -A31, -A32, -A33, and -A74 of HLA-A19 (**Table 1**) (18).

With the advancement of biochemical methodology using isoelectric focusing (IEF), it is also possible to distinguish variants of HLA-A, -B, and -C locus antigens. Immunoprecipitated HLA molecules, by the use of monoclonal antibodies and *Staphylococcus aureus* complex, are focused on a pH-gradient-incorporated agarose gel. Protein molecules of variants of a particular antigen, with various isoelectric points as a result of amino acid substitution, will migrate to certain positions on the electrophoresis gel. Bands representing variants of an antigen can be visualized when HLA proteins are metabolically labelled with a radio-isotope and exposed on X-ray film (65). However, preliminary determination of antigen specificities by serology is necessary to facilitate recognition of variants by IEF.

Another alternative to investigate HLA class I variants is by DNA typing using sequence-specific primers and synthetic oligonucleotide probes (66). In this particular approach, antigen-specific amplification of exon 2 and selective amplification of portions of exon 3 are accomplished with sequence-specific primers. After appropriate adjustments of the PCR conditions, hybridization patterns using products of the PCR reactions with a set of probes allow unequivocal assignment of defined nucleotides that

Table 1. Broad specificities with variants and associated antigens. Numerous antigens originally considered well-defined could be split into so-called variants (subgroups) with better discriminating antisera.

Original Broad Specificities	Variants and Associated Antigens
A9	A23, A24, A2403
A10	A25, A26, A34
A19	A29, A30, A31, A32, A33, A74
A28	A68, A69
B5	B51, B52
B7	B703
B22	B54, B55, B56
Cw3	Cw9, Cw10
DR1	DB103
DR2	DR15, DR16
DR5	DR11, DR12

characterize a given allele. The limitation of this method is that it is also necessary to know the serological antigen of interest in advance in order to select primers and probes.

Therefore, determination of variants serologically is by far the simplest and fastest approach in comparison to all other available methods.

2. RATIONALE AND OBJECTIVES

The overall goal of this research project was to explore ethnic diversity of genetic composition, in particular, diversity of the HLA system of the MHC complex and minor histocompatibility antigens. The risk of graft rejection and graft-versus-host disease is higher with increased genetic disparity between donors and recipients at this gene region. Analysis of the role of individual HLA loci and microvariants is confounded by the fact that other minor histocompatibility antigen systems are also important in transplantation. It is my belief that knowledge and understanding of the immunological role of an individual HLA antigen and its variants in association with disease susceptibility is dependent on the ability to define each antigen and its variants. As transplantation therapy has been widely accepted as the choice of treatment for patients with severe haematological disorders, such as acute leukemia, it is essential to match unrelated donor and recipient pairs for tissue antigens to reduce administration of immuno-suppressants and to avoid graft rejection or graft-versus-host disease.

DNA sequencing and cytotoxic T cell clone analyses have revealed many alleles or variants of HLA antigens in a random population. In addition, population studies have also indicated that ethnic diversity of HLA antigens is part of the nature of the HLA system. Many of the DNA sequencing alleles are not detectable by serology, mainly due to unavailability of a typing reagent.

The hypothesis for this study was that if graft rejections can be caused by humoral and cellular responses as a result of tissue antigen disparity, there will be alloantisera capable of recognizing some if not all HLA variants. Furthermore, evidence suggests that MHC is not the only set of genes involved in activities such as

graft rejection. There is a large series of minor histocompatibility loci which can provoke strong immunological reactions. Such reactions, if they involve humoral immune response, would probably include production of alloantibody in response to alloimmunization, such as blood transfusion, organ transplantation and pregnancy.

The specific objectives of this undertaking and the rationale for each objective are as the follows:

1. Identification and characterization of HLA-A2 variants.
2. Identification and characterization of HLA-A11 variants.
3. Identification of novel HLA-C locus antigens.
4. Identification of an ethnic-specific minor histocompatibility antigen.

A2 antigen is the most common HLA-A locus antigen in most ethnic populations. Are all A2 antigens in different races serologically identical? Are there antibodies capable of detecting A2 variants? The fact that the A2 antigen is present in high frequency in most populations provides an ideal area of research to investigate its heterogeneity. Information on A2 heterogeneity will be useful for further study of disease susceptibility or disease resistance in terms of the efficacy of this antigen in the binding of processed endogenous peptides, and its ability to present foreign peptides to immunocompetent accessory cells.

A11 antigen is known to be expressed in most ethnic groups with great frequency variation. Its frequency is approximately 2% in Black, 5% in Caucasian, 10% in East Indian, and 30% in Oriental populations (62). Recently, A11 was found to present peptide residues of Epstein-Barr virus (EBV) nuclear antigen-4, which is in

turn recognized by A11 restricted cytotoxic CD 8+ T lymphocytes in A11 healthy, virus-carrying individuals (67). Are there A11 variants in different ethnic groups? Are there alloantisera capable of recognizing A11 variants? If so, answers to these questions may be useful for future study to investigate differential ability of A11 antigen in EBV peptide presentation, and to analyze graft acceptance in matched or mismatched A11 variants in transplantation pairs.

Individuals with missing C-locus antigen(s) are referred to as having C-blank allele(s). While C-locus antigens have been intensively explored serologically, there remains a high frequency (20-50%) of unidentified C-blank alleles in HLA typed individuals (41, 68, 69). The inability to serologically detect C-locus antigens is believed to be due to the unavailability of specific typing sera and not to HLA-C gene deletion or mutation (70). The reasons for the scarcity of alloantisera specific to C-locus antigens is attributed to either low density or weak immunogenicity of this group of antigens. HLA-C gene transcripts have been found in lymphocytes of C-blank individuals at comparable levels to those in lymphocytes of individuals who have two serologically recognized C alleles. Indications of "new" C-locus antigens had been reported with serological procedure (68), isoelectric focusing analysis (71, 72), and nucleotide sequencing methodology (73, 74). Today, many of the DNA sequenced HLA-C alleles are unidentifiable by serology (64).

The fact that the serological assay was the first method used in discovering C-locus antigens in the early days of HLA typing, it is reasonable to believe that there are alloantibodies (which are yet to be found) capable of detecting those alleles identified by DNA sequencing. The only challenge is how many alloantisera can be found to

specifically define "new" C-blank antigens, since the amount of C-locus protein expressed on the cell surface is only 10% of what is expressed by genes in HLA-A or -B loci. Identification of "new" C-locus antigens will facilitate our understanding of its immunological function and its significance in transplantation.

Serological typing of HLA antigens with Caucasian alloantisera tends to underestimate ethnic differences and the number of alleles (75). It has been well appreciated that HLA class I alleles specific to particular ethnic groups do exist. Examples for such a distinction are: (a) HLA-B46 is limited to Oriental populations (76), (b) HLA-B13.1 is limited to individuals of Oriental origin while B13.2 is limited to Caucasian ethnic groups (77), and (c) HLA-A8001 is only found in African Americans in the United States (78, 79). The extent of ethnic diversification and heterogeneity can only be measured in population studies and molecular analysis, from the general major population to multicultural populations (ethnic minority groups and aboriginal populations).

In transplantation of organs or tissues between HLA identical siblings, matching entire HLA haplotypes and half of the many non-HLA transplantation antigens resulted in rejection unless immunosuppressive drugs were used. This indicates that there are other factors apart from MHC, e.g. minor histocompatibility (mH) antigens, that are important for transplantation; incompatibility of multiple non-HLA histocompatibility loci is extremely serious and life-threatening (80, 81, 82, 83).

In humans, T-cell immunity to mH antigens is of particular interest for its presumed role in the development of graft-versus-host disease and graft rejection after bone marrow transplantation from an HLA-identical sibling. mH antigens are able to

induce responses of both cytotoxic CD8+ and helper CD4+ T lymphocyte subsets. However, attempts to produce antibodies against mH antigens have been for the most part unsuccessful (1).

The general objective for a study in this area is to screen sera obtained from multiparous women against lymphocytes from Caucasian and non-Caucasian blood donors in order to select alloantiserum which might contain antibodies capable of recognizing mH antigens in a complement-dependent microlymphocytotoxicity test. Historically, the family of mH antigens was discovered at the same time as the MHC, but genetic and immunologic analysis of mH antigens has lagged far behind similar analyses of MHC associated alloantigens. Obviously, the major obstacle to an extensive understanding of the mechanisms of the T cell responses to mH antigens is their elusive identification. Although relatively little is known of the mechanism of these T cell responses, it is clear that a number of pathways are available, the selection of which depends upon a number of factors including multiplicity of mH antigens, importance of antigen presentation, and analysis of effector function. The molecular cloning of mH genes and the characterization of their antigenic products would be a major step toward unravelling the complexities of the T cell responses, which in turn would find direct application in developing techniques for specific modulation of responses to mH antigen that block successful clinical transplantation.

3. MATERIALS AND METHODS

3.1 Serology

3.1.1 Serum Samples

Serum samples from healthily voluntary blood donors with a history of at least two child births were screened for the presence of HLA antibody by testing against peripheral blood lymphocytes (PBLs) of known HLA phenotypes using the complement-dependent standard NIH microlymphocytotoxicity (LCT) test. Antibody specificities of sera with the positive antibody screening test were identified and confirmed with random and selected panel PBLs of known HLA typings. Sera were stored at or below -30°C to avoid denaturation.

3.1.2 PBL Samples

PBLs were isolated from acid citrate dextrose (ACD) or heparin-treated whole blood donated by healthy volunteers, using Ficoll Hypaque (Sigma) density gradient followed by thrombin treatment to remove excess platelets, granulocytes and monocytes. T and B lymphocytes were separated by nylon wool. PBLs were adjusted to a desired concentration and suspended in RPMI (Sigma) culture medium.

3.1.3 Standard NIH LCT Test

Serological typing for HLA-A, -B, and -C antigens and other LCT tests were performed according to the standard NIH technique (**Table 2**) in Terasaki microtitre wells (84). Briefly, 1μl of PBL ($2x10^6$/ml) was added to 1 μl of serum and incubated at room temperature (20-22°C) for 30 minutes. After incubation, 5 μl of rabbit complement (Cedarlane, Hornby, Ontario) were added to the mixture and incubated for a further 60 minutes. At the end of the incubation, 2 μl of 5% eosin solution were

Table 2. Procedure for the NIH LCT test. The two-stage LCT test required three components: target cells, antibodies, and complement. The cells were first incubated with antiserum and then incubated with complement. Staining of dead cells and fixation of the reaction were achieved by Eosin Y and formalin respectively. Differentiation of positive and negative reactions were observed using an inverted phase contrast microscope.

Procedures	
Step 1	Dispense alloantisera (1μl/well)
Step 2	Dispense PBL ($2x10^6$/well) T lymphocytes for Class I B lymphocytes for Class II
Step 3	Incubation RT 30' for Class I 37°C, 1 hr. for Class II
Step 4	Dispense rabbit complement (5μl/well)
Step 5	Incubation RT, 1 hr for Class I RT, 2 hrs. for Class II
Step 6	Staining (5% eosin; 2μl/well)
Step 7	Incubation RT, 2'
Step 8	Fixation (18.5% formalin; 5-10μl/well)
Step 9	Grade reaction

added to the test and fixed by 5-10 μl of 18.5% formalin solution. For HLA-DR and -DQ antigen typing, B lymphocytes were used in the procedure. In addition, serum and cells were first incubated at 37°C for 60 minutes, followed by a further incubation at room temperature for 120 minutes in the presence of rabbit complement.

3.1.4 Immunofluorescence Procedure

Serological determination of non-HLA antigen was confirmed by the immunofluorescence procedure (**Table 3**) using carboxyfluorescein diacetate (CFDA) for live lymphocytes and propidium iodide or ethidium bromide to counterstain dead lymphocytes. Briefly, to 1 ml of T (or B) lymphocytes at a concentration of $3x10^6$/ml was added 1 μl of CFDA (0.006%) solution. After gentle mixing, the mixture was incubated in the dark at 37°C in a water bath for 30 minutes followed by washing twice with RPMI culturing medium containing 5% fetal calf serum. The pellet was adjusted to a final concentration of $3x10^6$/ml in the same culture medium. CFDA-labelled lymphocytes were then used for LCT tests according to the standard NIH LCT test, except FluroQuench (One Lambda, Canoga Park, CA) was used in place of formalin. After the settling of cells, the reaction was examined manually or automatically in a fluorescent microscope (85).

3.1.5 Absorptions

Unless otherwise indicated, serum absorptions were performed by incubating 25 μl of sera and $25x10^6$ packed T lymphocytes or equal volume of serum with equal volume of packed red blood cells at 4°C for 60 minutes, with mixing at 15 minute intervals. Sera were retrieved by high speed centrifugation at 12,000 rpm for 15 minutes.

Table 3. Procedure for immunofluorescence LCT test. In immunofluorescence LCT test, the cells were incubated with CFDA first and followed by incubation with antiserum and complement as in the two-stage NIH LCT test. FluroQuench was added at the end of the test to stain dead cells instead of Eosin Y and formalin.

Procedures	
Step 1	Label T lymphocytes with CFDA 37°C, 30'
Step 2	Wash cells 2x, RPMI
Step 3	Adjust cell concentration $3x10^6$/ml
Step 4	NIH LCT test
Step 5	Add FluroQuench 5μl/ml
Step 6	Grade reaction

3.2 Red Blood Cell Antibody Screening Test

Alloantisera were tested with red blood cells of 12 individual donors in an Indirect Antiglobulin Test (86). These red blood cells comprehensively comprised antigens for the major erythrocyte blood group systems.

3.3 HLA Class I Biochemistry

3.3.1 One-Dimensional Isoelectric Focusing

The methods and reagents for one-dimensional isoelectric focusing (1D-IEF) (**Figure 6**) were based on the 10th International Histocompatibility Workshop (87). This procedure may be divided into several stages: (A) metabolic labelling of HLA class I antigens, (B) cell lysis, (C) immunoprecipitation of class I antigens with monoclonal antibody w6/32 (courtesy of the 10th IHW), (D) neuraminidase treatment, and (E) IEF analysis. The details of each are described below:

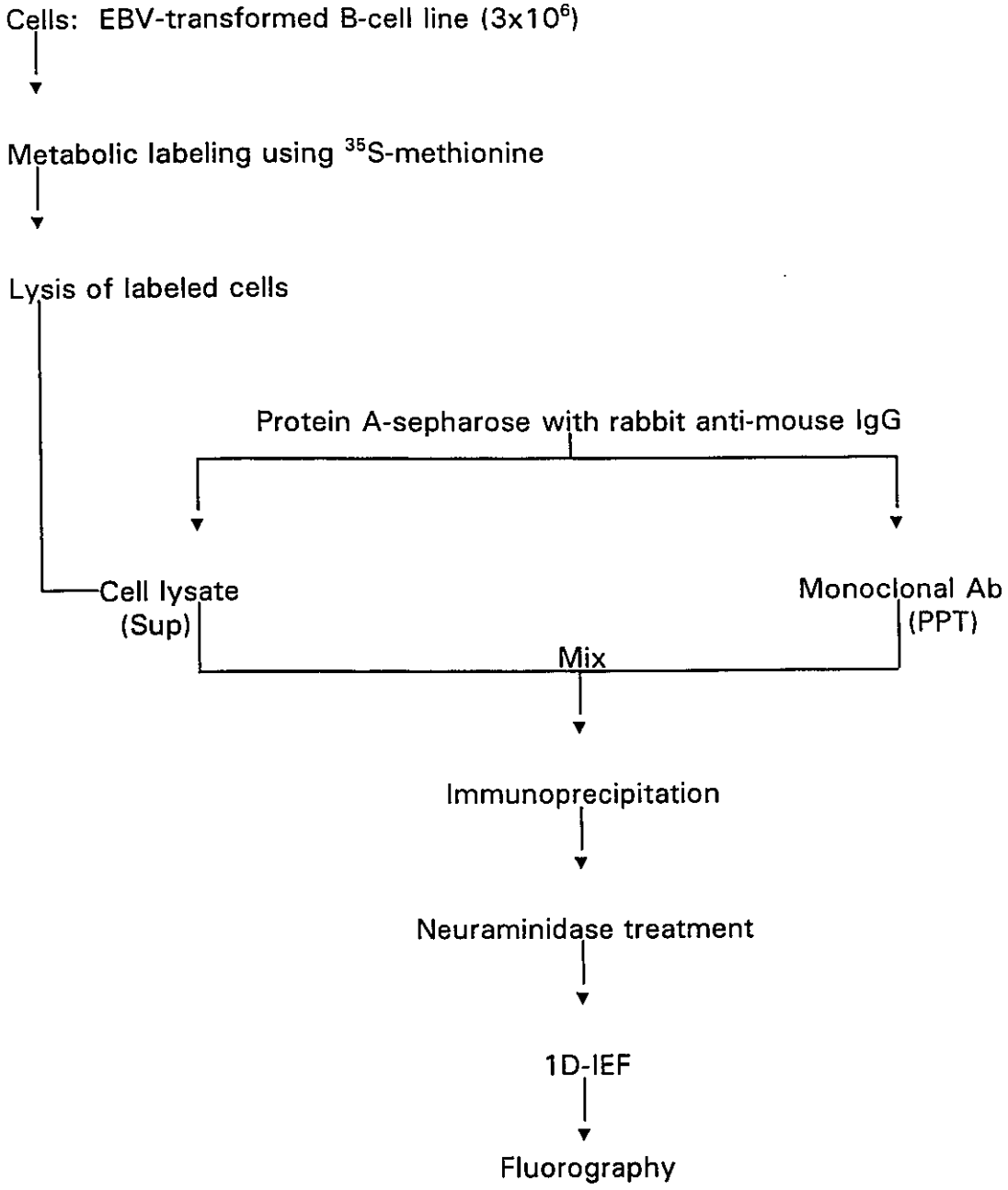
A. Metabolic Labelling of HLA Class I Antigens

Epstein-Barr virus transformed B-lymphoblastoid cells from donors of interest were kindly provided by the HLA Laboratory, National Office, The Canadian Red Cross Society, Ottawa. Control B-lymphoblastoid cell line with known IEF HLA-A and -B typing (A1, A2.2, B27, and B35) was obtained from the 10th IHW.

B-lymphoblastoid cells at a concentration of $3x10^6$ per ml were first pelleted by centrifugation in a Beckman TJ-6 centrifuge. After centrifugation, supernatant was decanted into 1% (v/v) bleach solution and the pellet resuspended in methionine-free RPMI-1640 supplemented with 12% FCS, glutamine and Penicillin-Streptomycin-Fungizone in a sterile 24-well culture plate ($3x10^6$/1 ml/well). To each well of cell culture, 50 μCi of ^{35}S-methionine were added and mixed with the cells using a Pasteur

Figure 6. Steps of 1D-IEF gel electrophoresis. The procedure of 1D-IEF was as described in the text (3.3.1). Sup, supernatant; PPT, precipitate.

1D-IEF


Cells: EBV-transformed B-cell line (3x10⁶)
Metabolic labeling using ³⁵S-methionine
Lysis of labeled cells
Protein A-sepharose with rabbit anti-mouse IgG
Cell lysate (Sup)
Monoclonal Ab (PPT)
Mix
Immunoprecipitation
Neuraminidase treatment
1D-IEF
Fluorography

pipette, then incubated in a 37°C CO_2 incubator for 18-19 hrs (overnight).

B. Preparation of Cell Lysates for IEF Analysis

Metabolically labelled cells were transferred to a 12 ml centrifuge tube and capped (contents of one well per tube). Pelleted cells were collected after centrifugation at 2,000 rpm for 15 min. Cells were washed with phosphate buffered saline and pelleted by centrifugation. To each pellet, 1 ml of lysis buffer was added, resuspended with a Pasteur pipette, transferred to an Eppendorf tube and incubated on ice for 30 min. After incubation, the lysed cells were centrifuged at 4°C for 10 min. in a refrigerated microfuge. After centrifugation, the supernatant was transferred to a prelabelled Eppendorf tube and the cell pellet discarded. The cell lysate was incubated in a 37°C water bath for 10 min. followed by centrifugation at 1,400 rpm for 3 min. The supernatant was discarded. To each lysate 0.7 ml of TNEN buffer (20 mM Tris-HCl, pH 7.6; 0.5% NP-40; 10mM EDTA; 0.1 M NaCl) was added. The sample was further processed or stored at -65°C.

C. Immunoprecipitation of Class I Antigen with MoAb w6/32

Class I antigen immunoprecipitation was performed by incubating the cell lysate with 5μl of hyperimmunized rabbit anti-mouse immunoglobulin (RAMIg). The lysate-RAMIg mixture was transferred to 0.25 ml pelleted *Staphylococcus aureus* cells and mixed thoroughly with a Pasteur pipette. The mixture was rotated at 4°C for 15 min. followed by centrifugation in a 4°C microfuge for 10 min. to obtain precleared lysate. The precleared lysate was added to an Eppendorf tube containing *Staphylococcus aureus* cells previously coated with MoAb w6/32 (IgG 2b). The tube containing the mixture was allowed to rotate at 4°C for 30 min. followed by centrifugation in a refrigerated

microfuge for 5 min. to obtain the pellet. The pellet was washed twice with 2 ml of the first wash solution (0.1 % SDS and 1% BSA in TNEN) and once with 1 ml of the second wash solution (0.5M NaCl in 1/10 dilution of TNEN).

D. Neuraminidase Treatment

The final pellet from the above was treated with 20 μl of type III neuraminidase (Sigma; in 50 mM EDTA, pH 7.0) containing 1mM fresh PMSF (phenylmethylsulfonylfluoride) in ethanol and incubated at 37°C for 3 hrs. At the end of the incubation, 1 ml of the second wash solution was added to each sample and the mixture centrifuged in a refrigerated centrifuge. The supernatant was discarded and 50 μl of IEF sample buffer were added to each pellet and allowed to stand at RT for 30 min.

E. IEF Analysis

To begin the IEF procedure, gel box apparatus, glass plates (Aquebogue Inc., New York), IEF gel mixture (containing ampholine, pH 3.5-10.0) and IEF running buffers (15 mM H_2PO_4 and 20 mM NaOH) must first be prepared. Samples (10 μl) from step C were placed in individual wells of the gel and overlaid with IEF buffer. H_2PO_4 was added into the lower chamber and NaOH into the upper chamber, and electrophoresis was performed at 200 volts for the first hr. and then at 400 volts overnight (16-18 hr.). Voltage was increased to 1,000 volts for the last hour. At the end of electrophoresis, the gel was removed from the apparatus and fixed in 15% acetic acid for one hr. and then treated with Amplify (Amersham) for 15-20 min. Lastly, gel was dried and stored with x-ray film (KODAK XAR) at -70°C for three days.

4. RESULTS

4.1 Identification and Characterization of HLA-A2 Variants

4.1.1 Identification of Alloantibodies Against A2 Variants

The experiments in this section characterized alloantibodies to the A2 variants and employed these reagents in determining A2 antigen variants. Six A2 alloantisera were selected for this purpose. All six sera were kindly donated by Caucasian donors. The criterion to select a serum was based on the reactivity of the serum versus A2-positive T lymphocytes. Based on their differential reaction patterns against T lymphocytes possessing A2 antigens, these sera were divided into three reactive groups. The first group of A2 antiserum (broad specificity; n=3) consisted of sera that reacted with T-lymphocytes of all A2 donors (n=301) from all ethnic groups tested. The second group of A2 antiserum (n=2; sera 02479 and 02782) reacted with T lymphocytes of some (n=52) but not all of Oriental donors (n=92). All A2 positive T lymphocytes from Caucasian (n=194), East Indian (n=9), and Black (n=6) donors reacted with this group of A2 antisera. The third group of A2 antiserum (n=1; serum 03035) reacted with all A2-positive cells from Caucasian donors (n=194), some Oriental donors (n=27), and most East Indian (n=8) donors. These serological A2 antigen variants were designated as A2.1 variant, A2.2 variant, and A2.3 variant. The reaction patterns of all variants against the three reactive groups of antisera are summarized in **Table 4**. From the above results, three A2 variants were defined.

4.1.2 Frequency of A2 Variants in Different Ethnic Groups

The frequency of the A2 variants described above was determined in different ethnic populations. The complement-dependent LCT test was employed for this

Table 4. Reaction patterns of A2 variants with three groups of alloantisera. Using the NIH LCT test, T lymphocytes from donors expressing A2 antigens were tested with three groups of alloantisera (broad specificity A2 sera = group 1; sera 02479 and 02782 = group 2; serum 03035 = group 3). Variants of A2 antigen were assigned based on the serological reaction pattern of each T lymphocyte donor versus each group of alloantiserum.

Groups	Sera	A2 Variants		
		A2.1	A2.2	A2.3
1	Broad specificity A2 sera (n = 3)	+	+	+
2	02479	+	-	+
	02782	+	-	+
3	03035	+	+	-

purpose. A total of 485 Caucasian donors was tested against sera 02782, 02479, and 03035. All Caucasian donors with A2 antigen (n=194) belonged to the A2.1 variant type. Of 160 Chinese and Vietnamese donors tested, 52 of 92 (56.5%) A2 positive individuals were serologically typed as expressing the A2.1 variant. Thirteen of 92 (14.1%) expressed the A2.2 variant, and the remaining 27 individuals (29.3%) belonged to the A2.3 variant type. In East Indian blood donors, out of a total of 44 individuals tested, eight of nine (89.9%) A2 positive individuals belonged to the A2.1 variant type. The remaining donor (10.1%) expressed the A2.3 variant. However, the A2.2 variant was not found in this ethnic population. With a limited number of Black donors (n=26) tested, it was found that all A2 positive individuals (n=6) were of the A2.1 variant. The frequency of each variant in each ethnic group is summarized in **Table 5**.

4.1.3 IEF Analysis of A2 Variants

In order to confirm the serological assignment of A2 variants in various ethnic groups in this study, IEF analysis of A2 antigen was performed employing EBV-transformed B lymphoblastoid cell-lines derived from selected donors expressing variants of interest. In the isoelectric focusing analysis, shown in **Figure 7** and **Figure 8**, all A2 variants are the most basic proteins among HLA-A and -B antigens identified. The IEF results illustrated that the pattern of the A2 antigen consisted of four distinctive protein bands which were more basic than the beta-2-microglobulin protein. Each band identified on IEF gels represented an A2 variant. These bands are designated as IEF variants A2.1, A2.2, A2.3, and A2.5 based on their descending order on the autoradiograph.

Table 5. Population distribution of serological A2 variants. Using the NIH LCT test, A2 T lymphocytes from donors of various ethnic background were tested against three groups of alloantisera that had been characterized previously to differentiate A2 variants. The percentage of each variant in each ethnic group of donors was calculated according to the ratio of number of donors reacting to each group of serum versus the total number of donors tested.

Ethnic Groups	A2 Variants %		
	A2.1	A2.2	A2.3
Oriental (n = 92)	56.5	14.1	29.3
East Indian (n = 9)	89.9	0	10.1
Caucasian (n = 194)	100	0	0
Black (n = 6)	100	0	0

Figure 7. IEF banding positions of HLA-A2 variants. MoAb w6/32 immunoprecipitated HLA proteins prepared from ^{35}S-methionine metabolically labelled EBV transformed B-lymphoblastoid cells were subjected to IEF analysis. Selected cells with serologically defined A2 variants were analyzed. IEF banding position of each A2 variant from each cell-line was assigned in comparison to control cell line in lane 1. The cell-line of lane 2 was derived from a Caucasian donor, while cell-lines of lanes 3 to 7 were derived from Oriental donors.

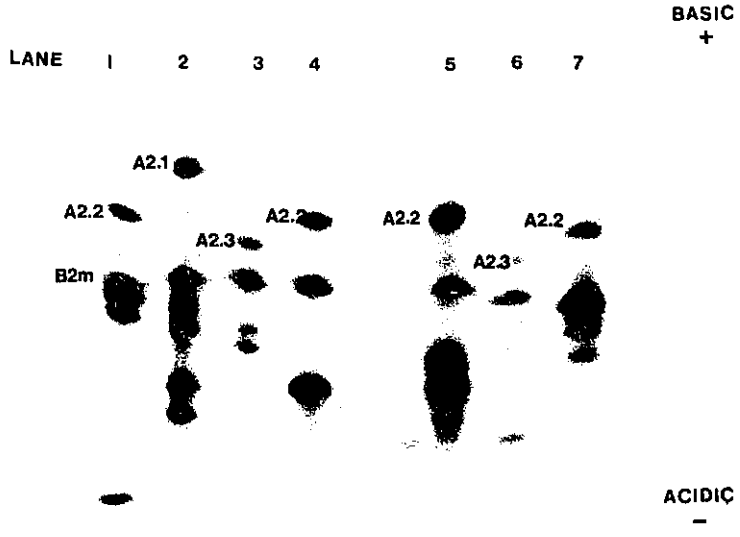
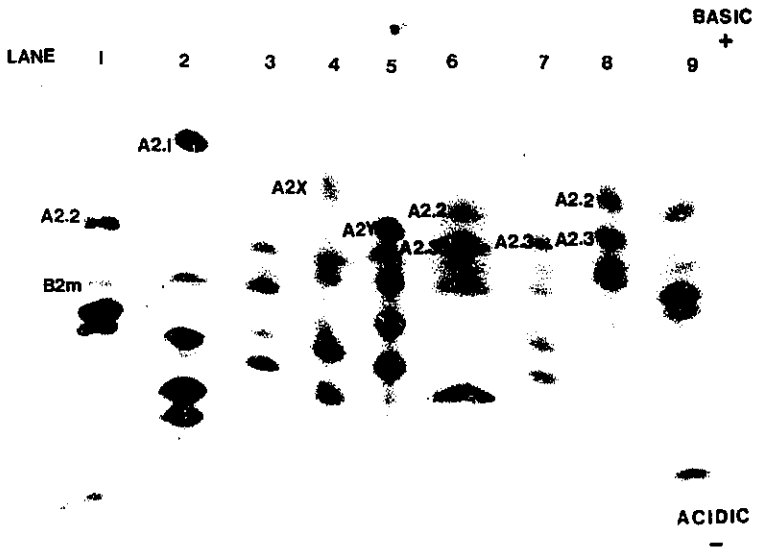

BASIC
+
LANE 1 2 3 4 5 6 7
A2.1
A2.2
A2.2
A2.3
A2.2
A2.2
A2.3
B2m
ACIDIC
−

Figure 8. IEF banding positions of possible HLA-A2.2 variants. IEF analysis to demonstrate further subgroups of A2.2 variant was performed according to the experiment described in Figure 7. Variants of A2.2 (A2X and A2Y) were assigned based on the variation of A2.2 banding positions in comparison with control cell line in lanes 1 and 9, and other serologically determined A2 variants from lane 2 to lane 8. The cell line in lane 2 was obtained from a Caucasian donor while lane 3 to lane 8 were from Oriental donors.


BASIC
+
LANE
1
2
3
4
5
6
7
8
9
A2.1
A2X
A2.2
A2.2
A2.2
A2Y
A2.3
A2.3
A2.3
B2m
ACIDIC
–

As shown in **Figure 7** and **Figure 8**, the most basic band, IEF variant A2.1, was identified in a Caucasian donor. IEF variant A2.2 was found in this study to be the most common variant in all ethnic groups including Caucasian, East Indian, and Oriental populations. The A2.3 IEF variant was found only in Oriental individuals particularly in Chinese and Vietnamese populations. In **Figure 9**, the most acidic IEF variant, A2.5 (serologically typed as A2.3), was found only in an East Indian donor. The banding position of A2.5 IEF variant migrated very closely together with the beta-2-microglobulin. In addition, **Figure 9** further demonstrates that A2 Caucasian donors serologically expressing only A2 antigen are indeed homozygous for IEF A2.2 variants, containing one single IEF A2.2 protein band on IEF gel.

4.1.4 Serological Testing of Cells with IEF A2 Variants

To correlate IEF A2 variants with serological A2 variants, T lymphocytes of donors expressing IEF variants A2.1, A2.2, A2.3, and A2.5 were tested in the NIH LCT test with alloantisera recognizing serological A2 variants.

Results in **Table 6** show that IEF variant A2.1 reacted with sera 02782, 02479, and 03035; IEF variant A2.3 reacted with serum 03035; IEF variant A2.5 reacted with sera 02782 and 02479; and IEF variant A2.2 reacted with sera 02782 and 02479 similarly to IEF variant A2.1. However, serum 03035 gave inconsistent results failing to react with some Oriental donors with IEF A2.2 variant.

4.1.5 Family Study of A2 Variants

In order to verify the specificity and reproducibility of the reagents recognizing A2 variants, alloantisera were tested in family studies. In the family study shown in **Figure 10A**, the mother was serologically typed as A2.1 and the father A2.2; the child

Figure 9. IEF banding position of HLA-A2.5 variant. IEF analysis to demonstrate A2.5 variant in an East Indian donor was performed as the experiment described in Figure 7. Assignment of A2.5 band in the East Indian donor in lane 3 was based on the comparison of all A2 bands of the control cell-line in lane 11 and all other lanes. The donor in lane 1 was an American Black, and donors in lanes 2 and 4 to 10 were of Caucasian origin.

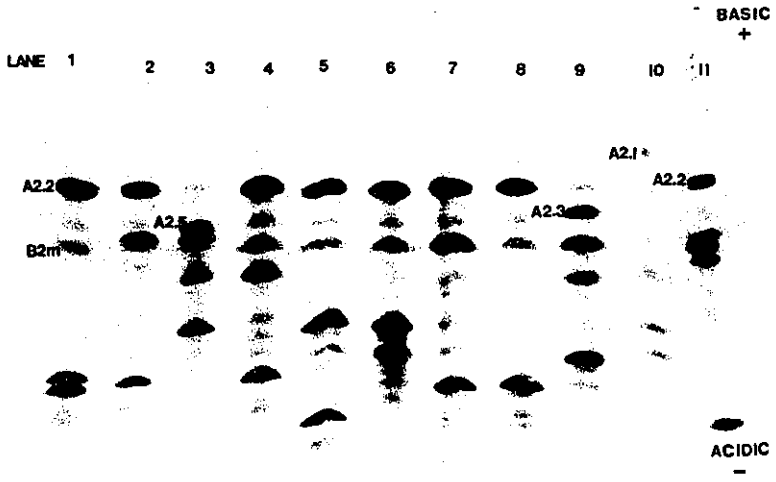

BASIC
+
LANE 1 2 3 4 5 6 7 8 9 10 11
A2.2
B2m
A2.5
A2.3
A2.1
A2.2
ACIDIC
−

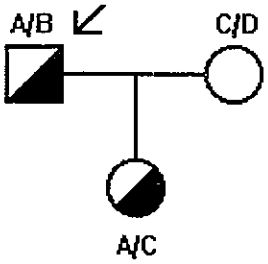
Table 6. Reaction patterns of IEF variants with A2 alloantisera. Using the NIH LCT test, T lymphocytes expressing IEF variants A2.1, A2.2, A2.3, and A2.5 from Caucasian (n=8), East Indian (n=1), and Oriental donors (n=13) were tested with sera previously identified (in Table 4) as recognizing serological variants A2.1, A2.2, and A2.3.

Sera	IEF Variants			
	A2.1 (n=1)	A2.2 (n=8)	A2.3 (n=3)	A2.5 (n=1)
02782	+	+	-	+
02479	+	+	-	+
03035	+	+/-*	+	-

*In Oriental donors with serological variant A2.3 phenotype (See section 4.1.4).

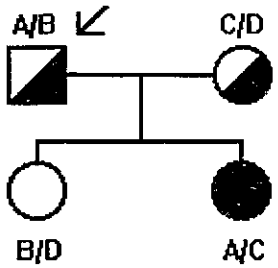
Figure 10. Family study of HLA-A2 variants using alloantisera. Using the NIH LCT test, T lymphocytes of all family members were tested with the three groups of alloantisera previously shown to differentiate serological A2 variants. The assignment of each A2 variant in each propositus (indicated by an arrow) was as described in Table 4. The assignment of haplotypes possessed by each donor was deduced based on serological tissue typing. (A) Pedigree of the family showing segregation of haplotypes bearing A2.1 and A2.2 (represented by half-shaded circles or squares); (B) Pedigree of the family showing segregation of haplotypes bearing A2.1 (represented by full or half-shaded circles).

A



A: A2.2, B62, Cw10
B: A11.1, B13, Cw4
C: A2.1, B13
D: A11.1, B62, Cw10

B



A: A2.1, B60, Cw7
B: A11.2, B13, Cw10
C: A2.1, B46, Cw1
D: A24, B62, Cw1

inherited both maternal A2.1 and paternal A2.2 variants, but was serologically typed as expressing only A2.1. No antiserum is available to distinguish between A2.1 and A2.2 variants when an individual is heterozygous for each antigen. Using isoelectric focusing, shown in **Figure 8** (in lane 6 and lane 8), both serological variants A2.1 (IEF A2.2 band) and A2.2 (IEF A2.3 band) were easily distinguishable in donors that had A2.1 and A2.2 serological variants. In another family, as shown in **Figure 10B**, both parents were serologically typed to express A2.1 variant (IEF variant A2.2). As expected, the offspring inherited paternal haplotype A2.1, B60 and maternal haplotype A2.1, B46, and expressed only A2.1 serological variant (IEF variant A2.2). Family study on serological variant A2.3, unfortunately, was impossible due to unavailability of the parents.

4.2 Identification and Characterization of HLA-A11 Variants

4.2.1 Identification of Alloantibodies Against A11 Variants

Experiments were also performed to characterize alloantibodies to the A11 variants and to employ these reagents to determine A11 antigen variants. Alloantisera against the target antigen A11 were first selected. Six antisera, obtained from Caucasian blood donors, displaying three groups of reaction patterns were chosen for this purpose. The first serum group (n=4), designated as anti-A11.1+A11.2, reacted with all A11 positive T lymphocytes from all ethnic groups tested (broad specificity). The second serum group (n=1), designated as anti-A11.1, reacted with all A11 positive T lymphocytes from all ethnic groups tested (n=122), except a portion of A11 positive T lymphocytes from Oriental blood donors (variant specificity). The third serum group (n=1), designated as anti-A11.2, reacted only with some Oriental A11 positive

individuals but failed to react with all Caucasian (n=12), all East Indian (n=35), and some Oriental blood donors. The serological patterns of these three groups of sera are summarized in **Table 7**. From the above results, two A11 variants could be defined. These serological A11 antigen variants were designated as A11.1 variant and A11.2 variant.

A11.1 variant is the common form of A11 variant observed in all ethnic groups including Caucasian, Oriental, and East Indian populations. However, the rarer variant A11.2 was found only in Oriental donors.

4.2.2 Family Study of A11 Variants

Alloantisera were tested in family studies in order to verify the specificity and reproducibility of the reagents recognizing A11 variants. Segregation of A11.2 and A11.1 variants were investigated in three families as depicted in **Figure 11(A, B, C)**. Results in **Figure 11A** show that the haplotype of A11.2, Cw10, B13 was inherited from the father by one of the daughters. The family study shown in **Figure 11B** indicates that the haplotype of A11.2, Cw -, B48 was the same haplotype for the two sisters; one of them can actually be serologically typed to express both A11.1 and A11.2 variants. **Figure 11B** also demonstrates that the transmission of A11.2, Cw -, B48 haplotype occurred from the mother to her son. Family study results illustrated in **Figure 11C** reveal that the transmission of A11.1 antigen from the A11.1 homozygous father to all four of his children. It also shows that, in this family, A11.1 was expressed in either of two haplotypes: A11.1 and B60 or A11.1 and B70 haplotypes.

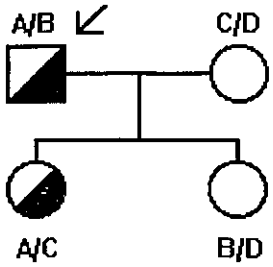
Results of the above-described family studies confirm that the inheritance of

Table 7. Serological reaction patterns of HLA-A11.1 and -A11.2 antigens. Using the NIH LCT test, donor T lymphocytes expressing A11 antigen were tested against broad specificity antisera (anti-A11.1+A11.2) and variant-specific antisera (anti-A11.1 and anti-A11.2). A11 variant was assigned based on the serological reaction pattern of T lymphocytes of each donor versus each group of antiserum.

Cells	Sera		
	Anti-A11.1 + A11.2 (n = 4)	**Anti-A11.1 (n = 1)**	**Anti-A11.2 (n = 1)**
A11.1 (n= 61)	+	+	-
A11.2 (n = 14)	+	-	+

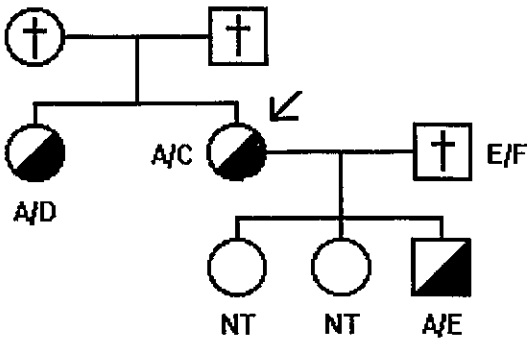
Figure 11. Family studies of HLA-A11 variants using alloantisera. Using the NIH LCT test, T lymphocytes of all available family members were tested with broad and variant-specific A11 alloantisera. Assignment of each variant was based on the serological reaction pattern of donor cells towards each group of antiserum as described in Table 7. The assignment of haplotypes borne by each propositus (indicated by an arrow) was deduced according to serological tissue typing. (A) and (B) Pedigrees of the families showing segregation of haplotype bearing A11.2 (represented by a half-shaded circle or square); (C) Pedigree of the family showing segregation of haplotypes bearing A11.1 (each is represented by a full or half-shaded square). Deceased family members are depicted by a cross. NT= not tested.

A



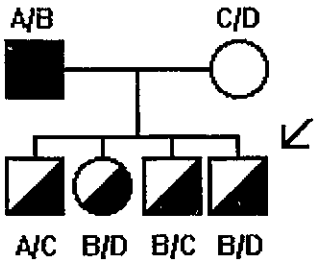
A: A11.2, B13, Cw10
B: A2, B60, Cw7
C: A24, B62, Cw1
D: A2, B46, Cw1

B



A: A11.2, B48
C: A33, B58, Cw3
D: A11.1, B75, Cw8
E: A33, B75, Cw8

C



A: A11.1, B60
B: A11.1, B70, Cw3
C: A2, B60
D: A24, B60

A11.1 and A11.2 variants was under genetic control and the reactions of the alloantisera defining each variant were indeed specific and reproducible. More importantly, each form of A11 variant within each family was identified.

4.2.3 Frequency of A11 Variants in Different Ethnic Groups

The frequency of the A11 variants described above was determined for different ethnic origins. The complement-mediated LCT test was again employed for this purpose. In a total of 146 Chinese donors tested, 75 were found to be A11 positive (51.36%). Out of these, 61 individuals (81.33%) were of the A11.1 subtype and the remaining 14 donors (18.67%) were of the A11.2 subtype. In a total of 125 East Indian blood donors tested, 35 (28%) A11 positive individuals were identified. In a total of 114 Caucasian blood donors tested, only 12 (10.5%) expressed A11 antigen. In contrast to Oriental blood donors, A11.1 variant was the only type of A11 variant observed in both Caucasian and East Indian blood donors (**Table 8**).

4.2.4 IEF Analysis of A11 Variants

In order to verify the serological assignment of A11 variants using variant-specific alloantisera in this study, IEF analysis of A11 antigen was performed. IEF results in **Figure 12** and **Figure 13** demonstrate that two distinctive bands corresponding to serological A11.1 and A11.2 variants are detected.

The band of the common variant A11.1 is more acidic than the less common variant A11.2. In other words, the banding position of A11.2 band is closer to the beta-2-microglobulin band than to the A11.1 band. Additionally, a donor serologically typed to express both A11.1 and A11.2 variants (**Figure 11B**) was confirmed by IEF to contain both A11.1 and A11.2 proteins (in lane 5 of **Figure 13**). Results obtained

Table 8. Population distribution of serological A11 variants. Using the NIH LCT test, A11 T lymphocytes from donors with different ethnic background were tested with broad specificity antisera and variant-specific antisera which were previously shown to characterize different A11 variants.

Ethnic Groups	All Variants %	
	A11.1	A11.2
Oriental (n = 75)	81.33	18.67
East Indian (n = 35)	100	0
Caucasian (n = 12)	100	0

Figure 12. IEF analysis on expression of HLA-A11.2 antigen in an Oriental family. MoAb w6/32 immunoprecipitated HLA proteins prepared from ^{35}S-methionine metabolically labelled EBV transformed B-lymphoblastoid cells of family members shown in Figure 11A were analyzed by IEF. IEF bands of the control cell-line are shown in lane 1. Lanes 2 and 5 are of maternal and paternal cells respectively. Lanes 3 and 4 are those of two offspring. Band of each serological antigen of each family member was deduced by comparison with IEF report of the 10th IHW (87).

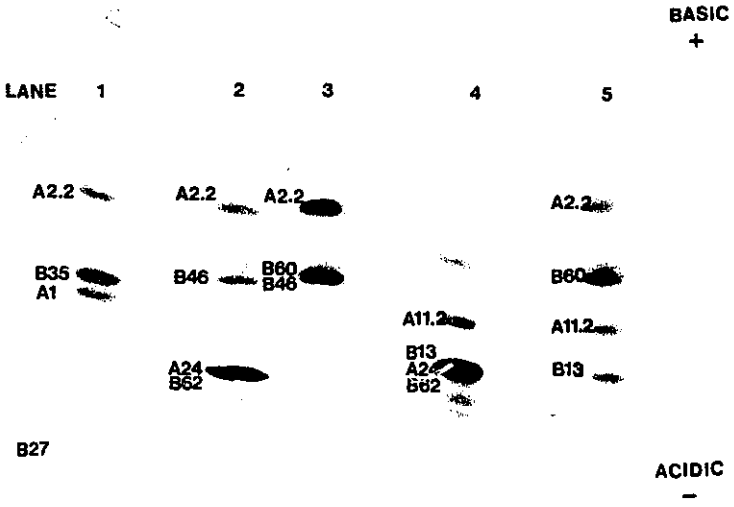
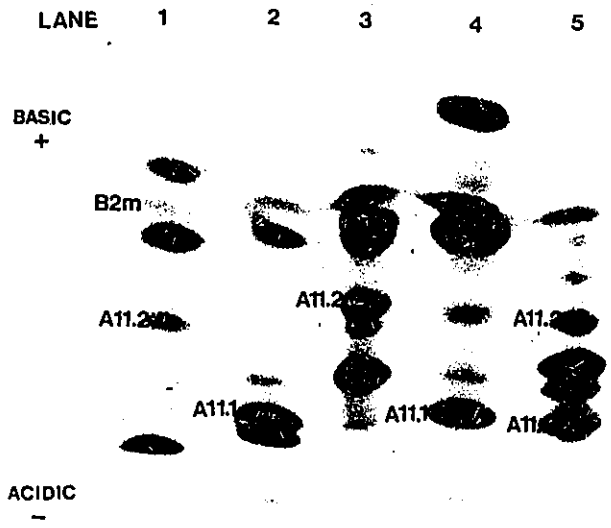

BASIC
+
LANE 1 2 3 4 5
A2.2
A2.2
A2.2
A2.2
B35
A1
B46
B60
B46
B60
A11.2
A11.2
B13
A24
B62
B13
B27
ACIDIC
−

Figure 13. IEF analysis of HLA-A11.1 and HLA-A11.2 antigens. MoAb w6/32 immunoprecipated HLA proteins prepared from ^{35}S-methionine metabolically labelled EBV transformed B-lymphoblastoid cells known to express A11.1 and A11.2 serological variants were subjected to IEF analysis. IEF band corresponding to each A11 variant is indicated. Lanes 1, 3, 4 and 5 are of Oriental donors; donor in lane 2 is Caucasian.


LANE 1 2 3 4 5
BASIC
+
B2m
A11.2
A11.2
A11.2
A11.1
A11.1
A11.1
ACIDIC
–

in the experiment described in this section substantiated the serological assignment of A11.1 and A11.2 antigen, and subsequently verified the specificity of the alloantisera used in the study.

4.3 Identification of Novel HLA-C Locus Antigens: Cw Hunt and Cw Grace

The strategy to select sera for the identification of serologically undetermined HLA-C locus antigens was to employ antisera containing characterized specificity(ies) against WHO-recognized C-locus antigens and un-characterized specificity(ies) against T lymphocytes from individuals with C-blank(s) phenotype.

4.3.1 Cw Hunt

4.3.1.1 Identification of an Alloantiserum Recognizing Cw Hunt

Experiments performed in this section were to characterize a selected alloantiserum to recognize a novel C-locus antigen: Cw Hunt. One serum (serum 3028) that contained anti-Cw2 antibody and an unknown specificity consistently reacting with C-blank T lymphocytes was employed for this investigation. Using the complement-dependent LCT test, crossmatch of serum 3028 with random and selected donor T lymphocytes (n=2,563) confirmed that the serum reacted with cells expressing Cw2 antigen and cells expressing either no serologically detectable HLA-C antigen or only one WHO-recognized HLA-C antigen. Additionally, the serum failed to react with T lymphocytes expressing two other C-locus antigens, except Cw2 antigen for which it was specific (**Table 9**). Crossmatch test of serum 3028 with T lymphocytes of the husband of the serum donor (A2, A24, B27, Cw2, DR1, DR4, DR53, DQ1, and DQ7) was positive. The crossmatch test of serum 3028 and red blood cells of the husband

Table 9. Reactivity of Cw Hunt antibody with selected and random panel donors. To demonstrate that the specificity of serum 3028 is directed against an antigen in C locus, serum 3028 was tested, using the NIH LCT test, with selected and random HLA typed individuals expressing one C locus antigen, no C locus antigen, or two C locus antigens. The total number of donors tested and the percentage of reactive donors in each category are indicated.

LCT Reaction	HLA-C Locus Haplotype						
	Single blank *		Double blank @		Full House #		Total No. Tested
	No.	%	No.	%	No.	%	
Positive	127	4.95	22	0.85	0	0	2,563

* One HLA-C antigen present serologically

@ No serologically detectable HLA-C antigen

\# Two HLA-C antigens present serologically

was impossible due to the ABO blood group incompatibility. However, absorption of serum 3028 by the husband's packed red blood cells failed to remove its specificity, whereas his T lymphocytes completely abolished its reactivity (**Table 10**).

4.3.1.2 Family Study

In order to confirm the specificity and reproducibility of the alloantiserum 3028 in detecting the new C-locus antigen, family studies using the complement-mediated LCT test were again performed. Results of the family studies are demonstrated in **Figure 14(A, B, C)**. The specificity of serum 3028 directed against T lymphocytes expressing a new C-locus antigen was confirmed as it only reacted with cells with either no serological detectable C-locus antigen or cells that possess only one WHO-recognized C-locus antigen, in the absence of Cw2 antigen. Serum 3028 also illustrated in these family studies that the segregation of the new C-locus antigen is in parallel with C-blank haplotype, and the expression of the new C-locus antigen is under genetic control.

4.3.1.3 Absorption Study

An absorption study was conducted to investigate further if the reactivity of serum 3028 against the new C-locus antigen of each donor was due to the same specificity. The serum was first absorbed by C-locus blank lymphocytes of a donor and tested with T lymphocytes from the serum donor's husband and other C-locus blank donors. Results of the absorption study shown in **Table 11** demonstrate that the specificity of the serum toward T lymphocytes of each C-blank donor previously shown to be detected by serum 3028 appeared to be the same. Therefore, each of these donors shared an antigen specificity expressing common public epitopes. A positive reaction

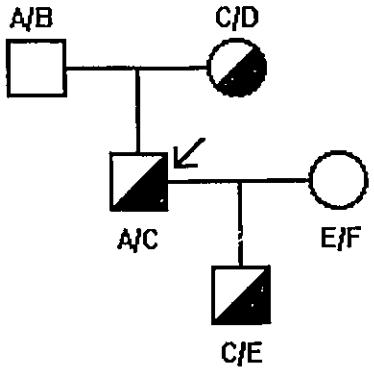
Table 10. Effect of absorption on the reactivity of Cw Hunt antibody. In order to determine that the specificity of serum 3028 is directed toward antigen in association with HLA class I antigen, serum 3028 was first absorbed by T cells or red blood cells of the serum donor's husband. Using the NIH LCT test, absorbed and unabsorbed serum 3028 was tested with T lymphocytes from the serum donor's husband and from donors known to express only one C- locus antigen and that had been previously shown to react with serum 3028.

Cell Tested	HLA Phenotype	LCT		
		Unabsorbed	Absorbed By	
			T Cells*	Red Cells*
1	A2, A11, B60, Cw7	+	-	+
2	A2, B38, B76, Cw4	+	-	+
3	A2, A29, B38, B7, Cw7	+	-	+
4	A1, B61, B37, Cw6	+	-	+
5	A31, A32, B52, B60, Cw1	+	-	+
6*	A2, A24, B27, Cw2	+	-	+

* Husband of serum donor

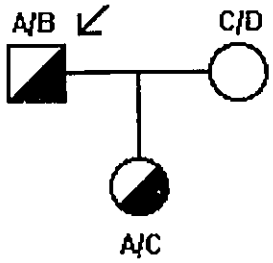
Figure 14. Family studies to verify the identification of Cw Hunt antigen using alloantiserum 3028. Using the NIH LCT test, T lymphocytes of all family members were tested with serum 3028. Assignment of haplotypes borne by each family member was deduced based on serological tissue typing. Propositi (indicated by arrows) expressing Cw Hunt antigen were assigned based on their positive reaction with serum 3028 and are represented by half-shaded circles or squares. (A) Pedigree of the family showing segregation of Cw Hunt on A2, B60 haplotype; (B) Pedigree of the family showing segregation of Cw Hunt on A2, B13 haplotype; (C) Pedigree of the family showing segregation of Cw Hunt on A1, B61 haplotype. NT= not tested.

A



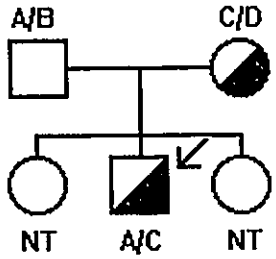
A: A11, B60, Cw7
B: B70, Cw3
C: A2, B60, Cw Hunt
D: A24
E: A24, B46, Cw1
F: A33, B58

B



A: A2, B13, Cw Hunt
B: A11, B62, Cw10
C: A2, B62, Cw10
D: A11, B13

C



A: A1, B61, Cw Hunt
B: B37, Cw6
C: A32, B52, Cw Grace
D: A31, B60, Cw Hunt

Table 11. Effect of absorption using cells with Cw Hunt phenotype. In order to illustrate that the reactivity of serum 3028 toward T cells of each reactive donor was due to the same Cw Hunt specificity, serum 3028 was first absorbed by T lymphocytes of the serum donor's husband and T lymphocytes from donors previously speculated to express Cw Hunt. Using the NIH LCT test, unabsorbed and absorbed sera were tested against the T lymphocytes of the same donors employed in the absorption.

Cell Tested		LCT			
		Unabsorbed	Absorbed By T Cells from Donor		
No.	Phenotype		No. 2	No.3	No.4
1*	A2, A24, B27, Cw2, (?Cw Hunt)	+	-	-	-
2	A1, A24, B61, Cw Hunt	+	-	-	-
3	A31, A32, B52, B60, Cw1, Cw Hunt	+	-	-	-
4	A2, A11, B60, Cw7, Cw Hunt	+	-	-	-

* Husband of serum donor

of Cw2 negative donor lymphocytes with serum 3028 indicated the presence of the new C-locus antigen, Cw Hunt.

In order to determine whether anti-Cw Hunt of serum 3028 is a separate specificity or a crossreactivity of the Cw2 antibody, the absorption assay was repeated using Cw2 positive cells, Cw Hunt cells, and B27 positive cells as a control. **Table 12** shows that unabsorbed serum and absorbed serum (by B27 cells) both retained antibody that reacted with the husband's cells and Cw Hunt positive cells. However, either Cw2 positive cells or Cw Hunt positive cells can remove such reactivity. This finding suggested that serum 3028 recognized a common epitope shared between Cw2 and Cw Hunt antigens. Based on this result, however, since the serum donor's husband has Cw2, it is not certain whether he also has the Cw Hunt antigen.

4.3.1.4 Frequency of Cw Hunt Antigen

Serum 3028 was employed in the complement-dependent LCT test to determine the frequency of Cw Hunt antigen in Caucasian, East Indian, and Oriental populations. The frequency of Cw Hunt was estimated to occur in approximately 6% of the populations investigated. However, this did not account for individuals with Cw2 alleles, since the serum was not monospecific.

Linkage disequilibria between C-locus antigens and B locus antigens are frequently observed. A possible linkage disequilibrium between Cw Hunt antigen and B60 or B61 antigens was observed in this study.

4.3.2 Cw Grace

4.3.2.1 Identification of Alloantisera Recognizing Cw Grace

Experiments described in this section were to characterize two selected

Table 12. Removal of Cw Hunt and Cw2 antibodies by T lymphocytes expressing Cw2, Cw Hunt, or B27 antigen. In order to determine if the antibody of serum 3028 against Cw2 and Cw Hunt antigens is a crossreactive antibody, serum 3028 was first absorbed by T cells known to express Cw2, Cw Hunt, or B27 antigens, followed by the NIH LCT test of the absorbed and unabsorbed sera with cells expressing Cw Hunt antigen and cells of the serum donor's husband. (see section 4.3.1.3 for further experimental details)

Cell Tested		Serum 3028			
		Unabsorbed	Absorbed By		
No.	Phenotype		Cw 2 Cells	Cw Hunt Cells	B27 Cells
1	A2, A11, B60, Cw7, Cw Hunt	+	-	-	+
2	A2, A29, B38, B7, Cw7, Cw Hunt	+	-	-	+
3	A1, B37, B61, Cw6, Cw Hunt	+	-	-	+
4	A31, A32, B52, B60, Cw1, Cw Hunt	+	-	-	+
5*	A2, A24, B27, Cw2, (?Cw Hunt)	+	-	-	+

* Husband of serum donor

alloantisera detecting a second new C-locus antigen, Cw Grace. Both sera were chosen because of their polyspecific reactivities against multiple C-locus antigens. Each serum contained different antibody specificities toward different C-locus antigens, and each exhibited a common specificity for reacting with T cells of donors with no serologically detectable C-locus antigen or with only one WHO-recognized C-locus antigen.

The first serum, 14072, was obtained from a Caucasian woman (HLA typed as A1, A2, B8, Bw6, Cw7, DR3, DR13, DR52, DQ1, and DQ2). Her husband was HLA typed as A2, A24, B60, B62, Bw6, Cw3, DR15, DR13, DR52, DQ1, and DQ2. Using the complement-mediated LCT test, crossmatch of the serum with the husband's T lymphocytes was positive. This confirmed a possible allo-sensitization of the serum donor by the husband's T lymphocyte antigen. Screening of the serum with selected and random T lymphocyte donors (n=978) revealed that this serum contained HLA antibody specificities for Cw1 and Cw3 antigens. In addition, an unidentified antibody specificity reactive with cells possessing only one or no HLA-C-locus antigen was observed. Crossmatch between serum donor and the husband's red blood cells was not possible due to the ABO blood group incompatibility. However the serum was found to contain no detectable levels of antibody against major erythrocyte blood group systems by indirect antiglobulin test.

The Caucasian donor of the second serum, 18869, was HLA typed as A32, B27, B61, Bw4, Bw6, Cw2, DR13, DR16, DR52, and DQ1. The husband's HLA phenotype was A1, A26, B8, B37, Bw4, Bw6, Cw6, Cw7, DR1, DR3, DR52, DQ1, and DQ2. Using the complement-mediated LCT test and the indirect antiglobulin test, serum 18869 was tested with the husband's T lymphocytes and red blood cells

respectively. It reacted with the husband's T lymphocytes but not with his red blood cells. This result eliminated the possibility of antibody specificity toward erythrocyte antigens. Screening of the serum with random and selected T lymphocyte donors (n=350) indicated that this serum contained antibody specificities for Cw6, Cw7, Cw4, Cw3, Cw1, Cw8 antigens, and an antibody with unknown specificity reacting with cells expressing no serologically detectable C-locus antigen or only one WHO-recognized C-locus antigen.

In addition to the first common feature where both sera reacted with cells expressing no or only one serologically defined C-locus antigen, both sera failed to react with T lymphocytes expressing two HLA-C locus antigens except the antigens for which they were specific. Most strikingly, both sera reacted with T lymphocytes from the same donors that lack either one or two HLA-C locus antigens.

4.3.2.2 Absorption Study

In order to ascertain whether the reactivity of sera 14072 and 18869 was directed toward a C-locus antigen, an absorption study was attempted using cells expressing various C-locus phenotypes. Results in **Table 13** show that unabsorbed and Cw7 absorbed serum 14072 retained antibody specificities against cells expressing Cw1, Cw3, or the new C-locus antigen, whereas serum absorbed by cells with Cw3, Cw1, or the new C-locus antigen, removed or substantially weakened the antibody reactivities.

Similarly, the reaction of serum 18869 against cells with Cw1, Cw9, Cw10, Cw4, Cw8, and cells expressing the new C-locus antigen was removed or weakened by absorption of T lymphocytes with Cw6, Cw7, Cw1+Cw7, Cw6+Cw7, Cw4, Cw4+C

Table 13. Reaction of serum 14072 absorbed by C-locus antigens. Experiments were performed to determine the crossreactivity of serum 14072. Serum was absorbed by cells expressing Cw1, Cw3, Cw Grace, or Cw7 (control). Unabsorbed and absorbed sera were tested by the NIH LCT test with cells known to express Cw1, Cw3, or Cw Grace antigens.

Cells	Serum Absorbed By				Unabsorbed
	Cw Grace	Cw1	Cw3	Cw7	
Cw1	-	-	-	+	+
Cw3	±	±	-	+	+
Cw Grace	-	-	-	+	+

blank, and Cw8 antigens. However, serum unabsorbed or absorbed by Cw2, Cw5, or Cw2+Cw5 cells, retained reactivity as demonstrated in **Table 14** and **Table 15**.

4.3.2.3 Family Study

In order to validate the specificity and genuineness of the alloantibody in detecting the C-locus antigen described above, a family study using complement-dependent LCT test was carried out. Since serum 18869 reacted with all serologically recognized C-locus antigens except Cw2 and Cw5, it was not included in family study. However, serum 14072, which was specific for Cw1, Cw3, and the new C-locus antigens, was further analyzed using members of five unrelated families. As shown in **Figure 15(A, B, C, D, E)** the segregation of this new C-locus antigen expressed on cells previously typed to express no C-locus antigen was confirmed in the five families studied. Furthermore, family studies verified that the expression of this new C-locus antigen is under genetic control. These findings demonstrate that cells previously typed to express C-blank are in fact expressing the "new" C-locus antigens, Cw Grace.

Testing of random donors suggested that it was probably in association with B18, B35, B38, B39, B51, and B52 antigens.

4.3.2.4 Frequency of Cw Grace Antigen

To determine the frequency of Cw Grace antigen in the general population, sera 14072 and 18869 were screened in the LCT test with 978 and 350 random and selected donors respectively. Both sera detected this new C-locus antigen in Black, Caucasian, East Indian, and Oriental populations. The frequency of the new C-locus antigen was approximately 5% in the general population of this study. It was expressed in Black, Caucasian, East Indian, and Oriental ethnic groups. A positive reaction of

Table 14. Reaction of absorbed and unabsorbed serum 18869 with cells expressing various C-locus antigens. Serum 18869 was first absorbed by cells expressing different C locus antigens. Using the NIH LCT test, unabsorbed and absorbed sera were tested versus cells expressing C-blank phenotype and cells with various C-locus antigens.

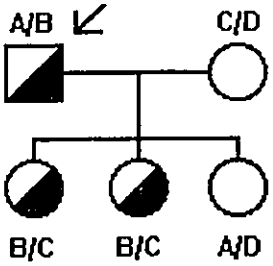
		Serum Absorbed By				Unabsorbed
		Cw6	Cw1+7	Cw6+7	Cw7	
Cells	Cw1	-	-	-	-	+
	Cw9	-	-	-	-	+
	Cw10	-	-	-	-	+
	Cw4	-	-	-	-	+
	Cw6	-	-	-	±	+
	Cw7	±	±	-	-	+
	Cw8	±	-	-	-	+
	C blank	-	-	-	-	+

Table 15. Reaction of serum 18869 with cells expressing crossreactive and non-reactive C-locus antigens. Serum 18869 was first absorbed by cells expressing crossreactive and non-reactive antigens, followed by the NIH LCT test with cells expressing C-blank phenotype and cells with crossreactive C-locus antigens.

		Serum Absorbed by Antigens					
		Cw4	Cw4+ C blank	Cw8	Cw2	Cw5	Cw2+5
Cells	Cw1	+	-	-	+	+	+
	Cw9	NT	-	-	+	+	+
	Cw10	-	-	-	+	+	+
	Cw4	-	-	-	+	+	+
	Cw6	+	-	±	+	+	+
	Cw7	+	-	±	+	+	+
	Cw8	-	-	-	+	+	+
	C blank	+	-	-	+	+	+

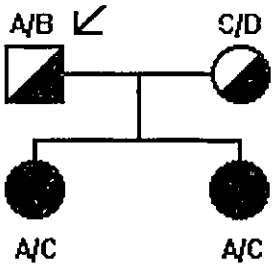
Figure 15. Family studies to verify the identification of Cw Grace antigen using alloantiserum 14072. Using the NIH LCT test, T lymphocytes of family members were tested against serum 14072. Propositi (indicated by arrows) expressing Cw Grace antigen were assigned based on positive reactions with sera 14072 and 18869 and are indicated by half-shaded circles or squares. Propositi with homozygous Cw Grace are represented by full-shaded circles. Assignment of haplotypes possessed by each propositus was deduced based on serological tissue typing. (A) Pedigree of the family showing segregation of Cw Grace on A28, B39 haplotype; (B) Pedigree of the family showing segregation of Cw Grace with both A34, B61 and A2, B62 haplotypes; (C) Pedigree of a Black family showing segregation of Cw Grace with A25, B18 haplotype; (D) Pedigree of the family showing segregation of Cw Grace travelling with A2, B38 haplotype; (E) Pedigree of the family showing segregation of Cw Grace linked to A32, B52 haplotype. NT= not tested.

A



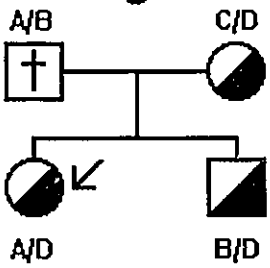
A: A1, B8, Cw7
B: A28, B39, Cw Grace
C: A2, B18, Cw7
D: A2, B62, Cw10

B



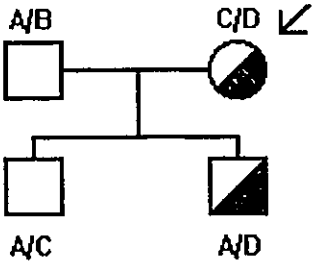
A: A34, B61, Cw Grace
B: A24, B51, Cw Hunt
C: A2, B62, Cw Grace
D: A34, B51, Cw4

C



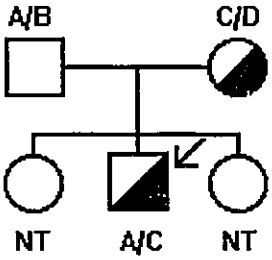
A: A2403, B35, Cw4
B: A2, B53, Cw4
C: A36, B53, Cw4
D: A25, B18, Cw Grace

D



A: A24, B27, Cw2
B: A2, B60, Cw10
C: A30, B13, Cw6
D: A2, B38, Cw Grace

E



A: A1, B61, Cw Hunt
B: B37, Cw6
C: A32, B52, Cw Grace
D: A31, B60, Cw Hunt

lymphocytes, previously typed to express only one or no C-locus antigen with both sera 14072 and 18869, is indicative of expression of the new HLA-C-locus antigen, Cw Grace.

4.4 Serological Identification of a Human Minor Histocompatibility Antigen (hmHΦ) Restricted to Oriental Populations

4.4.1 Identification of an Alloantiserum Recognizing hmHΦ

Experiments performed in this section were to characterize an alloantiserum recognizing a human minor histocompatibility antigen (mH) herewith named Φ. The serum (serum Mar) was selected from a routine HLA antibody screening procedure because of its peculiar serological characteristics, reacting with Oriental donor T lymphocytes but not with T lymphocytes from Caucasian donors. The donor, a Caucasian, had two children and was transfused with two units of blood in 1976. To identify the antibody specificity of the serum, antibody screening of the serum versus T and B lymphocytes and erythrocytes was performed. To completely rule out antibody specificity toward red blood cell antigens, extended red blood cell antibody screening against major blood group systems and low frequency antigens was necessary. Using the indirect antiglobulin test, serum Mar was found to contain no detectable level of red blood cell antibody against any major red cell blood group systems or low frequency red cell antigens, including Bg, C^w, Co^b, Kp^b, Wr^a, Yt^b, and St^a. It failed to react with her own red blood cells, her husband's, her children's, and with erythrocytes from donors whose lymphocytes gave positive reactions with serum Mar in a complement-dependent standard NIH LCT test. This suggested that the antibody detected in serum Mar was directed against an antigen expressed on lymphocytes but not on erythrocytes.

This antigen was tentatively referred to as human minor histocompatibility antigen Φ (hmhΦ), as it turned out to be a non-HLA antigen that was not associated with the MHC system.

HLA antibody screening of serum Mar with lymphocytes from selected and random donors (n=1,560) of Black, Caucasian, East Indian, and Oriental populations excluded its specificity toward all serologically recognized HLA class I and class II antigens. A crossmatch test of serum Mar versus her own lymphocytes, her husband's and her children's were all negative, suggesting that the occurrence of the antibody specific for lymphocyte antigens was most probably due to the blood transfusion instead of to fetal-maternal sensitization.

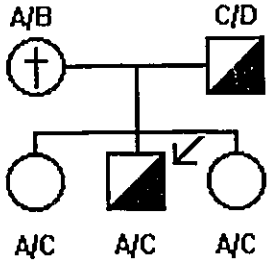
Serum Mar was initially found to react with T and B lymphocytes isolated from one of three siblings of a family with Oriental relations, all of whom had identical HLA-A, -B, -C, -DR, and -DQ antigens. Since the antigen hmHΦ was expressed on T lymphocytes, this ruled out the involvement of HLA class II antigens which are known to be expressed on B lymphocytes, monocytes and activated T lymphocytes, but not on resting peripheral blood T lymphocytes.

4.4.2 Family Study

In order to confirm that the reaction of serum Mar against one member of the three HLA-A, -B, -C, -DR, and -DQ identical siblings was not due to random reactivity, LCT tests on all living members of this family were repeated (**Figure 16A**). This family study again confirmed that the antibody of serum Mar was detecting an antigen that is not associated with the HLA system, since only one of the three HLA identical siblings expressed hmHΦ reacting with serum Mar. More importantly, the

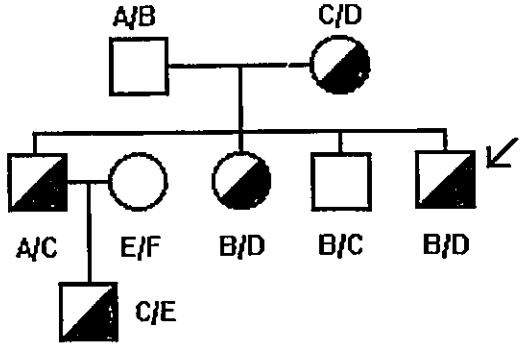
Figure 16. Family studies to confirm the identification of hmHΦ antigen using serum Mar. Using the NIH LCT test, T lymphocytes from selected families were tested versus serum Mar. Propositi (indicated by arrows) expressing hmHΦ antigen were identified based on positive reactions with serum Mar and are depicted by half-shaded circles or squares. Assignment of haplotype borne by each propositus was deduced according serological typing. (A) Pedigree of a Chinese family showing genetic transmission of hmHΦ; (B) Pedigree of a Taiwanese family showing genetic transmission of hmHΦ from the first generation to the third generation; (C) Pegigree of a Japanese family showing segregation of hmHΦ; (D) Pedigree of a Vietnamese family showing all siblings expressing hmHΦ. Deceased family members are denoted by a cross. NT= not tested.

A



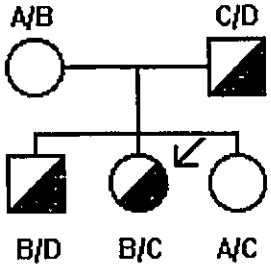
A: A3, B14, Cw8, DR4
C: A11, B75, Cw8, DR12
D: A31, B35, Cw4, DR8

B



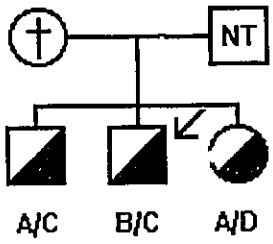
A: A11, B60
B: A11, B70, Cw3
C: A2, B60
D: A24, B60
E: A24, B46, Cw1, DR15
F: A33, B57, DR4

C



A: A2, B59, Cw1, DR4
B: A26, B62, Cw3, DR2
C: A2, B54, Cw1, DR4
D: A31, B48, Cw4, DR9

D



A: A11, B58, Cw10, DR3
B: A2, B46, Cw1, DR9
C: A33, B58, DR3
D: A2, B18, Cw7, DR2

father was found to express hmHΦ, confirming that the expression of this minor histocompatibility antigen Φ was under genetic control. The son had inherited hmHΦ from his father rather than derived it from an event of random mutation or recombination of genes.

Family studies from an additional three unrelated families (**Figure 16B, C, D**) further confirmed the observation that serum Mar was detecting an alloantigen completely unrelated to the HLA system. Results obtained from these family studies clearly indicate that the expression of hmHΦ antigen was not in segregation with an HLA haplotype shared between parents and their offsprings or among siblings of a family. Equally important was the observation that hmHΦ was consistently found to be expressed in blood-related family members once one of the family members with hmHΦ was identified.

4.4.3 Absorption Study

In order to ascertain that the antibody specificity of serum Mar against each family was directed towards the same antigen, an absorption study was performed using hmHΦ positive cells and hmHΦ negative cells as controls. Results in **Table 16** suggest that the antibody specificity of serum Mar against hmHΦ positive individuals was identical, since hmHΦ positive cells from unrelated individuals were able to remove the antibody specifically.

4.4.4 Ethnicity of hmHΦ

The country of origin and ethnic background of the families in **Figures 16A** to **16D** are summarized in **Table 17**. A common denominator for these four families was that all of them belonged to the Oriental persuasion, although they had arrived in

Table 16. Absorption of serum Mar by hmHΦ (+) and hmHΦ (-) T cells. Serum Mar was first absorbed by T cells with or without hmHΦ antigen. Absorbed and unabsorbed sera were tested with T cells of four hmHΦ (+) individuals from four different families and with T cells from ten hmHΦ (-) donors in NIH LCT tests.

		Serum Mar		
		Absorbed By		Unabsorbed
		Φ(+) (n = 2)	Φ(-) (n = 5)	
Cells	Φ(+) (n = 4)	-	+	+
	Φ(-) (n = 10)	-	-	-

Table 17. Ethnicity of the families with hmHΦ antigen. Country of origin and ethnic background of the families studied in **Figures 16A, B, C,** and **D.**

Family in Figure	Country of Origin	Ethnic Background
16A	Jamaica	Chinese
16B	Taiwan	Chinese
16C	Japan	Japanese
16D	Canada	Vietnamese

Canada from different geographical regions.

The family study results shown in **Figures 16B, 16C,** and **16D** also excluded the linkage association between the antigen hmHΦ and male restricted minor histocompatibility antigen H-Y. Some female individuals in these family studies do in fact express the antigen hmHΦ.

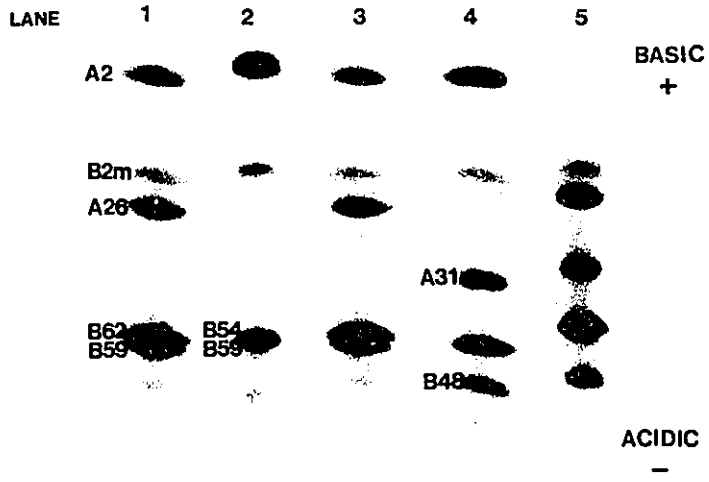
4.4.5 Frequency of hmHΦ

To determine the frequency of hmHΦ in the general population, serum Mar was used in the complement-mediated LCT test to screen random donors of various ethnic backgrounds. For a total of 300 random Oriental donors tested, it was revealed that the frequency of hmHΦ antigen was about 5.5% in this population. However for 60 Black, 200 East Indian, and 1,000 random Caucasian donors, there was an absence of any donor with hmHΦ antigen.

4.4.6 IEF Analysis of hmHΦ

IEF analysis was attempted to determine whether monoclonal antibody w6/32, which is specific for HLA-class I framework molecules, would immunoprecipitate the hmHΦ molecules. The autoradiograph (**Figure 17**) of the family depicted in **Figure 16C**, indicated that while molecules of HLA-A and -B antigens were unambiguously demonstrated, no indication of the hmHΦ molecule was visualized. Using the immunofluorescence procedure, it was confirmed that serum Mar can detect the hmHΦ antigen expressed on T lymphocytes.

Figure 17. IEF analysis of cells of family-study donors expressing hmHΦ antigen. MoAb w6/32 immunoprecipitated HLA proteins prepared from ^{35}S-methionine metabolically labelled EBV transformed B-lymphoblastoid cells from family members investigated in Figure 16C were analyzed by IEF. IEF bands in each lane represent HLA-A and -B antigens of each family member. Cells in lane 2 (child 1) and lane 5 (child 2), and lane 4 (father) were known to express hmHΦ antigen. Cells in lane 1 (mother) and lane 3 (child 3) were known to express no hmHΦ antigen.


LANE
1
2
3
4
5
BASIC
+
A2
B2m
A26
A31
B62
B59
B54
B59
B48
ACIDIC
–

5. DISCUSSION

5.1 HLA-A2 Variants

During the 10th IHW, five variants of HLA-A2 antigen were detected by IEF analysis, according to their banding positions on IEF gel electrophoresis. These variants were designated as IEF A2.1, IEF A2.2, IEF A2.3, IEF A2.4, and IEF A2.5. The IEF A2.1 variant was the most basic protein and was absent in Orientals. The IEF A2.2 variant was the most common variant of the A2 antigen and was found in all ethnic groups studied. The IEF A2.3 variant was primarily detected in individuals with Asiatic ancestry. The IEF A2.4 variant was detected only in American Indians (41). Out of a total of 918 blood donors, consisting of Blacks, North American Caucasians, European Caucasians, American Indians, Orientals, and Southeast Asians, only one donor expressing the IEF A2.5 variant was found in a donor of Southeast Asian origin (41). While IEF analysis had been quite successful in identifying HLA-A2 variants, serological definition of such variants was less fortunate due largely to a lack of specific antisera.

Serological data obtained from this study, as demonstrated in **Table 4**, indicate that there are at least three serological A2 variants. This confirms the observation made by IEF, cytotoxic T lymphocyte clones, and DNA sequencing, that HLA-A2 antigen appears to be extremely heterogeneous, consisting of various forms of variants (41). IEF analyses (**Figure 7** and **Figure 8**) are consistent with the 10th IHW report, and support the observed variations of A2 antigen with IEF (41).

Serological results obtained in this study clearly indicate that IEF variant A2.3 (serologically A2.2) is restricted to Oriental populations, and three alloantisera were

discovered in this study that were capable of recognizing IEF variants A2.3 and A2.5 of HLA-A2 antigen. In addition, serological data obtained from this report further suggest that the IEF variant A2.2 determined by IEF analysis may consist of additional microvariants in the Oriental population, since the reactions of alloantiserum 03035 with the IEF variant A2.2 displayed two distinctive patterns, as shown in **Table 6**. Furthermore, IEF analysis shown in **Figure 8** also demonstrates that A2X and A2Y bands on IEF gel appear more basic or more acidic respectively than other A2.2 bands. My hypothesis of further subgroups of this IEF A2.2 variant from serological findings coincided with the observation that DNA sequenced alleles HLA-A*0206, 0207, and 0208 all have an identical IEF band, A2.2 (88). Perhaps serologically determined A2.3 (IEF Variant A2.2) of Oriental individuals whose T lymphocytes failed to react with serum 03035 possess HLA-A* 0206, 0207, or 0208 allele of the A2 variant.

While IEF A2.5 variant was found only in a donor from Southeast Asia, among 918 donors tested during the 10th Workshop (41), it was found in the present study in a donor from Kenya with an East Indian ethnic background. This suggests that IEF A2.5 variant may not be restricted to Southeast Asian only. Like the workshop report, IEF A2.1 variant was found in a Caucasian donor, but was absent in the Oriental donors (n=12) tested.

During the 11th IHW, a MoAb BB 7.2 was reported to be able to differentiate A210 (A*0210), a serological variant of A2 found in Oriental populations. However, the IEF banding position of A210 remained unknown (88). **Table 18** shows the comparative reaction patterns of local A2 alloantisera 02782 and 02479 with the 11th IHW MoAb BB 7.2. Serum 02782 displayed an identical reaction pattern, reacting with

Table 18. Comparison of reaction patterns of local alloantisera 02782 and 02479 with the 11th IHW MoAb BB7.2. Local alloantiserum 02782 and 11th IHW MoAb BB7.2 displayed an identical reaction pattern with A2, A210, A203, A69, and A68 antigens. However, sera 02479 was found to have a different reaction pattern failing to react with A69 antigen.

		Antigen				
		A2	**A210 (A*0210)**	**A203 (A*0203)**	**A69**	**A68**
Sera	**02782**	+	-	+	+	-
	02479	+	-	+	-	-
	BB7.2	+	-	+	+	-

A2.1, A2.3, and A69 antigens, but failing to react with A2.2 (A210) antigen, as did the Workshop MoAb BB 7.2. This latter serum is known to recognize a tryptophan residue at position 107 of the HLA-A2 molecule (88). I speculate that serum 02782 is most likely detecting the same A2 residue as MoAb BB 7.2, and conversely the A210 variant recognized by MoAb BB 7.2 has an identical IEF migration as the serological A2.2 variant (IEF variant A2.3) recognized by serum 02782. In the same vein, serum 02479 exhibited a different reaction pattern than MoAb BB 7.2 and serum 02782, failing to react with A69 antigen. This suggests that serum 02479 is recognizing a different determinant(s) or even a different site(s) of the A2 molecule, and further that A210 has at least two distinct variant-specific determinants which can be recognized by alloantisera.

This study confirms that, similar to other ethnic groups, IEF A2.2 variant (serologically A2.1) was the most common A2 variant observed in Oriental donors. However, IEF A2.3 variant (serologically A2.2) was only observed in the Oriental population. The frequency of IEF A2.3 is estimated at 14% of the total A2 population, or approximately 8% of Oriental donors.

Alloantibodies are readily available and capable of detecting some subgroups of the HLA-A 2 antigen as defined by IEF analysis. In addition, alloantibodies are available to differentiate between some alleles identified by DNA sequencing, which was one of my objectives. Expanding the investigation to study the American Indian population may facilitate characterization of additional A2 alloantisera in recognizing the A2.4 IEF variant, which has been reported in this indigenous group, and may well facilitate the identification of new alleles. Large-scale screening of sera from every

population would certainly increase the chance of discovering more variant-specific antisera. Further study to reveal the underlying mechanism which controls the differential production of various variant-specific A2 antibodies in Caucasian donors may help to explain the nature of interaction between peptides and MHC molecules, and the efficacy of antigen recognition, processing, and presentation amongst various individuals.

5.2 HLA-A11 Variants

Results shown in **Table 7**, **Table 8**, **Figure 12**, and **Figure 13** demonstrate that HLA-A11 antigen consists of two serologically and biochemically distinct variants: A11.1 and A11.2. IEF showed that A11.2 protein is more basic than A11.1 protein. While A11.1 was found in all ethnic groups, A11.2 was limited to the Oriental population. This information is important in determining histocompatibility between organ donors and recipients, as graft rejection or graft-versus-host disease may occur as a consequence of HLA mismatch.

The frequency of A11 was approximately 51% in Oriental donors selected randomly. This frequency was about 20% higher than what was reported in the Workshop (62), and probably reflects the difference in sample group sizes and demographic regions of donors tested. The A11.2 variant represented about 18.7% of the A11 population, and the remaining 81.3% were A11.1.

Figure 11B shows that serologically typed homozygous A11 cells using broad-specificity antisera may in fact be heterozygous for both A11.1 and A11.2 antigens when tested with variant-specific antisera. This heterozygosity is more likely to occur in Oriental populations than in any other ethnic group. Results obtained here suggest

that with the availability of monospecific alloantisera, A11.1 and A11.2 variants can be easily and quickly identified by the complement-dependent LCT test (as shown in **Table 7**).

Segregation of A11.2 haplotype was shown in family studies, as depicted in **Figure 11A** and **11B**. These results clearly illustrate the genetic inheritance of A11.2 subtypes. Similarly, genetic segregation of A11.1 was demonstrated in a family study (**Figure 11C**).

Comparison of nucleotide sequences of A11 indicated that there is a single base difference in codon 43 between A*1101 (GAG) and A*1102 (AAG) variants (89). This nucleotide substitution results in a lysine residue encoded in A*1102 in place of a glutamic acid in position of A*1101. This difference may reflect the charge differences of A11.1 and A11.2 molecules on IEF. Furthermore, this difference probably constitutes an epitope variant recognized by specific alloantisera for A11.1 or A11.2.

Three distinct types of A11 alloantisera were produced in Caucasian women: firstly, the type of antibody recognizing a common public epitope shared by A11.1 and A11.2 variants, secondly, the antibody recognizing only the A11.1 private epitope, and thirdly, the antibody recognizing only the A11.2 private epitope. It would be interesting to explore the mechanism whereby different types of antibody molecules are generated in different individuals in response to allo-immunization by seemingly identical Caucasian A11 antigen.

5.3 "New" HLA-C Locus Antigens: Cw Hunt and Cw Grace

Unclassifiable "blank" specificities are observed in each HLA-A, -B, and -C locus. In HLA-A and -B loci, the "blank" allele ranges from 5-19% at most. In the

HLA-C locus, the frequency of unclassifiable "blank" specificities is estimated to be as high as 30-50 %. Serologically, there are ten official C-locus antigens (Cw1 to Cw10) approved by the WHO nomenclature commitee (18). However, DNA sequencing based on independent research from different laboratories confirmed 26 C-locus alleles (69). Why does serology fail to identify all HLA-C alleles? No doubt this is related to the unavailability of typing alloantisera. What is the reason for a lack of HLA-C locus antisera? One possible explanation for the scarcity of anti-C locus sera might be related to the functional ability of the HLA-C antigen in mounting an immune response, or might be due to feeble competence of the HLA-C molecule to provoke allostimulation. Another explanation may be that alloantisera are screened and characterized most of the time on the basis of reaction patterns against a screening panel of cells possessing well-characterized HLA alleles. In so doing, there is a tendency for sera directed against those well-characterized HLA antigens to be detected predominantly. Consequently, it is impossible to identify sera against poorly characterized or unknown alleles such as HLA-C locus antigens, especially those tightly linked to other HLA loci as common haplotypes. Therefore, the high frequency of blank specificities at the HLA-C locus may result from the fact that genes of the HLA-C locus tend to be in strong linkage disequilibrium with the genes of the HLA-B locus. Further, if sera used to characterize unknown C-locus antigens in the Black population were selected from the Caucasian population, limited C-antigen diversity or C-blank haplotype might be predominantly obtained. In fact, this was what happened when Amerindians were typed for HLA-A and -B antigens using antisera selected to define Caucasian HLA antigens (42).

In this report, a serum (3028) recognizing Cw2 antigen was found additionally

to detect a novel C-locus antigen, Cw Hunt. Similarly, two unrelated alloantisera (sera 18869 and 14072) were found to recognize a second new C-locus antigen, Cw Grace. The antibody absorption assay, shown in **Table 12**, suggests that serum 3028 contained an antibody recognizing a common epitope shared by Cw2 and Cw Hunt antigens. This crossreactivity between Cw2 and Cw Hunt is analogous to the crossreactivities displayed in alloantisera Cw4 and Cw6 (90), and in alloantisera Cw5 and Cw8 (91).

In the case of sera 18869 and 14072, each serum reacted with a combination of different C-locus antigens expressing private epitopes. Meanwhile, each serum also crossreacted with a common C-locus blank antigen which displayed public determinants resembling other C-locus antigens. Crossreactivity is a natural phenomenon frequently observed between HLA antigens and alloantisera. It is not unusual for HLA alloantisera to crossreact with antigens with resembling amino acid sequences or with antigens comprised of identical amino acid residues at certain locations on the molecule, since multiple determinants are expressed on each HLA antigen. Furthermore, multiple determinants may consist of highly polymorphic private epitopes and the public or supertypic determinants that are shared within a family of crossreactive groups.

Extensive analysis of the polymorphism of HLA-C alleles revealed that the serological description of HLA-C antigens generally reflects the underlying structures of the alleles, which vary on 10 basic motifs (69). Cw Hunt antigen is most likely in association with Cw2 antigen belonging to the same basic motif, since cells of the husband of the serum donor (3028) expressed Cw2 antigen. In addition, either Cw2 or Cw Hunt cells were capable of removing the specificities of both Cw2 and Cw Hunt in an absorption assay (**Table 12**). On the other hand, Cw Grace might be in

association with two different basic motifs: Cw1+Cw3 motif, and Cw6+Cw7 motif. This was confirmed firstly by the findings that either Cw1, Cw3, or Cw Grace antigen can actually remove or weaken the specificity of serum 14072 against Cw Grace antigen (**Table 13**). Secondly, the husband of the serum donor expressed only Cw3. The cells of the husband of serum donor 18869 expressed Cw6 and Cw7, and either Cw6 or Cw7 antigen were capable of abolishing Cw6, Cw7, and Cw Grace specificities in the serum (**Table 14** and **Table 15**). It is noteworthy that among the other five Cw2 alloantisera used to phenotype random donors, serum 3028 is the only alloantiserum known to crossreact with Cw Hunt antigen. The factors responsible for triggering the immune systems of different individuals to produce Cw2 antibodies crossreactive or non-crossreactive with Cw Hunt antigen are most puzzling, and this problem deserves further exploration.

The antibody reactivities of serum 14072 resemble those of a murine MoAb described by Dejelo, et al. (92) which also detected Cw1, Cw3, and C-blank cells. Again, which factor is responsible for generating crossreactive or non-crossreactive alloantisera remains to be carefully investigated.

It is not surprising that the donor of serum 18869, who expressed Cw2 on her lymphocytes, did not produce Cw2 alloantibody against herself. However, the observation that her alloantibody crossreacted with almost all serologically recognized antigens in C-locus except Cw5 may indicate a certain degree of homology in the structure, determinant, or sequence of Cw2 and Cw5 antigen.

The observation that Cw Hunt and Cw Grace are found in many different ethnic populations, suggests that the gene encoding Cw Hunt and Cw Grace molecules most

likely existed before the diversification of those ethnic groups.

Although the detection of Cw Hunt and Cw Grace antigens in this study revealed that about 11% of serologically-typed individuals were erroneously thought to lack one or two C-locus antigens, there exists a high percentage of donor cells which still lack one or two C-locus antigens. In light of the number of HLA-C alleles determined by DNA sequencing, more serologically undetected C-locus antigens await to be identified by newly purified antibodies. Nevertheless, results obtained from this study suggest that C-locus antigens probably evolved in the same fashion as the other HLA molecules, deriving their unique polymorphism from an ancestral molecule and retaining sequential epitopic or topographical epitopic similarities that account for the crossreactions.

5.4 Human Minor Histocompatibility Antigen: hmHΦ

In addition to MHC, it is generally accepted that a number of other antigens that are not controlled by the MHC loci can also induce an allograft rejection. The identification of MHC antigens, in the early period of immunogenetics, was based on their ability to provoke antibody synthesis and on their strong immunogenicity in skin grafting experiments between congenic-resistant strains of mice (1). Other antigens with less immunogenicity were named minor histocompatibility (mH) antigens. Soon, it became very clear that the distinction between minor and major histocompatibility antigens relies solely upon their immunogenicity. Some MHC antigens are not strong immunogens, while some mH antigens appear neither weak nor minor (93). There appears to be a large number of non-MHC antigens that can induce graft rejection (1).

The total number of mH loci is not known. In mice, over 50 mH genes have been described (94). In humans, several mH-specific cytotoxic T lymphocyte clones

have been reported in patients after multiple blood transfusions and bone marrow or kidney transplantation (95, 96). In humans and mice, mapping of the mH gene loci has been successful only for the male Y chromosome-associated mH antigen, H-Y (97).

In contrast to typing for HLA class I and II antigens that can be performed by serology, isoelectric focusing, oligonucleotide typing, or restriction fragment length polymorphism analysis, typing for mH antigen can as yet only be performed using mH-antigen-specific T lymphocyte clones. To date, there are five mH-antigen-specific cytotoxic T lymphocyte clones described that can be used for mH typing: the HLA-A2 restricted mH-1, mH-2, mH-4, and mH-5-specific clones, and the HLA-A1-restricted mH-3-specific clone (98).

Attempts to generate antibodies against mH antigens have, by and large, been unsuccessful. The few reported successes could not be exploited to characterize the antigens because the antisera were generally weak. The reason why it has been so hard to obtain mH-antigen-specific antibodies remains unknown (1).

In this study, a serum recognizing an Oriental-specific minor histocompatibility antigen, hmHΦ, was discovered in a Caucasian woman with a history of blood transfusions. To my knowledge, this is the first confirmative antibody known to detect an ethnic-specific alloantigen outside the MHC system. This antibody was produced most likely as a consequence of alloimmunization by blood transfusion, since a crossmatch test of serum Mar versus lymphocytes of the husband and the children were all negative. Unfortunately, information on these blood donors was unavailable because of record deletion.

Family studies performed on four different families, shown in **Figures 16A** to

16D, clearly indicate that the hmHΦ antigen detected in random Oriental donors is not the product of a gene conversion since some members of each donor family were found to express hmHΦ. The fact that hmHΦ was present in 5.5% of the Oriental population, large-scale antibody screening in Oriental women with a history of pregnancies may find additional sera containing antibodies with the same specificity.

hmHΦ was found in Chinese, Japanese and Vietnamese of the Oriental populations (**Table 17**) investigated. This result suggests that some people in these three ethnic groups may be closely related. Extended anthropological study may provide further information on the evolution and segregation of hmHΦ antigen.

If indeed hmHΦ antigen can induce alloimmunization, it is important to determine the status of hmHΦ antigen between donor and recipient for transplantation purposes. The degree of histocompatibility is essential to determine the acceptability of a graft in an organ transplantation.

More study is needed to determine the function of hmHΦ antigen. What role does it have in disease susceptibility or disease resistance? What role does it have in graft rejection or graft-versus-host disease? Are there any other ethnic-specific hmH antigens, and if so what are their functions? I am certain that further, more elaborated and intensive serological search will guarantee isolation and development of novel alloantisera for the detection of new hmH antigens.

6. CONCLUSION

Bare lymphocyte syndrome is a severe or lethal congenital immunodeficiency characterized by defective expression of HLA class I and class II antigens on the surface of peripheral blood lymphocytes (99, 100). This reiterates that the pivotal function of the HLA molecules is in the network of immune recognition, immune regulation and immune response. Clinically, HLA molecules constitute a vital element in disease susceptibility, disease resistance, graft rejection, and graft-versus-host disease.

To date, many ethnic diversities in allogenic HLA loci are not fully recognized due to the nature of extensive HLA polymorphism compounded by the unavailability of HLA typing reagents. In order to elucidate the essence of the individual HLA antigen and its variants in terms of clinical implications, it is imperative to unambiguously define variants of HLA alleles. Information from this will assist in evaluating the degree of compatibility between donor and recipient in transplantation, as well as provide a measurable criterion to make a prognosis for the outcome of a transplantation.

Results obtained from this study indicate that HLA-A2 antigen may be further split, using the isoelectric focusing technique, into various subgroups: IEF variant A2.1 (serologically A2.1), IEF variant A2.2 (serologically A2.1), IEF variant A2.3 (serologically A2.2), and IEF variant A2.5 (serologically A2.3). Two alloantisera were identified in this study to recognize IEF variant A2.3, and one antiserum was found to detect IEF variant A2.5. However, antisera specific for IEF variants A2.1 and A2.2 remain to be discovered. This study further discovered indications of heterogeneity in

Oriental IEF variant A2.2 using a serum defining IEF variant A2.5. Extended antibody screening is required to identify additional antibody specificities for other A2 variants.

This study also identified two HLA-A11 variants, A11.1 and A11.2. Isoelectric focusing confirmed the findings of the alloantisera which specifically recognized and differentiated both variants. A11.1 is the common form of variant found in all ethnic groups, but A11.2 is restricted to the Oriental population. Oriental donors homozygous for HLA-A11 antigen may in fact possess heterozygous HLA-A11.1 and HLA-A11.2 antigens. With the availability of monospecific antisera, phenotyping of HLA-A11 individuals can easily be performed.

While HLA-C locus antigens were extensively investigated, many of the DNA-sequenced HLA-C alleles are unidentifiable by serology. This study identified one antiserum recognizing a "new" HLA-C locus antigen, Cw Hunt, and two antisera recognizing another "new" HLA-C locus antigen, Cw Grace. Cw Hunt is in linkage disequilibria with HLA-B61 and -B60 antigens, while Cw Grace is in linkage disequilibria with B18, B38, B39, B51, B52 and B35 antigens. The reactions of both groups of C-locus antisera with pertinent groups of C-locus antigens are the result of antibodies reacting with public epitopes expressed on C-locus antigens. In combination, both groups of sera revealed that about 11% of HLA-typed individuals express the new C-locus antigens. Identification of new HLA-C antigens may be useful to assess transplantation rejection in HLA-A and -B matched donor and recipient pairs in whom C-locus antigens were not determined before transplantation, and to evaluate the involvement of C-locus antigens in immunology.

An intriguing observation emerged from the above three groups of alloantisera

(A2, A11 and C-locus); only a few alloantisera are capable of reacting with variants of A2 and A11 antigens or of crossreacting with new HLA-C locus antigens. What underlying mechanisms direct the immune components of different hosts to generate diverse antibody spectra and specificity profiles toward seemingly similar antigen is profoundly enticing.

In humans, T-cell immunity to minor histocompatibility (mH) antigens is of particular interest for its presumed role in the development of graft-versus-host disease and graft rejection after bone marrow transplantation from an HLA-identical sibling. mH antigens are able to induce responses of both cytotoxic (CD8+) and helper (CD4+) T lymphocyte subsets. However, attempts to produce antibodies against mH antigens have been largely unsuccessful. In this study, an alloantibody recognizing an Oriental-restricted hmHΦ antigen was found in a Caucasian woman who had received two units of blood. This marks the first antibody, to my knowledge, with reproducible reactivity in detecting an mH antigen found only in a particular population. This antibody is extremely unique and is most valuable to be used as a tool to measure the effect of mH antigen in HLA-matched transplantation. It may be employed for anthropological investigation on relatedness of diverse peoples.

Finally, it is my believe that the overall results obtained from this research project have achieved the four objectives that I endeavoured to accomplish during my graduate study.

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