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Transplantation Antigens and Histocompatibility Matching

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1. Introduction

Since the discovery of the major histocompatibility complex (MHC) in 1967, there has been significant development in the field of organ and tissue transplantation. In humans, the MHC is called the human leukocyte antigen system and is located on the short arm of chromosome 6, near the complement genes. These cell surface proteins are the principal antigenic determinants of graft rejection.

The presence of donor-specific HLA antibodies in kidney transplant recipients can be identified by crossmatch. Since 1969, pre-transplant crossmatch has become a mandatory component of the transplant work-up process. It has largely eliminated hyperacute rejection. Crossmatch techniques have expanded from basic complement-dependent microcytotoxicity (CDC) assays to additionally include flow crossmatches and virtual crossmatches derived using the luminex assay. The improved sensitivity and specificity of virtual crossmatch when compared to CDC and flow crossmatches has revolutionised the pre-transplant crossmatch process, but also greatly increased its complexity.

2. HLA antigens

HLA molecules are membrane bound glycoproteins that bind processed antigenic peptides and present them to T cells. The essential role of the HLA antigens lies in the control of self-recognition and thus defence against microorganisms. Based on the structure of the antigens produced and their function, there are two classes of HLA antigens, HLA Class I and Class II.

The overall size of the MHC is approximately 3.5 million base pairs. Within this is the HLA Class I genes and the Class II genes each spread over approximately one third of this length. The remaining section, sometimes known as Class III, contains loci responsible for complement, hormones, intracellular peptide processing and other development characteristics [1]. Thus the Class III region is not actually a part of the HLA complex, but is located within the HLA region, because its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA antigens.

2.1. HLA Class I antigens

The cell surface glycopeptide antigens of the HLA A, B and C series are called HLA Class I antigens [2]. HLA Class I antigens are expressed on all nucleated cells of the body. Additionally, they are found in soluble form in plasma and adsorbed onto the surface of platelets. Erythrocytes also adsorb HLA Class I antigens to varying degrees depending on the specificity (e.g. HLA-B7, A28 and B57 are recognizable on erythrocytes as so called "Bg" antigens). Immunological studies indicate that HLA-B (which is also the most polymorphic) is the most significant HLA Class I locus, followed by HLA-A and then HLA-C. There are other HLA Class I loci (e.g. HLA E, F, G, H, J, K and L), but most of these may not be important as loci for "peptide presenters".

The HLA Class I antigens comprise a 45 Kilodalton (Kd) glycopeptide heavy chain with three domains, which is non-covalently associated with β_2-microglobulin, which plays an important role in the structural support of the heavy chain [3]. The HLA Class I molecule is assembled inside the cell and ultimately sits on the cell surface with a section inserted into the lipid bilayer of the cell membrane and has a short cytoplasmic tail.

The general structure of HLA Class I, HLA Class II and IgM molecules show such similarity of subunits, that a common link between HLA and immunoglobulins, back to some primordial cell surface receptor is likely. The full 3-dimensional structure of HLA-A Class I molecules has been determined from X-ray crystallography [4]. This has demonstrated that the molecule has a cleft on its outermost surface, which holds a peptide. Consequently, if a cell becomes infected with a virus, the virally induced proteins within the cell are broken down into small peptides which are then inserted into this cleft during the synthesis of HLA Class I molecules. The HLA Class I molecules then translocate these virally (or self) induced peptides to the cell surface leading to activation of cytotoxic (CD8) T cells [5]. This role of HLA Class I, in identifying cells, which are changed (e.g. virally infected), is the basis for their expression on all cells [4]. Epitopes on certain expressed HLA Class I molecules also act as ligands for killer inhibitory receptors expressed on natural killer [6] cells, thereby influencing NK cell function [7].

2.2. HLA class II antigens

The cell surface glycopeptide antigens of the HLA DR, DP and DQ loci are termed HLA Class II [1]. The tissue distribution of HLA Class II antigens is confined to the "immune competent" cells, including B-lymphocytes, macrophages, and endothelial cells and activated T-lymphocytes. The expression of HLA Class II, on cells, which would not normally express them, is stimulated by cytokines like interferon-γ and is associated with acute graft rejection in the

setting of transplantation. HLA Class II molecules consist of two chains each encoded by genes in the "HLA Complex" on Chromosome 6 [3]. The T Cells, which link to the HLA Class II molecules, are Helper (CD4) T cells. This role of HLA Class II, in initiating a general immune response, is the rationale for their limited expression on "immunologically active" cells (B lymphocytes, macrophages, etc.) and not on all tissues [4].

2.3. Recent changes to HLA nomenclature

A new HLA nomenclature was introduced in April 2010, replacing a system which had been in use since the 1990's. The main drive for the change was that the old system could no longer accommodate the increasing number of HLA alleles that were being described. There are currently over 5,700 alleles described across all the classical and non classical HLA loci.

The old system was based on assigning significance to pairs of digits in the allele nomenclature (Fig 1). For example in the allele HLA-A*02010102L, the designation 'HLA' identifies the allele as a HLA allele. The dash (-) separates the HLA designation from the gene, in this case the 'A' gene. The '*' is a separator. Of the actual allele name, the first two digits (02010102L) represents the allele group and in most instances, was synonymous with the Serological type (A2 in this case). The third and fourth digits (02010102L) identified the specific allele. All alleles whose nomenclature differed in these first four positions (02010102L) must code for proteins with different sequences. Alleles whose nomenclature differed in the fifth and sixth position (02010102L) code for proteins with silent mutations within the coding sequences. A sequence which differed by mutations in the introns or in the untranslated regions flanking the 3' and 5' ends of the exons were identified by different digits in the seventh and eighth positions (02010102L). In addition, a number of suffixes were used to identify sequences that were null, i.e. not expressed (N), those that had low expression (L), those that were secreted (S), those found only in the cytoplasm (C), those with questionable expression and those with aberrant expression (A).
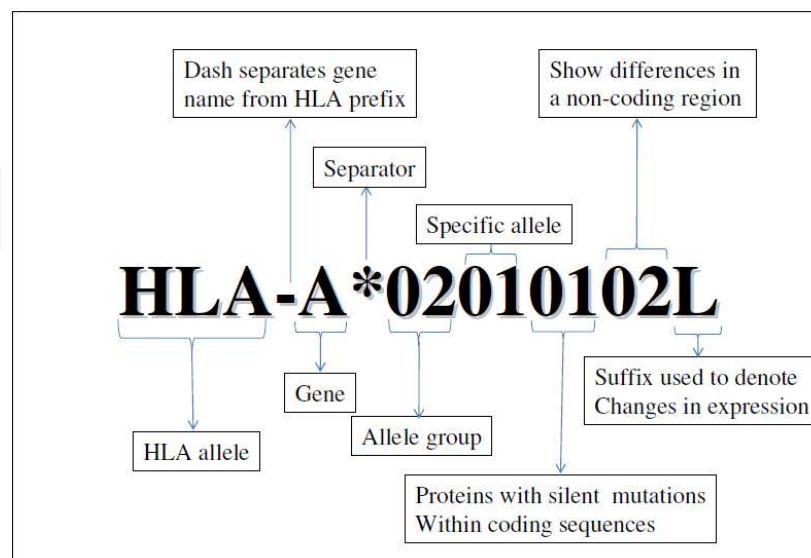

Figure 1. Old HLA nomenclature

A key limitation of this old system was that it only allowed for up to 99 alleles which differ in any of the pairs of positions. The HLA-A*02 and B*15 allele groups were the first to run into this problem when more than 99 alleles were detected. At that time, the WHO Nomenclature Committee for the HLA system decided to adopt the rollover sequences A*92 and B*95 respectively for A*02 and B*15. When A*0299 was identified, the next A*02 allele described was named A*9201. Similarly when B*1599 was identified the next B*15 allele described was named B*9501. Recently however, a number of other HLA types started to fast approach 99 alleles. These include A*03, B*40, B*44 and DRB1*11. Adopting rollover sequences for all of these was impractical. A rollover system of sorts had already been adopted for HLA-DPB1. When HLA-DPB1*9901 was identified, the next HLA-DPB1 allele was named 'within the existing sequences' as HLA-DPB1*0102.

In 2010, a new nomenclature system was adopted (Fig 2) [8, 9]. This introduced colons ':' as separators between pairs of digits. HLA-A*02010102L therefore became HLA-A*02:01:01:02L. The pairs of digits separated by colons are known as Fields. The first and second digits of the old nomenclature form the 1st Field of the new nomenclature. The third and fourth digits of the old nomenclature form the 2nd Field of the new nomenclature. To help reduce confusion in adopting the new nomenclature, the leading '0' in alleles 1-9 of each allele group was kept.
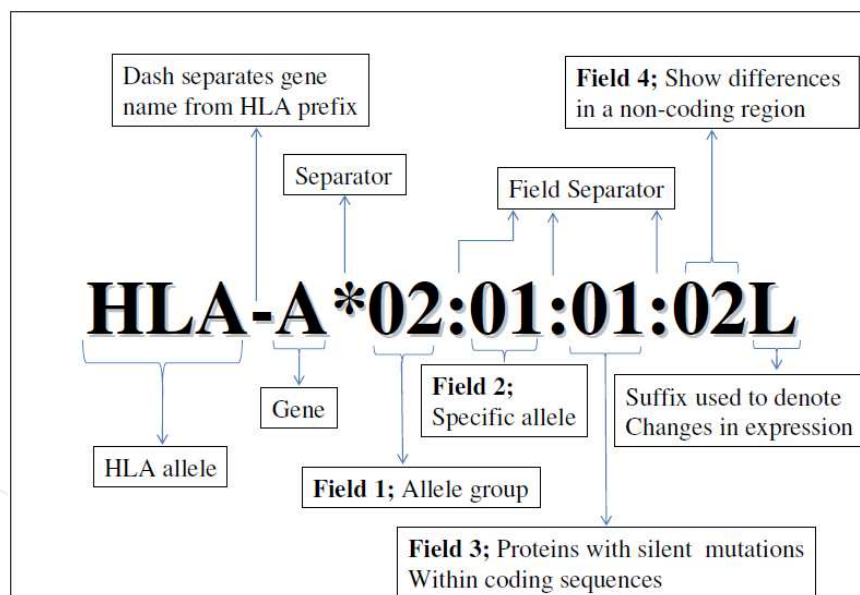

Figure 2. New HLA nomenclature

The introduction of the colons means that each Field is no longer restricted to 99 digits but can be expanded limitlessly. Once HLA-A*03:99 was identified, the next A3 allele could be named HLA-A*03:100.

With the introduction of colons and therefore the removal of the artificial restriction of 99 digits, there is no more need for rollover sequences. HLA-A*92 and B*95 were renamed A*02 and B*15 respectively and their associated alleles remapped. A*9201 became A*02:101. A*9202 became A*02:102 etc. HLA-B*9501 became B*15:101. HLA-B*9502 became B*15:102 etc. HLA-

A*02:100 and B*15:100 were not used to help make the remapping easier. However other HLA types which exceed 99 alleles will use allele 100. HLA-DPB1 alleles were also remapped. HLADPB1*0102 became HLA-DPB1*100:01.

A number of other changes were made to the nomenclature. The 'w' was dropped from HLA-Cw alleles but not from Cw antigens. HLA-Cw*0102 became HLA-C*01:02. The 'w' was kept in antigen names to avoid confusion with complement factors as well as with KIR ligand groups. For ambiguous allele strings, the codes 'P' and 'G' were introduced. A group of alleles that share the same nucleotide sequences within exons 2 and 3 for HLA class I and exon 2 for HLA class II were named after the first allele in the sequence and given a code of 'G' as a suffix. E.g. HLA-A*02:01:01 and HLA-A*02:01:02 could be named HLA-A*02:01G. A group of alleles that share the same protein sequences in the $\alpha2$ and $\alpha3$ domains, irrespective of the nucleotide sequence differences could be named after the first allele in the sequence and given a code of 'P' as a suffix e.g. HLA-A*02:01:01P.

2.4. Clinical relevance of the HLA system

The most important function of MHC molecule is in the induction and regulation of immune responses. T-lymphocytes recognize foreign antigen in combination with HLA molecules.

In an immune response, foreign antigen is processed by and presented on the surface of a cell (e.g. macrophage). The presentation is made by way of a HLA molecule. The HLA molecule has a section, called its antigen (or peptide) binding cleft, in which it has these antigens inserted. T-lymphocytes interact with the foreign antigen/HLA complex and are activated. Upon activation, the T cells multiply and by the release of cytokines, are able to set up an immune response that will recognize and destroy cells with this same foreign antigen/HLA complex, when next encountered. The exact mode of action of HLA Class I and HLA Class II antigens is different in this process. HLA Class I molecules, by virtue of their presence on all nucleated cells, present antigens that are peptides produced by invading viruses. These are specifically presented to cytotoxic T cells (CD8) which will then act directly to kill the virally infected cell. HLA Class II molecules have an intracellular chaperone network which prevents endogenous peptide from being inserted into its antigen binding cleft. They instead bind antigens (peptides) which are derived from outside of the cell (and have been engulfed). Such peptides would be from a bacterial infection. The HLA Class II molecule presents this "exogenous" peptide to helper T cells (CD4) which then set up a generalized immune response to this bacterial invasion. Thus it is apparent that MHC products are an integral part of immunological health and therefore it is no surprise to see a wide variety of areas of clinical and genetic implications.

2.5. HLA and renal transplants

HLA typing was applied to kidney transplantation very soon after the first HLA determinants were characterized [10-12]. The importance of reducing mismatched antigens in donor kidneys was immediately apparent with superior survival of grafts from HLA identical siblings compared to one haplotype matches or unrelated donors. It is apparent that the effect of HLA matching is significant, even with the highly efficient immunosuppression used today. The

important things include need for ABO compatibility and the need for a negative T-lymphocyte crossmatch (using cytotoxicity). Complement binding anti-HLA Class I antibodies present at the time of transplant will cause "hyperacute rejection" of the graft (i.e. when the T cell crossmatch is positive).

3. HLA antibody detection and identification

3.1. Lymphocytotoxicity (serological testing)

In this serological test, lymphocytes are added to sera, which may or may not have antibodies directed to HLA or other cell surface antigens [13]. If the serum contains an antibody specific to an HLA (Class I or Class II) antigen on the lymphocytes, the antibody will bind to this HLA antigen. Complement is then added. If there is a cell bound antibody that is able to fix complement, the complement pathwayis activated causing membrane damage. The damaged cells are not completely lysed but suffer sufficient membrane damage to allow uptake of vital stains such as eosin or fluorescent stains such as Ethidium Bromide. Microscopic identification of the stained cells, indicates the presence of a specific HLA antibody. The cells used for the test are lymphocytes because of their excellent expression of HLA antigens and ease of isolation compared to most other tissue. The most important use of this test is to detect specific donor-reactive antibodies present in a potential recipient prior to transplantation.

This test has long been used to type for HLA Class I and Class II antigens, using antisera of known specificity. However, the problems of cross-reactivity and non-availability of certain antibodies have led to the introduction of DNA-based methods.

3.2. Mixed Lymphocyte Culture (MLC)

When lymphocytes from two individuals are cultured together, each cell population is able to recognize the "foreign" HLA class II antigens of the other. As a response to these differences, the lymphocytes transform into blast cells, with associated DNA synthesis. Radio-labelled thymidine, added to the culture, will be used in this DNA synthesis. Therefore, radioactive uptake is a measure of DNA synthesis and the difference between the HLA Class II types of the two people. This technique can be refined by treating the lymphocytes from one of the individuals to prevent cell division, for example by irradiation. It is thus possible to measure the response of T lymphocytes from one individual to a range of foreign lymphocytes. It has thus proved possible by using the mixed lymphocyte culture (MLC) test to use T lymphocytes to define what were previously called HLA-D antigens. The "HLA-D" defined in this way is actually a combination of HLA-DR, DQ and DP.

An important use of the MLC is in its use as a "cellular crossmatch" prior to transplantation especially bone marrow. By testing the prospective donor and recipient, an in-vitro transplant model is established which is an extremely significant indicator of possible rejection or Graft-Versus-Host reaction.

3.3. Molecular genetic techniques

3.3.1. RFLP (Restriction Fragment Length Polymorphism)

Restriction Fragment Length Polymorphism (RFLP) methods rely on the ability of certain enzymes to recognize exact DNA nucleotide sequences and to cut the DNA at each of these points [14]. Thus, the frequency of a particular sequence will determine the lengths of DNA produced by cutting with a particular enzyme.

The DNA for one HLA (Class II) antigen, e.g. DR15, will have these particular enzyme cutting sites (or "restriction sites") at different positions compared to another antigen, e.g. DR17. Consequently, the lengths of DNA observed when DR15 is cut by a particular enzyme, are characteristic of DR15 and different to the sizes of the fragments seen when DR17 is cut by the same enzyme [15].

3.3.2. Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is a revolutionary system for investigating the DNA nucleotide sequence of a particular region of interest in any individual [16]. Very small amounts of DNA can be used as a starting point, such that it is theoretically possible to tissue type using a single hair root. Sequencing DNA has been transformed from a long and laborious exercise to a technique that is essentially automatable.

The first step in this technique is to obtain DNA from the nuclei of an individual. The double stranded DNA is then denatured by heat into single stranded DNA. Oligonucleotide primer sequences are then chosen to flank a region of interest. The oligo- nucleotide primer is a short segment of complementary DNA, which will associate with the single stranded DNA to act as a starting point for reconstruction of double stranded DNA at that site.

If the oligonucleotide is chosen to be close to a region of special interest like a hypervariable region of HLA-DRB then the part of the DNA, and only that part, will become double stranded DNA, when DNA polymerase and deoxyribonucleotide triphosphates are added. From one copy of DNA it is thus possible to make two. Those two copies can then, in turn, be denatured, reassociate with primers and produce four copies. This cycle can then be repeated until there is a sufficient copy of the selected portion of DNA to isolate on a gel and then sequence or type.

There are a number of PCR based methods in use. For example:

• *Sequence Specific Priming (SSP)* - In this test, the oligonucleotide primers used to start the PCR have sequences complimentary to known sequences which are characteristic to certain HLA specificities. The primers, which are specific to HLA-DR15, for example, will not be able to instigate the PCR for HLA-DR17. Typing is done by using a set of different PCR's, each with primers specific for different HLA antigens.

3.4. Sequence Specific Oligonucleotide (SSO) Typing

By this method, the DNA for a whole region (e.g. the HLA DR gene region) is amplified in the PCR. The amplified DNA is then tested by adding labeled (e.g. Radioactive) oligonucleotide

probes, which are complementary for DNA sequences, characteristic for certain HLA antigens. These probes will then "type" for the presence of specific DNA sequences of HLA genes.

3.5. Panel Reactive Antibodies (PRA)

PRA has been used to measure patient HLA sensitisation ever since pre-formed donor specific HLA antibodies were associated with hyperacute rejection in renal transplantation in the 1960's [17]. As traditionally defined, PRA refers to the percentage of an antibody screening panel with which the patient's serum reacts. A kidney patient with a PRA > 85% is considered highly sensitised. This measure of PRA however relies on the composition of the panel which may not necessarily reflect the antigen frequencies in the donor population. This measure of PRA is not therefore a good reflection of the chances of the patient finding a compatible donor. Variations in cell panels, both commercial and in house, result in wide variations in recorded PRA for patients on the waiting list.

The calculated PRA (cPRA) was introduced to overcome this problem [18]. The cPRA can be calculated in a number of different ways, but relies on the identification of a potential recipient's anti-HLA antibody profile. This has been made much easier by the wide adoption of solid phase assays such as Luminex. Luminex assays, especially those involving the use of single antigen beads (SABs) allow fine specificity definition and allow the strength of the reactions (MFI) to be used to assess immunological risk and help decide whether or not specificity should be listed. The cPRA is then calculated by defining a set of unacceptable mismatches for that recipient, and weighting those mismatches according to the frequency of the antigen in the donor population. This could be based on the frequency of different HLA antigens in the most recent 10,000 deceased donors.The cPRA therefore gives a measure of the chances of a patient finding a compatible donor in the donor pool.

cPRA removes some of the variability between laboratories using different panels and allows a PRA value to be assigned which reflects the patients' transplantability.

4. Non-HLA antibodies

Acute and chronic allograft rejection can occur in HLA-identical sibling transplants implicating the importance of immune response against non-HLA targets. Non-HLA anti-bodies may occur as alloantiboides, yet they seem to be predominantly autoantibodies. Antigenic targets of non-HLA antibodies described thus far include various minor histocom-patibility antigens, vascular receptors, adhesion molecules, and intermediate filaments. Non-HLA antibodies may function as complement and non-complement-fixing antibodies and they may induce a wide variety of allograft injuries, reflecting the complexity of their acute and chronic actions.

4.1. The KIR receptor complex

The adaptive immune response recognises infection through presentation of pathogen-derived peptides in association with MHC to the host T cells. One of the mechanisms

which pathogens use to evade this immune response is to down regulate their MHC cell surface expression. Natural Killer cells are able to detect altered expression of MHC through a number of cell surface receptors leading to target cell lysis [19]. These receptors include the killer immunoglobulin like receptors (KIR), which are also expressed on some effector T cells. In humans, the KIR gene cluster is located on chromosome 19. KIR genes are both polygenic and polymorphic [20]. The KIR gene cluster codes for 15 expressed KIR genes and 2 pseudo genes.

The ligands for KIR receptors are HLA class I molecules [21]. These include HLA-C locus antigens with either Asn (Group 1 HLA-C antigens) or Lys (Group 2 HLA-C antigens) at position 80, the HLA-Bw4 epitope and some HLA-A antigens. KIR receptors binding to HLA class I are either inhibitory or are stimulatory with the overall effect of NK cell interaction with the target cell dependent on the balance between these inhibitory and stimulatory signals. It is thought that the inhibitory KIR's bind class I with greater affinity than the corresponding activating KIR with the effect that under normal circumstances the inhibitory signal prevails. The 'missing self' hypothesis holds that NK cell alloreactivity occurs when the ligand for inhibitory KIR receptors is down regulated or 'missing', leading to activation. This however requires that KIR receptors engage their cogent HLA class I molecules during maturation to acquire effector function. NK cells that express only inhibitory KIRs for absent HLA class I molecules are hypo responsive in the non transplant setting.

Inhibitory KIR receptors possess long cytoplasmic tails with immunoreceptor tyrosine based inhibitory motifs (ITIMs). Activating KIR receptors have short cytoplasmic tails that pair with adaptor molecules with immunoreceptor tyrosine based activating motif (ITAMs). The nomenclature for KIR receptors therefore includes an 'L' (long tail) for inhibitory KIR's and an 'S' (short tail) for activating KIR's. The nomenclature also includes 'P' for pseudo genes. The inhibitory and activating KIR receptors share sequence and structural similarities in their extracellular domains. KIR's have either 2 or 3 extracellular immunoglobulin domains and this is reflected in their nomenclature as either '2D' or '3D', giving KIR receptors nomenclature such as KIR2DL1, KIR2DS2 and KIR3DL1, where the final digit indicates the order in which the genes were described.

The KIR genes assemble into haplotypes with two haplotypes described, 'A' and 'B'. The 'A' haplotype has only one activating KIR (2DS4), while the 'B' haplotype has a higher number of activating KIRs and generally possess more KIRs than the 'A' haplotype.

4.2. MICA/B

The major histocompatibility complex class I related chain was first described in the 1990's [22]. The genes are located centromeric to the HLA class I B gene. The only two MIC genes which are expressed are MICA and MICB. MICA and MICB share a significant amount of sequence homology with HLA class I and have some similarity in their conformation. MICA and MICB antigens have α1, 2 and 3 domains like classical HLA antigens but do not associate with β2 microglobulin and do not bind peptide for presentation to T cells. Instead, MIC antigens serve as ligands for the NKG2D receptor on NK cells and on some T cells.

MICA and MICB genes are polymorphic but not as much as the classical HLA class I genes. Over 70 MICA alleles and over 30 MICB alleles have been described [23]. Unlike HLA class I where the polymorphic residues are located mainly in the region that forms the peptide binding groove, polymorphism in MIC is more dispersed throughout the $\alpha2$ and $\alpha3$ domains. There is also polymorphism in the trans-membrane region. Many MIC antigens have the same extracellular domains with the only differences lying in the trans-membrane regions.

MICA and MICB antigens are constitutively expressed on epithelial cells, especially those of the gastrointestinal tract and on fibroblasts, monocytes, dendritic cells and on endothelial cells. They are not constitutively expressed on lymphocytes. They are however up regulated in stressed cells.

The structure of MICA is similar to that of HLA class I but has some striking differences. Like HLA class I, MICA has three extracellular domains (α1, 2 and 3), a transmembrane region and a cytoplasmic domain. Unlike HLA class I, the MICA protein does not associate with $\beta2$ microglobulin. The MICA α1 and 2 domains form a platform that is analogous to the platform formed by HLA class I α1 and 2 domains. In HLA class I, this platform forms the peptide binding groove. The MICA molecule however has extensive disordering of sections of the alpha helix in the α2 domain resulting in a very shallow groove, incapable of binding peptide. The MICA α1 and 2 platform domains do not interact with the α3 domain except for being linked together through a short linker chain. This allows for some flexibility in the structure.

The NKG2D receptor forms a complex with MICA by binding orthogonal to the alpha helices of the platform α1 and 2 domains.

4.3. Minor histocompatibility antigens

HLA presents the major genetic barrier to stem cell transplantation. However, evidence that other genetic systems are involved includes GvHD and some degree of rejection even when transplanting with HLA identical siblings. A non HLA system which is thought to contribute to this is the minor histocompatibility antigen (mHA) system. Minor histocompatibility antigens comprise of peptides derived from proteins in which some degree of polymorphism exists such there may be differences between the patient and donor repertoires. These peptides can be presented to the immune system by both HLA class I and II antigens.

The best characterised minor antigens are the Y chromosome derived HY peptide and the autosomal HA1 to HA5 peptides. Minor histocompatibility antigens such as HA1 and HA2 have restricted tissue distribution and are present normally only on haematopoietic cells. Others such as HY are more ubiquitously distributed, expressed for instance on gut epithelium. HA1 and HA2 are expressed on leukaemic cells and some tumour cells, making them potential targets for cellular therapy. Minor HLA antigens are restricted by certain HLA types such as HLA-A2 for instance.

5. Cross-matching techniques

Crossmatching was developed in an attempt to identify recipients who are likely to develop acute vascular rejection of a graft from a given donor. This phenomenon, hyperacute rejection (HAR) [24], is a result of preformed antibodies against the donor; referred to as donor-specific antibodies (DSA). Such antibodies are usually formed as the result of previous exposure to HLA, generally through pregnancy, blood transfusion or previous transplantation [25]. There are other debated forms of developing anti-HLA Abs such as via microbial exposure but the above three are thought to be the most prevalent. Particularly relevant is the exposure of women during pregnancy, to their partner's HLA. This commonly results in direct sensitization against the partner, potentially making him an unsuitable living donor. HAR may also occur in blood group incompatible transplantation or rarely as a result of other non-HLA antibodies.

Preformed antibodies cause rejection by binding to HLA antigens expressed on the endothelium of vessels in the transplanted kidney, resulting in activation of the complement cascade with resultant thrombosis and infarction of the graft. HAR can occur immediately upon reperfusion of the donor kidney. This catastrophic outcome necessitates the immediate removal of the graft. Clearly avoiding HAR is desirable and crossmatching helps predict and hence prevent this [17].

There are different types of crossmatch tests available.

5.1. Complement-Dependent Cytotoxicity (CDC) crossmatch

A CDC crossmatch involves placing recipient serum (potentially containing donor-specific anti-HLA antibodies) onto donor lymphocytes (containing HLA antigens). A cytotoxic reaction (deemed 'positive') suggests the presence of preformed DSA.

CDC crossmatching was pioneered by Terasaki and colleagues in the 1960s [13, 17]. It identifies clinically significant donor specific HLA antibody mediated responses for a given recipient. Lymphocytes from the donor are isolated and separated into T and B cells. Serum from the recipient is mixed with the lymphocytes in a multi-well plate. Complement is then added (usually derived from rabbit serum). If donor-specific antibody is present and binds to donor cells, the complement cascade will be activated via the classical pathway resulting in lysis of the lymphocytes.

The read-out of the test is the percentage of dead cells relative to live cells as determined by microscopy. The result can thus be scored on the percentage of dead cells, with 0 correlating to no dead cells; scores of 2, 4 and 6 represent increasing levels of lysis. On this basis, a score of 2 is positive at a low level, consistent with approximately 20% lysis (generally taken as the cut-off for a positive result). A score of 8 represents all cells having lysed and indicates the strongest possible reaction. The use of a scoring system allows a semi-quantitative analysis of the strength of reaction. Another way to determine the strength of the reaction is to repeat the crossmatch using serial doubling dilutions of the recipient serum (often known as a 'titred crossmatch'). In this way, dilutions are usually performed to 1 in 2, 4, 8, 16, 32, 64 and so on.

In the situation of a high titre of high avidity DSA it may be that many dilutions are required for the test to become negative (e.g. 1 in 128). With antibody at a low level or one with a low affinity, a single dilution may be enough to render the crossmatch result negative. This may also give an indication as to the likelihood that a negative crossmatch could be achieved with a desensitization protocol.

The basic CDC crossmatch can be enhanced by the addition of antihuman globulin (AHG). This technique increases the sensitivity of the CDC crossmatch as a result of multiple AHG molecules binding to each DSA attached to the donor cells thereby amplifying the total number of Fc receptors available for interaction with complement component 1, which increases the likelihood of complement activation and cell lysis.

It is also possible to have a negative crossmatch in the presence of a DSA and this can happen in the following conditions:

1. antibody titre is too low to cause complement activation

2. antibody is of a type that does not activate complement and

3. antigen for which the antibody is specific is expressed only at very low levels on the donor's lymphocytes.

A further consideration relates to variations in antibody levels in a given individual's serum samples, collected at different times. The most reactive serum is generally called the 'peak serum'. This may have been collected several years earlier, with the 'current serum' showing quite different reactivity. As an example, the peak serum may show a clear positive CDC crossmatch result, but as the antibody levels have fallen in subsequent sera, so too may the degree of cell lysis in the assay. This may render the CDC crossmatch negative. Nevertheless, the antibodies found in the peak sera may still be of relevance, indicating that re-exposure to the relevant antigen could initiate a memory response with the risk of early and aggressive rejection. For this reason, patients on transplant waiting lists have sera collected at frequent intervals; variations can be monitored and newly appearing HLA antibodies can be detected.

There are important differences in HLA expression between T and B cells, which influence the interpretation of the crossmatch. T cells do not constitutively express HLA class II so the result of a T-cell crossmatch generally reflects antibodies to HLA class I only. B cells on the other hand express both HLA class I and II, as well as a larger range of surface markers, icluding Fc receptors. Because of this, a positive B-cell crossmatch is more difficult to interpret than a positive T-cell cross match. It may be due to antibodies directed against HLA class I or II or both, or it may be due to antibody binding to other sites, that may or may not be clinically important. Hence, if the T- and B-cell crossmatches are positive the interpretation is that there may be either single or multiple HLA class I DSA/s or a mixture of HLA class I and II DSA. While a negative T-cell crossmatch in the setting of a positive B-cell crossmatch suggests either there may be one or more class II DSA/s but no class I antibodies or that there is a low-level DSA to a class I antigen with greater lysis of B cells relative to T cells. This is often due to the fact that B cells express higher levels of HLA class I than do T cells [26]. When class I complement fixing HLA DSA are present at a significant level one would expect both the T and B-cell

crossmatches to be positive. A negative B-cell crossmatch in the presence of a positive T-cell crossmatch therefore suggests a technical error. This is not unusual as B cells tend to be less resilient than T cells and their viability can often be a concern in the assays.

5.2. Positive T-cell CDC crossmatch

Transplanting in the setting of a positive T-cell crossmatch, which is not due to an autoantibody, is likely to generate a very poor outcome. Patel and Terasaki described the outcomes of 30 such transplants [17]. Twenty four (24) patients lost their grafts immediately to HAR while another three lost their grafts within 3 months. It is not clear why the other three patients had less severe reactions but it may relate to false positive crossmatches generated by autoantibodies given that dithiothreitol (DTT) which cleaves the multimers of IgM antibodies was not used in their assays. Other possibilities include false positive tests or lower immunogenicity of the antibodies or antigens in those cases.

A recent study investigated whether IVIg or plasma exchange was more effective at desensitizing crossmatchpositive recipients so that they might be crossmatch-negative at the time of transplant [27]. While most patients were successfully desensitized there was a group of 10 patients who did not achieve a negative crossmatch but were still transplanted. Of this group 70% developed AMR with 50% losing their grafts. Given this data, even after reducing the antibody titre with a desensitization protocol before transplant, a persistent positive T-cell crossmatch remains an absolute contraindication to transplantation.

5.3. Positive B-cell CDC crossmatch

B-cell CDC crossmatching is not as predictive of HAR as the T-cell CDC crossmatch and there has been much controversy about its role [28]. Many centres do not perform B-cell crossmatching for cadaveric renal transplantation because of uncertainty about the significance of a positive result. The major limitation is a rate of false positive results of up to 50% [29]. In some cases this reactivity may be due to non-HLA molecules on the surface of the B lymphocytes, including the presence of Fc receptors. While a negative result is reassuring a positive result may mean a transplant is cancelled when it was safe to proceed. Another argument against the routine use of B-cell crossmatching is that antibodies to class II antigens are of less significance in generating antibody-mediated rejection. But recently it has been found that they are not so benign [30].

B-cell crossmatches are often performed as part of the immunologic assessment before live donor transplantation when there is more time to determine the significance of the result. Paired with information about the presence of DSA, determined by more specific means such as antigen-coated beads (Luminex, discussed below) the B-cell CDC crossmatch results may be more meaningful [31]. If a B-cell crossmatch is positive and there are no detectable antibodies to class I or II antigens, the result may be falsely positive while a positive result in the presence of detectable DSA signifies that the identified DSA may be functionally relevant in that it can activate complement and were associated with increased risk of rejection [32]. This has led to the suggestion that the B-cell

CDC crossmatch should not be used alone to determine transplant suitability and that it be interpreted only in the light of accompanying Luminex results [31].

5.4. The flow crossmatching technique

A flow crossmatch involves adding recipient's serum to donor lymphocytes and then incubating them with fluorescein-labelled antibodies against human IgG (antihuman IgG fluorescein isothicyanate [FITC]). This fluorescein-labelled antibody will bind to all the IgG antibodies in the recipient serum. If a DSA in this serum then binds to the donor lymphocytes, it will be detectable by flow cytometry.

Flow crossmatching is performed using the same initial base ingredients as CDC crossmatching (i.e. donor lymphocytes and recipient serum) and was first described in 1983 [33]. The two are mixed to allow antibody binding and after washing, fluoresceinated AHG is added to bind attached DSA and hence allow detection by flow cytometry. The read-out may be reported simply as positive or negative or can be further quantitated. Intensity of fluorescence above control, referred to as channel shifts, may be reported while another means of quantitation is to determine the number of dilutions of recipient serum required to generate a negative result.

The subtype of antibody can also be determined by the isotype specificity of the fluorescently labelled detection antibody. Hence if only IgG antibodies are of interest the detection antibody chosen will be of the type that binds only to IgG and not IgM or IgA [34]. Furthermore the subtype of IgG can be elucidated by choosing a detection antibody that binds only to IgG1, 2, 3 or 4. Refining the analysis in this way provides information about the likelihood of complement activation *in vivo* as IgG4 does not activate complement.

The role of flow crossmatching in the pre-transplant assessment is controversial. The significance of a positive result is mainly of interest when the CDC crossmatch is negative. In this setting the positive flow crossmatch is likely to be caused by a non-complement fixing antibody, a non-HLA antibody or a low-level antibody that is below the threshold of sensitivity of the CDC methodology. In patients who are not known to be sensitized several studies have suggested that a positive T- or B-cell flow crossmatch was not predictive of increased rejection rates or worse graft survival while in sensitized patients other studies have suggested inferior graft survival [30, 34-39]. A possible reason for this difference is that there would be a higher false positive rate in non-sensitized patients than in sensitized patients given that they are not expected to have a positive result. Another factor determining the significance of the result is the cut-off values used to determine a positive test [34]. These are not applied uniformly between centres and those that apply a very low cut-off value will increase sensitivity at the expense of specificity.

Some transplant clinicians do not use flow crossmatching as part of their pre-transplant assessment and rely on CDC crossmatching along with defining DSA by Luminex, otherwise known as 'virtual crossmatching'. Others contend that flow crossmatching adds important information on the strength of donor-specific antibody reactivity and should be considered in the context of donor-specific antibody results and CDC crossmatching to help develop an

overall opinion on the likelihood of immune complications. The area remains controversial and no clear recommendation can be made at this time.

5.5. Virtual crossmatching

Virtual crossmatching refers to the comparison of the anti- HLA antibodies of the recipient, derived from Luminex, with the HLA of the donor [40]. If there is a DSA present this would represent a positive virtual crossmatch. Antibodies are defined against HLA class I and II antigens. Synthetic microspheres (beads) coated with HLA antigens are commercially available for this testing. Beads may be coated with multiple HLA antigens for screening purposes or a single HLA antigen for defining specificity of antibodies more precisely. For the virtual crossmatch, multiple beads each coated with a single HLA antigen are mixed with recipient serum. Anti-HLA antibodies present bind to the beads and are detected by an isotype-specific (e.g. IgG) detection antibody via flow cytometry. Unique fluorochromes within the beads mark the HLA antigen specificity of each bead. This technique is as sensitive as flow crossmatching and provides the specificity of the antibody [41].

It has long been established that the presence of antibodies that react with human leucocytes portend worse long-term graft survival [42]. This information has been further refined by more sensitive antibody detection systems, particularly Luminex. It has been shown that recipients with DSA have worse graft survival than those with third party anti-HLA Abs (antibodies against HLA antigens that are not donor-specific) who in turn have reduced graft survival compared with recipients without any anti-HLA antibodies [43]. Therefore, the presence of a DSA suggests inferior graft survival compared with no DSA even in the presence of a negative CDC crossmatch [44].

Luminex testing offers significant advantages over CDC and flow crossmatch in terms of defining the HLA specificity of identified antibodies. The presence of a DSA detected by Luminex in the setting of a negative or positive CDC crossmatch appears to have prognostic importance in terms of graft survival and acute rejection risk; however, there are insufficient data to determine the significance of a DSA with a negative flow crossmatch [40, 44-46].

In each assay, negative control beads provide a minimum threshold for a positive result. Positive results can then be graded as weak, moderate or strong on the basis of the degree of fluorescence of the positive bead. This result can be scored as a median fluorescence index (MFI) or molecules of equivalent soluble fluorescence. The molecules of equivalent soluble fluorescence of a DSA have been shown to correlate with antibody titre and predict graft failure [47].

While Luminex testing has added significantly to the understanding of crossmatching, the methodology has some significant limitations that can make interpretation difficult. Limitations include possible interference by IgM antibodies, variable antigen density on beads, conformational changes to antibodies in the process of binding to the beads, and gaps in the HLA antibody repertoire in bead sets. [45, 48, 49].

5.6. Cellular crossmatching

All of the above-mentioned crossmatching techniques attempt to detect a donor-reactive antibody likely to result in acute or chronic antibody-mediated rejection. The presence of sensitization of the cellular arm of the immune system, particularly T cells, can be assessed by cytokine assays such as ELISPOTs. These assays detect the number of recipient T cells producing cytokines such as interferon gamma when encountering donor antigen presenting cells. The assays are conducted in plates coated with a capture antibody for the cytokine of interest. The mixed donor and recipient leucocytes are added to the plate and incubated. After washing to remove the cells the reaction is developed by adding a second antibody for the cytokine of interest and then stained for that antibody [50].

6. Conclusion

Understanding of the transplantation antigens and crossmatching is a vital tool in transplant. Crossmatching plays a key role in assessing immune compatibility between a donor and recipient. A positive T-cell CDC crossmatch would usually mean that a particular pairing should not proceed. But in some cases, a desensitization protocol may allow such a transplant to occur, avoiding hyperacute or early acute rejection. However they have inferior longterm graft outcomes compared with patients who are not sensitized to their donor. The advent of flow crossmatching and Luminex assays has allowed the detection of very lower titre, anti-HLA antibodies of uncertain clinical significance.

CDC crossmatching along with Luminex should be used in determining anti-HLA antibodies. The role of flow crossmatching is less clear and its help in decision making is unclear. The ideal future crossmatch will be highly sensitive in identifying DSA and provide accurate prediction of the functional significance of the antibody. This will hopefully allow differentiation between transplants that can safely proceed in the face of a clinically irrelevant DSA while providing clear prognostic information in the setting of more serious antibodies.

Further studies are required to better define the significance of very low-level DSA, non-complement fixing antibodies, IgM antibodies and non-HLA antibodies as well as the importance of assessing T cellular sensitization.

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