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The HLA system: immunobiology, HLA typing, antibody screening and crossmatching techniques

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This review by Howell, Carter and Clark was intended to be an introduction to the system species reviews by Clark and Unsworth (*J Clin Pathol* 2010;63:21–25), Sage (*J Clin Pathol* 2010;63:194–198), and Carter (*J Clin Pathol* 2010;63:189–193). Unfortunately the articles became unlinked during the publication process.

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ABSTRACT

The Human Leukocyte Antigen (HLA) system plays a critical role in regulating the immune response. As a consequence of its role in immune regulation and exquisite polymorphism, the HLA system also constitutes an immunological barrier which must be avoided or otherwise overcome in clinical transplantation. This introductory review provides a brief summary of the immunobiology of the HLA system and methodology for HLA typing, antibody screening and patient-donor cross-matching. This constitutes a basis for consideration of the importance of these procedures in the system-specific reviews which follow.

INTRODUCTION

The human leucocyte antigen (HLA) system, encoded by two highly polymorphic gene families located within a 3600 kb region located at chromosome 6p21.3, plays a critical role in regulating the immune response. The 'classical' HLA genes are the most polymorphic in the human genome, with a large number of allelic variants at each locus. Allelic variation is maintained at the population level due to the survival advantage conferred, with marked inter-individual differences in immune responses to foreign antigens. As a consequence of its role in immune regulation, the HLA system also constitutes an immunological barrier which must be avoided or otherwise overcome in clinical transplantation. In this issue, the role of HLA matching and antibody responses are reviewed in stem cell, renal, pancreas (including islet), heart, lung and liver transplantation. This introductory review seeks to provide a brief summary of the immunobiology of the HLA system and methodology for HLA typing, antibody screening and identification and patient–donor crossmatching, as a basis for a consideration of the importance of HLA matching, antibody screening and crossmatching in the system-specific reviews which follow.

IMMUNOBIOLOGY OF THE HLA SYSTEM

HLA molecules are membrane bound glycoproteins that bind processed antigenic peptides and present them to T cells. Gene inheritance follows Mendelian principles with (usually) en-bloc HLA-A, B, C, DR, DQ haplotype transmission from each parental chromosome, although recombination within the HLA system can occur. All of the inherited antigens are codominantly expressed. HLA class I A, B and C antigens are expressed on almost all nucleated cells and platelets and present intracellularly processed peptides (of viral or self origin) to CD8+ cytotoxic T cells.[1] These peptides are generally 8–10 amino acids in length.[2] HLA class I gene polymorphisms mostly encode variant amino acids within the antigen binding cleft of the expressed class I molecules, which affects the repertoire of peptides which can be bound and presented to CD8+ T cells. T cell recognition of a non-self or aberrantly expressed self peptide complexed with a self HLA class I molecule can result in a cytotoxic T cell response against the target cell. Epitopes on certain expressed HLA class I molecules also act as ligands for killer inhibitory receptors expressed on natural killer (NK) cells, thereby influencing NK cell function.[3]

HLA class II DR, DQ and DP molecules are expressed on a more restricted range of cells, including B cells, activated T cells, and the monocyte/macrophage lineage; they are also interferon γ inducible on other cells, for example endothelial cells during inflammatory responses. Class II molecules present peptides, mostly of exogenous origin, to CD4 + helper T cells. These peptides typically vary from approximately 14 to 25 amino acids in length.[2] HLA class II gene polymorphisms mostly encode variant amino acids within the antigen binding cleft of the expressed class II molecules, which affects the repertoire of peptides which can be bound and presented to CD4+ T cells. T cell recognition of the HLA plus non-self peptide complex can lead to T cell activation and cytokine production, driving both antigen specific T and B cell responses against epitopes on the target antigen.

In stem cell transplantation, patient–donor HLA matching is of critical importance, since the regenerating immune system from the allograft is capable of recognising non-self HLA antigens on host tissues, resulting in a largely pathogenic graft versus host response, which is principally T cell mediated.[4]

In solid organ transplantation, patient–donor HLA mismatching has a variable but deleterious effect on allograft survival, due to recognition of non-self HLA molecules by the recipient's T cells. This allorecognition can be either 'direct', that is, direct recognition of intact expressed donor HLA molecules as non-self by the recipient T cell population, or 'indirect', in which donor HLA molecules are themselves processed into antigenic peptides by recipient antigen-presenting cells and recognised as non-self by recipient T cells.[5] Both pathways are important in cellular rejection processes, but the balance between the two is thought to change with time. A third pathway, termed 'semi-indirect' allorecognition has recently been described,[6] which involves acquisition of intact donor HLA by the recipient's dendritic cells, which then undertake presentation to recipient T cells. The in-vivo significance of this pathway remains to be elucidated.

Antibody responses against non-self HLA antigens expressed on donor organs can also occur and can result in hyperacute, delayed hyperacute and acute rejection processes and can also play a role in chronic allograft damage.[7] Anti-HLA antibodies can develop as a result of sensitisation events which include pregnancy, blood transfusions and previous transplants. The specific HLA antigens against which such antibodies are directed are normally regarded as unacceptable antigens for any future transplant, due to their known role in the above rejection processes. Organs expressing these antigens must be avoided. However, in some circumstances, carefully monitored antibody removal can allow transplantation to proceed, but only as part of an agreed protocol.[8]

Donor specific anti-HLA antibodies developing after solid organ transplantation have been linked with poorer outcome,[9] and regular antibody monitoring—especially in the early post-transplant period—is necessary.

DETERMINING HLA TYPE

Methods for determining individual HLA polymorphisms or 'HLA typing' have evolved enormously since the discovery of the human major histocompatibility complex and have developed in parallel with, and contributed to, the unravelling of the genetic complexity of this region, such that over 2000 alleles of the classical HLA class I (A, B and C) and class II (DR, DQ and DP) loci are now known. Methods for HLA typing are either based on detection of genetic variation in the expressed HLA molecules (serological typing), or now almost universally, at DNA sequence level (DNA typing).

Serological HLA typing

HLA typing methods were originally based on the detection of expressed HLA molecules on the surface of separated T cells (HLA class I) and B cells (HLA class II) using panels of antisera, usually obtained from multiparous women in a complement dependent cytotoxicity test.[10] Such 'serological' HLA typing suffers from a number of drawbacks. Live lymphocytes are required, and lymphocyte counts can be low in some transplant patients. Panels of antisera must be maintained, although commercial kits are now available. Finally, the typing resolution obtainable from serological methods is low. While good serology may provide a level of resolution adequate for renal transplant HLA typing, it is inadequate for stem cell transplant matching and therefore has been largely superseded by DNA-based typing in clinical HLA laboratories. However, serological typing still has a useful role as an adjunct to DNA-based typing, for example to determine whether a particular HLA allele is actually expressed at the cell surface. A number of such non-expressed 'null' HLA alleles are now known.[11]

DNA-based HLA typing

DNA-based typing methods offer a number of advantages over serological typing methods. Live lymphocytes are not required and DNA is easily extracted from any nucleated cell, although peripheral blood lymphocytes are the usual source. DNA is easily stored, allowing repeat sample testing when required.

A number of different DNA-based HLA typing methods are in everyday use in clinical HLA typing laboratories, all of which are based on PCR amplification of target sequences in the HLA genes under investigation. PCR primers and oligonucleotide probes can be designed and validated in-house, or purchased commercially. As such, unlike antisera, they are a renewable resource.

PCR with sequence specific primers (PCR–SSP)

One commonly applied approach is to use panels of 'sequence specific primers' which amplify particular HLA alleles or allele groups.[12] The presence or absence of a particular allele is determined by the presence or absence of DNA amplification by a particular primer pair, as determined by agarose gel electrophoresis. This method, termed PCR–SSP, is rapid and ideally suited to deceased donor typing. The method is usually used at 'low resolution' to detect allele groups, but secondary panels can be used to achieve allele level typing. However, allelic typing is cumbersome using this approach. Due to logistical considerations the method is unsuited to typing large numbers of samples.

PCR with sequence specific oligonucleotide probes (PCR–SSOP)

Another commonly employed approach is to detect HLA polymorphisms in locus-specific PCR products using short oligonucleotide DNA 'probes' in a hybridisation assay (reviewed in Krausa and Browning [13]). In its original form, PCR–SSOP typing is most appropriate for typing large numbers of samples in batches, since multiple oligonucleotide probes are required per locus.

A recent variant of this technique utilises probes coupled to fluorescently labelled microbeads in a flow cytometric assay using X-Map technology (Luminex),[14] and this approach is now being used increasingly for clinical HLA typing. In this format, the method is suitable for typing small or medium numbers of samples.

Sequence-based typing

Sequence-based typing (SBT) can also be used to achieve allelic level HLA typing as required for stem cell transplantation programmes. SBT is also required for investigation and confirmation of new allelic sequences.[15]

A number of other methods are in use some clinical histocompatibility & immunogenetics laboratories,[16] although none of these are widely used.

ANTIBODY IDENTIFICATION

The deleterious effect of antibodies in renal transplant hyperacute rejection is well documented. However, the deleterious role of antibodies in all forms of solid graft rejection has become more apparent over the last few years, driven by the development of new assays, such as detection of intragraft C4d deposition.[17] In addition, new assays have been developed, allowing the identification of previously undetectable antibody specificities. Antibody detection methods also need to be as sensitive as the crossmatch technique employed (see below). Methods in current use are reviewed briefly below.

HLA antibody screening by complement-dependent cytotoxicity

For several years the cornerstone of HLA antibody screening was the complement-dependent cytotoxicity (CDC) assay.[18] The CDC technique relies on live separated T cells for class I antibody identification and live B cells for class I and class II antibody identification. Class II antibody identification is often difficult in the presence of multiple class I antibodies. The CDC assay is relatively slow and cumbersome and involves either testing several panel donors to cover all major specificities, or relying on commercially available frozen cell trays or immortalised lymphoblastic cell lines (LCLs). These tests can only detect complement fixing antibodies. CDC assays—although relatively time consuming and insensitive—benefit from allowing easy determination of antibodies of both IgM and IgG isotype by the use of dithiothreitol (DTT; see below), and are the only test results with proven clinical significance.

Other cellular based serological assays have used flow cytometry to increase sensitivity and to detect non-complement fixing antibodies.[19] However, these assays suffer similar problems when patients are highly sensitised to class I or class II antibodies.

HLA antibody screening by ELISA

ELISA based methods[20] were the first assays to utilise antigenic determinants stripped off cells such as platelets for HLA class I assays, and from LCLs with a relevant capture antibody for HLA class II assays. These assays have several advantages over cellular assays: they do not rely on collection and separation of large numbers of cells, they detect both complement fixing and non-complement fixing antibodies, testing is rapid (results can be available within 2 h), results are semi-quantitative, and only HLA antibodies are detected. ELISA tests come in two different formats: either screening assays which are able to determine the presence of HLA antibodies, or identification assays which allow determination of allotypic specificities.

The main benefit of ELISA screening assays is the ability to screen large numbers of patient samples in a short period of time. Another benefit of ELISA identification assays is the ability to independently identify HLA class II specificities in the presence of high panel reactive class I specificities. In addition, ELISA assays are thought to be more sensitive than CDC, but may suffer from the formation of altered epitopes when antigens are stripped from the cell surface. ELISA is less sensitive than flow cytometric/Luminex assays.

HLA antibody screening by flow cytometry/X-map techniques

Currently considered to be the gold standard for antibody screening and identification, these assays rely on either soluble or recombined HLA molecules bound to polystyrene particles. The X-Map (Luminex) assay[21] relies on two different recording systems. The first is a laser which detects differences in 100 individual polystyrene particles, each stained with varying amounts of two different fluorochromes, giving a unique but reproducible gated position. Each bead set is then bound to either a mixture of HLA antigens or a single HLA antigen. Beads are then mixed with patient serum to allow antibody binding. A second anti-human antibody linked to a reporter molecule is then added to the reaction mixture, and the reporter fluorescence measured with another laser, giving a semi-quantitative level of antibodies in the patient sample.

Luminex and flow cytometric assays can be used for both antibody screening or identification in different formats. Both complement fixing and non-complement fixing antibodies can be detected by flow cytometric screening techniques. Luminex assays are able to independently identify HLA class II specificities in the presence of HLA class I specificities. Single antigen Luminex tests or flow beads can also be used to determine specific antigen reactions in high panel reactive samples, allowing determination of acceptable HLA mismatches and increasing chances of transplant.

New antibody identification techniques have allowed determination of HLA antibodies in transplant patients with failing grafts in all forms of solid organ transplantation. Furthermore, many previously unexplainable flow cytometric positive crossmatches can be explained by antibody identification using flow based antibody identification techniques.

THE PATIENT–DONOR CROSSMATCH

Preformed antibodies directed against donor HLA antigens and present in recipient serum at the time of transplant can cause hyperacute rejection. Such antibodies can be detected by careful and regular patient antibody monitoring as outlined above. In addition, before certain solid organ transplants may proceed, it is necessary to perform a functional assay or 'crossmatch' to determine that no potentially deleterious donor-specific antibodies are present—or have been present—in the intended transplant recipient. In other forms of solid organ transplant, the crossmatch may be performed retrospectively, as a guide to post-transplant patient management. These scenarios are described in more detail elsewhere in this issue.

Crossmatching is normally performed using one or two methods, namely complement dependent cytotoxicity (CDC) crossmatching[22] and crossmatching by flow cytometry (FC).[23] These crossmatching tests differ in both the types of antibody detected and in sensitivity, and, when performed together, give maximum information to the transplant team.

Crossmatching by complement-dependent cytotoxicity

In the CDC crossmatch, serum samples from the recipient are incubated with separated T and B cells from the potential organ donor. When donor reactive antibodies are present, the addition of complement results in cell killing. This is reported as a positive crossmatch. In the USA, the sensitivity of the technique is enhanced by the addition of anti-human globulin, but this is not widely used in Europe. Crossmatches are usually performed using separated T and B cells. T cell positive crossmatches are usually due to anti-HLA class I antibodies and are usually a contraindication to transplantation. B cell positive crossmatches in the absence of T cell positivity may be due to anti-HLA class II antibodies or weak class I antibodies, since class I is more strongly expressed on B cells than T cells.

CDC crossmatches are performed with and without the addition of DTT, which digests IgM antibodies by reduction. Thus positive crossmatches due to IgM antibodies will be abrogated, such that positive crossmatches due to IgG and IgM antibodies can be distinguished. Positive crossmatches due to IgM antibodies are not usually regarded as a contraindication to transplantation. However, it should be noted that class switching from IgM to IgG can occur, so such crossmatch results must be reviewed carefully. Many autoantibodies are of IgM subclass. Failure to distinguish crossmatch reactivity due to this cause from that attributable to alloantibodies may inappropriately deny a patient the chance of transplantation. To assist in this differentiation an autocrossmatch may be set up alongside the donor (allo) crossmatch to establish the autoantibody contribution to serum reactivity.

Crossmatch results can only be reliably interpreted in the context of a comprehensive antibody screening programme. Via such an approach, the specificity of anti-HLA antibodies can be fully defined and hence a patient's crossmatch reactivity against a potential donor of known HLA type can be predicted. This avoids unnecessary crossmatching and has dramatically reduced the incidence of unexpected positive crossmatches. In addition, when such positive crossmatches do occur, they are more easily interpreted as resulting from non-HLA or clinically irrelevant antibodies.

Crossmatching by flow cytometry

Crossmatching byFC is more sensitive than the traditional CDC approach.[24] In some centres, all renal transplant patients are also crossmatched by FC, while in others, FC is only used for regraft or sensitised patients (those with antibodies against a range of HLA antigens).

In the flow cytometric crossmatch, donor lymphocytes are incubated with the recipient sera, and after washing,

Take-home messages

- HLA genes are the most polymorphic in the human genome.
- HLA antigens play a critical role in regulating the immune response and constitute the principal barrier to successful transplantation of cells, tissues and organs between individuals.
- Techniques for accurate HLA typing, antibody detection and characterisation and final assessment of patient–donor alloreactivity have been developed and established in histocompatibility & immunogenetics laboratories in support of clinical transplant programmes.
- This mini-review provides a brief critique of these laboratory techniques.

anti-human Ig is added conjugated with a fluorochrome stain. A further monoclonal antibody is added to identify either T or B lymphocytes and is conjugated with a different fluorescent stain. The mixture, after incubation and washing, is passed through a flow cytometer, where a laser is used to excite the fluorochromes and the intensity measured by a photomultiplier tube. The intensity of fluorescence correlates with the amount of immunoglobulin on the labelled cells and hence reveals the amount of donor-specific antibody in the recipient.

The flow cytometric crossmatch is effective in detecting donor directed IgG antibodies. It is less good for IgM.

Maximum information is obtained when both CDC and FC crossmatches are performed, that is, some crossmatches may be FC positive, but CDC negative, indicative of weak antibodies. As for CDC crossmatches, results should only be reliably interpreted in the context of a comprehensive antibody screening programme.

Crossmatching by solid phase methods

In certain situations, confident interpretation of the CDC or FC crossmatch is precluded owing to antecedent patient treatment with modalities, in particular humanised monoclonal antibodies, that have a tendency to generate false positive results in these systems. Limitations on the availability of donor cells also represents a restriction on use of these assays in the setting of post-transplant monitoring. The recent availability of a commercial bead based assay system that involves donor cell HLA capture onto Luminex beads,[25] which can then be used to determine presence of donor HLA antibody in the recipient, avoids interference by biologics and is beginning to find application in the setting of antibody incompatible transplantation. Donor antigen coated beads also offer a means of performing follow-up studies in the absence of a source of viable donor cells.

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