Immunological risk assessment and human leukocyte antigen antibody testing in kidney transplantation

Immunological assessment in the early days of solid organ transplantation relied upon the complement dependent cytotoxicity crossmatch test (CDC). This was followed by flow cytometry and more recent solid-phase immunoassays (Luminex assay) that allow detection and characterization of low-levels of antibodies against human leukocyte antigens (HLA). In the 1980s and 1990s, effective immunosuppressive drugs primarily targeted to control T-cell alloimmunity resulted in significant reductions in rejection episodes and improvement in graft survival. The role of antibody-mediated rejection (AMR) in both acute and chronic graft failures has been increasingly recognized in recent years in a large number of publications. This has resulted in a change in management protocols and detection/monitoring of HLA alloantibodies using sensitive techniques such as the Luminex assay. There are detailed published guidelines on detection and management issues associated with HLA antibodies that the reader should refer to: Tait *et al*.[1] and British Transplantation Society Guidelines for the detection and characterization of clinically relevant antibodies in transplantation (http://www.bts.org.uk/Documents/Guidelines/Active).

Detection of clinically relevant antibodies is performed by cell-based assays that detect direct interaction between the antibodies present in the recipient serum with donor cells (crossmatch tests - CDC and flow cytometry) and tests to detect the presence of HLA antibodies using beads coated with HLA antigens either in flow cytometry or in solid phase assay platforms.[2-5] The latter include an ELISA and Luminex technology that is increasingly used worldwide.[5]

The Luminex technique can detect several HLA antigens (100 different HLA Class 1 and 100 different Class 2 alleles) in two reaction tubes. In addition, using beads coated with a single HLA antigen (single antigen beads [SABs]), HLA antibody specificity can be precisely characterized. There is no other currently available technique that can achieve this. However, widespread use of this highly sensitive assay can lead to clinically inappropriate decisions if not interpreted correctly. Both false positive and false negative results are known to occur, and if the test is used in a format that is not widely used across the world or recommended, it can yield rather confusing results. In this issue of IJN, Chacko *et al*.[6] describe their experience of false positive Luminex assay positivity due to nonspecific binding to antibody-coated beads. This report and previous reports of false positive and false negative results observed in a Luminex assay highlight the importance of understanding the Luminex assay using it in its most validated format and the need to take care while interpreting the results keeping in mind clinical details and the results of other more routinely used crossmatch tests.

The CDC crossmatch is the most widely used test to determine whether a kidney transplant can proceed or not. While it is adequate in the majority of cases, the test is subject to inter-observer variation, it is not automated and most importantly it is not very sensitive as it only detects high levels of HLA antibodies. The advantage of this test is that it is widely used, most laboratories feel comfortable with it, and it detects only clinically relevant complement fixing HLA antibodies. However, as it does not detect low-level HLA antibodies, AMR can occur in patients transplanted with negative CDC crossmatch. With regards to sensitization status of the patient, similar technique was used to assess the panel-reactive antibody (PRA), in which panels of donor cells were used to represent common HLA antigens found in potential deceased donor population. The results were dependent upon composition of cells. PRA estimation using donor cells is no longer used routinely.

Flow cytometry crossmatch is more sensitive than the CDC crossmatch and has replaced CDC crossmatch in some centres. It detects lower levels of HLA antibodies (immunoglobulin G [IgG] only), and it is semi-automated with a resultant reduction in inter-observer variability. It must be emphasized that while a positive CDC crossmatch is considered an absolute contraindication to a kidney transplant, flow cytometry crossmatch positivity is a relative contraindication. False positive results may occur with flow cytometry crossmatch, in particular with B-cells due to nonspecific binding.

Solid-Phase Assays

Solid-phase assays, such as ELISA and Luminex assays, are sensitive methods that detect lower levels of HLA

antibodies and allow precise determination of antigen and allelic specificities of HLA antibodies (Reviewed in British Transplantation Society guidelines for the detection and characterization of clinically relevant antibodies in transplantation). HLA antibodies can be detected using polystyrene beads coated with HLA antigens. These are commercially available for use either in a conventional flow cytometer or a dual laser fluoroanalyzer. The most widely used technique is the X-map (Luminex) technique. The detailed technology of Luminex is beyond the scope of this review, and the reader is referred for details to the available literature and internet. Briefly, microbeads are coloured with a combination of two dyes. Each set of beads has the dye in different proportions such that the bead sets can be distinguished by a dual laser system. HLA-specific antibody binding to the microbeads is detected using R-phycoerythrin conjugated anti-human immunoglobulin and a flow analyzer.

There are three types of panels available commercially:

- Pooled antigen panels coated with either affinity - purified HLA Class I (HLA-A, HLA-B, and HLA-C) or HLA Class II (HLA-DR, HLA-DQ, and HLA-DP) protein molecules obtained from multiple cell lines and are used as a screening test for the detection of HLA antibody. Pooled antigen beads are relatively inexpensive and indicate the presence or absence of antibody to a particular HLA class, but they do not provide specificity and do not represent all possible antigens. A typical example of commercially available bead set would have a set of 12 beads coated with a pool of purified HLA Class I glycoproteins and another set of 5 beads with HLA Class 2 glycoproteins. The results are interpreted as either HLA IgG Class I and/or Class II antibodies positive or negative
- Phenotype panels in which each bead set bears either HLA Class I or HLA Class II proteins of known specificities derived from a single cell line. These are available as Class I and II bead sets. The read-outs from these beads provide PRA (panel reactivity) expressed as percentage of beads showing a positive result. The composition of the bead panel (i.e., the specificities of Class I and Class II antigens) is given with each kit
- Single-antigen beads (SABs) in which each bead population is coated with a molecule representing a single recombinant allelic HLA Class I or II antigen. Here, HLA Class I and II recombinant single antigens from transfected cell lines are used to coat the beads (microparticles). The Luminex assay using SABs is the only technique that allows precise characterization of HLA antibody specificities to enable precise antibody specificity analysis. In addition to detecting antibodies against HLA A, B, C, DR, and DQB antigens, Luminex-SAB is capable of detecting antibodies against HLA, DQA, DPA, and DPB antigens not detectable by currently available ELISA. This allows the definition of unacceptable antigens (donor HLA antigens against which recipient has an antibody) facilitating organ allocation in deceased donor allocation programmes, such as in the UK. In addition, the assay provides a semi-quantitative fluorescence value (mean fluorescence intensity [MFI]) that is meant to represent the amount (titer) of the antibody. However, it must be emphasized that the MFI value represents the amount of antibody bound to the beads and not the serum levels (titer) of the antibody. The variables that influence the binding are: The amount (titer) of the antibody, the affinity of the antibody to the antigen, the antigen density on the bead and denatured antigen as opposed to intact antigens found on cells. The antigen density varies between the beads both within an assay and between different kits. In spite of these limitations, Luminex SAB (L-SAB) is the best available assay currently for identification and quantification of HLA-specific antibodies, including identification of antibodies against HLA-DQA1 and DPB1 and allele-specific antibodies.

The beads (microparticles) coated with soluble HLA antigen of known specificities can also be used in a conventional flow cytometer to determine PRA (flow-PRA). The kit provides composition of the panel (i.e. HLA antigens coated onto the beads).

While the commercially available kits provide their own positive and negative controls, it is a good practice for each laboratory to use their own internal positive control serum (from a pool of known sensitized patients) and a negative control serum from non-alloimmunized individuals.

The Luminex solid phase assay offers the following advantages:

- No requirement for viable lymphocytes and complement
- Detects only HLA specific antibodies
- Objective and partially automated
- Commercially available
- Provides quantification of antibody titer (crude).

Limitations of the Luminex assay

There are certain clinically relevant technical aspects and limitations that the clinician and the transplant immunology laboratory need to be aware of and these include:

False positive test results

The problem with any test that is very sensitive is that antibodies detected may or may not be clinically relevant. Denatured proteins on the bead surface could have antibody binding epitopes that are not normally expressed *in vivo* leading to immunologically irrelevant binding.[7] Also, during the process of coating of HLA antigens onto the bead surface, conformational changes to the protein can potentially lead to the exposure of neo-epitopes leading to false-positive binding., In this edition of IJN, Chacko *et al*.[6] describe high MFI values due to nonspecific binding of antibodies to the beads coated with a capture antibody. The authors used donor cell lysate to coat the HLA antigens to the beads coated with capture antibody trying to create a bead equivalent of the CDC crossmatch. Although this kit is commercially available, the test is neither standardized nor universally used in this format to detect HLA antibodies and it may produce results that are hard to interpret. In addition, it could lead to wrong clinical decisions with disastrous consequences to the patient such as declining a transplant. Therefore, using the Luminex assay in this format is not recommended.

Other reasons for false positive results include nonspecific binding of non-HLA antibodies and other serum proteins (e.g. IVIg, during infection/systemic inflammatory disorder). These can increase the background MFI although any such change would occur for both control and test beads.

What mean fluorescence intensity (MFI) values should worry the clinician? Lack of good correlation between donor-specific antibody MFI values and crossmatch positivity

One of the difficulties with the Luminex assay is that the donor-specific antibody (DSA) levels determined using the MFI values do not always correlate with crossmatch positivity (CDC or flow cytometry). The widely used cut-off MFI in the Luminex assay is 1000. It is well-known that MFI values of well above this level can be associated with a negative crossmatch (even the more sensitive flow cytometry crossmatch). In our centre, we have had two cases with DSA MFI values on >10,000 with a negative flow cytometry crossmatch. The first patient underwent a living donor kidney transplant (without desensitization). She developed acute antibody-mediated rejection (AMR which was successfully treated and currently has stable allograft function and a serum creatinine of 140–150 μmol/l (1.6–1.7 mg/dl) with persistent DSA of around 4000 MFI. The second patient has been placed in the UK kidney sharing scheme (KSS). While such scenarios are not common, they are well-recognized. It must also be noted that there is a poor correlation between pretransplant DSA MFI values and graft survival.[8] Based on the current evidence, it is not possible to assign an MFI cut-off value that correlates with the occurrence of AMR and graft survival. Modifications to the Luminex assay to detect complement fixing (C1q binding) have been used to further characterize pathologic role of the DSA with some studies suggesting that C1q+ DSAs are associated with worse graft survival compared to C1q negative DSAs.[9]

Therefore, each laboratory wishing to use the Luminex assay should try to establish their own clinically relevant MFI values setting the positive threshold to where they commonly see crossmatch positivity (flow cytometry crossmatch). Furthermore, it must be noted that clinically relevant MFI values can be different for different HLA antigens. In other words, some HLA antibodies need to be present in higher concentrations (higher MFI values) than others to cause crossmatch positivity. Finally, MFI levels from one laboratory cannot be compared to the other, and there can be lot to lot variation in MFI values even if kits from the same company are used.[10] Therefore, the results of L-SAB assay should be interpreted in conjunction with clinical history (sensitization status) and crossmatch results.

False negative test results

When the HLA antibody titres are high, a false negative test can occur due to the prozone phenomenon.[11] In this scenario, high titre antibodies lead to complement activation and deposition of complement proteins on the bead which then prevents HLA antibody from binding to the HLA antigen on the bead. A similar situation can arise due to the binding of IgM antibodies or other serum factors to the beads. These issues can often be resolved by serum dilution and treatment with dithiothreitol (DTT). Drugs such as IvIg and nonspecific binding by serum proteins can also interfere with the specific binding of HLA antibodies to the HLA antigens on beads.

Another reason for a false negative result is epitope sharing. Here, different HLA antigens on different beads share common antibody binding epitopes leading to binding of an anti-HLA antibody to more than one bead with consequent reduction in the MFI on a single bead. Finally, during the process of attachment of HLA molecule, the bead, immunologically relevant epitope, can become hidden and un-accessible to antibody binding.[7]

It must be emphasized that the clinical relevance of pretransplant DSAs detected by highly sensitive SAB Luminex assay in the absence of CDC or flow cytometry crossmatch positivity remains unclear.[12,13] However, accumulating evidence does suggest that the development

of *de novo* DSAs against mismatched HLA antigens is associated with an increased risk of chronic AMR, transplant glomerulopathy, and graft loss.[14,15]

In the UK, the transplant Immunology laboratories screen for HLA antibodies using Luminex in all recipients (both on deceased donor wait list and for living donor transplant) irrespective of their sensitization status. If HLA antibodies are found on the screening test, further characterization of specificities is done using the SAB Luminex assay. Final immunological stratification is made based on these results and the results of crossmatch testing (CDC or CDC + flow cytometry or flow cytometry crossmatch alone). However, this approach may not be applicable to all transplant centres in countries like India as not all may have fully-equipped transplant immunology laboratories and centres vary in the expertise required to standardize and optimise the Luminex assay (and interpret the results in association with the crossmatch results). In some centres, resources would be limited, and perhaps most important consideration would be the financial constraints that the patients face. Most often patients and their families pay entirely for the cost of transplantation and drugs. The utility of money saved by avoiding unnecessary expensive tests or tests performed and interpreted wrongly, should not be underestimated. This is unlike the situation in countries like the United Kingdom where the cost of treatment is borne entirely by the state (National Health Service).

It can be argued that in low-risk cases, it is not necessary to perform the Luminex assay and that the crossmatch tests are adequate in deciding if a transplant can proceed or not.

A suggested guideline applicable to the Indian transplant centres and other centres from the developing world

- CDC and where available flow cytometry crossmatch for all patients irrespective of HLA match and sensitization status
- In high-risk patients (previous blood transfusions, second or subsequent transplants, husband to wife donation, multiple pregnancies, and child to mother donation) - assess the level of sensitization (look for pre-existing anti-HLA antibodies):
  This is done by either flow cytometry (flow PRA) or Luminex solid phase assay (Luminex PRA)
  Both of these tests will give PRA for Class 1 and Class 2 antibodies expressed as percentage
- If the screening Luminex PRA or flow cytometry PRA demonstrates anti-HLA antibodies, then do an SAB Luminex assay to identify the antibody specificities and to identify if there are DSAs
  If the screening PRA test is negative, no further assessment with SAB Luminex assay is required
- Low/standard risk patients: HLA antibody testing may not be necessary.

Once crossmatch results are available

If positive (either T-cell, B-cell or both), proceed to Luminex to look for anti-HLA antibodies.

L-SAB assays will confirm presence or absence of Class 1 or Class 2 antibodies and their specificities.

Look at the positive test results (MFI > 1000). Check if these antibodies are against mismatched donor HLA antigens as the donor HLA type would be known. Most of the relevant DSAs tend to have an MFI of >3000.

Donor-specific antibody present and crossmatch positive

This is an HLA incompatible situation. Do not transplant. Look for an alternative donor or register on to a KSS (kidney swap or paired exchange) to get a compatible kidney.

If there are no other options

Single DSA with MFI < 10,000 - consider de-sensitization (double filtration or cascade plasmapheresis (DFPP) with IVIg, aim for crossmatch negativity. Once the crossmatch is negative, proceed to transplantation with ATG induction. The other alternative induction agent is Campath (Alemtuzumab). Rituximab can be used as a part of the desensitization regime, but this makes interpretation of subsequent B-cell crossmatch difficult due to false positivity.

In these cases, a test 1.5 plasma volume DFPP with pre- and post-Luminex assay is essential to assess how effectively the DSA can be removed and to get an idea of how many DFPP sessions are likely to be required.

Single DSA with MFI > 10,000: The same protocol as above can be used, but it is a very high-risk approach.

Multiple DSAs each with MFI > 5000: Transplant not recommended.

It must be emphasized that HLA incompatible transplant (DSA+ve, crossmatch+ve) performed after desensitization carries a very high risk of AMR. Postoperative monitoring includes repeated Luminex assays to monitor antibody titres, and these patients are likely to require further sessions of DFPP and heavier post-transplant immunosuppression. The risk of sepsis and death are significantly higher in these patients. Overall, the risks are significantly higher compared to

ABOi transplantation, and the long-term results are not satisfactory.

Adequate counseling for patients and family is essential both with respect to the risk of graft loss and financial burden. The latter is very significant in situations where the cost of the therapy is entirely the responsibility of the patients and their families.

Positive current donor-specific antibodies with negative crossmatch (complement dependent cytotoxicity and flow cytometry)

This is not a contraindication for transplantation but is high immunological risk transplantation with high risk of acute AMR. These DSAs may or may not lead to early graft loss.[13] Based on the current literature and experience, this is not an indication for desensitization. Where possible, patients should be advised to go on a kidney sharing scheme (kidney swap).

If transplant needs to proceed, transplant with either Basiliximab or ATG induction, ATG being the preferred option. There is no clear evidence or guidelines regarding the choice of induction agent in this situation. Some centres transplant these patients with basiliximab induction and reserve ATG for the treatment of severe rejection if required. Careful posttransplant monitoring is required in these cases.

Recommended posttransplant monitoring for donor-specific antibody

- HLA incompatible transplant recipients (who underwent pre-Tx desensitization) – alternate days until D10, 1-month, 3 months, 6 months, and 12 months
- At the time of rejection, [if the rejection was AMR or cellular with vascular component (Banff 2A and above)], 1-month, 3 months, and 6 months postrejection
- Routine post-Tx monitoring for DSAs for compatible transplant recipients cannot be recommended in current time as this is not cost effective, and it is unclear what to do if DSA is detected (or rising) with stable allograft function. If this situation arises, in my opinion, appropriate management is – (1) Do not reduce immunosuppression, (2) optimize tacrolimus levels, (3) Consider doing a transplant kidney biopsy. Further management should be guided by biopsy findings.

Conclusion

The detection and characterization of HLA antibodies are crucial for the appropriate management of renal transplant recipients. The Luminex assay provides the most sensitive technique to achieve this. Transplant clinicians and laboratory personnel should be aware of its limitations and should be able to interpret it in association with clinical picture and crossmatch test results.

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