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# Post-transplantation antibody monitoring and HLA antibody epitope identification

Junchao Cai and Paul I Terasaki

We earlier reviewed the reasons to believe that HLA antibodies are the *cause* of chronic rejection. In this review we shall focus on two questions: first, *why is it important to test transplant recipients for alloantibody post-transplantation?* Recent large-scale prospective studies on the effect of alloantibodies on graft survival showed that rates of graft failure more than doubled in recipients who had alloantibodies compared with antibody-free patients. The causal relationship between alloantibody and transplant rejection — especially chronic rejection — has been demonstrated both experimentally and clinically. We recommend universal testing of allograft recipients for antibodies since that will help clinicians identify this obvious risk factor and take appropriate action to minimize deterioration of transplant function. Second, *why is it important to identify HLA antibody epitopes?* Since HLA antibody is a potential cause of graft rejection, identifying the epitope — or antigenic determinant — to which an antibody binds, becomes very important. Such identification lets clinicians target the real transplant antigen and may lay a foundation for the development of new treatments and/or new matching strategies to reduce the occurrence of antibody-mediated rejection (AMR). Now that natural HLA antibodies have been identified; it is important to distinguish these from donor-specific epitopes. The establishment of 103 HLA class I epitopes, 83 class II, and 7 major-histocompatibility-complex class I-related chain A (MICA) epitopes is reviewed. Single antigen bead technology has been important in identifying the epitopes by experiment.

**Address**
Terasaki Foundation Laboratory, 11570 West Olympic Boulevard, Los Angeles, CA 90064, USA

Corresponding author: Terasaki, Paul I (terasaki@terasakilab.org)

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Four decades ago, to argue the relationship between smoking and lung cancer, Sir Austin Hill and Sir Richard Doll posited nine criteria to justify moving from a claim of association to one of causations: strength, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy [1]. In our recent review, we demonstrated that most of these criteria are met and that HLA antibodies can be considered a primary cause of chronic rejection [2•]. Here, we expound the importance of antibody monitoring and antibody epitope identification for transplant recipients.

## Antibody monitoring for transplant recipients

The updated Banff 07 diagnostic categories of antibody-mediated rejection (AMR) have been modified and expanded from the original two (hyperacute and accelerated acute) to the current three: acute AMR, chronic active AMR, and, now, C4d deposition without morphologic evidence of active rejection [3•]. Including as a diagnostic category of rejection a condition that does not, in fact, involve immediate evidence of rejection — but has so often been found to predict rejection as to impel inclusion — emphasizes the importance of early identification of potential tissue damage.

*In situ* C4d deposition is alloantibody dependent. It develops, post-transplantation, as a continuous process, starting as focal staining and progressing to diffused PTC staining [3•]. Dickenmann's retrospective study demonstrated that the presence of C4d in kidney transplants — absence of any histological evidence of acute rejection — is not associated with *rapid* deterioration of graft function. However, anti-rejection therapy results in the improvement of kidney function in these patients. So even in grafts with normal histology, the detection of C4d in diagnostic biopsies can be interpreted as a sign of 'smoldering' rejection that will benefit from therapy [4••].

Acute and/or chronic AMR is a sequential process. It begins with donor-specific antibody (DSA) that generates antibody-mediated endothelial damage (e.g. C4d deposition) leading to arterial and parenchymal damage and, finally, transplant dysfunction. In this pathological process, development of antidonor antibody is the primary and essential step, C4d deposition being dependent on the presence of antidonor antibodies. Since it is obviously best to arrest the process as early as possible, detection should start with monitoring for the formation of DSA, rather than waiting until C4d staining shows that the pathology has already begun. Besides that, the following facts suggest further advantages of post-transplantation antibody monitoring as compared with C4d staining:

1. Development of antidonor antibodies is the initial humoral response against allograft, while complement activation (evidenced by C4d deposition) is one of the secondary events; AMR may also involve noncomplement-dependent mechanisms [5].
2. Multicenter large-scale prospective studies confirmed that the presence of HLA and/or MICA antibodies in patients with well-functioning grafts predicted later graft failure [6••,7,8] even though AMR may not appear until 3–4 years after antibody appearance [9,10] — or even 13 years after [11•].
3. More sensitive techniques have recently become available for the identification of HLA and/or MICA antibodies and the determination of donor specificity [12,13].
4. Antibody testing is easier on patients than protocol biopsy since it is much less invasive and requires only microliters of serum/plasma.
5. Antibody testing is less expensive, and can be repeated often.

Our previous prospective studies indicated that patients with HLA antibody had lower graft survival than patients without HLA antibody [7,8] did. In those studies, antibodies were identified with screening assays that did not also identify donor specificities. By using single antigen beads, our most recent prospective study [6••] demonstrated for the first time that for patients with DSA the one-year death-censored survival rate was lower than for patients with strong nondonor-specific antibody (NDSA), moderately strong NDSA, or no antibody. The rate was 64.3% for DSA patients versus 66.2%, 85.8%, and 95.2% for the others. These data demonstrated that identification of donor specificity and classification of patients with antibody based on their antibody levels seems advantageous. Further investigation must determine why NDSA is associated with lower graft survival and why this negative association seems to depend on antibody level. But this phenomenon does not change the conclusion that monitoring of transplant recipients for antibodies will help predict later graft loss.

Currently, there are several questions need to be addressed when monitoring patients for antibodies:

1. *Time points and/or frequencies of antibody monitoring.* When we retrospectively examined serial samples of sera from 93 kidney patients who had failed grafts, antibodies were noted before graft failure in 65 cases. Some antibodies were formed as little as 1 month after transplantation, though the mean time appears to be about 11 months. An average of 29 months elapses before graft failure is detected by a rise in serum creatinine. After the emergence of antibody, the graft is completely lost after an average of 44 months [14]. On the basis of these results, we think biannual or annual monitoring of antibody might be a reasonable suggestion for most patients. However, since the time from antibody appearance to a noticeable effect upon the graft varies significantly between patients, for high-risk patients with high titer of alloantibodies, more frequent antibody testing might be required.
2. *Antibody classes and subclasses.* Even though there are publications demonstrating the protective effect of IgM [15,16], and IgG1 subclass is associated with a significantly worse clinical outcome [17], studies regarding the effect of different classes and subclasses of antibody on transplant outcome are limited. Most publications using commercially available solid-phase antibody testing kits detect the IgG form of HLA and MICA antibodies. Further research needs to be performed to investigate the role of different classes and subclasses of antibody in transplantation and to reveal the underling mechanisms.
3. *Whether all antibodies detected by bead technology are relevant.* Alloantigen-coated beads are widely used to detect antibodies in transplantation. There has been a concern that antigens coated on the surface of beads may be structurally different from membrane expressed antigens and some 'unnatural' antigens may detect irrelevant antibodies. Our experiments with monoclonal antibodies W6/32 (recognizing intact HLA class I molecule) and HC10 (recognizing peptide and β2m-free heavy chain) demonstrated that both intact and peptide and β2m-free heavy chain were expressed on the surface of beads. Morales-Buenrostro *et al.* have recently identified natural antibodies in the sera of normal males. These antibodies occur in up to 60% of un-immunized subjects, and the majority seems to target infrequent HLA antigens [18••]. The antibody titers for most of these natural antibodies seemed to increase when elution buffer (which removes peptide and β2m from class I heavy chain)-treated beads were used. These data suggest that these natural antibodies may target peptide and β2m-free heavy chain of class I molecules. Further investigation is needed to discover how these natural antibodies develop and whether they complicate antibody monitoring of allograft recipients. If they do, we must find out how to differentiate them from alloantibodies — whether pre-existing or developed post-transplantation.

## Identification of HLA antibody epitopes

Laux *et al.* reported on conventional mismatching (CMM) at the allelic level. They found that a transplant group with one DP mismatch showed a significantly better graft outcome than a group with two mismatches. A refinement produced an interesting result. The researchers looked at a subset of the recipients who had only one conventional mismatch — but also had three or more epitope mismatches (EMMs) — comparing that group with a subset of those who had two allele mismatches but no more than two EMM. This time, the one-allele-mismatch group

(but with ≥3EMM) had a significantly *lower* survival rate than the two-allele-mismatch group with ≤2EMM. These data strongly suggested that epitope mismatches are of greater biological significance than conventional mismatches [19]. Therefore, identification of antibody epitopes — the 'transplantation antigens' responsible for AMR — becomes very important.

Site-mutation of protein sequence by molecular biology techniques is commonly used to identify residues/epitopes that may play a crucial role in protein–protein or protein–DNA/RNA interaction [20]. The potential epitopes of HLA-specific antibodies can now also be deduced by comparing the amino acid sequences of HLA antigens. After many early attempts to use amino acid sequence matching for transplants, Duquesnoy formalized the process with his HLAMatchmaker program [21•]. This program views each HLA antigen as a string of epitopes represented by triplets of polymorphic amino acid residues in antibody-accessible positions. HLAMatchmaker determines which triplets are different between donor and recipient. Antibodies are induced only against a proportion of the epitopes–immunogenic epitopes, whereas epitopes shared by the mismatch and the patient's own HLA alleles are not immunogenic. This algorithm has been reported to be clinically useful in determining HLA compatibility at the humoral-immune level [21•,22]. The recent 'Eplet' version of the program considers the structure of epitopes more comprehensively and it provides a more detailed assessment of HLA compatibility [23,24]. A different strategy based on a computer program has been used to predict HLA epitopes in our recent studies [25].

However, studies have shown that the potential epitopes predicted purely by computer-based comparison of HLA sequences may not always be the 'real' antibody epitopes. As we reported previously [20], antibody analysis of one graft-failure patient who received a DR-mismatched kidney (recipient: DR 17, –; donor: DR 15, 17) showed that the recipient developed not only DSA — anti-DR 15 — but also many NDSAs. Sequence-based epitope analysis indicated that donor and recipient DR molecules are mismatched at 12 amino acid residues that are distributed on both β-1 and β-2 domains. However, the antibody profile suggested that only 1 of these 12 mismatches seems to play a crucial role in mediating antibody–antigen interaction. Similarly, a study by Adeyi *et al.* delineated the different immunogenicities of theoretically deduced epitopes (triplets), in which serum analysis after transplant nephrectomy showed restricted antibody specificity patterns against donor triplets. Many donor triplets were nonreactive whereas others were apparently recognized by antibodies [26]. Much the same conclusion can be drawn from the Laux study, in which hypervariable regions on the β chain of HLA DP antigens were grouped into six polymorphic regions designated as potential epitopes A–F. Graft survival analysis demonstrated that matching for epitopes located in regions A, B, E, and F had an influence on graft outcome whereas matching for epitopes in regions C and D had no effect [19]. All of the above findings demonstrated that only some of the theoretically deduced epitopes were immunogenic.

The advent of single antigen bead technology [12] has made it possible to identify epitopes experimentally — rather than deriving them theoretically — by having either monoclonal or alloantibodies react with an array of single antigen beads. Potential epitopes can be deduced from the reaction pattern of HLA antibodies. These identified epitopes can be further confirmed with absorption and elution experiments using recombinant cell lines that have a single HLA antigen. El-Awar *et al.* tested a large series of antibodies against many specificities, empirically establishing antibody epitopes of HLA class I molecules [27]. These epitopes are the exact sites or determinants to which the antibodies are directed. Since 2006, experimentally derived epitopes — using recombinant single specificity lines and single antigen beads for the identification of the antibodies — have been used to identify 101 epitopes on class I antigens, 83 epitopes on class II antigens, and 7 epitopes on MICA antigens [20,25,28,29,30••,31].

## Actions to take after antibody detection post-transplantation

### Antibody-positive patients

Studies have repeatedly shown that about 20% of patients have antibodies, despite immunosuppressive therapy [6••,8]. Detection of antibodies in patient peripheral blood implies that the patient is in danger of later graft loss because of possible AMR. For these antibody-positive patients — even though they have well-functioning grafts — we recommend that action be taken not only to remove circulating anti-donor antibodies but also to inhibit/deplete cells that produce antibody. As reviewed previously, therapeutic strategies to prevent and treat AMR include first, inhibition or depletion of antibody-producing cells; second, removal or blockage of pre-existing or newly developed antibodies; and third, impediment or postponement of antibody-mediated tissue injury [5]. Many currently available approaches to AMR treatment have proven effective for different patients under different circumstances [32–37]. Much work needs to be done to better understand how current therapies can be best used to prevent and/or treat AMR. It may require time, but it is very important to test whether antibody removal will indeed prolong graft survival. Such testing is also very crucial for the final establishment of a causal relationship between antibody and graft rejection.

### Antibody-negative patients

When a patient is identified as being free of alloantibodies, the possible explanations may include: first, effective immunosuppression; second, low immunogenicity of

allograft; and third, immune tolerance of recipient. Although it is tempting to reduce immunosuppression in these patients, we believe that it serves patients better to wait until clinicians have more certain ways to remove antibodies once they form. That means *intensive* effort needs to be placed on removing antibodies from those who have them whenever they appear. Of course, eventually, one of the most compelling reasons for antibody monitoring will be the ability to identify those in whom immunosuppression can be reduced.

## Conclusions

Evidence that HLA antibodies are likely to be the main *cause* of chronic graft rejection has been presented [2•]. We believe that since this is a hypothesis than can be proved or disproved, it is time now to test it. This means that we should use antibody testing to monitor transplants post-transplantation. Whenever the antibody is found, it needs to be removed in order to prevent chronic rejection. In many instances, since the antibody appears much earlier than the increase in serum creatinine [14], many may hesitate to be involved in an extensive attempt to remove the antibodies. However, as shown now by extensive prospective studies [6••,7,8], it is probable that antibodies are causing damage that is not revealed by serum creatinine measurements because the kidney has considerable reserve to compensate for damage. Identification of the HLA epitopes should be helpful in distinguishing donor-specific antibodies from natural antibodies, which appear to be produced in response to non-HLA environmental stimuli.

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