Donor Reactive HLA Antibodies in Renal Allograft Recipients: Considerations, Complications and Conundrums

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Abstract

Whether sensitized patients wait for a compatible crossmatch with a deceased donor, enter a paired exchange program with the hope of finding a compatible living donor or go through a desensitization protocol depends on a number of factors, not the least of which is the overall philosophy of the transplant center. Centers such as ours take the position that donor directed antibodies detected by solid phase assays (even those that are “weak”) present an unacceptable risk factor to the patient. This philosophy is predicated on the biological role of the immune system, specifically that antibodies were generated in response to a non-self (allo) antigen and that a successful immune response eliminates that which caused its stimulation. While obviously an oversimplification, this philosophy mandates a comprehensive evaluation of HLA antibodies in sensitized recipients. This paper addresses the challenges and conundrums associated with HLA antibody identification.
Overview

Following the landmark report of Patel and Terasaki [1], a positive cytotoxic crossmatch between donor cells and recipient serum was considered a contraindication to renal transplantation. The high rate of immediate graft loss among patients transplanted across this barrier was unacceptable. It soon became apparent that the clinically relevant antibodies in a lymphocyte crossmatch were those directed against antigens encoded by the human major histocompatibility complex (MHC) henceforth referred to as the Human Leukocyte Antigen (HLA) complex. The paradigm to not cross the positive crossmatch barrier was modified when it was recognized that cytotoxic crossmatches due to non-HLA antibodies (e.g., autoantibodies) had no impact on allograft survival and could be safely ignored (reviewed in [2]). However, there was a growing appreciation that complement fixing donor directed HLA antibodies undetectable by standard cytotoxicity assays were clinically relevant (reviewed in [3]). Such observations led to the development of more sensitive antibody detection tests including the antiglobulin enhanced cytotoxicity [AHG-CDC] and flow cytometric crossmatch assays [4-8]. While more sensitive, the shortcoming of these tests was their (relative) lack of specificity [9, 10]. Subsequently, the development and implementation of solid phase assays that specifically identified HLA antibodies represented a major advancement [3, 11, 12]. These sensitive and specific assays provided the transplant community with analytical tools not previously available and, in many situations, actually changed how crossmatch data were interpreted. For example, in a single center study by Kerman et al, no differences were initially reported in the number of rejection episodes and/or graft losses among recipients transplanted with renal allografts from donors whose flow crossmatches were positive or negative as long as the AHG-CDC crossmatches were negative [13]. Subsequently, retesting of sera from these patients by solid
phase technology revealed that several of the “Positive” flow cytometric crossmatches were actually due to non-HLA antibodies [14]. Importantly, the above patients experienced no rejection episodes or graft loss during the study period. Thus, it should not be surprising that solid phase assays have supplanted cell based assays and become the “gold-standard” to identify HLA antibodies.

Nonetheless, how information from solid phase assays is translated into clinical practice is quite controversial. For example, in terms of risk, should “weak” (low titered, low fluorescence intensity) donor directed antibodies be given the same clinical significance as “strong” (high titered, strong fluorescence intensity) donor directed antibodies [15-18]? Can non-complement fixing donor directed HLA antibodies be considered less problematic than complement fixing antibodies as suggested by Bohmig et al [19] or should they be considered to confer the same degree of risk to long term graft survival as proposed by Cai and Terasaki [20]? Such disparate data beg the question of how antibodies are classified as “Strong” or “weak”, or complement / non-complement fixing. Indeed, we recently demonstrated that HLA antibodies considered to be non-complement fixing by AHG-CDC assays were in fact complement fixing when assayed by a more sensitive flow cytometric based assay [21]. Thus, the fact that an antibody does not fix complement in vitro should not automatically be taken as evidence that the same antibody will not fix complement in vivo.

There are numerous other controversies involving antibodies detected by solid phase assays with literature supporting either side of the argument. Can donor directed antibodies detected in a solid phase assay that do not result in a positive lymphocyte crossmatch, even by the most sensitive of assays, be ignored [22, 23]? Are antibodies directed against certain HLA
antigen specificities less problematic than others (reviewed in [24])? Are donor directed class II antibodies more tolerable than donor directed class I antibodies [25, 26]?

Over the past three years the concept of virtual crossmatching (predicting the crossmatch outcome without actually performing a physical crossmatch) has taken center stage as an approach to more efficient allocation and distribution of deceased donor organs [27-29]. While virtual crossmatching has proven successful for predicting actual negative crossmatches between specific donor:recipient combinations, it can be argued that, if the threshold for calling an antigen “unacceptable” is set too low, those patients will never be considered for donors that might be suitable. Without question, not all patients with low, moderate or even high levels of donor directed antibody have poor outcomes if transplanted with donor organs that express the corresponding antigens. Unfortunately, other patients with characteristics virtually identical to those just described will experience severe episodes of rejection and may lose their grafts if transplanted. There are currently no assays that unequivocally categorize patients into one group or the other.

Over the last decade, desensitization strategies have been advocated as an approach to transplant highly sensitized patients (especially those who present with crossmatch incompatible donors) [30, 31]. While an excellent strategy for some patients, there is clear evidence that patients who are desensitized before being transplanted will not all have a good long term prognosis, with up to 40% of these patients experiencing antibody mediated rejection, transplant glomerulopathy and/or graft loss [32-34]. Why some desensitized patients are more likely to experience antibody mediated injury than others is not known. One explanation is that susceptible patients have a higher number of memory B cells and/or plasma cells dedicated to the production of donor directed HLA compared to patients with a good prognosis [35]. New tests
recently developed that identify HLA antibody producing cells in vitro may eventually help
discern sensitized patients into high and low risk categories [36, 37].

Should sensitized patients wait for a compatible crossmatch with a deceased donor, enter
a paired exchange program [38] with the hope of finding a compatible living donor or go through
a desensitization protocol? This is not always a simple decision. How sensitized is the patient?
How long has s/he been waiting? What is their overall health status? Another major factor is the
philosophy of the transplant center. Our center takes the position that donor directed HLA
antibodies (even those that are “weak”) are an unacceptable risk to the patient. This philosophy
is predicated on the biological role of the immune system, namely, that these alloantibodies were
generated in response to a non-self antigen and that a successful immune response will eliminate
that which caused its stimulation. While an oversimplification, this philosophy requires a
thorough evaluation of HLA antibodies in sensitized recipients.

**HLA antibody detection and identification:** Methods to detect and categorize HLA antibodies
have steadily evolved during the past 40 years. Initially, a complement dependent cytotoxicity
(CDC) assay was the only assay available to identify antibody activity. Although easy to perform
and relatively inexpensive, allograft recipients with pre-transplant HLA antibodies undetectable in
CDC assays experienced early (accelerated) antibody-mediated episodes of rejection and graft
loss [1]. These clinical ramifications accelerated the development and implementation of more
sensitive assays to identify (HLA) antibodies. Early variations included additional wash steps
and/or longer incubation times (to eliminate anti-complementary factors and/or increase the
binding of low avidity antibodies) [4]. Subsequently, the use of a secondary reagent, namely anti-
human immunoglobulin (AHG), permitted the detection of non-complement fixing antibodies [5-7].
Even so, it was soon recognized that these approaches were limited. For example, when
assaying for HLA antibodies using a panel of frozen cells, panel composition could skew the results. A given panel may indicate that the patient’s serum reacts with every cell (100% PRA) indicating that the patient would need a “perfect” match in order to have a negative crossmatch. But what if the patient had only one antibody, (e.g., A*02) and every target cell expressed HLA-A*02. Obviously, this would be an extreme example, but indicates how panel composition could provide misinformation. Similarly, analysis of patient sera with a panel of cells might reveal that the patient has antibodies to only HLA-A*01. But, if several of the HLA*01 target cells also expressed HLA-B*08 and no cells expressed HLA-B*08 without also expressing HLA-A*01, antibodies to HLA-B*08 would be masked. Under these circumstances, a positive crossmatch with a cell expressing HLA-B*08 without HLA-A*01 would be interpreted as being due to a non-HLA antibody. Another limitation was the requirement for viable cells as targets. Thus, if a patient possessed a lymphocyte-reactive, non-HLA antibody, an incorrect assumption would be made about that individual’s chances of finding a compatible donor. Lastly, most of the antibody identification assays using lymphocyte panels were limited to T cells and therefore, could only detect antibodies detected to class I HLA antigens.

In 1983, Garavoy et. al. took advantage of a relatively new technology (flow cytometry) in clinical laboratory practice and observed that a flow cytometry-based crossmatch (FCXM) could detect levels of HLA antibodies on lymphocytes undetectable by other techniques [8]. While clearly more sensitive than other crossmatch assays, since the FCXM was a cell-based assay, positive T cell and/or (especially) B cell FCXMs lacked antigen specificity. That some positive crossmatches were due to HLA and others to non HLA antibodies contributed to a conflicted and confused literature. For example, when donor:recipient pairs transplanted after a negative CDC or AHG-CDC crossmatch were re-evaluated based on whether their flow cytometric crossmatches
were positive or negative, some centers reported significant differences in episodes of rejection/graft loss, while other centers reported no differences between the two groups [13, 39-42]. The underlying assumptions were that a positive T lymphocyte FCXM was due to HLA class I antibodies while a positive B cell FCXM (with a negative T cell FCXM) was considered the result of class II directed HLA antibodies. It is important to recognize that numerous other antigens are also expressed T and B lymphocytes. Thus, positive crossmatches could result from: 1) antibodies to self-antigens on recipient lymphocytes which bind to the same epitope on donor lymphocytes; 2) Fc receptor binding of immunoglobulin independent of antigen specificity, and 3) binding of antibodies to lymphocyte antigens other than those encoded by the major histocompatibility complex [43-45]. In any of the above circumstances, a positive crossmatch is not considered a contraindication to transplantation [3].

In the current era, new tools have become available that provide a rationale to help interpret these exquisitely sensitive cellular assays. In today’s HLA laboratory, methods to identify, reduce and eliminate non-HLA antibodies in FCXM assays are routinely applied. Such approaches include ultracentrifugation of patient serum to eliminate immune complexes that non-specifically bind to donor lymphocytes and treatment of donor cells with pronase, a proteolytic enzyme that cleaves Fc receptors from the cell surface [44]. Elimination (or at least reduction) of surface Fc receptors significantly decreases non-specific binding of immunoglobulin and reduces the rate of false positive B cell crossmatches (Figures 1A and 1B). However, the most significant breakthrough was the development of assays using solid phase matrices coated with purified HLA class I or class II antigens. These new technologies have completely transformed the histocompatibility laboratory’s approach and ability to detect HLA antibodies. In these assays, HLA class I (or class II) antigens are isolated from transformed or transfected cell lines (either as
an entire cluster or single allele, respectively) and attached to solid phase matrices such as a plastic plate or inert microparticles [11, 12].

**Confounding issues: Pre-Transplant conundrums/challenges**

Recent evidence revealed that patients awaiting allograft transplantation can produce antibodies not only to HLA antigens but to specific alleles of HLA antigens [14, 46]. Our first experience with allele-specific antibodies occurred in a patient who displayed antibodies to HLA-A*68. Importantly, this patient expressed the HLA-A*68 antigen. Was this an example of HLA autoantibodies? Further investigation using high resolution histocompatibility typing revealed that the patient possessed HLA-A*6802, an allele of HLA-A*68, while her husband and son (with whom she was crossmatch positive) both possessed the HLA-A*6801 allele. Assessment of the protein sequences of A*6801 and A*6802 revealed that these two alleles differed by five amino acids. More recently, we identified a patient who was homozygous for HLA-A*02 who was found to have antibodies only to HLA-A*25,26,34,43 and 66 (the so called 10 CREG antigens) using a flow based detection assay. Interestingly, this patient also had an antibody to HLA-A*0203 when the serum was tested on an extended bead panel using Luminex technology. High resolution typing of the patient documented the presence of HLA-A*0201. Assessment of protein sequences revealed that HLA-A*0203 shares an identical sequence common to HLA-A*25, 26,34,43 and 66 which is not expressed on A*0201 or A*0205. Moreover, this potential epitope occurs on an antibody accessible part of the HLA molecule (alpha-helix). This particular case clearly indicates that epitopes can be shared among HLA antigens previously not considered to be in the same CREG. More importantly, the antibody to HLA-A*0203 we detected was “strong” and would be considered an unacceptable antigen. However, since this antibody was not
identified with what was then our routine panel for antibody identification, we would certainly have accepted any donor that did not express the A10 CREG antigens, especially donors that were HLA-A*02 (since our patient was homozygous for that antigen), including donors who expressed HLA-*0203. A positive crossmatch with such a donor would most likely be attributed to non-HLA antibodies. These data underscore the need to be vigilant for allele-specific antibodies (at the very least alleles that are common in the donor population) and support the concept that high resolution typing of donors (again, for the common alleles) is warranted in solid organ transplantation. In addition to antibodies against HLA-A*6801 and A*0203, several other allele specific antibodies have been identified by our laboratory (Table I). The implications of allele specific antibodies for organ allocation are significant. Currently, in the United States, patients with antibodies to HLA-B*4402 but not HLA-B*4403 are not distinguished from patients who have antibodies to both alleles. This is because data entry of “unacceptable/avoid” antigens into the United Network for Organ Sharing (UNOS) database is typically limited to antigens, not alleles. As a result, a patient listed as having antibodies to HLA-B44 but who only has antibodies to HLA-B*4403 will not be offered kidneys from any HLA-B44 positive donor, some of whom they would be predicted to have a negative crossmatch. The only option available for these patients is not to consider HLA-B44 as “unacceptable” and crossmatch with them with all HLA-B44 positive donors. Based on the frequencies of HLA-B*4402 and 4403, positive crossmatches would be expected approximately half of the time. Neither of the above approaches is acceptable. A better solution would be to allow for the entry of allele-specific antibodies in the UNOS matching system. This would necessitate high resolution typing of all donors (at least for those antigens to which allele specific antibodies have been identified), not for the purpose of HLA matching but rather, for identification of acceptable mismatches.
Instead of waiting (perhaps too long) for a crossmatch compatible donor, many centers will apply desensitization strategies to remove/inhibit HLA antibodies [30, 31]. The most successful of these approaches treats patients with either plasmapheresis and a low dose (100mg/kg) of intravenous immunoglobulin (IVIG) or a high dose (2gm/kg) without plasmapheresis. For either approach, the goal is to convert donor reactive crossmatches from positive to negative or, at the very least, from strongly positive to weakly positive. There are two underlying premises here namely: 1) by reducing the strength of the antibody the transplant can be performed without fear of hyperacute rejection; and 2) desensitized patients who develop antibody-mediated rejection post-transplant can be rescued with appropriate therapy.

In monitoring for effective desensitization, it is important to understand the limitations of in vitro assays. For example, while low dose IVIG does not interfere with the detection of HLA antibodies on lymphocytes or microparticles [47], however, the same cannot be said for high dose IVIG [48]. An example of how high dose IVIG can interfere with the detection of HLA antibodies begins in Table II. Here, it appears as though the addition of high dose IVIG to a patient’s serum converted a flow cytometric T and B cell positive crossmatch with his donor to negative. These data suggest it would be reasonable for this donor:recipient combination to proceed to transplant. However, upon closer examination (Figures 2A, 2B), additional information changes the interpretation. Specifically, when comparing flow cytometric crossmatch results performed with normal human serum (NHS; contains no HLA antibodies) “spiked” with IVIG, it became clear that the background binding had increased substantially above that of NHS without IVIG. Thus, quantifiable differences (e.g., channel shifts, MESF values) between the control and patient specimens will not be reliable. Obviously, for the example shown here, it is inappropriate to interpret the crossmatch between donor lymphocytes
and recipient serum containing IVIG as negative. A more accurate approach is a recently described assay wherein donor cells are treated with recipient serum + IVIG followed by monitoring C3b deposition on the donor cells as a means to determine whether IVIG treatment will be beneficial to a particular donor:recipient pair [49].

Confounding issues: Post-Transplant conundrums/challenges

While a large number of patients have been successfully desensitized and transplanted at centers around the world, many of them experience acute antibody-mediated rejection and/or transplant glomerulopathy [32, 50-52]. As a result, the use of Thymoglobulin® as an induction and/or rescue agent has steadily increased [53]. Thymoglobulin® is a polyclonal rabbit anti-human reagent which possesses at least 23 different antibody specificities, including antibodies to human HLA class I and class II antigens as well as antibodies against β2-microglobulin [54]. Hence, when monitoring post-transplant for the presence of HLA antibodies it is critical to know that Thymoglobulin® has been administered. Since Thymoglobulin® contains antibodies against both Class I and Class II antigens, Thymoglobulin® can directly interfere with the detection of human HLA antibodies (Figure 3). We recently demonstrated that the presence of a therapeutic dose (100 ng/ml) of Thymoglobulin® in a patient’s serum with known HLA alloantibodies masked the detection of HLA antibody even when using appropriate secondary reagents [55]. In order to be certain that Thymoglobulin® will not interfere with HLA alloantibody detection, Thymoglobulin® must first be removed from the sample prior to testing. One method involves the use of goat-anti-rabbit immunoglobulin coated beads [56]. For the sake of completeness, the adsorbed serum should then be tested to determine if any rabbit immunoglobulin (i.e., Thymoglobulin®) remains post adsorption. Only by completing this rather tedious process is it
feasible to reliably determine whether low levels of HLA antibody are present in sera obtained from patients receiving Thymoglobulin®. It is important to follow these protocols because of the clinical implications. Specifically, consider patients already receiving Thymoglobulin® who present with symptoms of acute rejection: If they are monitored for HLA antibodies without Thymoglobulin® removal, they would be considered to be antibody negative and the therapeutic strategies to treat these patients may not include antibody depletion.

Another immunosuppressive agent, Rituxan, a chimeric monoclonal antibody directed against CD20, also presents a challenge to the detection of clinically relevant HLA antibodies [57], specifically when performing B cell crossmatches. In the presence of Rituxan B cell crossmatches will always appear positive since Rituxan present in recipient serum will bind to donor cells expressing CD20. Recent studies have demonstrated that rather than removing the antibody, CD20 can be removed from the B cell surface via proteolytic cleavage with pronase [58]. Thus, under the appropriate conditions (and with the appropriate controls), positive B cell crossmatches can be interpreted even in the presence of Rituxan. Other therapeutic monoclonal antibodies, such as Campath (anti-CD52), cannot be adsorbed from patient sera. In these circumstances, a crossmatch will always appear positive, although positive SPADS, including a recently developed immunosorbent crossmatch assay [59] will not be affected. It is critical that the laboratory be informed when patients have received any monoclonal or polyclonal antibody treatments so that the appropriate procedures can be implemented and crossmatches are not misinterpreted.

Conclusions:
Over the past forty years, many advances have taken place in the detection and identification of HLA antibodies. The most notable of these has been the introduction of sensitive and specific SPADS. The more exacting identification of HLA alloantibodies has significantly altered the manner in which the histocompatibility community interprets positive crossmatches between potential allograft recipients and their donors. In a similar manner, significant advances in immunosuppression and antibody modulation have created opportunities to transplant highly sensitized patients. For many transplant programs, the presence of pretransplant, donor-directed alloantibody does not necessarily portend the same contraindication to transplantation as it once did. Rather, the presence of donor-directed alloantibody represents one risk factor among many that must be considered. At present, how to assess the degree of risk is incompletely defined. For example, while it is certainly conceivable that some donor-directed antibodies are more detrimental to patient outcome than other antibodies, this distinction is not clearly understood. Similarly, while target antigens such as MHC class I and/or class II may be differentially expressed on an allograft, how antigen expression affects outcomes in a sensitized patient is unknown. The relative strength (titer) of class I/class II HLA antibodies is also likely to play a role in graft outcome but how, when and for who must still be determined. One fact remains unambiguous. The human immune response is protective and its major function is to generate effectors (humoral and cellular) to eliminate the target(s) that provoked its response. With that in mind, the detection of donor-directed HLA antibody in an allograft recipient, regardless of the quantitative level should be viewed as an indication that the immune system has been primed to allogeneic MHC antigens. At the very least, recipients who present with such antibodies should be considered to be a different risk level than subjects devoid of HLA antibodies.
Bibliography


Legends

Figure 1A. At least three different types of glycoproteins are expressed on the surface of B cells, namely, MHC class I antigens, MHC class II antigens (which can bind to MHC class II antibodies) and Fc receptors. Low titer antibodies class I antibodies can bind to B cells and not T cells based on B cells expressing more class I than T cells. Antibodies to class II will bind to B cells and not T cells (which, under normal circumstances, do not express class II). Fc receptors (which have a significantly higher expression on B cells than T cells) can bind the Fc portion of antibodies independent of their antigen specificity. Thus, in a flow cytometric crossmatch, B cells will have a higher background than T cells.

Figure 1B. Reduction/elimination of Fc receptors from lymphocytes prior to performing a crossmatch. Cells are treated with pronase, a proteolytic enzyme that will digest Fc receptors without effecting the expression of class I or class II antigens (44). Pronase also digests CD20 (a target of Rituxan) from the surface of B cells (58).

Figure 2: Highlighted portion reveals a shared amino acid sequence (epitope) between HLA-A* 0203, 2501, 2601, 3401, 4301 and 6601. Note that HLA-A*0201 and HLA-A*0206 do not possess this sequence.

Figure 3A: Flow cytometric T (top) and B cell (bottom) crossmatches in the presence of normal human serum (NHS) or patient serum diluted 1:1 with carrier. The numbers in the upper right hand quadrant of each histogram represent the median channel value (MCF) of the cells on a 1024 scale. The difference (Δ) between the NHS and patient serum is 196 channels for T cells and 128 channels for B cells. A crossmatch is considered positive when the Δ MCF is >60 for T cells and >100 for B cells.

Figure 3B: Flow cytometric T (top) and B cell (bottom) crossmatches in the presence of normal human serum (NHS) or patient serum diluted 1:1 with IVIG. The Δ MCF between the NHS and patient serum is 43 for T cells and 55 for B cells, suggesting the crossmatch became negative. Note, however, that the MCF of T cells and B cells in NHS diluted in carrier vs NHS diluted in IVIG are significantly different (T cells: 258 vs 441; B cells: 279 vs 525). With such a high background, the crossmatches (T and B) cannot be interpreted.

Figure 4: The presence of Thymoglobulin® can interfere with the detection of low levels of HLA antibodies. As shown, a high concentration of Thymoglobulin® can bind to surface HLA antigens and effectively block human HLA alloantibodies from binding to the same targets. The FITC conjugated rabbit-anti-human Ig would have nothing to bind, as all human alloantibodies would be in solution, not cell bound. The result would be interpreted as negative.
Table I

HLA CLASS I and CLASS II ALLELE SPECIFIC ANTIBODIES

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### Table II

#### FCXM pre and post-IVIG

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**INTERPRETATION:**

IVIG converted the FCXM from Positive to Negative
Explanations of B cell positive/T cell negative crossmatches

**A**

- **MHC II** - Anti-Class II HLA antibody binds to B cells
- **MHC I**
  - Low titer anti-Class I HLA antibody binds B cells, not T cells
  - *FcγR*
  - Auto-antibody, I-C or IgG aggregates can non-specifically bind to FcγR

**B**

- **MHC I**
- **MHC II**
- **FcγR**
- **Pronase**
  - Pronase removes Fc receptors (not HLA antigens)
  - Lowers background
  - Increases sensitivity and specificity
Figure 2
Figure 3A

NHS+CARRIER

T Cells

Patient serum + CARRIER

T Cells

B Cells

B Cells

Counts

Counts

Counts

Counts

ANTI-IgG FITC

ANTI-IgG FITC

ANTI-IgG FITC

ANTI-IgG FITC

258

454

279

432
NHS + IVIG

PT SERUM + IVIG

T Cells

B Cells

Figure 3B

Counts

ANTI-IGG FITC

Counts

ANTI-IGG FITC

Counts

ANTI-IGG FITC

Counts

ANTI-IGG FITC

M1

M1

M1

M1

441

484

525

555
Where Thymoglobulin Interferes