

The problem of transplanting the sensitized patient: Whose problem is it?

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TABLE OF CONTENTS

1. Abstract
2. The patient's problem
3. The physician's problem
4. The laboratory's problem
5. Conclusion
6. Acknowledgements
7. References

1. ABSTRACT

Transplantation is the treatment of choice for end-stage renal failure and a life-saving treatment for failure of other major organs. Improvements in surgical techniques, histocompatibility testing, and immunosuppressive drugs have significantly improved both patient and graft survival (1-4). However, there are formidable barriers to the successful transplantation of patients who possess HLA-specific antibodies. Sensitized patients wait longer for a transplant and, once transplanted, experience more rejection episodes and have decreased graft survival compared to non-sensitized recipients (5-8). Improvements in HLA-specific antibody detection have expanded the donor pool available to sensitized patients (9-14) and desensitization protocols designed to reduce the breadth and amount of HLA-specific antibody have found increased success during the last decade (15-20). Determining the appropriate course of treatment for the sensitized patient requires accurate immunologic characterization and clinical assessment of the patient. Together, the transplant physician and histocompatibility expert must determine what constitutes a compatible donor and assess the patient's risk for a particular transplant. Thus, sensitization to HLA antigens is a problem for the patient, the transplant physician, and the histocompatibility laboratory.

2. THE PATIENT'S PROBLEM

There are over 95,000 patients awaiting transplantation currently in the United States (US) (8). The majority (75%) of these patients are waiting for kidneys and face a median wait-time of 3 years. The renal waiting list is predicted to increase 20% per year with no corresponding increase in donor organs (8,21). This situation is not unique to the US as the disparity between the number of patients needing a transplant and the number of available donors exists worldwide. Transplantation is life saving for patients with heart, lung, or liver failure, but transplant candidates on the wait-list suffer high mortality rates, ranging from 7% in kidney candidates to 15% for those waiting for a heart transplant (8). Further, long-term dialysis for patients waiting for compatible renal organs is associated with serious health problems that impact long-term graft survival following transplantation (4).

Due to the acute shortage of organs from deceased donors, the number of kidneys transplanted from living donors increased dramatically during the 1990s (8). In 2006, of the 15,751 kidney transplants performed in the US, over one-third used living donor organs. Access to a living donor circumvents the long wait-time for deceased donor organs and the health problems associated with long-term hemodialysis. Furthermore, the graft survival rates for

Transplanting the sensitized patient

living donor kidney transplants is significantly higher than deceased donor kidneys when measured at one year (95% versus 89%) and five years post-transplant (79% versus 66%). However, in many cases blood group (ABO) and histocompatibility (HLA) incompatibilities between the potential donor and recipient preclude the use of a living donor (20). Given the increase in donation from unrelated living donors, the 36% probability of ABO incompatibility between unrelated recipient-donor pairs could substantially impact live donor transplantation (8,22). Therefore, despite the availability of a living donor some patients are forced to wait for a deceased donor organ.

Humoral sensitization to HLA alloantigens, which can occur following exposure to HLA antigens via transfusion, pregnancy, or transplantation, presents a significant obstacle to transplantation (8,20,23-25). The extent and duration of sensitization from transfusion or pregnancy correlates with both the number and timing of those events while sensitization from transplantation appears to be the longest lasting (26-28). Approximately 25% of patients on the US renal waiting list are sensitized, with higher rates of sensitization found in previously transplanted patients, females, and African-Americans (8,26,29-30). On average, sensitized patients wait twice as long for a renal transplant as do non-sensitized patients with the impact on waiting time increasing as the breadth of sensitization increases (8). Renal candidates with a prior transplant are five-times more likely to be sensitized to HLA antigens compared to candidates who have not been previously transplanted (8). The increase in HLA sensitization with each subsequent transplant poses a significant problem for pediatric kidney transplant recipients who will likely receive multiple transplants over the span of their life (31). The introduction of recombinant human erythropoietin in 1989 reduced the number of blood transfusions received by dialysis patients, resulting in a significant drop in patient sensitization rates and mean transplant waiting times (30,32). Unfortunately, the need for transfusions has not been eliminated completely and patients with chronic renal failure still suffer higher sensitization rates compared to other solid organ candidates (8,30). However, sensitization rates for candidates of other solid organ transplants may be on the increase. Recent data show an increase in sensitized heart candidates due to transfusions associated with left ventricular assist device (LVAD) surgery, allogeneic valve transplants, and an increase in the number of patients receiving second transplants (24,33). Another factor contributing to an "apparent" increase in sensitization among all transplant candidates has been the development and expanded use of more sensitive methods for the detection of HLA-specific antibodies (34-36).

African Americans comprise 12% of the US population yet represent 35% of the candidates awaiting kidney transplantation due to the higher rates of renal failure in this population (8,29). Sensitization rates for African Americans are also higher than those of other races, resulting in a longer median wait-time on the kidney transplant list (8). Reasons for this increased sensitization may include a genetic propensity for a stronger immune

responder status in addition to an increased likelihood of exposure to disparate HLA antigens from blood transfusions from a primarily Caucasian donor pool (29,37). Exposure to paternal HLA antigens during pregnancy places women at a higher risk for sensitization. Women on the kidney wait-list are three times more likely to develop HLA-specific antibodies compared to male candidates (8). Parous women are also more likely to develop HLA-specific antibodies following blood transfusions (30). Interestingly, there may be other routes of HLA sensitization in addition to those traditionally recognized, *ie*, transfusion, pregnancy, and transplantation. There have been reports of non-transfused, non-transplanted males who expressed HLA-specific antibodies. The possibility of sensitization by environmental agents is supported by known sequence homology between HLA antigens and microbial agents, although the ability of these agents to induce a primary HLA-specific antibody response has not been proven (38-41). Also, inflammation resulting from infection or trauma may result in reactivation of a senescent response or expansion of ongoing antibody production (42 and J.E. Locke unpublished data).

In addition to reduced access to transplantation, the sensitized patient who receives a transplant has an increased risk of rejection episodes and reduced graft survival compared to the non-sensitized patient (8,17,24). Antibodies to donor HLA have been long established as detrimental to renal transplants (43-45). Correlations between donor HLA-specific antibody (DSA) and reduced graft survival and demonstrations of the deleterious effect of such antibody on vascular endothelium continue to be reported in transplants of other organs (1,46-50). Thus, the sensitized patient will require more extensive pre-transplant evaluation and post-transplant follow-up to reduce the risk of rejection and graft loss.

3. THE PHYSICIAN'S PROBLEM

The transplant physician is confronted with several problems posed by the sensitized patient: the patient's deteriorating health while awaiting transplantation, assessing the immunologic risk of a particular transplant, and either finding a compatible donor or decreasing the patient's level of sensitization. The health care of the patient awaiting transplantation is a broad subject not appropriate for this chapter and will not be discussed here.

Assessment of the immunologic risk for transplantation involves determining if HLA-specific antibody is present and, if so, defining the antibody specificities which, in turn, define incompatible donor antigens. Antibodies are assessed in two ways: tests against a panel of HLA phenotypes or antigens to determine antibody specificity and crossmatch tests with donor cells to confirm donor specificity and assess DSA strength. Historically, the complement-mediated or complement-dependent cytotoxicity (CDC) crossmatch assay had been the sole tool to assess the patient's risk and determine if transplantation was a viable treatment option. The significance of lymphocytotoxic HLA-specific

Transplanting the sensitized patient

antibodies in transplantation was revealed in 1969 by Patel and Terasaki, who demonstrated that 80% of crossmatch positive renal transplants resulted in hyperacute rejection (51). Following this study, the CDC crossmatch became the “gate-keeper” in renal transplantation, nearly eliminating the incidence of hyperacute rejection. Crossmatches performed against cells from a panel of individuals selected to represent all available HLA antigens, referred to as “antibody screening” was used to characterize the specificity of antibodies present and obtain a rough assessment of the extent or breadth of sensitization. The level of sensitization is expressed as a percent PRA (panel of reactive antibodies) and is determined by the number of positive reactions within the panel. Generally, patients regarded as highly sensitized have PRAs of greater than 80%, these patients possess a broad range of HLA antibodies directed toward many HLA specificities. However, the CDC assay has several technical and logistical problems. The test requires a sufficient number of viable lymphocytes, is of relatively low sensitivity, and, most importantly, is not specific for antibodies against HLA antigens. Improvements in cell isolation methods have improved the ability to isolate adequate numbers of T and B lymphocytes however, even with augmentation with an antiglobulin (AHG) reagent, the test remains fairly insensitive (52). Also, therapeutic anti-lymphocyte antibodies, auto-antibodies, and various other non-HLA-specific antibodies will yield positive reactions in the assay, particularly with B cell targets. Therefore, using the CDC crossmatch alone to determine the level of risk is not always straightforward. Crossmatch interpretation requires information from sensitive antibody screening tests and the patient’s sensitization history should also be considered in assessing the immunologic risk associated with a particular transplant.

A positive T cell CDC (or AHG-CDC) crossmatch is usually considered a strong contraindication to renal transplantation. However, the importance of a B cell crossmatch has been controversial (7,53-55). Antibodies specific for HLA class I antigens (HLA-A, B, C), which are expressed on all nucleated cells including T and B lymphocytes, can be detected in T cell crossmatches. HLA class II antigens (HLA-DR, DQ, DP) are constitutively expressed on only a subset of cells including B but not T lymphocytes. Therefore, a B cell crossmatch may detect antibodies specific for HLA class I or class II antigens. Interpretation of any crossmatch can be confounded by the presence of auto-antibodies (primarily of the IgM class) or non-HLA antibodies which may not be deleterious to the allograft. However, B cells seem to be particularly susceptible to lysis by non-HLA-specific antibodies (55). As a result, the credibility and utility of B cell crossmatches has suffered. Improved techniques have reduced the complexity of interpreting B cell crossmatches, these include the reduction of IgM antibodies using heat or dithiothreitol, the inclusion of autologous B cell crossmatches, and most importantly, the use of solid-phase antibody screening techniques to identify the presence of antibodies specific for HLA class II antigens (10,52). Furthermore, reports of hyperacute or accelerated rejection in HLA-DR incompatible renal transplants cannot be

ignored (56-59). More recently, the predictive power of a B cell crossmatch was re-evaluated within a multicenter cohort of 9031 kidney recipients transplanted between 1994 and 1995 and for which both a T cell and B cell crossmatch was performed (54). This study found that there was a small but significant reduction in the one-year graft survival in patients transplanted with a negative T cell, positive B cell crossmatch (82.4%) as compared to patients negative for both T and B cell crossmatches (86.7 %). This reduction in graft survival was found to be more pronounced in recipients of regrafts (74.6% versus 83.5%). Interestingly, while this study shows the significance of antibodies detected in B cell crossmatches, particularly in regrafts, the target antigens (HLA class I, class II, non-HLA) that contributed to this reduced graft survival were not delineated. To better characterize the antibodies responsible for B cell crossmatch positivity, Le Bas-Bernardet *et al* performed a detailed analysis of sera from 62 kidney recipients transplanted with a negative T, positive B cell CDC crossmatch (55). HLA class II-specific antibody was found in only 23% (14/62) of the patients. Of these 14 patients, one suffered a hyperacute rejection and 2 more lost their allograft in the first 3 months post-transplant. However, in a majority (77%, 48/62) of the patients the positive B cell CDC crossmatch was due to antibodies of unknown specificity or auto-antibodies but not HLA-specific antibodies. In these 48 patients, graft survival was similar to those with negative B cell crossmatches. Most recently, the relevance of a positive B cell crossmatch when substantiated by the presence of HLA class II antibodies specific for donor antigens was investigated. Antibody mediated rejection (AMR) occurred in 86% (6/7) of kidney recipients transplanted with positive B cell crossmatches and class II DSA compared to 40% (2/5) patients that tested negative for both (60). Therefore, while positive B cell crossmatches can result from a variety of antibodies, many of which are irrelevant to allograft survival, positivity due to HLA class II specific antibodies is highly relevant to transplant outcome.

In the 1980s, flow cytometric crossmatches were introduced, providing a very sensitive method for detecting the presence of donor-reactive antibody (61-63). In addition to increased sensitivity, this method has several advantages over CDC including the ability to determine multiple parameters, such as cell type and immunoglobulin class and the lack of a requirement for a second phase reaction (cell death). Unfortunately, this method does not increase but, rather, may reduce specificity by detecting additional irrelevant antibodies not detected in the CDC assay. Thus, false positive results due to auto-antibodies, antibodies specific for non-HLA antigens, or non-specific binding of the fluorescent conjugate to surface Fc receptors can occur (35). Furthermore, determining what level of fluorescence represents a positive reaction is also somewhat subjective and varies between laboratories. Of great debate is the clinical relevance of the very low levels of antibodies that the flow cytometric crossmatch detects (64-67). Thus, although improvements in prospective donor-recipient crossmatches have played an important role in reducing the incidence of hyperacute rejection and early graft loss, the dramatic increase in sensitivity may also

Transplanting the sensitized patient

prevent safe transplants from occurring. This is serious problem for the sensitized patient who may miss the opportunity to be transplanted with a compatible donor. Therefore, each center must develop interpretation strategies for determining recipient-donor compatibility and these should include analysis of HLA antibody screening data to reduce false positive flow cytometric crossmatch interpretations.

In contrast to kidney transplantation, the utility of a prospective recipient-donor crossmatch in other solid organ transplants has been less clear (68-71). In heart and lung transplant recipients, patients have a lower incidence of HLA sensitization, therefore the increased ischemia time imposed to perform prospective recipient-donor crossmatches may be deemed unjustified (48,72). Nevertheless, in heart transplant recipients the presence of HLA-specific antibodies is a risk factor for rejection which is in turn associated with increased mortality (2,6,9,24,72-75). Similarly, the loss of lung allografts to chronic rejection as defined by the development of bronchiolitis obliterans syndrome (BOS) was found to be significantly higher in sensitized patients than in nonsensitized patients (9,23,48). The significance of a positive lymphocyte crossmatch may also be dependent on organ-specific differences allowing some organs to be more resistance to damage from HLA-specific antibodies. In liver transplantation, hyperacute rejections are rare and published reports show no significant correlation between a positive CDC crossmatch and reduced graft survival (76-77). Reasons for these observations may include low level HLA antigen expression on liver allografts or a large production of soluble HLA antigen that complexes with antibody in the circulation (70). In spite of this resistance to hyperacute rejection, there have been reported differences in the clinical courses of liver transplants in patients with positive crossmatches. Analysis of 1520 liver transplants from a single center revealed no effect of CDC crossmatch results on long-term graft survival (76). However, recipients transplanted with a positive crossmatch had increased early graft failure rates. Opelz and colleagues described a similar correlation between early graft loss and a positive flow cytometric T cell crossmatch (78). However, this study also found the flow cytometric crossmatch to be an important predictor of acute and chronic rejection in liver transplant recipients. Therefore, due to improvements in crossmatch and antibody screening techniques the importance of prospective crossmatches in all solid organ transplants appears to be increasing (6,7,25,79-81).

Perhaps the most important advancement facilitating transplantation of the sensitized patient has been the ability to accurately characterize HLA-antibodies (9-10,24). There are two types of solid-phase immunoassays for HLA-specific antibody screening which are differentiated by the platform on which they are performed: an enzyme-linked immunosorbent (ELISA) based system and a flow cytometric system using color-coded beads (10,34-35,82). Both techniques use soluble HLA antigens that are captured onto a solid-phase consisting of microtiter plates or glass slides for the ELISA assay and beads for the

flow cytometric assay. These assays abrogate many of the problems found in cell-based antibody screening by identifying only HLA-specific antibodies (including non-complement-fixing antibodies), increasing the speed of testing from days to hours, and eliminating the need for viable cells thereby allowing screening in the presence of lymphotoxic therapeutics. Most importantly, characterizing the level and specificity of HLA antibodies allows for a more accurate interpretation of crossmatch results (83-85). As mentioned earlier, these methods have lead to a better characterization of HLA antibodies directed against class II antigens which have previously been obscured by difficulties in B cell crossmatch interpretations (49,55,72).

New techniques have allowed for greater specificity in assigning HLA-specific antibody reactivity but have also led to an increase in the number of patients identified as sensitized (10,34,86). This poses a "double-edged sword" for the sensitized patient; the increased sensitivity in detecting antibody may deter some viable transplants from taking place while increased characterization of antibody may facilitate identifying a compatible donor. Determination of the specificity of HLA antibodies, even in patients with broad HLA reactivity, allows laboratories to perform "virtual crossmatches" to predict compatibility with potential donors, allowing more efficient and broader searches for organ donors (9,11-14). In 1985, Eurotransplant began listing "acceptable mismatches" in patients with PRA>85%, these represent HLA antigen mismatches for which the patient did not have circulating alloantibody (87). These "acceptable mismatches" were determined by analyzing the negative cytotoxicity reactions within an extensive PRA cell panels containing cells with well characterized HLA phenotypes. This effort dramatically increased the transplantation of highly sensitized patients, but also proved very labor intensive.

In the US, donor selection criteria for highly sensitized patients are based on determining "unacceptable mismatches" depending on the HLA-specific antibodies of a particular patient. Characterization of the specificity and amount of HLA-specific antibodies, using solid-phase based antibody screening, allows laboratories to generate a list of unacceptable HLA antigens that would likely produce a positive crossmatch. The increased specificity of solid-phase assays has greatly improved the ability to predict negative crossmatches when compared to what was previously achieved using CDC antibody screening methods to define unacceptable antigens or using % PRA. Molecular information regarding the polymorphic amino acid residues that create both private epitopes (specific for a single HLA antigen) or public epitopes (shared between HLA antigens) on HLA antigens have also been utilized to determine unacceptable mismatches (11,82,88-90). Evidence shows that the majority of HLA-specific antibodies are directed against public epitopes not private epitopes contained on foreign HLA antigens (45,91-92). Importantly, patients are less likely to generate HLA-specific antibodies against foreign HLA antigens that share epitopes with their own HLA phenotype (92). Therefore, by taking advantage of epitopes shared between the patient's

HLA phenotype and that of the potential donor and avoiding those shared between the HLA antigens to which the patient has antibodies and the potential donor, laboratories have increased their ability to predict negative recipient-donor crossmatches in sensitized patients. Increasing the number of negative crossmatches for highly sensitized patients could, in turn, impact donor allocation and provide the opportunity to utilize donors from a broader geographic region (9). These new tools have greatly improved the sensitized patient's access to transplantation.

Recent improvements in the ability to quantify HLA-specific antibodies and accurately determine specificity has facilitated the increased use of two new avenues for transplanting sensitized patients: desensitization and paired donor exchanges (18,20). In cases where compatible donors cannot be found for sensitized patients, desensitization protocols have been developed to eliminate or substantially reduce alloantibody thereby increasing access to transplantation. Two approaches have been proven to be efficacious; the use of high-dose, pooled, intravenous pooled human immunoglobulin (IVIg) (18,93) or low-dose anti-CMV hyperimmune IVIg (CMVIg) paired with plasmapheresis (15,94). The proposed mechanisms for the immunosuppressive effect of IVIg are many and involve multiple components of the immune system (20,95-98). IVIg has been shown to effect the activation, differentiation, and effector functions of macrophages, dendritic cells, and T and B lymphocytes. Neutralization of alloantibodies has been proposed to occur through the presence of anti-idiotypic antibodies or soluble HLA antigens. The inhibition of complement activation, the up-regulation of inhibitory Fc receptors, and the down regulation of activating Fc receptors is believed to be modulated by the Fc portion of immunoglobulin proteins contained within IVIg. Together these effects are dramatic in reducing inflammation and restoring homeostasis of the immune system within sensitized transplant candidates and patients with autoimmune disease.

Preemptive high-dose IVIg (2g/kg body mass) protocols have been shown to reduce allosensitization, reduce acute rejection episodes, and improve graft survival in sensitized renal and heart allograft recipients (18,99). A randomized, multicenter, double-blinded clinical trial was performed between 1997 and 2000 to examine the efficacy of high-dose IVIg in lowering allosensitization and improving transplantation rates in highly sensitized renal transplant candidates (100). One hundred and ten patients with PRAs of >50% were randomized to receive 4 monthly treatments of IVIg (at a dose of 2g/kg) or placebo. High-dose IVIg proved significantly better at reducing HLA-specific antibodies, resulting in a transplant rate of 35% (16 patients) in the IVIg group compared to 17% (8 patients) in the placebo group. However, graft survival at 2 years post-transplant was similar in both groups; with an 80% graft survival in the IVIg group and 75% in the placebo group. Glotz *et al* reported a higher transplantation rate (87%, 13/15) using 3 monthly courses of high-dose IVIg (93). A similar transplantation rate has also been reported by

Jordan *et al* using 4 monthly treatments of high-dose IVIg protocol (18,101). Reduction of HLA antibodies to achieve negative CDC crossmatches occurred in 83% (67/77) of these patients, resulting in the transplantation of 42 living and 25 deceased kidney allografts. The incidence of allograft rejection in these patients was 28%, with a 3 year graft survival of 87% and 3 year post-transplant mean creatinine value of 1.4 mg/dl. IVIg treatment has also been successful in reducing HLA-specific antibodies in heart candidates sensitized following LVAD surgery, resulting in a significant reduction in the wait-time to cardiac transplantation (99,102). Therefore, the use of high-dose IVIg to reduce alloreactivity in sensitized patients increases both access to transplantation and long-term transplant outcome.

The desensitization protocol employed at the Johns Hopkins Comprehensive Transplant Center utilizes alternate day, single volume plasmapheresis paired with low-dose CMVIg (100mg/kg body mass) and quadruple, sequential immunosuppression (15,20,103). The addition of plasmapheresis is believed to facilitate the removal of alloantibodies as well as inflammatory cytokines and complement components. The use of CMVIg provides some replacement of depleted IgG and also provides the additional advantage of reducing viral reactivation and its associated inflammation. The immunologic risk of each patient is assessed according to immunologic history, as well as the titer of the DSA. The number of pre- and post-transplant treatments is then determined based on this assessment. Using this protocol, Montgomery *et al* has successfully reduced alloreactivity permitting transplantation of more than 80 sensitized kidney transplant patients (15). There have been no hyperacute rejections and graft survival at 1 and 3 years post-transplant are similar to those of non-sensitized patients. The majority of patients achieve negative AHG-CDC crossmatches prior to transplant. However, 9 patients have been transplanted with a positive CDC crossmatch, following preconditioning. DSA was eliminated in these 9 patients post-transplant after continued treatment (15,104). Unexpectedly, a small number of patients, for whom treatment was aborted for medical or logistical reasons, experienced a return of DSA, rebounding to levels higher than those measured prior to treatment. Unlike high-dose IVIg, which has an effect independent of transplant, the Hopkin's protocol appears to require the presence of donor antigen to maintain the elimination or reduced levels of DSA. Therefore, protocols utilizing low-dose CMVIg and plasmapheresis for preemptive use are best applied in transplantation with incompatible live donors.

A direct comparison between the efficacy of a single dose of high-dose IVIg or low-dose IVIg paired with plasmapheresis, anti-CD20 antibody and thymoglobulin therapy has also recently been reported (105). This center found that although high-dose IVIg reduced donor-specific alloantibody in all cases, only 38% (5/13) achieved low enough levels to allow for a negative AHG-CDC crossmatch. In contrast, 85% (41/48) of patients who received low-dose IVIg and plasmapheresis achieved a negative crossmatch and proceeded to transplant.

Transplanting the sensitized patient

Moreover, rejection rates were significantly lower in patients who received plasmapheresis in addition to IVIg as compared to IVIg alone, 29% and 80% respectively.

The implementation of desensitization protocols is not without cost and difficulties for both the patient and the institution. Adverse reactions to IVIg such as headaches, fatigue, and increased blood pressure do exist but are found to affect less than 5% of patients (95). However, the use of IVIg has recently been linked to an increased risk in thrombosis (106). Potential transmission of blood-borne pathogens is also a concern, however, improvements in screening plasma donations for hepatitis C, hepatitis B, West-Nile virus, and HIV have substantially reduced this risk (95). Complications associated with plasmapheresis include an increased risk of infection and depletion of coagulation factors causing an increased risk of bleeding (15,94). Desensitization protocols require extensive support beyond what is normally needed for transplantation. Success requires a highly qualified staff of transplant physicians, nurses, and coordinators. The histocompatibility laboratory will be required to provide data to assess the patient's risk for successful desensitization and will need the expertise and resources for monitoring treatment efficacy. When plasmapheresis is part of the protocol, a hemopheresis expert must be available to oversee the treatments and deal with possible side effects. The program's pharmacy must monitor the availability of IVIg or CMVIg supplies, which are sometimes limiting due to the complexity of production. Finally, all individuals involved in the care of the patient must communicate in a timely and effective manner. While there is a cost, that may be substantial, associated with desensitization treatment of any sort, it should offset the cost of dialysis over the many years a sensitized patient waits for a transplant (15).

Paired kidney exchanges provide a second and newer strategy to finding compatible living organ donors for highly sensitized patients. Using this approach, recipients who are HLA or ABO incompatible with their respective live donors proceed to transplant through an exchange of donor organs, resulting in compatible live organ transplants for each recipient (17,20,107). Montgomery *et al* has reported the early experience at Johns Hopkins, in which 22 patients were transplanted in 10 paired kidney exchanges (107). After a median follow-up period of 13 months, the graft survival rate in these patients is 95.5% and equal to that of recipients transplanted with traditional crossmatch negative live donors. Importantly, these exchanges have allowed for the transplantation of highly sensitized patients, without increased incidence of AMR. However, performing paired kidney exchanges requires the coordination of multiple, simultaneous transplants. It requires careful assessment of each patient's alloantibody and careful evaluation of potential live donors in order to identify an exchange that is advantageous to all recipients. All recipient-donor pairs must be available for transplant on the same day. In addition, there are a number of logistical details such as the availability of operating rooms, transplant personnel, and post-operative care personnel. Nevertheless, this option

could significantly impact the estimated 6000 kidney transplantation patients who have available but incompatible live donors (107-108).

For decades, research has been performed to determine the utility of xenografts (tissues transplanted between different species) to alleviate the shortage of transplant organs (109-110). Pigs have been determined to be the most likely source of xenografts due to the similarities, between pigs and humans, in organ size and physiology (109,111). Moreover, the use of pigs over non-human primates elicits fewer ethical objections, reduces the risk of cross-species disease transmission, and provides a more rapidly replenishable resource. However, the existence of natural antibodies directed toward carbohydrate moieties found in pigs but not in humans has proven to be a formidable hurdle to successful engraftment. The use of pig strains genetically engineered to eliminate the prominent carbohydrate epitope or inhibit complement activation has abrogated hyperacute rejection of pig xenografts in non-human primate models (109-112). This has led to the suggestion that xenotransplantation may provide a solution for highly sensitized patients for whom a compatible human donor cannot be found (112-113). Unfortunately, there have been multiple reports of crossreactivity between HLA antibodies and antigens expressed on pig cells (113). Studies using both flow cytometric and CDC crossmatches show that, following treatment to remove natural antibodies, sera from sensitized patients were more reactive with pig lymphocytes than sera from non-sensitized patients (114-117). These crossmatch studies were further supported by the sequence homology between HLA antigens and swine leukocyte antigens (SLAs), the binding of HLA-specific antibodies to affinity-purified SLA, and the elution of HLA-specific antibodies from pig tissue that had been perfused with sera from sensitized patients (115,118-119). More recently, Wong *et al* have shown that crossmatch strength was reduced when patient sera was tested with pig lymphocytes from an alpha-1,3 galactosyltransferase deficient strain (120). Using this model, crossmatch reactivity did not correlate with sensitivity to HLA and the antibodies responsible were largely of the IgM isotype which are not uniformly considered deleterious in human allografts. Despite these recent breakthroughs, robust innate and adaptive immunological responses toward pig xenografts and complications due to coagulation dysregulation have resulted in poor graft survival statistics in non-human primate models (109-110). Therefore, at present, desensitization protocols offer greater promise for successful transplantation of the highly sensitized patient than does xenotransplantation.

Now armed with highly sensitive techniques that can identify very low-levels of DSA at the time of transplant, histocompatibility laboratories and transplant physicians are faced with new dilemmas regarding risk assessment in transplanting highly sensitized patients.. These advances have generated much debate concerning the relevance of HLA antibodies when detected at levels that are well below those detected with CDC (14,64-67). A large prospective study compared graft survival and

Transplanting the sensitized patient

incidence of rejection in kidney recipients transplanted with positive versus negative T or B cell flow cytometric crossmatches (65). The incidence of rejection at one month post-transplant, particularly steroid resistant rejection, was higher in flow cytometric crossmatch positive patients (26%, 26/100) compared to flow cytometric crossmatch negative patients (12%, 12/100). Yet, the majority of the recipients with positive flow cytometric crossmatch results did not experience a humoral rejection. Furthermore, graft survival rates between these two groups were not found to be different at 1 year post-transplant. A second prospective study analyzed 257 kidney transplant recipients, 31 of whom were transplanted with a positive flow cytometric T cell crossmatch, and found no correlation between flow crossmatch results and incidence of rejection or graft survival at 1 year post-transplant (66). Recipients within the positive flow cytometric crossmatch group did have a higher median PRA value (63% versus 0%) and a higher percentage of regrafts (42% versus 21%) and deceased donors (52% versus 18%). Finally, in a retrospective analysis of 80 consecutive deceased donor kidney transplants, pre-transplant DSA was found in 17 of 80 recipients (21%) using flow cytometric crossmatches and solid-phase antibody screening (67). Six of these 17 recipients suffered an early antibody mediate rejection episode. All rejections were successfully treated with IVIg and plasmapheresis. Thus, the literature contains uncertainty as to the relevance of low level DSA present at the time of transplant.

Perhaps one reason for this uncertainty is that the humoral response toward a transplanted allograft is not static and therefore cannot be evaluated through a single pre-transplant time-point. Instead, alloimmune responses may need to be viewed as dynamic processes, subdued through immunomodulation in some cases or activated during episodes of inflammation in others. Studies that have evaluated the relevance of low-level DSA in recipients at a post-transplant time-points support this hypothesis (5,43,121-127). Christiaans *et al* analyzed 143 kidney recipients and found that 11% (16/143) tested positive in a pre-transplant flow cytometric crossmatch (122). However, when these recipients were retested post-transplant (less than 6 month post-transplant), 13 patients had no detectable alloantibody while 3 patients maintained DSA. While the majority of patients that tested negative in pre-transplant flow cytometric crossmatches remained negative (79%, 113/143), 14 patients converted to a positive result post-transplant. Graft survival was then compared between recipients that maintained or developed detectable levels of DSA post-transplant and patients that tested negative post-transplant. The 5 year graft survival rate for recipients with positive post-transplant flow crossmatch results was 35% compared to 60% in recipients without detectable DSA. Cox regression analysis revealed the presence of post-transplant DSA to be the only risk factor contributing to reduced graft survival.

The development of HLA-specific antibodies post-transplant has also been reported to exert deleterious effects on transplanted lung and cardiac allografts (9,47,49,128-129). To determine the role of HLA-specific

antibodies in the development of chronic rejection, as defined by the development of BOS, post-transplant antibody analysis was performed on 27 lung allograft recipients (129). Lung recipients were tested at 6, 12, 24, 36, and 48 months post-transplant using a solid-phase ELISA based antibody screening assay. HLA-specific antibody was detected in 66% (10/15) of recipients that developed BOS. In contrast, none of the recipients (0/12) that remained rejection-free had detectable HLA-specific antibody. Importantly, detection of HLA-specific antibody occurred at a mean of 20 months prior to the onset of BOS. A similar study performed on 285 cardiac transplant recipients, monitored over a 2 year post-transplant period, showed that 37% (97/285) of recipients developed HLA class I-specific antibodies and 51% (146/285) developed antibodies specific for HLA class II antigens (49). The development of chronic rejection, in the form of coronary artery disease, occurred in 10% of recipients without detectable post-transplant HLA-specific antibodies and in 35% of recipients who did develop HLA-specific antibodies. These studies stress the importance of post-transplant monitoring of HLA-specific antibodies to identify patients at risk for chronic allograft rejection, however, detection of these antibodies post-transplant does not guarantee rejection.

Low levels of DSA may also persist following desensitization protocols (94,104,130). Gloor *et al* has reported the persistence of DSA four months post-transplant in a majority of the 12 patients that have been successfully transplanted following desensitization with low-dose IVIg, plasmapheresis, anti-CD20, and splenectomy (130). Initially, all 12 patients tested AHG-CDC crossmatch positive with their perspective donors, following desensitization all tested AHG-CDC negative and proceeded onto transplant. Four months post-transplant, 50% (6/12) of these patients maintained DSA as detected by flow cytometric crossmatch, and 82% (9/11) remained positive when tested with a flow cytometric solid-phase antibody assay. The persistence of DSA did not correlate with humoral rejection or early graft loss. Similar results were reported by Zachary *et al* for 67 patients treated with plasmapheresis, CMVIG and quadruple immunosuppression (94). Elimination of DSA occurred in only 53% of patients, as determined by a sensitive flow cytometric solid-phase antibody detection assay. Two factors were found to be associated with the predictive persistence of HLA-specific antibody, the strength of antibody at initiation of treatment and the class of antigen to which the antibody had specificity. Difficulty in eliminating high titer DSA may reflect the involvement of a larger number of B cell clones. Elimination of DSA categorized by specificity correlated with the level of expression and distribution of the target antigen. For example, 75% of HLA class I antibodies, 60% of HLA class II antibodies specific for DR and DQ, and 20% of HLA antibodies specific for DR51,52, and/or 53 were eliminated. HLA class I antigens have a much broader tissue distribution than the class II antigens DR and DQ, while DR51,52, and 53 are expressed at much lower levels on the cell surface. The impact of these persistent DSAs on the transplanted allograft was related to their strength.

Transplanting the sensitized patient

Recipients with DSA strong enough to be detected by ELISA based assays had an increased incidence of AMR compared to recipients without persistent DSA (20). While recipients with DSA detected only by flow cytometric solid-phase assays, did not show this increased risk.

Growing experimental and clinical evidence now suggest that some low level DSA may not be detrimental to transplanted allografts, in fact, low levels may even have beneficial effects. The term “accommodation” was first used to describe observations that ABO incompatible allografts could survive and function normally in the presence of low-level ABO specific alloantibodies (131). The basis for accommodation may involve multiple mechanisms, including changes in the host’s immune response as well as changes in the allograft itself (131-132). Qualitative changes in the host’s immune response may include a change in immunoglobulin class or subclass, such that the predominant antibodies are those that are less efficient in activating complement. Changes in the T cell component of an alloimmune response (Th1 versus Th2) may affect the cytokine milieu, thereby reducing inflammation and increasing the production of anti-apoptotic proteins such as Bcl2, BclxL, and heme-oxygenase-1 (132-133). Modifications within the graft may include the loss of antigen expression or alterations in the antigenic epitope (132). Tighter control of complement activation may allow more efficient removal of terminal complement complexes or an inhibition of complement activation through increased expression of inhibitory proteins. Alternatively, changes within the endothelium may allow resistance to complement damage. Gene expression studies suggest that class I DSA at low levels may result in alternative signaling cascades resulting in the induction of survival proteins in endothelial cells (133-135). The ability to detect DSA at very low levels has left us with a predicament of determining what constitutes a deleterious antibody from one that is irrelevant or potentially advantageous following transplantation. Thus, while the sensitization poses many problems for the transplant physician, determining the risk posed by DSA is less clear than ever before.

Although HLA antibodies have proven to be the most deleterious to transplanted allografts, the clinical importance of non-HLA antibodies in allograft rejection is still under investigation (136-137). The rejection of allografts from HLA identical siblings or in the absence of HLA antibodies implicates a role for non-HLA antibodies in some rejections (138-140). Non-HLA antigens that have been associated with graft rejections include: the glomerular basement membrane protein agrin, the protein filament vimentin, phospholipids, and the major histocompatibility complex class I-related chain A (MICA) (141-144). Many of these antigens are expressed on endothelial cells, which serve as a barrier between the recipient’s immune system and the transplanted allograft (80,145). Early studies, performed in the 1980s, identified endothelial cell reactive antibodies in kidney transplant recipients with failed allografts (146-148). In 1997, Sumatran *et al* reported two consecutive hyperacute kidney rejections in a pediatric recipient that had no detectable

HLA-specific antibody (138). Further testing showed that this patient had IgM antibodies reactive with a 97-110 kDa protein expressed on cultured endothelial cells. Antibodies specific for MICA have been eluted from rejected allografts and found to be associated with irreversible rejection, and long-term graft loss (142,149-151). However, despite the detrimental impact that these non-HLA antibodies have on transplanted allografts, the cell specific expression of these non-HLA antigens preclude their detection via conventional lymphocyte crossmatch assays.

Once transplanted, the sensitized patient could be at higher risk for acute and chronic AMR (5-7,20). Post-transplant monitoring may be needed to prevent or reduce graft loss in these patients. Unfortunately, it is not yet known at what level DSA is deleterious to the allograft. Furthermore, post-transplant monitoring is problematic in the US due to reimbursement issues. Accurate diagnosis is crucial because AMR is typically refractory to conventional therapy used to treat cellular rejection (7). Currently, immunosuppressive rescue therapies used to treat AMR of transplanted renal and heart allografts include: IVIg alone (152), plasmapheresis alone (153), or plasmapheresis paired with CMVIG or IVIg (103,154-155). Rocha *et al* reported successful reversal of AMR in 16 renal transplant recipients (154). The majority the patients received plasmapheresis paired with high or low-dose IVIg (14/16), the remaining 2 patients were treated with either IVIg or plasmapheresis. The one year graft survival in these patients was 81%, similar to graft survival rates in recipients treated for cellular rejection. Jordan *et al* has utilized high-dose IVIg alone to reverse antibody mediated allograft rejection (156). In this study, 7 renal and 3 cardiac transplant recipients showed a complete reversal of severe antibody mediated rejection following IVIg infusion. Nine of these 10 patients experienced no further rejection episodes. In addition, post-transplant splenectomy has also been successful in rescuing kidney allografts from severe AMR (157-158).

New markers of complement activation have increased the ability to diagnose AMR within allograft biopsies (6,7,15,159). Immunohistological techniques have identified deposition of C4d, a complement split product of complement component C4, in biopsies of early and late rejecting kidney and heart allografts (159-164). Moreover, diffuse staining of C4d within the peritubular capillaries of kidney biopsies has been correlated with a significant reduction in 1 year graft survival, resistance to both steroid and T cell directed immunotherapies, and the presence of circulating DSA at the time of the biopsy (159,162,165-166). With the use of these new tools, it is now appreciated that humoral rejection is responsible for approximately 20-30% of acute rejection episodes following kidney transplantation (159). Improvements in the diagnosis of humoral rejection have also lead to the diagnosis of “subclinical” AMR in which there are signs of rejection despite stable graft function. A recent report by Haas *et al* discusses 10 cases of subclinical AMR in highly sensitized renal transplant patients who were transplanted following desensitization (167). Of concern is that subsequent biopsies on these individuals showed “chronic

Table 1. Antibody screening assays: strengths, weaknesses, and utility

Category	Assay Type	Strengths/ Advantages	Weaknesses/Shortcomings
Cell Based Assays	General properties	Can test the donor directly; can test for auto-antibodies.	Requires access to sufficient number of viable cells. Not specific for HLA.
	Cytotoxicity	Inexpensive, widespread expertise	Low sensitivity. Subjective scoring of test results.
	Flow cytometry	High sensitivity; can assay multiple parameters simultaneously.	Requires expensive equipment and reagents; expertise more limited.
Solid Phase Immunoassays	General properties	Commercially available; highly sensitive; high throughput; unaffected by many irrelevant antibodies; can test plasma or serum; can control for Ig class tested; partially automated with objective scoring of reactions; no special treatment needed to differentiate between antibodies to class I and class II antigens.	No control over panel composition; no way to determine if assigned phenotypes are correct; no way to determine distortion or contamination of target molecules or amount of antigen present (except for single antigen targets), increased cost per sample.
By Method	ELISA	More sensitive than cytotoxicity; modest capital equipment investment required.	Some sera yield high background; may be affected by high IgM levels.
	Bead based, laser detected	more sensitive than ELISA; higher throughput than ELISA; small sample volume requirement.	Same as for ELISA plus higher capital equipment cost; may be too sensitive for broadly sensitized patients.
By Target	Pooled antigens	Very high throughput; least expensive of solid phase assays.	Antigen composition unknown; may miss antibodies to rare antigens; no specificity determination.
	Phenotypes	Provides specificity determination.	Identifying or tracking DSA may be difficult in highly sensitized patients
	Single antigens	Uncomplicated recognition of specificity; most sensitive of the solid phase assays.	Failure of any bead may result in missing key antibody; doesn't provide insight into strength of reactivity against phenotypes with >1 target antigen.
	Donor antigens	Provides direct testing of donor.	Not possible to determine if there is sufficient amount of each antigen in its native conformation.

changes" consistent with chronic allograft nephropathy. One limitation to this study and that of Gloor *et al* is that long-term follow-up of desensitized transplant recipients is not yet available (167-168). Thus, new protocols for diagnosing and treating AMR have also improved graft outcomes in sensitized patients.

4. THE LABORATORY'S PROBLEM

The sensitized patient presents the histocompatibility laboratory with numerous responsibilities: identifying who is sensitized; accurately determining the specificity of the antibodies present and the titer of those antibodies specific for a given donor; identifying factors that may affect the risk of AMR in a patient; and, at centers with desensitization programs, establishing protocols for monitoring patients undergoing desensitization.

Today there are a variety of methods available for the detection and characterization of HLA-specific antibodies and each has its strengths, weaknesses, and particular utility. An overview of the characteristics of the various assays is provided in (Table 1). We believe that the information in this table suggests that no one test can provide all the information necessary on all patients and that it is best to maintain several test methods in the laboratory whenever possible. The solid-phase immunoassays have increased the ability to detect and characterize HLA-specific antibodies exponentially. The cost of a test using pooled antigen targets make it financially feasible to screen all sera sent to the laboratory. When the results of tests against phenotypes are listed in order of reaction strength, it is possible to define most if not all antibodies present in sera that have restricted reactivity - *ie*, sera that are not pan-reactive. This can be augmented, appreciably, with tests using single antigen targets. However, these tests are not foolproof. It is impossible to identify all the antibody specificities present in a serum that

reacts with all phenotypes in a panel and some single antigen targets may perform suboptimally or fail detection (169). Failure maybe due to problems in production or may reflect that some antigens are more labile than others. Failed reactions could result in missing the identification of critical specificities. In other cases, a distorted or misfolded HLA molecule may have an epitope that is not found on properly conformed molecules, resulting in a false positive reaction with a non-HLA-specific antibody and an incorrect assignment of antibody specificity (82). Single target platforms may have varying amounts of antigen on each bead or microtiter well (J. Crompton, unpublished data and A.A. Zachary, unpublished data). This may deter the assessment of antibody strength between different HLA specificities and between the products of different companies. Certain sera will yield high background, making interpretation of test results very difficult. These problems are also likely to exist in the solid-phase crossmatch tests that are becoming available (170). Therefore, despite their extremely high sensitivity and accuracy, it would seem that solid-phase immunoassays do not replace cellular assays but should be used as an adjunct to cell-based crossmatch tests.

Another aspect of interpreting antibody tests is to correlate reaction strength among the various assays with clinical outcome. In this way, solid-phase assays, while not a surrogate for a donor cell crossmatch, can provide a reasonable prediction of the crossmatch results and can be used to define unacceptable donor antigens (85). This is significant because proficiency tests indicate that there is a high degree of correlation among laboratories in the identification of antibody specificities but not in interpreting crossmatch results (82). This may be less of a problem than it seems since there is great variability among patients and among the clinical protocols of various programs. What is important is that the laboratory be able to interpret the crossmatch tests with respect to the clinical outcomes of their own transplant program.

Transplanting the sensitized patient

While the timing and severity of AMR correlates with the amount of DSA present, *ie*, titer, it is not the only factor relevant to the risk of such rejection. A patient who has lost a previous graft rapidly to immunologic rejection and/or who has made antibody to every previous HLA mismatch may be an aggressive responder at high risk for rejection of subsequent transplants. Further, patients may be sensitized but not making HLA-specific antibody at the time of testing. Such patients may be at high risk for a rapid anamnestic response following transplantation. Therefore, laboratories should investigate the sensitization history of patients. This would include, at a minimum, determining mismatches of previous transplants. However, additional information regarding the number of pregnancies and age of youngest child as well as the dates of any large numbers of transfusions may also prove important. This information reflects a potential for sensitization but does not guarantee that sensitization occurred, as indicated by the number of successful child to mother and husband to wife transplants and successful transplantation following donor-specific transfusion (171-173). Our laboratory has recently reported that assessing expanded clones of HLA-specific B cells by staining B cells with HLA tetramers may be useful in predicting production of HLA-specific antibody in the early post-transplant period (174-175). What is important is that the laboratory's evaluation of each patient should be based on all available information and not just on the results of antibody tests.

The laboratory has a significant role in monitoring patients undergoing desensitization - *ie*, treatment to reduce or eliminate HLA-specific antibody(ies). The type of monitoring will depend on the specific treatment protocol. If the treatment is designed to reduce the breadth of reactivity without consideration of particular specificities, the laboratory must be able to identify all specificities present and determine the persistence or elimination of each, over the treatment period. This can usually be achieved with a comprehensive antibody screening protocol. If the program is designed to reduce or eliminate antibodies to a particular donor, the laboratory must first identify each DSA present, the titer of the donor reactivity, and monitor all changes in donor reactivity. If the antibody is reactive in the CDC assay, periodic crossmatch testing during the treatment period may be an inexpensive way to assess DSA strength provided there is adequate access to donor cells. However, if the antibody is below the strength detectable by CDC or if interpretations of CDC crossmatch tests are confounded by auto-antibody in the donor and/or recipient or the presence of lymphocyte-depleting therapeutic antibodies, antibody screening by solid-phase immunoassay may be more practical and yield results in a timely fashion. In treatment protocols that utilize plasmapheresis, testing should be done at sufficient intervals to determine if the projected number of pre-transplant treatments will be sufficient. Of course, post-transplant monitoring is absolutely necessary following desensitization, since there is a high risk of antibody rebound as a result of the inflammatory response to surgical trauma. Thus, the laboratory's initial assessment of a patient will help formulate a treatment plan which may then be modified

according to the efficacy of the treatment as assessed by the laboratory's antibody tests. This is a very interactive process with the laboratory providing information that helps guide the treatment and the clinical team providing information that helps the laboratory determine its testing protocol.

5. CONCLUSIONS

The problem of sensitization is, thus, a problem for the patient, the physician, and the laboratory. The sensitized patient must understand how their condition impacts the opportunity for transplantation, must appreciate that waiting time will be greater than for the non-sensitized patient, and may be confronted with decisions about treatment protocols. Once transplanted, the patient must be aware of the increased risk of rejection, the increased importance of compliance with medication, and the need to report any health problems in a timely fashion. The physician must be aware of the risks associated with transplanting a sensitized patient, of treatment modifications that are appropriate for such patients, and of options for overcoming or circumventing the patient's immunologic barrier to transplantation. The laboratory is responsible for providing the information that will guide the decisions and actions of both the patient and the physician. Sensitization poses both a formidable barrier to transplantation and a significant risk to the transplanted organ, but it should not be viewed as insurmountable. While sensitization to HLA antigens remains everyone's problem, overcoming this barrier will be everyone's victory.

6. ACKNOWLEDGEMENTS

We thank J.E. Locke for helpful comments on the manuscript.

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Abbreviations: US: United States; DSA: donor HLA-specific antibody; CDC: complement-dependent cytotoxicity; PRA: panel of reactive antibodies; AHG: antiglobulin; AMR: antibody mediated rejection; ELISA: enzyme-linked immunosorbent assay; LVAD: left ventricular assist device; IVIg: intravenous pooled human immunoglobulin; CMVlg: anti-CMV hyperimmune IVIg; BOS: bronchiolitis obliterans syndrome; SLA: swine leukocyte antigen; MICA: major histocompatibility complex class I-related chain A.

Key Words: Transplantation, HLA, PRA, Sensitization, Alloantibodies, Rejection, Review

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