Quantifying HLA-specific antibodies in patients undergoing desensitization

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Purpose of review
Two major desensitization protocols have been used to eliminate or reduce HLA antibodies to a level that allows transplantation with a low risk of antibody-mediated rejection (AMR). This review will focus on the antibody testing methods used to assess changes in the breadth and strength of antibody levels and the relative strength of donor HLA-specific antibodies (DHSAs).

Recent findings
Correlations of solid-phase immunoassay (SPI) class I and II levels with the donor-specific T and B cross-match results have shown the acceptable levels of DHSA that correlate with a low risk for AMR. The DSHA levels determined by SPI correlate with cross-match results and with clinical outcome. Therefore, the results of either assay can be used to determine the risk of AMR and when treatment has reduced DSHA to a level safe for transplantation. Monitoring DSHA is important for guiding the number of treatments as well as the timing of additional treatments needed to achieve these acceptable levels.

Summary
DSHA monitoring, in both protocols, uses the correlation of solid-phase antibody testing and the donor-specific cross-match to determine the efficacy of the protocol and when the acceptable level of DSHA is achieved permitting transplantation with minimal likelihood of AMR.

Keywords
crossmatch, desensitization, donor HLA-specific antibody, IVIg, solid-phase immunoassays

Introduction
There are two general formats for eliminating or reducing human leukocyte antigen (HLA) antibodies in patients: high-dose intravenous immunoglobulin (HD-IVIg) and plasmapheresis combined with low-dose anti-cytomegalovirus (anti-CMV) globulin or IVIg (PP-IVIg) (reviewed in [1,2]). Many centers have made small modifications to these programs but the general approach remains the same. In the HD-IVIg protocol, patients receive a bolus of IVIg, 2 g/kg body weight, monthly. Recently, this protocol has been modified to include the administration of rituximab and two doses of IVIg in a 1-month period [3•]. This approach can be done to reduce the overall sensitization of patients on the waiting list allowing deceased-donor organ transplantation or to eliminate or reduce donor HLA-specific antibodies (DHSA) to a well tolerated level, for living donor organ transplantation. In the PP-IVIg protocol, patients receive a plasmapheresis treatment three times a week followed by 100 mg/kg body weight of CMVIg [4]. This protocol is applied, preemptively, to patients awaiting transplantation from

a living donor and immediately following transplantation from a deceased donor to whom the patient has antibodies. The schedules for monitoring DSHA are different for these two protocols but both depend on the ability to determine the relative strength of DSHA.

The article will review the various methods for determining antibody strength, including their strengths, weaknesses, and utility and monitoring schedules for the two types of desensitization protocol.

Antibody assays
The two types of assays used to monitor HLA antibody are the crossmatch, which tests reactivity with donor cells directly, and antibody screening and characterization, which assesses the breadth and relative strength of a patient's HLA-specific antibodies [5]. Importantly, both types of assay measure the relative amount but not exact amount of HLA antibody present in the serum. Crossmatch tests can measure either the secondary effect of DSHA by assessing complement-mediated cytotoxicity

or can measure binding of antibody to donor cells in a flow cytometric method. The advantage provided by the crossmatch assay is that it is a direct measure of the relative strength of donor-reactive antibody, accounting for variability in antigen expression by the donor cells. However, non-HLA antibodies such as auto-antibody and therapeutic antibodies will render the cross-match uninterpretable. There are multiple variations of the crossmatch tests and, importantly, there is no absolute standard for defining positivity. Among different laboratories, the flow cytometric crossmatch results are read on different scales, using different units of measurement and different antiglobulin reagents. Thus, it is the responsibility of each transplant program to correlate test results with clinical outcome.

Antibody screening and characterization had been performed for decades using crossmatch tests against a panel of cells from individual donors but today is done predominantly by solid-phase immunoassays (SPIs). Thus, the discussion will focus on SPIs. SPIs are commercially available using a variety of platforms and in a variety of formats. There are multiplexed bead assays that are performed either on a standard flow cytometer or on a fluoroanalyzer. Enzyme-linked immunosorbent assays (ELISAs) are available using traditional microtiter plates or microtest plates. The targets of these assays are soluble HLA antigens and occur in three formats: a pool of antigens derived from multiple individuals and containing most HLA antigens, phenotype panels in which each target contains all the antigens of the phenotype of an individual, and single HLA antigens. The applicability of these various formats has been discussed extensively elsewhere and will only be mentioned briefly here [5]. The pooled antigen format determines the presence or absence of HLA-specific antibody but does not provide specificity. However, when testing sequential sera from a patient, the pooled antigen test in the multiplex bead assay does provide an indication of changes in breadth and strength of the antibodies. Phenotype panels are excellent for determining antibody specificities in most cases and because a phenotype may contain more than one donor antigen, providing an assessment of the collective strength of DSHAs. However, with broadly reactive sera, some antibodies may be masked by others and certain specificities such as HLA-Cw are not detected well in these panels. Single antigen panels provide maximum detection of the various antibodies present, to the extent that the target antigens are present in the panel. However, the collective strength of multiple antibodies cannot be determined by summing the values of the reactions of the different antibodies. Further, interpretation of these assays must take into account variability in both the condition and amount of each antigen. SPIs are commercially available from a limited number of vendors which affords some degree of consistency in results from

laboratories using the same assays. However, lot to lot changes in sensitivity and panel composition are known to occur and can affect the interpretation of changes in antibody strength when monitoring patients over time. Further, the high degree of sensitivity of these assays makes them more susceptible to environmental factors that affect test results. Therefore, some leeway should be applied in evaluating differences in reaction strength among sequential sera [6].

Test applications
As crossmatch assays monitor the reactivity of the patient's antibodies with donor target antigens in their native conformation, they are perhaps the most informative about changes in DSHA strength. Further, when DSHA level is high enough to saturate the target in SPI, the relative strength and changes in the strength of DSHA cannot be determined by SPI and must be assessed by crossmatch testing. However, there are multiple drawbacks to using the crossmatch as the only monitoring assay. First, they require an adequate supply of viable donor cells which is not always possible. Second, as noted above, these tests are not informative about DSHA when interfering factors are present. Third, crossmatch tests, particularly the flow cytometric crossmatch, use larger serum volumes than do the multiplex bead assays. However, when they yield accurate results, they are incomparable for confirming clinically relevant levels of DSHA suggested by SPI results.

SPIs provide rapid assessment of the relative strength of DSHA and afford greatly increased specificity compared with the crossmatch test. SPIs provide information about antibody specificity which can be used to determine the calculated panel reactive antibody (CPRA), the percentage of the donor population that is deemed incompatible by the criteria of an individual transplant program. These tests can be correlated with the results of crossmatch tests and thus can be used as a virtual crossmatch with a specific donor [7–11]. This approach, in turn, provides an assessment of the efficacy of desensitization on patients awaiting deceased donor transplant [12,13]. Also

SPIs provide an assessment of changes in relative reactivity with a specific donor which, in turn, is an excellent tool for monitoring during and subsequent to desensitization. However, these tests are also subject to interference by immune complexes, IgM antibodies, and some therapeutic agents such as high-dose IVIg, thymoglobulin, eculizumab, and bortezomib [14[•],15]. Therefore, accurate interpretation of SPI test results must take this interference into account and, when possible, serum treatments to eliminate or reduce the interference should be performed. Further, the inherent test variability must be assessed. As can be seen from Fig. 1, the positive control reactivity can be used to assess variability in test sensitivity so that, changes in the strength of DSHA is suspect when it parallels changes in the strength of the positive control. Monitoring may continue for days, weeks, or months. As the sensitivity of SPI has been known to change between lots of test kits, it is worthwhile to sequester sufficient reagents from a single lot for testing during the critical peri-transplant period.

Monitoring protocols

Goals common to both types of desensitization protocol are to monitor the efficacy of treatment and assess when the level of DSHA has been reduced sufficiently to permit transplantation with a high likelihood of success and a minimized likelihood of antibody-mediated rejection. For patients undergoing treatment with high-dose IVIg while awaiting deceased or living donor transplantation, monitoring of antibody strength determined by both SPI and donor-specific crossmatch is used to determine acceptable levels of DSHA. The effect of desensitization is transient prior to transplantation. Therefore, it is unfortunate that as the treatment eliminates certain unacceptable antigens, patients will also have a reduced CPRA. In the USA when the CPRA is below 80, the patient no longer receives points for sensitization thus making them less competitive for transplantation than they would have been before desensitization. This practice is contradictory to the very purpose of desensitization. For patients being treated with the plasmapheresis protocol for living donor transplantation, pretreatment tests are applied to predicting the number of pretransplantation and post-transplantation treatment that are needed, to assess the need for additional treatments, and to determine when the DHSA is at a level sufficiently low for transplantation.

Monitoring for the high-dose IVIg protocol

The combination of high-dose IVIg and rituximab (IVIg/R) immunotherapy has become the standard of care at Cedars-Sinai Medical Center for broadly sensitized patients awaiting living donor transplantation, if no HLA or ABO-compatible donor is available, and for deceased donor transplantation, if no living donor is available [2,3[•],16]. We have presented our guidelines for acceptable

Figure 1 Pre-transplant and post-transplant monitoring
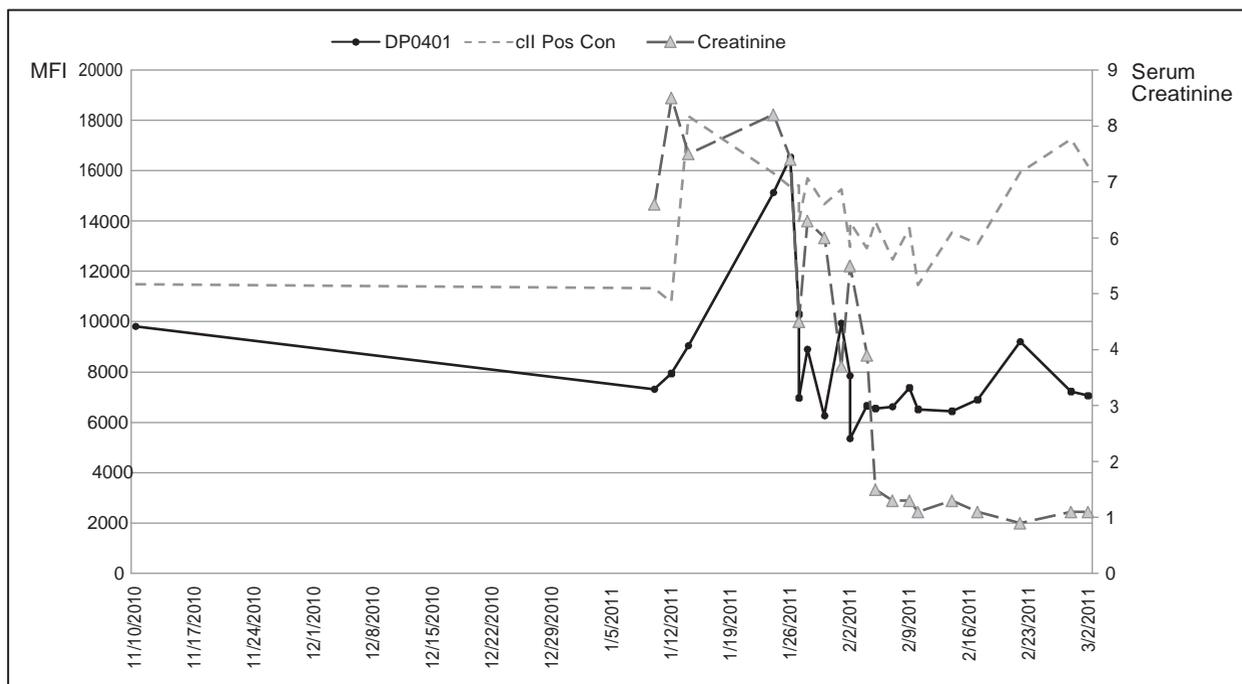
There is an initial increase, post transplant, in DHSA in the face of decreasing reaction strength of the positive control. Following the decline in DHSA strength, the patient's antibody strength parallels that of the positive control, suggesting that the apparent changes in DHSA strength are due, in large part, to differences in day-to-day test sensitivity. DHSA, donor HLA-specific antibody.

antibody levels leading to the identification of compatible living and deceased donors [13,17••]. This approach gives broadly sensitized patients the opportunity for transplantation with compatible donors.

We have reported that patients undergoing immunotherapy show a significant decrease in T-cell flow cytometric crossmatch results and complement-dependent cytotoxicity (CDC) T-cell PRA results compared with those of sera obtained before the initiation of therapy and immediately pretransplant [3•]. The patients are selected for transplant based on achieving acceptable levels of DSHA including binding to single antigen class I beads defining DSHA at less than 100 000 SFI/5000 median fluorescence intensity (MFI) and a positive T-cell donor median fluorescence intensity (FXM) less than 250 mean channel shift (MCS) [13] We have further established the correlation between B-cell flow cross-match and class I and II SPI [17••]. The use of pronase-treated cells allows monitoring of changes in donor-specific B-cell crossmatches subsequent to high-dose IVIg/R immunotherapy. Thus, our guidelines for selection of an appropriate compatible include a positive pronase B-cell-treated flow cytometry crossmatch below 300 MCS, and no DSHAs in the high binding range of above 200 000 SFI/10 000 MFI. If the T and B-cell crossmatches and DSHA SPI binding levels are no more positive than the values indicated, the patients are at low risk for early AMR.

The protocol involves two doses of IVIg and one of rituximab administered over a 1-month period. For living donor transplantation, the strategy for monitoring the antibody levels is to obtain pretreatment and post-treatment specimens for both Luminex single antigen and donor flow cytometry and CDC crossmatch testing. If the desired crossmatch level is not achieved, a second test is performed 1 month later. Usually, the desired level is achieved by that time. Rarely plasmapheresis may be performed prior to the transplant. If the desired antibody level is not achieved after a second round of treatment, the patient is provided the opportunity to enter into a paired donor exchange and is also placed on the deceased donor waitlist.

For deceased donor transplantation, the patients broadly sensitized to HLA who have reached the top of the waitlist (usually after 7–9 years on the waitlist) are treated with IVIg/R. The Luminex single antigen bead testing is performed pretreatment and post-treatment and at monthly intervals until transplant. After the initial level, there is a period of 30–120 days when the antibody levels reach the lowest levels for most patients [17••]. After that time period, the antibody levels either increase or appear to remain constant. These results indicate that the antibody profiles for all patients do show some impact of the IVIg/R therapy; however, the decrease observed does

appear to rebound over time. Thus, if transplantation has not occurred by 6 months, the patient receives the IVIg/R treatment again and the same testing protocol is followed. We have reported that of 108 patients treated with this strategy, 74% received a deceased donor transplant; 53% were transplanted with a positive flow crossmatch; 35% were transplanted with a negative flow crossmatch; and 12% received zero HLA-ABDR mismatched grafts. Figure 2 illustrates the overall antibody profile and the DHSA profile for a patient who received a deceased donor graft after IVIg/R therapy. Antibody levels decreased with therapy and dropped even more post transplant. The flow cytometry crossmatch results at the time of transplant were 177 mean channel shifts over background (MCS) and 322 MCS for pronase-treated T cells and B cells, respectively. For all protocols, the antibody levels are monitored by SPI at 2 weeks, 1 month, 3 months and quarterly for a year post transplant.

Monitoring for the plasmapheresis–low-dose CMVIg protocol

Here we will describe the monitoring protocol used at the Johns Hopkins University Immunogenetics Laboratory which can be used as a guide and modified according to the treatment protocols practiced at other centers [1,4,5]. As noted above, the results of initial tests are used to determine the number of pretreatments and post-treatments that will be needed. This prediction is based on cross-match results but SPI are also performed at this time to confirm the correlation of the results of these tests with those of the crossmatch test. If a patient has a positive CDC crossmatch with the donor, CDC crossmatch tests and SPI are used on selected sera until the cytotoxicity crossmatch is negative. If the patient has a negative cytotoxicity crossmatch prior to treatment, monitoring is conducted by SPI and crossmatch tests are performed to confirm antibody strength, when indicated. The phenotype panel format is used as the major tool for assessing DSHA, when it is sufficient to detect changes in DSHA; otherwise the single antigen panel is used alone or in combination with the phenotype panel.

Once treatment is started, each pre- and postplasmapheresis serum specimen is tested with the pooled antigen assay as a general monitoring tool. The pre- and post-plasmapheresis sera from the first plasmapheresis treatment and the preplasmapheresis serum from the second treatment are tested for DSHA strength. This test is done to measure the efficacy of the plasmapheresis and the amount of rebound of antibody seen prior to the second treatment. In the vast majority of cases, there is an initial drop in antibody strength followed by some degree of rebound as DSHA in the extravascular spaces equilibrates with that in the serum. However, as assessed by the pooled antigen assay, there is, in most cases, an overall continuing decrease in DSHA strength. In the

Figure 2 Donor HLA-specific antibody levels for serum
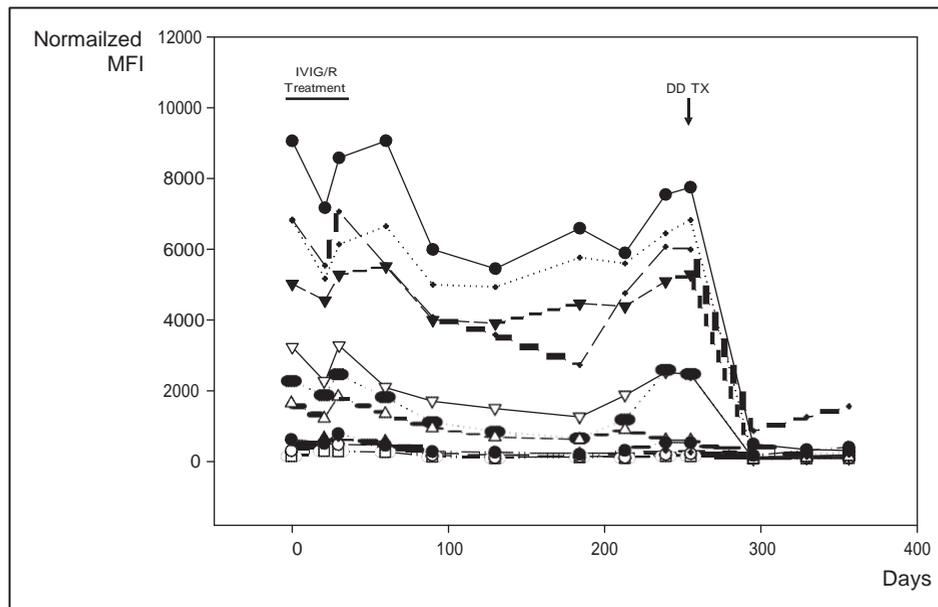
The DHSA levels for serum obtained pre-IVIg/R treatment, during treatment, at the time of deceased-donor transplant, and post transplant. The patient's serum contained DHSA to HLA-A23, B27, B81, DR12, DR52, and DQ5. The patient has not experienced any AMR post transplant. DHSA, donor HLA-specific antibody.

exceptional patient, a persistent rebound of antibody occurs. There may also be a rebound because of some proinflammatory event, despite a previous consistent decline in antibody level [18]. In these cases, the monitoring information may indicate the need for changes in the treatment protocol such as more frequent treatment or the use of additional immunosuppressive agents. Following transplantation, the same monitoring protocol is followed until treatment is discontinued. Subsequently, sera are tested twice weekly in the first post-treatment week, weekly during the first month, at 2 months, 3 months, and quarterly thereafter. Indications for additional testing are changes in the clinical status of the transplant, infection, trauma, or allergic reaction.

It is important to note that post-transplant testing is worthwhile for any transplantation, occurring with or without desensitization, as it provides the earliest indication of possible AMR and/or chronic changes due to DSHA [19].

Other considerations
It is desirable to know when it is reasonable to transplant and when it is safe to discontinue treatment post transplantation. In general, the risk of AMR is proportional to the level of DSHA prior to treatment [20] or at the time of transplantation [21]. Thus, the best scenario is the elimination of DSHA prior to transplantation. However, this approach is not always possible and continued treatment must be balanced with the health status of the patient who may be losing access for dialysis or have other risk factors.

Reinsmoen et al. [13] have correlated the results of pre-transplant SPI with the results of crossmatch tests and with the incidence of AMR. The highest class I DSA level measured by SPI was correlated with the T-cell flow cytometric crossmatch MCS values. We observed that all three patients with DSA levels greater than 100 000 SFI/5000 MFI and T-cell MCS greater than 250 experienced early AMR. Likewise, for the pronase-treated B-cell flow cytometry crossmatch and the corresponding sum of the class I and II DSA, four of six patients with MCS above 300 and class I and II DSA sums above 600 000 SFI/ 30 000 MFI experienced AMR within 42 days of transplant [17••]. The other two patients had AMR diagnosed within 200 days post transplant. These values provided the basis for our guidelines for acceptable antibody levels following desensitization therapy.

For patients undergoing desensitization by PP-IVIg, continued treatment can increase the risk of infection which may, in turn, result in a significant rebound in DSHA strength [18]. Therefore, the time to transplant must take into account both the risk of AMR and the risk associated with continued treatment. However, continued development of therapeutic agents has substantially reduced the risks associated with DSHA, making it possible to transplant safely in the presence of increased amounts of DSHA.

Ideally, treatment would be discontinued only when the patient has good graft function and DSHA has been

eliminated. However, one of us [22] has shown that the complete elimination of DSHA following transplantation is determined by the initial titer and specificity of DSHA with class I, DRB1 and DQ, and DR51-specific, DR52-specific, and DR53-specific antibodies being eliminated in 75, 53, and 20% of cases, respectively. DSHA elimination is also impacted by the extent and specificity of donor mismatch. Thus, the likelihood of DSHA elimination, the strength of DSHA, and graft function must all be considered in determining when to discontinue treatment.

The question of the clinical relevance of low levels of DSHA has not been determined conclusively and is outside the scope of this article [23–26].

Finally, although there are readily available methods and tools for measuring HLA-specific antibodies, it is clear that there are non-HLA antibodies that are clinically relevant. The impact of desensitization on these antibodies needs to be established. Additionally, the specific targets and methods for measuring these antibodies are being developed but need to be expanded further. Hopefully, future articles will be able to address monitoring for these antibodies.

Conclusion

Two major desensitization protocols have been employed to reduce or eliminate HLA-specific antibodies in patients awaiting solid organ transplantation. The antibody strength and specificity is assessed by SPI and crossmatches for both protocols enabling the identification of compatible donors. Both protocols have been used pre-emptively to lower HLA-specific antibodies prior to living donor transplantation. For deceased donor transplantation, the IVIg/R protocol is used prior to transplant, whereas the low-dose IVIg-PP protocol is used after transplant. With proper antibody profiling, both methods have been show to be efficacious and give patients broadly sensitized to HLA the opportunity for transplantation.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
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