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SPECIAL THEME ISSUE:

C1q Antibodies in Thoracic Transplantation

Key points:

- Single antigen bead (SAB) flow cytometry methods are sensitive and detect both complement and non-complement fixing antibodies (Ab). The C1q assay identifies a subset of HLA antibodies that are complement-fixing and shown to be more clinically relevant than those that do not fix complement.
- Evidence for the clinical utility of the C1q assay exists in the post kidney and heart transplant settings. Less evidence exists for its pre-transplant use, or its use in other solid organ transplant settings (e.g., lung).

- Post renal transplant, the presence of C1q-binding donor specific HLA antibodies (DSA) is strongly associated with allograft loss. There is controversy whether C1q binding is superior or not to standard IgG to assess immunologic risk for other adverse outcomes.
- The C1q assay has the potential in the pre heart transplant setting to be used as a tool for judiciously decreasing the number of antigens deemed unacceptable in highly sensitized patients who cannot wait for a perfectly compatible donor. However, more published evidence to support C1q use in this setting is needed.
- Screening for DSA to HLA is recommended post heart transplant due to the known risk for early and late AMR and graft failure. Based on current evidence, the C1q assay may further stratify risk for graft loss beyond the standard IgG assay.
- The clinical utility of the C1q assay is not firmly established in heart transplantation. The sensitivity of the commercial C1q assay and the lack of consensus on how to define relevant IgG and/or C1q-fixing DSA levels (by MFI and/or titer) may explain some of the conflicting results.

Introduction

Antibody (Ab) mediated complement fixation and intravascular thrombosis in organ transplant rejection have been recognized and studied since the 1960s[1]. It is known that preformed and de novo circulating donor specific antibodies (DSAs) to human leukocyte antigens (HLA) are associated with acute and chronic antibody mediated rejection (AMR) and graft failure. [1,2] It is also a widely held view that the most harmful DSAs are IgG antibodies to HLA that are capable of activating the classical complement cascade. Non-HLA Ab as well as IgM and non-complement fixing antibodies have also been reported to be associated with graft loss but to much less degree than the IgG Ab to HLA that are capable of activating the classical complement cascade. Non-HLA Ab as well as IgM and non-complement fixing antibodies have also been reported to be associated with graft loss but to much less degree then the IgG Ab to HLA[3]. However, these have not been as rigorously or as extensively studied. This is likely due to the fact that all of the standard assays performed for identification of HLA Ab or DSA are technically designed to detect only IgG. Thus, the potential/real effect of these other Ab on transplant outcomes has been either not known or not investigated for many years, and conclusions have been drawn based on methodologies in place well before current technological and scientific advances. Nevertheless, for the purpose of this review, DSA refers to HLA DSA of the IgG isotype, unless specified otherwise.

The shift from cellular assays (CDC, complement dependent cytotoxicity) to bead technology (e.g, SAB, Flow or Luminex single antigen beads) for identification of HLA antibodies was both a paradigm shift and an exponential change in sensitivity. When comparing antibodies by CDC and IgG on SAB, the CDC assay only detects about 9% of the antibodies detected by IgG on SAB (Tyan, unpublished observations). The CDC is known to be clinically relevant, whereas in the early period of adoption of the SAB, and before the development of modifications to the assays, the beads were considered to be overly sensitive for detecting antibodies and their clinical relevance was uncertain.

Though they are very good to predict the absence of rejection when no (potential) DSA is found, a positive result indicates a risk rather than a contraindication to transplantation[4]. Taking advantage of the sensitivity and specificity of the beads, modifications to the SAB assays were explored in attempts to identify the clinically relevant subset of preformed antibodies (Ab) pre-transplant and/or DSAs post-transplant that could better predict the risk for allograft loss and guide treatment of AMR. For that purpose, complement binding assays using C1q[5], the first component in the classical complement activation cascade, or other downstream complement components such as C4d[6] or C3d[7], have been developed to identify complement fixing antibodies which are known to be more harmful[1]. The objective of this report is to review the literature regarding the utility of C1q assay for identification of clinically relevant antibodies pre- and post-transplant.

Methods to identify HLA and Complement-Fixing Donor Specific Ab.

Cellular assays. Complement dependent cytotoxicity (CDC) is a functional assay measuring target cell killing after mixing serum from a candidate or recipient with either donor or HLA-typed third party T cells, B cells, and/or unseparated mononuclear leukocytes in the presence of rabbit complement. It requires completion of the complement pathway to the formation of the membrane attack complex (MAC). CDC assays were traditionally used (and still are in many countries) to assess immunologic compatibility between donor and recipient, and the results correlated with the risk for allograft rejection and failure.

A positive CDC crossmatch precludes transplantation since it highly predicts hyperacute and accelerated acute rejection immediately post-transplant[1]. However, CDC has low sensitivity and a negative CDC crossmatch does not completely eliminate the risk of acute rejection and graft failure, especially when preformed DSA is present in the recipient's serum. Furthermore, it has been shown that certain preformed non-HLA Ab, not routinely and in fact rarely tested for prior to transplant, are associated with allograft failure. [3]

Flow cytometric crossmatch is another cellular assay that can determine whether antibody to the donor is present, but it cannot define what the antibody is or whether it is to HLA. Typically performed near the time of transplant, this assay mixes lymphocytes of the donor (typically from blood, but also from lymph nodes) with the intended recipient's serum. Secondary antibodies labeled with different fluorescent probes discriminate donor T-cells, B-cells, and any recipient IgG antibodies that bind to these lymphocyte subsets. In heart transplantation, a prospective positive flow cytometric crossmatch may not prevent a transplant from proceeding, but it may drive intervention in the OR (e.g., plasmapheresis) and/or alter the transplant physician's choice of induction therapy or immunosuppression.

Solid phase assays. Microspheres (beads) coated with cloned or membrane extracted HLA antigens have now been widely adopted in many transplant centers and have replaced cellular assays for pre-transplant alloantibody screening in the US, though they are less prevalently used in other countries. The beads are individually distinguished based on uniquely graded internal fluorescence. Bead coatings can be customized, and these can precisely identify specific HLA and non-HLA antibodies and alleles. Solid phase assays using single antigen beads (SAB) and reagents to detect IgG binding are very sensitive and specific, and are dependent only on antibody binding without respect to complement. These assays typically detect many more Ab than CDC assays as noted above, but are less informative with respect to their clinical relevance. In order to detect complement fixing Ab, different components of the complement activation cascade such as C1q, C4d or C3d can be added to the beads[5,7] and detected by second step antibodies specific to them (or, in the case of C1q, by direct labeling). The intensity of the reaction between recipient/candidate serum

antibodies and specific HLA antigens (+/- C1Q, C4d, or C3d) is quantified by grouping reactions based on two signals: the internal fluorescence of each unique bead population and the fluorescence intensity of second step antibody bound to each bead. Results are reported as mean fluorescence intensity (MFI) for each population. There is no uniform standard for the threshold of positivity, and this is determined individually by center, based on its clinical preferences. It is highly variable.

The assays may be performed on a flow cytometer or using the Luminex® platform, which is a high throughput microfluidic system capable of analyzing multiple fluorescent signals and their relative intensities quickly.

Preformed HLA Antibodies, UA and VXM prior to heart transplant.

It has been suggested that C1q binding could be useful to increase the donor pool in highly sensitized patients awaiting heart transplant [8]. Due to time restrictions, a prospective crossmatch in heart transplantation is rarely performed and only for local and/or stable donors. Recipient/donor immunologic compatibility is usually assessed via a virtual crossmatch (VXM) which is a crossmatch performed electronically comparing a patient's antibodies with the donor's antigens to determine any incompatibilities.

Panel reactive antibody (PRA) is obtained using variable panels by CDC or by beads having multiple HLA antigens on the surface of each uniquely distinguishable population. It is independent of specificity and only reflects the number of positive reactions over the total tested. PRA is therefore variable depending on the method and the panel construction and cannot be compared center to center. Antibody analysis is performed routinely pre-transplant to determine if the intended recipient has pre-formed circulating antibodies directed against HLA. Both cellular and solid phase immunoassays can be used, as long as they screen against a wide range of HLA antigens. In practice, it is not possible to construct a panel that is representative of the actual potential donor pool and the PRA is not an accurate estimate of potential compatible donors.

The calculated PRA (cPRA) is based on the actual specificity of the antibodies found rather than the percent reactivity on panels of variable composition. Based on center specific preferences, the antibody specificities are entered into UNet as "unacceptable" (UA) which generates a cPRA. CPRA is based on two years of the actual donor pool, ethnically weighted. It is based on genetic frequencies found in the pool rather than on the percent of positive reactions in a given test and it is globally uniform once the UAs are entered, i.e., entering the same list of UAs from different centers will always yield the same cPRA. In general, the more UAs listed and the higher the frequency of a particular HLA antigen in the general population, the higher the cPRA. However, this is not always true since a donor may carry more than one UA so that the UAs are not strictly additive. The decision to enter UAs into UNet is challenging since there is a need to balance the risk of rejection with the likelihood of finding a donor. It is possible to obtain the cPRA without having to list the UAs into UNet by using the CPRA Calculator [https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/#calctop] and make individualized choices of UAs for any given patient given their urgency. These can then be listed as UAs in UNet. The main difficulty in selecting which UAs to list stems from the lack of consensus on MFI threshold to define relevant HLA Ab. Arbitrary MFI cutoffs are typically 1000 - 3000 for kidney and 3000 - 5000 for heart but can vary widely from center to center or for individual patients depending on the urgency of the transplant. For instance, a cPRA of 90% calculated using a MFI cutoff of 5000 will exclude ~90% of the potential donors via a VXM. Higher MFI cutoffs can be used for highly sensitized patients to expand the donor pool but at the cost of a higher risk of rejection if this is not selectively evaluated.

The C1q assay is a potentially attractive method to detect the more clinically relevant complement-fixing antibodies amongst all those found by IgG and reduce the number of UAs based

on clinical evidence rather than IgG MFI. The initial clinical study of C1q was in 18 pediatric heart transplant patients and showed an excellent association between DSA detected by the C1q assay within one week post-transplant and AMR in the first month post-transplant. Positive predictive value (PPV) and negative predictive value (NPV) for early (within 1 week) post-transplant C1q were each 100% (PPV= 5 C1q+/5 AMR, NPV=9 C1q-/9 no AMR) with 100% sensitivity and specificity. DSA by the C1q assay always preceded the presence of AMR, and the biopsy was always negative in the absence of C1q Ab. Presence of C1q DSA so early post-transplant suggests that these antibodies were present at the time of transplant. On the other hand, preformed and early-post transplant IgG DSA did not correlate well with AMR suggesting that C1q positivity to stratify patients could potentially enhance donor access via VXM without compromising survival [9]. It was also acknowledged that these patients remained at risk for late AMR and would require close monitoring due to the possibility of antibody conversion from a complement non-fixing state to a complement fixing state [9]. Another small study of 15 pediatric and adult sensitized patients (cPRA > 50%) showed that HLA IgG Ab MFI > 8000 correlated with a positive CDC-XM and concluded that risk assessment for a positive CDC-XM and early AMR could be improved by assessing C1q binding [10]. In this study, it appears that 11/15 patients were tested for C1q: IgG+/C1q+ DSAs were present in 7/7 patients diagnosed with clinical AMR (cAMR) in the first month post-transplant, whereas in the four patients who were AMR free, 4/4 had IgG+ DSA but none had C1q+ DSA. They also found that persistent C1q+ DSA, refractory to desensitization, associated with persistent C4d+ biopsies and resistant clinical AMR. Patients who responded to desensitization and became C1q negative showed resolution of their clinical graft dysfunction symptoms but remained C4d+ on biopsy.

In a recent study of 49 endomyocardial biopsies (EMB) performed in 44 heart transplant patients with mild to moderate acute cellular rejection (ACR), 84% of the 43 patients with IgG DSA had C1q+ DSA and C1q+ DSA was observed in 16 of the 17 cases with C4d IF+ biopsies. C1q+ DSA was better correlated with a C4d IF+ biopsy than IgG+ DSA (40% vs 24%, respectively (p=.02). Additionally, 82% of 28 EMB evaluated during a period of EF decline correlated with having C1q+ DSA. It was reported that there was not a direct correlation between the presence of C1q+ DSA and C4d deposition which could be due to C1q+ DSA preceding the C4d deposition and/or the development of AMR[11].

In contrast to the above studies, a retrospective study of 28/264 adult heart patients with pre-transplant DSA who were transplanted on the basis of a negative CDC crossmatch, 19 of whom developed AMR, reported a correlation of AMR with preformed DSA and elevated peak PRA assessed by IgG but not with C1q[12].

Preformed C1q-binding DSAs in kidney transplant (KT).

Three studies in kidney transplant patients did not find an association between pre-transplant complement-fixing antibodies identified by C1q and early AMR or graft survival[13-15]. In a cohort of 837 KT patients[13], it was shown that the presence of preformed IgG DSAs (n=290, MFI > 500) predicted graft survival while C1q+ DSA (n=30, MFI > 300 over background) was inconclusive, possibly due to the low prevalence of C1q+ DSA (4%) compared to IgG+ DSA (35%). In another study of 355 KT recipients[14], 28 patients with preformed IgG+ DSA (MFI>2000) were tested for their capacity to fix C1q. Of the 28, 15 had C1q+ DSA (raw MFI>500), but C1q binding did not predict AMR or graft loss. The third retrospective study in 48 KT recipients[15] positive for pre-transplant DSA by IgG (MFI ≥1000) and tested by C1q (12 C1q+, 36 C1q-, MFI ≥ 500) did not find an association between C1q binding and AMR or graft loss, but IgG MFI was higher in patients with C1q+ DSA (n=12) compared to C1q- DSA (n=36; p<0.001). The conclusion of these 3 papers is that, in contrast to the post-transplant setting, C1q testing in pre-transplant sera has limited use for immunologic risk assessment.

Using a somewhat different approach, Arreola-Guerra et al assessed the association between a positive flow cross match (FXM) and C1q binding in pre-transplant serum from 50 patients with potential living donors who had positive IgG DSA and negative CDC XM (25 positive FXM, 25 negative FXM)[16]. Factors associated with a positive FXM were the presence of C1q+ Ab, C1q+ DSAs and IgG DSA MFI. An interaction was observed between the presence of C1q+ DSAs and IgG MFI such that an association between C1q+ Ab with a positive FXM was observed only for IgG MFI < 5000 while there was no association for MFI > 5000. For the FXM prediction, IgG DSA MFI = 2300 had the best sensitivity and specificity (72% and 75%, respectively), C1q+ DSA was the most specific (~96%) and the combination of IgG DSA-MFI > 2300 and C1q+ Ab was the most sensitive (92%). The authors concluded that C1q+ Ab has high specificity but low sensitivity to predict FXM results in KT patients with DSA and negative CDC XM[16].

AMR and allograft failure post kidney transplant (KT): IgG versus C1q DSAs.

The development of de novo DSA is clearly associated with AMR and graft loss as well as AMR resolution after treatment. Because not all DSAs lead to bad outcome, different groups investigated whether C1q binding DSA could be a better predictor than standard IgG DSA. The first reports to show the clinical utility of C1q binding in KT were published by the group at Stanford in two studies of adult[17] and pediatric[18] KT recipients showing a significant correlation with adverse outcome and graft failure. In the first study of 31/274 adult KT recipients with IgG+ DSA, patients with C1q+ DSA (n=12) had more acute rejection (83% vs 42%, p=0.03), C4d deposition (67% vs 26%, p=0.06), transplant glomerulopathy (50% vs 0%, p=0.001) and graft loss (67% vs 16%, p=0.007) than those who were negative for C1q[17]. The second study in 35/193 pediatric KT recipients with documented de novo DSA identified by IgG SAB showed that the C1q+ DSA patients (n=15) were much more likely to have graft loss than the C1q-DSA patients (46.7% vs 15%, p=0.04; OR=5.8). In 22 of 35 patients with documented biopsy, C1q+ patients (n=10) were more likely to have C4d deposition on the biopsy (50% vs 8%, p=0.03) and acute rejection (60% vs 17%, p=0.03) than C1q patients (n=12)[18].

Other small studies followed these two initial reports to demonstrate the clinical utility of C1q binding post kidney transplant[19-21]. Freitas et al found that the presence of de novo C1q binding DQ DSA was associated with a 30% lower 5-year allograft survival[19]. Patients with de novo DQ DSAs and acute rejection had more C1q-binding antibodies than those without acute rejection (100% vs 37%, p=0.001). Another retrospective study[20] of 35 HK patients with both preformed and de novo DSAs showed that in those woth de novo DSA (n=18), transplant glomerulopathy occurred more in C1q positive compared to C1q negative patients (80% vs 0%, p=0.001). In a larger cohort of 284 DSA negative KT patients monitored prospectively [21], 31 (11%) developed de novo DSA during a mean follow up of 2.5 years. AMR was significantly higher in the C1q positive group (45%) compared to C1q negative and DSA negative groups (5% and 1% respectively). The study also included a cohort of 405 patients tested retrospectively that showed similar results.

The largest single study to demonstrate C1q clinical utility included a group of 1,016 KT recipients as well as an external validation cohort of 643 patients. This large study showed that assessment of complement binding capacity of DSAs using C1q was very useful to identify patients at high risk for kidney allograft loss and a much better predictor than the standard IgG assay[22]. Five-year survival of 54% in the C1q+ DSA group (n=239) was significantly lower compared to 93% in the C1q- DSA (n=77) and 94% in the no DSA groups (n=700). The results were similar in the external validation cohort. In this robust study, MFI cutoff > 500 was used to define DSA for both the standard IgG and the C1q assays. A detailed analysis to compare the predictive power of the standard IgG and C1q assays at different MFI thresholds was not performed as noted by others [23]. Thus, it is possible that a higher MFI threshold more commonly used in renal transplant might have improved IgG

performance. This was analyzed in a recent cross-sectional study of 86 DSA+ patients identified from a group of 741 KT recipients[24]. For an MFI threshold of 1000, sensitivity and specificity for AMR diagnosis were 82% and 62%, respectively, for IgG binding and 36% and 99%, respectively, for C1q binding. For an MFI threshold of 5000, sensitivities and specificities were 34% and 86% for IgG and 32% and 99% for C1q. The Authors estimated a 50% prevalence of silent AMR in DSA+ long term KT recipients and concluded that assessment of IgG MFI may add predictive accuracy without independent diagnostic advantage of complement binding[24].

A few recent studies did not find an association between C1q binding and AMR or graft loss and questioned the clinical utility of the C1q assay. A study performed in 35 KT patients with transplant glomerulopathy (TG), and de novo DSAs at the time of biopsy, showed a trend for a higher rate of graft loss in C1q positive compared to C1q negative patients (60% vs 40%, p=0.3)[25] and poor correlation with C4d[25]. Sicard et al[26] analyzed 69 KT recipients with AMR and assessed the ability of C1q and C3d binding DSAs to predict graft loss. The authors found a strong tendency for worse graft survival with C1q+ DSA that almost reached statistical significance (p=0.06) while no correlation was found with C4d deposition. In this study, C3d binding DSA correlated strongly with graft survival[26].

Another small study in 34 KT recipients concluded that C1q binding by de novo DSA in patients with AMR largely reflects differences in antibody strength[27]. AMR was more prevalent in C1q+ (n=12) compared to C1q- (n=22) patients (53% vs 13%, p=0.04) as a few earlier studies suggested[9,17,22]. However, AMR was also more prevalent in patients with DSA MFI > 7000 (n=18) compared to DSA MFI < 7000 (n=16) patients (78% vs 31%, p=0.017). The authors suggested that the association between C1q binding and AMR could be explained by significantly higher MFI levels in C1q+ patients with AMR than in C1q- patients with AMR. MFI>7000 had higher diagnostic sensitivity for AMR than C1q binding (74% vs 53%) but lower specificity (73% vs 87%)[27].

Some questioned the clinical utility due to technical limitations such as antibody level, complement interference and denatured HLA on SAB that could affect the clinical interpretation of the C1q binding[28-30]. It is unlikely that these would be more variable for the C1q assay than for the IgG assay and it seems reasonable that comparisons between the two methods can be made and conclusions drawn without invoking unidentified variable factors.

Conclusions. Immunologic risk assessment remains a clinically important problem in solid organ transplantation, perhaps the more so in thoracic transplantation where survival options are limited and the risk of error is grave. What methods to use and how to interpret the widely disparate data from the retrospective studies for the benefit of patients is a major challenge. Even with cutting edge technology, we have not been successful at finding an IgG threshold on the beads where there is consensus regarding the clinical significance. The body of evidence supporting the adverse consequences of C1q+ DSA continues to grow, suggesting that it has a useful role in transplant decisions. There is not a strict correlation between IgG MFI at any given titer and C1q positivity such that the former could be substituted for the latter. This suggests that the two may be correlated but are independent. Together they are more informative than either alone. Current evidence in kidneys also suggests that the subclass of the IgG may be just as important as the MFI and subclass plus C1q may be more clinically informative.[31]

Moving forward will require leaving behind outdated methodologies that may be comfortable and being more succinct in our data descriptions. In the almost 50 years since Patel and Terasaki demonstrated that cytotoxic antibodies to the donor were correlated with hyperacute and accelerated acute rejection of kidneys, the transplant community has become comfortable with terms like PRA, antibody and crossmatch, as if each of these were a unique entity. In fact, PRA is not uniformly determined and doesn't equal cPRA, antibody detection is widely variable and dependent on method

(even on the beads), and crossmatches may be virtual or physical. As we move forward in our understanding of the relationship of all of these components, we need to be much more specific when using these terms, both in written form and in conversation, so that the best data can be accumulated and the best studies done to advance our understanding of the interplay between and among them and develop the most effective treatment strategies for our patients.

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