Clinical Relevance of Pre-transplant HLA Donor Specific Antibodies (DSA) in Renal Patients waiting for a Transplant: A Risk Factor

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Abbreviated title: Clinical Relevance of pre-transplant DSA

Key words: Pre-transplant antibodies Sensitisation Kidney transplant Clinical relevance Solid phase assays

Abbreviations: CDC: Complement Dependent Cytotoxicity DSA: donor specific antibody NSPs: Non Sensitised Patients SPs: Sensitised Patients HSPs: Highly Sensitised Patients

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Abstract

The use of highly sensitive solid-phase antibody detection assays, including x-MAP multiple bead-based technology (Luminex) has greatly enhanced our ability to accurately detect and define very low levels of HLA antibodies. These developments have led to patients having increasing list of antibody specificities (which may not be clinically relevant), resulting in a new ‘technological barrier’ to transplantation in sensitised patients (SPs). Alloantibodies are known to play a major role in all types of solid organ rejection; the presence of low titre DSA-identified pre-transplant is associated with an increased risk of antibody mediated rejection (AMR). However, these low titre antibodies do not represent an absolute contraindication to transplant. Improvement in the diagnosis and treatment of AMR will allow sensitised patient with DSA to be successfully transplanted in the short term but extended follow up is required to ensure acceptable long-term graft survival in this group. These factors need to be integrated into the decision algorithms for immunosuppressive treatment in patients at immunologic risk.

Introduction

Serology formed the basis for clinical Human Leukocyte Antigen (HLA) testing when it was described by Dausset in 1954 (1). Its relevance to transplantation was uncovered by the work of Friedman and Terasaki in the 1960s (2, 3) and histocompatibility testing improved significantly when the agglutination assay was replaced with the complement-dependent microlymphocytotoxicity (CDC) test (4). By the end of 20th century, the preferred platform for HLA antibody testing in many laboratories is changing from CDC to solid phase antibody assays: however, the clinical relevance of antibodies defined by these assays is still a matter of debate and this will be discussed in some detail.

In the UK, short to medium term graft survival is 93% at 2 years and 83% at 5 years (5): However, despite progress in the development of immunosuppressive therapy, there has been little impact on long term graft survival. UK Transplant (UKT) data show that 23% of patients awaiting their first renal transplant are sensitised to HLA (6). In addition, 52% of patients waiting re-transplant are sensitised, with a larger proportion being highly sensitised having greater than 85% calculated reaction frequency (cRF). This cRF (compared to PRA) is determined using patient’s unacceptable antigens and HLA frequencies derived from the HLA types of 10,000 donors, thereby standardising patient sensitisation status across centres in UK.

Prior sensitisation due to blood transfusion, pregnancy and organ transplantation can lead to sustained production of alloantibodies. The occurrence of natural antibodies to HLA has also been reported but their clinical significance is not yet known (7). Alloantibodies have been shown to play a role in all types of solid organ rejection; hyperacute, acute and chronic (8, 9). The clinical relevance of the humoral response was clearly demonstrated as early as 1969 showing that early alloantibody-mediated tissue injury is caused by the binding of antibody to HLA antigen on endothelial cells (3). Subsequently, a number of studies have shown that patients with pre-transplant HLA antibodies have an increased risk of graft failure and it is widely acknowledged that these antibodies form a significant ‘biological’ barrier to transplantation (10, 11). The role of non-HLA antibodies and the non-immunological factors have been shown to play a role; their clinical relevance is discussed elsewhere.
The development of solid-phase antibody detection assays, including flowcytometric methods and x-MAP multiple bead-based (Luminex) technology, has revolutionised the detection and definition of HLA antibodies with high degree of sensitivity and accuracy (12, 13-15). These technologies have led to patients having greater number of antibody specificities being defined. Consequently, many previously non sensitised patients (NSPs) have become apparently sensitised patients (SPs) and many already SPs have become ‘highly’ sensitised patients (HSPs). Detection of these alloantibodies, often of unknown clinical relevance has lead to discrimination against sensitised patients with respect to organ allocation.

To determine the clinical relevance of alloantibodies defined by solid phase assays is a major challenge facing the H & I community. Various attempts to answer this question have led to some confusion as to what type and how much donor reactive antibody is clinically relevant in any given transplant situation. To reach universal consensus on these parameters will require greater national and international collaboration in determining standard practices.

**Role of HLA antibodies in renal allograft rejection**

Acute, accelerated acute and chronic rejections are believed to have humoral components. Numerous studies have suggested a two to ten fold higher incidence of acute rejection in the presence of HLA antibodies (16, 17-22). The CDC method is a functional test as it detects complement fixing antibodies (IgM, IgG1 and IgG3) and these antibodies have been regarded as detrimental to the graft (3). In biopsy, the detection of complement split product C4d deposition on vascular endothelial cells of peritubular capillaries is considered highly indicative of AMR (9, 23).

Studies of renal recipients using flow cytometry have reported that only IgG and not IgM HLA antibody are associated with acute and chronic rejection (16, 22). Other studies have reported that IgG is associated with more severe rejection than IgM antibody (32), while anti-HLA class II responses have been strongly associated chronic rejection in recipients of living donor kidneys (24). In patients without HLA antibodies, chronic rejection was less frequent and graft survival higher than in patients with HLA antibodies (24, 25). In a multicenter prospective study, HLA antibodies were demonstrated to be strongly predictive of long-term graft failure (26). However, other data is at odds with this. The finding that up to 45% of patients with de novo antibody had a functioning graft five years later indicates that these antibodies do not necessarily herald graft failure. One hypothesis is that a process of accommodation occurs, a condition in which an organ transplant functions normally despite the presence of anti donor antibodies (27). The mechanism of this accommodation is unclear.

Humoral, acute and cellular rejection episodes generally appear in the early post transplant period and can be successfully reversed with early intervention (28). The treatment of chronic antibody mediated rejection is more difficult and to date there are no proven therapies for this condition.

**Antibody Identification**

**Complement Dependent Cytotoxicity (CDC)**

The long established CDC assay is over 30 years old and is still widely used (29, 30). In our centre, patient samples are tested using a well characterised selected panel of cells from 40 donors which includes most HLA antigens. Our technique is a variant of the standard NIH
method (4), employing extended incubation times, 60 minutes with patient serum and target lymphocytes followed by 120 minutes with complement for increased sensitivity.

CDC is a robust method for the detection and definition of complement-fixing IgG (IgG1 and IgG3) and also IgM antibodies, directed against HLA and/or non-HLA targets (including autologous reactive antibodies). Although criticised for its low sensitivity (31), the pattern of CDC panel reactivity as a measure of anti-HLA sensitisation (in vivo), is a useful indicator of an increased risk of rejection and a potential hyperacute rejection. Its inherent disadvantages include, requirement of a large panel of viable lymphocytes to cover most commonly occurring HLA specificities, is subjective, requires manual reading of the test, expert analysis and is unable to detect noncomplement-fixing antibodies. The use of commercially available frozen cell trays has been one approach to overcome some these problems associated with CDC (30).

**Solid Phase Assays:**

(a) **ELISA**
Affinity purified HLA class I and class II antigens of known HLA type, either pooled or specific are bound directly to wells of microtitre plates. Antibody reactivity is detected by the addition of a secondary antibody, an enzyme-conjugated anti-human immunoglobulin that induces a colour change on addition of the enzyme substrate, detected by measuring optical density. This is a quick, objective, semi-quantitative assay which detects both complement and non-complement fixing HLA antibodies with a reported greater sensitivity than CDC tests (32).

(b) **Flow PRA/x-MAP Multi beads based Technology (Luminex)**
These flowcytometric based assays involve the use of micro-particles or beads coated with pooled or specific purified HLA class I and class II antigens. Up to 100 beads can be combined in a single test. HLA specific binding can be detected using a fluorescence-conjugated secondary antibody directed against human immunoglobulin and fluorescence is measured in a flow analyser (33). Standard solid phase assays are generally designed to detect IgG (IgG1, IgG2, IgG3 and IgG4) antibodies but can also be modified to detect other immunoglobulin isotypes (IgM, IgA). Single antigen (SA) assays have single recombinant HLA antigen on each bead. These SA assays are invaluable for accurate assignment of antibody specificities in highly reactive sera (34) and hence can be applied to the monitoring patient antibody profiles in relation to DSA pre-transplant following antibody removal and identification of DSA post transplantation (35, 36). Application of this technology has been crucial in the identification of the HLA class I epitopes, which explains why donor mismatch of a single antigen can result in antibody against a series of antigens (due to an epitope mismatch) previously defined as serologic cross-reactive groups (CREGS) (37). In future, we conjecture that we may be assigning antibody specificity to the relevant target epitope(s) rather than to antigens (38). Recent reports show that these assays can also be modified to potentially identify clinically relevant complement C4d fixing antibodies (39).

In these solid phase assays, some caution must be exercised as HLA extraction and binding to the carrier may result in subtle conformational changes of tertiary protein structure, thereby modifying the structure of the molecules and the accessibility of epitopes. Instances of impaired binding of HLA antibodies have subsequently resulted in false-positive or false-negative reactions (40, 41). Further antigen density on beads may not correlate with the natural HLA expression on cells in vivo. It is important that the Luminex SA beads are ‘normalised’ for the quantity of antigen per bead by their reaction with the monoclonal w6/32 antibody. The reactions are then compared with standard beads to derive the molecules of equivalent soluble fluorochrome (MESF) values (42); this might allow inter-assay comparison of results.
Antibody screening information is essentially a means of facilitating transplantation in sensitised patients and reducing the rate of positive crossmatches thereby minimising cold ischemia times. This can be achieved by identifying the pre-transplant risk factors associated with the presence of DSA; we must consider the type of antibody – complement fixing or not, HLA or non-HLA, present in current and/or historic samples and the titre. It is extremely important that they are detected pre-transplant to categorise patients groups as NSP, SP or HSP. This allows appropriate donor selection and immunosuppression as appropriate to minimise the risk of AMR. Antibody identification is important but the absence of a reaction is equally important and can be analysed and exploited especially in a sensitised patient to facilitate transplantation. An approach, widely used within Eurotransplant is to utilize the ability of the HLAMatchmaker computer algorithm to predict acceptable mismatches to enhance transplantation in these patients (43, 44). In our centre (perhaps controversially), Luminex defined (CDC negative) non complement fixing antibodies are listed as ‘acceptable antigens’ in relation to organ allocation based on our previous findings (45).

Clinical Relevance of pre-transplant DSA: Day 0 (D0) and in historic sample.

There is some evidence that enabled us to evaluate the clinical impact of low titre pre-transplant DSA at D0 or in historic samples. Consistent to our finding in a recent retrospective study where HSPs were transplanted on the basis of negative CDC crossmatch at (D0), it has been shown that DSA detected by Luminex but not CDC was associated with ‘treatable’ rejection episodes but with the preservation of long-term graft survival (45, 46). Other studies have illustrated the clinical significance of HLA antibodies detected only in historic samples, overriding the importance of D0 sample (47). Also, early graft loss in patients with current CDC negative but historically positive (48). Others have shown that patients who developed AMR were more likely to have DSA pre-transplant (47, 49, 50). Further, patients with DSA without AMR have been shown to have the same graft survival as patients without DSA (50). Others have suggested that preformed DSA at any titre at the time of transplant are clinically important (51). More recently in a cohort of living donor renal transplant recipients, it has been demonstrated that the presence of pre-transplant low titre DSA is associated with an increased risk of AMR, despite negative pre-transplant cytotoxicity and flowcytometric crossmatches (52). Taken together these data suggest that the presence of DSA-identified pre-transplant at low titres is associated with an increased risk of AMR: However, this does not represent an absolute contraindication to transplant. Improvement in the diagnosis and treatment of AMR will allow sensitised patient with DSA to be successfully transplanted. These factors need to be integrated into the decision algorithms for immunosuppressive treatment in patients at immunologic risk.

In our centre, we have recently carried out a retrospective study on patients transplanted during the era 1999 to 2001 with at least five years post transplant follow up, in an effort to demonstrate clinical relevance of pre-transplant DSA solely detected by Luminex assays (45).

Prior to 2001, CDC was the main method for antibody detection. A total of 121 recipients were retrospectively screened using sera taken immediately prior to transplant for the presence of HLA antibodies using Labscreen mixed LSM™ assays (One Lambda inc., Conga Park, CA). Patients positive for HLA antibodies were further tested using Luminex single antigen beads. A negative cut off was derived using receiver operator characteristic (ROC) curve analysis. Based on the results of Luminex analysis 83 patients had no antibodies (NAB), 22 patients had non donor specific antibodies (NDSA) and 16 had donor specific antibodies (DSA) (Table 1).
There were no cases of hyperacute rejection despite the DSA group having measurable circulating donor specific antibody (CDC negative) present on the day of transplant. The number of patients with acute rejection episodes and rate of delayed graft function (DGF) was similar in the all three groups irrespective of antibody status. One year graft survival was similar across the three groups with (Table 1); in addition one year patient survival was similar (100% for DSA, 100% for NDSA and 98% for NAB; (p=NS). Censored graft function at one year also showed no differences (Table 1). It appears, therefore, that the presence of DSA at the time of transplant has little impact on first year graft outcome. Five year patient survival was again similar in the three groups in those for which survival data was available (DSA 83%, NDSA 100%, NAB 94%; p=NS). Graft survival at five years was inferior in DSA group compared to others but this did not reach significance (Table 1).

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<td>5-yr graft survival</td>
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Table 1: one and five year outcomes – there were no significant differences in one and five year graft survival in the three groups on univariate analysis. In addition, creatinine levels at one and five years were also similar among groups.

Transplantation across DSA at time of transplant: Short and long term outcomes

It has been shown that the presence of DSA detected only by Luminex in the context of a negative CDC and flowcytometric crossmatch is not associated with hyperacute rejection or poorer early outcome as measured by DGF, acute rejection rates and graft survival and function at 1 year. Greater than 60% graft survival beyond five years is probably acceptable even today in a high risk group of patients (45, 46), given the relatively poor survival of patients on dialysis compared to those receiving a transplant (53). In our study 16 transplants would not have occurred, as the patients in the DSA group would not have been offered the kidney they received. These results show that we need to be very careful in defining unacceptable specificities, as sensitised patients are potentially being denied organs which, if transplanted are likely to yield reasonable short to medium term results (54-56). Long term outcomes for these transplants are yet to be seen; follow up studies are urgently needed.

Detection of alloantibodies by solid phase assays which may not be clinically relevant could lead to discrimination against sensitised patients with respect to organ allocation: However, in the modern era, these ‘biological’ and ‘technological barriers’ can sometimes be overcome by the careful use of plasma exchange, administration of IVIg and anti CD20 without compromising transplant outcomes in the short term. These techniques have allowed transplant to proceed in what might have been previously considered to be ’untransplantable’ patients (57-61)

Based on our findings, we have recently adopted a policy change with regards to listing of ‘unacceptable’ antigens for sensitised patients with UKT. The sensitised patients are at greater risk of hyperacute rejection (identified as immediate and irreversible graft loss within first 24 hours of reperfusion) and subsequently AMR, and acute cellular rejection. Now that AMR can be effectively treated (62), hyperacute rejection which remains untreatable may pose the only major contraindication to transplanting the sensitised patient.
In order to assess the risk of hyperacute rejection the more sensitive the test is at detecting HLA antibodies, the less is its specificity at predicting hyperacute rejection. More sensitive tests may pick up antibodies present at very low titres or that bind with less affinity. Also, non complement binding antibodies may be detected in non cytotoxic assays. These antibodies may not lead to immediate graft loss but can be viewed as a marker of increased risk of rejection or graft loss over time (63). Accordingly, this has led us to review the antibody status of patients on our local transplant waiting list. In some sensitised patients we have been able to identify Luminex defined (CDC negative) antibodies and have listed them as ‘acceptable antigens’ with UKT. In non sensitised patients where we are confident that the patient is antibody negative by CDC and Luminex, we have adopted a ‘virtual crossmatch’ strategy i.e. no prospective crossmatch performed at the time of transplant, thus reducing cold ischemia time significantly. At our centre, since August 2008, twenty such transplants have been successfully carried out and as predicted retrospective crossmatches were negative in all cases (BTS abstract 2009).

**Conclusion**

Results derived from the new sensitive solid phase antibody detection methods can potentially discriminate against sensitised patients. It is, therefore, essential that antibody screening results are correctly interpreted in light of new interventions that can be used to enable transplantation in these circumstances. Antibodies defined by these assays are useful predictors of probable AMR, which can be effectively treated. However, their effect on long term graft survival is still unknown but effective desensitisation, vigilant monitoring, and early identification and treatment of AMR may be critically important in attaining the best possible long-term results for sensitised patients.

Since January 2008, we have successfully transplanted 19 SPs including 15 HSPs in whom the HLA specificities defined by solid phase assay were listed as ‘acceptable’ instead of as ‘unacceptable’. In these patients the CDC crossmatch was negative but flowcytometric positive in some cases (BTS abstract 2009); to date all patients have functioning graft. Patients with DSA at the time kidney transplant are considered to be of high immunological risk and are subjected to more intensive immunosuppression regimens. Improvements in diagnosis and treatment of AMR allow patients to be transplanted with reasonable outcomes despite a positive flow cytometric crossmatch. Extended follow up is required to ensure acceptable long-term graft survival in this group.

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