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Clinical Relevance of Complement Fixing Antibodies in Cardiac Transplantation

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Abbreviated title: Antibodies in cardiac transplantation
Abstract
In this review, the relative importance of pre-transplant complement fixing and non-
complementing fixing HLA and non-HLA antibodies is reviewed. Sera from 565
adult cardiac transplant recipients was retrospectively analysed for the presence of
HLA antibodies using CDC, HLA coated Luminex beads and C4d deposition on
Luminex beads and results correlated with graft survival. Whereas 14/565 patients
had CDC positive donor specific antibodies (DSA) before their transplant, this
number was increased by 53 using Luminex beads, of these 19 had DSA by Luminex.
Patients negative for CDC cross-match, but Luminex +ve with DSA had poor one
year survival (42%) compared to 77% one-year survival of patients with CDC-ve,
Luminex +ve non-donor specific antibodies. The effect of donor specific antibodies
on allograft survival was further analysed according to titre, immunoglobulin sub –
class and ability to fix C4d onto Luminex beads. Ability to fix C4d, but not antibody
titre or Ig subclass was strongly associated with poor allograft survival (P=0.0002). A
retrospective of sera form 616 cardiac transplant patients revealed presence of IgM
complement fixing non-HLA antibodies was associated with early graft failure and a
diagnosis of Primary Graft Failure. In conclusion, complement fixing antibodies to
relevant antigens are associated with poor allograft survival after heart transplantation.
Introduction
It is well established that transplantation of hearts into patients with pre-formed donor specific complement fixing HLA antibodies results in hyperacute or accelerated acute rejection [1], in much the same was as would happen if kidneys were transplanted into sensitised recipients [2]. Until about 1995, the cytotoxic cross match, which measures complement fixing antibodies was used to determine whether patients had antibodies to donor leukocytes. Since that time there have been major advances in the technology used to detect antibodies, and a greater understanding of importance of non-HLA antibodies in causing early damage to the graft. The use of solid phase assays has increased sensitivity such that approximately 5 times as many patients make HLA antibodies compared to the cytotoxic method [3]. The increased sensitivity raises the question about the clinical relevance of these antibodies, since many of the antibodies detected will not be complement fixing; further stratification of antibodies may be necessary to avoid depriving patients of transplants due to antibodies which may not be important. The purpose of this review is to summarise about what is known about the clinical relevance of pre-transplant antibodies detected by solid phase assays. In addition the role of complement fixing non-HLA antibodies will be reviewed.

a) HLA antibodies.
Studies from renal centres have demonstrated that presence of pre-transplant donor specific HLA antibodies detected by solid phase assays [4,5], in the absence of a positive cross-match with donor cells, are associated with higher rates of graft failure. In order to investigate whether this was the case for heart transplant recipients, we undertook a large retrospective study of pre-transplant serum from 565 adult cardiac transplant recipients transplanted between 1991-1995. All patients had been transplanted with a negative cross-match to donor T cells, except 4 who were transplanted prior to 1992 and a recent patient who became HLA antibody positive due to a unreported pregnancy. All sera were tested for HLA antibody reactivity using standard complement dependent cytotoxicity (CDC) assays and solid phase Luminex based assays. Patients were initially screened using Luminex multiple antigen beads; positive sera was further analysed for HLA specificity using single antigen beads, and donor specificity was assigned. Prior to transplantation 14 patients were known to have produced HLA antibodies, detectable by CDC assays. Retrospective analysis using the Luminex assays revealed a further 53 patient samples contained HLA antibodies. CDC assays detected HLA class I antibodies in 12 serum compared with 54 using Luminex and class II antibodies in 4 compared with 25 by Luminex (Table 1). All sera that were positive by CDC were also positive by the Luminex assay (Table 1). Actuarial graft survival was analysed according to pre-transplant HLA antibody status (Fig 1); it can be seen that the 5 patients transplanted with CDC+ve cross-match and Luminex +ve donor specific antibodies (DSA) showed poor one year survival (41.7%). Similarly, patients negative for CDC cross-match, but Luminex +ve with DSA also had poor one year survival (42%) compared to 77% one–year survival of patients with CDC-ve, Luminex +ve non-donor specific antibodies. The survival of patients with non-donor reactive HLA antibodies, detected by Luminex, is equivalent to patients negative for antibodies (Fig 1). These results attest to the importance of donor specific HLA antibodies as determinant of allograft survival. The majority of graft failure within the donor specific groups occurred within the first 3 months after transplantation.
Properties of HLA Abs that influence pathogenicity

Little is known about the properties of antibodies that contribute to their pathogenicity in a transplant setting. Factors likely to be important include, ability to fix complement, titre, immunoglobulin subclass and antigen specificity.

C4d fixing antibodies: It is known that antibody mediated activation of complement and deposition of various split components (C1q, C4b, C4d, C3a, C3d) contribute to graft damage in the absence of assembly of the terminal lytic complex (C5-C9) and cell lysis[6,7]. Thus deposition of C4d along peritubular capillaries of renal biopsies [8] and to a lesser extent in cardiac biopsies [9] has become a useful adjunct to diagnosis of acute humoral rejection. We therefore devised an assay to determine ability of Luminex binding antibodies to fix C4d on to Luminex beads [3]. Pre-transplant serum from sixty-seven patients (from the 565 patients in the 1991-2005 study described above) were found to contain HLA abs detected by Luminex, of these 22 cause C4d deposition on Luminex beads. Of the 22 Luminex positive C4d fixing sera, 11 contained DSA and 11 had no detectable DSA. The one year graft survival for C4d+ DSA (n=11) was 20% compared to 91% for C4d-ve non-donor specific abs (n=11), 54% for C4d-ve DSA (n=13) and 75% for antibody negative recipients (p=0.0002). Fig 2. These results demonstrate that pre-transplant DSA that fix C4d in this test, are as poor a prognostic indicator as an IgG cytotoxic cross-match. It is interesting that graft survival of patients with DSA that do not fix C4d is in between those who are antibody negative and those with C4d fixing DSA. It may be that pre-transplant antibodies which do not fix C4d become complement fixing after transplantation. On the other hand, antibodies that do not fix C’ may be damaging due to their ability to activate endothelial cells [10] and cause exocytosis of weibel palade bodies and hence producing a pro-coagulation phenotype [11].

Antibody Titre: An analysis of 145 serum samples containing HLA antibodies as defined by Luminex assays was performed comparing Mean Fluorescent Intensity (MFI) values to C4d binding results. The mean MFI of the highest bead value in the 51 serum which demonstrated C4d fixation in the Luminex assay was 13069 +/- 5200, compared to 4982 +/- 3335 for the C4d negative group p<0.0001. However, 12 of 89 samples with an MFI below 8317 (Mean MFI of C4d Negative + 1SD) had fixed complement, whilst 17 of 56 samples with MFI >8317 were unable to fix complement, suggesting the association between MFI and C4d is inconsistent.

Furthermore, analysis of patients who had undergone transplant with donor specific Luminex detected antibodies revealed that C4d fixing antibodies were a better predictor of poor graft survival than patients with high MFI DSA. Patients who were C4d positive had a 90 day and 1 year graft survival of 30% and 20% respectively compared to 58% and 42% for patients with high MFI (defined as MFI>8317). These results suggest an inconsistent association between MFI and ability to fix C4d; they also suggest that C4d fixation is a better predictor of poor graft survival than MFI of DSA.

Imunoglobulin subtypes: In humans, IgG1 and IgG3 are the IgG subtypes known to activate complement via the classical pathway. This response is triggered when C1q binds to the Cγ2 region of IgG1 or IgG3 or the Cμ3 region of IgM. Identification of
the IgG subtypes in 50 HLA antibody positive serum samples was performed using secondary antibodies to IgG1, IgG2, IgG3 and IgG4 in Luminex assays. PE-conjugated secondary antibodies (diluted 1/50) were used instead of the PE-labelled anti-human IgG, which is normally used. IgG1 was the predominant isotype, being present in 36/50 of the samples; it was the sole IgG isotype in 18 cases. IgG2 was present in 15 samples of which only 4 contained IgG2 alone. IgG3 was detected alone in 7 samples, with IgG3 also present in a further 13 samples in combination with other subtypes. IgG4 was found in 10 samples although on only one occasion as the sole subtype.

There was no correlation between IgG subclass and the ability to fix complement in the Luminex assay, with only 19 of the 44 samples that were either IgG1 or IgG3 able to fix C4d. Of the 18 samples that contained IgG1 alone, only 6 fixed C4d. It is interesting to note however, in the 12 samples which were both IgG1 and IgG3, 8 fixed complement in the Luminex assay. The samples with IgG4 alone did not fix complement. Studies from renal centres have also reported that IgG1 is the dominant isotype in serum from sensitised patients and that mixtures of IgG1 and IgG3 are better able to fix complement than IgG1 alone ([12,13]).

Multivariate analysis of sera containing IgG1 (n=36), IgG2 (n=15), IgG3 (n=20) and IgG 4 (n=10) donor specific HLA antibodies, and graft survival at 1, 3 and 5 years revealed no difference in graft survival. In view of the small number of samples studies (n=51) further studies are warranted on effects of Ig subclass and graft survival. Further work needs to done to explore synergy between Ig subtypes and synergy between multiple antibodies binding to the same cell, since they may help define their clinical relevance. Currently, it appears that the C4d fixing assay, together with determination of donor specificity, is a useful test for predicting early graft failure after cardiac transplantation.

b) Non-HLA antibodies

It is well established that antibodies to non-HLA antigens, such as vimentin [14] MICA [15] and angiotensin-receptor antibodies [16] are associated with rejection after solid organ transplantation. Use of solid phase assays for determining HLA antibodies, has resulted in a reduced incidence of accelerated acute rejection after renal transplantation [17], although when it occurs the causes remain unknown. Recent data from our laboratory has re-evaluated the role of complement fixing IgM non-HLA antibodies in pre-transplant serum as a risk factor for early graft failure after heart transplantation [18].

Complement fixing IgM non-HLA antibodies

One of the advantages of performing a cytotoxic cross-match with donor leukocytes is that presence of autoreactive cytotoxic antibodies can be discovered. These antibodies are revealed as an IgM positive cytotoxic cross-match with donor leukocytes, which are also positive against a panel of HLA typed leukocytes, but with no particular specificity. Some centres will check the sera against patients’ own cells, and if positive, these are called cytotoxic autoreactive non-HLA IgM antibodies. The majority of renal centres have reported that this antibody is not damaging to renal allografts [19,20]. A single study of heart transplant recipients reported that appositive IgM cross-match had no effect on graft survival [21] but whether these were non-HLA abs was not determined. We performed a large retrospective study of pre-transplant sera from 616 adult heart transplant recipients transplanted between 1991
and 2003 [18]. All sera were tested with Luminex beads to assess presence of HLA antibodies. Of these 616 patients, 488 were Ab negative, 59 were IgM non-HLA +ve, 58 were IgG HLA Ab +ve, 11 were IgM HLA Ab +ve. Actuarial graft survival demonstrated that survival of grafts in patients transplanted with IgM non-HLA antibodies (n=59) was 55.9, 54.2, 49.9, and 43.4% at years one, two five and ten, compared to 75.8, 73.7, 66.6 and 52.8% respectively for patients who were antibody negative (Fig 3, p=0.0085). Indeed, it can be seen from Fig 3, that survival is no worse for patients with non-HLA IgM antibody than for those with donor specific IgG HLA antibodies (p=0.194). Multivariate analysis demonstrated that pre-transplant IgM non-HLA abs remain a significant risk factor for poor survival after adjusting for other poor prognostic indicators (HR 2.359, p=0.0002). The figure demonstrates that the main effect of this IgM non-HLA ab occurs in the first year after transplantation.

Prior to 1999, solid phase immunoassays were not available to detect HLA antibodies and it was possible to miss HLA specificities; in this study we had re-analysed all IgM non-HLA antibody positive sera in this study with Luminex beads and confirmed that none of them had HLA reactivity. The specificities of the IgM antibody are not known. In many centres these IgM non-HLA antibodies are called autoreactive antibodies. Of the 59 sera with IgM non-HLA antibodies 54% had reactivity to recipient leukocytes. This is likely to be an underestimate of autoreactive cases; some of the peripheral blood samples from recipients had been stored at 4 degrees, a procedure which, in our experience, inhibits detection of autoreactive antibody. Some of the sera contained IgM antibodies to the autoantigen vimentin [14](14% of our cases). It is likely that many of them were autoantibodies reacting to non-polymorphic autoantigens, although some may be directed at polymorphic non-HLA antigens found on leukocytes. In this study, MICA antibodies were not investigated. Antibodies to MICA are not detected by the lymphocytic cell cross-match [15] making it unlikely that MICA is responsible for the effects described here. Although one might speculate that presence of autoimmune antibodies prior to transplantation may be associated with pre-transplant disease, such as dilated cardiomyopathy which is associated with autoimmunity [22], in this study this was not found to be the case.

The histological criteria of antibody-mediated rejection (AMR), published in 2005 [23] include endothelial swelling, presence of intravascular macrophages as well as capillary C4d positivity, with the presence of interstitial haemorrhage, acute inflammation, vasculitis and myocyte necrosis in the later stages. Biopsies or post-mortem specimens from 17 patients with IgM non-HLA antibodies and 7 patients with IgG HLA were re-reviewed for evidence of AMR, using these criteria.

Retrospective staining for C4d on all available material from IgM non-HLA and IgG positive patients demonstrated that IgM non-HLA antibodies are associated with complement fixation in the allograft; 5/17 specimens had strong capillary staining. Others have also shown that non-HLA IgM antibodies are associated with C4d deposition in allografts, thus 75% of renal allografts from ABO incompatible recipients demonstrate C4d peritubular capillary deposits A detailed review of the histology, cardiac pathology and consensus cause of death (COD) failed to demonstrate that any of the 17 patients with IgM non-HLA could be said to have died of AMR, but the histology of 3 of 7 patients with IgG HLA was consistent with the 2005 criteria for AMR. All three IgG HLA patients had DSA and 2 of them were
positive for C4d on their post-mortem (PM) specimens. The detailed histological analysis of PM or ‘last biopsies’ revealed an association between IgM non-HLA antibodies and presence of ischaemic damage in the heart, shown in 8/17 cases. There was also an association between IgM non-HLA antibodies and Primary Graft Failure. There is emerging evidence for a role for the classical pathway of complement fixation and IgM autoantibodies in the pathology of ischaemic reperfusion injury [24,25]. For example, immunodeficient RAG-/- mice are resistant to myocardial and intestinal re-perfusion injury, but this is restored by infusion of either natural IgM antibodies [25,26] or IgM autoantibodies to phospholipids [27,28] or ribonuclear-proteins[29]. It has been suggested that IgM natural antibodies and IgM autoantibodies bind to neo-antigens exposed on the surface of cells exposed to ischaemia [30]. It is likely that the antibodies in our study are low avidity polyspecific antibodies because they work better in the cold than at room temperature (Smith, JD, unpublished, 2008). The latter property suggests they may have specificity for carbohydrate antigens [31]. The fact that they work better in the cold suggests their pathogenicity may be unique to cardiac transplantation, where patients are subjected to moderate hypothermia during cardiopulmonary bypass, during the transplant procedure, and the transplanted heart is subjected to cold storage. These antibodies are very effective at fixing complement. We suggest that the presence of IgM autoantibodies in patients at the time of implantation may result in binding of IgM to de-novo expressed autoantigens exposed on the allograft during ischaemia. It has been shown that IgM co-localises with complement in infarcted human myocardium, indicating the presence of autoantigens in damaged cardiomyocytes [32]. Other possible targets would be apoptotic circulating cells including activated neutrophils [33] and platelets [34] which express autoantigens on their surface [35,36]. Fixation of complement is a highly inflammatory process, having many down stream effects [6,37] including xenograft [38] and allograft rejection [39]; it is associated with platelet aggregation and endothelial activation [40]. PGF is the most common cause of death in the first 30 days after heart transplantation [41]. It is a poorly understood entity, but is significantly associated with hypoxic injury of the donor heart [42] making it likely that ischaemia reperfusion injury is an important causative factor.

**In conclusion:** Detection of complement fixing antibodies by the cytotoxic cross match has served the transplant community well for forty years. Use of solid phase assays to determine specificity of antibodies has advanced our knowledge of the range of HLA antibodies which are produced but leaves a gap of knowledge regarding function and pathogenicity. Further research, using a combination of assays to determine specificity and function will pave the way for better understanding of the clinical relevance of antibodies in transplantation.
Table 1.
Comparison of CDC and Luminex assays (565 sera)

<table>
<thead>
<tr>
<th>HLA Abs</th>
<th>Class I</th>
<th>Class II</th>
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<tbody>
<tr>
<td>CDC +ve/Luminex +ve</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>CDC +ve/Luminex -ve</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CDC -ve/Luminex +ve</td>
<td>53</td>
<td>42</td>
</tr>
</tbody>
</table>
Figure Legends

**Fig 1. Comparison of detection method and donor specificity on graft survival.**

Actuarial graft survival according to antibody status of pre-transplant sera: sera contained CDC +ve Luminex + ve DSA (n=5), CDC+ve Luminex +ve non DSA (n=4), CDC-ve/Luminex +ve DSA (n=19), CDC-ve/Luminex +ve non DSA (n=39) and patients with no detectable HLA antibodies (n= 498). Both CDC DSA and Luminex DSA have significantly reduced graft survival compared with other groups, p=0.0039. Reproduced from Smith et al [3], with permission from Blackwell Munskaard.

**Fig 2. Effect of C4d depositing DSA on graft survival.** Actuarial graft survival according to antibody status of pre-transplant sera; sera contained C4d+ve DSA (n=11), C4d+ve non DSA (n=11), C4d-ve DSA (n=13), C4d-ve non DSA (n=32) and no detectable HLA antibodies (n= 498),p=0.0002. Reproduced from Smith et al [3], with permission from Blackwell Munskaard.

**Fig 3. Effect of IgM non-HLA antibodies on graft survival.** Kaplan-Meier survival for allografted hearts (n=616) according to pre-transplant antibody status. Patients were either antibody negative (n=488), IgM non-HLA positive (n=59), IgG HLA non-donor specific (n=36), IgG donor-specific (n=22) or IgM HLA positive (n=11). Reproduced from Smith et al [18] with permission from Lippincott Williams &Williams.
References


Fig 1

- CDC+ve/Luminex+ve/DSA N=5
- CDC+ve/Luminex+ve/No DSA N=4
- CDC-ve/Luminex+ve/DSA N=19
- CDC-ve/Luminex+ve/No DSA N=39
- Negative N=498
Fig 2

- C4d -ve/No DSA N=32
- C4d +ve/No DSA N=11
- C4d -ve/DSA N=13
- C4d +ve/DSA N=11
- Negative N=498
Fig 3.