

C4d Fixing, Luminex Binding Antibodies—A New Tool for Prediction of Graft Failure After Heart Transplantation

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The standard method to detect pretransplant antibodies has been the complement dependent cytotoxicity (CDC) test of donor leukocytes. Solid phase assays to detect HLA antibodies in pretransplant serum reveal a greater number of sensitized patients, but their clinical impact is less certain. Here we have developed a method of detecting C4d fixing HLA antibodies on Luminex beads. Pretransplant serum from 565 cardiac transplant patients was retrospectively tested for the presence of HLA antibodies using CDC, HLA coated Luminex beads and C4d deposition on Luminex beads, and the results correlated with graft survival. Whereas 5/565 patients had CDC positive donor specific antibodies (DSA) before their transplant, this number was increased by 19 using Luminex beads. The 1-year survival of CDC -ve/Luminex +ve patients with DSA (n = 19) was 42% compared with 77% for CDC -ve/Luminex +ve without DSA (n = 39, p = 0.0039). Fixation of C4d (22/67 Luminex positive sera) had a negative effect on graft outcome; 1-year graft survival was, C4d +ve/DSA +ve (n = 11) 20%, C4d +ve/DSA -ve (n = 11) 91%, C4d -ve DSA +ve (n = 13) 54%, C4d -ve DSA -ve (n = 32) 75%, compared with 75% for antibody-negative patients (p = 0.0002). In conclusion, detection of Luminex +ve DSA in pretransplant serum provides a powerful negative predictor of graft survival, especially if they bind C4d.

Key words: Antibody mediated rejection, cardiac allograft, HLA antibodies, sensitized patients

Received 05 June 2007, revised 09 August 2007 and accepted for publication 12 August 2007

Introduction

Preformed antibodies to graft antigens is a major risk factor for rapid humoral rejection after renal (1) and heart or lung transplantation (2). The major risk factors for humoral sen-

sitization are pregnancy, blood transfusions and allotransplantation. Until recently, the gold standard method to test for preformed antibodies was the complement dependent cytotoxicity assay (CDC); this assay consists of incubating leukocytes from the prospective donor with serum from the recipient in the presence of rabbit complement and determining leukocyte survival (3). The advantage of this test is its high positive predictive value for rapid humoral rejection (1,2), making it clear which patients not to transplant with a particular donor. The disadvantage of the test is that the rapid graft failure can still occur as a result of antibodies to antigens, not present on leukocytes (4); in addition the end point of the test, *in vitro* complement-mediated lysis, is a stringent requirement which may not be of physiological relevance. In recent years, solid phase assays using plastic plates or beads coated with HLA antigens have proved to be robust methods of monitoring production of HLA antibodies by transplant recipients (5–7). These assays can identify the precise specificities of antigens, and donor cells are not required. These assays cannot distinguish between complement fixing and noncomplement fixing antibodies, so it is not surprising that they detect many more sensitized patients than the CDC assay (8,9); this brings into question whether it is safe to transplant patients who may be CDC negative but positive for HLA antigens detected by solid phase assays. Recent studies of renal transplant recipients (all with negative CDC cross matches to their donor) have shown that those with donor specific antibodies (DSA) detected by flow cytometry (10) or Luminex (8) have higher rates of graft failure than antibody negative patients. Wahrmann et al. first described cell-independent detection of HLA antibody-triggered classical complement activation, using Flow PRA coated microbeads (11,12). Here, we have modified this assay for use with HLA antigen coated Luminex beads and applied it in a retrospective study of heart transplant recipients.

Methods

Patients

Between April 1991 and January 2005, 653 consecutive adult primary heart transplants were performed at the Royal Brompton & Harefield NHS Trust. Patient samples were excluded if they were known to contain IgM HLA antibodies (n = 14) or had IgM nonHLA antibodies present prior to transplant (n = 44). All recipient sera underwent a lymphocytotoxic cross match against donor T and B cells at the time of transplant. All patients except

Table 1: Characteristics of study patients (N = 565)

Mean age (range) years	49 (17–67)
Gender (M:F)	467:98
Diagnosis	
Cardiomyopathy	269
Ischemic disease	254
Others	42
	No HLA mismatches
A MM	1.35
B MM	1.57
DR MM	1.29

five were transplanted with a negative cross match to donor T cells, of which four were transplanted before 1992 and one more recently (the patient had become antibody positive due to an unreported pregnancy that was terminated shortly before the transplant).

All patients treated after 1997 received induction therapy with rabbit ATG (Thymoglobulin). The maintenance immunosuppression prior to 2000 was a combination of cyclosporine, corticosteroids and azathioprine (AZA), with mycophenolate mofetil (MMF) replacing AZA from 2000. The characteristics of the study group are shown in Table 1.

CDC assay

All patients had undergone pretransplant complement dependent lymphocytotoxic antibody screening (CDC). Sera were tested using an HLA Class I (A, B, C) and Class II (DR and DQ) typed panel of peripheral blood mononuclear cells (PBMC) from 30–40 healthy volunteers and 10–20 chronic lymphocytic leukaemia cells. All sera were screened by a modified microcytotoxicity technique (13), in the presence and absence of 0.01 M Dithiothreitol (DTT) to distinguish between IgG and IgM antibodies. A patient was considered to be antibody positive if panel reactivity was greater than 5% and the strength of reaction was greater than 10% above background levels.

In addition, all recipient sera underwent a lymphocytotoxic cross match against donor T and B cells at the time of transplant. The CDC cross match was reported retrospectively shortly after the transplant.

Luminex detection of HLA antibodies

Pretransplant sera were retrospectively screened for the presence of HLA antibodies using the Labscreen Mixed assay (One Lambda, Canoga Park, CA). This kit consists of a pool of six Luminex microbeads each coated with purified HLA Class I molecules from six cell lines, and three different beads coated with purified HLA Class II molecules. Sera were incubated with the beads for 30 minutes, after washing, they were incubated with an antihuman IgG phycoerythrin (PE) conjugated monoclonal antibody for 30 minutes. Results were recorded according to median fluorescence intensity of PE staining. Labscreen software was used to determine positivity.

Sera found to contain HLA antibodies underwent further testing to assign specificity of the target antigen(s) using the Lifecodes Class I and/or Class II ID kits (Tepnel, Stamford, CT) or Labscreen Single Antigen kits. All tests were performed in accordance with the manufacturer's instructions.

C4d binding using Luminex technology

Assay optimization: The assay developed is a modification of the standard Lifecodes protocol. Briefly, 50 μ L sera (at different dilutions; see Results) was added to the HLA coated Luminex microbeads and incubated for 30 minutes. The wells were then washed four times with 200 μ L wash buffer, before the addition of 50 μ L undiluted normal human serum (NHS) as a source of complement. After 30-minute incubation, the wells were

washed and 50 μ L of a mouse antihuman C4d monoclonal antibody (Ab Serotec, Oxford, UK; diluted 1:100) added for 30 minutes. The wells were washed and 50 μ L of pretitrated PE conjugated donkey antimouse Ig (Jackson ImmunoResearch Laboratories, Inc., USA used at 1:75) added for 30 minutes before the beads were passed through the Luminex fluoroanalyzer.

Source of complement: Serum samples from eight different normal volunteers, checked to be negative for HLA antibodies, were used as a source of complement.

HLA antibody positive serum: Twenty-five pretransplant serum samples from cardiac transplant recipients, previously screened by CDC and Luminex assays were selected for use in the development of this assay. Twelve were known to be cytotoxic by the CDC assay, the remaining 13 were CDC negative, but contained Luminex detectable HLA antibodies.

Univariate and Multivariate analysis of risk factors

The following variables were considered: recipient gender, recipient age, number of mismatches at HLA locus A, B and DR, indication for transplantation (cardiomyopathy, ischemic disease, others), recipient height, recipient weight, recipient ABO status, recipient CMV status, preoperative (pre-op) diabetes, pre-op creatinine, pre-op creatinine clearance, New York Heart Association (NYHA) status, inotropes pre-op (yes/no), intraaortic balloon pump pre-op (yes/no), left ventricular device (LVAD) (yes/no), donor age, donor female (yes/no), recipient/donor sex (effect of gender mismatch between donor and recipient), donor CMV status, ischemic time.

Statistical analysis

Actuarial graft survival was estimated using Kaplan–Meier analysis and statistical differences calculated with the log-rank statistic. Univariate and multivariate analysis was performed using Cox regression analysis. A p-value <0.05 was considered to be significant.

Results

C4d assay

Initially, a single strong CDC positive serum with specificity for HLA-B7 by CDC and HLA-B7,27,60,61,13,55,56,48 by Luminex was used to develop the assay conditions, in combination with eight different sera from normal human volunteers as a source of complement. Figure 1 illustrates a range of complement activity detected by C4d deposition onto HLA B7 coated Luminex microbeads.

Mean fluorescence intensity (MFI) signal intensity varied between the eight sera; NHS G gave the strongest signal and was used in subsequent assay development.

Different conditions were used to test the ability of (neat) HLA positive serum to cause deposition of C4d, in the presence and absence of exogenous C' (NHS) (Table 2; Figure 2). In the absence of serum containing HLA antibodies specific for antigens bound to the beads, (Row 2, Table 2) no C4d was deposited. Row 3 illustrates that some HLA positive sera cause C4d deposition in the absence of exogenously added C', while others require addition of exogenous C' (Row 4). Row 5 illustrates that heat inactivation of HLA specific sera does not inhibit binding to beads, but heat inactivation of NHS (the source of complement)

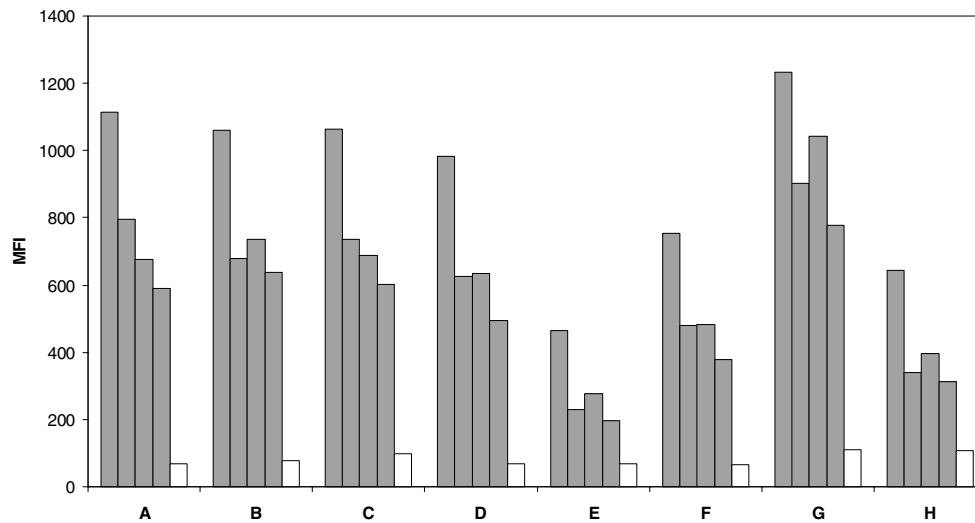


Figure 1: C4d deposition on Luminex beads. Four Luminex specific microbeads coated with HLA-B7 (shaded) and a negative control bead (white) were incubated with a HLA-B7 cross-reactive groups (CREG) antibody positive serum with eight different (A–H) normal human serum (NHS) as a source of complement. C4d deposition was visualized using monoclonal antibody to C4d as described in Materials and Methods. The amount of C4d, measured by mean fluorescence intensity (MFI) is shown on the y-axis. Individual bars represent four specific Luminex microbeads and the negative control bead (no HLA present).

inhibits C4d deposition (Row 6). In Row 7, HLA antibody and NHS was mixed with the beads, but in the absence of antibody to C4d, the secondary PE labeled antimouse did not detect signal. Serum was tested neat, and at 1:2 and 1:5; the strongest binding occurred using neat serum (Figure 2). In subsequent assays patient serum was used at 1:2.

Comparison of CDC and C4d deposition

Twenty-five serum samples previously screened by CDC and Luminex assays were selected to directly compare the assays. Twelve were known to be cytotoxic by the CDC assay (CDC +ve) and the remaining 13 were CDC negative, but contained Luminex detectable HLA antibodies (CDC -ve/Luminex +ve). Twenty-one serum samples contained Class I antibodies, two contained Class II reactive antibod-

ies, and two both Class I and II antibodies. Of the four Class II positive sera, two were detectable by CDC.

All of the CDC positive sera were found to bind human complement determined by the detection of C4d bound to the Luminex beads. In addition, 7/13 of the CDC negative/HLA antibody positive sera showed demonstrable levels of C4d binding (Fig. 3). C4d deposition was assessed as having positive reactions determined by the analysis software and also displaying HLA specificities previously found by Luminex antibody screening. The raw MFI readings in the C4d assay were reduced compared with normal antibody binding at 15–50% of MFI for antibody binding.

Comparison of CDC and Luminex for effect of graft survival

A cohort of 565 adult cardiac transplant recipients pretransplant serum samples were evaluated for HLA antibody reactivity using a standard CDC assay and solid phase Luminex based assays.

Prior to transplantation, 14 patients were known to have produced HLA antibodies, detectable by CDC assays. Retrospective analysis using the Luminex assays revealed that 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 four compared with 25 by Luminex (Table 3).

Donor specific antibodies and graft survival: All sera that were CDC +ve were also positive in the Luminex assay (Table 3). Here, the patients are analyzed according

Table 2: Conditions used to develop C4d fixing assay on HLA coated Luminex beads

Condition	αHLA		αC4d	PEαmouse	C4d deposition
	C' fixing	NHS ¹			
1	+	+	+	+	Pos
2	-	+	+	+	Neg
3 ²	+	-	+	+	Pos
4 ³	+	-	+	+	Neg
5	HI	+	+	+	Pos
6	HI	HI	+	+	Neg
7	+	+	-	+	Neg

¹Normal human serum (NHS), added as source of complement.

²Infers that patient serum contains active C'.

³Infers that patient serum does not contain active C'.

HI = heat inactivation of serum.

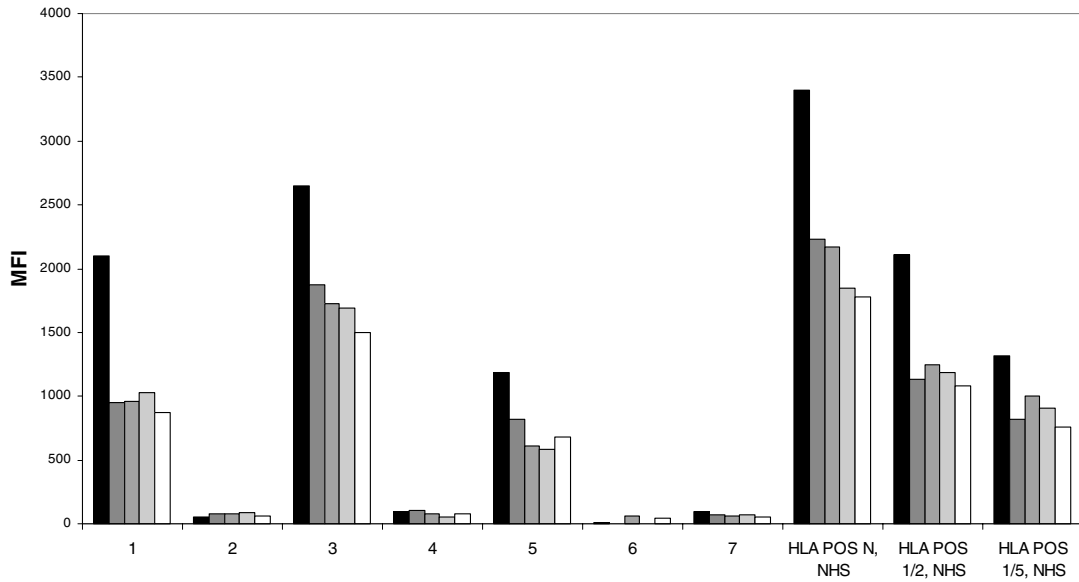


Figure 2: C4d deposition induced on five different Luminex microbeads coated with HLA molecules with seven different assay conditions and dilutions of test serum. Panels 1–7 represent the different conditions (see Table 2 for details). The last three panels show C4d staining using patient serum neat, 1/2 and 1/5 dilution. Mean fluorescence intensity (on y-axis) represent the intensity of C4d deposition.

to whether they were CDC +ve or Luminex +ve, and whether they made DSA (Figure 4). The 1-year actuarial graft survival for DSA +ve recipients (combined CDC and Luminex assays) was 41.7% compared with 76.6% for antibody positive (combined assays) without DSA and 74.8% for negative recipients ($p = 0.0007$).

There were five patients who, when tested at the time of transplant, were deemed CDC +ve with no DSA; these were later found to have DSA after retrospective analysis with the Luminex assay and were therefore included in the CDC –ve/Luminex +ve DSA group for the following analysis (Figure 5).

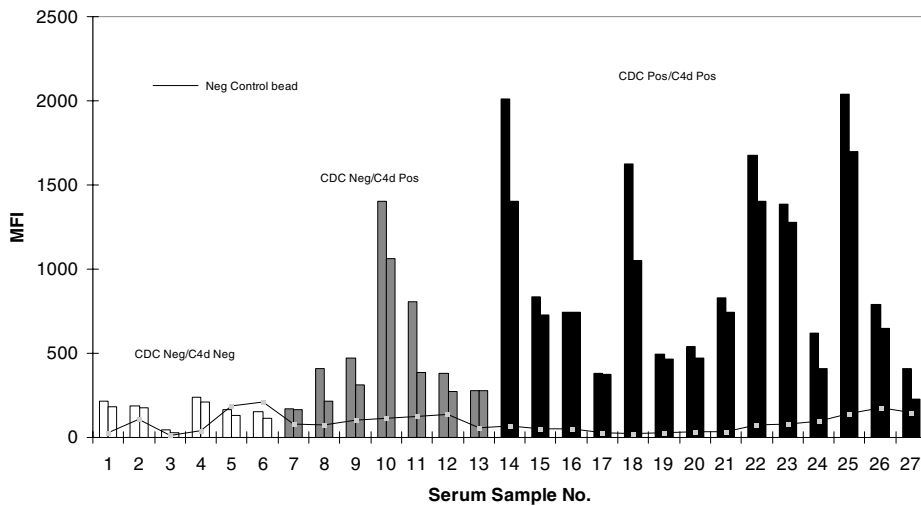


Figure 3: C4d deposition on two HLA coated microbeads induced by the 25 serum used to validate the assay. HLA coated Luminex beads (from the LifeMatch kit) were incubated with 25 different patient sera; 23 of these contained either Class I or Class II antibodies; two samples (numbered 15/26 and 22/27) contained both Class I and Class II antibodies. Mean fluorescence intensity (MFI) of the two highest reading beads, on the y-axis represents the amount of C4d deposited under different conditions described by shading of the bars. The white bars represent CDC neg/Luminex pos/C4d neg serum, grey bars CDC neg/Luminex pos/C4d pos and the black bars CDC pos/Luminex pos/C4d pos serum. The line graph represents the negative control bead value for each test.

Table 3: Comparison of CDC and Luminex assays (565 sera)

	HLA Abs	Class I	Class II
CDC +ve/Luminex +ve	14	12	4
CDC +ve/Luminex -ve	0	0	0
CDC -ve/Luminex +ve	53	42	21

When patients were divided into those showing donor specificity according to CDC or Luminex (Figure 5), the results were as follows: patients who were CDC +ve/Luminex +ve with DSA (n = 5) showed poor 1-year survival (41.7%) compared with CDC +ve/Luminex +ve but lacking DSA (n = 4, 75%). Of the 58 patients who were CDC -ve/ Luminex +ve, those with DSA (n = 19) showed 1-year survival of 42% compared with 77% for CDC -ve/Luminex +ve without DSA (n = 39). The survival of patients with antibodies but not of donor specificity was equivalent to antibody negative patients (p = 0.0039, Fig. 5). The majority of graft failure within the donor specific groups occurred within the first 3 months after transplant with CDC +ve DSA having a 90-day graft survival of 40%, CDC -ve/Luminex +ve DSA of 57.9% compared with 78.5% for negative recipients.

Of the 24 patients identified as being donor specific in the Luminex assay, DSA were directed to Class I in 14 cases, Class II in eight cases and showed specificity for both Class I and II antigens in two cases.

Luminex C4d deposition and graft survival (Figure 6):

In total, 67 recipient samples contained HLA antibodies detected by Luminex, of these 22 caused C4d deposition. Of the 22 Luminex positive C4d fixing sera, 11 contained DSA and 11 had no detectable DSA.

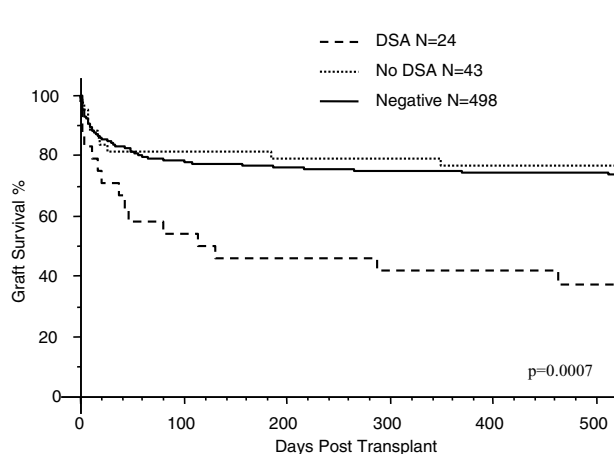


Figure 4: Effect of donor specific HLA antibodies on graft survival. Actuarial graft survival according to antibody status of pretransplant sera: sera contained CDC or Luminex detected DSA (n = 24), CDC or Luminex nondonor specific antibodies (n = 43) and patients negative for HLA antibodies (n = 498), p = 0.0007.

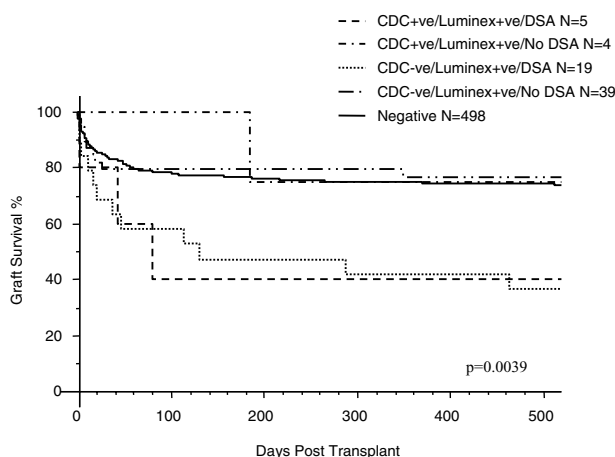


Figure 5: Comparison of detection method and donor specificity on graft survival. Actuarial graft survival according to antibody status of pretransplant sera: sera contained CDC +ve Luminex +ve DSA (n = 5), CDC +ve Luminex +ve non-DSA (n = 4), CDC -ve/Luminex +ve DSA (n = 19), CDC -ve/Luminex +ve non-DSA (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.

The 1-year graft survival for C4d +ve DSA (n = 11) was 20% compared with 91% for C4d +ve nondonor specific (n = 11), 54% for C4d -ve DSA (n = 13) and 72% for C4d -ve nondonor specific (n = 32) and 75% for negative recipients, p = 0.0002. (Fig. 6). Of the 11 patients with C4d +ve DSA, five of these had been transplanted against a positive cross match to donor T cells; their 1-year survival was 40% the survival of the other six patients was 0% at 1 year.

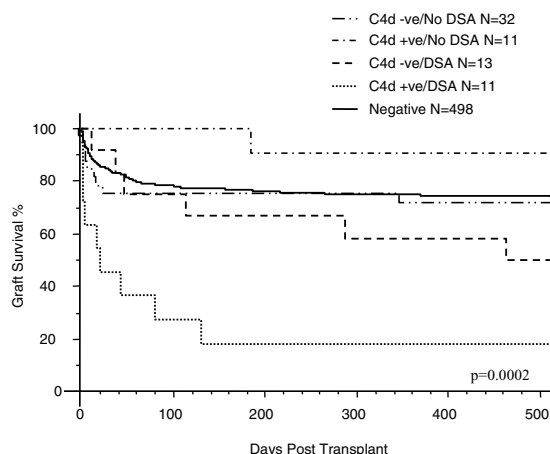


Figure 6: Effect of C4d depositing DSA on graft survival. Actuarial graft survival according to antibody status of pretransplant sera; sera contained C4d +ve DSA (n = 11), C4d +ve non-DSA (n = 11), C4d -ve DSA (n = 13), C4d -ve non-DSA (n = 32) and no detectable HLA antibodies (n = 498), p = 0.0002.

Fifteen samples contained Class I HLA antibodies, whilst one sample contained Class II antibodies causing C4d deposition. C4d deposition in the remaining six samples was due to both Class I and Class II reactive HLA antibodies.

Interestingly, at 5 years posttransplant, the C4d –ve DSA group of patients graft survival has decreased to 40%, with the C4d +ve DSA graft survival of 18% compared with 68% and 82% for the C4d –ve without donor specificity and C4d +ve without donor specificity, respectively.

Multivariate analysis: In order to check for confounding factors that could influence the effect of DSA on graft survival, a multivariate analysis was carried out. Data was available from 564 patients for a univariate analysis of risk factors (described in Materials and Methods). In this set, 10 patients were C4d +ve DSA, 11 were C4d +ve non-donor specific, 13 were C4d –ve DSA, 32 were C4d –ve nondonor-specific and 498 patients were antibody negative. Univariate analysis demonstrated that apart from DSA, five other factors significantly affected graft survival ($p \leq 0.05$), these were recipient age ($p = 0.014$), number of mismatches at HLA-A, ($p = 0.050$) and HLA-DR ($p = 0.011$), indication for Tx ($p = 0.008$, cardiomyopathy being associated with longer survival) and female donor/male recipient ($p = 0.005$). Ischemic time was not quite significant ($p = 0.060$). These factors were selected as possible confounders for a multivariate Cox regression model analysis (Table 4). The presence of DSA was found to be an independent risk factor for graft survival ($p = 0.0002$) as was female donor/male recipient ($p = 0.0004$), indication for transplant ($p = 0.0317$), mismatches at DR ($p = 0.0130$) and recipient age ($p = 0.0409$). DSA +ve C4d positivity was not an independent risk factor ($p = 0.1733$), possibly due to the strong confounding factor of DSA alone and the limited number of cases available for analysis.

Discussion

This study has described a new method of detecting C4d fixing HLA DSA using Luminex beads. The results demonstrate that Luminex is more sensitive than CDC, and with the added refinement of including C4d fixation, the assay is better able to detect clinically relevant antibodies.

Table 4: Impact of pretransplant DSA on cardiac allograft survival in a multivariate Cox proportional hazards model

Variable	Hazard ratio	95% CI	p-Value
DSA	2.70	1.60–4.57	0.0002
Female donor/ male recipient	1.64	1.25–2.15	0.0004
Recipient age	1.01	1.00–1.03	0.0409
Indication for Tx	0.73	0.55–0.97	0.0317
HLA-DR mismatches	1.33	1.06–1.67	0.0130

DSA = positive for donor specific antibody.

Analysis of 565 pretransplant sera demonstrated only 14 to be sensitized by conventional standards ($>5\%$ PRA in the CDC assay) and a further 53 to be positive in the Luminex assay; all sera positive for CDC activity could also fix C4d onto HLA coated Luminex beads.

Donor specificity had a significant effect on 1-year cardiac allograft survival, regardless of whether tested by the CDC or Luminex method; 1-year graft survival was 41.7% for DSA +ve patients (CDC or Luminex figures) compared with 76.6% for DSA –ve patients (CDC or Luminex) and 74.8% for antibody negative patients (Fig. 4). When sera are analyzed according to presence of DSA and method of detection, Luminex is able to detect patients who were CDC –ve, but had poor graft survival. Thus, 24/67 Luminex +ve sera contained DSA. Five of these were also positive for CDC DSA (in Figure 5) and had been transplanted with a positive cross match to donor T cells (four of them prior to 1992 and one recently, due to an unreported pregnancy). Nevertheless, 19 patients who were CDC –ve/Luminex +ve DSA had equally poor survival at 1 year (Figure 5). Multivariate analysis confirmed the presence of pretransplant DSA to be a strong risk factor for poor survival ($p = 0.0002$) independent of other known risk factors. Similar to these results, Gibney et al. have reported higher rates of primary nonrenal function, delayed graft function and lower 6-month graft survival in patients with pretransplant DSA detected by Luminex beads (8). The detrimental effect of CDC +ve DSA appears to be more rapid than CDC –ve/Luminex +ve DSA; 90-day survival being 40% compared to 57.9%, respectively.

A further level of stratifying these antibodies is provided by the ability of Luminex +ve DSA to cause deposition of C4d. The 1-year survival of patients with C4d +ve DSA ($n = 11$) was only 20% compared with 91% for C4d +ve non-DSA ($n = 11$). Five of the patients with C4d +ve DSA had a positive cross match with donor T cells and their 1-year survival was 40%; additionally, the survival of the other six patients, transplanted against a negative cross match was 0%. Patients with DSA that do not fix C4d had 54% survival at 1 year; it is interesting to note that the survival of these patients at 5 years is poor, namely 40% compared with DSA –ve patients (Figure 6), confirming the importance of donor specificity as a determinant of graft survival. The multivariate analysis did not find a significant difference between C4d +ve DSA and C4d –ve DSA, however the numbers of patients in these two sub-groups was small (10 vs 13, respectively). Our results support the preliminary conclusion that the C4d fixing DSA predict more rapid graft failure than DSA that do not fix C4d. However, this hypothesis should be tested in other centers. Our studies support the findings of Wahrman et al. (14), using sensitized renal transplant recipients, that fixation of C4d in a cell free assay is a better prediction of early graft failure than detection of antibodies alone. The conclusion from this part of the study is that presence of C4d +ve DSA detected by Luminex is as bad a prognostic indication for

poor survival as the positive CDC cross match test to donor T cells, and should be avoided.

The question then arises whether patients with C4d –ve DSA should be transplanted; it is possible that these patients will continue to produce antibodies after their transplant and they may become complement fixing. Should these patients be transplanted, they would require close monitoring and perhaps augmented immunosuppression (10).

A number of features other than ability to fix complement could be used to stratify pretransplant antibodies as risk factors for transplantation. These include immunoglobulin subclass, titre and antigenic specificity (15). It is recognized that antibody-mediated activation of C' and deposition of the 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 (16,17). Thus, deposition of C4d along peritubular capillaries of renal biopsies (18) and, to a lesser extent, in cardiac biopsies (19) has become a useful adjunct to diagnosis of acute humoral rejection. The majority of C4d +ve DSA patients lost their grafts within the first 90 days of their transplant which implicates humoral rejection as a mechanism (20). It may well be that the titre of DSA increases after transplantation, adversely affecting graft survival. Staining for C4d on surveillance cardiac biopsies has not been performed routinely at this institute, it is therefore not possible to comment on the presence of C4d in biopsies from these patients. In view of the chemotactic properties of split C' components, there may well be a mixed histology of cellular and humoral rejection in failed grafts of patients with C4d +ve DSA. The incidence, grade and type of rejection occurring in our patients are currently being investigated.

In conclusion, this retrospective study of pretransplant serum has described a new method of detecting C4d fixing donor specific anti-HLA antibodies on Luminex beads. The test has a strong predictive value for poor survival. It is anticipated that this test will provide a powerful additional tool for stratifying antibody status of patients.

Acknowledgments

We would like to thank Dr. Derek Robinson (University of Sussex) for statistical analysis. The study has been supported by a grant from the Clinical Research Committee of the Royal Brompton and Harfield NHS Trust and a Research Grant from Roche UK.

No conflict of interest to report.

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