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Original Article

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Impact of sensitivity of human leucocyte antigen antibody detection by Luminex technology on graft loss at 1 year

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Abstract

Background. The clinical relevance of the detection of human leucocyte antigen (HLA) antibodies in sera of renal transplant recipients by highly sensitive methods such as Luminex alone is uncertain and a matter of debate. The choice of output thresholds affects antibody detection and thus organ allocation, yet there are no internationally agreed threshold levels. This study aims at evaluating our current practice of using an MFI threshold of 1000 in antibody detection.

Methods. We carried out a case–control study by looking at 761 renal transplant recipients at one unit between 2000 and 2010. Of these, there were 93 cases of graft loss within 1 year and stored serum samples of 40 cases were available for testing. Controls were selected (graft function >2 years) and individually matched according to age, sex, number of transplants and date of transplant. All 40 cases and 40 controls had negative crossmatch by complement-dependent cytotoxicity (CDC) at the time of transplant, and pre-transplant sera were re-analysed for the presence of detectable HLA and donor-specific antibodies (DSAs) using Luminex screen and single-antigen beads and MFI threshold values of 1000, 2000 and 4000.

Results. In nearly 48% of cases with graft loss within a year, HLA antibodies were detectable by Luminex when using a 1000 MFI threshold. This was 25% greater than in controls ($P = 0.017$). There was also a 15% increase in detected DSAs; however, statistical significance depends on the inclusion or exclusion of one specific case. Using MFI thresholds of 2000 and 4000, no DSAs were found in any long-term surviving grafts.

Conclusions. Selection of appropriate MFI cut-off values influences the detection of DSAs and, thus, organ allocation. Using a threshold of 1000 led to the detection of DSAs in 5% of long-term graft survivors in our population and should be considered too sensitive. Using a detection threshold of 2000 is sufficiently sensitive and leads to clinically relevant detection of DSA.

Keywords: crossmatch; HLA; Luminex; renal transplantation

Introduction

The realization that the presence of antibodies in the serum of renal transplant recipients against donor lymphocytes causes immunogenic graft loss forms the basis of 'crossmatching' in solid organ transplantation [1]. The manner of detection of these antibodies is ever evolving. The complement-dependent cytotoxicity (CDC) test [2] was the first such test and in the modified form remains in use today. Since the 1990's, flow cytometry (FC) is increasingly being used as it has been shown to have superior sensitivity in detecting antibodies against donor lymphocytes [3]. This has already led to circumstances where transplants have gone ahead with a negative CDC crossmatch, but positive FC which in turn has been linked to increased graft loss in the long term [4].

A more recent innovation has been the advent of single-antigen-sensitive beads, which allow for a very sensitive and specific identification of the presence of antibodies in the recipient's serum [5–7]. It also allows for 'virtual crossmatching', or the identification of donor-specific antibodies (DSAs) in transplant recipients and forming a judgement of rejection risk based on the knowledge of the donor's human leucocyte antigen (HLA) type, rather than a physical crossmatch [8]. It appears that the identification of DSAs in pre-transplant sera can be linked with increased immunogenic graft loss, even in the presence of negative CDC and/or FC crossmatches [9, 10]; however, this remains under debate. In particular, the degree of fluorescence (mean fluorescent intensity or MFI) that is of clinical relevance remains unclear, with large retrospective series showing no apparent difference in graft loss in recipients with or without DSA as detected by Luminex at an MFI of 1000, 2000 or 3000 [11], and smaller series also questioning the relevance of DSA as detected by the exquisitely sensitive Luminex technique [12, 13].

The choice of the threshold MFI level influences the reported presence or absence of antibodies in a recipient's

serum and, therefore, may impact the organ allocation process significantly. In our own unit, for example, we are trialling a 'virtual crossmatch' system, whereby the donor HLA type is matched virtually against a database of known recipient HLA-antibodies as detected by Luminex in order to reduce cold ischaemia time. This study aims to assess our own practice of using a cut-off value of 1000 MFI when deciding on the presence or absence of antibodies in recipients' sera. It furthermore aims to reproduce the negative results previously published in relation to graft loss from a single transplantation centre [11].

Materials and methods

Graft survival data from all renal transplants performed at a single centre over a 10-year period of time (761 transplants performed between 2000 and 2010) were analysed and all cases of graft loss within 1 year following surgery were selected retrospectively. The point of graft loss was defined as the first day back on dialysis. Cases were defined as graft loss within 1 year and controls as having functioning grafts for more than a year. Controls were selected matching for age, sex, number of previous transplants and date of transplant. All 80 cases and controls received deceased donor transplants. Where available, donor/recipient CMV status was recorded, but not formally matched to assess the possibility that *de novo* CMV infections and CMV graft nephropathy may have played a role in graft loss. Despite no formal matching, there were no significant differences in the donor CMV status. Due to the retrospective nature of the project, we were unable to record numbers of pregnancies and/or blood transfusions pre-transplant, however we hope that due to age-matching, the number of pregnancies would be similar between the groups. Characteristics of cases and controls are further displayed in Table 1. All recipients were geographically from the north west of England, North Wales or Isle of Man. Data on donor demographics were not included in the selection procedure or analysis.

Frozen pre-transplant sera from the unit sample stores (stored at −80°C) were sourced and defrosted. Out of 93 identified cases, 40 samples were available for testing. All cases and controls were tested using Luminex LABScreen kits from One Lambda and sera screening positive for either HLA Class I or II were analysed further with Luminex SA1 or SA2 (single-antigen) beads. Assays were performed as per manufacturer's instructions, except at half the suggested bead volume (2.5 µL beads per 20 µL serum). Samples were tested twice on the same screening plate to establish the tolerance of the test.

All patients were transplanted with a negative CDC crossmatch (T and B cell) and all but two samples (both cases of graft loss, one no data, the other B cell positive) also had negative FC crossmatching.

Virtual crossmatching was performed comparing donor tissue typing as determined by a single specific primer-polymerase chain reaction (SSP-PCR) with HLA antibodies as identified after performing a single-antigen test on pre-transplant sera.

Statistical analysis was performed using IBM SPSS Statistics version 19 software for Mac. Normally distributed data were reported as mean ± standard deviation (SD) and mean values were compared using Student's *t*-test. Non-normally distributed data were reported as median ± IQR, with the exception of 'number of transplants received' in Table 1, where the decision was made to report the mean to emphasize that the vast majority of cases had received their first transplant. Probabilities for this sample set were calculated using Wilcoxon's signed-rank analysis for related samples. Nominal data were reported as proportions ± 95% confidence interval and the differences were analysed using the Chi-squared test. $P < 0.05$ was considered significant.

Results

A total of 761 renal transplant recipients were reviewed. Ninety-three cases of graft loss were identified in this cohort and 40 of these were matched with controls and analysed. The remaining cases had insufficient stored samples for analysis. The median graft survival in cases was 3 (interquartile range, IQR 0.57–13) weeks and that of controls 310 (IQR 210–440) weeks, and 88% of controls had functioning grafts at the end of the study period. The demographic variability and matching criteria were checked and there was no significant difference

Table 1. Demographic summary of cases and controls

Summary of demographic	Graft failure within 1 year	Graft survival >1 year	Difference (95% CI)	P value
Age (years), mean (SD)	48.2 (14.7)	49.9 (12.7)	1.64 (−4.62–7.88)	0.603
Sex				
M, number (%)	23 (57.5%)	23 (57.5%)	Matched	N/A
F, number (%)	17 (42.5%)	17 (42.5%)		
Number of transplants received—mean (SD)	1.18 (0.15)	1.19 (0.13)	0.0119 (−0.110–0.0859)	0.793

M, male; F, female.

Table 2. Detected HLA antibodies

Outcomes	Graft failure within 1 year ($n = 40$)	Graft survival >1 year ($n = 40$)	Difference (95% CI)	P value (Chi-square)
DSA using an MFI cut-off of 1000 (incl special case)	8 (20%)	2 (5%)	15% (7.8–22)	0.044
DSA using an MFI cut-off of 1000 (excl special case)	7 (18%)	2 (5%)	12.5% (5.6–19)	0.077
DSA using an MFI cut-off of 2000	4 (10%)	0	10% (5.3–15)	0.058
DSA using an MFI cut-off of 4000	2 (5%)	0	5% (1.6–8.4)	0.25

found between the groups. There was also no significant difference in the number of CMV-positive donors (P = 0.279; see Table 1).

Using single-antigen beads as described in the Materials and methods section and an MFI cut-off of 1000, 19 cases (47.5%) and 9 controls (22.5%) were classed as positive [difference 25% (12.5–37.5), P < 0.05] for HLA antibodies. In the case group, seven (18%) were also positive for DSAs, compared with only two (5%) in the control group. One case had a DSA with an MFI of 866. Given a tolerance of between 10 and 15% of the Luminex assay in our experiments, this sample may or may not reach a cut-off of 1000 if retested. Analysis was undertaken both including and excluding this case to assess its impact (see Table 2).

For the purpose of analysis, both scenarios were evaluated. If this sample is excluded from analysis, there appear to be 12.5% (5.57–19.4; P = 0.077) more DSA in the graft-loss group, although this does not reach statistical significance. If this case is included, there appear to be 15% (7.80–22.2; P = 0.043) more DSA in the graft-loss group, which would be significant at the 5% level (see Table 2).

If the MFI threshold for DSA is lifted to 2000, the number of detected antibodies reduces to 4 (10%) in the graft-loss group and 0 in the graft survival group (P = 0.058). Raising the threshold to 4000 reduces the DSA detection rate further to 2 (5%), with no significant difference between the groups (P = 0.25).

Discussion

Luminex is a new and powerful tool in the allocation of kidneys to recipients. As with any test, the setting of the output threshold defines its sensitivity and specificity. The lower MFI threshold of 1000 leads to the detection of DSA in recipient sera that is significantly higher in the early graft-loss group. Worryingly, however, it also detected the DSA in 5% of the graft survival group, meaning that had Luminex been available at the time, these individuals may not have received their transplant. Setting the threshold at 2000 increases the specificity of the test and in our cohort would not have reported any DSA in the graft survival group, while still identifying DSA in four patients in the early graft-loss group. Of course, the argument could be made that had we used a threshold of 2000, 3 cases of early graft failure with DSA identified at 1000 would have been transplanted. In reality, we do not advocate selecting renal transplant recipients based on a single Luminex output alone; however, we would suggest that the additional information provided by Luminex may help decide between two otherwise similar candidates, or indeed guide decisions about immunosuppressive or follow-up regimens and should thus form a part of the complex decision-making process when matching donor organs to potential recipients. Indeed, rather than relying simply on ‘positive’ or ‘negative’ DSA detection by Luminex, reporting the strength of positivity (i.e. MFI) may add value in this decision-making process.

There are a number of reasons why our results appear to contradict those published in earlier studies. First, the smaller studies [12, 13] chose to test sera from consecutive transplant patients. Consequently, the number of cases of graft loss in an already small cohort became even smaller, well beyond the capability of meaningful statistical analysis (Phelan *et al*.’s research included 64 renal transplants with 10 cases of graft failure, van den Berg-Loonen *et al*. presented 27 transplants with 7 cases of graft failure). Second, the larger, multi-centre study of Susal *et al*. [11] analyses its data in separate categories of 11 HLA subtypes, thereby greatly reducing statistical power. Similarly, it may be the case that weakly complement-fixing DSAs are more damaging than non-complement-fixing DSAs, but a previous series of 837 transplantations concluded that due to their low prevalence the clinical relevance of complement fixing DSA could not be clarified. Based on the prevalence reported in their series (∼10% of DSAs are complement fixed), we estimated that no more than one case in our series would be complement fixed, thereby making meaningful analysis impossible and therefore, we decided not to test for complement fixation in our series [14]. Indeed, complement fixing adds another layer of complexity to the analysis. Other series have reported much higher levels of C1q-fixing at nearly half of all measured HLA antibodies [15], while more recent series find there is little correlation between the MFI of IgG-positive sera and the ability of these sera to fix completement [16], thus relevance of complement fixation on graft loss might be evaluated separately to detection of HLA antibodies by Luminex.

There are of course a number of limitations to our study. First and foremost, it is retrospective and based entirely on the use of frozen pre-transplant serum. It is conceivable that antibodies in older samples have degraded/denatured more than that of fresh samples, however this need not necessarily lead to a bias between the groups (which were evenly matched in time for that very reason). Also, it is still a relatively small series, albeit all from one single centre, thereby eliminating some of the confounding factors introduced by multi-centre studies. Another drawback of this study is the absence of histological confirmation of immunological rejection. Again, however, there is no reason to suspect that this should bias the presence or absence of antibodies detected by Luminex in either group. Furthermore, the timing of graft failure (0.5–13 weeks) in the early graft-loss group suggests an antibody-mediated rejection as a primary cause of graft failure. Also, while we did include all types of graft failure (including thromboses), the timing of the failure suggests at least a significant proportion were due to immune-mediated rejection. Finally, we have made no attempt to investigate differences in immunosuppressive regimens. It is the fact that these data are from a single centre that allows us to speculate that each individual in either group will have had similar immunosuppressive regimens, given that cases and controls were matched according to the time of transplant with a median difference of 8.5 weeks.

The results presented here do suggest that there is an increased risk of early graft loss (at 1 year) in patients with DSA as detected by Luminex using threshold MFI values of 1000 and 2000. Using a threshold of 2000 eliminated the detection of DSA in the graft survival group in our series, suggesting this level may lead to the clinically relevant detection of DSA without leading to excessive denial of organs to patients who need them. The availability of this information needs to be taken into account when taking organ allocation decisions, but it may also call for alterations to induction and/or maintenance regimens in these recipients. Based on our results we recommend the use of a threshold of MFI 2000 when reporting on the detection of DSA by Luminex, but the

implications of DSA reported in such a way on organ allocation or immunosuppressive regimen deserve further exploration and investigation.

Author contributions

P.S. participated in research design, writing of the manuscript and the performance of research and data analysis. Departmental support was received in the form of reagents and laboratory time. No external support was received. J.J. participated in the performance of research. A.H. and D.M. participated in research design and data analysis and are departmental heads of transplantation and transplant immunology units using the Luminex technology.

Conflict of interest statement. None declared

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