Improved Graft Survival in Highly Sensitized Patients Undergoing Renal Transplantation After the Introduction of a Clinically Validated Flow Cytometry Crossmatch

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Background. Flow cytometric techniques are increasingly used in pretransplant crossmatching, although there remains debate regarding the clinical significance and predictive value of donor-specific antibodies detected by flow cytometry. At least some of the discrepancies between published studies may arise from differences in cutoffs used and lack of standardization of the test.

Methods. We selected cut-off values for pretransplant flow cytometric crossmatching (FCXM) based on the correlation of retrospective results with the occurrence of antibody-mediated rejection. The impact on long-term renal graft survival of prospective FCXM was determined by comparing graft survival between patients crossmatched with complement-dependent cytotoxicity (CDC) only with those prospectively crossmatched with both CDC and FCXM.

Results. Chosen cut-off values gave a positive predictive value of FCXM for antibody-mediated rejection of 83%, and a negative predictive value of 90%. After the introduction of prospective B- and T-cell crossmatching by flow cytometry in addition to CDC in our center, there was a significant improvement in renal graft survival in highly sensitized patients ($P=0.017$). Four-year graft survival in highly sensitized patients after the introduction of FCXM was 89%, which did not differ significantly from that seen in nonsensitized patients (93%; $P=0.638$).

Conclusions. Our data demonstrate that prospective FCXM improves renal transplant outcome in highly sensitized patients, provided that cut-off values are carefully validated and results interpreted in the context of sensitization history and antibody screening results.

Keywords: Flow cytometry, Crossmatching, Validation, Renal transplant.

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Complement-dependent cytotoxicity (CDC) techniques with or without enhancement are now used in pretransplant crossmatching after Patel and Terasaki's publication in 1969 describing antidonor reactivity in recipient sera (*1*). CDC crossmatching detects preformed, donor-specific, complement-fixing antibodies and was initially introduced to prevent the devastation of hyperacute transplant rejection. However, acute humoral or antibody-mediated rejection (AMR) in the presence of donor-specific alloantibodies is now increasingly recognized as a cause of early renal graft dysfunction with a reported incidence of up to 8% (*2, 3*). As CDC assays do not detect noncomplement-fixing antibodies, nor all complement-fixing antibodies, newer techniques with greater sensitivity are gaining favor and often used in conjunction with CDC.

Flow cytometric crossmatching (FCXM), as first described by Garovoy et al. in 1983 (*4*), has considerably greater sensitivity than the basic or enhanced CDC assays (*4–6, 7*), allows identification of antibody isotype, and detects low-level cytotoxic as well as noncytotoxic antibodies. FXCM techniques are routinely designed to detect IgG donor-specific antibody (DSA). The clinical significance of IgM DSA remains controversial, and IgA DSA is not known to be of clinical significance. Renal transplants performed across a positive flow cytometric crossmatch have been shown to have higher rates of acute rejection, early graft loss, and lower 1-year graft survival (*8*). However, there are conflicting reports in the literature regarding the clinical significance of DSA detected by flow cytometry and not by CDC.

In 2000, 50% of tissue typing laboratories in the United States performed the final pretransplant crossmatch by flow cytometry (*7*), yet the definition of a positive result is not

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standardized and may account for discrepancies between studies. Positive cut-off definitions from published research studies include more than 2SD shift above mean channel shift from the negative control (*9, 10*), variable mean channel displacement for T and B cells (*11, 12*), or more than 3SD increase in sample median fluorescence intensity from negative control values with SD derived from previous crossmatches between nonsensitized sera and donor lymphocytes (*13*). Furthermore, flow cut-off values used to determine a positive or negative crossmatch are not reported as having been validated by retrospective correlation with clinical outcome, with the exception of Kotb et al. (*14*). To date there have been no studies reporting the clinical impact of the introduction of FXCM on graft survival.

We sought to determine optimal flow cytometric cut-off values for B- and T-cell crossmatches by correlating results obtained from crossmatches performed retrospectively with the occurrence of AMR in renal transplant recipients in our center. We also assessed the impact of the introduction of pretransplant crossmatching by flow cytometry using these cutoffs on graft survival.

MATERIALS AND METHODS

All laboratory investigations were performed in the Histocompatibility and Immunogenetics (H&I) laboratory of Beaumont Hospital, which is the national H&I service for solid organ transplantation in Ireland.

All patients diagnosed with AMR between 1998 and 2000 for whom sufficient frozen donor lymphocytes were available for crossmatch studies were included. The clinical outcome of these patients has been previously published (*3*). Control sera from 50 patients receiving renal transplants in the same time period who had not experienced AMR were also included.

The panel-reactive antibodies (PRA) of transplant recipients was assessed on peripheral blood lymphocytes using the National Institute of Health basic CDC technique (*15*) on a selected panel to encompass donor antigens commonly encountered in the Irish population. Potential transplant recipients all received regular screening for anti-human leukocyte antigen (HLA) antibodies by CDC and ELISA (LAT-M/LAT, One Lambda Inc., Canoga Park, CA) every 3 months in line with the European Federation for Immunogenetics standards.

AMR was suspected if there was a clinical evidence of acute graft dysfunction within 6 weeks of transplantation, and confirmed by typical histologic findings of capillary or peritubular polymorphonuclear leukocytes, together with visualization of immunoglobulin or C4 deposits by direct immunofluorescence, or the presence of DSA in patient serum by ELISA or flow cytometry, as per amended Banff criteria (*16*).

Retrospective crossmatches were performed using pretransplant sera of the above renal transplant recipients, all of whom had a negative CDC before transplantation. Flow cytometric crossmatch analysis was performed by modification of a previously described dual-color technique (*17*). In brief, donor cells were incubated with patient or control serum for 30 min at 22°C. Cells were then washed and stained with anti-human IgG conjugated to fluorescein isothiocyanate as well as either anti-CD3 conjugated to phycoerythrin (T-cell stain) or anti-CD19 conjugated to phycoerythrin (B-cell stain) for 30 min at 4°C in the dark. Cells were then washed with FACSFlow, pelleted by microcentrifugation, and finally resuspended in 250 μL FACSFlow. Fluorochrome-conjugated antibodies bound to the cell surface were detected by two-color analysis on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data analyzed with Cellquest software. Results for T- and B-cell crossmatches were expressed as a ratio compared with the corresponding result obtained from male AB serum.

Graft survival was followed up for renal transplants performed at Beaumont Hospital from 1998 to 2005 with information obtained from the National Renal Transplant Registry of Ireland and stratified according to semiquantitative PRA values. To determine the impact of the introduction of prospective FCXM on graft survival, data were divided into two distinct 4-year periods of 1998–2001 and 2002–2005. Renal transplant recipients in the first group all had a negative CDC. Prospective crossmatching by flow cytometry in addition to CDC was introduced into the laboratory in late 2001. Thus, any patients in the second group who had ever had detectable anti-HLA antibodies on screening or who had a sensitizing event within 6 months of transplant received a pretransplant crossmatch by both CDC and flow cytometry.

Immunosuppression regimens given to low-risk patients evolved over the study period from combined cyclosporine and azathioprine, to tacrolimus and azathioprine, and finally, tacrolimus and mycophenolate together with corticosteroids. There was no change in the immunosuppressive regimen given to a subgroup of highly sensitized (PRA ≥50) patients or in surgical or postoperative management between the two time periods. However, from 2002, all positive or equivocal crossmatches were discussed with a consultant immunologist before proceeding to transplantation. Crossmatch results were interpreted in the context of sensitization history and antibody screening results. Transplantation was permitted to proceed with a positive FCXM if the presence of donor-specific anti-HLA antibodies could be excluded.

For statistical analysis, log-rank tests were used to determine differences in graft survival of high PRA patients between the two time periods 1998–2001 and 2002–2005. Cox proportional hazards methods were used in multifactorial models to determine independence of effect of confounding variables on graft outcome. Fisher Exact and Wilcoxon Rank-Sum tests were used to determine differences in demographic and clinical variables. A P value less than 0.05 was considered a significant result. The statistical software used for all analyses was Stata (version 8, College Station, TX).

RESULTS

Twenty patients met criteria for the diagnosis of AMR between 1998 and 2000, of which five were excluded from analysis due to lack of availability of frozen donor cells. Five patients were primary graft recipients and 10 were recipients of second or subsequent renal transplants. Of the 50 selected control sera, one was excluded from analysis due to insufficient cells for accurate analysis, thus a total of 64 retrospective flow cytometric crossmatches for both T and B cells were performed.

Results for T- and B-cell crossmatches were plotted on each axis of a scatter chart with additional identification of the

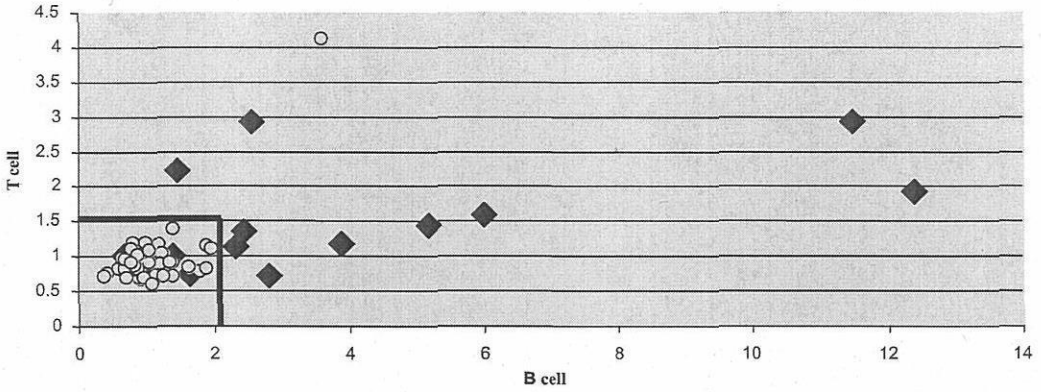

FIGURE 1. Scatter plot of B- and T-cell FCXM results. Patients diagnosed with antibody-mediated rejection are identified with ♦ and controls identified with ○. Cut-off values for a positive crossmatch (T cell >1.5, B cell >2) are delineated and were selected by visual analysis of data distribution.

presence or absence of AMR (Fig. 1). Positive cutoffs were determined by visual analysis of the data and set as a T-cell ratio more than 1.5 and B-cell ratio more than 2. Using these cutoffs, 10 of 15 patients with AMR had a positive crossmatch, giving a sensitivity of 67%. Forty seven of 52 patients with a negative result did not demonstrate features of AMR, giving a negative predictive value of 90%. Of 12 patients with a positive T- or B-cell crossmatch, 10 were diagnosed with AMR, giving a positive predictive value of 83%. Patient 11 was a nontransfused male, whose ELISA screening results were repeatedly negative for anti-HLA antibodies, but had an antilymphocyte antibody detectable by CDC. Hence, appropriate clinical interpretation of results further enhances positive predictive value. Of the 64 patients in this study, only one would have inappropriately denied a transplant because of an FCXM that seemed to be clinically significant.

Graft survival data were obtained from a total of 523 renal graft recipients from 1998 to 2001 and 554 recipients from 2002 to 2005, representing all renal transplants performed within each time period. An increase in graft survival was detected for patients in the latter time period, with a 4-year graft survival of 77% in 1998–2001 compared with 92% in 2002–2005 ($P<0.001$).

When graft recipients in the first time period were stratified into groups of low (≤10%), medium (11%–49%), and high (≥50%) PRA, there were significant differences in graft survival for the different patient groups, with 4-year graft survival of 80%, 75%, and 65%, respectively ($P=0.015$). In contrast, patients who received renal transplants between 2002 and 2005, after the introduction of prospective flow cytometric crossmatching, did not show an inverse correlation between PRA and long-term graft survival, with 4-year survival rates of 93%, 92%, and 89% for low, medium, and high PRA groups, respectively ($P=0.638$) (Fig. 2A). Thus, the introduction of FCXM led to the greatest improvement in 4-year graft survival in patients with high PRA, which increased from 65% to 89%. In addition, there was a significant decrease in biopsy-proven cellular rejection in high PRA patients, from

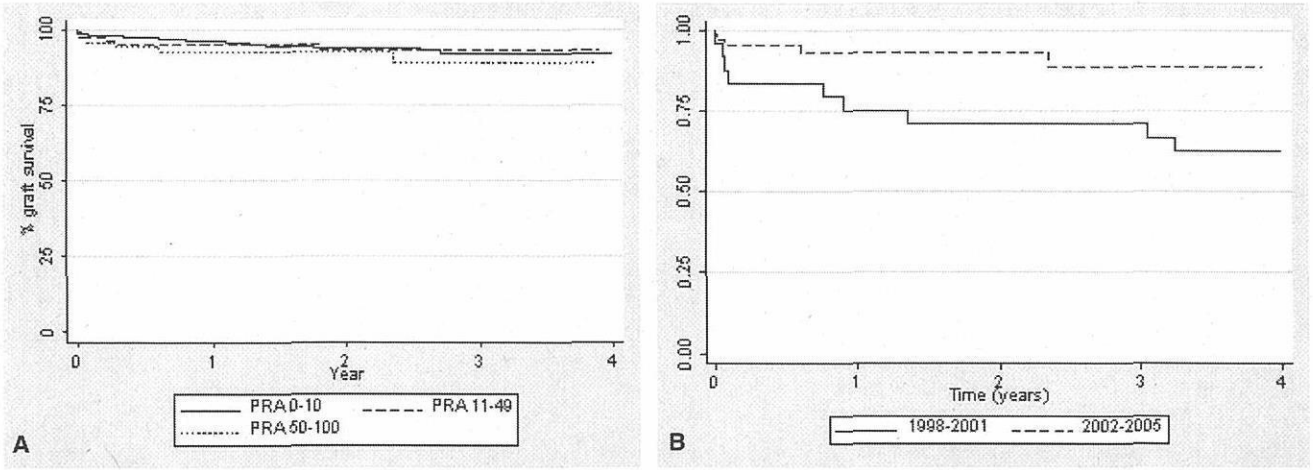

FIGURE 2. (A) Four-year graft survival of renal transplants performed between 2002 and 2005, after the introduction of prospective FCXM. There is no significant difference in graft survival at 4 years in patient groups stratified by PRA. (B) Four-year graft survival of renal transplants in highly sensitized recipients performed in the two time periods before (1998–2001) and after (2002–2005) the introduction of FCXM. All patients received immunosuppression with tacrolimus, mycophenolate, and corticosteroids. Graft survival is significantly higher for patients receiving transplants between 2002 and 2005, after the introduction of prospective FCXM ($P=0.017$).

40.8% in patients receiving transplants in 1998–2001 to 14.9% in those receiving transplants from 2002 to 2005 ($P=0.042$). The two patient groups with high PRA showed no significant difference in mean donor and recipient age, sex, match grade, length of cold ischemic time nor in the distribution of primary versus regrafts between the two time periods.

A Cox multifactorial regression model was constructed for a number of possible confounders including the above variables as well as posttransplant complications of delayed graft function and biopsy-proven rejection. The change in graft survival between the two time periods remained significant in the presence of these possible confounders ($P=0.009$). Thus, a reduced risk of graft failure was predicted for the latter time period.

To exclude any change in immunosuppression as a confounder affecting outcome, graft survival between the two time periods was compared between patients with high PRA for whom there was clear documentation of ongoing immunosuppression with tacrolimus, mycophenolate, and corticosteroids.

Figure 2(B) shows that within this group, there was a significant increase in 4-year graft survival from 62.5% between 1998 and 2001 (24 patients) to 88.6% between 2002 and 2005 (64 patients) ($P=0.017$).

DISCUSSION

Despite a number of studies showing inferior graft outcomes in patients with a positive FCXM, the clinical significance of DSA detected by flow cytometry and not CDC remains controversial. At least one study has shown no difference in the number of rejection episodes or 1-year graft survival among transplant recipients with positive or negative FCXM (*12*, *18*). More recently, Vasilescu et al. (*11*) demonstrated that a positive flow crossmatch performed retrospectively was not invariably associated with increased rejection or graft loss. Even studies that have reported increased graft loss in recipients with a positive flow cytometric crossmatch did not demonstrate an increased risk in all patients, raising the possibility of oversensitivity, lack of specificity of a positive result, and the inappropriate exclusion of patients for transplantation. Thus, it is vital that the cutoff values that determine a positive reaction are carefully validated to ensure clinical relevance.

Our data support the well-known increased sensitivity of FCXM compared with CDC crossmatching. Of 64 patients with a negative CDC, 12 patients, or 19% demonstrated a positive T- or B-cell flow cytometric crossmatch. This is consistent with previous reports demonstrating the considerably greater sensitivity of FXCM compared with CDC (*4*, *5*). In renal transplant recipients with a negative anti-human globulin (augmented)-CDC, a median of 15% primary grafts and 34% regrafts demonstrate a positive crossmatch by flow cytometry (*8*).

We have shown that the use of cut-off values selected by retrospective correlation of FCXM values with the occurrence of AMR, results in higher positive and negative predictive values than previously reported (*11*). The implementation of these cut-off values in prospective flow cytometric crossmatches in our center resulted in an improvement in long-term graft survival in highly sensitized patients. In agreement with this, it has been reported that graft survival among regraft recipients, a population that usually includes a significant percentage of highly sensitized patients, is significantly increased by prospective crossmatching by flow cytometry (*9*). Because of the evolution in immunosuppression regimens over the study period, the contribution of FCXM to the improvement in graft survival seen in less-sensitized patients cannot be determined.

A prospective study evaluating FCXM showed that transplant candidates with low or negative PRA and a positive flow cytometric crossmatch had significantly greater rates of early rejection and steroid-resistant rejection when compared with FCXM negative controls. Despite this, there was no difference in 1-year graft survival between the two groups. Thus, a prospective positive FCXM in unsensitized patients identified those at increased risk of rejection, although the authors concluded that patients with a positive FCXM should not be excluded from transplantation without consideration of other risk factors such as donor age and degree of sensitization of the recipient (*19*). However, the crucial difference between our study and previous data is the clinical validation of the cutoffs used to determine a positive, equivocal, and negative result.

In our center, the combined intervention of the introduction of prospective, clinically validated FCXM and on-call consultant input has improved graft survival in highly sensitized renal transplant recipients. Since 2001, we routinely performed prospective B- and T-cell flow cytometric crossmatches in patients with any level of detectable anti-HLA antibodies. Unsensitized patients (no anti-HLA antibodies detected by CDC and ELISA or Luminex) do not receive a prospective FCXM; therefore, our findings cannot be extended to this group. Cross-match results are interpreted together with the results of antibody screening, sensitization history, and donor-recipient matching. A positive FCXM attributable to anti-HLA antibodies is a contraindication to transplantation in the absence of augmented immunosuppression. Our data demonstrate that prospective crossmatching by flow cytometry is a useful technique to identify sensitized renal transplant recipients undetected by CDC crossmatching and that the predictive value of the test can be maximized by clinical validation of cutoff values. The decision to proceed with transplantation can be further optimized by interpreting pretransplant cross-match results in the context of the patient's sensitization history and antibody screening results.

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