The clinical significance of flow cytometry crossmatching in heart transplantation

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 Received 6 September 1999; received in revised form 17 December 1999; accepted 25 January 2000

 Abstract

 Objective: Flow cytometry crossmatching (FCXM) is more sensitive than the cytotoxic crossmatch in identifying preformed antibodies to donor alloantigens, but its clinical importance is controversial. The objective of this study was to determine the association of a FCXM with survival and incidence of vascular rejection in cardiac transplant recipients with a negative cytotoxic crossmatch.

 Methods: Between 1993 and 1998, 357 heart transplant recipients with a negative T cell cytotoxic crossmatch were studied by three-color FCXM to quantify anti-donor IgG reactions against B and T lymphocytes. Reactions positive against both were consistent with human leukocyte antigen (HLA) Class I reactivity, and those against B cells only were considered to be against HLA Class II antigens. Endpoints were episodes of vascular rejection, death from acute and chronic rejection and overall survival.

 Results: Fifty patients were FCXM for Class I-positive, 144 for Class II-positive, and 163 were negative. At 1 month, freedom from vascular rejection was 64% in Class I patients, but 90% and 96% in Class II or negative crossmatch patients (P < 0.0001). Survival of the negative crossmatch group was higher than either Class I or II groups (94%, 74% and 76%, respectively, at 3 years; P < 0.0001). Death from acute rejection was 3% and 2% at 3 years in negative or Class II-positive patients, but 19% in Class I patients (P < 0.0001). Death from chronic rejection occurred only in Class II patients (P = 0.002).

 Conclusions: Despite a negative T-cell cytotoxic crossmatch, a positive flow cytometry crossmatch correlates with important clinical events after heart transplantation.

 Keywords: Flow cytometry; Crossmatch; Survival; Vascular rejection

 1. Introduction

 Presently the complement-dependent cytotoxicity test remains the ‘gold standard’ transplant crossmatch technique. However, acute graft rejection and transplant related coronary artery disease still occur and continue to influence early and late recipient survival respectively, despite a negative cytotoxic crossmatch [1–3]. This suggests a need to investigate alternative techniques to detect preformed antibodies. The flow cytometry crossmatch (FCXM) is a more sensitive technique used to detect anti-donor antibodies and has been shown to be a better predictor of outcome in renal re-transplantation than the cytotoxic crossmatch [4,5]. However, there is a paucity of literature pertaining to its use in cardiac transplantation, and less regarding the association of a positive B-cell crossmatch with graft survival.

 Vascular rejection in heart transplant recipients is becoming recognized as a cause of cardiac dysfunction leading to mortality in the early post-transplantation period and may be a risk factor for the subsequent development of accelerated graft atherosclerosis [6–9]. Since humoral immunity is felt to be largely responsible for this form of rejection, pretransplant detection of preformed donor-specific anti-human leukocyte antigen (HLA) antibodies, by a sensitive technique such as flow cytometry, may help in avoiding this type of rejection.

 This study was performed to determine whether a positive
flow cytometry crossmatch was associated with (1) the development of vascular rejection and (2) decreased survival following heart transplantation.

2. Materials and methods

2.1. Patient characteristics

Of the 496 patients undergoing heart transplantation between March 1993 and August 1998, 357 with a negative Amos-modified T-lymphocyte cytotoxic crossmatch and in which both a T- and B-cell flow cytometry crossmatch was interpretable were studied. Of these, 285 (80%) were men and 72 (20%) were women recipients with a mean age of 51 ± 14 years (range 1–73 years). All had end-stage heart failure due to ischemic cardiomyopathy in 211 (59%) and non-ischemic cardiomyopathy in 146 (41%). Eighty-five (24%) recipients were bridged to transplantation on an implantable left ventricular assist device (VAD). Mean ± SD follow-up for survivors was 2 ± 1.5 years (range 2 months–5.4 years).

2.2. Flow cytometry

Quantitative FCXM was performed as previously described [10–12]. In brief, donor lymphocytes (2 × 10^5) obtained from peripheral blood, lymph nodes or spleen, were incubated with 20 μl of recipient serum or with negative or positive control sera. Unbound antibody was washed away, and the cells were incubated with monoclonal antibodies to CD3 and CD20 labeled with perCP and phycoerythrin respectively (Coulter Corp. or Becton Dickinson), to identify B and T cells, and fluorescent-coupled F (ab')2 fragments of goat antibody (Jackson Immunoresearch) to identify the heavy chains of bound human immunoglobulin G (IgG). The reactions were analyzed on a fluorescence-activated cell analyzer (FACSCAN) (Becton Dickinson). IgG binding was recorded as the median channel number which was converted to molecules of equivalent soluble fluorochrome (MESF) by calibrating the instrument using fluorescent microbeads using the QuikCal system (Flow Cytometry Standards Corp., Research Triangle Park, NC). All reactions were compared with normal control sera. A shift from control values of > 500 MESF was considered positive for T-cell reactions, and > 2000 MESF was considered positive for B-cell reactions. The average time taken to perform a flow cytometric crossmatch was 1.5 h (compared with 3.5 h taken to perform a cytotoxic crossmatch). Positive reactions against both T and B cells were considered to be against HLA Class I antigens, and B cell-only reactions were considered to be against HLA Class II antigens.

The flow cytometry crossmatch result was known prior to transplantation in 39 (11%) patients and therefore was not used as part of the decision to transplant process in the majority of patients. However, it was always known post-transplant and helped tailor the recipient’s post-transplant immunosuppression management.

2.3. Panel-reactive antibody assay

Assessment of panel-reactive antibodies (PRA) against B and T lymphocytes was examined just before transplantation. The recipients’ sera were heat-treated to remove immunoglobulin M reactivity and tested by complement-dependent lymphocytotoxicity against a comprehensive 25- to 50-member cell panel of HLA-typed donors selected to represent most of the defined HLA specificities. In this report the screening test was considered positive when at least 10% of the cells in the wells showed cytotoxicity by standard dye-exclusion assay.

2.4. Vascular rejection diagnosis

Routine surveillance endomyocardial biopsies were performed weekly for the first 4 weeks after transplantation, every 2 weeks during the second month, and then the interval increased to 6–8 weeks until the patient was 1–3 years post-transplant when biopsies occurred every 4 to 6 months. The diagnosis of vascular rejection was based on the demonstration of immunoglobulin and complement on the coronary vascular endothelium by immunofluorescent staining, according to the criteria defined by Hammond and colleagues, in addition to the presence of endothelial cell swelling and activation on light microscopy [13]. Immunofluorescent staining was performed on all endomyocardial biopsy specimens from patients who showed persistent findings of vascular rejection or hemodynamic compromise during follow-up.

2.5. Definitions

Recipient cause of death was obtained from postmortem findings and clinical record review and defined according to criteria laid down by the United Network for Organ Sharing (UNOS). These were limited to six causes (acute rejection, chronic rejection, nonspecific graft failure, infection, malignancy and other). Death resulting from acute rejection was defined as death with a proven autopsy finding of acute rejection or death of cardiogenic shock soon after an endomyocardial biopsy specimen. Death resulting from acute rejection was defined as death with a proven autopsy finding of acute rejection or death of cardiogenic shock soon after an endomyocardial biopsy finding for acute rejection, when an autopsy was not available. Death caused by chronic rejection was defined as death from cardiogenic shock in a recipient with angiographic or autopsy proof of significant graft atherosclerosis.

2.6. Immunosuppression

Maintenance immunosuppression consisted of a triple-drug combination of cyclosporine, azathioprine and prednisone, with mycophenolate mofetil largely replacing azathioprine since 1997. Patients with compromised renal function were treated selectively with OKT3 monoclonal antibody for induction, followed by conversion to cyclos-
porine-based immunosuppression when renal function improved. Post-transplant plasmapheresis was used in 16 (32%) Class I-positive recipients, 6 (4%) Class II-positive recipients and two (1%) negative crossmatch recipients. As well as the transplant crossmatch result, the decision to use plasmapheresis post-transplant was based on the recipient’s postoperative clinical course and the results of the post-transplant donor-specific flow cytometry crossmatch monitoring.

Episodes of acute rejection were initially treated with intravenous methylprednisolone for 3 days. Recurrent or refractory rejection was treated with steroids and OKT3. Other treatment modalities, such as plasmapheresis, immunoglobulin and cytolytic therapy, were used in cases of acute rejection where a strong humoral component was suspected. OKT3 was used in 32 (9%) of patients, either for induction or for the treatment of recurrent or refractory rejection.

2.7. Data analysis

The two primary endpoints examined in this study were (1) vascular rejection after transplantation, and (2) mortality from the time of transplantation. The objective of the analysis was to determine the association between these outcomes and the flow cytometry crossmatch result.

Time-related analyses were conducted using the method of Kaplan and Meier. The log-rank test was used to test equality of the survivorship function when vascular rejection or survival were stratified by flow cytometry crossmatch result.

Potential donor and recipient risk factors for vascular rejection or death were examined with parametric, multivariable analyses in the hazard function domain [14]. The following variables were entered into the multivariate analysis: recipient variables (age, gender, race, etiology of cardiomyopathy, ventricular assist device, intra-aortic balloon pump, extracorporeal membrane oxygenation, OKT3 use, flow cytometry crossmatch result, T-and B-cell PRA antibody status); donor variables (age, gender, race, cause of death, and ischemic time); recipient/donor mismatch (age, gender, race, and HLA, A, B and DR mismatches); surgical variables (retransplantation, and the flow cytometry crossmatch result).

Variables were retained in the final model if \( P < 0.05 \).

The investigation of survival was further refined by a competing risks (multiple decrement) analysis of death from acute versus chronic versus other causes, according to flow cytometric crossmatch result. These are presented simply as freedom from death in acute rejection and freedom from death in chronic rejection.

A secondary objective of the study was to characterize patients with a positive flow cytometry result. For this, logistic regression was used. The variables considered in this analysis included age, gender, race, etiology of cardiomyopathy (ischemic vs. non-ischemic), donor and recipient mismatches with respect to gender and race, the use of a left ventricular assist device as a bridge to transplant, and B- and T-cell PRA results.

3. Results

3.1. Characteristics of FCXM positive recipients

Variables that characterized recipients with a positive flow cytometry crossmatch are shown in Table 1. Women recipients bridged to transplantation on a ventricular assist device, and recipients with elevated pretransplant T and B cell panel reactive antibodies were more likely to be Class I-positive. The only variable found to characterize patients with a positive Class II crossmatch was the use of a ventricular assist device.

3.2. Risk factors for vascular rejection

Forty-six patients experienced vascular rejection, 40 of them within the first 6 weeks of transplantation. Thirty-eight of the 46 patients (83%) experiencing vascular rejection had a positive FCXM. At 1 month, freedom from vascular rejection was highest in patients with a negative flow cytometry crossmatch (96%), somewhat lower in patients with a Class II-positive result (90%), but only 64% freedom in patients that were Class I-positive (Fig. 1, \( P \) (log-rank test for equality across all strata) \(< 0.0001 \). Risk factors associated with the development of vascular rejection included a positive flow cytometry crossmatch (with a Class I result, more likely to be associated with vascular rejection than a Class II result), B cell PRA > 10%, and female recipients of male hearts (Table 2). The use of OKT3 was not found to be a risk factor for the development of vascular rejection (coefficient ± SE = 0.31 ± 0.51, \( P = 0.55 \)). Also of importance, the year of transplant, which takes into account the change in immunosuppression protocol in 1997, did not have any impact on the incidence of vascular rejection.

<table>
<thead>
<tr>
<th>Recipient variable(^{a})</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient ± SE</td>
<td>( P )</td>
</tr>
<tr>
<td>Female</td>
<td>2.2 ± 0.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>VAD</td>
<td>2.3 ± 0.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>% T-cell PRA</td>
<td>0.05 ± 0.2</td>
<td>0.005</td>
</tr>
<tr>
<td>% B-cell PRA</td>
<td>0.04 ± 0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^{a}\) \( P \)-values and coefficients expressed vs. negative flow cytometry crossmatch group.

\(^{b}\) VAD, ventricular assist device; PRA, panel-reactive antibodies.
3.3. The impact of FCXM result on survival

There were 48 deaths among the 357 patients with an overall 1-, 2-, and 3-year survival of 89%, 86% and 84%, respectively. Forty of the total 48 deaths (83%) were recipients with a positive FCXM. At 3 years, survival was highest among patients with a negative flow cytometry crossmatch (94%), but was 74% and 76%, respectively, in patients with a Class I- or Class II-positive result (Fig. 2, \( P \) (log-rank test for equality across strata) < .0001). Risk factors for death included a positive flow cytometry crossmatch, either Class I- or Class II-positive, younger recipient age, and older donor age (Table 2). Similar to the situation described above for vascular rejection, the year of transplant did not have any impact on survival.

3.4. Autopsy results of recipient death by FCXM

The cause of death by each flow cytometry crossmatch result, obtained from autopsy and clinical record review, is presented in Table 3. Although the overall number of deaths from acute and chronic rejection is small, Class I-positive recipients were more likely to die from acute rejection whereas Class II-positive recipients were more likely to die from chronic rejection (Fig. 3). Patients dying from chronic rejection displayed diffuse concentric atherosclerotic stenosis of the coronary arteries with substantial luminal narrowing throughout. Interestingly, four of the total eight deaths from chronic rejection were within the first year and the remaining four by the second year. The mean donor age for those recipients dying of chronic rejection was 31 (range 17–42) years.

### Table 2

<table>
<thead>
<tr>
<th>Vascular rejection</th>
<th>Risk factor</th>
<th>Coefficient ± SE(^a)</th>
<th>( P )</th>
<th>Death</th>
<th>Risk factor</th>
<th>Coefficient ± SE(^b)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive FCXM(^c)</td>
<td>1.0 ± 0.2</td>
<td>&lt; 0.0001</td>
<td>Positive FCXM</td>
<td>1.9 ± 0.5</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female recipient of a male donor</td>
<td>1.5 ± 0.3</td>
<td>&lt; 0.0001</td>
<td>Younger recipient age(^d)</td>
<td>−1.6 ± 0.4</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-cell PRA &gt; 10%</td>
<td>1.0 ± 0.3</td>
<td>0.007</td>
<td>Older donor age</td>
<td>0.03 ± 0.01</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Early hazard phase coefficient.

\(^b\) Late-phase coefficient.

\(^c\) Negative, Class II, Class I in ascending order of importance.

\(^d\) Log transformation.
4. Discussion

4.1. Sensitization

Alloimmunization to HLAs may occur after previous organ transplantation, pregnancy and blood transfusions. Our data are consistent with these principles by finding a positive transplant crossmatch in a higher proportion of women and patients bridged to transplantation on implantable left ventricular assist devices. We have previously shown that the presence of a ventricular assist device prior to transplantation, through the multiple blood transfusions and infections associated with this procedure, sensitizes the recipient to HLA antigens [15]. These data also demonstrate that patients with elevated panel reactive antibodies against T and B cells were more likely to have a positive Class I crossmatch than those with a low PRA.

4.2. Flow cytometry vs. cytotoxic crossmatch

Evidence is mounting that despite a negative cytotoxic crossmatch, a positive flow cytometric crossmatch predicts poor outcome in both renal and cardiac transplantation [3–5]. This suggests that the cytotoxic crossmatch technique is missing clinically relevant antibodies as demonstrated in this study by the strong association of the flow cytometry crossmatch result with recipient survival and development of vascular rejection.

A three-color flow cytometric crossmatch enabled us to simultaneously study recipient IgG binding to both donor T and B cells. By selective receptor targeting, this technique eliminates both the need to remove recipient IgM reactivity, and to separate the donor cells into B- and T-cell populations, both of which are time consuming procedures essential in the cytotoxic crossmatch technique.

Furthermore, being quantitative, flow cytometry allows surveillance of changes in humoral immunity over time and with different interventions such as pre- and post-transplant plasma exchange. Donor lymphocytes from either lymph nodes or spleen fragments are frozen, stored and used for post-transplant donor specific flow cytometry crossmatch monitoring. We have previously shown that

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Cause of death by FCXM result a</th>
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</thead>
<tbody>
<tr>
<td>Cause of death (n = 48)</td>
<td>Total</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>12 (25)</td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>8 (17)</td>
</tr>
<tr>
<td>Nonspecific graft failure</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Infection</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Other</td>
<td>21 (44)</td>
</tr>
<tr>
<td>Total</td>
<td>48 (100)</td>
</tr>
</tbody>
</table>

a Results are expressed as the number of recipients followed by (percentage of total dead). *P < 0.0001 (log-rank). **P = 0.002 (log-rank).
patients failing to demonstrate a significant decrease in donor specific flow cytometry crossmatch antibody titers with plasma exchange post-transplant, had a poor prognosis [16].

In this study, in 39 (11%) of the transplants the FCXM result was known prior to transplantation. This was mainly due to the often urgent need to transplant and the distant procurement of the donor heart. The crossmatch result was always known within hours of transplantation and helped tailor both the immunological surveillance and treatment of the recipient. However, if the potential recipient is stable and particularly if they are very sensitized, as evidenced by a large panel reactive antibody result, it is our policy to await a local donor to become available in order to obtain a negative prospective FCXM before transplantation is allowed to proceed.

4.3. Vascular rejection

The role of humoral immunity in acute cardiac allograft rejection has only recently been recognized. Patients with circulating antibodies to HLA antigens have been shown to have a greater risk of vascular rejection [7]. Antibodies against donor-specific HLA antigens would be expected to
interact with graft coronary endothelium, which constitutively express HLA Class I and II antigens [17,18]. Our findings support these observations by demonstrating an increased incidence of vascular rejection in patients with a positive Class I and II crossmatch. Furthermore we identified that a B cell panel reactive antibody >10% was a risk factor for the development of vascular rejection, further supporting the involvement of the humoral immune system in its etiology.

Recently, vascular rejection has also been associated with OKT3 induction and the subsequent development of human anti-mouse antibodies [19]. However, Ratliff and colleagues described a population who had vascular rejection, but had not received OKT3. This suggests that exposure to OKT3 is not necessary for the occurrence of vascular rejection [20]. Our results support these previous findings by finding no association between the use of OKT3 and the development of vascular rejection.

### 4.4. B-cell crossmatch

Most literature points to the detrimental effects of transplantation across a positive T-cell crossmatch, largely neglecting the B-cell crossmatch and its impact. Looking at both B- and T-cell crossmatch results, we assigned antibody specificity based on our knowledge of the differential HLA Class I and II expression on these two cells; where T-cells express only HLA Class I antigens, B cells express both Class I and II antigens. With this, we found that anti-HLA Class I antibodies (both T- and B-cell positive FCXM) were associated with death from acute rejection and anti-HLA Class II (only B-cell positive FCXM) antibodies with death from chronic rejection. Furthermore, both a positive B-cell crossmatch and elevated B-cell PRA status were clinically relevant in identifying recipients at risk of developing vascular rejection.

The association between antibodies directed against B lymphocytes and death from chronic rejection has been previously noted in heart transplantation [21]. Similar to the latter series quoted, we observed a rapid accelerated progression of atherosclerosis resulting in death in the presence of a positive B-cell crossmatch with most deaths occurring within 2 years of transplantation. Autopsy results in these patients demonstrated diffuse concentric involvement of the coronary arteries, which is more typical of transplant related atherosclerosis than localized eccentric disease present in pre-existent donor atherosclerosis. Furthermore, the mean donor age for patients dying of chronic rejection was 31 years and together with the pattern of disease, makes it unlikely that death in this group was from pre-existent donor atherosclerosis.

### 4.5. Study limitations

The endpoint examined for the incidence of chronic rejection was recipient death by chronic rejection. Both angiographic and intravascular ultrasound documentation of transplant related coronary artery disease would be informative. Transplant coronary artery disease may, however, be underestimated by angiography and there is no general consensus as to the degree of coronary artery stenosis used to define its presence. Therefore, we chose to use death by transplant related coronary artery disease as a more definitive endpoint.

### 4.6. Conclusions

Despite a negative T-cell cytotoxic crossmatch, flow cytometry crossmatching detects clinically relevant antibodies. If transplantation across a positive flow cytometry crossmatch cannot be avoided, this knowledge can guide the clinician in closer rejection surveillance and in tailoring post-transplant immunosuppression. Furthermore, both the B-cell crossmatch and the B-cell panel-reactive antibody status are clinically relevant and should be considered an integral part of the transplant work up.

### Acknowledgements

We thank Dr James B. Young of the Department of Cardiology for his enormous help and suggestions with this manuscript.

### References


Appendix A. Conference discussion

Dr Joachim Hasse (Freiburg, Germany): Thank you very much for these interesting findings and clear presentation. May I ask which clinical implications your findings have now in your practice of transplantation?

Dr Bishay: I think the importance of flow cytometry crossmatching is not just at the transplant level but also at the post-transplant monitoring of these patients. For example, if a patient has a positive flow crossmatch at the time of transplantation, we are more aware to watch these patients, particularly for vascular rejection, and to perhaps treat more aggressively, and also then to use flow cytometry crossmatching to monitor them and to monitor their response to therapies used post-transplant.