Impaired renal graft survival after a positive B-cell flow-cytometry crossmatch

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Abstract

Background. The clinical and immunological relevance of a positive B-cell flow-cytometry (B-FCXM) crossmatch in renal transplantation is still controversial.

Methods. We retrospectively analysed 145 consecutive cadaveric renal transplantations performed from May 1991 to September 1995 in our institution. All grafts were transplanted following a negative IgG T-cell complement-dependent cytotoxicity crossmatch (T-CDCXM). Concomitantly to CDCXM, B-cell and T-cell FCXM were performed and results were expressed as a mean fluorescence index (FI). Two groups were compared: 116 recipients grafted with a negative B-FCXM vs a group of 19 patients grafted with a positive B-FCXM.

Results. The two groups were similar for length of cold ischaemia, donor and recipient’s age and degree of HLA mismatching. The proportion of patients with pre-transplant anti-HLA class I antibodies or a retransplantation was significantly increased in the positive B-FCXM group vs the negative B-FCXM group. Recipient survival at 48 months was not significantly different in the two groups. However, graft survival at 12 and 48 months was significantly poorer in the positive B-FCXM than in negative B-FCXM (68% vs 90% at 12 months: \( P = 0.007 \), and 57% vs 79% at 48 months: \( P = 0.02 \)). Within the positive B-FCXM group, no differences were found in pre-transplant anti-HLA class I or II alloimmunization as well as retransplantation frequency between the patients who lost their graft and the patients who did not.

Conclusion. Our results suggest that a pretransplant positive B-FCXM is associated with an impaired long-term graft survival in renal allotransplantation.

Key words: B cell; flow-cytometry crossmatch, graft survival, renal transplantation

Introduction

Pretransplant crossmatch (XM) detection of anti-donor IgG antibodies to HLA class I molecules con-traindicates renal transplantation. The presence of such antibodies is associated with hyperacute graft rejection as well as reduced graft survival. Presently, complement-dependent cytotoxicity (CDC) test remains the standard pretransplant crossmatch technique. However, graft rejection and overall poor graft survival can be observed despite a negative CDCXM. Development of alternative methods for the detection of recipient serum antibodies directed against donor determinants is therefore necessary. Several teams perform the anti-globulin assay, increasing the sensitivity of the standard CDC by the addition of a complement-fixing anti-human IgG. Flow-cytometry crossmatch (FCXM) is a more sensitive technique that allows the detection of low titres of both complement-dependent and -independent anti-donor antibodies, and is therefore increasingly used in addition to the standard CDC test [reviewed in 1]. FCXM has been reported to be at least as sensitive as the anti-globulin assay [2].

A positive T-FCXM has been recognized as associated with higher rejections episodes and lower graft survival after renal retransplantation [3,4], as well as after primary renal transplantation [5–7]. However, in two recent studies, T-FCXM showed no advantage over the CDCXM [8,9]. Performing crossmatches on T and B lymphocytes is justified by the differential expression of HLA class I and II molecules on lymphocytes. In contrast to T lymphocytes, B lymphocytes express HLA class II molecules constitutively and HLA class I molecules at a higher density than T cells. Positive B-cell crossmatches may be due to antibodies directed against non-HLA antigens (e.g. autoantibodies), or to HLA class II molecules [10]. Another possibility is the presence of low titres of antibodies to HLA class I molecules [11]. This heterogeneity might contribute to the controversy surrounding the clinical and immunological relevance of positive B-cell crossmatch in renal transplantation [1,12]; in particular
with respect to FCXM because of the high B-cell fluorescence background. The purpose of our study was to evaluate the influence of a pretransplant positive B-cell FCXM (B-FCXM) on the survival of a renal graft.

Subjects and methods

Patients

We analysed 145 consecutive cadaveric renal transplantations performed in our institution from May 1991 to September 1995. All grafts were transplanted after a negative IgG T-CDXCXM with both current and historical sera. Pretransplant transfusion was not systematically performed. Immunosuppressive therapy included corticosteroids, azathioprine, cyclosporine, and anti-thymocyte globulin (ATG 3mg/kg/day) for 10 days after transplantation until June 1993; after that date, one bolus of 9 mg/kg was added at the time of the transplantation surgery). Three patients were excluded from the analysis due to non-compliance to immunosuppressive therapy. FCXM data of seven other recipients were not available and were therefore excluded from the analysis. Graft loss was recorded on the date of return to dialysis, transplant nephrectomy, or death with a functioning graft.

Complement-dependent cytotoxicity crossmatch (CDXCXM)

Donor lymphocytes were obtained from lymph nodes and/or spleen after Ficol-Hypaque centrifugation and evaluated for cell viability by Tripan Blue exclusion. Cells were used to concomitantly perform CDXCXM and FCXM with current and historical sera. The CDXCXM were performed using a fluorescence modified NIH test [13] as follows: 1 µl of a donor cell suspension of unseparated total lymphocytes (at 2 × 10^6 cells/ml) were incubated with 1 µl of recipient serum in Terasaki trays under oil. Following 30 min incubation at 4°C, 22°C and 37°C, 3 µl of rabbit complement was added and incubated for 60 min at 22°C. Cytotoxicity was then evaluated on fluorescence inverted microscope after addition of 3 µl of acridine orange/ethidium bromide. In parallel, T and B lymphocytes (separated with immunomagnetic beads, Dynabeads®) were used as target cells (at 22°C for first incubation and 30 min for the complement step). Results were obtained by evaluating the proportion of dead cells (red fluorescence) among the viable cells (green fluorescence) on an arbitrary scale of 0 (no dead cells) to 4 (100% of dead cells). Recipient serum was treated with Dithiothreitol (DTT) to reduce IgM antibodies (36 µl of serum + 4 µl of DTT 0.1 M incubated for 30 min at 22°C). DTT treated serum was then used to perform the crossmatch with unseparated lymphocytes as described above. Five minutes before addition of complement, 1 µl of 0.02 M cysteine was added to block complement inactivation by DTT. Positive and negative controls using known polyspecific and negative anti-HLA antibodies respectively were performed for all crossmatches.

Flow-cytometry crossmatch

FCXM were performed as follows: 10^6 donor lymphocytes were centrifuged and 3 µl of phycoerythrin (PE)-conjugated CD3 (Immunotech, Marseille) or 100 µl of PE-conjugated CD19 (Becton-Dickinson, Le Pont de Claix) diluted 1/20 were added to the cell pellet. The cells were then incubated with 100 µl of the recipient serum for 30 min at 37°C. After two washes with normal saline (5 min at 600 g) a further incubation of 30 min at 4°C was performed with 100 µl of fluorescein isothiocyanate (FITC)-conjugated F(ab)2 goat antihuman IgG (Kallestad Diagnostics, Marnes La Coquette), diluted 1/200 in normal saline. The cells were then washed twice, resuspended in 200 µl of normal saline and analysed. Results with current sera were retained for analysis in this study. A pool of sera negative for anti-HLA antibodies from donors of AB blood type was used as negative control. The positive control consisted of a pool of sera from highly sensitized patients (PRA > 90%).

Flow-cytometry analysis

The analysis was performed on a FACScan (Becton-Dickinson), using a Lysis software. The data were collected using logarithmic amplification and viable lymphocytes were gated on forward and side scatter profiles. A gate was then set on cells bearing PE fluorescence (minimum of 1000 events). Logarithmic amplification was used to detect the FITC fluorescence, and fluorescence intensity was displayed on a 1024-channel four-decade log scale. A cut-off value for positivity was established at 40 channel mean fluorescence shift to T-FCXM and 60 channel shift to B-FCXM. Results were then expressed as a mean fluorescence index (FI) (negative control: 0%; positive control: 100% control), calculated as follows:

\[
\text{mean FITC fluorescence of the positive control} - \text{mean FITC fluorescence of the negative control}
\]

A FI > 10% was considered as a positive flow-cytometry crossmatch.

Anti-HLA antibodies screening

The recipients’ sera were tested regularly for the presence of anti-HLA class I and class II antibodies (panel reactive antibodies, PRA) using the complement-dependent cytotoxicity technique described above. A recipient’s serum was first tested at his or her registration on the renal transplantation waiting list. The serum was then tested every 3 months except if a sensitization event occurred (e.g. blood transfusion). In this case, samples of serum collected before transfusion and after 8, 15, and 21 days were tested. For anti-HLA class I antibodies screening, the sera were tested by incubation with a panel of 30 cells, selected to represent all frequently encountered HLA specificities as well as most of the rarer ones. If a positive screening was found, the serum was tested on an additional panel of 30 different cells to further identify the HLA specificities of the antibody. For anti-HLA class II antibodies screening, the serum was incubated with platelet pools of 50 donors for 1 h at 22°C and at 4°C (twice with two different pools), in order to absorb the anti-HLA class I antibodies eventually present. The serum was then tested over a panel of 36 selected cells, separated in B and T lymphocytes (used as absorption control). Anti-HLA class I and II antibodies screening was performed with and without DTT reduction of IgM antibodies and only DTT-resistant antibodies were included in the PRA.
Impaired renal graft survival following positive B-cell crossmatch

B-FCXM results and correlation with the CDCXM

Nineteen recipients were grafted with a positive B-FCXM. One hundred and sixteen recipients were grafted with a negative B-FCXM. Mean donor’s age (35.9 and 40.1 years, respectively positive and negative B-FCXM; $P < 0.05$) as well as mean recipient’s age (48.8 and 45.9 years, respectively positive and negative B-FCXM; $P$: ns) were similar in both groups of recipients. The degree of HLA mismatching and length of cold-ischaemia time were also similar in both groups (Table 1). The B-CDCXM was positive for five recipients in the positive B-FCXM group (26%) and for eight patients in the negative B-FCXM group (7%), $P < 0.05$. All the eight recipients in the negative B-FCXM group with a positive B-CDCXM had a negative post-DTT CDCXM. In contrast, CDCXM using unseparated lymphocytes (with or without DTT) was negative in the five recipients with both a positive B-CDCXM and B-FCXM. Of the 19 recipients grafted with a positive B-FCXM, six (31%) also had a positive T-FCXM, while only three recipients (2%) grafted with a negative B-FCXM had also a positive T-FCXM ($P < 0.001$).

Influence of gender, previous transplants and/or presence of anti-HLA class I antibodies

There was a greater number of females in the positive B-FCXM than in the negative B-FCXM (63% vs 32%, respectively, $P < 0.05$). The incidence of a positive B-FCXM was greater in patients previously transplanted compared with first transplants recipients (36% vs 14%, $P:0.04$). Patients with pre-transplant anti-HLA class I antibodies (current PRA >10%) had a higher incidence of a positive B-FCXM compared with nonsensitized recipients (PRA <10%) (56% vs 10%, $P < 0.001$).

Statistical analysis

Statistical analysis was performed using SigmaStat and SigmaPlot software 1.02 version (Jandel Scientific). Mann-Whitney rank sum test and $\chi^2$-test (with Yates correction factor) were performed when indicated. Recipient and graft’s cumulated survival were generated by Kaplan–Meyer analysis and compared by log-rank test at 12 and 48 months post-transplantation ($\geq$10 recipients at risk). A $P$ value $<0.05$ was considered as statistically significant.

Recipient and graft’s survival

Median recipient follow-up was 58.6 months (0.1–83.6 months). Recipient’s survival (Kaplan–Meyer analysis) in positive and negative B-FCXM groups were similar at 48 months (84% vs 91% respectively, $P$:ns). In contrast, graft survival at 48 months was poorer in positive B-FCXM group (57%, 10 recipients at risk) than in negative B-FCXM group (79%, 74 patients at risk; $P:0.02$). Furthermore, earlier graft survival analysis shows that at 12 months post-transplant there was already a significant difference between positive and negative B-FCXM groups (68% vs 90% respectively, $P:0.007$, Figure 1).

Influence of immunological risks factors in the positive B-FCXM group

Nine recipients lost their graft in the positive B-FCXM group, all but one (death with a functioning graft) as a consequence of acute and/or chronic rejection. No differences were apparent in pretransplant anti-HLA class I alloimmunization as well as retransplantation frequency between the patients who lost their graft and the patients who did not (Table 2), but because of small numbers, no statistical analysis was performed. Among the 19 recipients with a positive B-FCXM, graft loss was observed both in recipients with a positive T-FCXM (four out of six) and negative T-FCXM (five out of 13, $P$:ns). Autologous crossmatches (CDCXM) were performed in 13 recipients and were found to be negative. When sera used for the crossmatch were available (13 recipients), we performed a retrospective screening for anti-HLA class II antibodies by the standard CDCXM technique. In the positive sera, no anti-donor specificity could be

Table 1. Pretransplant characteristics of the recipients

<table>
<thead>
<tr>
<th></th>
<th>Positive B-FCXM group ($n=19$)</th>
<th>Negative B-FCXM group ($n=116$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold ischaemic delay (minutes)$a$</td>
<td>1218</td>
<td>1390</td>
<td>ns$^b$</td>
</tr>
<tr>
<td>HLA mismatches (A, B, DR)$a$</td>
<td>3.16</td>
<td>3.05</td>
<td>ns$^b$</td>
</tr>
<tr>
<td>Previous transplantation</td>
<td>7 (36%)</td>
<td>17 (14%)</td>
<td>$&lt;0.05^c$</td>
</tr>
<tr>
<td>Pretransplant anti-HLA class I antibodies</td>
<td>9 (47%)</td>
<td>10 (8%)</td>
<td>$&lt;0.001^d$</td>
</tr>
<tr>
<td>Female</td>
<td>12 (63%)</td>
<td>38 (32%)</td>
<td>$&lt;0.05^e$</td>
</tr>
<tr>
<td>Positive B-CDCXM</td>
<td>5 (26%)</td>
<td>8 (7%)</td>
<td>$&lt;0.05^e$</td>
</tr>
<tr>
<td>Positive T-FCXM</td>
<td>6 (31%)</td>
<td>3 (2%)</td>
<td>$&lt;0.001^d$</td>
</tr>
</tbody>
</table>

$^a$Mean.

$^b$Mann–Whitney rank sum test.

$^c$$\chi^2$ test.
Complement-dependent cytotoxicity is a technique with a strong clinical correlation, widely used as the standard technique for pretransplant crossmatching in renal transplantation. Flow-cytometry crossmatch has several advantages over classical CDCXM: less time-consuming, more sensitive, less technician-dependent and allows a reliable distinction between IgG and IgM antibodies as well as between anti-T-cell or anti-B-cell antibodies [1,14]. However, the interpretation of the test may be difficult, due to the different affinities and specificities of the polyclonal antibodies in the recipients’ sera as well as structure and expression level variability of the target HLA antigens. In addition, the use of an indirect immunofluorescence method increases the difficulty of interpretation. Most critical is the distinction of a weak positive from a negative result. Several methods have been used to distinguish the positive from negative results: a channel shift of fluorescence is the most used criteria [3,15,16]; other laboratories use a fluorescence ratio between the test result and the mean +2 or +3 standard deviations of the negative control result [14,17]. Efforts have been made to standardize the flow cytometry crossmatches by the estimation of the number of cell-bound fluorescein molecules [18]. Using different cells and different sera, we determined a cut-off of positivity as a 40 channel shift for the T-FCXM and 60 channel shift for the B-FCXM (results not shown). After verifying that this technical criteria is fulfilled, we calculated a fluorescence index (FI) as a ratio between the recipient’s result and the positive control result, both corrected by the exclusion of the background negative control result. This allows to include the intrinsic variability of the expression of the HLA antigens on the target cells and to express the final result by a quantitative approach, rendering result’s analysis easier.

Several studies, using CDCXM or FCXM techniques showed an adverse impact on graft survival or a greater incidence of rejection episodes after a positive B-cell crossmatch [11,12,16,17,19]. In contrast, others studies showed no adverse effects [20,21]. In our cohort of 145 consecutive renal transplantations, a positive pretransplant B-FCXM is significantly associated with a lower graft survival at 12 and 48 months when compared to graft survival after a negative pretransplant B-FCXM. Pretransplant alloimmunization anti-HLA class I, as well as a history of previous transplantation, were associated to positive B-FCXM (Table 1). This association has been also described in other studies [12,16]. No difference was found in cold ischaemic time or degree of HLA mismatch between both groups, suggesting these factors were not involved in the observed difference in graft survival.

In the positive B-FCXM group, as one could expect from the high sensitivity of FCXM, the five recipients with a positive B-CDCXM were also found positive by FCXM. However, B-cell positivity was detected in two patients out of 10 with no anti-HLA class I antibodies were found to be positive for anti-HLA class II antibodies. No difference was apparent in the proportion of anti-HLA class II alloimmunization between the patients who lost their graft and the patients who did not (Table 2). In addition, the fluorescence index (FI) of the B-FCXM was equivalent in the two subgroups of patients.

**Discussion**

Complement-dependent cytotoxicity is a technique with a strong clinical correlation, widely used as the standard technique for pretransplant crossmatching in renal transplantation. Flow-cytometry crossmatch has several advantages over classical CDCXM: less time-consuming, more sensitive, less technician-dependent and allows a reliable distinction between IgG and IgM antibodies as well as between anti-T-cell or anti-B-cell antibodies [1,14]. However, the interpretation of the test may be difficult, due to the different affinities and specificities of the polyclonal antibodies in the recipients’ sera as well as structure and expression level variability of the target HLA antigens. In addition, the use of an indirect immunofluorescence method increases the difficulty of interpretation. Most critical is the distinction of a weak positive from a negative result. Several methods have been used to distinguish the positive from negative results: a channel shift of fluorescence is the most used criteria [3,15,16]; other laboratories use a fluorescence ratio between the test result and the mean +2 or +3 standard deviations of the negative control result [14,17]. Efforts have been made to standardize the flow cytometry crossmatches by the estimation of the number of cell-bound fluorescein molecules [18]. Using different cells and different sera, we determined a cut-off of positivity as a 40 channel shift for the T-FCXM and 60 channel shift for the B-FCXM (results not shown). After verifying that this technical criteria is fulfilled, we calculated a fluorescence index (FI) as a ratio between the recipient’s result and the positive control result, both corrected by the exclusion of the background negative control result. This allows to include the intrinsic variability of the expression of the HLA antigens on the target cells and to express the final result by a quantitative approach, rendering result’s analysis easier.

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only by FCXM in 14 patients. We also found eight recipients with a positive B-CDXM and negative B-FCXM. However, CDXM in all these recipients was also positive against unseparated lymphocytes as target cells and was found to be negative against these same cells after DTT serum treatment. These findings suggest that the positive B-CDXM crossmatch in these recipients was due to IgM antibodies, and could explain the negative FCXM, as only IgG antibodies are detected by our FCXM technique.

Previous studies have suggested that a positive FCXM was relevant only in the case of retransplantation [3,4]. Within our positive B-FCXM patients, a retransplantation, as well as pretransplant alloimmunization anti-HLA class I were apparently not associated with an increased risk of graft loss. The specificity of the antibodies detected by FCXM, rather than their mere presence, is probably the determining factor to predict an adverse outcome [22,23]. Several reports have suggested that IgM antibodies, auto-antibodies and antibodies directed against non-HLA antigens have no deleterious effects on graft outcome [11,23–25]. For 13 recipients in the positive B-FCXM group the results of autologous crossmatch (performed by the CDCX technique) were available and were found negative (data not shown). These data, as well as the detection of antibodies of IgG isotype by FCXM, strongly suggest that the positive B-FCXM in these recipients is not in relation with the presence of auto-antibodies.

Antibodies to HLA class II molecules are not usually associated with hyperacute graft rejection, despite some reported cases when present in high titres [12,26]. When available, sera used for the crossmatch in the positive B-FCXM group were retrospectively analysed (by CDC) for the presence of anti-HLA class II antibodies. The presence of such antibodies was not apparently associated with a graft loss.

Several findings in our study suggest that a positive B-FCXM was mainly in relation with the presence of low levels of anti-donor HLA class I molecules: there is a higher proportion of anti-HLA class I alloimmunization and retransplants in the positive B-FCXM group, and the presence of anti-HLA class II antibodies is irregular in these recipients. Furthermore, the T-FCXM was found to be positive in six recipients in the positive B-FCXM, supporting the presence of anti-donor HLA class I molecules as responsible for the positivity of the B-FCXM. The T-FCXM was found to be positive for three recipients in the negative B-FCXM, suggesting an artefact or the presence of non-HLA antibodies. This further supports the importance of the B-FCXM as a complement of the T-FCXM when deciding whether to transplant or not.

Overall, our results suggest that a pretransplant positive B-FCXM is associated with an impaired graft survival in renal allotransplantation. Based on these results, in the presence of positive B-FCXM we have adopted the policy to not transplant; or in individual cases with no other risk factors (such as a female recipient, known prior alloimmunization, prior transplantation), to consider to transplant with the understanding that there is an increased risk for poor graft survival.

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