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Lack of correlation between IgG T-lymphocyte flow cytometric crossmatches with primary renal allograft outcome

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Abstract. The flow cytometric crossmatch (FCXM) has been reported to be more sensitive and capable of detecting very low levels of antibodies than the normally used complement dependent cytotoxicity test. We studied both the two colour IgG T cell FCXM and CDC-XM in 146 renal allograft recipients, 111 primary and 35 regrafts, of which 26% (29/111) of 1st and 20% (7/35) of regrafts had a positive FCXM. There was no overall correlation between the FCXM results and early graft outcome in primary renal allografts. The FCXM did not appear to have any advantage over the CDC-XM in predicting graft outcome in unsensitized first grafts. In the small number of regrafts studied, a positive FCXM was associated with a higher degree of graft failure. FCXM can exhibit false negative results if sera are used solely neat although these prozone phenomena do not influence subsequent graft outcome.

Key words: Flow cytometry – Crossmatch – Renal transplantation – Antibodies – Prozone phenomenon

Following the introduction of pre-transplant crossmatching for recipients of renal allografts by complement dependent cytotoxicity (CDC) tests, the incidence of antibody mediated early graft failure, especially hyperacute rejection, fell dramatically [1]. However both hyperacute and accelerated acute rejections are still seen, even with a negative CDC crossmatch [7], and it has been suggested that a significant proportion of early failures including immediate graft non-function are due to undetected humoral rejection [8]. These findings have lead to attempts to either increase the sensitivity of current crossmatch techniques [9, 12, 15] or to search for alternative crossmatch target cells [3].

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The flow cytometric crossmatch (FCXM) is a very sensitive technique for measuring low levels of anti-donor antibodies which are not detectable by standard crossmatch techniques [2, 4]. The use of FCXM, however, in primary and secondary allograft recipients has been questioned as being too sensitive and not correlating with graft outcome [10]. Other reports have shown that positive FCXM is associated with an increased risk of rejection episodes especially in retransplant patients, allowing the identification of a high risk patient group which have a poor clinical course [2, 9, 12, 14].

In this report we examined the two colour IgG T-cell flow cytometric crossmatch (FCXM) in 146 renal allografts and identified a possible cause of false negative results if recipient sera are used solely undiluted. In addition we performed IgG B cell FCXM in 33 of these transplant recipients.

Patients and methods

Patients. A total of 146 cadaveric donor renal allografts in 143 patients (101 male and 42 female) transplanted between February 1987 and July 1991 were studied. Of these, 111 were first and 35 were regrafts (20 second, 10 third, 4 fourth and 1 fifth). Average age at transplant was 42.4 ± 14.4 years for male patients (range 19–75 years) and 41.2 ± 15.1 years for female patients (range 18–67 years). The mean number of HLA-A, -B and -DR antigen mismatches in the study group was 0.81 ± 0.69, 1.0 ± 0.68 and 0.73 ± 0.65 respectively. Of the patients studied 14.4% (21/146) were beneficially matched according to the criteria of Gilks et al. [5].

Immunosuppression. Primary immunosuppressive therapy consisted of conventional azathioprine and prednisolone (4 cases), cyclosporin monotherapy or cyclosporin with prednisolone (15 cases), triple therapy with azathioprine (95 cases), triple therapy with mizoribine (9 cases), quadruple therapy comprising primary ATG/ALG tailoring onto triple therapy (21 cases), and two cases of primary graft failure.

Lymphocytotoxic assays. Donor lymphocytes were isolated from spleen or lymph node. Splenic lymphocytes were carbonyl iron-treated to remove phagocytic cells. Separated T and B lymphocytes

Table 1. Graft survival after first month post transplant in primary recipients

NIH CDC crossmatch		FACS crossmatch	
	Negative	Negative	Positive
	$N = 111$	$N = 82$	$N = 29$
> 30 day survival	86% (95/111)	84% (69/82)	90% (26/29)
		N5 $P = 0.2$	

Table 2. Graft survival after 1st month post transplant in retransplant recipients

NIH CDC crossmatch		FACS crossmatch	
	Negative	Negative	Positive
	N = 35	$N = 28$	$N = 7$
> 30 day survival	86% (30/35)	89% (25/28)	71% (5/7)
		N5 $P = 0.2$	

were obtained by neuraminidase-treated sheep red blood cell rosetting and lysis of sheep cells with ammonium chloride.

The extended NIH two-stage microlymphocytotoxicity test was performed. Recipient serum (1 µl) was added to a microtitre plate followed by 1 µl donor cells (2×10^6/ml) and incubated for 60 min at 22°C. Then 5 µl rabbit complement (Biotest) was added, followed by further incubation for 120 min at 22°C. The cytotoxicity test reactions were assessed by fluorescent microscopy, using acridine orange and ethidium bromide staining. The criterion for a positive test result was defined as a 5–10% or greater proportion of killed cells above background.

Panel reactive antibodies (% PRA). Recipient serum samples were routinely screened against a panel of at least 70 cells from 50 individuals; this panel comprised 20 isolated T and B cells, 20 peripheral blood lymphocytes and 10 chronic lymphocytic leukaemia cells. A serum was considered to have panel reactive antibodies if greater than 3% of the cell panel was positive. Unsensitized recipients were defined as having < 10% PRA with a current serum sample at the time of transplant; 79.5% (116/146) patients were classified in this category. Sensitized patients were regarded as having > 10% PRA with 20.5% (30 recipients) in this group and only 6 patients (4%) being highly sensitized, > 85% PRA.

Flow cytometric crossmatch. Donor spleen cells were either isolated from fresh splenic material or retrieved from cyropreservation. Washed spleen cells were subsequently incubated at 37°C for 30 min in RPMI 1640 at 10^7 cells/ml to ensure removal of any cytophilic immunoglobulin. Mean donor spleen cell viability was 78.0% ± 15.4 (range 40–100%) prior to use. Spleen cells were washed and resuspended in cold phosphate buffered saline (PBS), pH 7.2, containing 0.1% sodium azide, at 10^7 cells/ml. We added 100 µl cell suspension (10^6 cells) to a FACS sample tube, it was spun and the supernatant discarded; 100 µl recipient's serum was added to the cell pellet, cells resuspended, and incubated for 30 min at 22°C.

In order to avoid possible false negative results due to 'prozone phenomena' patient sera were also tested at a 1:4 dilution in PBS. All tests included a positive control, consisting of a pool of 4 highly sensitized renal patient sera (> 95% PRA) and a negative control, pooled human AB serum (minimum 6 individuals) that had been screened for the absence of erythrocyte antibodies, lymphocytotoxins and blocking activity in mixed lymphocyte cultures. Following the primary antibody incubation stage, the cells were washed 3 times in cold PBS/azide, the total wash volume being approximately 10 ml. Subsequently, cells were pelleted and 20 µl FITC conjugated $F(ab')_2$ rabbit anti-human IgG (Dakopatts), diluted 1:10 in PBS was added, followed directly by either 5 µl R-phycoerythrin (PE) conjugated mouse monoclonal anti-human CD3 (Serotec) for T cells, or 5 µl PE-conjugated anti-CD19 (Dakopatts) for B cells; this was incubated for 30 min at 4°C. Following a second wash cycle, cells were resuspended in 200 µl cold PBS/azide followed by the addition of 300 µl 0.5% paraformaldehyde in PBS. Cells were left at 4°C prior to FACS analysis.

Data analysis. The labelled samples were analysed on a FACScan flow cytometer (Becton Dickinson) with a 15 mW argon Laser at 488 nM. Band pass filters of 530 nM and 585 nM were used for fluorescein and phycoerythrin fluorochromes respectively. Fluorescence detectors were on logarithmic amplifiers with 4 log decade scales. Forward angle (FSC) and side angle (SSC) light scatter profiles were collected and a lymphocyte gate constructed on the basis of these FSC/SSC characteristics. For T cell crossmatches, FITC staining was assessed for both CD3 + ve and CD3 – ve cells using histograms constructed for each group.

For B-cell crossmatches CD19 + ve and CD19 – ve histograms were used. In the initial phase of the study Consort 30 software was used for the analysis. In the later stages cells were analysed using Becton Dickinson Lysys 2 software. Median T cell or B cell fluorescence intensity was obtained for each histogram using geometric statistics, and the sample median (mean with Consort 30) then compared to both negative and positive controls.

A positive FACS crossmatch was defined as a shift in the median channel of fluorescence of > 20 channels to the right of either the T cell peak (CD3) or B cell peak (CD19) in the patients sera compared with the human AB serum negative control [9].

Statistical analysis. Graft survivals were calculated using acturial life-table methods [6] over the first 90 days in cohorts of 5 days and at 1, 3, 6 and 12 months post transplant. Patients were followed for a minimum of one month and were included in the analysis irrespective of the cause of graft failure, including patient death with a functioning graft. Analysis of results were carried out using chi-square and Fisher's exact 2 × 2 contingency tables, *t*-tests and Mann-Whitney U-tests using the University of Southampton Faculty of Medicine MEDSTAT programme.

Results

All 146 renal allograft recipients had a negative NIH extended T cell crossmatch (Tables 1 and 2). One 1st graft recipient, 0.9% (1/111), had a positive B cell crossmatch due to an IgM non-HLA auto-antibody. The graft is currently surviving > 11 months. In first graft recipients 26% (29/111) had a positive IgG T cell FCXM with 90% (26/29) surviving > 30 days post transplant (Table 1). There was no statistically significant difference between 30 day graft survival in FCXM + and – groups ($P = 0.2$, Table 1). At 90 days post transplant the actuarial graft survival was 83% for T cell FCXM + compared to 79% for FCXM – recipients, $P = 0.15$ (Fig. 1). The 6 month and 1 year graft survivals were 83% and 78% for FCXM + recipients compared to 78% and 78% for FCXM-recipients respectively. On testing the recipient serum at neat and at a 1:4 dilution against donor spleen cells, 48% (14/29) of 1st graft recipients were shown to be Nt – and 1:4 + . This prozone phenomenon could have led to these grafts being regarded as FCXM negative giving false negative FCXM results if the patients serum had not been tested in dilution. However the presence of these T cell FCXM Nt –

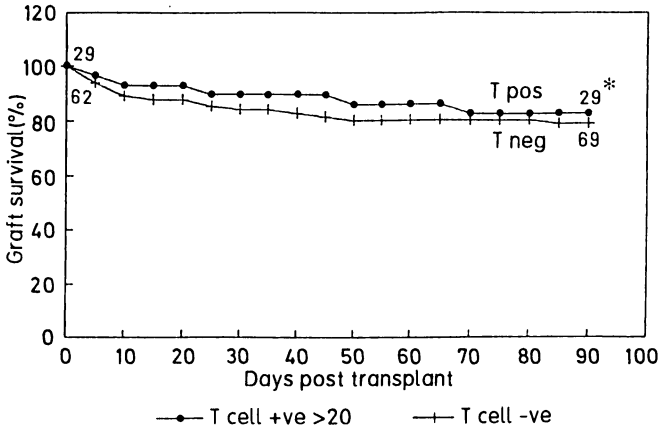

Fig. 1. IgG T cell FACS crossmatch: primary grafts. The *asterix* indicates the number of patients followed-up at each time interval

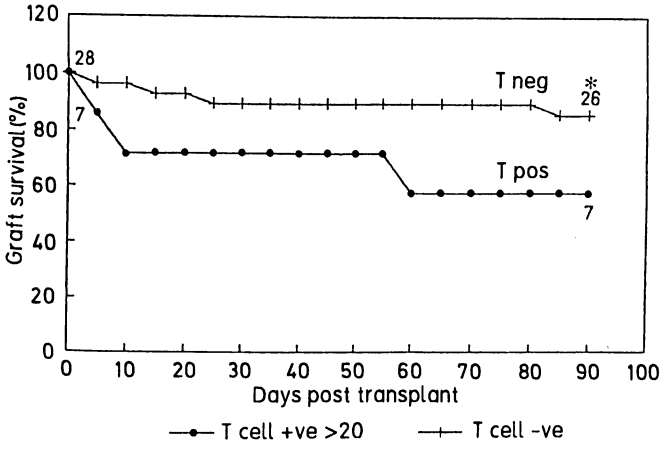

Fig. 2. IgG T cell FACS crossmatch: regrafts. The *asterix* indicates the number of patients followed-up at each time interval

1:4+ recipients did not alter the lack of correlation between T cell + recipients and graft outcome. Analysis of several patient parameters in first grafts revealed no statistical differences in HLA-A, -B, -DR locus mismatches, % peak and current PRA, % beneficially matched grafts, male : female ratio, the serum creatinine at 90 days and the number of rejection episodes post transplant in T cell FCXM + and FCXM – groups. However in the FCXM + grafts mean age at transplant was 48.8 ± 13.7 years compared to 42.3 ± 15.0 years in the FCXM – group ($P = 0.03$). The donor spleen cell viability also showed a significant difference, being 73.1 % ± 15.6 in the FCXM positive group compared to 80.3 % ± 14.7 in the FCXM negative group of patients ($P = 0.03$).

In the regraft recipients 20 % (7/35) had a positive T cell FCXM crossmatch with 71 % surviving > 30 days post transplant (Table 2). As with the first grafts there was no significant difference between 30 day graft survival in the FCXM + and – groups. At 90 days post transplant the actuarial graft survival was 57 % for T cell FCXM + compared to 85 % for T cell FCXM – recipients, $P = 0.28$ (Fig. 2). The number of FCXM positive regrafts of seven is too small to be of statistical significance, but the trend is towards graft failure in the FCXM + group. The 6 month and 1 year graft survivals were 57 % for FCXM + recipients compared to 85 % and 73 % for FACS-recipients respectively. In the regraft patient group there were 29 % (2/7) prozone phenomena seen. Analysis of patient parameters as for first grafts (see above) revealed no significant differences between FCXM positive and negative groups. The IgG B cell FCXM was positive in 6 % (2/33) of patients studied (Table 3). There was insufficient data to examine the effect of B cell FCXM on graft outcome.

Table 3. Graft survival after 1st month post transplantion in 33 allograft recipients (23 1st and 10 regrafts)

NIH CDC crossmatch	FACS B-cell (CD19 +) crossmatch	
Negative	Negative	Positive
N = 33	*N* = 31	*N* = 2
> 30 day survival	94 % (31/33)	94 % (29/31)	100 % (2/2)
	N5 $P = 0.9$	

Discussion

The crossmatching of recipient sera and donor lymphocytes in order to avoid or reduce the incidence of antibody mediated rejection is still one of the major considerations prior to renal transplantation. It is arguably the most important role of tissue typing and histocompatibility laboratories. The incidence of hyperacute rejection, seen following the introduction of the CDC lymphocytotoxicity test as a pre-transplant crossmatching technique, fell from 10–12 % of all grafts in 1967 to less than 0.5 % in 1988 [1].

The criteria for, and the significance of, a positive crossmatch has been revised considerably. The current concensus about complement dependent cytotoxicity crossmatching is that a positive crossmatch on a current serum sample against donor T lymphocytes due to IgG HLA class I (A, B and C) alloantibodies is an absolute contraindication to transplantation [15].

The introduction of flow cytometric crossmatching to detect low levels of donor reactive antibodies [2, 4, 9, 11, 12, 14] has lead again to a reappraisal. In this study first grafts were successfully transplanted across a positive FXCM suggesting that renal transplantation can occur without hyperacute rejection even if low titer donor-reactive preformed antibodies are present. The incidence of false positive results in primary recipients was 27 % at 30 days post transplant (number of grafts FXCM + > 30 days/total number of grafts > 30 days). This suggests that the FXCM is over-sensitive and has a high rate of false-positive results not correlating with graft outcome. Similar findings have been reported by other groups [2, 11, 14].

One caveat to this is that in our study group 82 % (91/111) of primary graft recipients were unsensitized (< 10 % PRA). In primary allograft recipients FCXM appears to have no advantage over the NIH CDC crossmatch in predicting graft outcome (Table 1). Thus we feel that the prospective use of FXCM in primary allografts and denying a transplant on the basis of a positive FXCM

is not warranted. In addition we were unable to identify groups of patients with a poor clinical course (high number of rejection episodes, serum creatinine at 90 days) as has been shown in other studies [11, 14]. With only 3% (4/146) of allograft recipients receiving conventional azathioprine and prednisolone, the remainder having either CyA, CyA-Pred, Triple or Quadruple immunosuppressive therapy, the clinical relevance in the cyclosporin era and modern immunosuppressive regimen of detecting these low levels of weak donor reactive antibodies has to be re-evaluated [9, 11].

It has been suggested that FCXM should be confined solely to regraft patients [9, 12]. We had a limited number of grafts available to study (7 FCXM +) with no statistically significant difference between the FCXM positive and negative groups although there appeared to be a trend towards graft failure in the positive FCXM group. These findings require confirmation in a larger cohort of patients.

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