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The value of flow cytometric crossmatch in cardiac transplantation

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Abstract One of the major clinical problems in cardiac transplantation is that of moderate rejection of the graft, and over the past few years there is increasing evidence that humoral antibody may be important in graft prognosis. The sensitivity of the conventional cytotoxic crossmatch has been questioned, and an increased significance of there of the flow cytometric crossmatch (FCXM) to detect the presence of antibodies before transplantation has been reported. In this study we have examined the sera of 138 cardiac transplants (1988–1992) for the presence of donor-directed IgG and IgM antibodies using FCXM. Sera were collected immediately before transplantation and before the institution of immunosuppressive therapy. All pretransplant cytotoxic crossmatches were negative. After a minimum follow-up period of 3 months, the performance of the transplants was graded by endomyocardial biopsy: 1, no or mild evidence of rejection; 2, patients showing moderate rejection requiring increased immunosuppression. Of the 138 patients studied, 10 patients were excluded as they died within the first week of transplantation. Eight children were excluded since they were given prophylactic ATG (Merieux). A positive FCXM result was defined as showing values in excess of that found for the AB control sera. A significant association was found between the presence of both IgG to T and B cells and IgM to T cells and graft performance ($P = 0.02$ and 0.93, respectively). Indeed, IgM-directed T-cell antibodies were only found in patients with moderate rejecton.These two groups were mutually exclusive, so that the FCXM was able to identify the presence of moderate rejection in 55% of the patients. In conclusion, results show that pretransplant FCXM in cardiac transplantation provides a more sensitive assay of antibody status in recipients and has proved to be of prognostic value.

Key words Crossmatch
Cardiac transplantation
Flow cytometry

Introduction

Over the past few years, the prognostic value of the pretransplant flow cytometric crossmatch (FCXM) has been confirmed in clinical renal transplantation [1]. Correlations between the presence of "binding" antibodies detected by flow cytometry and both renal graft survial and graft performance have been noted [2]. Preliminary results have also indicated that flow cytometric assays detect IgG in the sera of heart graft recipients to donor T and B cells, which are undetected by the conventional cytotoxic crossmatch [3]. Such patients also show poorer graft performance as defined either by endomyocardial biopsy or on the basis of clinical criteria. The aim of the present study was to test the relationship between the presence of pretransplant immunoglobulins (IgG and IgM) in the sera of cardiac transplant patients detected by FCXM and subsequent graft performance.

Materials and methods

A total of 138 consecutive cardiac transplant patients as studied and followed for a minimum period of 3 months after transplantation. Patients were given cyclosporin A, azathioprine and prednisone as their maintenance therapy post-operatively, and all patients had conventional cytotoxic crossmatches performed between their immediate pretransplant sera and donor splenic T cells using the standard NIH technique. Rejection of the transplanted graft was diagnosed on the basis of both endomyocardial biopsy [4] and clinical performance.

Cell/sera preparation

Donor mononuclear cells were isolated from spleen samples taken at organ retrieval. After suspension in Earles BSS, the lymphocytes were separated by Ficoll-Hypaque gradient centrifugation. Donor cells in 10% DMSO and recipient sera were stored, respectively, in liquid nitrogen or at −20 °C until required.

Conventional crossmatch

A standard method (CXM) [2] was employed which consisted of the incubation of test sera with separated donor T and B cells in the presence of rabbit complement. Cell viability was assessed by ethidium bromide and acridine orange double staining, and if more than 10% of the cells as estimated as being dead, compared with the negative control (AB) sera, the cell/sera combination under test was reported as positive. Panel reactivity was determined by incubating the pretransplant recipient serum with a lymphocyte panel of adult healthy volunteers using CXM. Panel reactivity was considered positive if the recipient serum reacted with any of the lymphocytes tested.

Flow cytometric crossmatch

The dual-colour flow cytometric binding assay for the detection of IgG in the recipient directed against donor lymphocytes was used [2]. This assay has proven to be a very sensitive and quantitative method for detecting T- and B-cell-bound antibody. Briefly, donor lymphocytes were incubated with the recipient's pretransplant serum, and then after washing, fluorescein-conjugated sheep $F(ab)_2$ anti-human IgG (Sigma Dorset) was used to detect bound IgG. At this stage a further wash was performed to minimise any cross-reaction with the mouse monoclonal anti-CD3 used in the next stage. Anti-Leu-4 (CD3, T cell) or anti-Leu-16 (CD20, B cell) monoclonal antibodies conjugated with phycoerythrin (Becton Dickinson, Oxford) were then added to detect either donor T cells or B cells. To identify IgM antibodies which may have bound to the cells of the donor, fluorescein-conjugated anti-human IgM (Caltag, San Francisco) was added in place of the anti-human IgM.

Flow cytometric analyses were performed on a FacScan (Becton Dickinson, Oxford) with fluorescence excitation being produced by an argon laser (15 mW of 488 nm light). The instrument was calibrated with FITC- and PE-labelled beads (Flow Cytometry Standards, Becton Dickinson, Oxford). List mode data were collected using linear amplification for the forward and side scatter parameters and logarithmic amplification for the fluorescent parameters. The median of 530 nm fluorescence (i.e. anti-IgG or anti-IgM) or 575 nm phycoerythrin-positive cells (i.e. either T cells or B cells) was compared between the test serum (in duplicate) and five AB sera from normal, healthy, adult volunteers. Data acquisition and analysis were performed with LYSYS II software. Comparisons were made between the fluorescence intensity of the test and control sera using the equation:

$$\text{Relative fluorescence} = \frac{\text{Median channel of fluorescence of test sera}}{\text{Median Channel of fluorescence of AB controls}}$$

In the analysis of the data, relative fluorescence values greater than 1.01 were considered positive.

Results

Ten of the patients were excluded from the analysis because they died within 1 week of transplantation. In the IgG crossmatch studies 36 of the patients were either to have at least one episode of "moderate" rejection according to Billingham's criteria [4] on endomyocardial biopsy ($n = 27$) or to show clinical evidence of rejection which required increased immunosuppressive therapy ($n = 9$, group 2). In the IgM FCXM studies 18 patients showed rejection (biopsy confirmed $n = 13$, clinically confirmed $n = 5$). All episodes of rejection responded to bolus methylprednisolone. The other 56 or 22 patients, in the IgV or IgM study groups, (group 1), respectively, never showed more than "minimal" or "mild" rejection by the same criteria, and none of these patients required augmented immunosuppression.

Comparison between recipient variables is summarized in Table 1. As can be seen, no correlation was found between graft performance and any of the standard recipient variables. Similarly, no correlations were found between HLA compatibility and graft performance

Table 1 Summary of recipient variables in cardiac transplant patients (group 1, patients with either mild or no rejection; group 2, patients with moderate biopsy-proven or recurrent clinical rejection)

Recipient variables	Rejection status		Significance [a]
	1	2	
Age (years)	45.9 ± 11.3	45.2 ± 11.9	NS
Sex (% male)	92% (66/72)	84% (47/56)	NS
Blood group (% matched)	72% (48/67)	69% (34/49)	NS
CMV status (% mismatch-positive donor to negative recipient)	33% (19/57)	31% (11/36)	NS
Ischaemic time (min)	178.2 ± 40.8	179.2 ± 39.1	NS
Days in hospital	22.0 ± 8.2	22.5 ± 8.0	NS

[a] Association analysis was performed with the Fisher test

Table 2 Summary of histocompatibility and panel reactive status of cardiac transplant patients

Tissue match and antibody status	Rejection status		Significance
	1	2	
HLA, -A, -B and -DR mismatches			
A. locus	1.36 ± 0.71	1.46 ± 0.71	NS
B. locus	1.65 ± 0.56	1.61 ± 0.62	NS
DR locus	1.47 ± 0.64	1.42 ± 0.68	NS
Total mismatches	4.49 ± 1.15	4.46 ± 1.21	NS
Panel reactivity			
T cell (%)	6.7 (1/15)	14.3 (3/21)	NS
B cell (%)	20.8 (3/15)	33.3 (7/21)	NS
T and B cell (%)	6.7 (1/15)	14.3 (3/21)	NS

Table 3 Summary of crossmatch results in cardiac transplant patients

	Rejection status		Significance
	1	2	
Conventional cytotoxic crossmatch			
T cell (% positive)	8.0 (2/25)	19.2 (5/26)	NS
B cell (% positive)	12.0 (3/25)	34.6 (9/26)	NS
T and B cell (%)	8.0 (2/25)	19.2 (5/26)	$P = 0.04$
FACS crossmatch			
IgG positive to T cell (%)	26.8 (15/56)	27.7 (10/36)	NS
IgG positive to B cell (%)	26.8 (15/56)	41.7 (15/36)	NS
IgG positive to T and B cells (%)	3.6 (2/56)	19.4 (7/36)	0.015*sig
IgM positve to T cell (%)	0 (0/19)	27.8 (5/18)	0.020*sig
IgM positive to B cell (%)	31.8 (7/22)	55.6 (10/18)	NS
IgM positive to T and B cells (%)	4.5 (1/22)	16.7 (3/18)	NS

(Table 2). Whilst no correlation between graft performance and panel reactivity was seen, it is interesting that an increased incidence of raised reactivities (either T cell, B cell or both) was found in patients showing rejection. Comparisons between panel reactivity results and FCXM results were difficult to make as the number of tests performed on the same immediate pretransplant sample as low. With regard to the conventional CXM (Table 3), the presence of cytotoxic antibodies showed a definite trend towards an association with the presence of rejection. This achieved a significant correlation value in patients who showed both T- and B-directed antibodies ($\hat{P} = 0.04$; Table 3). In analysing these data the results of samples not taken immediately before transplant were omitted. Several examples of a change in antibody status were found between the previous and pretransplant samples. In a study conducted by Smith et al. [5], a pronounced effect of the presence of panel reactive cells

was found. In their study, panel reactive antibody values in excess of 10% were considered important.

With regard to the FCXM, significant associations were found between the presence of IgG to donor T and B cells and IgM to donor T cells. With respect to IgG positivity to donor T and B cells, the incidence of FCXM-positive results was only 3.6% in non-rejecting patients and 19.4% in patients with rejection ($P = 0.015$). IgM positivity to T cells was found in none of the non-rejecting patients and in 27.8% of patients with rejection ($P = 0.02$). None of these combinations was associated with antibodies detected in the conventional CXM. In all cases the association between the presence of IgG or IgM antibodies was mutually exclusive. When the results were combined, it was found that if patients possessed either IgG or IgM antibodies to donor cells in their pretransplant sera, 55% showed rejection problems. On the other hand, only 2.7% of patients with no rejection problems exhibited antibodies ($P > 0.001$).

Discussion

Results from this study have confirmed the value of FCXM in cardiac transplantation in predicting patients at risk from moderate or severe rejection. The time taken for the FCXM to be completed is 3–4 h if donor spleen cells are used, and as such its routine use is precluded in heart transplantation. If donor peripheral blood is available before organ retrieval, the FCXM may be performed before the operation. Currently in Newcastle, the FCXM is performed on all heart transplant recipients, and the surgeon is informed of the result, either during transplantation or on the first postoperative day. It may be that modified postoperative immunosuppression should be given in cases where a positive crossmatch is found. The presence of such antibodies may well need to be considered when postoperative immunosuppression is given. It is impossible to make a good correlation between the results of panel reactivity and the FCXM due to the low number of patients studied.

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