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Posttransplant donor-specific antibody characterization and kidney graft survival

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Abstract This study was designed to investigate the clinical relevance of donor-specific antibodies (DS-Abs) and their influence on graft survival. Among 106 patients who underwent cadaveric kidney donor transplantation and were monitored by flow cytometry crossmatch (FCXM) during the 1st posttransplantation year, 25 (23.6%) resulted positive for DS-Ab production. During a 2-year follow up only 12 of the 81 FCXM-negative patients (14.8%) suffered rejection vs 17 of 25 FCXM-positive patients (68%; $P = 0.00001$). Correlating graft loss to DS-Ab production, 9 FCXM-positive patients lost the graft vs only 1 among the FCXM-negative patients. A worse graft function was evidenced in FCXM-positive subjects who had also suffered rejection episodes than in those which had acute rejection but did not produce DS-Abs. A high incidence of HLA-AB mismatches was found in FCXM-positive subjects which produced anti-class I antibodies. FCXM appears useful in estimating posttransplant alloimmune response. Moreover our findings confirm the harmful effects of anti-class I DS-Abs on long-term graft survival.

Key words Kidney transplantation immunology · Kidney transplantation monitoring · Flow cytometry crossmatch · Donor-specific antibodies · Class I histocompatibility antigens immunology

Introduction

Many authors agree in considering early acute rejection (ARj) occurrence and donor-specific antibody (DS-Ab) production to be the most important risk factors for the development of chronic graft failure [1–3]. In order to reveal the presence of alloantibodies, many techniques have been used, but flow cytometry crossmatch (FCXM) has revealed a higher sensitivity, especially in evidencing the presence of low titre antibodies which cannot be assessed by complement-dependent lymphocytotoxicity (CDC) [4–6]. Among the advantages of FCXM is its ability to detect complement-fixing as well as non-fixing antibodies and to simultaneously identify the alloantibody class of the (IgG and/or IgM) and their type of target cells (T and/or B lymphocytes) [7].

To exactly characterise the anti-HLA class I or II specificity of such alloantibodies, a new cytometry technique, which uses microbeads coated with purified HLA antigens (FlowPRA Screening Test; One Lambda, Calif., USA) has been introduced. There is plenty of evidence about the clinical utility of FCXM before renal transplantation, while only limited information has been supplied regarding the role of posttransplant FCXM in monitoring donor-specific humoral immune response [8, 9] and in predicting chronic rejection (CRj) occurrence [10, 11]. This study was designed to analyse the posttransplant humoral response to mismatched HLA antigens of transplanted kidneys in order to evaluate the role played by such DS-Abs in the occurrence of CRj and, consequently, renal graft survival.

Materials and methods

We enrolled in our study the 106 recipients who underwent cadaveric renal transplant at the Transplant Unit of Clinical Surgery, Tor Vergata University of Rome, and whose donor lymphocytes were available. Prior to transplantation none of the patients showed the presence of antibodies to donor cells using both CDC-XM and FCXM analysis. Best donor-recipient HLA matching was the basic criterion for selecting kidney transplant recipients; matching priority was HLA-DR, HLA-B, HLA-A. The patients' immunosuppressive regimen was based on a triple-therapy protocol: cyclosporine/prednisone/azathioprine or mycophenolate mofetil. All patients received 500 mg methylprednisolone on the day of surgery. ARj episodes were treated by administering three boluses of methylprednisolone (0.5–1 g/dose).

Close clinical monitoring was performed on the recipient population for at least 2 years after transplantation. ARj episodes were diagnosed evaluating clinical symptoms and confirmed by needle core biopsy. Serum creatinine levels were considered the main parameter for assessing graft function. Deteriorated renal function with a stable serum creatinine level higher than 4.0 mg/dl was established as the parameter for defining graft failure. In some cases the diagnosis of CRj was based on pathological data.

Serum samples were collected from the recipient before transplantation, then regularly during the 1st posttransplant year (10 samples per patient minimum) and every time clinical symptoms led to suspect the occurrence of ARj. FCXM monitoring was performed using a three-colour fluorescence technique [7]. Briefly, 2.5×10^5 donor spleen lymphocytes were incubated with 75 µl undiluted serum for 30 min at room temperature. After two wash steps the lymphocytes were incubated for 30 min at 4 °C with 50 µl diluted fluorescein isothiocyanate (FITC)-conjugated F(ab')2 goat anti-human IgG or IgM (Dakopatts, Denmark), 5 µl peridinin chlorophyll protein-conjugated anti-CD3 monoclonal antibody and 5 µl phycoerythrin-conjugated anti-CD20 (Beckton Dickinson, Calif., USA). The cells were then washed twice and resuspended in 200 µl 1% paraformaldehyde in PBS until analysis. In all FCXM samples, donor lymphocytes were incubated with test serum, a positive control serum (a pool of patients' sera with > 90% panel reactive antibodies) and a negative control serum (a pool of > 5 sera from healthy male subjects). FACScan flow cytometer and Cell Quest software (Becton Dickinson) were used to analyse FCXM. The crossmatch was considered positive when a median channel shift of more than 2 SD above the predetermined median channel shift from the negative control was evidenced.

All FCXM IgG-positive sera were retrospectively analysed using the FlowPRA class I and II screening test which consists of a pool of microbeads coated with purified HLA class I or class II antigens from 30 cell lines covering all common HLA antigens. Five microlitres of class I or II beads were incubated with 20 µl serum sample for 30 min at room temperature. After two washes, 1 µl FITC-conjugated goat anti-human IgG (Fcγ) was added and the samples were then incubated for 30 min at room temperature. The beads were then washed twice and resuspended in 0.5 ml fixing solution. Negative and positive control sera were tested in the same manner. A two-colour analysis was performed using Cell Quest software. Two gates were set on FL2 histogram to analyse separately class I (= FL2 negative particles) and class II (= FL2 high fluorescent particles) beads. The percentage of positive reactions, due to antibodies binding the microbeads, were represented in the FITC histogram by the percentage of beads shifted to the right of the cut-off point set on FL1 histogram of negative control serum. The chi-squared test and the Mann-Whitney two-sample test were used for statistical comparison. A P value <0.05 was considered significant.

Table 1 Flow cytometry crossmatch (FCXM) results compared to acute rejection (ARj) occurrence and graft outcome

	FCXM positive (25 patients)	FCXM negative (81 patients)	Significance
ARj positive	17 (68%)	12 (14.8%)	$P = 0.00001$
ARj negative	8 (32%)	69 (85.2%)	
Graft failure	9 (36%)	1 (1.2%)	$P = 0.00001$
Good function	16 (64%)	80 (98.8%)	

Results

Out of 106 subjects enrolled in this study, all but three received primary cadaveric renal transplantation. The occurrence of ARj was evidenced in 29 subjects. ARj or CRj caused graft failure in 10 patients; only 1 patient lost the graft for severe ARj resistant to immunosuppressive therapy. Two more cases of failure were registered due to infectious causes, one of which eventually led to the patient's death.

During the 1st year after transplantation, FCXM monitoring showed the production of DS-Abs in 25 patients (23.6%), in 23 of whom the appearance of alloantibodies occurred within the first 3 months after transplantation. All but 3 FCXM-positive patients (88%) produced IgG DS-Abs while only IgM DS-Abs were found in the sera of the remaining patients (12%). Analysing FCXM results in relation to ARj occurrence, a significantly higher incidence of graft rejection in FCXM-positive patients (17 patients out of 25; 68%) vs FCXM-negative patients (12 patients out of 81; 14.8%; $P = 0.00001$) was evidenced. Moreover the appearance of antibodies preceded by some days any clinical evidence of rejection in 6 of the 17 (35%) FCXM-positive/ARj-positive patients. As far as graft survival is concerned, a higher incidence of renal failures was ascertained in FCXM-positive patients when compared to negative ones (36% vs 1.2%; $P = 0.00001$; Table 1).

Serum creatinine level analysis was performed 3, 6, 12 and 24 months after transplantation demonstrating marked differences in our patient population (Fig. 1). FCXM-positive patients constantly showed significantly higher mean creatinine values than those of FCXM-negative subjects (3 months: 3.26 vs 1.77, $P < 0.0001$; 6 months: 2.43 vs 1.77, $P < 0.0001$; 1 year: 2.55 vs 1.66, $P < 0.001$; 2 years: 2.63 vs 1.46, $P < 0.003$).

Correlating both FCXM status and ARj occurrence with graft function, a remarkably higher incidence of graft failure was evidenced in FCXM-positive/ARj-positive patients (47.1%) than in subjects showing only FCMX positivity (12.5%) or only ARj occurrence (8.3%; Table 2). When considering the trend of serum creatinine levels throughout our observation period, this parameter clearly appears to be more closely linked to FCXM status than to the occurrence of ARj episodes

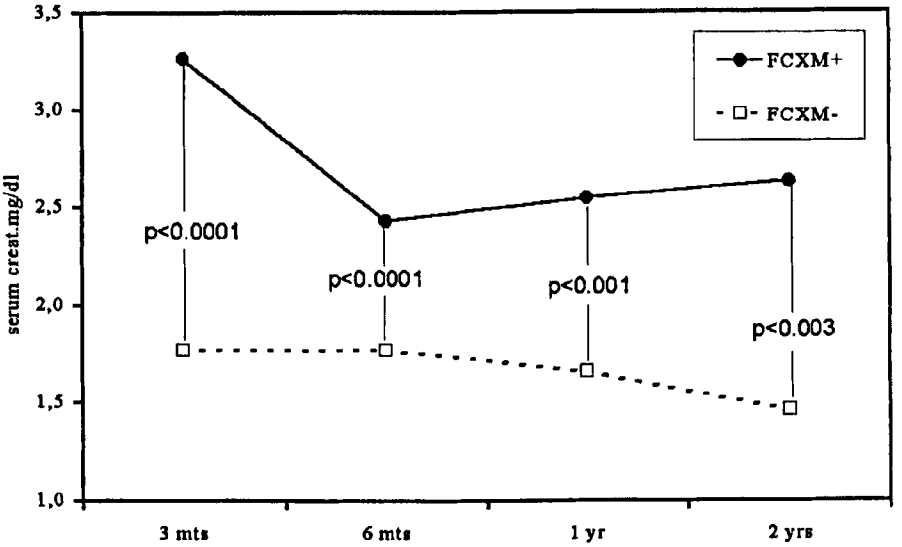

Fig. 1 Mean serum creatinine level trend: a comparison between flow cytometry crossmatch (FCXM)-positive (*FCXM +*) and FCXM-negative (*FCXM–*) patients throughput out observation period

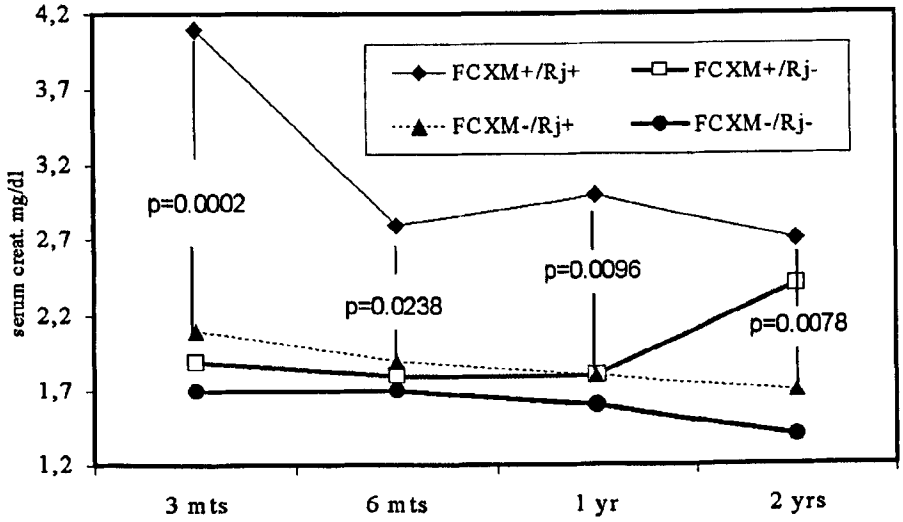

Fig. 2 Mean serum creatinine level trends in our patient population grouped according to FCXM status and acute rejection (*Rj*) occurrence

(Fig. 2). In fact, FCMX-positive/ARj-positive patients have shown constantly higher serum creatinine levels than the FCXM-negative/ARj-positive patients (3 months: 4.07 vs 2.14, $P = 0.0002$; 6 months: 2.80 vs 1.94, $P = 0.0238$; 1 year: 2.95 vs 1.80, $P = 0.0096$; 2 years: 2.72 vs 1.75, $P = 0.0078$). Moreover, FCXM-positive patients which had not suffered ARj episodes, evidenced an increasing trend of mean serum creatinine levels, rising from 1.85 mg/dl 1 year after transplantation to 2.45 mg/dl at the end of the second year.

The sera of 18 out of 25 FCXM-positive patients were examined by the FlowPRA class I and II test. Antibodies directed against class I antigens were evidenced in 44.4% of patients (8 out of 18), while anti-class I and II Abs were found in 50% cases (9 patients). Exclusive anti-class II antibody production was found in only 1 patient (Fig. 3). Seventeen percent of the samples containing anti-B IgG antibodies at FCXM analysis presented exclusively anti-class II antibodies at FlowPRA analysis. A significant incidence of HLA-AB mismatch was evidenced in anti-class I-positive patients vs negative patients (mean value 2.23 ± 1.20 vs 1.79 ± 0.92; $P = 0.0489$) when correlating HLA-A, B and DR mismatch to the presence of anti-class I and II antibodies (Table 3). On the other hand, no correlation between DR mismatch and the presence of anti-class II was evidenced.

Discussion

This study was designed to evaluate the relevance of DS-Abs on graft outcome in 106 kidney transplanted patients. The correlation between humoral immune re-

Table 2 Role of FCXM status and/or ARj occurrence on graft outcome. FCXM positive/ARj positive vs FCXM negative/ARj positive: $P = 0.043$

	Number of patients	Good function	Graft failure
FCXM positive/ ARj positive	17	52.9%	47.1%
FCXM negative/ ARj positive	12	92.7%	8.3%
FCXM positive/ ARj negative	8	87.5%	12.5%
FCXM negative/ ARj negative	69	97.1%	2.9%

Table 3 Donor-specific antibody production in relation to HLA class I and II mismatches *(MM)* (data expressed as mean values ± SD)

	Antibody positive	Antibody negative	Significance
MM A	0.88 ± 0.69	0.98 ± 0.70	NS
MM B	1.35 ± 0.70	1.06 ± 0.61	$P = 0.0853$
MM AB	2.23 ± 1.20	1.79 ± 0.92	$P = 0.0489$
MM DR	0.41 ± 0.62	0.60 ± 0.58	NS

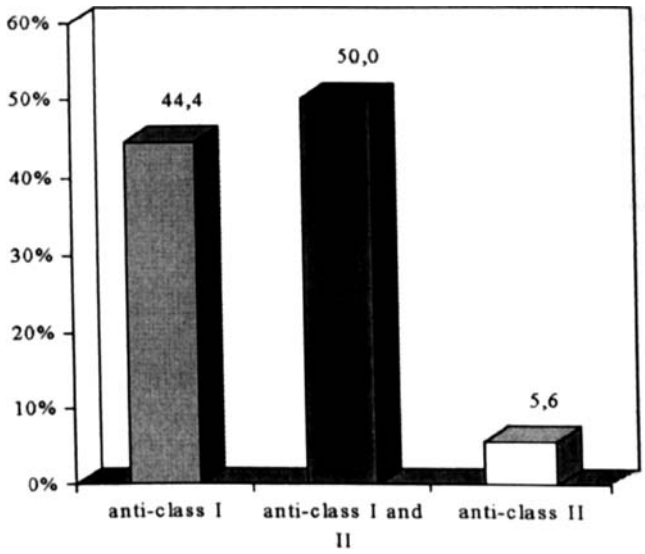

Fig. 3 Anti-class I and/or II antibody characterisation in IgG-positive sera

sponse and rejection occurrence was analysed by means of FCXM monitoring during the 1st posttransplant year and a follow-up period of 2 years. Recent literature has indicated that DS-Ab production is strongly associated with the occurrence of acute rejection [8, 9, 12]. Our data confirm these findings, since 68% of the 25 FCXM-positive patients suffered ARj episodes, compared to only 14.8% among the FCXM-negative patients ($P = 0.00001$). Furthermore, DS-Ab monitoring has been predictive of the onset of a clinically evident rejection episode in 35% of the FCXM-positive patients.

A strong link between FCXM positivity and graft outcome is further evidenced when comparing serum creatinine levels and graft function to FCXM status. In fact, FCXM-positive patients showed significantly higher serum creatinine levels than negative patients throughout our observation period. The presence of DS-Abs appears even more fundamental in determining graft failure since 90% of graft losses took place among the 25 FCXM-positive subjects while only 10% happened among the 81 FCXM-negative subjects ($P = 0.00001$). It is therefore possible to consider the production of DS-Abs to be one of the main causes of CRj, as stated also by other authors. The ineffectiveness of DS-Abs appearing within 1 month after transplantation in determining graft rejection has been recently proven [10]. Our data evidenced the onset of a humoral response within the 1st month in 4 out of 9 cases of FCXM-positive patients who eventually suffered graft loss.

The importance of ARj episodes and DS-Abs on graft function was also evaluated. A higher incidence of graft failure was evidenced in FCXM-positive patients who had also suffered ARj occurrence than in those subjects who had ARj episodes but did not produce DS-Abs ($P = 0.043$). The predominant role of antibody-mediated damage can be further evidenced by analysing the importance of the same parameters on serum creatinine levels throughout the period of our observation. Patients which were both FCXM and ARj positive and those which were FCXM positive/ARj negative showed similar values of serum creatinine 2 years after transplantation while all FCXM-negative subjects presented markedly lower values, regardless of ARj occurrence during the same period.

A better characterisation of DS-Ab production was achieved by retrospective analysis of all IgG-positive patients' sera in order to evidence the incidence of anti-HLA class I and/or II antibodies. Our findings demonstrated a marked predominance of anti-class I antibodies, at even higher levels than those reported in literature [13]. About 45% of the examined samples were found to contain exclusively anti-class I antobodies, while the exclusive presence of anti-class II antibodies was evidenced in a very low percentage of our subjects (5.6%).

The relationship between anti-class I and II antibodies and the mean value of mismatches for A, B, AB and DR antigens was investigated to correctly define the importance of HLA mismatches on DS-Ab production and eventually on long-term graft survival. A strong link

was ascertained between the presence of anti-class I antibodies and AB mismatches ($P = 0.0489$). No significant correlation was found between mean DR locus mismatches and anti-class II Ab production.

Our study evidences the role of FCXM as a basic means of investigation in monitoring the onset of an alloimmune response. In fact, FCXM is often able to anticipate the occurrence of ARj episodes before they become clinically evident. Moreover, it supplies useful information in ill-defined clinical statuses, such as those lacking clinical evidence of rejection. As far as graft outcome is concerned, our data have showed FCXM monitoring to be a primary tool for revealing the presence of DS-Abs in posttransplantation care, since such immune activation represents a strong negative prognostic factor on long-term graft survival. The high incidence of anti-class I DS-Abs in FCXM-positive patients (94.4%) confirms the harmful effect of HLA class I antigen mismatch on graft outcome.

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