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The relevance of more sensitive ancillary crossmatch techniques in predicting early cadaver renal graft outcome

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Abstract. The predictive value of varying levels of antibody activity, its class and antigen specificity in sera of 81 recipients of cadaver renal allografts was evaluated. Recipients for transplantation were selected on the basis of a negative dye uptake T-cell crossmatch, after which the more sensitive ^{51}Cr release technique was employed in a blind study using unseparated donor target cells. Recipient sera with peak panel reactivity and current samples were evaluated before and after reduction with dithiothreitol to destroy the IgM subclass. Double absorption with pooled platelets allowed antibodies against HLA class I antigens to be distinguished from those against HLA class II/non-HLA antigens. Optimal levels of cytotoxicity were established, giving a sensitivity of 73%. Data were assessed in terms of positive predictive value, and showed that conventional T-cell crossmatching is adequate for the primary transplant group, but more sensitive ancillary tests are indicated for regrafts. In this category of patients, IgG antibodies, whether against HLA class I antigens or HLA class II/non-HLA antigens, were highly predictive of early graft loss (positive predictive value 50%–100%). Using this protocol for patient selection, 1-month graft survival would have improved from 73% to 96%.

Key words: Crossmatch, outcome renal transplantation – Antibodies, preformed – Platelet absorbed crossmatch

The highest rate of loss of kidney allografts occurs in the early weeks post-transplantation followed by a log-linear drop-off after 1 year [28]. Important short-term factors influencing graft survival include centre differences, HLA-matching, first or subsequent transplant, degree of recipient presensitization, race, transfusion history and donor age [28]. Of these, the presence of preformed antibodies directed against donor HLA class I antigens is associated with immediate rejection of the graft [23]. This necessitates meticulous crossmatch testing to exclude such patients. In recent years workers have made several attempts to increase the sensitivity of crossmatch tests [1]. These have included longer incubation times [7], addition of antiglobulin reagents [13] and flow cytometric measurement of antibodies [6, 8, 9]. Several workers have found flow cytometry too sensitive with a high rate of false-positive results not correlating well with graft outcome [18, 27, 29], whilst others have suggested that the technique should be restricted to predicting rejection of regrafts [16, 19]. The confounding influence of IgM antibodies in crossmatches has recently been highlighted by Ting [30] who suggested that a transplant may successfully be performed in the presence of an IgM-positive crossmatch.

We have used a donor T-cell ^{51}Cr-release technique in addition to the standard dye uptake crossmatch in an attempt to increase sensitivity [20]. In addition, this study utilized unseparated spleen or lymph node donor target cells to exploit increased HLA antigen density on the B-cell fraction [25], and to establish the importance of antibodies against HLA class II antigens. Patients were selected for transplantation using the conventional dye uptake technique, and results from ancillary tests were only disclosed at the end of the study to test the data and to prevent clinical decisions being based on the findings. The aim of this study was to determine the role of antibody class, IgG specificity and degree of cytotoxicity in peak and current sera in predicting early outcome of cadaver renal allografts.

Patients and methods

A total of 81 recipients of cadaveric renal allografts (35 female and 46 male; 51 Caucasian, 25 Negroid and 5 Asian) with a median age of 34 years (range 1–61) were studied, of whom 65 (80%) were considered presensitized as measured by panel reactivity. Fifty-five of the patients received primary transplants and the remainder regrafts (19 second, 6 third and 1 fourth transplant). Seven patients had one, seven had two, 34 had three and 33 had four HLA-A or HLA-B mismatches. Eight patients received total lymphoid irradiation administered as previously described, with the remainder receiving triple therapy [21].

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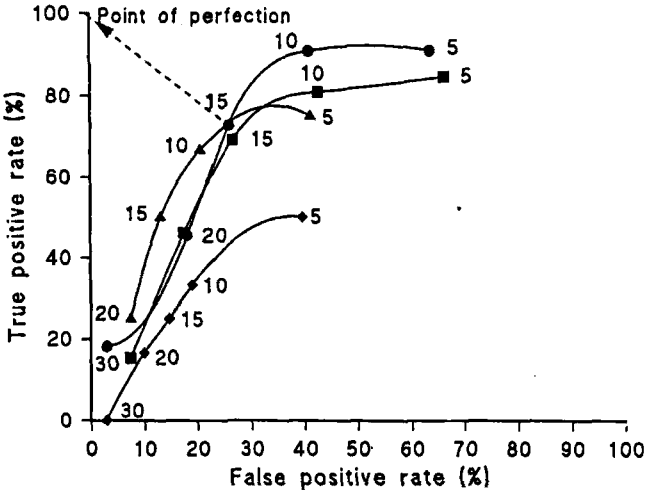

Fig. 1. Receiver operating characteristic curves (ROC) for ancillary crossmatch tests performed on current recipient sera. ■—■ Untreated; ●—● IgG component; ▲—▲ HLA class II and non-HLA antibodies; ◆—◆ HLA class I directed antibodies. Numbers on curves refer to threshold values of percent ^{51}Cr release. The point of perfection refers to maximum true-positive and minimum false-positive rates

^{51}Cr crossmatch technique

Viable donor spleen or lymph node cells were obtained by density gradient centrifugation on Ficoll-Hypaque [2] and were labelled with $Na_2{}^{51}CrO_4$ (3.7 MBq/20 × 10^6 cells) for 1 h at 37 °C. When required, T-cell targets were separated by elution from a nylon wool column [14] prior to labelling. The labelled cells were washed four times and suspended in RPMI-1640 medium. Target cells (5 × 10^4 in 25 µl) were dispensed into round bottommed microtitre plates, incubated with low toxicity rabbit serum (25 µl) as a source of complement and patients peak or current sera (25 µl) at 37 °C. Donor serum served as a control while Brij-35 detergent released maximum isotope. All tests were performed in quadruplicate. After 1 h 125 µl RPMI-1640 was added to the wells. After centrifugation (100 *g*, 5 min) supernatants (100 µl) were harvested and counted in a gamma counter. Antibody activity was expressed as percent ^{51}Cr release according to the formula (test – control release)/(total – control release) × 100.

Selection of serum samples for crossmatching

Serum samples were collected from patients at monthly intervals, tested against a panel of lymphocytes from 30 normal volunteers for panel reactive antibodies (PRA), and stored at −20 °C. Peak sera from presensitized patients were available in 65 patients. Current sera were the most recent available and the majority tested were obtained within 2 months of transplantation. Crossmatch using untreated sera measured total antibody activity.

IgM in sera was reduced into non-complement fixing 7 s monomeric subunits by reduction with dithiothreitol (DTT, 5 m*M* final concentration, 45 min at 37 °C) and cytotoxicity after DTT treatment was considered to be due to IgG [22] isotypes γ_1, γ_2 and γ_3. IgM activity was quantified by subtracting the percentage ^{51}Cr release obtained with reduced serum from the value obtained with untreated serum. A decrease in cytotoxicity greater than 10% was taken as a positive IgM-containing serum.

Antibodies with HLA class I antigen specificity were removed from sera by double absorption for 30 min at 0 °C using equal volumes (250 µl) of outdated, pooled, washed, packed platelets from more than 100 blood donors, and were then treated with DTT to remove the IgM subclass. Non-HLA antibodies, which crossreact with platelet surface antigens, would also have been lost during this absorption, but such loss was not quantified or controlled for. Residual cytotoxicity was ascribed to HLA II and/or non-HLA antibodies. IgG HLA class I antibodies were quantified by subtracting the percentage ^{51}Cr release obtained with platelet-absorbed serum from the value obtained with reduced serum.

Statistical methods

The significance of varying degrees of antibody activity, class and specificity in peak and current sera with respect to early graft survival were determined by Fisher exact statistics. Receiver operating characteristic (ROC) curves, which display true-positive and false-positive antibody thresholds, were drawn according to Komaroff and Berwick [17].

Results

Renal graft survival

Of the 81 patients selected for transplantation, 13 (16%) rejected their grafts within the first month. The rejection rate for primary grafts was 11% (6/55) and for regrafts 27% (7/26). Three grafts were rejected hyperacutely and another four in accelerated fashion. Biopsies showed that all hyperacute rejections were antibody mediated. Cytological examination indicated that the remaining grafts were lost by a combination of humoral and cellular rejection. Another four presensitized patients lost their grafts within 3 months, but despite elevated creatinine levels at the cut-off time of 1 month were defined as non-rejectors.

Defining positive crossmatch thresholds

If the crossmatch is used as a predictor for early graft rejection, a decision must be made as to the level of ^{51}Cr release above which a presensitized patient is considered positive and denied transplantation. Figure 1 shows the ROC curves [17] for the ancillary crossmatch tests performed on current recipient sera. Due to the paucity of patients with pure IgM antibodies, this curve was not included. As the isotope-release threshold value, depicted on each curve, rose from 5% to 30%, both the true-positive and the false-positive rates tended to decline. The point where the true-positive prediction was maximal and false-positive prediction minimal, i.e. closest to the point of perfection, was reached at 15% cytotoxicity for untreated and reduced (IgG activity) current sera. This level of cytotoxicity gave a true-positive rate (sensitivity) of 73% and a false-positive rate of 26% (specificity 74%). The optimal value for platelet-absorbed sera (IgG activity against donor HLA class II/non-HLA antigens) was just under 10%. ROC values for peak sera were similar, as were values obtained when patients were analysed in terms of primary or regraft categories (data not shown). In this study, cytotoxicity against donor HLA class I antigens was not a good predictor of graft survival at any degree of isotope release, due to patients being selected on the basis

Table 1. Predictive value of ancillary crossmatch tests using the optimal cytotoxicity threshold in current and peak sera. TP, FN, FP, and TN: number of patients at the optimal cytotoxicity threshold with true-positive, false-negative, falşe positive and true negative antibody activity; PPV and NPV: positive predictive value (%) and negative predictive value (%); P = significance level

Serum fraction and cytotoxicity threshold (%)		All grafts							Primary grafts							Regrafts						
		TP	FN	FP	TN	PPV	NPV	P	TP	FN	FP	TN	PPV	NPV	P	TP	FN	FP	TN	PPV	NPV	P
Current sera:																						
Untreated	(>15%)	9	4	18	50	33	93	0.005	3	3	15	34	17	92	0.301	6	1	3	16	67	94	0.002
IgG component	(>15%)	8	3	17	49	32	94	0.004	3	1	15	33	17	97	0.114	5	2	2	16	71	89	0.007
IgM component	(>10%)	0	11	3	62	0	85	0.626	0	4	3	45	0	92	0.787	0	7	0	17	0	71	1.000
HLA I antibodies	(>10%)	4	8	13	55	24	87	0.226	2	3	11	38	15	93	0.347	2	5	2	17	50	77	0.287
HLA II/non-HLA antibodies	(>10%)	8	4	14	54	36	93	0.003	5	0	11	38	31	100	0.001	3	4	3	16	50	80	0.175
Peak sera:																						
Untreated	(>15%)	8	3	20	34	29	92	0.033	3	2	18	23	14	92	0.415	5	1	2	11	71	92	0.010
IgG component	(>15%)	7	4	18	31	28	89	0.098	2	3	16	22	11	88	0.657	5	1	2	9	71	90	0.018
IgM component	(>10%)	2	7	5	46	29	87	0.281	0	3	4	36	0	93	0.741	2	4	1	10	67	71	0.272
HLA I antibodies	(>10%)	4	6	14	39	22	87	0.303	0	4	13	28	0	88	0.241	4	2	1	11	80	85	0.022
HLA II/non-HLA antibodies	(>10%)	5	3	17	38	23	93	0.090	2	0	17	25	11	100	0.181	3	3	0	13	100	81	0.021

of a negative dye uptake T-cell crossmatch (Fig. 1), but a realistic cytotoxicity level of 10% was selected for comparative purposes.

Predictive value of peak and current recipient sera

In practical terms, what the clinician needs to know is not the sensitivity, specificity, or even statistical significance of a crossmatch test, but the predictive value [17]. Table 1 summarizes the number of patients with true- and false-positive and negative results as well as positive and negative predictive values for all five ancillary crossmatch tests in both peak and current sera. The IgM component is included for completeness, but did not contribute to prediction of graft outcome, as is discussed below. The positive predictive value (PPV) for all transplants was marginally better using current sera, while negative predictive value (NPV) was almost identical with both peak and current sera. Despite the high statistical significance ($P = 0.003$, 0.004 and 0.005) of three of the crossmatch tests in current sera, the PPV demonstrates that only one-third of patients testing positive would lose their grafts by 1 month, thus denying two-thirds of positive patients the opportunity of a transplant. Patients testing negative on either peak or current sera, had an 89%–94% chance of graft function at 1 month. When patients were categorized in terms of primary and regrafts, a different picture emerged. Ancillary crossmatch tests in primary grafts did not contribute to predicting early graft rejection with the possible exception of antibodies in current sera directed against HLA II/non-HLA donor antigens (PPV 31%, NPV 100%).

Clearly the value of ancillary crossmatch tests lies in the selection of patients for regrafts. In this group the PPV was consistently better in peak sera (71%–100%) than in current sera (50%–71%). The practice of accepting peak positive, current negative conventional T-cell crossmatch patients for transplant during the course of this study is reflected in the discrepant values for HLA I antibodies in current and peak sera (Table 1; PPV = 50% and 80%; $P = 0.287$ and 0.022, respectively) and suggests that this approach should be reconsidered.

Effect of immunoglobulin class

Seven patients exhibited IgM antibodies in peak and/or current sera which did not significantly affect early graft survival and could not be considered of predictive value (Table 1). Only one patient had a pure donor-directed IgM antibody (>25% Cr-release), in peak and current sera with HLA class II/non-HLA specificity, and a clinically uneventful course. The value of removing IgM activity may lie in eliminating the non-specific effects of autoantibodies but the low number of patients with this class of antibody precluded any firm conclusions. Activity of the IgG component remaining after DTT treatment was as predictive of early graft outcome as untreated sera.

Effect of antibody specificity

^{51}Cr-release assays using T-cell targets showed that 90% of recipients with donor-directed HLA class I antibodies were screened out by the primary cross match. It was not surprising therefore that ROC curves applied to antibodies with this specificity were not relevant (Fig. 1), and prediction of graft outcome was not statistically significant except in the case of peak sera of regrafted patients. The latter were often selected on the basis of a negative current crossmatch, and peak sera with HLA I antibodies were not necessarily screened out (Table 1; PPV = 80%, $P = 0.022$). The 2/7 rejections identified using current sera against class I antigens were not statistically significant ($P = 0.287$) but with a PPV of 50%, it may be argued that more sensitive crossmatching is justified in regrafted patients, while in primary grafts, conventional T-cell crossmatching is adequate.

IgG antibodies reactive with donor HLA class II and/or non-HLA antigens was the only ancillary test of value in primary graft patients (Table 1; PPV = 31% in current sera). In the regraft group, all three patients with antibodies of this specificity in peak sera lost their grafts within 1 month (PPV = 100%).

Table 2. Predictive value of ancillary tests in presensitized patients (PRA > 20%) using current sera. TP, FN, FP, and TN: number of patients at the optimal cytotoxicity threshold with true-positive, false-negative, false-positive and true-negative antibody activity; PPV and NPV: positive predictive value (%) and negative predictive value (%); P = significance level

Serum fraction	TP	FN	FP	TN	PPV	NPV	P
Primary grafts:							
Untreated	2	0	6	9	25	100	0.206
IgG component	2	0	5	10	29	100	0.154
HLA I antibodies	1	0	3	12	25	100	0.250
HLA II/non-HLA antibodies	1	0	2	13	33	100	0.188
Regrafts:							
Untreated	5	0	0	4	100	100	0.008
IgG component	5	0	0	4	100	100	0.008
HLA I antibodies	1	4	0	4	100	50	0.556
HLA II/non-HLA antibodies	3	2	0	4	100	67	0.119

Value of ancillary tests in presensitized patients

In the light of the relevance of the ancillary tests in regrafted patients we analysed our data with respect to another indicator of presensitization, namely > 20% PRA activity. As shown in Table 2, the PPV was low for patients undergoing first transplants, with the possible exception of antibodies against donor non-HLA/HLA class II antigens (PPV = 33%). Patients testing negative, however, had a 100% chance of graft survival at 1 month. For regrafts, there was 100% PPV with all tests.

Discussion

The results of kidney transplantation have improved during the last few years [28], but presensitization remains a serious problem best illustrated by the progressive deterioration of the 1-month allograft failure rates from 8% for first grafts to 14% for second grafts and as high as 20% for third grafts [11]. Our results were comparable with other centres, with 1-month failure for primary grafts 11% (6/55), regrafts 27% (7/26) and both groups combined 16%, including three hyperacute and four accelerated antibody-mediated rejections, all undetected by conventional crossmatching. It is generally believed that early humoral rejection is preventable if antibodies against donor HLA antigens are detected [12] and this has led to growing interest in more sensitive crossmatching assays. The assessment of subliminal sensitization afforded by flow cytometry which measures both complement binding and non-cytotoxic antibodies [6, 8, 9] has highlighted the pitfalls of increasing test sensitivity which inevitably results in higher false-positive rates [18, 27] especially in primary grafts [16].

Recently, Mahoney et al. [19] constructed ROC curves to relate early graft loss with the degree of preformed antibody activity, reflected by flow cytometer channel shifts, to identify high risk patients with a sensitivity of 71% and a 16% false-positive rate. We used a sensitive ^{51}Cr-release method as an objective measure of complement-dependent antibodies, and applied ROC curves to the data to establish optimal thresholds of cytotoxicity for each ancillary test. This resulted in 73% sensitivity or true-positive rate and 26% false-positive rate (Fig. 1). Using these tests as selection criteria, one-quarter of patients with normal graft function at 1 month would have been denied transplant, though three of the four patients who subsequently lost their grafts were included in this category.

We elected to assess our data using predictive values, believing this to be of most practical relevance to the clinician faced with selecting suitable recipients on the basis of crossmatch tests. For example, despite the high statistical significance of three ancillary tests in current sera for all grafts (Table 1; $P = 0.003$, 0.004 and 0.005), PPV demonstrated that in reality only one-third of positive patients would lose their grafts by 1 month. In the combined transplant group, PPV ranged from 23% to 36%, but when grafts were separated into primary and retransplants, it became clear that the value of more sensitive crossmatching lies in the regrafted group with PPV more than doubling and ranging from 50% to 100% (Table 1). The PPV for antibodies against HLA class I and II in current sera was lower (50%) than those obtained from peak sera (80% and 100%), almost certainly due to the practice followed during this study of accepting patients for transplant with a peak positive/current negative T-cell crossmatch [3]. These data suggest that there is merit in using a more sensitive detection technique in regrafted patients and confirms Ting's findings [30] that positive historical antibody levels should never be ignored.

It is evident that conventional T-cell crossmatching is adequate for patients awaiting first transplants, but clinicians should be alerted to the fact that patients with HLA II/non-HLA antibodies, not routinely assessed, have a one-third chance of irreversible rejection within 1 month (Table 1, PPV = 31%). This also held true for primary graft patients with a PRA sensitization status > 20% (Table 2, PPV = 33%). There were no false-positive results in six regrafted patients with PRA > 20%, resulting in 100% PPV for all four ancillary tests, again indicating how important more sensitive crossmatching is in this group.

Our attempts to establish the importance of immunoglobulin class were curtailed by the low incidence of IgM antibodies. In this study only one of 81 patients tested had a pure IgM antibody with specificity for HLA II/non-HLA antigens which was associated with an uneventful clinical course. This is probably a consequence of the incubation temperature used in ^{51}Cr-release assays (37 °C) which diminishes the contribution of IgM autocytotoxins frequently observed by workers who employ low temperatures. The PPV of immunoglobulin class G generally equalled that of untreated sera (Table 1) probably because IgM antibodies were not a confounding factor.

In addition to the undisputed importance of HLA class I antibodies [7], this study has emphasized the importance of IgG antibodies against class II/non-HLA antigens, with all three positive crossmatch patients in the regrafted group losing their grafts. We can only speculate on the relevance of a particular specificity in this immunoglobulin G subclass. It is possible, but unlikely, that residual class I antibodies were present in the sera after platelet absorption, as it has been shown that the platelet absorption technique for

measuring class I antibodies correlates well with the inhibition of cytotoxicity using $F(ab')_2$ fragments of anti-class I monoclonal antibodies [15]. Secondly, the antibody specificity may well have been directed against class II antigens, as patients were not matched for these, and unseparated donor target cells, relatively rich in class II antigens, were used. The relevance of IgG class II antibodies to graft outcome remains controversial [15, 30].

Finally, Ting has shown that non-HLA antibodies against lymphocyte targets are not detrimental to renal grafts [30], but this is not true of non-HLA antibodies against vascular endothelial cell antigens which have been incriminated in irreversible renal graft rejection in HLA identical combinations [5], and in patients with negative HLA crossmatches [4, 10, 24, 26]. We concede that the latter antibodies might be implicated in our study, as the target cells used may well have included some macrophages/monocytes (especially when isolated from donor spleen), which share an antigen with vascular endothelial cells [4, 5, 24, 26].

In conclusion, we have shown the clinical relevance of the more sensitive ^{51}Cr-release crossmatch technique in regrafted patients, while conventional crossmatching for first transplants remains adequate. If applied in the selection of regrafted patients, the 1-month survival would have improved from 73% to 96%.

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