Transpl Int (1996) 9 [Suppl 1]: S20–S24

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Anticipation of highly sensitised renal patients' immunoadsorption requirements by prescreening using protein A minicolumns

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Abstract We are able to subdivide highly sensitised renal patients who wish to enter our immunoadsorption programme into two groups; those who will require acute pretransplant immunoadsorption only and those requiring regular immunoadsorption prior to transplantation. This division of patients is based on the results obtained from laboratory assessment using protein A minicolumns. Patient's plasma is passed down a minicolumn for 6 × 10 min cycles, a sample of plasma is kept after each cycle for analysis by cell flow cytometric cross-match (FCXM). The samples are screened against cells from two normal volunteers, one expressing a previously mismatched Class I HLA antigen (MMA) to which the patient has raised persistent IgG antibodies, the other, whilst not expressing any MMAs, should express a cross-reactive HLA Class I antigen (XRA) to which the patient has formed persistent IgG antibodies. Patients are allocated into the acute pretransplant immunoadsorption group if, after 6 minicolumn cycles, the T cell FCXM vs XRA and MMA is reduced to less than 1 Log median fluorescence intensity shift above the negative control and that both these values have been reduced by at least 15 % from the preimmunoadsorption figure. If these criteria are not met, regular immunoadsorption is required under cover of cyclophosphamide. Eleven patients who have been allocated by these criteria have subsequently been transplanted without any incidence of hyperacute rejection.

Key words Renal transplantation · Immunoadsorption · Minicolumn · Cross-match

Introduction

Dialysis-dependent patients in end-stage renal failure with high levels of anti-HLA panel-reactive antibodies (PRA) owing to previous blood transfusions, pregnancy or previous failed organ transplants have a greatly reduced likelihood of obtaining a graft which does not carry the HLA antigens to which they have raised antibodies. These antibodies may be removed and their resynthesis reduced by immunoadsorption (IA) and the administration of cyclophosphamide, respectively. IA has proved to be well tolerated and is efficient at lowering PRA levels and anti-HLA titres against specific antigens [1, 5]. However, there is some resynthesis of anti-HLA antibodies despite cyclophosphamide cover [2, 3]. Previously, patients with only narrow ranges of anti-HLA specificities have been immunoadsorbed and transplanted in our region [6]. In our current programme, patients with a broad range of anti-HLA antibody specificities are offered treatment, thus making immunoadsorption available to most highly sensitised patients.

IA to remove anti-HLA antibodies from sensitised renal patients is often carried out over several sessions before a potential donor has been identified [3, 4]. If a donor is not found during, or within a short period after, the schedule of IA treatments then the patient's anti-

HLA antibodies will often 'rebound' to, or beyond their pre-IA levels [3, 6]. It was felt that sensitised patients who would require only one or two acute IA sessions immediately pretransplant, to remove sufficient quantities of their anti-HLA antibodies in order to achieve a negative cross-match against a suitable donor, could be identified. These patients would be considered for acute IA only prior to transplantation without the necessity of their undergoing a series of IA sessions, with concomitant immunosuppression, in the hope of a suitable donor becoming available within this period.

The aim of this study was to develop a predictive laboratory test capable of distinguishing between those patients requiring acute pretransplant IA only and those requiring repeated IA sessions over several weeks in order to obtain a negative cross-match prior to transplantation.

Patients and methods

Patients

Between August 1993 and August 1995, 11 patients (5 males, 6 females; age range 11–54 years) in total renal failure with high titre anti-HLA antibodies were studied. The major reactivity of these patients' anti-HLA antibodies was to Class I antigens, demonstrated by a high T cell PRA value. However, 2 of the patients with low PRA values (18 % and 45 %) did in fact have high titre, broadly reactive antibodies to HLA Class II antigens. The anti-HLA antibodies present in all but 1 of the patients had primarily been induced by previous renal allografts.

Protein A minicolumn assessment

A protein A minicolumn (Excorim, Lund, Sweden) was set up in circuit with a flow cell spectrophotometer set to read at 280 nm and a pump set at a flow rate of 1.8 ml/min. The column was washed through with 25 ml of phosphate-buffered saline (PBS), pH 7.2. Patient's plasma (13 ml) was centrifuged at 750 *g* for 10 min to remove any debris, and then 12 ml was applied to the column and 1 ml was reserved for cross-match (XM) analysis. The PBS eluted from the column was discarded. The plasma was continuously circulated through the column for 10 min before being recovered and 100 µl was removed for XM analysis. Unbound protein was washed from the column with PBS until the absorbance measured in the UV flow cell had returned to baseline. Glycine-HCl buffer, 0.1 M pH 2.5, was added to the column in order to elute the bound IgG from the column. The addition of buffer was continued until all the bound immunoglobulin had been eluted (absorbance returned to baseline). The column was rinsed with PBS until the pH of the eluate had reached 7.2. The collected plasma was reapplied to the column and the cycle of protein binding/elution repeated for a minimum of 6 cycles, ensuring that 100 µl of plasma was stored from each cycle.

Extracorporeal immunoadsorption

All patients except one received a 'trial' long (> 6 hours) extracorporeal immunoadsorption session after their minicolumn assessment to check the veracity of the minicolumn results. Subsequently, all patients underwent IA treatments in accordance with their minicolumn results. Extracorporeal immunoadsorption was performed using a Citem 10 system (Excorim, Lund, Sweden). The system consisted of a parallel arrangement of two Immunosorba 62.5 ml staphylococcal protein A columns. The patient's blood, anticoagulated with heparin, was separated by a Gambro PF2000 filter into plasma and cellular fractions. Acid citrate dextrose was also used at 10 % of flow rate as additional anticoagulation and also to prevent complement activation. The protein A columns were perfused alternately with 200–400 ml of plasma per 10 min cycle. The columns were regenerated using an acidic buffer followed by washing with buffered saline. The treated plasma was recombined with the other blood constituents and returned to the patient. An IA session was usually of at least 7 h duration. Aliquots of serum were collected before, at hourly intervals during, and also after the IA procedure. Samples from the 'trial' IA sessions were stored at −20 °C prior to screening for their cytotoxic antibodies (CAB) by CXM and also for CAB/non-cytotoxic IgG anti-HLA antibodies by TFCXM and BFCXM against two normal lymphocyte donors carrying MMA and XRA, respectively. Samples from the immediate pretransplant IA sessions were snap-frozen before being screened against donor spleen and auto peripheral blood T and B cells by CXM and FCXM.

Normal volunteer and patients' autologous lymphocytes

Peripheral blood lymphocytes (PBL) were separated from the blood of two normal volunteers and also from the patient being screened by density gradient centrifugation. The volunteers were selected so that one of them expressed at least one previously mismatched Class I HLA antigen to which the patient being screened had raised IgG antibodies. The second volunteer, who did not express any previously mismatched HLA antigens, expressed at least one cross-reactive antigen to which the patient had formed IgG antibody. The two lymphocyte populations were each resuspended (separately) in two separate aliquots for CXM and FCXM screening.

Cytotoxic cross-match (CXM)

Screening against MMAs and XRAs

The lymphocyte populations from the normal volunteers and the patient (to check for the presence of autoantibodies) were resuspended (separately) at 2×10^6/ml in RPMI 1640 medium. A standard NIH microcytotoxicity assay was used (false positive CXMs owing to autoantibodies were excluded by the addition of DTT or by setting up a CXM using autologous PBL). Briefly, 1 µl aliquots of the normal donor cells were incubated with 1 µl aliquots of the patient's serum (double diluted in RPMI 1640 from 1:1 to 1:512) at 22 °C for 30 min in Terasaki plates. Rabbit complement (5 µl) was added to each well followed by 60 min incubation at 22 °C. The percentage of cells killed was assessed by the addition of an EB/AO mix. The plates were viewed using an inverted UV microscope.

Screening against potential donors

Single cell suspensions of donor spleen cells were separated by density-gradient centrifugation, with the mononuclear cell layer being harvested. The CXMs were set up as detailed above.

T and B cell flow cytometric cross-match (T & B FCXM)

Screening against MMAs and XRAs

The normal donor cells and the patient's own lymphocytes were resuspended at between 2 and 4×10^7/ml in PBS – 0.1 % bovine serum albumin – 0.1 % sodium azide (PBA). Duplicate 20 µl aliquots of the stored serum samples were each added to 30 µl of each of the donor cells, normal AB serum was used as a negative control. The cells and serum were incubated for 30 min at 22 °C. PBA (2 ml) was added to each tube which were then centrifuged for 5 min at 150 *g*. The supernatant was decanted and the cell pellet resuspended in the residual liquid. This 'washing' process was repeated. Normal mouse serum (5 µl) was added to each tube which was then mixed and incubated at RT for 10 min. Mouse anti-CD3-phycoerythrin (4 µl) (Dako Ltd.) was added to one set of each of the XRA and MMA cells, mouse anti-CD19-phycoerythrin was added to the second set. Rabbit anti-human IgG-fluorescein isothiocyanate (4 µl) (Dako Ltd., non-crossreacting with mouse IgG) was added simultaneously to all of the cell pellets in the tubes, followed by gentle mixing and 20 min incubation at 4 °C. The cells were washed twice in PBA and then resuspended in approximately 300 µl of PBA. Ten thousand events from each tube were acquired using a live lymphocyte gate, set on forward and side scatter, on a 'Facscan' flow cytometer. Lysis II data management software was used for the analysis. Using a dot plot, a gate was set to include all the CD3-PE$^+$ cells in the positive and negative controls. This defined the T cell gate. Histograms were generated of the FITC fluorescence intensity for the T cells in the test samples and the data was analysed by overlaying the FITC histograms. The peak to peak distances for each of the histograms from the AB negative control were measured. This gave the median fluorescence intensity for each sample, as a measure of the amount of binding of the patient's IgG on the T cells. Fluorescence intensity was measured on a Log scale, a shift in fluorescence intensity of greater than 0.5 of a Log above the AB control was considered to be positive. The analysis was repeated using a B cell gate which was set on the positive and negative controls to which CD19-PE had been added.

Some patients exhibit an 'unmasking' of non-cytotoxic IgG autoantibodies during IA. This has also been demonstrated using the minicolumns. These autoantibodies would affect a FCXM, therefore, an autoFCXM was also set up when a patient was being screened. Any positive values obtained from the autoFCXM were subtracted from the FCXM values obtained against the MMA and XRA volunteer cells.

Screening against potential donor cells

The density-gradient separated spleen lymphocytes were resuspended in PBA at between 1–1.5 $\times 10^7$/ml. The FCXMs were set up as detailed above, including an autoFCXM.

Immunosuppression and transplantation

Patients undergoing acute IA only prior to transplantation commenced their associated immunotherapy immediately prior to the IA treatment (oral cyclophosphamide 2 mg/kg per day and prednisolone 0.5 mg/kg per day). Other patients undergoing a regular programme of IA received the same immunosuppression commencing 3 days prior to the first IA treatment, the cyclophosphamide dose was adjusted according to the neutrophil counts. Following transplantation, all patients received a 10-day course of antithymocyte globulin (ATG, Fresenius, FRG or Merieux at the manufacturers' recommended dose) in addition to triple therapy (cyclosporin A was given to achieve whole blood trough levels of 200–300 ng/ml, prednisolone at 20 mg/day and cyclophosphamide). Three months posttransplantation, azathioprine at 50–100 mg/day was substituted for the cyclophosphamide.

Rejection was diagnosed on the basis of a rising creatinine level (when there was urine output) confirmed by renal biopsy.

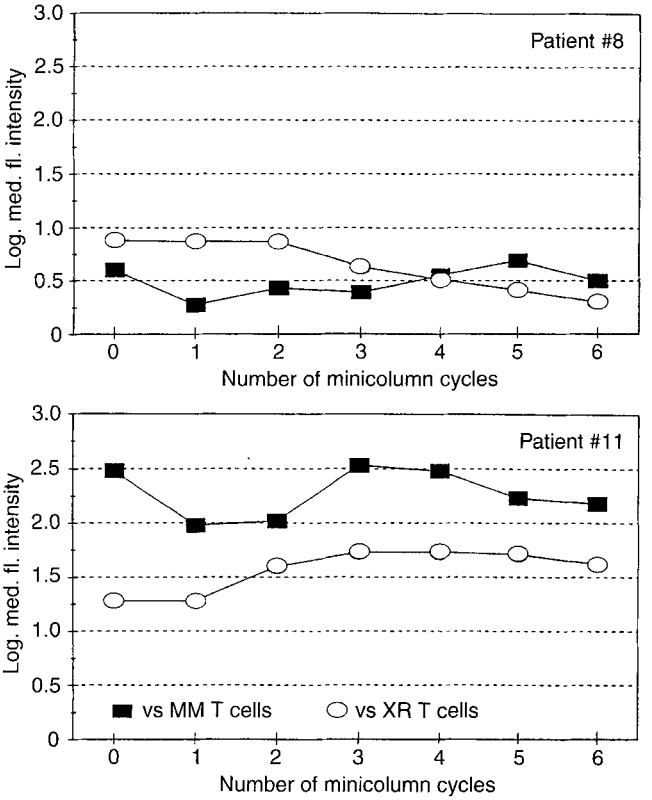

Fig. 1 Minicolumn analysis

Results

Statistical analysis

Graft loss was considered to be at the time of nephrectomy or return to dialysis. Graft survival figures excluded death with a viable graft. Transplant survival figures included death with a viable graft.

Minicolumns

All patients in this study underwent a minicolumn analysis prior to extracorporeal IA. Figure 1 shows the TFCXMs obtained from two patient's minicolumn analysis. The upper panel shows the results from patient # 8 who was assigned to the acute IA group. The lower panel of Fig. 1 is patient # 11's results who was assigned to scheduled IA. Of the 11 patients studied, 5 fulfilled

Table 1 Patient data prior to transplantation (*Tx* transplantation, *PRA* panel-reactive antibodies, *IA* immunoadsorption, *CXM* cytotoxic cross-match, *XM* cross-match)

Patient	Sex	Age at Tx (years)	Number positive XMs prior to Tx	Minicolumn result	% PRA pre-IA	% PRA post-IA	CXM titre pre-IA/pre-Tx	CXM titre post-IA/pre-Tx
1	M	50	27 (42 months)	Scheduled IA	18	16	1:256	0
2	F	54	4 (29 months)	Acute IA	98	76	1:8	0
3	F	45	76 (98 months)	Scheduled IA	88	20	1:32	0
4	M	11	55 (72 months)	Scheduled IA	100	51	> 1:512	0
5	F	45	9 (28 months)	Acute IA	45	11	1:2	0
6	M	36	100+ (156 months)	Acute IA	64	19	0	0
7	M	25	61 (88 months)	Scheduled IA	100	36	1:4	0
8	F	32	30 (23 months)	Acute IA	82	7	1:2	0
9	M	43	92 (94 months)	Scheduled IA	90	58	0	0
10	F	38	84 (58 months)	Acute IA	95	13	1:4	0
11	F	40	31 (67 months)	Scheduled IA	100	13	1:16	0

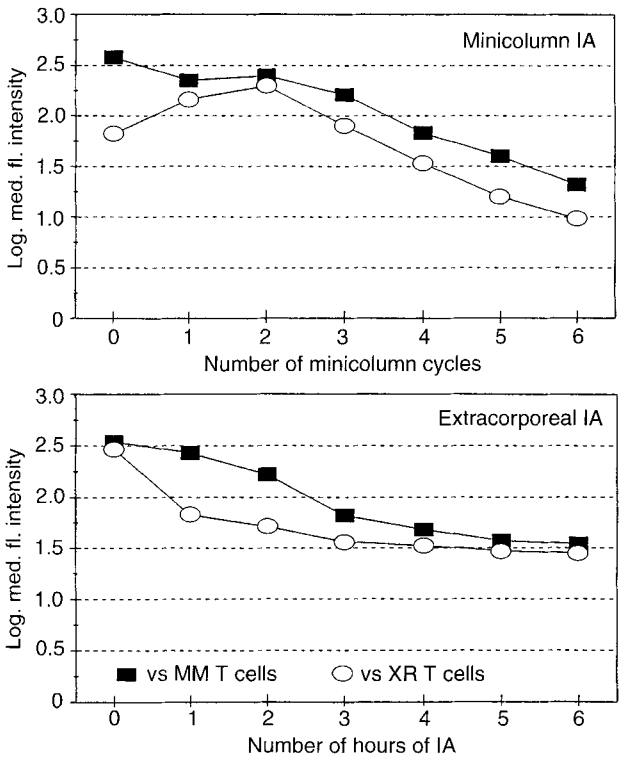

Fig. 2 T cell flow cytometric cross-match results (patient # 7)

the criteria for acute pretransplant IA only whilst the remainder, who did not, were assigned to a schedule of IA sessions (Table 1). Figure 2 shows the T cell flow cytometric cross-match results (TFCXM) of the minicolumn and also the first extracorporeal IA samples for patient # 7. It can be seen that the results obtained from the minicolumn are very similar to those of the extracorporeal IA.

The mean PRA of the 'acute' group prior to extracorporeal IA was 77 % (± 9.8 SEM), this was reduced to a mean of 25 % (± 22.6 SEM) by the acute pretransplant immunoadsorption. The mean PRA of the 'scheduled' group was 83 % (± 13.1 SEM), reducing to a mean of 32 % (± 18 SEM) after the schedule of immunoadsorptions had been carried out. There was no significant difference in PRA reduction between the groups. The average of the fall in the % PRA for each group was 52 % (acute IA) and 51 % (scheduled IA) (Table 1).

Table 2 HLA matching and graft function (*MM* mismatch, *ATG* antithymocyte globulin)

Patient	Donor MM A/B/DR	Current plasma creatinine (μm/l)	Outcome
1	00/01/11	Not applicable	Non-immunol-related death
2	01/01/01	167	Function
3	01/01/01	264	Function
4	11/11/11	200	Function
5	11/11/11	186	Function
6	11/01/11	147	Function
7	11/11/11	1007	Rejected (ATG withdrawn)
8	11/01/11	1459	Technical failure
9	01/01/01	285	Function
10	01/01/00	827	Technical failure
11	00/11/11	250	Function

The one year graft survival for all patients is 75 % (n = 8), with 13 % (n = 1) being lost to rejection within this period and the one year transplant survival is lower at 64 % (n = 7), as shown in Table 2. The functioning grafts, all of which are continuing to show a gradual reduction of plasma creatinine levels with time, have a range of plasma creatinine values from 147 to 285 μM/l (Table 2). Three of the patients were completely mismatched at the A, B and DR loci; the other patients had some degree of HLA matching (Table 2).

Discussion

The present single centre study indicates that we are able successfully to identify, using the formula devised, the pretransplant IA requirements of sensitised patients in order to render them cross-match negative once a potentially suitable donor kidney has been identified. The level of reduction in the %PRA for each patient group following their prescribed programme of immunoadsorption was essentially the same, indicating that the desired 'windows of opportunity' regarding the removal of cross-reactive anti-HLA antibodies had been attained for both groups. Following IA and transplantation, the 1-year graft survival figures for these patients is only slightly lower (75 %) than for the equivalent period for regrafted patients in UKTSSA-participating centres (77 %, average for all regrafts). Our transplant survival figure of 63 % is, however, lower than the national figure of 74 % for regrafted individuals [7]. The small degree of HLA matching that occurred in this study was entirely fortuitous as a compatible blood group with a negative cross-match against the donor were the only selection criteria used. This lack of matching does not appear to have compromised the graft survival figures when compared to the national average obtained for sensitised renal patients within the same period [7].

The results obtained from this small study would seem to indicate that the patients were assigned to the correct treatment groups. The preassessment of patients by minicolumn analysis prior to entering our immunoadsorption programme allows us to maximise resources and to avoid patients undergoing unnecessary treatments. These results should ideally be further tested in a multicentre study.

Acknowledgements We would like to thank Excorim (Lund, Sweden) for the gift of the protein A minicolumns.

References

1. Brocard JF, Farahmand H, Fassi S, Plaisant B, Fries E, Cantarovich M, Bismuth A, Lambert T, Hiesse C, Lantz O, Fries D, Charpentier B (1989) Attempt at depletion of anti-HLA antibodies in sensitized patients awaiting transplantation using extracorporeal immunoadsorption, polyclonal IgG, and immunosuppressive drugs. Transplant Proc 21: 733–734
2. Charpentier BM, Hiesse C, Kriaa F, Rousseau P, Farahmand H, Bismuth A, Fries D (1992) How to deal with the hyperimmunized potential recipients. Kidney Int (suppl) 38: S176–S181
3. Esnault V, Bignon JD, Testa A, Preud'homme JL, Vergracht A, Soulillou JP (1990) Effect of protein A immunoadsorption on panel lymphocyte reactivity in hyperimmunized patients awaiting a kidney graft. Transplantation 50: 449–453
4. Gil-Vernet S, Griñó JM, Martorell J, Castelao AM, Serón D, Díaz C, Andrés E, Gonzalez-Castellanos L, Alsina J (1990) Anti-HLA antibody removal by immunoadsorption. Transplant Proc 22: 1904–1905
5. Palmer A, Taube D, Welsh K, Brynger H, Delin K, Gjörstrup P, Konar J, Söderström T (1987) Extracorporeal immunoadsorption of anti-HLA antibodies: Preliminary clinical experience. Transplant Proc 19: 3750–3751
6. Palmer A, Welsh K, Gjorstrup P, Taube D, Bewick M, Thick M (1989) Removal of anti-HLA antibodies by extracorporeal immunoadsorption to enable renal transplantation. Lancet 1: 10
7. United Kingdom Transplant Support Service Authority (1995) Renal transplant audit 1984–1993