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ORIGINAL ARTICLE

Evaluation of flow cytometric panel reactive antibody in renal transplant recipients – examination of 238 cases of renal transplantation

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Summary

In Japan, the complement-dependent cytotoxicity (CDC-crossmatch) test and the anti-donor antibody flow cytometric assay (FCXM) are used to evaluate presensitization among transplantation candidates. We introduced the flow cytometric panel reactive antibody method (FlowPRA) at our institution, and in this paper, we compared the results of FCXM and FlowPRA. Sera of a total of 238 patients receiving the first graft were analyzed by FlowPRA retrospectively. Specimens from 125 of these patients were also analyzed by FCXM, and the results obtained using the two methods were compared. In addition, postoperative pathological findings by graft biopsy were examined in patients with PRA class 1(+) or PRA class 2(+). (i) Class 1 antibodies were detected in 36 of the 238 patients (15%), class 2 antibodies in six patients (3%), and both class 1 and class 2 antibodies in five patients (2%). (ii) Totally 125 patients analyzed by both FCXM and FlowPRA, 28 patients (22%) who tested negative by FCXM were, however, found to be positive by FlowPRA, and 16 of these 28 patients (57%) had shown evidence of humoral rejection suspected of antibody-mediated in the early postoperative stage. A large proportion of patients who tested negative by FCXM but positive by FlowPRA experienced rejection. Thus, for detecting 'high responders' in patients receiving the first graft, use of FlowPRA to detect antibodies may be superior to that of FCXM.

Introduction

With the development of ciclosporin (CsA) and tacrolimus (FK) in the late 1980s, and the development of mycophenolate mofetil (MMF) in the year 2000, the survival rate of transplanted kidneys has improved significantly. By using more than one of these immunosuppressants in combination, our center has achieved excellent results in living renal transplantation, even by global standards, with 1-year and 5-year survival rates of transplanted kidneys of 95% and 90%, respectively [1].

In the 1980s, the identity of the human leukocyte antigen (HLA) complex was gradually revealed [2,3], and an association was suggested between the transplantation outcome and the mixed lymphocyte response (MLR) [4,5]. In Europe and America, as a method to detect the humoral activity in recipients prior to transplantation, the results of the panel reactive antibody (PRA) assay are reflected on the order of the patients on the waiting list. The patients' sera are examined preoperatively for the presence or absence of anti-HLA antibodies against class 1 and class 2 HLA antigens, using beads coated on their surface with HLA antigens [6]. Those positive for the antibodies are listed higher on the list. Generally, the frequency of the so-called high responders who test positive by the PRA assay, is believed to be high among patients

with a history of exposure to nonself-antigens through blood transfusion, pregnancy, infection, organ transplantation, etc. [7]. While the HLA type and the MLR determine the cellular immunocompatibility between the donor and the recipient, the PRA method evaluates the humoral immunocompatibility. In the case of transplantation of cadaveric donor allograft, in particular, patients in the waiting list of the Japan Organ Transplant Network are prioritized based on the blood type, the HLA type and the humoral activities detected by complement-dependent cytotoxicity (CDC-crossmatch).

In this study, we analyzed the preserved serum specimens of 238 patients who underwent renal transplantation by the PRA method and compared the results with those obtained by the conventional method.

Materials and methods

Patients

The subjects of the study were 238 patients who underwent renal transplantation at the Kidney Center of Tokyo Women's Medical University between 1991 and 2002, whose serum specimens were stored frozen at −70 °C (Table 1). While 220 patients received living renal grafts, 18 received cadaveric renal grafts. The blood types matched in 210 cases, and were either not matched or incompatible in 28 cases. Patients receiving a second transplant were excluded from this study, because the immunological background including immunosuppressive regimen are so much different and the number of retransplants are too small to draw the overall conclusions.

Immunosuppressive therapy

Our center's protocol for immunosuppressive therapy in kidney transplant cases basically consists of the use of three drugs in combination [1]. Either CsA or FK is used as the calcineurin inhibitor (CNI), and azathioprine, mizoribine or MMF (since the year 2001) is used as the antimetabolite. Since 2002, basiliximab has also been administered in addition. Steroids are administered to all patients.

In blood-type-incompatible transplantation (from a non-O type to O type, AB type to non-AB type, A type to B type, or B type to A type group), elimination of the blood type antibodies by the preoperative double blood filtration method, perioperative splenectomy, postoperative administration of deoxyspergualin (DSG) or irradiation of the transplanted kidney with a radiation dose of 4.5 Gy used to be conducted until 1998. At present, however, the practice of administering DSG and irradiation of the transplanted kidney has been discontinued. In another group of cases with blood-type-incompatible transplantation (from non-AB to AB and O to non-O group), the transplanted kidney is locally irradiated with a total radiation dose of 4.5 Gy on the first, third and fifth postoperative days, in order to prevent hemolysis as a result of graft-versus-host disease (GVHD)-like reaction [8].

Table 1. Subjects of the study who underwent renal transplantation ($n = 238$).

Male	155
Female	83
Living	220
Cadaveric	18
Blood type compatible	210
Blood type incompatible or mismatch	28

Anti-donor antibody flow cytometry and FlowPRA

The crossmatch test using flow cytometry (FCXM) is superior to the conventional CDC-crossmatch in terms of the sensitivity of the test for detecting donor-specific anti-donor antibodies. Ever since this finding was reported in 1983 [9], we have conducted the examination using both methods. In our present analysis, we compared the advantages and limitations of FCXM and FlowPRA. FCXM was performed using recipient's serum immediately before transplantations, and FlowPRA was also performed using recipient's serum immediately before and 1 week after transplantation.

FCXM

Donor lymphocytes are added to the patient's serum, followed by incubation for 30 min at room temperature. After washing, phycoerythrin (PE)-labeled CD19 (Pharmingen, CA, USA) and cytochrome-labeled CD3 (Pharmingen) are added, reaction allowed to occur for 30 min at 4 °C. After washing three times, fluorescein isothiocyanate (FITC) labeled anti-human IgG antibody (Pharmingen) was added as a second antibody and the cells are fixed in 2% formalin–phosphate-buffered saline. The FCXM was performed using fluorescence-activated cell sorter (FACS) (Becton Dickinson, CA, USA) and a positive FCXM was defined as channel shift >10. All 238 patients in this study who were T-cell CDC-crossmatch and T-cell FCXM negative were considered as the suitable indication for renal recipients.

FlowPRA

Beads coated with the HLA antigens were added to the patient's serum using the FlowPRA Screening Kit (One Lambda, Inc., CA, USA), and left at room temperature for 30 min. Then, the secondary antibody, FITC-labeled anti-human-IgG antibody, was added, and the reaction mixture was allowed to stand for another 30 min. After washing twice, determination was started using the FACS.

When 10% or more beads were stained in comparison with that in the negative control and when multimodal staining was observed, the reaction was considered as positive. The serum was allowed to react with 10 mM of dithiothreitol at 37 °C for 10 min to eliminate immunoglobulin M (IgM). The disappearance of IgM from the serum was confirmed using rabbit anti-human IgM antibody (DAKO, Osaka, Japan) as the secondary antibody, before conducting the analysis by the ordinary FlowPRA.

Postoperative pathological findings by graft biopsy ($n = 230$)

We routinely perform the protocol/episode biopsy using a 16-gauge needle within 2 weeks after renal transplantations with informed consents. Unfortunately, informed consents were not obtained from eight recipients among 238 in this study. C4d staining is as follows: 4μm thick cryostat sections from each stored frozen specimen were stained using an indirect immunofluorescence method. The primary antibody was a purified mouse monoclonal antibody to human C4d (Quidel, CA, USA) and the secondary antibody was a FITC-conjugated polyclonal goat antibody to mouse IgG (Jackson Immunoresearch Lab, PA, USA). The slides were examined using a fluorescence microscope (Olympus, CA, USA) by a renal pathologist. We investigated these pathological results in patients with class 1(+), class 2(+), or both class 1(+) and class 2(+). The criteria of antibody-mediated humoral rejection were proposed at the sixth Banff conference of 2001. The following three factors are essential for the diagnosis of acute-mediated rejection: (i) serological evidence of anti-donor antibody; (ii) morphological evidence of tissue injury such as neutrophil and/or monocyte infiltration in peritubular capillary (PTCs) and/or glomeruli; (iii) immunopathological evidence for antibody action such as diffuse staining of C4d on PTCs suggesting the activation of complement via the classical pathway.

Results

Results of FlowPRA

Of the 238 patients, 36 patients (15%) were positive only for class 1 antibodies, six patients (3%) were positive only for class 2 antibodies, and five patients (2%) were positive for both class 1 and class 2 antibodies. The remaining 191 patients (80%) were negative for both class 1 and class 2 antibodies.

Comparison of FlowPRA and FCXM

Out of the 238 patients, the preserved sera of 125 patients who received one transplant were tested by both methods, FlowPRA and FCXM. T-cell-FCXM(+) patients were excluded from Table 2 because these patients were not considered as indications for renal recipients at our institution. Twenty-eight patients (22%) tested positive by FlowPRA method despite testing negative by FCXM. On the contrary, three patients (2%) tested negative by FlowPRA despite testing positive by FCXM. While no particular changes were observed after the transplantation in the three PRA(−)/B-cell-FCXM(+) patients, the 16 patients (16/28, 57%) of the 28 PRA(+)/FCXM(−) patients showed biopsy proven antibody-mediated rejection as presented in Table 3.

Ten of 16 patients showed preoperative-positive for only class 1 antibodies, two of 16 patients did positive for only class 2 antibodies, and four of 16 patients did positive for both class 1 and class 2 antibodies. In 12 patients (12/16, 75%), intense rejection associated with oliguria, with a daily urine volume of 500 ml or less or even anuria occurred during the first or second week after transplantation. The remaining four patients (four of 16, 25%) showed subclinical C4d staining by protocol biopsy. One of these patients (patient 9) was resistant to all treatment administered against rejection, and was discharged from the hospital with no success in achieving diuresis. Patients 1 to 12 were treated for the rejection by all of the existing approaches, including plasma exchange, muromonab-CD3 (OKT3) and steroid pulse therapy, and recovered. However, the serum creatinine level at discharge was high (average value of 2.45 mg/dl), and the mean PRA titer was 73% for class 1 antibodies and 18% for class 2 antibodies. Thus, no tendency towards improvement was noted in these patients, and they continue to be under careful follow-up at our outpatient clinic. Patients 15 and 16 received anti-CD25 antibody, i.e. basiliximab as a preemptive therapy. Among these 16 patients, only patients 15 and 16 exhibited a decrease in the PRA titer after the transplantation. Patient 1 received a bolus injection of gamma globulin (IVIG) for the treatment of rejection. While the PRA titer was not modified by conventional immunosuppressive therapy, such as steroids or CNI, after IVIG, the titer of class 1 antibodies decreased first, and then that of class 2 antibodies also decreased by the

Table 2. Comparison of the PRA and FCXM methods (patients receiving the first graft) ($n = 125$).

	Class 1(−) class 2(−)	Class 1(+) class 2(−)	Class 1(−) class 2(+)	Class 1(+) class 2(+)
T(−)B(−)	92 (74)	20 (16)	3 (2)	5 (4)
T(−)B(+)	3 (2)	1 (1)	1 (1)	0 (0)
T(+)B(−)	0 (0)	0 (0)	0 (0)	0 (0)
T(+)B(+)	0 (0)	0 (0)	0 (0)	0 (0)

Percentage values are given in parentheses.

Table 3. Details of the recipients with PRA(+)/FCXM(−) who demonstrated antibody-mediated rejection ($n = 16$).

Name	Age	Gender	PRA value (before Tx) Class 1	PRA value (before Tx) Class 2	PRA value (1 week after Tx) Class 1	PRA value (1 week after Tx) Class 2	Cr on discharge	Comment
YI	52	F	17.19	1.04	59.34	51.6	0.9	AMR (POD5), IVIG
KS	48	M	83.78	0.74	99.73	11.54	2.8	AMR (POD4)
TS	51	M	17.57	0.37	93.29	10.12	2.1	AMR (POD4)
MS	48	M	98.37	9.1	99.35	17.83	2.6	AMR (POD4)
RT	11	M	7.4	62.4	53.76	68.19	2.7	AMR (POD7)
MS	44	M	16.27	1.56	65.92	23.93	2.9	AMR (POD5)
KT	28	F	15.21	2.18	57.93	4.92	1.8	AMR (POD10)
TY	48	F	34.08	68.91	45.12	30.43	2.9	AVR (POD5)
NU	51	M	47.54	29.43	99.29	43.21	HD	HD
KM	34	M	52.4	9	65.12	7.4	2.3	AMR (POD7)
IH	40	F	32.8	5.6	90	4.5	2.3	AMR (POD12)
IY	24	M	14.3	32	99	3.5	2.1	AMR (POD4)
TK	32	M	18.2	24.5	97	3.2	1.6	Subclinical AMR
MI	46	F	86.17	0.79	94.09	4.08	1.1	Subclinical AMR
TF	50	M	80.85	0.46	20.17	1.2	1.6	Stable, basiliximab, AMR
SK	46	F	2.06	85.23	0.89	23.21	1.5	Stable, basiliximab, AMR

Tx, transplantation; AMR, antibody-mediated rejection; POD, postoperative day; HD, hemodialysis; IVIG, gamma globulin high dose administration.

third day after IVIG. The creatinine level in this patient decreased to 0.9 mg/dl and the patient was discharged from the hospital. The clinical course has been uneventful to date.

Comparison study between postoperative pathological findings and FlowPRA

Since 2 years ago, we have adopted the protocol biopsy at the regular duration after transplantations. In 230 of total 238 patients, pathological findings were examined in correlation with PRA positivity. As shown in Fig. 1, the rate for rejection-free was 166 of 199 patients (83%) with PRA(−) while it was only 10 in 31 patients (33%) with PRA(+). In the analysis of rejection type, humoral rejection suspected of antibody-mediated was observed in 17 of 31 patients (55%) with PRA(+) and in only 11 of 199 patients (6%) with PRA(−). In all cases with humoral rejection, C4d deposition on PTCs was also shown.

ABO mismatched renal transplantation

All 28 blood type-mismatched recipients did not show any elevation of anti-blood type antibodies during follow-up. Among them, four recipients were positive only for class 1 antibody (mean; class 1, 14%). However, four recipients did not experience antibody-mediated humoral rejection clinically or subclinically because of preoperative low PRA titer.

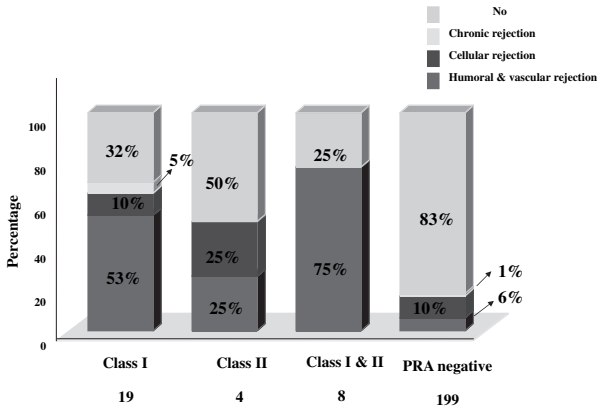

Figure 1 Postoperative pathological findings by graft biopsy ($n = 230$). A large portion of the patients with PRA(+), especially class 1 Ab, demonstrated humoral and vascular rejections while the patients with PRA(−) did not.

Discussion

In recent years, advances in immunosuppressive therapy have led clinicians to ask whether antibodies identified by more sophisticated crossmatch techniques represent a contraindication to transplantation. To answer this question, it is essential to prove that antibodies specific for donor HLA antigens are present. Many literatures revealed that the majority of studies failed to provide sufficient evidence to ensure that positive crossmatches were correctly assigned. Indeed, few investigators performed the labor-intensive

studies necessary to document that positive crossmatches were the result of antibodies specific for donor HLA antigens. Moreover, the testing methodology used in these studies was insensitive. The recent development of HLA antigen assay (i.e. ELISA and microparticle-based flow cytometric assay) has revolutionized our ability to detect HLA antibodies. Thus, we believe that it is essential to re-examine the conclusions of studies that formed the basis of our current crossmatch paradigms.

In 2001, the Department of Urology of our center introduced FlowPRA to determine antibody titers for the evaluation of humoral immunocompatibility. Comparison of FlowPRA and FCXM yielded interesting results. Twenty-two per cent (28 patients) of patients who tested negative by FCXM and underwent transplantation were found to test positive by the FlowPRA. Sixteen of these patients (16/28, 57%) experienced antibody-mediated humoral rejection within 2 weeks after the operation. The daily urine output decreased to 500 ml or less, about, on average, 6.1 days after the transplantation, and marked increase in the serum creatinine levels was observed. Diuresis was established following OKT3, plasma exchange, and steroid pulse therapy in almost all of these patients, however, the condition of the transplanted kidneys did not remain entirely satisfactory after the recovery. Moreover, the PRA titers remained almost the same as before the operation. Patients 15 and 16 in our study were treated with anti-CD25 antibody. It is noteworthy that the PRA titers in these patients decreased to below the baseline titer after the transplantation. This finding suggests that basiliximab is also effective for inhibiting the activity of B lymphocytes expressing the CD25 antigen [10].

Further increase of the PRA titer was observed at 1 week after renal transplantation in 14 of the 28 patients (50%) who were FCXM(−)/PRA(+) before the operation. Moreover, increases in the titers of both class 1 and class 2 antibodies were seen in eight of these patients (data not shown). A probable basis for these observations is activation of B lymphocytes expressing the receptor BCR which recognizes the chain common to class 1 and class 2 antigens, i.e. the $\alpha 1 \alpha 2$ chain.

FlowPRA has numerous advantages over the conventional CDC-crossmatch and FCXM, in that freeze-preserved serum specimens can be assayed using commercially available beads, and that more than one sample can be processed simultaneously within a short time. On the contrary, due to the high cost, only a small number of institutions in Japan have routinely adopted this technique. Moreover, although CDC-crossmatch and FCXM can also detect non-HLA antibodies besides HLA antibodies, FlowPRA does not require the use of rabbit complement, which is required in the CDC-crossmatch. Furthermore, the technique for lymphocyte extraction does not pose a problem in FlowPRA, as unlike in FCXM, viable lymphocytes are not handled directly. Thus, each method has its own merits and demerits.

Recently, the pathogenesis of antibody-mediated humoral rejection has aggressively investigated. The methods for detecting anti-donor specific antibodies have been significantly improved. The analysis of ABO incompatible renal transplantation contributed to clarifying the pathogenesis of antibody-mediated rejection. C4d deposition on PTCs is recognized as a reliable sensitive diagnostic indicator of antibody-mediated rejection. As we expected, in the pathological analysis, humoral rejection is likely to occur in a large population of PRA(+) patients while only 6% of PRA(−) patients revealed humoral rejection. These 6% PRA(−) patients with pathological humoral rejection did not show typical clinical manifestations of humoral rejection such as oliguria. Their humoral activities are not so much enough to induce the poor influence on the graft function. It is noteworthy that one patient receiving the allograft from his wife lost the graft accompanied by anuria at the very early period after transplantation despite of no activity in FlowPRA. Other humoral factor except for anti-HLA antibody that was not detected by FlowPRA, may be associated with his graft dysfunction. Unfortunately, the graft specimen could not be taken as a sample because of his disagreement.

In this study, it is unclear which class of anti-HLA antibody plays a more important role to induce antibody-mediated humoral rejection because of the small number of class 2(+) patients. However, we are impressed from the pathological results, that the class 1(+) patients seems to have a poor graft function [11,12].

Further studies are in progress using PRA-ELISA assay or Single beads antigen PRA to detect donor-specificity [13,14].

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