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ORIGINAL ARTICLE

Characterization of rejection episodes in patients following positive crossmatch and ABO-incompatible live donor renal transplantation

Liise K. Kayler,[1] John L. Farber,[2] Beth Colombe,[3] Deborah LaCava,[3] John J. Friedewald[4] and Lloyd E. Ratner[5]

1 Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
2 Department of Pathology, Thomas Jefferson University Hospital, Philadelphia, PA, USA
3 Department of Medicine, Thomas Jefferson University Hospital, Philadelphia, PA, USA
4 Department of Medicine, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA
5 Department of Surgery, Columbia University, New York, NY, USA

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Correspondence
Liise K. Kayler, Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA. Tel.: +412 215 6829; fax: +412 647 5480; e-mail: liisekayler@yahoo.com

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Summary

For kidney transplant recipients with donor-specific antibody (DSA) to HLA- (+XM) or ABO-antigens (ABOI), there is a need to improve detection and treatment of antibody-mediated rejection (AMR). The methods included a retrospective review of consecutive patients that received plasmapheresis and immune globulin (PPIVIg) to abrogate +XM or ABOI. Twelve patients were transplanted after PPIVIg (+XM = 9, ABOI = 2, +XM/ABOI = 1). No hyperacute rejections occurred. Rejection occurred in seven patients [four AMR, three acute cellular rejection (ACR)]. In four +XM patients, DSA was detected during graft dysfunction despite lack of histologic and C4d features of AMR. In one patient, DSA preceded the histologic and immunofluorescent features of AMR. In another patient with borderline changes and DSA, graft function improved after PPIVIg, despite lack of histologic or immunofluorescent evidence of AMR. One patient with Banff IIA ACR and DSA treated with antithymocyte antibody but not PPIVIg had recurrent rejections and poor graft function. In +XM and ABOI recipients with graft dysfunction: (i) DSA may represent AMR in the absence of C4d or histologic features of AMR; (ii) DSA can precede C4d or light microscopic features of AMR; (iii) A poor outcome may result if DSA or continued allograft dysfunction is present and not treated despite a negative biopsy.

Introduction

Potential live kidney donors are commonly prohibited from donating if their prospective recipients have preformed donor-specific allo- or natural antibody (DSA) detected by current crossmatching techniques or due to blood type incompatibility, respectively. Our center and others have successfully utilized plasmapheresis (PP) combined with intravenous immune globulin (PPIVIg) and tacrolimus plus mycophenolate mofetil-based immunosuppression to achieve ABO- and crossmatch- incompatible kidney transplantation. While antibody reduction protocols have successfully reduced hyperacute rejection to a rare event, acute rejection remains problematic, occurring in approximately 19–64% [1–4] of cases, with the majority of rejection episodes being antibody mediated [1].

In patients with renal transplant dysfunction, the diagnosis of antibody-mediated rejection (AMR) is generally made in the presence of three criteria – allograft biopsy findings of polymorphonuclear leukocytes in peritubular capillaries, circumferential monoclonal anti-C4d antibody staining of the peritubular capillaries, and serologic evidence of donor-specific antibody (DSA) [5]. Difficulty in

establishing a diagnosis of AMR often occurs when two of these criteria are not present or when these criteria do not present concurrently. Additionally, while AMR may coexist with acute cellular rejection (ACR) [6,7], the clinical relevance of DSA during episodes of ACR and/or borderline rejection remains unknown. To optimize incompatible kidney transplant outcomes, there is a need to improve the detection and early treatment of AMR and combined AMR/ACR.

To characterize acute rejection in the setting of ABO- and crossmatch- incompatible renal transplantation, we performed a detailed comparative analysis of biopsies obtained during episodes of allograft dysfunction and by surveillance protocol. Characterization of rejection episodes was performed by evaluation of biopsies with histopathology and immunofluorescence with C4d, the determination of DSA, antirejection therapy administered, and renal functional outcome.

Materials and methods

Study group

This study was conducted in accordance with the Institutional Review Board of the Thomas Jefferson University Hospital. Between September 2002 and November 2003, we performed 12 consecutive live donor renal transplants in recipients with donor-specific antibodies against HLA antigens ($n = 9$), ABO antigens ($n = 2$), and both types of antigens ($n = 1$). Sensitization against the donor was determined when the patient had at least one of the following characteristics: a positive T-cell antiglobulin-enhanced complement-dependent cytotoxicity crossmatch (AHG-CDC), a positive B-cell NIH CDC crossmatch (NIH CDC), a positive T- or B-cell flow cytometric crossmatch (FXM), or the presence of anti-a IgG antibody to non-A_2 donors.

Conditioning and immunosuppression protocol

Patients underwent a pretransplant conditioning regimen consisting of a series of two to seven PP on a Monday, Wednesday, Friday schedule, with one volume plasma exchange reconstituted with 5% albumin, followed by intravenous immunoglobulin (Cytogam® cytomegalovirus immune globulin intravenous; MedImmune, Gaithersburg, MD, USA) at 100 mg/kg. The number of PPIVIg treatments was based on initial DSA titer (Fig. 1). At the commencement of PPIVIg, immunosuppression with tacrolimus (0.1 mg/kg/day) (Prograf®; Fujisawa Healthcare Inc., Deerfield, IL, USA) and mycophenolate mofetil (2 g/day) (Cellcept®; Hoffman-La Roche Inc., Nutley, NJ, USA) was initiated. Steroids and daclizumab (2 mg/kg)

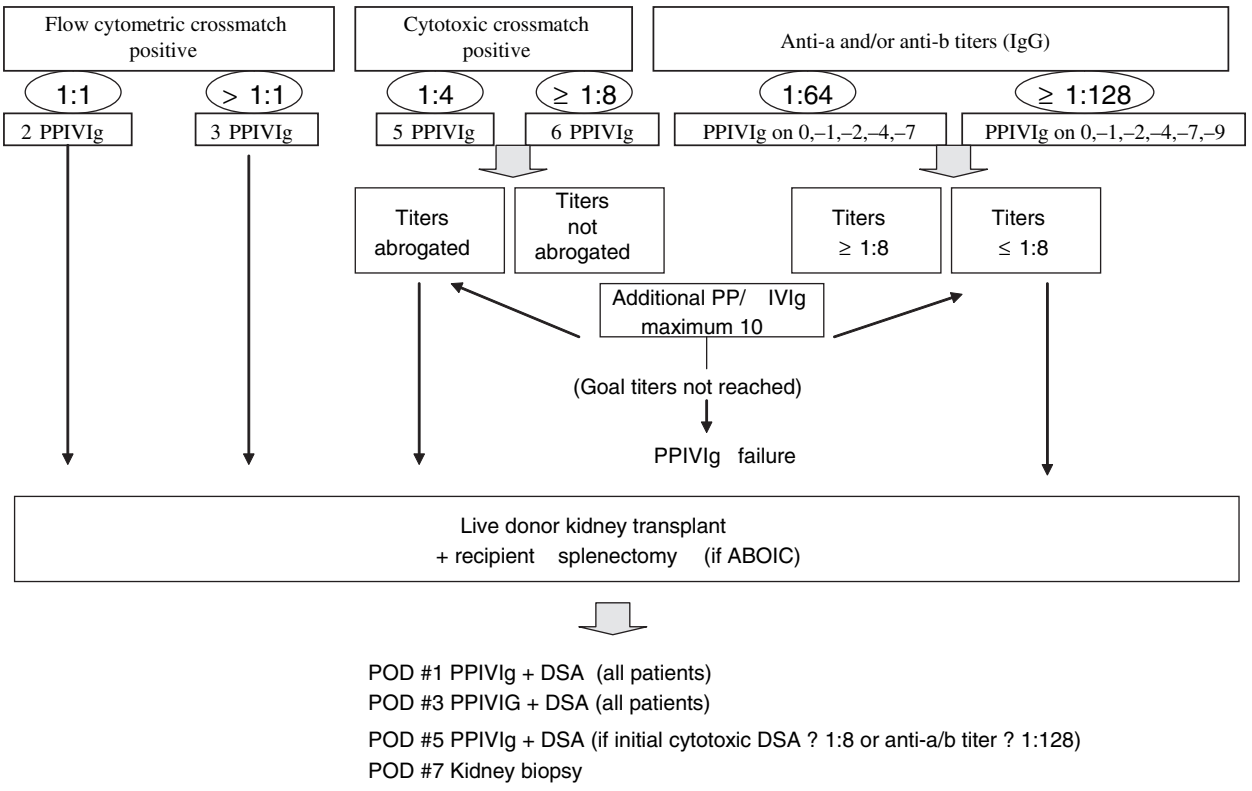

Figure 1 Desensitization and postoperative surveillance protocol.

(Zenapax®; Hoffmann-La Roche Inc.) were administered at transplantation. Daclizumab was repeated in the majority of patients at 2 weeks post-transplantation (1 mg/kg). ABO-incompatible kidney transplant recipients additionally underwent laparoscopic splenectomy at the time of transplantation. After transplantation, PPIVIg treatment, to reduce the rate of return of DSA, was administered on alternate days in all patients except one; the patient had already received PPIVIg to desensitize against a donor who in the interim was diagnosed with *Mycobacterium avium intracellulare*. An alternate cross-match incompatible (XMIC) donor was chosen. As DSA to the second donor were suppressed, PPIVIg was not administered.

Detection and treatment of acute rejection

Kidney biopsies were obtained on postoperative day 7 by surveillance protocol and otherwise for graft dysfunction defined as creatinine ≥0.3 mg/dl above baseline. Our original protocol intended surveillance biopsies to detect antibody-mediated injury on postoperative days 7, 14, 30, 90, and 365. Initial surveillance biopsy results were consistently negative for rejection among patients without graft dysfunction, therefore the protocol was amended to perform surveillance biopsy only on postoperative day 7.

In addition to light microscopic evaluation, staining for the complement degradation product C4d was performed using standard direct immunofluorescence techniques [8] with a monoclonal mouse anti-human C4d (Quidel, San Diego, CA, USA). All biopsies fulfilled minimal criteria of tissue adequacy (at least one arterial cross sections and six or more glomeruli) and were re-reviewed by one experienced transplant pathologist (J.F.) for classification according to the morphological features of acute cellular and AMR following the revised Banff 2001 criteria [9].

Treatment of AMR consisted of steroids (500 mg intravenous methylprednisolone daily for 3 days followed by a taper) and PPIVIg until return of creatinine to baseline or until DSA was rendered undetectable or resistant to further decrement. A repeat crossmatch was performed at least weekly during PPIVIg. Treatment of ACR was initially with steroids (500 mg intravenous methylprednisolone daily for 3 days followed by a taper). If the creatinine did not return to baseline after steroid treatment, repeat biopsy was performed. If rejection persisted, then antithymocyte globulin (Thymoglobulin®; SangStat, Fremont, CA, USA) for a total dose of 6 mg/kg was administered over 6 days.

Donor-specific cytotoxic crossmatch assays

Donor lymphocytes were isolated for T-cell cytotoxicity crossmatching using One Lambda (One Lambda, Canoga Park, CA, USA) magnetic beads and for B-cell cytotoxicity with Dynabeads magnetic beads (Dynal, Inc., Oslo, Norway). For the T-cell crossmatch, 1 μl containing approximately 2×10^3 of donor T cells was added to sextuplicate 1 μl volumes of serial dilutions of recipient sera (undiluted through 1:512). Histocompatability trays were incubated for 60 min at room temperature then washed three times with 5 μl of N-2-hydroxyethylpiperazine-N^1-2-ethane sulphuric acid (HEPES) (RPMI-HEPES). One microliter of AHG (goat antihuman kappa light chain) was added and incubated for 1 min, after which 5 μl of rabbit complement was added. The trays were incubated for 60 min at room temperature, 5 μl of Fluoroquench (One Lambda) was added, and trays were read using a fluorescent microscope. Cell death was identified by penetration of the fluorescent dye into the cell.

For the B-cell crossmatch, 1 μl of donor cells (approximately 2×10^3) was added to sextuplicate 1 μl volumes of serial dilutions of recipient serum (undiluted through 1:64). Trays were incubated for 30 min at room temperature. After the cell/serum incubation, 5 μl of rabbit complement was added followed by 60 min incubation at room temperature. Five microliters of Fluoroquench was added, and trays were read using an inverted fluorescence microscope.

In each well, any cell death read as 10% above background was considered positive.

All patients were evaluated for the presence of serum autoantibodies by testing the patient's lymphocytes with their own serum by the same procedure used for the T-cell crossmatch. Autoantibodies that are IgM in isotype are removed by the pre-crossmatch treatment of the serum by heating it at 63 °C for 10 min, or by treatment with dithiothreitol.

Flow cytometric crossmatch assays

For flow cytometric crossmatching, donor lymphocytes were isolated from peripheral blood using the Ficol-Hypaque density gradient technique and adjusted to a concentration of 2.5×10^6 cells/ml.

Flow cytometric crossmatches were performed as described previously [10]. Reactivities with T and B lymphocytes were analyzed separately on a Becton-Dickinson FACScan cytometer (Franklin Lakes, NJ, USA). Reactivity was assessed using the median channel shift obtained with the test serum normalized to the value for the negative control. The median channel shift for a positive TFXM and BFXM was considered to be 15 and 30 channels, respectively, above the negative control value using a 256-channel scale.

Detection and characterization of HLA-specific antibodies

Per protocol, DSAs were tested prior to and after PPIVIg administration at least weekly when undergoing PPIVIg treatment of AMR.

Detection and characterization of HLA-specific antibodies was performed by AHG-enhanced complement-dependent cytotoxicity using frozen class I 60 cell panel trays from GenTrak (Liberty, NC, USA) and by enzyme-linked immunoadsorbent assays using GTI Quik-Screen Class I and class II Screening kits for detection of panel reactive antibody, and by GTI Quik-ID Class I and Class II kits (GTI, Brookfield, WI, USA) to identify the antibody specificity

Determination of specificity and strength of antibody to donor HLA

Sera were obtained and tested for HLA-specific antibodies before and immediately after each PPIVIg, at a minimum. For specificity to donor HLA to be assigned unequivocally, it was necessary to identify the antibody by screening and confirm donor reactivity by crossmatch. All patients were tested at evaluation for the presence of autoantibody. The titer of sera positive by CDC was considered to be the highest dilution yielding cell death 10% or greater above background. For antibodies that were negative in the cytotoxic crossmatch, strength was assessed by titration of the sera against the cells of the donor by the flow cytometry assay referenced above. The titer was the greatest dilution that was positive against the donor cells.

Determination of strength of antibody to donor ABO

Antibody titer is obtained by testing serial dilutions of serum against selected red cells. Incubation is performed at: (i) room temperature for 30 min, (ii) 37 °C for 30 min without enhancement media, and (iii) 37 °C for 30 min with the addition of anti-IgG. The three incubations are performed to detect anti-a IgM, mixture of IgM and IgG, and isolated IgG, respectively. The titer is reported as the reciprocal of the highest serum dilution producing macroscopic agglutination.

Results

Twelve incompatible renal transplants from live donors were performed at the Thomas Jefferson University Hospital between September 2002 and November 2003. One-year patient and graft survival of 100% was achieved. At an average follow-up of 497 days (range 382–773) mean creatinine is 1.6 ± 0.4 mg/dl (range 0.9–2.2) with one graft loss (Tables 1 and 2). At baseline, four patients had a positive AHG-CDC T-cell crossmatch against their living donor, one patient had a positive B-cell NIH CDC crossmatch, one patient had a positive T-cell FXM, three patients had a positive B-cell FXM, two patients were ABO incompatible, and one patient was simultaneously ABO incompatible and had a positive T-cell AHG-CDC.

Table 1. Anti-aB isoagglutinin titers prior to and after (→) treatment with PPIVIg.

No.	Donor-to-recipient blood type	Anti-a IgG & IgM (room temperature)	Anti-a IgG (37°)	Anti-a IgG (Coombs)	Current creatinine (mg/dl) (days post-TX)
1	A_1B-to-O	64 → 2	64 → 2	128 → 8	1.5 (499)
2	A_1-to-O	64 → 1	64 → 2	256 → 32	1.7 (588)
3	A_1-to-O	128 → 2	256 → 2	1024 → 32	1.4 (410)

TX, transplant.

Table 2. Positive crossmatch titers (by modality) prior to and after (→) treatment with PPIVIg.

No.	Donor-specific antibody	T cell titer (AHG)	B cell titer (CDC)	T cell titer (FXM)	B cell titer (FXM)	Current creatinine (mg/dl) (days post-TX)
3	Unknown	256 → 0		16 → 16	8 → 1	1.4 (410)
4	B44	4 → 0		64 → nt		1.7 (681)
5	DR13		16 → 0		8 + 16 → 0	0.9 (428)
6	A28, B57	16 → 2	4 → 1			2.2 (430)
7	B58	8 → 0	2 → 0	4+ → 2	4 → 0	1.7 (490)
8	Unknown	1 → 0	1 → 0		1 → 1	1.6 (421)
9	Unknown			1 → 0	1 → 0	0.9 (382)
10	DQ7				1 → 2	HD (489)
11	A11				2 → 0	2.0 (773)
12	DQ2				16 → 2	2.0 (441)

nt, not tested; TX, transplantation; AHG, anti-human globulin-enhanced complement-dependent cytotoxicity crossmatch; CDC, cell-dependent cytotoxicity; FXM, flow cytometric crossmatch; HD, hemodialysis.

Table 3. Risk factors for AMR among kidney transplant recipients.

No.	Donor-specific antibody		Anti-HLA antibody	PRA >30%	Previous pregnancy	Husband to wife or son to mother	Retransplant
	Positive crossmatch	ABO incompatible					
1		+					
2		+					
3	+	+	+	+			+
4	+		+				+
5	+		+		+		
6	+		+	+			+
7	+		+	+	+		+
8	+		+				+
9	+		+	+	+		
10	+		+		+	+	
11	+		+	+			+
12	+		+	+	+		

PRA, panel-reactive antibody; HLA, human leukocyte antigen.

Titers of DSA prior to and after are shown in Tables 1 and 2. Among the 12 patients, the majority were Caucasian ($n = 8$) and the remainder were African-American ($n = 4$). There were six males and six females with a mean age of 45 ± 11 years. Risk factors for AMR among the recipients are shown in Table 3. Six LD transplants were first grafts and six were second grafts. In most of the recipients the peak PRA was over 30% (mean 52%, range 25–100%). All female recipients, except one, had been previously pregnant. The specificity of DSA to one or more donor antigens was determined in all but three patients.

There were 35 biopsies among 12 patients. None of the recipients developed hyperacute rejection; seven (58%) had at least one episode of AMR or ACR (Tables 4 and 5, respectively) and five (42%) have remained free of acute cellular or AMR (Table 6).

Antibody-mediated rejection

Antibody-mediated rejection was diagnosed in four patients (no. 3, 4, 6, and 12) on postoperative days 7, 7, 23 (re-confirmed on day 28) and 441, respectively (Table 4). Recurrent AMR developed in one patient (no. 6) on post-transplant day 175 and 421. In all six biopsies, the diagnosis of AMR included the presence of allograft dysfunction and both neutrophilic infiltration and C4d staining of the peritubular capillaries. AMR on biopsy presented concurrently with DSA detected at low levels (FXM) in two cases (no. 3 and 12). In one case (no. 6), rapid DSA elevations (detected first by FXM and 1 day later by cytotoxicity predicted AMR 23 days before histologic changes compatible with AMR; based on the DSA detection, the patient immediately underwent PPIVIg therapy. However, two biopsies taken 5 and 11 days after the detection of DSA had shown only borderline changes and the latter was also negative for C4d. In another case (no. 4), DSA was not detectable at the time of C4d+ biopsy. PPIVIg was given and DSA remained undetectable. As graft function had not improved, a repeat biopsy was obtained and showed only Banff borderline changes (C4d negative). The presence of ongoing graft dysfunction provided a heightened suspicion of an ongoing antibody-mediated process and PPIVIg treatments were continued. Contemporaneous with ongoing PPIVIg treatments low titer DSA became consistently detectable. PPIVIg was continued until optimal graft function was achieved. As DSA were still detectable after a total of PPIVIg treatments, anti-CD20 was given as adjunctive therapy in an attempt to induce suppression of DSA.

Cell-mediated rejection

Acute cellular rejection occurred in three patients (no. 10, 11, and 8) on post-transplant days 7, 14, and 90, respectively (Table 5). Two of the biopsies were C4d negative and one biopsy, which had not been immunostained, was repeated 5 days later and found to be C4d negative. DSA were detected in low levels concurrent with ACR in two cases. In one case (no. 11), the elevated DSA led to the presumption of mixed AMR/ACR. Treatment with pulse steroids and PPIVIg reduced DSA to non-detectable levels, although allograft function did not improve. Repeat biopsy 9 days later showed persistent ACR from grade Ib to IIa. Treatment with anti-thymocyte globulin therapy was successful. In the other case (no. 10), ACR was refractory to several courses of anti-thymocyte therapy and the allograft eventually failed. Although immunostaining for C4d was repeatedly negative when performed (five of six biopsies), DSA were intermittently detected by

Table 4. Patients with AMR: DSA titers during protocol PPIVIg.

			Donor-specific antibody titers* pre-PPIVIg → post-PPIVIg (treatment)						
No.	Event	POD	T cell	B cell	TFXM	BFXM	Anti-a IgG	Cr	Tac level
3	PPIVIg	−15	256 →	0 → 0	16+ → 16+	8 → 4	→ 1024		
3	PPIVIg	−13	2 → 1	0 → 0	32 → 16	2+ → 1	512 → 256		
3	PPIVIg	−11	1 → 1	0 → 0	64+ → 32	1 → 1	128 → 128		
3	PPIVIg	−8		0 → 0	128 → 64	8+ → 4	64 → 64		
3	PPIVIg	−6	0 → 0	0 → 0	64+ → 64+	32 → 16	128 → 64		
3	PPIVIg	−4	0 →	0 → 0	64+ → 16	1 → 1	512 → 128		
3	PPIVIg	−1	0 →	0 → 0	32+ → 32+	2+ → 2+	256 → 32		
3	PPIVIg	0	0 →	0 → 0	16 → 16	1 → 1	256 → 32	7.6	
3	PPIVIg	1				1 → 0	64 → 16	5.7	17.4
3	PPIVIg	3			8 → 4	0 → 0	16 → 8	2.3	27.9
3	PPIVIg	5			16 → 8	0 → 0	16 → 8	2.0	30.6
3	Biopsy	7	AMR, C4d + (steroids)					2.1	18.9
3	PPIVIg	9			16 → 8		16 → 8	1.8	13.2
3	PPIVIg	11			1 → 4		16 → 4	1.6	13.3
3	PPIVIg	13			16 → 8		16 → 8	1.5	13.7
3	PPIVIg	15			16 → 8		16 → 8	1.4	7.8
4	PPIVIg	−13	4 → 2	0 → 0	64 → 8				
4	PPIVIg	−11	8 → 2	0 → 0					
4	PPIVIg	−8	4 → 2	0 → 0					
4	PPIVIg	−6	2 → 1	0 → 0					
4	PPIVIg	−4	0 → 0	0 → 0					
4	PPIVIg	−1	1 → 0	0 → 0				4.8	
4	PPIVIg	1	0 → 0	0 → 0				2.8	15.2
4	PPIVIg	3	0 → 0	0 → 0				2.7	10.5
4	PPIVIg	5	0 → 0	0 → 0				3.9	22.8
4	Biopsy	7	AMR, C4d + (steroids)					2.4	16
4	PPIVIg	8						2.4	15.9
4	PPIVIg	10							
4	Biopsy	24	Borderline changes, c4d−					2.5	16
4	DSA	24	0	0	0	0			
4	PPIVIg	25						1.8	13.3
4	PPIVIg	27				4 → 2		1.8	17.4
4	PPIVIg	29						1.4	12.6
4	PPIVIg	31						1.3	16.5
4	PPIVIg	34			4 → 2	8 → 2		1.4	15.3
4	PPIVIg	36							
4	PPIVIg	38			4 → 2	4 → 2		1.3	16.6
4	PPIVIg	41						1.7	14.5
4	PPIVIg	43			2 → 1	2 → 1		1.6	
4	PPIVIg	45			4 → 1	1 → 0		1.8	
4	Biopsy	48	Tubular vacuolization, c4d− (anti-CD20)					1.7	15.4
6	PPIVIg	−13	16 → 16	4 → 2					
6	PPIVIg	−11	32 → 8	4 → 2					
6	PPIVIg	−8	8 → 4	2 → 2	64 → 16	8 → 4			10.8
6	PPIVIg	−6	4 → 4	2 → 0	32 → 16	8 → 2			
6	PPIVIg	−4	2 → 1	1 → 0	32 → 2	2 → 1			
6	PPIVIg	−1	2 → 2	2 → 1				10.8	8.7
6	PPIVIg	1	0 → 0	0 → 0				8.7	7.4
6	PPIVIg	3			1 → 1	2 → 1		8.5	40.2
6	PPIVIg	5	2 → 4	32 → 64	8 → 8	8 → 4		7.6	7.3
6	Biopsy	8	Borderline changes, C4d not tested (steroids)					8.7	12.6
6	PPIVIg	10	4 →	64 →	32 →	128 → 0		8.3	10.6
6	PPIVIg	12						9.4	9.8
6	Biopsy	14	Borderline changes, C4d−					10.1	9.8
6	PPIVIg	15						7.5	16.6

Table 4. (contd)

			Donor-specific antibody titers* pre-PPIVIg → post-PPIVIg (treatment)						
No.	Event	POD	T cell	B cell	TFXM	BFXM	Anti-a IgG	Cr	Tac level
6	PPIVIg	17	0 → 0	0 → 0				7.6	15.5
6	PPIVIg	20	0 → 0	1 → 0				7.9	10.2
6	PPIVIg	22	0 → 0	1 → 0				6.5	17.9
6	Biopsy	23	AMR, C4d+					8.2	15.3
6	PPIVIg	24	0 → 0	0 → 0	1 → 0	2 → 1		8.0	
6	PPIVIg	27	1 → 0	1 → 0	1 → 1	1 → 0		5.5	17.7
6	Biopsy	28	AMR, C4d+					6.2	10.4
6	PPIVIg	29	1 → 0	1 → 0	1 → 1	1 → 1		7.9	36
6	PPIVIg	31	1 → 0	1 → 0	1 → 1	2 → 1		8.4	40.2
6	PPIVIg	34			2 → 2	2 → 2		9.8	25.2
6	PPIVIg	36			4 → 2	2 → 1		6.9	12.9
6	PPIVIg	38			4 → 1	8 → 2		5.3	9.4
6	PPIVIg	41			4 → 1	8 → 2		4.2	12.6
6	DSA	173	0	0	1	2		2.3	10.6
6	Biopsy	175	AMR, C4d+ (steroids, IVIg 500 mg/kg)					2.3	22.9
6	PP/IVIg	175	0 → 0	0 → 0	1 → 0	2 → 1		2.3	19.3
6	Biopsy	421	AMR, C4d+					2.3	14.0
12	PPIVIg	−4	0 → 0	0 → 0	0 → 0	16 → 4			
12	PPIVIg	−1	0 → 0	0 → 0	0 → 0	8 → 2		9.1	
12	PPIVIg	1			0 → 0	1 → 0		8.2	8.0
12	Biopsy	5	Normal, C4d−					3.8	8.9
12	DSA	26	0	0	2	32		1.5	9.4
12	Biopsy	441	AMR, C4d+ (IVIg 500 mg/kg)					1.7	
12	DSA	447	0	0	0	4		2.1	
12	PP/IVIg	454	0 → 0	0 → 0	0 → 0	4 → 1		1.8	5.0
12	PP/IVIg	457	0 → 0	0 → 0	0 → 0	2 → 1		1.4	10.0

POD, postoperative day; Cr, creatinine; Tac, Tacrolimus; T cell, T cell anti-human globulin cytotoxic crossmatch titer; B cell, B cell complement-dependent cytotoxicity titer; TFXM, T cell titer by flow cytometry; BFXM, B cell titer by flow cytometry; DSA, donor-specific antibody; ACR, acute cellular rejection; AMR, antibody-mediated rejection; ATN, acute tubular necrosis; TMA, thrombotic microangiopathy; anti-CD20, anti-CD20 monoclonal antibody (Rituximab^{TM}; Genentech, Inc., San Francisco, CA, USA; 375 mg/m^2); HD, hemodialysis; IVIg, intravenous immune globulin.
*Blanks indicate absence of testing, numbers with '+' indicate titers were not tested beyond the indicated titer.

flow cytometry and cytotoxicity and may have represented mixed AMR/ACR. The third case (no. 8) of ACR occurred at day 90 during tacrolimus reduction to treat severe thrombotic microangiopathy (TMA). The ACR resolved with pulse steroids and PP; the latter was given for treatment of TMA.

Discussion

Pretransplant patient conditioning prior to ABO- or crossmatch-incompatible kidney transplantation is sufficient to allow kidney engraftment and prevent hyperacute rejection. [1–4,10–14]. The incidence of early rejection, however, is approximately 50%, indicating that recipients of incompatible kidneys remain at high immunologic risk. The excellent results that have been achieved, despite the increased immunologic risk of these patients, is largely due to improved detection and treatment of rejection, particularly AMR [3,15–18].

The 12 patients presented in this study have different combinations and titers of donor-specific antibodies, all of which represent a high immunologic risk and contraindication to transplantation. This report verifies the feasibility of transplanting patients with preformed donor-specific (anti-HLA) and/or natural antibody (ABO) and describes a preconditioning protocol that has resulted in excellent graft function at 12 months post-transplant despite a 58% incidence of rejection. Our excellent results are most likely due to an aggressive surveillance and therapeutic approach, with particular emphasis on the utilization of DSA levels and graft dysfunction to guide treatment for rejection.

To abrogate a positive crossmatch between a live donor and a prospective recipient, our preconditioning protocol consists of PP to remove anti-HLA antibody and isoagglutinins followed by low-dose immunoglobulin (IVIg) which reduces HLA sensitization through the activation of immunomodulatory pathways and anti-idiotypic

Table 5. Patients with ACR: DSA titers during protocol PPIVIg.

			Donor-specific antibody titers* pre-PPIVIg → post-PPIVIg (treatment)						
No.	Event	POD	T cell	B cell	TFXM	BFXM	Anti-a IgG	Cr	Tac level
8	PPIVIg	−11	1 → 0	1 → 1	0 → 0	1 → 1			7.8
8	PPIVIg	−8	1 → 0	1 → 0	0 → 0	0 → 1			
8	PPIVIg	−4	0 → 0	0 → 0	2+ → 1	4 → 2			
8	PPIVIg	−1	0 → 0	0 → 0	0 → 0	1 → 1		6.9	
8	PPIVIg	1	0 → 0	0 → 0	16+ → 16+	16+ → 16		5.2	<2.5
8	Biopsy	2	TMA (reduce Tac dose, PPIVIg × 5)					HD	
8	PPIVIg	3	0 → 0	1 → 0	1 → 0	8 → 4		5.2	18.4
8	Biopsy	6	ATN, less TMA, C4d−					HD	5.4
8	Biopsy	25	ATN, less TMA					HD	
8	Biopsy	83	Severe TMA (PPIVIgx15, stop Tac, start sirolimus)					4.9	6.3
8	Biopsy	90	ACR 1a, C4d−, no TMA (antithymocyte globulin)					1.7	6.9
8	DSA	90	0	0	0	0			
10	PPIVIg	−6	0 → 0	0 → 0	0 → 0	1 → 2			
10	PPIVIg	−4	0 → 0	0 → 0	0 → 0	1+ → 0			
10	PPIVIg	−1	0 → 0	0 → 0	0 → 0	1+ → 2		3.8	
10	PPIVIg	1						2.2	14.8
10	PPIVIg	3						2.4	16.5
10	DSA	7	0	0	1	0		3.2	12.5
10	Biopsy	7	ACR IIa (antithymocyte globulin)					3.2	12.5
10	Biopsy	12	Borderline changes, C4d−					1.4	3.7
10	DSA	24	0	0	1			4.5	28.2
10	DSA	37	0	0	0			3.7	6.7
10	Biopsy	145	ACR Ib, C4d− (steroids)					2.6	
10	DSA	150	0	0	0	0		2.0	6.4
10	Biopsy	191	ACR Ib, C4d− (antithymocyte globulin)					2.7	7.7
10	DSA	195	2	0	8+	0		2.5	7.7
10	Biopsy	246	ACR Ib, C4d− (steroids, IVIg 500 mg/kg)					3.5	18.3
10	Biopsy	290	ACR Ia, C4d− (anti-CD3)					5.5	12.0
11	PPIVIg	−48							
11	PPIVIg	−46							
11	PPIVIg	−43							
11	PPIVIg	−41							
11	PPIVIg	−39							
11	PPIVIg	−36							
11	PPIVIg	−34	0 → 0	0 → 0	0 → 0	1 → 0			
11	PPIVIg	−1	0	0	0	0		11.6	
11	DSA	−2	0	0	0	0		5.1	8.8
11	Biopsy	3	Borderline changes, C4d− (steroids)					5.2	11.9
11	DSA	4	0	0	0	0		5.7	12.5
11	DSA	4	0	0	0	0		4.8	28.2
11	Biopsy	14	ACR Ib, C4d− (steroids)					3.3	9.9
11	DSA	14	0	0	2	1			
11	PPIVIg	15	0 → 0	0 → 0	0 → 0	0 → 0		3.1	8.4
11	PPIVIg	17	0 → 0	0 → 0	0 → 0	0 → 0		3.2	11.2
11	PPIVIg	19	0 → 0	0 → 0	0 → 0	0 → 0		3.0	16.7
11	DSA	21	0	0	0	0		3.6	15.4
11	Biopsy	23	ACR IIa, C4d− (antithymocyte globulin)					3.3	16.5

POD, postoperative day; Cr, creatinine; T cell, T cell anti-human globulin cytotoxic crossmatch titer; B cell, B cell complement-dependent cytotoxicity titer; TFXM, T cell titer by flow cytometry; BFXM, B cell titer by flow cytometry; DSA, donor-specific antibody; ACR, acute cellular rejection; AMR, antibody-mediated rejection; ATN, acute tubular necrosis; TMA, thrombotic microangiopathy; HD, hemodialysis; IVIg, intravenous immune globulin; anti-CD3 (Orthoclone OKT®3; Ortho Biotech Inc., Raritan, NJ, USA 5 mg i.v. ×10 days).

*Blanks indicate absence of testing, numbers with '+' indicate titers were not tested beyond the indicated titer.

Table 6. Patients without rejection: DSA titers during protocol PPIVIg.

			Donor-specific antibody titers* pre-PPIVIg → post-PPIVIg (treatment)						
No.	Event	POD	T cell	B cell	TFXM	BFXM	Anti-a IgG	Cr	Tac level
1	PPIVIg	−11					128 → 64		
1	PPIVIg	−8					64 → 32		
1	PPIVIg	−6					128 → 32		
1	PPIVIg	−4					64 → 32		
1	PPIVIg	−1					32 → 8	4.9	
1	PPIVIg	1					16 → 4	4.2	37.4
1	PPIVIg	3					8 → 8	4.0	25.4
1	Biopsy	7	ATN, C4d−					3.6	14.2
1	Biopsy	14	TMA, C4d−					2.5	15.0
1	Biopsy	261	TMA, C4d−					1.6	
2	PPIVIg	−11					256 → 32		
2	PPIVIg	−8					512 → 128		
2	PPIVIg	−6					128 → 64		
2	PPIVIg	−4					256 → 64		52.5
2	PPIVIg	−1					128 → 32		
2	PPIVIg	0					128 → 32	9.1	
2	PPIVIg	1					16 → 8	7.2	56
2	PPIVIg	3					8 → 8	3.7	44
2	Biopsy	7	Normal, C4d−					2.3	21.8
2	Biopsy	44	Borderline changes, C4d− (steroids)					2.4	20.8
5	PPIVIg	−11	0 → 0	16 → 8	0 → 0	16 → 8			
5	PPIVIg	−8	0 → 0	8 → 4	0 → 0	8 → 2			
5	PPIVIg	−6	0 → 0	4 → 2	0 → 0	4 → 1			
5	PPIVIg	−4	0 → 0	2 → 2	0 → 0	2 → 0			19.7
5	PPIVIg	−1	0 → 0	4 → 1	0 → 0	1 → 0		9.6	
5	PPIVIg	1	0 → 0	0 → 0	1 → 0	0 → 0		1.9	
5	PPIVIg	3	0 → 0	0 → 0	0 → 0	0 → 0		0.8	18.8
5	PPIVIg	5	0 → 0	0 → 0	0 → 0	0 → 0		0.8	23.8
5	Biopsy	7	Normal, c4d−					0.8	17.4
7	PPIVIg	−4	8+ → 4	4 → 0	4+ → 4+	1 → 2			
7	PPIVIg	−1	0 → 0	0 → 0	4+ → 2	2 → 0			
7	PPIVIg	1	0 → 0	0 → 0	0 →	0 →		9.1	26.9
7	PPIVIg	3	0 → 0	0 → 0	0 → 0	0 → 0		7.3	28.8
7	Biopsy	7	Normal, C4d−					5.9	19.4
7	Biopsy	372	Borderline, C4d− (steroids)					2.4	9.5
9	PPIVIg	−4	0 → 0	0 → 0	1 → 0	1 → 0			
9	PPIVIg	−1	0 → 0	0 → 0	0 → 0	0 → 0		5.5	
9	PPIVIg	1	0 → 0	0 → 0	0 → 0	0 → 0		3.2	
9	PPIVIg	3	0 → 0	0 → 0	2 → 0	0 → 0		1.2	12.4
9	Biopsy	7	ATN, C4d−					0.9	17.7

POD, postoperative day; Cr, creatinine; Tac, tacrolimus; T cell, T cell anti-human globulin cytotoxic crossmatch titer; B cell, B cell complement-dependent cytotoxicity titer; TFXM, T cell titer by flow cytometry; BFXM, B cell titer by flow cytometry; DSA, donor-specific antibody; ATN, acute tubular necrosis; TMA, thrombotic microangiopathy; HD, hemodialysis.

*Blanks indicate absence of testing, numbers with '+' indicate titers were not tested beyond the indicated titer.

networks [12]. As antibodies frequently re-emerge after cessation of PP, IVIg is used to effect a durable reduction of DSA. A low dose of immunoglobulin is utilized as high doses interfere with assays that use an antiglobulin reagent including flow cytometric crossmatches and all solid phase immunoassays. The number of preconditioning PPIVIg for each patient in our protocol was chosen based on initial DSA titers. The kinetics of antibody removal was predictable and, in most cases, achieved isoagglutinin reduction to a titer of 1:8 and/or abrogation of the cytotoxic crossmatch. Difficulty with antibody reduction was seen in patients with high initial DSA (≥32) and isoagglutinin titers (≥256); the Monday–Wednesday–Friday schedule allowed DSA titers in these patients to rebound over the weekend that PPIVIg was not administered. The reduction of highly elevated DSA may require a strict

adherence to alternate day PPIVIg or an individualized approach of PPIVIg based on a target titer, rather than a fixed treatment schedule.

Early detection of rejection is vitally important. Surveillance biopsies obtained within the first week diagnosed several cases of AMR or ACR. The surveillance biopsies were obtained on postoperative days 5, 7, or 8. Two non-surveillance biopsies were obtained on days 2 and 3 for immediate anuria (result was TMA) and during re-operation for bleeding, respectively. Surveillance biopsy results were consistently negative for rejection among patients with steadily falling creatinine values and demonstrated various types of rejection among those with allograft dysfunction at the time of biopsy. Surveillance biopsy may not be necessary for early detection of rejection as allograft dysfunction would have prompted biopsy. In our protocol, however, allograft dysfunction can easily be attributed to elevated tacrolimus levels leading to delays in biopsy and rejection diagnosis and treatment.

Post-transplant PPIVIg was administered to prevent DSA reappearance in the early postoperative period. We observed that post-transplant PPIVIg was generally successful in maintaining a completely abrogated cytotoxic crossmatch; AMR and graft dysfunction can still develop despite undetectable DSA. Persistently elevated DSA or increasing titers of DSA despite post-transplant PPIVIg may be prognostic for antibody-mediated injury and early biopsy is recommended. Among the two highly sensitized ABOIC recipients who were transplanted under high titers, post-transplant PPIVIg effected additional decrements in isoagglutinin levels possibly preventing AMR. Ishida *et al.* found that all patients whose postoperative titer remained below 1:8 exhibited stable renal function (70/93, 75%), whereas all of the patients whose postoperative titer increased to above 1:64 lost their grafts (7/93, 8%) [11].

Donor-specific antibody testing was performed prior to and following each protocol PPIVIg and at least weekly during PPIVIg treatment for rejection. We noted several different trends in the presentation of acute rejection after incompatible kidney transplantation: (i) High level DSA elevations can predict AMR prior to the demonstration of classic features of AMR on biopsy. (ii) Following treatment of AMR, the presence of ongoing graft dysfunction and low levels of DSA may signify ongoing AMR even when histologic changes of AMR have resolved. (iii) The clinical significance of low levels of DSA is unclear, however low levels of DSA concurrent with ACR or borderline changes may represent mixed AMR/ACR or impending AMR; if treatment with anti-T cell therapy does not result in resolution of graft dysfunction, treatment for presumed AMR should be considered.

A firm diagnosis of AMR can be made in the presence of allograft dysfunction when three criteria are concordant: (i) light microscopy features consistent with AMR, (ii) C4d staining of peritubular capillaries (C4d positive), and (iii) the presence of DSA [5]. However, establishing the diagnosis of AMR may be difficult when all the data are not available, time lags occur between each of the findings, or one or more of the criteria are not present.

Each of the three criteria for the establishment of AMR has limitations. AMR is missed by light microscopic examination alone in 25% of cases [19]; the specificity of capillary C4d deposition, although high (93%) as a marker of DSA (FlowPRA), has a low sensitivity (33%) [20]; and DSA detection during allograft dysfunction has a low specificity (69%) and sensitivity (60%) as a marker for C4d staining [20]. Of these three factors, C4d positivity has been the most reliable predictor of antibody-mediated injury and has emerged as the gold standard for the diagnosis of AMR [21]. We have observed, however, that reliance on C4d alone can result in the delayed or failed detection of AMR; identification of DSA in C4d-negative cases is an important adjunct to the diagnosis AMR.

Donor-specific antibody in high titers strongly suggests AMR. It is only a matter of time before complement deposition becomes manifest. The detection of DSA prior to C4d positivity in AMR has previously been seen with time lags of 1 week and 1 month [13]. We noted a time lag of up to 3 weeks. The absence of C4d in the presence of DSA can be explained by several potential mechanisms. C4d binds covalently to the local site after being generated in the early stages of activation of the classic complement pathway. C4d may not be identified at the time of DSA elevation if the titer of circulating antibodies is low, or if the accumulation of antibodies within the graft is insufficient for complement activation and deposition of C4d [7,22]. Alternatively, non-complement binding antibodies may have developed, C4 or other complement components may have been reduced to insufficient quantities due to PP or binding by IVIg, or complement inhibitory proteins may have been upregulated.

Donor-specific antibody in low titer may be detectable at any point post-transplantation in normally functioning kidneys, either due to accommodation or a latent antibody response. During periods of allograft dysfunction however, low titer DSA elevations may represent re-synthesis of DSA and signify antibody-mediated injury or impending AMR. We found DSA elevations detected by flow cytometry to be helpful in guiding the duration of PPIVIg therapy for AMR. Even though histological changes of AMR and C4d positivity had resolved during PPIVIg treatment for AMR, the presence of persistent allograft dysfunction and sudden DSA elevations seen on repeat FXM testing may have indicated ongoing

antibody-mediated injury. Allograft dysfunction persisted until further PPIVIg treatments were given and DSA decreased substantially. In this case, C4d may have washed out of the allograft. The detection of C4d follows a dynamic course with presumed build-up and breakdown within days to a few weeks. While loss of C4d has generally been noted to occur over weeks, it has also been documented as early as 8 days [7].

Donor-specific antibody assessment may also be useful toward the decision not to initiate PPIVIg therapy. In three instances of late-onset AMR, diagnosed on postoperative days 175, 421, and 441, DSA titers were low, detectable only by flow cytometry, and unchanged compared with previous levels seen during baseline allograft function. As PP is unlikely to abrogate low DSA titers, moderately high-dose IVIg was administered. The need for any treatment at all is uncertain. The presence of low levels of DSA appears to have minimal effect on the renal allograft in the short term [23]. In the long term, it is possible that the presence of DSA will negatively correlate with long-term outcomes, more follow-up is needed. Several studies in previously unsensitized patients have demonstrated an association between the development of alloantibody and chronic allograft nephropathy [24–27]. The return of DSA detected by single-antigen flowbead analysis has been noted in 82% of patients at 4 months post-transplant after desensitization with PP/anti-CD20/low-dose IVIg/splenectomy [23]. Alternatively, not all patients with circulating alloantibody develop chronic allograft nephropathy [28] and some evidence suggests that endothelium can develop resistance to antibody-mediated damage in the presence of low levels of antibody. This resistance, termed 'accommodation,' has been suggested to result from a decreased sensitivity of endothelial cells to injury upon continuous stimulation by antibodies and/or complement [29], or perhaps continued stimulation of endothelial cells results in the production of decay-accelerating factor, which inhibits complement activation [30].

Donor-specific antibody may be helpful toward the diagnosis of mixed AMR/ACR. Nickileit *et al.* found that approximately 50% of biopsies with histological signs of acute rejection and half of those accompanied by elevated panel-reactive antibody titers were C4d negative [31]. We noted two cases of C4d-negative ACR accompanied by DSA. In one case, the patient received both PPIVIg for the presumed AMR and steroid treatment for ACR. While the DSA titers were successfully abrogated, allograft dysfunction continued and repeat allograft biopsy demonstrated more severe ACR. Treatment with anti-thymocyte therapy was successful. In this case, the elevated DSA may have been false positive or the prompt PPIVIg treatment averted frank AMR. In the second case, the patient developed recurrent ACR refractory to antilymphocyte therapy. Both low and high DSA titers had been intermittently detected at the time of repeat allograft biopsies. However, because C4d was consistently absent, antibody depletion therapy was not administered. The allograft eventually failed. We suspect that intermittent DSA elevations in this case were markers of active, and possibly transient, antibody-mediated responses.

Conclusions

We present favorable outcomes in a cohort of ABO- and crossmatch-incompatible allograft recipients with unique presentations of allograft rejection after transplantation. Our excellent results are most likely due to an aggressive surveillance and therapeutic approach, with particular emphasis on the utilization of DSA levels to guide treatment for rejection. DSA should also be assessed when biopsy (with C4d) is performed for allograft dysfunction to help guide diagnosis and treatment. DSA should first be assessed with both cytotoxicity and flow-based assays. When the titer is high and therefore detected by cytotoxicity, DSA can be followed until the cytotoxic assay becomes negative, then a switch to flow-based assays should be done to continue to follow the titers until they become negative or resistant to further lowering by PPIVIg.

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