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Influence of preoperative anti-HLA antibodies on short- and long-term graft survival in recipients with or without rituximab treatment

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acute antibody-mediated rejection, anti-HLA antibodies, B-cell targeting protocol, B-cell-activating factor belonging to the tumor necrosis factor family, chronic antibody-mediated rejection, *de novo* antibodies, desensitization protocol, graft loss, graft survival rate, renal transplantation, rituximab.

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Summary

We investigated the relationship between preoperative anti-HLA antibodies (donor-specific antibody, DSA) and the graft survival rate in recipients who had or had not received rituximab (Rit) treatment. The subjects were categorized into four groups as follows: DSA+Rit−, $n = 39$; DSA−Rit−, $n = 121$; DSA+Rit+, $n = 74$; and DSA−Rit+, $n = 47$. We examined the influence of preoperative DSA on the incidence of graft rejection and the survival rate of recipients who had or who had not received rituximab before transplantation. The 6-month acute rejection rates based on graft biopsies were 39%, 19%, 15%, and 0% for the DSA+Rit−, DSA−Rit−, DSA+Rit+, and DSA−Rit+ groups. The rates of chronic antibody-mediated rejection after more than 6 months were 50%, 22%, 18%, and 0%. The 5-year graft survival rate was significantly lower in the DSA+Rit− group (84%) than in the other groups (95% for DSA−Rit−, 98% for DSA+Rit+, and 91% for DSA−Rit+). The rate of the appearance of *de novo* anti-HLA antibodies was higher in the groups that did not receive rituximab treatment. The rate of graft loss associated with chronic antibody-mediated rejection was also higher in the DSA+Rit− group than in the other groups ($P = 0.01$). The presence of DSA and the administration of rituximab had strong impacts on not only short-term graft rejection, but also long-term graft rejection and its association with the graft survival time.

Introduction

For renal transplantation, the preoperative identification of anti-HLA antibodies capable of causing graft loss and the prevention of antibody-mediated rejection using an aggressive desensitization protocol are essential. In particular, the new development of antidonor HLA antibodies after transplantation (*de novo* anti-HLA antibodies) seems to be strongly associated with the occurrence of acute rejection and the graft survival rate [1,2]. Moreover, nondonor HLA antibodies are also reportedly related to graft prognosis [3]. However, we have no global standards for desensitization protocols, such as rituximab administration or plasmapheresis, according to sensitization status [4].

In the present study, we retrospectively investigated the influence of preoperative anti-HLA antibodies detected using a solid-phase assay (SPA; Luminex assay) on graft survival in patients treated with or without rituximab.

Materials and methods

Patient characteristics and immunosuppressive regimens

Figure 1 shows a flow chart summarizing patient enrollment. We performed a total of 520 transplantations

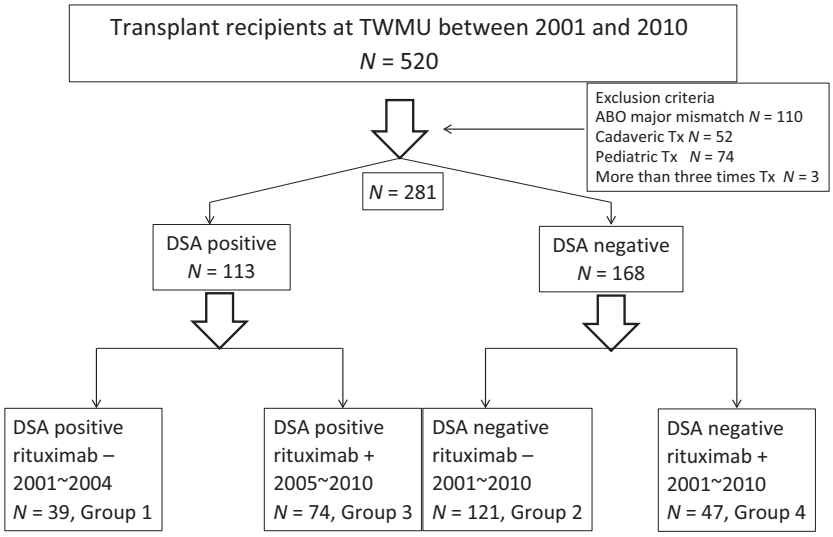


Figure 1 Patient flow chart. We performed 281 kidney transplantations. Among these 281 cases, 113 patients (113/281, 39%) were donor-specific antibody (DSA) positive when examined using a solid-phase assay (SPA, Luminex assay). Among these 113 recipients, 74 recipients received rituximab and underwent plasmapheresis between 2005 and 2010. The remaining 39 recipients did not receive this treatment between 2001 and 2004. One hundred and sixty-eight recipients were DSA negative when examined using SPA. Among these 168 recipients, 121 recipients did not receive rituximab treatment. The remaining 47 recipients received rituximab treatment without undergoing plasmapheresis.

between 2001 and 2010 at our single institution. Finally, 281 recipients were enrolled in this study.

Table 1 shows the characteristics of the patients in this study. All the recipients in this study were divided into four groups according to their preoperative donor-specific antibody (DSA) and rituximab treatment status as follows: Group 1 (n = 39, DSA+Rit−), patients with a positive DSA status who did not receive rituximab treatment; Group 2 (n = 121, DSA−Rit−), patients with a negative DSA status who did not receive rituximab treatment; Group 3 (n = 74, DSA+Rit+), patients with a positive DSA status who received rituximab treatment; and Group 4 (n = 47, DSA−Rit+), patients with a negative DSA status who received rituximab treatment.

Briefly, the Group 1 patients had undergone transplantations between 2001 and 2004. During this period, FCXM, LCT, and a panel reactive analysis assay (PRA), but not SPA, were used as immunologic methods. The DSA status of the Group 1 recipients was retrospectively confirmed to be positive using SPA and pooled serum samples. Despite DSA positivity, the Group 1 recipients underwent transplantation without undergoing desensitization, although they were treated using the triplicate immunosuppressive regimen consisting of tacrolimus (FK), mycophenolate mofetil (MMF), and methylprednisolone (MP) plus the administration of anti-CD25 antibody (Simulect®; administered twice; Fig. 2a).

The Group 2 patients had undergone transplantations between 2001 and 2010. DSA was not detected using any of the available immunologic methodologies, including SPA. All these recipients underwent transplantation after completing the triplicate immunosuppressive regimen and the administration of anti-CD25 antibody (Simulect®; administered twice; Fig. 2a).

The Group 3 patients had undergone transplantations between 2005 and 2010. DSA was detected using SPA during this era. As shown in Fig. 2b, rituximab and three sessions of plasmapheresis in addition to the triplicate immunosuppressive regimen were administered to these recipients as a desensitization protocol.

The Group 4 patients had undergone transplantations between 2001 and 2010. DSA was not detected in these recipients. However, these patients received the rituximab protocol for reasons other than desensitization, such as the prevention of hemolysis after transplantation using blood type minor-mismatch donors. The Group 4 recipients did not receive plasmapheresis prior to surgery (Fig. 2c). Between 2001 and 2004, transplants from ABO-minor-mismatched donors were performed using local irradiation of the graft [5]. Use of the rituximab protocol to prevent hemolysis in patients with minor blood incompatibilities was initiated in January 2005 (data, not shown). Serum samples were prepared from blood samples obtained from the recipients and donors after obtaining their informed consent. All the study procedures were approved by the Ethics Committee of Tokyo Women's Medical University. Immunosuppressive regimens were shown in Fig. 2a–c. The maintenance triplicate immunosuppressive regimen is as described before [6].

Table 1. (a) Recipient background, (b) cause of renal failure and (c) donor background.

	Group 1 (DSA+RIT−)	Group 2 (DSA−RIT−)	Group 3 (DSA+RIT+)	Group 4 (DSA−RIT+)
(a)				
n	39	121	74	47
Age (years)	43.4 ± 12.2	40.2 ± 13.9	44.7 ± 13.8	46.0 ± 15.2
Sex (M/F)	27/12	84/37	43/31	26/21
Dialysis period (months)	62.6 ± 58.5	49.4 ± 48.7	57.9 ± 60.4	58.4 ± 63.8
HLA-AB mm	3.1 ± 1.4	3.4 ± 1.6	3.1 ± 2.1	3.0 ± 1.4
HLA-DR mm	2.9 ± 1.2	3.8 ± 2.1	2.8 ± 1.7	3.1 ± 1.2
PRA (%)	32.6 ± 4.5	8.6 ± 4.4	41.6 ± 7.8	7.8 ± 3.3
Hypertension (%)	31 (79.5)	80 (66.1)	40 (54.1)	25 (53.2)
Dyslipidemia (%)	3 (7.8)	6 (5.0)	4 (5.4)	4 (8.5)
Diabetes (%)	5 (12.8)	14 (11.6)	7 (9.5)	6 (12.8)
WIT (min)	5.7 ± 2.7	6.0 ± 8.7	4.3 ± 1.1	4.6 ± 1.2
TIT (min)	98.1 ± 32.4	98.1 ± 32.9	108.7 ± 29.0	99.5 ± 34.2
Follow-up time in months	107 ± 22	83 ± 35	50 ± 20	52 ± 17
(b)				
CGN (%)	10 (25.6)	26 (21.5)	18 (24.3)	10 (20.8)
Reflux nephropathy (%)	2 (5.1)	5 (4.2)	3 (4.1)	1 (2.1)
Acute renal failure (%)	1 (2.6)	1 (0.8)	0	0
Polycystic kidney (%)	2 (5.1)	3 (2.5)	4 (5.4)	3 (6.4)
Hypoplastic kidney (%)	0	4 (3.3)	3 (4.1)	1 (2.1)
FSGS (%)	3 (7.3)	6 (5.0)	6 (8.1)	4 (8.5)
MN (%)	1 (2.6)	2 (1.7)	1 (1.4)	1 (2.1)
Lupus nephritis (%)	0	0	2 (2.7)	1 (2.1)
IgA nephropathy (%)	6 (15.4)	30 (24.8)	13 (17.6)	7 (14.9)
Nephrosclerosis (%)	1 (2.6)	3 (2.5)	1 (1.4)	2 (4.3)
DM (%)	4 (10.3)	13 (10.7)	5 (6.8)	5 (10.6)
Alport syndrome (%)	0	0	0	1 (2.1)
Toxemia pregnancy (%)	1 (2.6)	3 (2.5)	0	0
Unknown (%)	6 (15.4)	23 (19.0)	18 (24.3)	11 (21.9)
(c)				
n	39	121	74	47
Donor age	55.1 ± 10.5	56.9 ± 9.3	56.7 ± 9.6	57.4 ± 8.1
Sex (M/F)	12/27	34/87	30/44	15/32
Donor relationship				
Father/Mother	4/13	21/52	7/22	3/17
Siblings	5	18	16	5
Others	17	30	27	21

Statistical analysis was performed between Group 1 and each group.

$P < 0.05$ was considered as significant.

There were no significant differences between Group 1 and any groups in this table.

Detection of antidonor HLA antibody using a single-phase assay (SPA, Luminex)

Before transplantation, we determined the sensitized status of all the patients using an LCT/FCXM crossmatch assay, as previously reported. Patients with positive LCT/FCXM crossmatch assay results were excluded from this study. SPA has been used at our center since 2005; thus, the DSA status of the recipients who underwent transplantation prior to 2005 was analyzed retrospectively using serum that had been stored at −80 °C, as previously reported. Briefly, 20 μl of sera was added to 5 μl of Class I or Class II antigen beads; the beads were then incubated in the dark for 30 min at room temperature and then rinsed twice in a wash buffer. Next, 100 μl of 1:100 diluted phycoerythrin (PE)-conjugated goat anti-human IgG secondary antibody was added to the beads, and the beads were incubated for 30 min in the dark at room temperature and washed. The luminescence was read using a LABScreen™ 100 Luminex system (One Lambda Inc., Canoga Park, CA, USA). Data were analyzed using LABScreen analysis software HLA Fusion 2.0 (One Lambda), and a mean fluorointensity (MFI) of over 800 was considered positive. In this study cohort, none of the recipients had an MFI of over 2500.

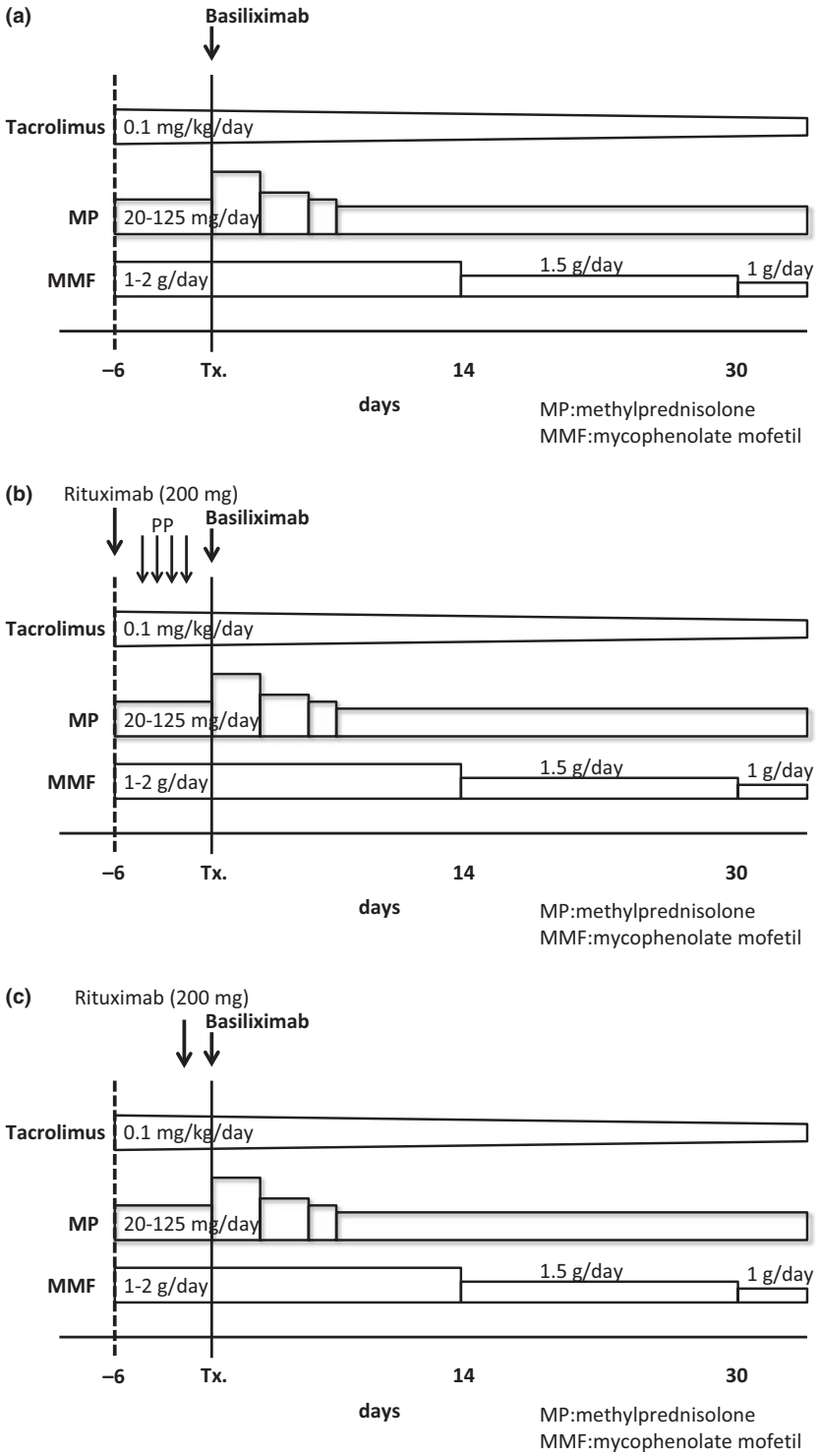


Figure 2 (a) Immunosuppressive protocols for Groups 1 and 2. (b) Desensitization protocol using rituximab and plasmapheresis for Group 3. (c) Desensitization protocol using rituximab alone for Group 4. MMF, mycophenolate mofetil; MP, methylprednisolone.

Therefore, we defined these patients as weakly sensitized recipients. We also examined the patients' DSA status using a Luminex assay during the postoperative follow-up period.

Pathological findings

Postoperative protocol biopsies (at 0 h, 6 months, and more than 6 months) as well as episode biopsies were performed for all the recipients, and the diagnoses were confirmed at the time of the detection of anti-HLA Abs. All the biopsy specimens were evaluated using light microscopy and immunofluorescence staining for C4d. Patients with complications, peri-renal infection, or a bleeding tendency were excluded. Whenever a rejection was suspected, an episode biopsy was performed. Two or three core biopsy samples were obtained using a spring-loaded 16-gauge biopsy gun under ultrasound guidance. In all the cases, a diagnosis of rejection was made in a blinded manner by the same central pathologist. The type of rejection was classified according to the Banff '07 criteria. The pathological findings were classified according to the Banff 1997 working classification and the Banff 2005 Update Edition and were evaluated comparatively in the recipients with and those without *de novo* anti-HLA Abs.

Renal function

The renal allograft function was evaluated by the serum creatinine level (sCr) and the estimated glomerular filtration rate (eGFR). GFR was estimated using the Cockroft's formula. Glomerular filtration rate (GFR) was estimated based on the serum creatinine level using the Filler equation and expressed in ml/min/1.73 m^2.

Statistical analysis

All the statistical analyses were performed using the JMP 8.0.1 software (SAS Institute, Cary, NC, USA). We used time-dependent statistical methods, such as Kaplan–Meier survival, as well as the log-rank test and the multivariate Cox regression model. Quantitative parameters were compared using an unpaired two-sample *t*-test and Mann–Whitney test, while qualitative parameters were compared using the chi-square test. *P*-values less than 0.05 were considered to indicate statistical significance.

Results

Patient characteristics

As shown in Fig. 1, we performed 281 kidney transplantations. Among the 281 cases, 113 cases (113/281, 39%) were DSA positive when examined using a solid-phase assay (SPA, Luminex assay). Among these 113 recipients, 74 recipients (74/113, 66%) received rituximab and plasmapheresis between 2005 and 2010. In contrast, the remaining 39 recipients (39/114, 34%) did not receive this treatment between 2001 and 2004 because we had not yet adopted the rituximab protocol during this era. One hundred and sixty-eight recipients (168/281, 61%) were DSA negative according to SPA. Among these 168 recipients, 121 recipients (121/168, 72%) did not receive rituximab treatment. The remaining 47 recipients (47/168, 28%) received rituximab treatment without plasmapheresis because rituximab has been routinely administered to recipients with a blood type minor mismatch to prevent hemolysis into the graft after grafting. In this study, hemolysis after ABO-minor-mismatched transplantations was not observed in any cases. Some researchers reported that ABO minor mismatch itself had no influence on the graft survival rate, regardless of hemolysis [7–9]. Until now, severe adverse events from rituximab have been observed in no cases [10].

As shown in Table 1a, no significant differences in age, sex, dialysis period, mismatch number of HLA-AB and –DR, or frequency of diabetes were observed among the groups. No significant differences in the PRA results were observed between Groups 1 and 3. As shown in Table 1b, original ESRD disease in recipient's characteristics between Groups 1 and 4. No differences in the donor characteristics were observed among the groups, as shown in Table 1c.

Patient survival rate and graft survival rate

Figure 3a and b shows the patient survival rate and the graft survival rate. No significant differences in the patient survival rate were observed among the groups ($P = 0.375$). The 5-year graft survival rate was significantly lower in the DSA+/Rit− group (84%) than in the other groups (95% for DSA−/Rit−, 98% for DSA+/Rit+, and 91% for DSA−/Rit+; $P = 0.034$).

Incidence of rejection before and after 6 months after transplantation

Figure 4a shows the incidence of rejection within 6 months after transplantation. Within 6 months after kidney transplantation, acute antibody-mediated rejection (AAMR) occurred in 12 of the 39 recipients (31%), eight of the 121 recipients (7%), 11 of the 74 recipients (15%), and 0 of the 47 recipients (0%) in Groups 1–4, respectively. The incidence rate of acute antibody-mediated rejection was significantly higher in Group 1 than in other groups ($P = 0.02$). T cell-mediated rejection (TMR) was observed in 16 of the 39 recipients (39%), 22 of the 121 recipients (17%), 0 of the 74 recipients (0%), and 11 of the 47 recipients (23%) in the above-mentioned groups, respectively. No rejection was observed in 53 of the 74 recipients (72%) in Group 3. In

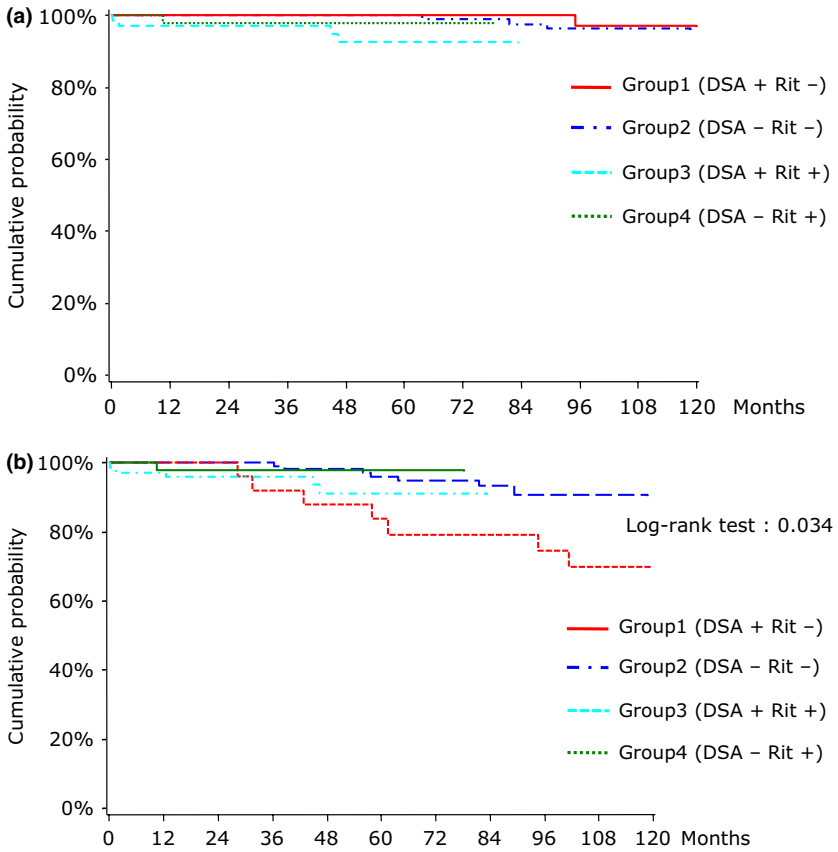


Figure 3 (a) Patient survival. (b) Graft survival. (a) and (b) Show the patient survival rate and the graft survival rate. No significant differences in the patient survival rates were observed among the groups ($P = 0.375$). The 6-month graft survival rate was significantly lower in the DSA+/Rit− group (84%) than in the other groups (95% for DSA−/Rit−, 98% for DSA+/Rit+, and 91% for DSA−/Rit+; $P = 0.034$) .

contrast, no rejection was observed in only 17% (7/39) of the recipients in Group 1.

At more than 6 months after transplantation (Fig. 4b), chronic antibody-mediated rejection (CAMR) occurred in 20 of the 39 recipients (50%) and interstitial fibrosis/tubular atrophy (IFTA) occurred in 10 of the 39 recipients (27%) in Group 1, while CAMR did not occur in any of the recipients (0/47) in Group 4. The incidence rate of chronic antibody-mediated rejection was significantly higher in Group 1 than in other groups ($P = 0.006$).

Cause of graft loss

As shown in Table 2, among the Group 1 recipients, seven of the 39 patients (17.9%) lost their grafts. All the grafts were lost because of chronic antibody-mediated rejection. The rate of graft loss associated with chronic antibody-mediated rejection was higher in the DSA+Rit− group than in the other groups ($P = 0.01$). As shown in Table 3, three of the 7 recipients with graft loss produced *de novo* antibodies, and the remaining four recipients continued to have preformed DSA. The rate of graft loss in Group 2 was 9.2% (11/121). Eight of the 11 recipients exhibited chronic rejection when graft biopsies were examined, all of which were caused by *de novo* antibodies, as shown in Table 3. The rate of graft loss was 5/74 (6.8%) and 1/47 (2.1%) in Groups 3 and 4, respectively. The main cause of graft loss in patients from Groups 3 and 4 was acute rejection and death with a functioning graft, not chronic rejection.

Change in antidonor-specific antibody (DSA) levels before and after kidney transplantation

Figure 5 shows the change in DSA levels before and after kidney transplantation. As shown in Fig. 5, the DSA level decreased in all the recipients within 6 months after transplantation, probably because of antibody adsorption on the graft. Among the patients with DSA positivity from Groups 1 and 3, the Group 1 recipients who did not receive rituximab exhibited a gradual elevation in the DSA level on a year-by-year basis, while the Group 3 recipients who received rituximab showed a decrease in DSA. However, a

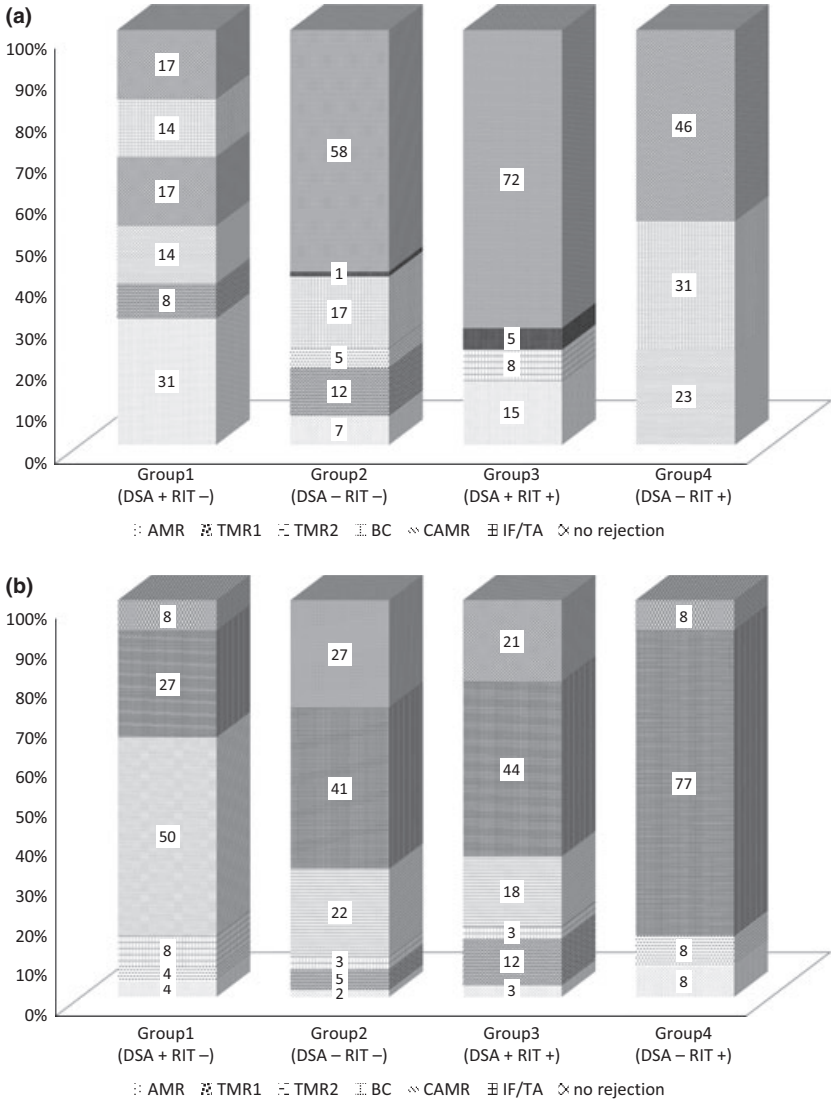


Figure 4 (a) Incidence of rejection within 6 months after kidney transplantation. (b) Incidence of rejection at more than 6 months after kidney transplantation. (a) Shows the incidence of rejection within 6 months after transplantation. Within 6 months after kidney transplantation, acute antibody-mediated rejection (AAMR) occurred in 12 of the 39 patients (31%), eight of the 121 patients (7%), 11 of the 74 patients (15%), and 0 of the 47 patients (0%) in Groups 1–4, respectively. T cell-mediated rejection (TMR) was observed in 16 of the 39 patients (39%), 22 of the 121 patients (17%), 0 of the 74 patients (0%), and 11 of the 47 patients (23%) in Groups 1–4, respectively. No rejections were observed in 53 of the 74 patients (72%) in Group 3. In contrast, no rejections were observed in only 17% (7/39) of the patients in Group 1. At more than 6 months after transplantation, chronic antibody-mediated rejection (CAMR) occurred in 20 of the 39 patients (50%) and interstitial fibrosis/tubular atrophy (IFTA) occurred in 10 of the 39 patients (27%) in Group 1, whereas CAMR did not occur in any of the patients (0/47) in Group 4.

Table 2. Cause of graft loss.

	Group 1 (DSA+RIT−)	Group 2 (DSA−RIT−)	Group 3 (DSA+RIT+)	Group4 (DSA−RIT+)
Death with functioning graft (%)	–	3 (2.5)	4 (5.4)	1 (2.1)
Acute rejection (%)	–	–	1 (1.4)	–
Chronic rejection (%)	7 (17.9)	8 (6.7)	–	–
Nonadherence (%)	–	–	–	1 (2.1)

Table 3. Patients with postoperative *de novo* DSA 5 years after transplantation.

Group	Name	Gender	Appearance (day)	Pathology	Pre-DSA	Post-DSA (MFI)	Graft
G1	KT	Male	1821	CAMR	B56	***DR11 (11983)***	Functioning
G1	FI	Male	1879	CAMR	DR15	DR15 (5817), ***DR10 (1879)***	Graft loss
G1	TT	Male	708	CAMR	A11	A11 (976), ***B54 (3288), DR4 (708)***	Graft loss
G1	FM	Male	374	CAMR	A24, DR9	***B61 (3544)***, DR9 (6806)	Graft loss
G1	NM	Female	3296	CAMR	A26	A26 (9827), ***DR8 (1945), DQ4 (15443)***	Functioning
G2	OS	Male	358	CAMR	None	***DQ8 (Unknown)***	Graft loss
G2	YM	Male	2311	CAMR	None	***DR15 (11452)***	Graft loss
G2	HS	Male	2398	CAMR	None	***DQ9 (17171)***	Functioning
G2	SY	Male	1936	CAMR	None	***DR9 (615)***	Graft loss
G2	AK	Female	1449	CAMR	None	***DQ9 (689)***	Graft loss
G2	SM	Male	1460	CAMR	None	***DR8 (1232)***	Graft loss
G2	TY	Male	837	CAMR	None	***DR15 (772)***	Graft loss
G2	KS	Male	2984	CAMR	None	***A26 (1626), DQ6 (15268)***	Graft loss
G2	ST	Female	353	CAMR	None	***DR15 (3577), DQ6 (10794)***	Functioning
G2	NM	Male	1655	Unknown	None	***DR9 (1739)***	Functioning
G2	AH	Male	424	CAMR	None	***DR15 (9034)***	Functioning
G2	ST	Male	205	CAMR	None	***DR9 (6180), DQ9 (21893)***	Functioning
G2	OM	Female	1742	IFTA	None	***DR9 (2230)***	Functioning
G2	ST	Male	505	CAMR	None	***DR4 (6995)***	Functioning
G2	WT	Male	1238	CAMR	None	***A2 (4535)***	Graft loss

CAMR, chronic antibody-mediated rejection; DSA, donor-specific antibody; IFTA, interstitial fibrosis/tubular atrophy.
Italic bold DSA means *de novo* DSA.

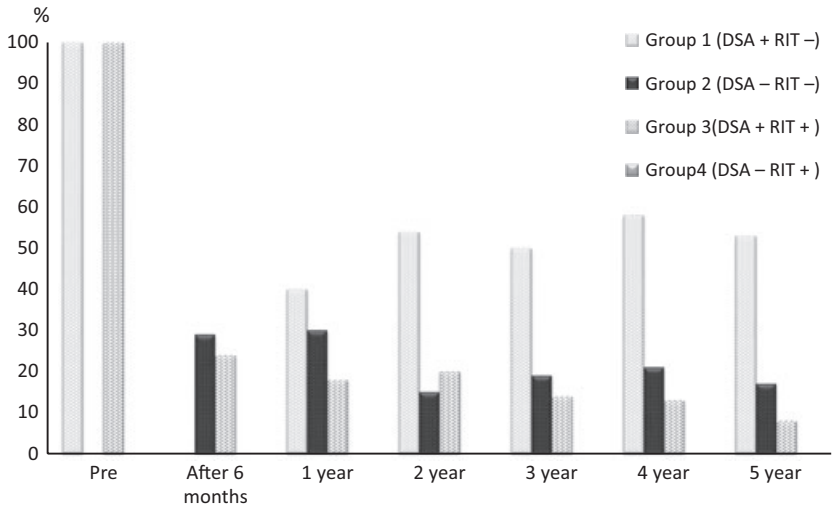
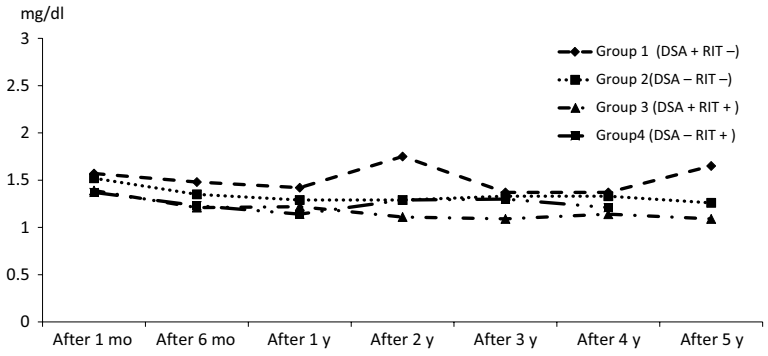


Figure 5 Changes in donor-specific antibody (DSA) status before and after transplantation. DSA decreased in all the recipients within 6 months of transplantation. In patients with DSA in Groups 1 and 3, the Group 1 recipients showed a gradual elevation in DSA from year to year, while the Group 3 recipients showed a decrease in DSA. In the patients without DSA from Groups 2 and 4, the Group 2 recipients without rituximab treatment exhibited the sudden appearance of DSA during the follow-up period, while the Group 4 recipients who were treated with rituximab did not exhibit the appearance of DSA until the end of the observation period. In Group1,the rate of *de novo* antibody among all DSA; 42% at 1 year, 53% at 2 years, 68% at 3 years, 77% at 4 years, 78% at 5 years. All DSA in Group 2 is *de novo* antibody at any time points. All DSA in Group 3 is preformed DSA at any time points.

DSA level with a very strong mean fluorescence intensity (MFI) that remained at a high level was observed in 3 recipients in Group 3, despite the administration of rituximab. In contrast, in the patients from Groups 2 and 4 who exhibited DSA negativity, the Group 2 recipients who did not receive rituximab exhibited the sudden appearance of DSA during the follow-up period, while DSA positivity had not been observed as of the end of the observation period in the Group 4 recipients who received rituximab.



	1 m	6 m	12 m	24 m	36 m	48 m	60 m
Group 1	1.57 (36)*	1.50 (40)	1.48 (42)	1.68 (32)	1.47 (42)	1.48 (42)	1.59 (34)
Group 2	1.51 (40)	1.45 (45)	1.39 (48)	1.43 (44)	1.45 (45)	1.47 (43)	1.42 (45)
Group 3	1.35 (45)	1.23 (51)	1.25 (53)	1.18 (55)	1.16 (57)	1.18 (55)	1.17 (56)
Group 4	1.35 (45)	1.22 (50)	1.13 (59)	1.43 (44)	1.44 (43)	1.35 (45)	–

*Value is expressed as sCr (mg/dl)(eGFR ml/min/1.73 m^2).
P<0.05 was considered as significant.
There were no significant differences between Group 1 and any groups.

Figure 6 Change in serum creatinine (sCr) level during the 5 years after transplantation. Figure 2 shows the renal graft function, as shown according to the serum creatinine level (sCr) and the evaluated glomerular filtration rate (eGFR). No statistical differences in sCr and eGFR were observed among any of the groups at any time point.

De novo DSA appearance after kidney transplantation

Table 3 shows the patients with *de novo* DSA at 5 years after transplantation. Overall, *de novo* DSA developed in 20 of the 160 cases (20/160, 13%). All 20 cases with the development of *de novo* antibodies had not received rituximab treatment (Groups 1 and 2). *De novo* DSA appeared in five of the 39 recipients (14%) in Group 1, while *de novo* DSA appeared in 15 of the 121 recipients (12%) in Group 2. No significant difference in the rate of *de novo* antibody development was observed between Groups 1 and 2. The subtype of *de novo* antibodies belonged to Class II in 18 of the 20 recipients and to Class I in only two of the 20 recipients. The mean fluorescence intensity (MFI) for these *de novo* antibodies was 7277 in Group 1 and 7458 in Group 2 ($P = 0.67$). In Group 1, preformed anti-HLA antibodies disappeared in one case and persisted in four cases with an MFI of 5856.

De novo antibodies developed a mean of 1616 days after transplantation in Group 1 and 1323 days after transplantation in Group 2 ($P = 0.55$). *De novo* antibodies were not observed after kidney transplantation in any of the patients in Groups 3 or 4. Three of the five recipients (3/5, 60%) with *de novo* antibodies in Group 1 lost their grafts, while eight of the 15 recipients (8/15, 53%) in Group 2 lost them. Pathologically, the graft biopsies showed chronic active antibody-mediated rejection in all five recipients in Group 1 and in 13 of the 15 recipients in Group 2 (5/5, 100% vs. 13/15, 87% for Groups 1 and 2, respectively; $P = 0.78$).

Renal function

Figure 6 shows the renal graft function, as evaluated based on the latest serum creatinine level (sCr) and the eGFR. No significant differences in sCr or eGFR were observed among the groups at any time point, because renal function in patients with graft loss was not included in this analysis.

Discussion

Many researchers have previously reported a clear association between the presence of antidonor HLA antibodies (DSA) and the graft survival rate [11]. Qualitative and quantitative analyses for DSA are essential prior to kidney transplantation to avoid acute rejection and to achieve an excellent graft survival rate [12]. Generally, anti-HLA antibodies are produced by exposure to nonself antigens. Reports have indicated that HLA Class I antibody is related to pregnancy and blood transfusion, HLA Class II antibody is related to a poor graft survival rate, and both Class I and II antibodies are related to graft loss [13,14]. In the present study, we found the higher appearance of the Class II HLA subtype among *de novo* anti-HLA antibodies.

The graft survival rates in the present study were similar to those in a previous report by Leckman *et al.* [15], in

which DSA+/Rit− patients had a poorer graft survival rate than any of the other groups that were examined. This poorer graft survival was thought to be linked to the new appearance of antidonor antibodies in DSA+/Rit− patients. Late-onset antibody-mediated rejection (AMR) due to the appearance of *de novo* antibodies is reported to be associated with worse prognosis rather than acute-onset AMR by many researchers. Everly, *et al.* [16] reported that, after dnDSA development, 24% of the patients will fail within 3 years.

Within 6 postoperative months, the incidence of AAMR was significantly higher in DSA+ patients: 12 of the 39 recipients (31%), eight of the 121 recipients (7%), 11 of the 74 recipients (15%), and 0 of the 47 recipients (0%) in Groups 1–4, respectively. At more than 6 postoperative months, the incidence of CAMR was also significantly higher in DSA+ patients: 20 of the 39 recipients (50%) in Group 1 and 14 of the 74 recipients (18%) in Group 3. The patients in Group 2, who exhibited DSA negativity, also exhibited a relatively high incidence of CAMR (26/121, 22%), probably because of the high rate of *de novo* antibody appearance [17]. The presence of other non-HLA antibodies such as anti-angiotensin receptor antibodies, not *de novo* HLA antibody, may have also some great influences on this high incidence rate of CAMR in immunologic low-risk Group 2 [18]. Also, there are some possibilities of lower doses of immunosuppressants such as tacrolimus and mycophenolate mofetil in our department, compared with other institutions, although this protocol yielded excellent graft survival rate in terms of rejection and nephrotoxicity [19].

We investigated the change in DSA in each group during a 5-year follow-up period. Group 1 recipients showed the temporal disappearance of DSA, followed by its reappearance and an increase in DSA until the end of the observation period. Group 3 recipients showed a gradual decrease in DSA. Group 2 recipients showed the new production of DSA at 6 months after transplantation, followed by the persistent presence of DSA in the recipients' sera. Group 4 recipients had not shown any production of DSA as of the end of the observation period. Many researchers have described an association between *de novo* antibodies and a poor graft survival time [20–22]. In the present study, the incidence of *de novo* antibodies was 14% (5/39), with an average MFI of 7277, in Group 1 and 12% (15/121), with an average MFI of 7458, in Group 2 ($P = 0.32$). No significant differences in the incidence or MFI of the *de novo* DSA antibodies were seen between the groups. On the other hand, *de novo* antibodies did not develop in any of the patients in Groups 3 or 4, who were treated with rituximab. However, three recipients in Group 3 experienced antibody-mediated rejection very early after transplantation, despite the administration of rituximab. These three patients showed the persistent presence of DSA with an MFI of over 15 000, which was much higher than that in nonrejection recipients [23].

As we described previously [24], chronic antibody-mediated rejection is remarkably reduced by targeting B-cell immunity through methods such as a splenectomy and rituximab treatment. Our previous study had a heterogeneous design, as the patient populations included blood type-identical patients and blood type-nonidentical patients. In the present study, only blood type-identical patients without ABO-incompatible transplantations were included, enabling a more homogeneous patient population.

Recently, the efficacy of rituximab for suppressing the production of anti-HLA antibodies has been reported by many researchers. Billing *et al.* [25], Hong *et al.* [26], and Barnett *et al.* [27] also reported the efficacy of rituximab for the treatment of chronic antibody-mediated rejection in kidney transplantation, which was similar to our previous research [24], although they adopted a combination of rituximab and IVIG. For heart transplantation, Aggarwal *et al.* [28] reported the effectiveness of low-dose rituximab for antibody-mediated rejection. Taken together, these recent lines of evidence suggest that rituximab may prevent the development of chronic antibody-mediated rejection by suppressing anti-HLA antibodies, including *de novo* antibodies. A recent paper [29] reported the prevention of anamnestic responses in patients with cryptic sensitization to HLA. In that paper, they confirmed a reduction in the incidence of antibody-mediated rejection and the development of antibodies post-transplantation.

Rituximab, a chimeric anti-human CD20, is approved for the treatment of B-cell lymphoma in adults. In transplant recipients, it is used for the treatment of post-transplant lymphoproliferative disease, to reduce preformed anti-HLA and anti-AB antibodies, and to prevent acute rejection. Three main pathways explaining the action of rituximab have been postulated. First, rituximab may act as a nonspecific intravenous immunoglobulin. Second, rituximab may deplete specific antidonor antibodies. Third, rituximab may act by eliminating B cells, which are very efficient antigen-presenting cells, particularly after they have been activated [30].

In the present study using an ABO-identical and ABO-minor-mismatched patient population, we observed a notably lower incidence of AMR and the development of DSA postoperatively. The incidence of AMR was lower in Group 3 (DSA+Rit+) than in Group 1 (DSA+Rit−), suggesting that rituximab played an important role in blocking T–B cell interactions during the very early phase after transplantation. On the other hand, the incidence of the development of *de novo* DSA was lower in Group 4 (DSA−Rit+) than in Group 2 (DSA−Rit−), suggesting

that rituximab also played a role in establishing an immunologic reset by depleting most of the B lymphocytes residing in the spleen and the secondary lymph nodes, leading to a remarkable decrease in chronic antibody-mediated rejection accompanying the decrease in antibodies. The rituximab protocol may play an excellent role in inhibiting humoral pathways during both the short term and the long term.

Clatworthy *et al.* [31] reported an open-label randomized controlled trial, conducted in Europe, in which the rate of acute rejection was higher in an anti-CD20 antibody (rituximab)-treated group than in an anti-CD25 antibody (daclizumab)-treated group, which led to the suspension of the study. These authors also reported elevated levels of B-cell-related cytokines (B-cell-activating factor belonging to the tumor necrosis factor family, BAFF) that were closely associated with a higher incidence of acute rejection in the rituximab group, although they also adopted a normal-dose rituximab regimen (375 mg/m^2 × three times). We also observed a significant elevation in BAFF after low-dose (150 mg/m^2 × one time) rituximab treatment [32]; however, no association between the elevation in BAFF and the incidence of acute rejection was observed postoperatively. The main difference between these two trials was the dose of rituximab given to the recipients.

A limiting factor in the present study is the difference in the observation period between Groups 1 and 4, although the difference was not significant. The reason for the shorter observation period in Groups 3 and 4 was that the rituximab protocol was begun in 2005. However, the decrease in DSA and the lower incidence rate of chronic antibody-mediated rejection were observed for 5 years after transplantation. Secondly, another limiting factor is that this study is a comparative study using historical data. While the immunosuppressive regimens and biopsy policies used during this study period were exactly the same throughout the study, a prospective randomized trial is needed for an accurate investigation.

In conclusion, the incidence of biopsy-proven graft rejection was significantly lower in patients who were treated with rituximab than in patients who were not treated with rituximab. The presence of DSA and the administration of the anti-CD20 antibody rituximab have strong impacts on not only short-term graft rejection, but also long-term graft rejection and its association with the graft survival time.

Authorship

HI: designed the research/study, performed the research/study, and wrote the paper. MF: performed research. TS: pathological work. TN: wrote the paper. KT: contributed important reagents. HI and MF: contributed equally to this study.

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