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Significance of low-level DSA detected by solid-phase assay in association with acute and chronic antibody-mediated rejection

Toshihito Hirai,[1,2] Naoki Kohei,[1] Kazuya Omoto,[1] Hideki Ishida[1] and Kazunari Tanabe[1]

1 Department of Urology, Tokyo Women's Medical University, Tokyo, Japan
2 Department of Urology, Kanazawa University, Kanazawa, Japan

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Correspondence
Kazunari Tanabe, Department of Urology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-Ku, Tokyo, 162-8666, Japan.
Tel.: +81-3-3353-8111;
fax: +81-3-5269-7353;
e-mail: tanabe@kc.twmu.ac.jp

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Summary

We sought to clarify the controversial issue of whether detecting low-level anti-donor-specific HLA antibody (HLA-DSA) by single-antigen flow-bead assay (SAFB) may have a potential role in reducing acute and chronic antibody-mediated rejection (AMR). We retrospectively studied the preoperative serum of ABO-compatible living kidney transplantation recipients transplanted between 2001 and 2004 by SAFB using a Luminex platform. HLA-DSA was detected only by SAFB in 24 patients, although all of them showed negative T-cell and B-cell complement-dependent cytotoxicity (CDC) crossmatches. The HLA-DSA patients went on to have surprisingly high levels of acute and chronic AMR despite being only weakly sensitized (acute AMR, 33.3%; chronic AMR, 41.7%). After 2005, we implemented SAFB routinely and any patient having a positive HLA-DSA was considered to be a desensitization candidate. The 52 patients found to have HLA-DSA underwent kidney transplantation after prior treatment with a single dose of rituximab (RIT) and three or four sessions of double-filtration plasmapheresis (DFPP) in addition to regimens commonly used between 2001 and 2004. After 2005, there was a significant reduction in the occurrence of acute and chronic AMR (acute AMR, 4.7%, $P < 0.001$; chronic AMR, 4.7%, $P < 0.001$). The 5-year graft survival rate also improved after implementing SAFB (83.3–98.1%, $P = 0.032$). The RIT/DFPP-induction protocol may improve graft survival even in patients with low-level DSA.

Introduction

The introduction of solid-phase assays, including ELISA, and multiple beads technology, of which the single-antigen flow-bead assay (SAFB) is one type, enables more sensitive detection of anti-donor-specific HLA antibody (HLA-DSA). However, evidence on the relevance between the clinical outcome and low-level HLA-DSA, which is detected by these sensitive assays, is still conflicting [1–6]. Amico *et al.* reported a high incidence of acute antibody-mediated rejection (AAMR, 55%) in patients with positive HLA-DSA detected by SAFB [1], and Patel *et al.* also reported a high incidence of AAMR (20%) in patients with positive HLA-DSA detected by ELISA [3], whereas all their patients showed negative cytotoxic assays. Amico *et al.* also reported a high incidence rate of clinical/subclinical AAMR in patients with low-level HLA-DSA prospectively, even though those patients were treated with anti-thymocyte globlin (ATG) and intravenous immunoglobulin administration (IVIG) [2]. Moreover, Lefaucheur *et al.* reported poor 8-year graft survival following a high incidence of AAMR among patients with pretransplant HLA-DSA detected by SAFB [4]. In contrast, Vlad *et al.* reported that low-level HLA-DSA detected by SAFB did not correlate to graft survival in their short-term follow-up period [5]. They concluded

that SAFB is too sensitive to exclude a transplant candidate from deceased- donor transplantation. However, they also recognized that SAFB is invaluable in predicting AAMR risk, confirming reports that support the relevance of SAFB. Once anti-HLA antibody bound to endothelium has caused transplant vasculopathy in a patient, it is difficult to control its progression [7,8], so the occurrence of AAMR is associated with a significantly decreased graft outcome [9].Therefore, desensitization for these patients with low-levels of HLA-DSA is now seen as being of considerable potential importance.

In our department, HLA-DSA is detected by only SAFB and not by complement-dependent cytotoxic crossmatch assay (CDC-XM), which is considered to be low level. SAFB-MFI of these low-level HLA-DSAs showed a range of more than 800 and less than 2500. Retrospective research shows that our patients with these low-level HLA-DSAs had surprisingly high rates of acute and chronic AMR. Consistent with other reports, our patients with HLA-DSA showed a high frequency of incidence of AAMR. We also report significant improvements in our transplant outcomes after implementing routine testing for SAFB and desensitization of those patients with positive test results. From these results, we speculated that low-level HLA-DSA detected by only SAFB should be eliminated as part of the treatment plan.

Materials and methods

All the study procedures were approved by the Ethics Committee of the Tokyo Women's Medical University (approved ID: 2516). Written informed consent was obtained from all the study participants, who were drawn from our kidney transplant program. The study was performed according to the principles of the Helsinki Declaration.

Patients

Before transplantation, we determined the sensitivity status of all patients using CDC-XM and flow cytometory crossmatch (FCXM) according to the manufacturer's protocol as previously reported [10–12]. Patients with a positive CDC-XM and/or T-cell FCXM were considered to be contra-indicated for receiving a transplant and were excluded from this study. Then, between 2001 and 2004, 152 cases of ABO-compatible kidney transplants were performed at our department. We retrospectively examined the preoperative serum of those patients using SAFB, and 24 patients showed a positive HLA-DSA despite their negative CDC-XM. We then retrospectively compared these weakly sensitized SAFB(+) patients (Group 1, $N = 24$) with SAFB(–) patients in the same period (Group 2, $N = 128$). After 2005, all of our transplant patients were examined using SAFB to evaluate their preoperative sensitized status. In 169 cases of ABO-compatible transplantation performed between 2005 and 2009, 52 patients were detected with the presence of HLA-DSA (Group3, $N = 52$), whereas 117 patients were not (Group4, $N = 117$). We considered those patients with low-level HLA-DSA, which was defined as CDC(–)/SAFB(+), to be suitable candidates for desensitization. These patients were treated with a single-dose administration of rituximab (RIT) and three or four sessions of double-filtration plasmapheresis (DFPP) preoperatively for desensitization. The patients' background is shown in Table 1.

Detection of anti-donor HLA antibodies

The FCXM was measured using FACS-Caliber (Becton Dickinson, CA, USA), and a positive FCXM was defined as a mean channel shift >10. FCXM was also performed on day-1 to evaluate the desensitization status and confirm negative conversion. Examinations using Pronase were conducted after administration of RIT. SAFB has been in use at our center since 2005; thus, the HLA-DSA levels of recipients receiving transplantation before 2005 were analyzed retrospectively using serum stored at −80 °C as previously reported [11]. Briefly, 20 μl of sera were added to 5 μl of class I or class II antigen beads; the beads were 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. Luminescence was read on a LABScreen^{TM} 100 Luminex system (One Lambda Inc., Canoga Park, CA, USA). Data were analyzed using LABScreen analysis software HLA Fusion 2.0 (One Lambda), and mean fluorointensity (MFI) over 800 was considered as positive. In this study cohort, no recipients had an MFI over 2500. Therefore, we defined these patients as weakly sensitized recipients. We also examined their status using SAFB during the postoperative follow-up period. Polymerase chain reaction-sequence specific primer (PCR-SSP) technology (low and high resolution Olerup SSP Geno Vision VertriebsmbH kits, Vienna, Austria) was used for HLA class I and class II DNA typing.

Immunosuppressant and desensitization protocol

All of the patients received a triple immunosuppressive protocol consisting of tacrolimus or cyclosporin, mizoribin or mycophenolate mofetil, and methylprednisolone (MP) from 1 week before surgery. Basiliximab was administered intravenously at the dose of 20 mg on day 0 and day 4.

Table 1. Patients' background.

	2001–2004		2005–2009		
	Group 1 SPA(+) *N* = 24	Group 2 SPA(–) *N* = 128	Group 3 SPA(+) *N* = 52	Group 4 SPA(–) *N* = 117	*P*-value
Recipient age	43.0 ± 11.6	38.7 ± 13.3	40.6 ± 12.8	43.8 ± 12.7	0.019
Recipient sex, *N* (%)					
Male	18 (75.0%)	85 (66.4%)	30 (57.7%)	84 (71.8%)	0.266
Female	6 (25.0%)	43 (33.6%)	22 (42.3%)	33 (28.2%)	
Duration of HD, months	74.2 ± 71.1	49.2 ± 43.4	57.7 ± 63.1	50.7 ± 55.0	0.173
WIT	5.8 ± 3.0	6.3 ± 8.5	4.3 ± 1.0	4.4 ± 1.2	0.032
TIT	91.5 ± 21.3	93.5 ± 34.7	111.1 ± 25.6	111.1 ± 40.5	<0.001
Pregnancy	4 (16.7%)	14 (10.9%)	15 (28.8%)	17 (14.5%)	0.027
Blood transfusion	8 (33.3%)	41 (32.0%)	20 (38.5%)	32 (27.4%)	0.540
No. of KTx, *N* (%)					
First	23 (95.8%)	121 (94.5%)	46 (88.5%)	112 (95.7%)	0.268
Second	1 (4.2%)	6 (4.7%)	3 (5.8%)	5 (4.3%)	
Third	0 (0.0%)	1 (0.8%)	3 (5.8%)	0 (0.0%)	
Donor age (range)	55.1 ± 11.5	56 ± 8.8	56.5 ± 9.9	57.8 ± 10.0	0.433
Donor gender, *N* (%)					
Male	7 (29.2%)	39 (30.5%)	23 (44.2%)	40 (34.2%)	0.335
Female	17 (70.8%)	89 (69.5%)	29 (55.8%)	77 (65.8%)	
HLA- AB mismatches (*N* of 0/1/2/3/4)	2.3 ± 1.0 (0/5/11/3/5)	1.7 ± 0.9 (12/42/56/12/6)	1.9 ± 0.8 (0/17/23/10/2)	1.6 ± 1.1 (19/33/42/16/7)	0.007
HLA- DR mismatches (*N* of 0/1/2)	1.0 ± 0.5 (3/17/4)	0.9 ± 0.6 (34/78/16)	1.0 ± 0.6 (10/33/9)	0.8 ± 0.7 (36/64/17)	0.480
ABO-blood type matched, *N* (%)	18 (75.0%)	96 (75.0%)	44 (84.6%)	86 (73.5%)	0.458
ABO-blood type mismatched, *N* (%)					
A to AB	1 (4.2%)	13 (10.2%)	1 (1.9%)	15 (12.8%)	0.445
B to AB	2 (8.3%)	9 (7.0%)	2 (3.8%)	7 (6.0%)	
O to A/B/AB	3 (12.5%)	10 (7.8%)	5 (9.6%)	9 (7.7%)	
CDC-XM					
T-cell positive, *N* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–
B-cell positive, *N* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–
FCXM					
T-cell positive, *N* (%)	0 (0.0%)	NA	0 (0.0%)	NA	–
B-cell positive, *N* (%)	6 (25.0%)	NA	18 (34.6%)	NA	0.402
SAFB					
Number of HLA-DSA (*N* with 1/2/3/4)	1.0 ± 0.5 (10/7/6/1)	no positive reaction	1.0 ± 0.6 (42/9/1/0)	no positive reaction	<0.001
Class of HLA-DSA					
Class I, *n* (%)	19 (79.2%)	0 (0.0%)	36 (69.2%)	0 (0.0%)	0.368
Cass II, *n* (%)	12 (50.0%)	0 (0.0%)	24 (46.2%)	0 (0.0%)	0.755
Class I + II, *n* (%)	7 (29.2%)	0 (0.0%)	8 (15.4%)	0 (0.0%)	0.161
Immunosuppression					
Tacrolimus	24 (100.0%)	117 (91.4%)	52 (100.0%)	114 (97.4%)	0.020
Cyclosporin	0 (0.0%)	11 (8.6%)	0 (0.0%)	3 (2.6%)	
MZR	0 (0.0%)	16 (12.5%)	0 (0.0%)	10 (8.5%)	0.018
MMF	24 (100.0%)	112 (87.5%)	52 (100.0%)	107 (91.5%)	
RIT	0 (0.0%)	0 (0.0%)	52 (100.0%)	0 (0.0%)	–
DFPP	11 (45.8%)	19 (14.8%)	52 (100.0%)	20 (17.1%)	<0.001
Follow-up period (month)	101 ± 18	106 ± 16	54 ± 15	49 ± 20	<0.001

KTx, Kidney transplantation; HD, hemodialysis; WIT, warm ischemic time; TIT, total ischemic time; CDC-XM, complement-dependent cytotoxicity crossmatch; FCXM, flow cytometory crossmatch; SAFB, single-antigen flow-bead; MZR, Mizoribin; MMF, Mycophenolate mofetil; RIT, Rituximab; DFPP, double-filtration plasmapheresis.

P-values were calculated among four groups by omnibus test.

Three or four sessions of DFPP were performed before transplantation for patients with history of sensitization according to a previously reported protocol, which we used for ABO-incompatible transplantation [11]. Occasionally, some patients also received preoperative DFPP to prevent recurrence of their original kidney diseases. In the Group 3 patients, DFPP was routinely undergone, although the durations, exchanged plasma volumes, and number of DFPP sessions in these patients were not different from those of the other groups. RIT was also administered at a single dose (200 mg) within 7 days prior to transplantation in the Group 3 patients. In all these cases, the population of CD19 cells in peripheral blood was decreased immediately to less than 1% and repeated administration of RIT was not needed at all. ATG was never used for induction in this study.

Diagnosis of rejection

Protocol biopsy was planned to take place within 6 months (early phase) and around 1 year (late phase) post-transplantation. Patients with complications, perirenal infection, or a tendency to bleed were excluded. Each time rejection was suspected, episode biopsy was performed. Two or three core biopsy samples were obtained using a spring-loaded 16-gauge biopsy gun under ultrasound guidance. The type of rejection was classified according to the Banff'07 criteria. Briefly, AAMR was defined as (i) diffuse C4d deposition in the peritubular capillaries (>50%); (ii) serologic evidence of circulating antibodies to donor HLA and so on; and (iii) morphologic evidence of acute tissue injuries such as capillary and/or glomerular inflammation (ptc/g>0) and/or thromboses. Cases with v3 were categorized in TMR III in this study. Chronic active AMR (CAMR) was defined by C4d+, presence of circulating anti-donor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours, and/or peritubular capillary basement membrane multilayering, and/or interstitial fibrosis/tubular atrophy, and/or fibrous intimal thickening in arteries. C4d staining in peritubular capillaries was routinely performed: cryostat sections were stained through indirect immunofluorescence using a mouse monoclonal anti-human C4d antibody (1:40; Quidel, San Diego, CA, USA), followed by fluoresceinisothiocyanate (FITC)-conjugated goat anti-mouse IgG. All patients with acute rejection including both TMR and AMR received bolus MP 500 mg intravenously for 2 days. When patients were diagnosed as AAMR or CAMR, they were treated using plasma exchange and/or DFPP to remove DSA (11 patients of Group 1, five patients of Group 2, four patients of Group 3, and one patient of Group 4).

Diagnosis of adverse events

Monitoring for cytomegalovirus (CMV) infection was performed using CMV pp65 C10/C11 antigenemia assay to detect CMV viremia. CMV infection and disease were defined according to previously published criteria [13].

The diagnosis of BK virus infection was based on histopathologic examination of renal biopsy specimens and detection of BK virus DNA in plasma and urine using polymerase chain reaction assay. When decoy cells were detected on urine cytology in outpatients, we checked for BK virus DNA in plasma and urine. Values of more than 10^7 BK virus copies per milliliter obtained repeatedly were considered indicative of BK virus infection. Moreover, renal biopsy was performed with immunohistochemical staining for simian virus 40 large T antigen.

Leukopenia was defined in our study as grade 2 (2000–3000/mm^2) or more severe (−2000/mm^2) according to National Cancer Institute-Common Toxicity Criteria.

Statistical analysis

All the statistical analyses were performed using the JMP 8.0.1 software (SAS Institute, Cary, NC, USA). Quantitative parameters were compared using the unpaired two-sample *t* test and Mann–Whiteney test; qualitative parameters were compared using the chi-square test. The *P*-values of less than 0.05 were considered to indicate statistical significance. Graft survivals were calculated using the Kaplan–Meier method.

Results

Patients' population and background

The background of the patients is shown in Table 1. Based on the different study periods, there is a significant difference in the length of the follow-up for Group 1/2 and Group 3/4. However, even in the later period, all of the patients were followed up for at least 3 years, so we considered the early and mid-term outcomes of these cohorts to be comparable. The duration of pretransplant hemodialysis was longer in Group 1 patients than those of the other groups, although there was no statistical significance.

In our department, HLA typing of HLA-AB and DR is routinely conducted, but the information on HLA-Cw and DP is not available, so only anti-HLA-AB or DR antibodies detected by SAFB were considered as HLA-DSA. The number of HLA-DSA types varied in both Group 1 and Group 3, although patients with only one type of HLA-DSA were in the majority in each group. This accords with their weakly sensitized status as predicted from the results of their negative cytotoxic assays.

Table 2. Incidence rate of biopsy proven rejection.

	2001–2004			2005–2009		
	Group 1 SPA(+) *N* = 24	Group 2 SPA(–) *N* = 128	*P*-value	Group 3 SPA(+) *N* = 52	Group 4 SPA(–) *N* = 117	*P*-value
Early phase						
No rejection	7 (29.2%)	89 (69.5%)	<0.001	46 (88.5%)	78 (66.7%)	0.003
BL	3 (12.5%)	22 (17.2%)	0.570	3 (5.8%)	15 (12.8%)	0.170
TMR I/II	7 (29.2%)	11 (8.6%)	0.004	1 (1.9%)	15 (12.8%)	0.026
TMR III	1 (4.2%)	1 (0.8%)	0.182	0 (0.0%)	1 (0.9%)	0.504
AAMR	6 (25.0%)	5 (3.9%)	<0.001	2 (3.8%)	8 (6.8%)	0.447
g score	1.0 ± 1.1	1.0 ± 1.2	>0.999	2.0 ± 1.4	1.1 ± 1.1	0.348
ptc score	1.8 ± 0.8	1.6 ± 0.9	0.705	2.0 ± 0.0	1.5 ± 1.1	0.556
Late phase						
No rejection	8 (33.3%)	92 (71.9%)	<0.001	33 (63.5%)	77 (65.8%)	0.767
IF/TA	5 (20.8%)	27 (21.1%)	0.977	14 (26.9%)	33 (28.2%)	0.864
CTMR	3 (12.5%)	1 (0.8%)	0.001	1 (1.9%)	3 (2.6%)	0.800
CAMR	8 (33.3%)	8 (6.3%)	<0.001	4 (7.7%)	4 (3.4%)	0.227
cg score	2.0 ± 0.8	1.5 ± 1.0	0.288	1.5 ± 0.6	1.3 ± 1.3	0.789

Early phase, within 6 months after surgery; Late phase, more than 6 months after surgery.
BL, Borderline change; TMR, T-cell mediated rejection; IF/TA, interstitial fibrosis and tubular atrophy; CTMR, chronic T-cell mediated rejection.
AAMR, Acute antibody-mediated rejection *All cases of AAMR were Type II.
CAMR, chronic active antibody-mediated rejection *There were no cases of CAMR in the early phase.
g/ptc/cg score: mean values of Banff score in the patients with AAMR/CAMR.
P-values were calculated using chi-square test and unpaired *t* test.

Incidence of biopsy proven rejection

Between 2001 and 2004, a high incidence rate of AAMR followed by a high incidence of CAMR was shown in the SAFB(+) patient group compared with those of the SAFB(–) patients in the same period (Table 2). According to this result, we decided to implement RIT/DFPP induction for patients with positive SAFB, even though their cytotoxic assays were negative. After implementing our new strategy, the incidence rate of AAMR and CAMR in the SAFB(+) patients significantly decreased (AAMR: from 25.0% to 3.8%,$P = 0.005$; CAMR: from 33.3% to 7.7%, $P = 0.004$, respectively), and the difference between the AAMR and CAMR rates of the SAFB(+) patients and the SAFB(–) patients disappeared later on. Interestingly, the incidence rate of TMR also decreased after implementing our new strategy (from 33.3% in Group 1 to 1.9% in Group 3, $P < 0.001$).

Anti-HLA antibody after kidney transplantation

During the follow-up period, postoperative SAFB was examined. Positive rates of HLA-DSA in each follow-up year are shown in Fig. 1. Positive rates of HLA-DSA in Group 1 and in Group 3 were 36.4% and 28.8% after 1 year ($P = 0.616$), 45.5% and 30.6% after 2 years ($P = 0.345$), 52.9% and 16.2% after 3 years ($P = 0.005$), and 63.6% and 18.2% after 4 years ($P = 0.009$), respectively. Reduction in a positive rate of HLA-DSA was observed in 71.2% patients after 1 year in the RIT-treated patients, in contrast with being observed in only 63.3% patients without RIT treatment. This depletion of HLA-DSA was also observed long-term in the Group 3 patients, even though high HLA-DSA positive rates continued in the Group 1 patients. Once we observed a recurrence of HLA-DSA, we usually increased the dosage of MMF to eliminate HLA-DSA. When clinical CAMR was diagnosed, tighter management, such as intermittent DFPP, additional administration of RIT, and splenectomy, was required.

Adverse events

To evaluate adverse effects of our induction therapy, we compared the incidence of infectious disease and leukopenia between the Group 1 (untreated patients) and the Group 3 (RIT-treated patients) (Table 3). There were no tuberculosis and EBV infections in these two groups. Only one patient in Group 3 showed BK virus infection, although this patient easily recovered after reduction in MMF. The incidence rates of Cytomegalovirus (CMV) viremia and infection were higher in Group 1 than those in Group 3, although the preoperative status of CMV was not significantly different between the two groups

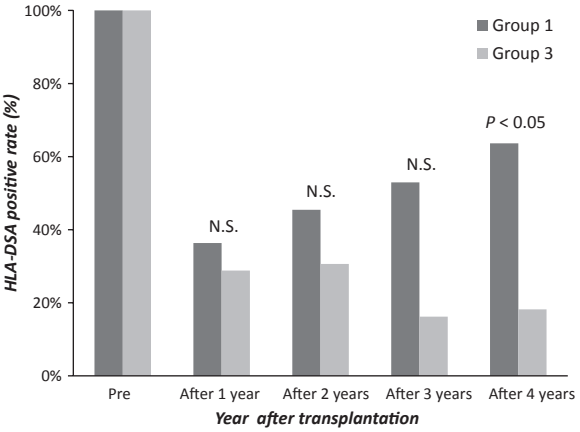

Figure 1 Positive rate of HLA-DSA after kidney transplantation. Positive rates of HLA-DSA detected by SAFB at every follow-up year are shown. *P*-values were calculated by the chi-square test. HLA-DSA: donor-specific anti-donor HLA antibody.

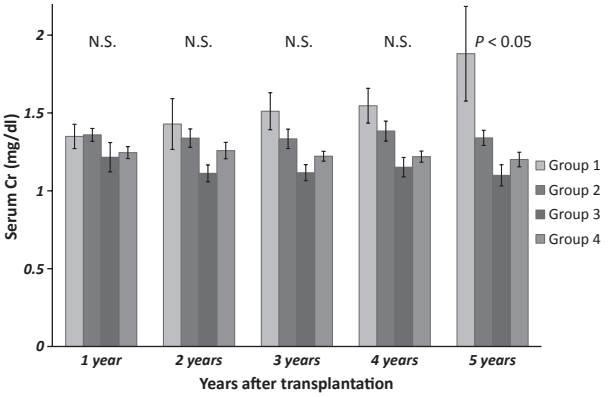

Figure 2 Post-transplant graft function. The mean serum creatinine concentrations of each group in every follow-up year are shown. The *P*-value was calculated by nonparametric one-way ANOVA.

(combination of a CMV positive donor and a CMV negative recipient occurred in four pairs of Group 1 patients (16.7%) and in seven pairs in the desensitized group (13.5%), $P = 0.712$), and no preoperative prophylaxis against CMV infection was administered to any of the patients. CMV infections (one gastritis, one hepatitis, and two CMV fevers occurred in Group 1 patients and one gastritis and two CMV fevers occurred in Group 3) were treated by intravenous injection of ganciclovir, reduction in the MMF dose, or occasionally by intravenous γ-globulin therapy.

The frequency of leukopenia observed among both groups was not significantly different. Leukopenia improved with reduction in the MMF dose or administration of G-CSF in both groups.

Graft function and survival

No delayed graft function was observed in this study. The mean serum creatinine concentrations of each group were almost same within 4 years post-transplantation, although patients in Group 1 showed significantly higher Cr levels than those in the other groups in year 5 after transplantation (Group 1/Group 2/Group 3/Group 4(mg/dl); after 1 year: 1.35 ± 0.08, $n = 24/1.36 \pm 0.04$, $n = 128/1.21 \pm 0.09$, $n = 52/1.24 \pm 0.04$, $n = 111$, $P = 0.169$; after 2 years: 1.43 ± 0.16, $n = 24/1.34 \pm 0.06$, $n = 127/1.11 \pm 0.05$, $n = 50/1.26 \pm 0.05$, $n = 86$, $P = 0.094$; after 3 years: 1.51 ± 0.12, $n = 23/1.33 \pm 0.06$, $n = 126/1.11 \pm 0.05$, $n = 35/1.22 \pm 0.03$, $n = 68$, $P = 0.219$; after 4 years: 1.54 ± 0.11, $n = 21/1.38 \pm 0.06$, $n = 123/1.15 \pm 0.06$, $n = 25/1.22 \pm 0.04$, $n = 42$, $P = 0.457$; and after 5 years: 1.88 ± 0.30, $n = 21/1.34 \pm 0.05$, $n = 117/1.10 \pm 0.07$, $n = 12/1.20 \pm 0.05$, $n = 22$, $P = 0.025$; Fig. 2). Ahead of this increase in Cr level, patients in Group 1 showed more frequent detection of moderate or severe proteinuria compared with the other groups. This had already became significant in year-3 after transplantation (16.7% in Group1, 8.3% in Group 2, 7.7% in Group 3, and 2.6% in Group 4 patients, $P = 0.035$).

Five patients in Group 2 and five patients in Group 4 died with a functioning graft. All cases with acute rejection were rescued, whereas six cases in Group 1 and seven cases in Group 2 had graft failure because of the development of chronic rejection. One patient in Group 2 lost

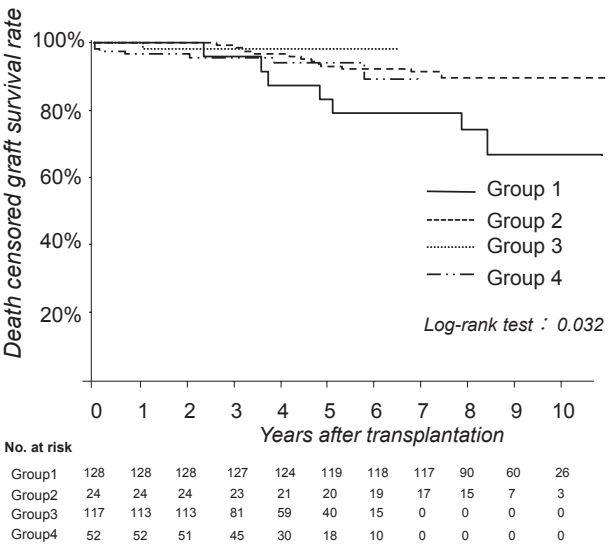

Figure 3 Kaplan–Meier Estimates (Graft Survival). The Kaplan–Meier curve showed a poorer graft survival rate in Group 1 compared with the other groups ($P = 0.032$). The *P*-value was calculated using the Log-rank test.

Table 3. Adverse events compared between two groups.

	Group 1 $N = 24$	Group 3 $N = 52$	*P*-value
CMV viremia	5 (20.8%)	3 (5.8%)	0.047
CMV infection	4 (16.7%)	3 (5.8%)	0.127
BK virus infection	0 (0.0%)	1 (1.9%)	0.494
Leukopenia	4 (16.7%)	12 (23.1%)	0.524

CMV, Cytomegalovirus.
P-values were calculated using chi-square test.

his graft because of noncompliance. Only one graft failure occurred in Group 3, and was also caused by noncompliance. In Group 4, two patients had graft failure, with one being caused by recurrence of IgA nephropathy and the other because of BK nephropathy.

The graft survival curve is shown in Fig. 3. The 5-year graft survival rate was 83.3% in Group 1, 93.0% in Group 2, 98.1% in Group 3, and 94.2% in Group 4, respectively. The graft survival rate of Group 1 was significantly poorer than the other groups ($P = 0.032$). Despite their positive HLA-DSA, the graft survival rate of patients in Group 3 was not different from that of the Group 4 patients, who did not have any pre-existing HLA-DSA.

Discussion

We previously reported on the impact of employing a B-cell depletion strategy for prevention of CAMR [14]. In the previous study, CAMR was found in 28.9% of cases of ABO-compatible kidney transplantation (ABO-C). In contrast, it was hardly observed in the cases of ABO-incompatible transplantation (ABO-I) in the same period, which were transplanted after receiving B-cell depletion therapy to eliminate anti-blood-type antigen antibodies, especially in the group treated by RIT (splenectomy group, 8.8%; RIT group, 3.5%). In this study, B-cell depletion therapy was also indicated for HLA-DSA(+) patients in the same manner as for the ABO-I patients. The incidence rate of CAMR in the RIT-treated group was as low as that in ABO-I cases in the same period, and it also became as same as that of the nonsensitized group. At the same time, it is noticeable that the incidence of CAMR in the SAFB(-)/ABO-C transplantation patients in this study was far lower (6.3% in 2001–2004 and 3.4% in 2005–2009) than in our previously reported results with ABO-C patients (28.9%), which might include SAFB(+) patients. This means that it is important to use SAFB to identify those patients who require B-cell depletion therapy. So, we consider that our strategy of identifying weakly sensitized recipients with SAFB and treating them by low-dose RIT is justified and valuable.

Lefaucheur *et al.* reported that 34.7% of the transplant candidates in 502 patients registered on the waiting list for deceased-donor kidney transplantation at their institution had antibodies against class I or class II HLA in at least one pretransplantation serum [15]. Highly sensitized recipients such as CDC-XM(+) or FCXM-T(+) were already excluded from our study cohort, so a positive rate of HLA-DSA in our study was comparable to their reports (24.1%; 15.8% in 2001–2004 and 30.8% in 2005–2009). Remarkably, 11 patients in Group 1 (45.8%) and 22 patients in Group 2 (42.3%) did not have any sensitizing history despite their positive SAFB. This means that it is difficult to predict the existence of HLA-DSA from patients' history only. One compelling reason for this observation is that these antibodies are occurring naturally with crossreactivity to epitopes on some HLA molecules, and are developed by stimulation of microbial and colonial antigens [16]. The clinical significance of natural anti-HLA antibodies has yet to be determined. One more concern is the problem of false positives of SAFB. It is now well known that during the production process of SAFB, HLA molecules may be denatured, leading to false positive SAFB results [17]. Fortunately, most of these technology-related false positive results occur with SAFB carrying rather infrequent HLA molecules limiting the clinical relevance of this issue [17,18]. Finally, the values of additional qualitative and quantitative parameters are currently required [19–21].

For these reasons, it is possible that we may be overtreating these weakly sensitizing recipients using intensive immunosuppression. However, our induction therapy showed a low incidence rate of CMV infection, as has been frequently observed in ATG-based induction regimens [22]. Although development of CMV infection was reported after RIT treatment for autologous hematological stem cell transplantation [23] and postkidney transplant lymphoproliferative disorder [24], there are many reports that have demonstrated the safety of RIT with infection rates similar to those seen in kidney transplant recipients who did not receive RIT [25–27]. In our data, the incidence of CMV infection is paradoxically less in RIT/DFPP-induction patients than in those who did not receive RIT (Group 1), although we never administered any prophylaxis. This might be because the administration of steroids for treatment of rejection was more frequently indicated in Group 1 than in Group 3, RIT/DFPP-induction group. Likewise, in our previous study, increases in infection with the BK virus or reactivation of HBV after RIT induction were also not observed [28]. So, we consider our induction regimen is safe and suitable for weakly sensitized recipients.

In this study, improvement in acute phase rejection in the later period was observed in not only humoral

rejection but also in TMR. Because the incidence levels of TMR and AAMR were almost the same in the SAFB(–) patients in 2001–2004 and in 2005–2009, these improvements in rejection cannot be wholly explained by the difference in historical background. Actually, the incidence of TMR in Group 3 patients was lower than in Group 4 patients transplanted in the same period. Consistent with our results, Tyden *et al.* reported improvement in cellular rejection in their RCT of RIT-induction therapy patients [29]. It is possible that RIT could be preventing TMR by elimination of B-cells acting as antigen-presenting cells. Our results show that RIT/DFPP induction significantly reduced the incidence of AAMR in Group 3 compared to Group 1, confirming other investigators' reports [25,30–33]. Although RIT is not able to deplete plasma cells, which are producing antibodies, RIT is able to prevent AAMR by depletion of memory B-cells that are necessary to AAMR as a source of antibody-producing plasma cells [30]. Furthermore, DFPP which was routinely undertaken in this group is well known as a preventative of AAMR. Stegall *et al.* reported the importance of reducing the DSA titer by preoperative multiple plasmapheresis (PP) for successful desensitization [34]. Overall, the combination of RIT with DFPP achieved excellent prevention of acute rejection.

Furthermore, in the long-term follow-up, the incidence of CAMR was effectively prevented in those with RIT/DFPP induction compared with the group without desensitization (7.7% vs. 33.3%). It is considered that this improvement in the incidence rate of CAMR is correlated with the reduction in postoperative HLA-DSA observed in the RIT/DFPP-treated group, and resulted in better graft function and graft survival in this group than those in the untreated group. It is well known that chronic rejection is related to HLA-DSA. Terasaki and Cai [35] reported that DSA was found in 86% of patients before graft failure, and patients could retain good graft function in the long-term if no DSA existed [36,37]. Therefore, maintaining the reduction in DSA is considered to be crucially important for long-term good graft function and survival. Gloor *et al.* reported that reduction in DSA was observed 4 months after transplantation in crossmatch positive recipients desensitized with RIT/IVIG/PP [38]. Loupy *et al.* also reported the reduction in DSA 1 year after transplantation and less incidence of CAMR in sensitized recipients desensitized with combination RIT/IVIG/PP [31]. Although almost all of our recipients were not 'highly' sensitized compared to in their reports, longer term depletion of DSA than in those reports was observed in our induction therapy with RIT/DFPP.

Although such a long-term depletion of postoperative HLA-DSA may be because of prevention of acute rejection by RIT/DFPP-induction therapy, one more hypothesis is that RIT itself prevents rerising pre-existent DSA. Chengyang *et al.* speculate that a preponderance of immature and transitional B-cells in the reconstituting B-cell compartment after RIT treatment may contribute to a tolerogenic effect in the islet transplantation of nonhuman primates [39]. Indeed, in human kidney transplantation, Kopchaliiska *et al.* showed the majority of the recovering cells had the phenotype of transitional CD38+ B-cells and the percentages of mature, memory CD27+ B-cells remained significantly depressed after RIT desensitization [40]. Actually, we also confirmed that repopulation of B-cells after RIT treatment was started from CD19 + CD24 + CD38 + transitional B-cells (data not shown). It is possible that the long-term depletion of DSA in this study would be followed by these transitional B-cells induced by RIT treatment. More long-term follow-up and analysis of repopulated cells are required.

In this study, only four patients in the RIT/DFPP group progressed to CAMR. Two of them were second-transplantation cases, and one other patient was a third-transplantation case. Their preoperative HLA-DSA was detected only by SAFB, although they had many types of anti-HLA antibody against a third-party. Desensitization with our RIT/DFPP-induction regime is considered to be insufficient for such highly sensitized cases. More intensive desensitization, such as IVIG, bortezomib, and eculizumab, is considered to be more appropriate for these cases [21,41].

The major limitation is that this study was not designed as a randomized-controlled trial, and contained a retrospective historical control group. The difference of follow-up duration between the two groups is a major concern. The higher rate of chronic rejection observed in Group 1 might be because of the longer follow-up time of this group. However, 66.7% of CAMR patients were diagnosed around 1 year after transplantation and the other cases were diagnosed within 3 years after transplantation. So, we considered that the follow-up period of the patients transplanted in latter period (32 months–60 months, median 43 months) was enough to evaluate the rate of CAMR. Indeed, the median times of the biopsy dates of each period were not significantly different (567.5 ± 126.7 days after surgery in 2001–2004, and 528.2 ± 45.6 days after surgery in 2005–2009, $P = 0.641$). Furthermore, the incidence of CAMR was not significantly different between Group 2 and Group 4. It means that this improvement in the rate of CAMR is not simply based on the difference in the period. Nevertheless, it is not possible to be ignored the fact that SAFB(+) patients in the latter period were managed more tightly than the other patients, because we had already recognized those patients as being at high risk, and it contributed their

favorable outcome. In addition, there are several statistical differences among the four study groups shown in the patients' background. It is undeniable that poorer graft survival in Group 1 was related to those differences, such as older recipient age, longer HD duration, and a larger degree of HLA mismatches.

As described above, successful transplantation of highly sensitized recipients has already been reported [25,30–32,34,38]. Although our historical control group was not highly sensitized compared to their reports, the incidence rate of AAMR in this group was surprisingly high. Even at low-DSA level, it is likely to encourage memory B-cell repopulation and foster the development of acute and chronic rejection. This is the first report to have shown the benefit of desensitization for patients with low-level DSA. On the basis of our findings, we are now considering initiating a randomized-controlled trial in our department.

Authorship

TH: participated in the writing of the paper. NK: participated in the performance of the research. KO: participated in the performance of the research. HI: participated in the performance of the research. KT: participated in research design.

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