Clearance of Intra-graft Donor Specific Anti-HLA Antibodies in the Early Stage of Antibody-Mediated Rejection Following Rituximab and Apheresis Therapy in Renal Transplantation

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ABSTRACT

Background. The management of acute or, in particular, chronic antibody-mediated rejection (AMR) resulting from donor-specific HLA antibodies (DSA) is a critical barrier to obtaining better long-term graft survival. To ascertain the efficacy of anti-AMR therapies, the transition of intra-graft DSA (g-DSA) was assessed.

Methods. Allograft biopsy specimens were analyzed by graft immunocomplex capture fluorescence analysis, as previously described. One hundred recipients who underwent graft biopsies between April 2016 and December 2017 were enrolled for this study. Fifteen recipients diagnosed with g-DSA positive (+) received anti-humoral treatments and underwent follow-up biopsies. g-DSA levels were assessed again by a follow-up biopsy at 6–12 months following the treatments.

Results. With anti-humoral treatments, 9 out of 15 recipients comprised a g-DSA negative (-) (3.59 ± 2.82-.58 ± .25): g-DSA6-12- group, while the remaining 6 recipients comprised a g-DSA +(20.6 ± 17.0-14.9 ± 14.1): g-DSA6-12+ group. The initial g-DSA scores were significantly higher in the g-DSA6-12+ group ($P = .01$). All samples were diagnosed as chronic AMR in the g-DSA+ groups, whereas there were 3 chronic AMR, 4 acute AMR, and 2 incomplete AMR samples in the g-DSA- group. Interestingly, the frequency of responsible DSA belonging to class II tended to be higher in the g-DSA6-12+ group (4/6) compared to the g-DSA6-12- group (2/9) ($P = .14$).

Conclusion. These results imply that chronic exposure to DSA causes significant and irreversible damage to the allograft. Timely and adequate anti-humoral intervention might reverse the early phase of AMR with complete clearance of g-DSA.

ANTIBODY-MEDIATED rejection (AMR) due to anti-HLA antibodies seems to be a considerable impediment to achieving an excellent long-term outcome in organ transplantation [1]. Generally, detection of donor-specific anti-HLA antibodies (DSA) in serum (s-DSA) is a prerequisite for confirming the diagnosis of AMR [2–5]. However, we previously described a graft immunocomplex capture fluorescence analysis (ICFA) technique to detect intra-allograft DSA (g-DSA) [6,7]. This novel approach has possibly contributed to accurate diagnoses and improved AMR outcomes in organ transplantation by means of monitoring g-DSA regularly as a sensitive marker for AMR. Thus, it is clearly important to review the significance and outcomes in g-DSA positive (+) patients. However, there

This study was funded by Wakunaga Pharmaceutical (grant no. 29collaboration004) and JSPS KAKENHI (grant no. 17K10518).

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0041-1345/19
https://doi.org/10.1016/j.transproceed.2019.01.126

are only a few reports discussing the treatment and outcomes of g-DSA+ recipients, especially cases diagnosed as chronic active AMR [8,9]. Thus, it is worthwhile to investigate the effectiveness of anti-humoral treatment with regard to g-DSA. Whether liver allografts protect kidney allografts in the context of liver and kidney transplantation continues to be debated [10–12]. Regarding AMR, the basis of this phenomenon seems to be that a large proportion of DSA is adsorbed in the liver. However, there was no precise report that visualized DSA deposition in the liver in a patient who had undergone a liver and kidney transplantation. To investigate whether a liver allograft protects a subsequent kidney allograft, we assessed a non-simultaneous liver and kidney transplant recipient with the presence of s-DSA. The influences of DSA on liver and kidney allograft from the same donor in a non-simultaneous liver and kidney transplant recipient are clearly relevant to understanding how DSA causes allograft injury.

In this report, we will present outcomes of anti-humoral treatment for AMR with graft ICFA results in renal transplant recipients and a case of non-simultaneous liver and kidney transplantations.

MATERIAL AND METHODS

Patients

A total of 100 Japanese renal transplant recipients were included in this study. They underwent graft biopsies and were examined by graft ICFA, as previously described [6,7]. According to the results of the graft ICFA, these patients were divided into g-DSA+ and g-DSA negative (-) groups. The regimens of anti-humoral treatments for 15 recipients are shown in Table 1. Ethics Committee approval was obtained from the internal research ethics committee of our university. The clinical trial registration number is UMIN000023787.

Single Antigen Bead Assay

A single antigen bead assay was performed based on the manufacturer's protocol and our previous report [6]. LABScreen Singles Antigen HLA Class I/II beads (One Lambda, Canoga Park, Calif, United States) were utilized in this research.

Graft Immunocomplex Capture Fluorescence Analysis

Graft ICFA was performed as previously described by Nakamura et al [6,7] (WAKFlow HLA antibody classes I and II, Wakunaga Pharmaceutical, Osaka, Japan).

Data Interpretation

The median fluorescent intensity (MFI) of the samples was evaluated by using the Luminex system (Luminex Corporation, Austin, Tex, United States). The ratio of sample MFI/blank beads MFI $\geq$1.0 was considered a positive result [6,7].

Histopathology

The allografts were biopsied and tissues were fixed in 10% buffered formaldehyde, paraffin-embedded, sectioned. In order to assess histopathologic lesions, samples were stained with hematoxylin eosin, C4d, Masson's trichrome, periodic acid-Schiff, and periodic acid-methenamine silver. Allograft histopathologic changes were evaluated based on the Banff 2013 classification. For immunohistochemistry, samples were blocked by using Blocking 1 solution (Nakarai-Tesque, Kyoto, Japan) and then reacted with anti-blood group antigen A (T36) (GeneTex, Irvine, Calif, United States), followed by reaction with a horseradish peroxidase polymer-conjugated system and 3,3'-diaminobenzidine.

Table 1. Anti-humoral Treatment Regimens for g-DSA+ Recipients

Patients		DSA Classes		Graft ICFA		Anti-humoral Treatments				
g-DSA6-12+	AMR type	Class I	Class II	Pre g-DSA	Post g-DSA	Reinforcement of IS	Rit (200–300 mg)	Steroid Pulse	IVIG	DFPP/PE
1	Chronic	-	+	39.0	41.1	+	+	-	-	+
2	Chronic	+	-	41.7	19.2	+	+	-	-	+
3	Chronic	-	+	6.1	5.8	+	+	-	-	+
4	Chronic	+	-	24.4	11.9	+	+	-	-	+
5	Chronic	-	+	8.2	1.7	+	+	+	-	+
6	Chronic	-	+	3.9	9.5	+	+	-	-	+
Mean				20.6	14.9					
SD				17.0	14.1					
g-DSA6-12-										
1	Acute	+	-	3.4	0.9	+	+	-	-	+
2	Incomplete	-	+	1.5	0.9	+	-	-	-	-
3	Acute	+	-	1.1	0.2	+	+	-	-	+
4	Acute	+	-	10.0	0.8	+	+	+	-	+
5	Chronic	-	+	1.5	0.6	+	+	-	-	+
6	Acute	+	-	2.0	0.3	+	-	+	-	-
7	Chronic	+	-	4.6	0.5	+	+	-	-	+
8	Incomplete	+	-	2.8	0.5	+	-	-	-	-
9	Chronic	+	-	5.4	0.5	+	+	+	-	+
Mean				3.59	0.58					
SD				2.82	0.25					

Abbreviations: AMR, antibody mediated rejection; DFPP, double-filtration plasmapheresis; IS, immunosuppression; IVIG, intravenous immunoglobulin; PE, plasma exchange; Rit, rituximab; SD, standard deviation.

Statistical Analysis

The student's t-test was applied for analysing the means of continuous values. P values <.05 were considered statistically significant. All statistical evaluations were performed using GraphPad Prism 6 (GraphPad Software, San Diego, Calif, United States).

RESULTS

Study Population

This study included 100 renal transplant recipients who underwent graft biopsies between April 2016 and December 2017. Their graft biopsies and serum obtained on the same day were collected and analyzed. The prevalence of g-DSA detected by graft ICFA was 17% (17/100) in renal transplant recipients. Of this population, s-DSA was detected in 15 out of 17 recipients. Fifteen recipients (13/15 s-DSA+) were treated with anti-humoral therapies and underwent follow-up graft biopsies.

The Existence of g-DSA Reflects Microvascular Lesions in Allografts

To ascertain whether or not graft ICFA results truly indicate AMR, the correlations between g-DSA and well-known microvascular lesion scores were investigated using the glomerulitis (g), glomerular basement membrane double contours (cg), peritubular capillaritis (ptc), and peritubular capillary basement membrane (ptc-bm) scores from the Banff classification. First, in g-DSA+ recipients, the scores g, cg, ptc, and ptc-bm were significantly correlated with the presence of g-DSA. Next, C4d revealed that there was also a significant difference between the 2 groups. Excluding the ABO-incompatible recipients made it even more clear that C4d scores were higher in the g-DSA+ group compared to the g-DSA- group ($P < .0001$) (Fig 1A, B). To consider the meaning of g-DSA levels, we divided the g-DSA+ recipients as follows: g-DSA+ < 10; and a high g-DSA group, g-DSA+ ≥ 10. Interestingly, cg scores were strongly associated with high g-DSA and, as expected, other microvascular lesion scores were exacerbated by a higher g-DSA concentration (Fig 1C). Taken together, g-DSA reflected microvascular lesions due to AMR and g-DSA accumulation eventually results in chronic changes.

Short-term Outcomes in g-DSA + Renal Transplant Recipients

To investigate the effects of g-DSA on allograft function, Δs-Cr and ΔeGFR during the preceding 12 months were calculated. Δs-Cr and ΔeGFR were significantly higher in the g-DSA+ group compared to the g-DSA- group. Furthermore, patients were again separated into the following groups: 1) g-DSA-; 2) a low g-DSA group, g-DSA+ (<10); 3) a high g-DSA group, g-DSA+ ≥ 10. Similarly, Δs-Cr and ΔeGFR were determined. As a result, high g-DSA recipients demonstrated significant deterioration in allograft function (Fig 2), indicating that g-DSA accumulation clearly had a negative impact on graft function.

Anti-humoral Therapy for g-DSA+ Recipients

Of the recipients, 15 were treated with anti-humoral treatments (rituximab 200–300 mg/body, or apheresis–double-filtration plasmapheresis/plasma exchange with reinforcement of daily immunosuppression) (Table 1) and underwent a follow-up biopsy at between 6–12 months. At the time the follow-up biopsy was taken, these 15 recipients were divided into the following 2 groups based on the results of g-DSA status: g-DSA6-12+ group (g-DSA identified in the follow-up biopsy, n = 6 [graft ICFA scores: 20.6 ± 17.0 (pre-treatment) to 14.9 ± 14.1 (post-treatment)]); g-DSA6-12- group (g-DSA not evident in the follow-up biopsy, n = 9 [graft ICFA scores: 3.59 ± 2.82 (pre-treatment) to 0.58 ± 0.25 (post-treatment)]). Although the

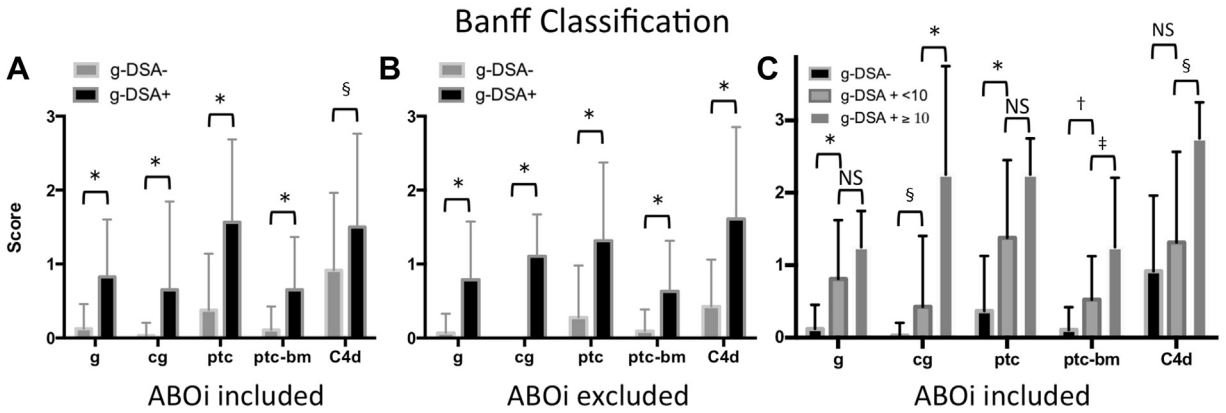

Fig 1. Histopathological impacts of g-DSA existence on g, cg, ptc, ptc-bm, and C4d based on the Banff classification. **(A)** Each of the Banff scores was analyzed according to the patient's status (g-DSA- or g-DSA+). **(B)** Each of the Banff scores was analyzed according to the patient's status (g-DSA- or g-DSA+, without ABO-incompatible cases). **(C)** These scores were reanalyzed according to g-DSA levels (g-DSA-, g-DSA < 10, and g-DSA ≥ 10, including ABO-incompatible cases). $^{*}P < .0001$, $^{\dagger}P < .0005$, $^{\ddagger}P < .005$, $P < .05$. NS, not significant.

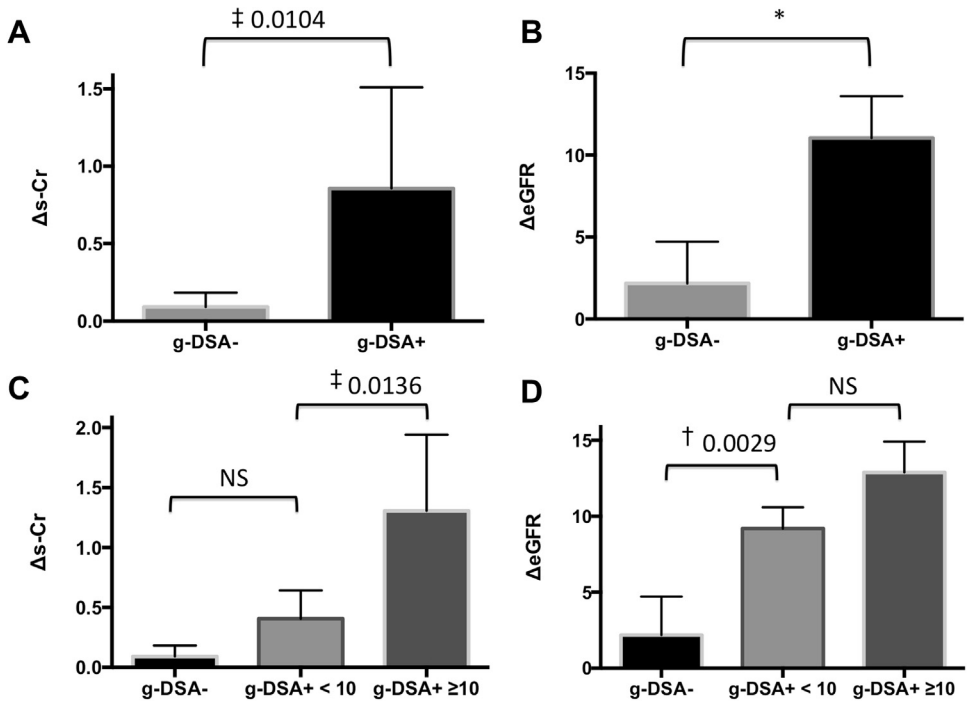

Fig 2. Short-term outcomes of renal graft function according to the g-DSA status. The deterioration in s-Cr and eGFR during the previous 12 months was calculated according to the presence or absence of g-DSA (**A** and **B**). Δs-Cr and ΔeGFR were calculated according to g-DSA levels (g-DSA-; g-DSA < 10; g-DSA ≥ 10 [**C** and **D**]). $^{*}P < .0001$, $^{\dagger}P < .005$, $^{\ddagger}P < .05$. NS, not significant.

initial pathological Banff scores were not significantly different between the 2 groups (Fig 3A), the initial g-DSA scores were notably higher in the g-DSA6-12+ group than they were in the g-DSA6-12- group (20.5 ± 17.0 vs 3.59 ± 2.82). Furthermore, in terms of DSA classes, recipients in the g-DSA6-12+ group tended to have class II DSAs (4/6), whereas the g-DSA6-12- group tended to have class I DSAs (7/9) ($P = .14$), prior to anti-humoral treatments (Fig 3B, C). Taken together, it can be argued that anti-humoral therapy is an effective treatment for recipients with relatively lower g-DSA, especially class I DSA.

One Accommodation Mechanism From s-DSA: Presentation of a Case

Among the g-DSA+ cases, we herein present the case of a 65-year-old Asian man who underwent non-simultaneous liver and kidney transplantation. Fifteen years after liver transplantation (DSA negative) owing to hepatitis C cirrhosis, he underwent kidney transplantation (DQ DSA positive) due to diabetic nephropathy with desensitization and was treated with rituximab 200 mg and double-filtration plasmapheresis/plasma exchange from the same donor (blood type: A-O). Following kidney transplantation, his liver graft function was stable despite the existence of de novo DSA, but his kidney graft function had slightly deteriorated. Histopathological analyses of the kidney and liver revealed chronic active AMR in the kidney allograft, whereas there was no sign of AMR in the liver. Interestingly, graft ICFA revealed only g-DSA existence in the kidney (3.9), and no g-DSA presence in the liver (.5), while s-DSA mean fluorescence intensity was over 10,000. Immunohistochemical analyses indicated loss of blood type antigen A expression of the interlobular venous/arterial epithelium and ductules, while antigen A expression was abundant in the kidney (Fig 4). To ascertain whether the graft totally lost reactivity with DQ DSA, the liver graft and his own serum were incubated together. Interestingly, graft ICFA revealed a weak positive result of 1.3 (>1.0). These findings suggest that there is an accommodation mechanism for DSA, perhaps similar to ABO-incompatible transplantation, and that the epithelium is an important barrier against DSA.

DISCUSSION

Previously, we reported that a graft ICFA technique firmly contributed to the detection of g-DSA as immune complexes in a renal allograft [6,7]. In the present study, the rate of g-DSA prevalence seems to be high, mainly because several patients with graft insufficiency were included and indicated for graft biopsies. s-DSA is considered an important serological marker to assess immunological reaction, and s-DSA detection is included as an integral part of AMR diagnosis [2]. However, it has also been reported that these values do not truly reflect a transplant outcome [13]. In fact, our results demonstrated that g-DSA values clearly correlate with a decrease in the graft renal function.

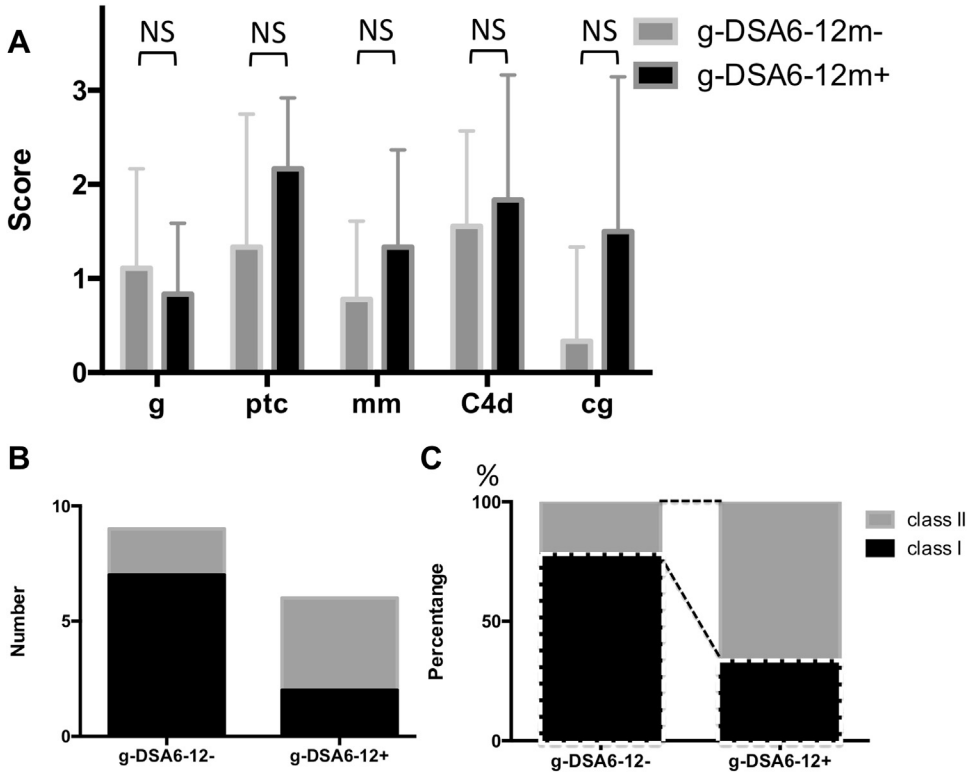

Fig 3. **(A)** The initial pathological Banff scores (g, cg, ptc, ptc-bm, and C4d) were not notably different between the g-DSA6-12+ and g-DSA6-12- groups. **(B)** The absolute number of patients with class I and II g-DSA in the g-DSA6-12+ and g-DSA6-12- groups. **(C)** The percentage of patients with class I and II g-DSA in the g-DSA6-12+ and g-DSA6-12- groups. NS, not significant.

Furthermore, g-DSA levels might be an effective marker to predict therapeutic responses, because they were notably lower in the g-DSA6-12– group. It can be argued that the levels of s-DSA might be saturated to some extent to maintain homeostasis of protein metabolism, but g-DSA accumulates as long as free HLA antigens exist in the allograft. The therapeutic effects on AMR recipients might be different depending on g-DSA scores. Thus, relatively low levels of g-DSA could be eliminated by anti-humoral therapy, but currently chronic lesions seem to be irreversible. In addition, cg scores tended to be higher in the g-DSA6-12+ group, despite there being no apparent differences in the initial Banff scores between the g-DSA6-12+/- groups. Considering the poor prognosis of chronic active AMR, the early diagnosis and complete clearance of g-DSA is required.

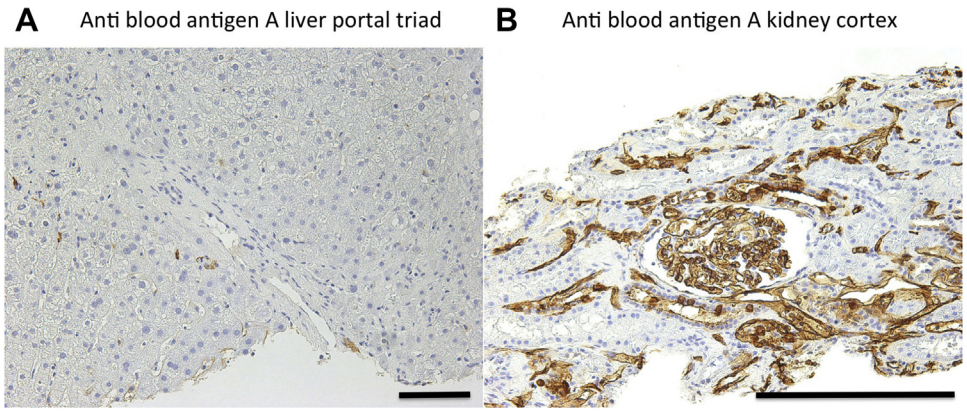

Fig 4. Immunohistochemical staining of human liver and renal allograft biopsies. Diaminobenzidine-peroxidase staining of blood antigen A or CD 68 indicates brown positive reaction. **(A)** The allograft from a donor of blood group A demonstrated almost no positive stain in the portal area. **(B)** There are rich A antigens in the renal allograft from the same donor. Scale bars: 500 μm.

Regarding histopathological changes, the presence of g-DSA detected by graft ICFA correlated closely with microcirculation lesions, which are pathological markers of AMR. Given the high sensitivity and specificity of this graft ICFA technique, it is clinically applicable and useful for diagnosing AMR. By performing graft ICFA routinely at the time of biopsy, an early and accurate diagnosis can be obtained, which could lead to improved graft survival. Compared to relevant articles, which occasionally reported the situation of s-DSA+/g-DSA- by the biopsy elution method [13,14], the high detection sensitivity of graft ICFA seems to be a clear advantage. Thus, basically there was no recipient with g-DSA-/s-DSA+, excluding a patient group discussed later. Although the presence of s-DSA has a negative impact on graft survival in renal [15] and even in liver transplantation [16], there are patients who have stable graft function despite the presence of a high s-DSA level. Those recipients seem to be a good research target in the field of DSA.

There seems to be several mechanisms by which liver allografts avoid DSA-AMR. Generally, the common idea appears to be that once liver allografts adsorb DSA, it is eliminated and hepatocytes are regenerated [17]. On the other hand, it is reasonable to believe that s-DSA does not react with allografts in low-susceptibility AMR patients. Theoretically, the epithelium accommodation might explain the hypothesis [18]. In this study, we clearly showed that the absence of g-DSA was one of the reasons that explained the stability of graft function in patients with high s-DSA by graft ICFA. Furthermore, the epithelium barrier might prevent g-DSA deposition. Given the fact that ICFA is very accurate and sensitive, allografts of recipients with g-DSA -/s-DSA+ may benefit from some mechanisms that prevent DSA deposition. Further research on these mechanisms should lead to an understanding of immunological tolerance in terms of DSA. Graft ICFA would be a good technique to study recipients with DSA-related AMR. At the very least, g-DSA -/s-DSA+ demonstrated by graft ICFA might be a low risk factor in terms of AMR.

CONCLUSION

This study was the first to use ICFA to visualize intra-graft DSA clearance in human renal transplantation. This novel technique would improve the results of AMR treatment.

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