A closer look at rituximab induction on HLA antibody rebound following HLA-incompatible kidney transplantation

Annette M. Jackson[1], Edward S. Kraus[1], Babak J. Orandi[2], Dorry L. Segev[2], Robert A. Montgomery[2] and Andrea A. Zachary[1]

[1]*Department of Medicine, Johns Hopkins University, Baltimore, Maryland, USA and* [2]*Department of Surgery, Johns Hopkins University, Baltimore, Maryland, USA*

Rituximab has been used to increase the efficacy of desensitization protocols for human leukocyte antigen (HLA)-incompatible kidney transplantation; however, controlled comparisons have not been reported. Here we examined 256 post-transplant HLA antibody levels in 25 recipients desensitized with and 25 without rituximab induction, to determine the impact of B-cell depletion. We found significantly less HLA antibody rebound in the rituximab-treated patients (7% of donor-specific antibodies (DSAs) and 33% of non-DSAs) compared with a control cohort desensitized and transplanted without rituximab (32% DSAs and 55% non-DSAs). The magnitude of the increase was significantly larger among patients who did not receive rituximab. Interestingly, in rituximab-treated patients, of the 39 HLA antibodies that increased post transplant, 34 were specific for HLA mismatches present in previous allografts or pregnancies, implying limited efficacy in memory B-cell depletion. Compared with controls, rituximab-treated patients had a significantly greater mean reduction in DSA (−2505 vs. −292 mean fluorescence intensity), but a similar rate of DSA persistence (52% in rituximab treated-and 40% in non-treated recipients). Thus, rituximab induction in HLA-incompatible recipients reduced the incidence and magnitude of HLA antibody rebound, but did not affect DSA elimination, antibody-mediated rejection, or 5-year allograft survival when compared with recipients desensitized and transplanted without rituximab.

Kidney International advance online publication, 23 July 2014; doi:10.1038/ki.2014.261

KEYWORDS: B cells; desensitization; HLA antibody; kidney transplantation; rituximab

Correspondence: *Annette M. Jackson, Immunogenetics Laboratory, 2041 E. Monument Street, Baltimore, Maryland 21205, USA.*
E-mail: ajackson@jhmi.edu

Received 24 March 2014; revised 22 May 2014; accepted 5 June 2014

B-cell depletion protocols using rituximab, a chimeric murine/human monoclonal antibody specific for CD20, were developed to treat B-cell malignancies[1] but have also been utilized to treat antibody-mediated autoimmune diseases[2,3] and to prevent or combat humoral rejection in solid organ transplantation.[4–7] In transplantation, B-cell depletion has been used before transplantation in desensitization protocols to reduce human leukocyte antigen (HLA) sensitization, allowing access to transplantation,[8–11] and perioperatively to prevent the development of *de novo* donor-specific HLA antibodies (DSA) or to prevent an anamnestic response.[6,12–14] It has also been utilized post transplant, during active antibody-mediated rejection (AMR), to dampen the immune response.[15–17]

The efficacy of desensitization protocols that include rituximab to decrease DSA has been reported in both ABO and HLA live donor–incompatible renal transplantation.[8,14,18–23] Kohei *et al.*[24] also reported a decreased incidence of *de novo* DSA and chronic AMR among ABO-incompatible recipients transplanted with rituximab induction compared with an ABO-compatible cohort transplanted without rituximab. However, the efficacy of rituximab in preventing post-transplantation DSA rebound and enhancing post-transplantation DSA elimination after desensitization protocols has not been analyzed in controlled cohorts. Reports to date have compared patients transplanted with rituximab treatment with those who had undergone no or less intensive desensitization treatment. Moreover, a limited number of post-transplant time points and HLA antibodies were included in previous studies.[14,18,23,25,26]

This study evaluates the impact of rituximab induction on HLA-specific antibody production in patients undergoing desensitization for HLA-incompatible live donor kidney transplantation. Our goal was to gain insight into the efficacy of B-cell depletion in preventing the activation and differentiation of HLA-specific B cells, particularly in sensitized recipients who may harbor HLA-specific memory B cells.

RESULTS

We compared the incidence of post-transplant HLA antibody rebound in 50 patients undergoing HLA-incompatible

transplantation using a desensitization protocol that either did or did not include a single dose of rituximab (375 mg/m^2) the day before transplantation. Patient demographics are provided in Table 1 and reflect our practice of using rituximab for patients with a higher risk for AMR.[27,28] The 25 patients who received rituximab induction had broader sensitization (mean calculated panel reactive antibody (CPRA) = 80 vs. 60%, $P = 0.02$), a higher incidence of previous transplants (76 vs. 28%, $P = 0.002$), and repeat HLA mismatches (80 vs. 0%, $P < 0.0001$). However, the two cohorts had similar DSA levels before desensitization and received a similar number of plasmapheresis treatments (Table 1, $P = 0.20$).

HLA antibody monitoring within the first 2 weeks post transplant revealed an increase in DSA for 36% (nine of 25) of rituximab-treated patients and 44% (11 of 25) of non-treated patients transplanted without rituximab ($P = 0.77$). Elevated DSA was treated with continued plasmapheresis and low-dose intravenous immunoglobulin (IVIg); however, all patients completed desensitization treatments within 2 weeks of transplant. An extended analysis was performed on 256 HLA antibodies (DSA and non-DSA) to examine HLA antibody levels following the cessation of plasmapheresis/IVIg treatments. The percentage change, comparing HLA antibody levels before desensitization (time zero) with four time points (1, 3, 6, and 12 months) post transplant, is plotted in Figure 1. The mean fluorescence intensity (MFI) for each antibody was normalized to the positive control bead value, to account for inter-run variability, and the percentage change from time zero was calculated. Among rituximab-treated patients, 7% (two of 29) of DSAs examined and 33% (37 of 111) of non-DSAs were increased at 1 month post transplant. In patients transplanted without rituximab, more HLA antibodies were increased at 1 month post transplant, 32% (eight of 25) of DSAs and 55% (50 of 91) of non-DSAs. The frequency of HLA antibody rebound was significantly higher in patients transplanted without rituximab induction for both DSA, $P = 0.03$, and non-DSA, $P = 0.003$. Moreover, the magnitude of the antibody increase was also larger in patients transplanted without rituximab induction. The mean percentage increase at 1 month for all HLA antibodies examined was 294 (median = 70) among the lower immunologic risk patients transplanted without rituximab, compared with 207 (median = 10) for those transplanted with rituximab induction ($P = 0.02$).

In both cohorts, HLA antibody rebounds detected at 1–3 months post transplant were transient, with 95 of 105 antibodies (90%) subsiding without any further plasmapheresis treatments. Of the 10 DSAs detected at 1 month post transplant, seven remained at a low level detectable only by bead immunoassays. The remaining three DSAs were of moderate levels, detected at a level sufficient to yield a weakly positive FCXM. These stronger rebounds occurred in three patients, two of whom received rituximab induction. Protocol biopsy surveillance at 1 month identified subclinical rejection (histopathology evidence of AMR without graft dysfunction) in seven patients with DSA rebound, including the three patients with the strongest DSA levels. Two patients showed no evidence of rejection and one patient, with normal allograft function, was not biopsied because of anticoagulation therapy.

No distinction between HLA class I vs. class II specificities was observed in antibodies (DSA or non-DSA) that increased post transplantation. For rituximab-treated patients, the

Table 1 | Patient demographics

	Rituximab $N = 25$	No rituximab $N = 25$	P-value
Recipient age (mean, s.d.)	41 ± 15	48 ± 13	0.08
Male gender (no. of patients, %)	8 (32%)	7 (28%)	1.0
Previous Txn (no. of patients, %)	19 (76%)	7 (28%)	0.002
Previous Txn ⩾3	5 (20%)	0	0.06
HLA-A;B;DR;DQ mismatch (mean)	4.8	5.0	0.61
Repeat HLA mismatch (No. of patients, %)	20 (80%)	0	0.0001
CDC CPRA[a] (mean, median)	48, 50	26, 3	0.02
FCXM CPRA (mean, median)	80, 89	60, 60	0.02
Crossmatch strength (no. of patients)			
CDC +	2	1	1.0
FCXM +	9	11	0.77
FCXM −, DSA +	14	13	1.0
Number of DSAs[b] (mean, median)	2.0, 2.0	1.7, 1.0	0.59
Donor age (mean, s.d.)	38 ± 12	46 ± 11	0.03
No. of pre-transplant plasmapheresis (mean)	3.7	2.3	0.08
No. of post-transplant plasmapheresis (mean)	4.1	3.9	0.81
Anti-CD25 induction (no. of patients, %)	10 (40%)	12 (48%)	0.78
Thymoglobulin induction (no. of patients, %)	15 (60%)	13 (52%)	0.78

Abbreviations: CDC, complement-dependent cytotoxicity; CPRA, calculated panel reactive antibody; DSA, donor-specific antibodies; FCXM, flow cytometric crossmatch; HLA, human leukocyte antigen; Txn, transplantation.
[a]CPRA was determined for HLA-specific antibodies of sufficient strength to yield a positive cytotoxicity (CDC) or FCXM.
[b]Number of DSAs before desensitization.

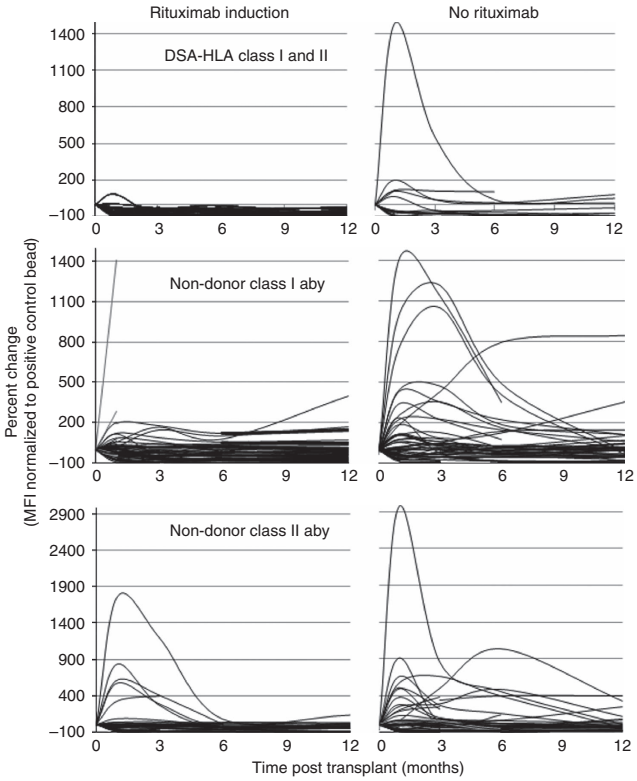

Figure 1 | Human leukocyte antigen (HLA) antibody rebound following HLA-incompatible transplantation: decreased incidence and magnitude in recipients receiving rituximab induction. HLA antibodies (54 DSA and 202 non-DSA) were measured before desensitization (time 0) and at four post-transplant time points (1, 3, 6, and 12 months) in patients transplanted with or without rituximab induction. Mean fluorescence intensity (MFI) values were normalized to the positive control bead, and percentage change from time zero was plotted. The incidence of post-transplant DSA ($P = 0.03$) and non-DSA class I and class II HLA antibody ($P = 0.0027$) rebound and the magnitude of the antibody rebound ($P = 0.02$) were lower in patients transplanted with rituximab induction compared with those without induction. aby, test bead MFI; DSA, donor-specific antibodies.

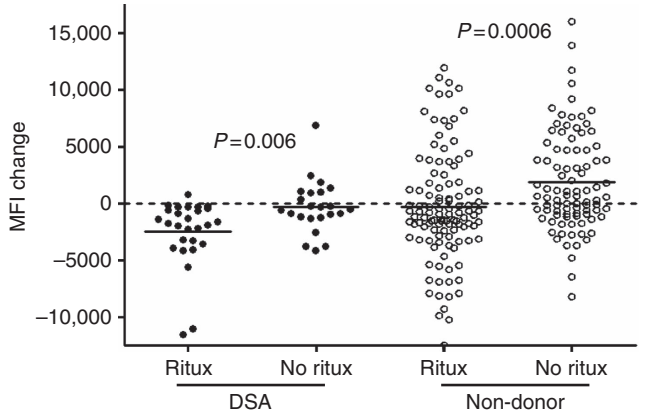

Figure 2 | Rituximab induction reduces donor-specific (DSA) and non-DSA human leukocyte antigen (HLA) antibody strength and incidence of rebound post transplantation. The change in pretreatment MFI and peak post-transplant MFI for DSA and non-DSA HLA antibodies were compared between patients transplanted with or without rituximab induction. Values plotted above the dotted line represent a post-transplant antibody rebound above pretreatment levels. The mean MFI change for DSA (-2505 vs. -292, $P = 0.006$) and for non-DSA HLA antibodies (-309 vs. $+1938$, $P = 0.0006$) are denoted by solid lines and were statistically lower in patients transplanted with rituximab induction.

incidence of non-DSA HLA class I vs. class II antibody rebound was 36 and 30%, respectively, and in patients transplanted without rituximab induction the values were 50 and 62%, respectively (Figure 1).

The assessment of HLA antibody levels using a percentage change calculation, which normalizes for inherent test-to-test variability, tends to emphasize changes in weaker antibodies more than strong antibodies. For that reason, we reanalyzed the data calculating the absolute difference in MFI (without normalization) of the pre-desensitization serum and the peak post-transplant serum, with zero representing no change from pre-desensitization baseline levels. Patients transplanted with rituximab induction showed a greater reduction in DSA from baseline levels compared with the control group (-2505 vs. -292 MFI, $P = 0.006$). Rituximab treatment also affected the mean MFI change of non-DSA HLA antibodies (-309 vs. $+1938$ MFI, $P = 0.0006$) post transplantation (Figure 2). Moreover, the impact of rituximab induction to decrease antibody levels was observed across HLA antibodies of different baseline (time zero) levels (categorized as: weak = MFI < 4000; moderate = MFI 4000–9000; strong = MFI > 9000, data not shown).

Thirteen (52%) of the rituximab-treated recipients and 10 (40%) non-rituximab recipients had detectable DSA at 1 year post transplantation. In the majority of these patients (19 of 23), these antibodies were of weak levels and detectable only by bead immunoassays. However, in four recipients, three in the rituximab-treated cohort and one in the non-rituximab group, DSA at 1 year post transplantation was of moderate levels and directed toward HLA class II antigens. Two of these four patients developed transplant glomerulopathy within 2 years of transplant, and both had received rituximab induction.

Nine rituximab-treated (36%) and seven (28%) non-rituximab-treated patients showed no increase in HLA antibodies post transplantation. For the remaining patients, post-transplant increases were seen for some, but not all, HLA antibody specificities. We analyzed factors that may predict which HLA specificities would increase post transplant and found that baseline levels of the HLA antibodies before desensitization were not predictive of post-transplant rebound in either rituximab-treated ($P = 0.65$) or non-rituximab-treated ($P = 0.17$) cohorts. Previous HLA mismatches did affect the likelihood of rebound. In 19 rituximab-treated patients with previous transplants, 34 of 39 (87%) HLA antibodies that increased post transplant were specific for HLA epitopes shared with previous allografts or pregnancies. Figure 3 provides a detailed example of post-transplantation HLA antibody increases in a representative rituximab-treated recipient. This patient had no increase in DSA toward his current (3rd) transplant; however, all of the

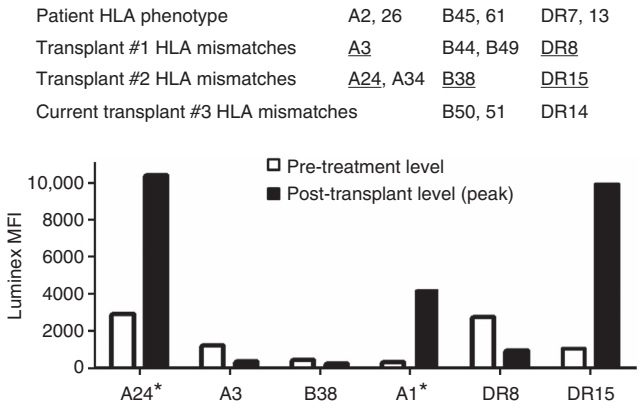

Figure 3 | Post-transplantation human leukocyte antigen (HLA) antibody rebound reflects previous HLA antigen mismatches but not pre-transplantation baseline HLA antibody strength. HLA phenotypes for the recipient and three renal allografts are provided. This patient received rituximab induction and experienced rebounds in non-DSA HLA antibodies but no increase in DSA directed toward the current (3rd) allograft. MFI values for HLA antibodies detected before desensitization and 1 month following the third transplantation are shown. All HLA antibodies specific for previous HLA mismatches are shown, and the asterisk highlights antibody reactivity toward a known cross-reactive epitope in a previous mismatched antigen (HLA-A1 and HLA-A24). DSA, donor-specific antibodies; MFI, mean fluorescence intensity.

non-DSA HLA antibodies that increased could be attributed to previously mismatched HLA antigens/epitopes.

Post-transplant clinical outcomes and complications are shown in Table 2. The incidence of infection (bacterial, viral, and fungal) during the first year post transplant was 44% in patients treated with rituximab and 28% in non-depleted patients, but this difference was not significant ($P = 0.24$). The total number of infections was greater in the rituximab-treated group (27 vs. 10), but reflects multiple infections in a limited number of patients.

Despite the increased number of immunologic risk factors among rituximab-treated patients, no significant difference was observed in the incidence of acute rejection within the first 3 months of transplant between the cohorts (Rituximab: 76% and Non-Rituximab: 56%, $P = 0.23$; Table 2). Many of these rejection episodes were cellular rejections (28 of 38 were cell-mediated rejection or mixed AMR/cell-mediated rejection), and cellular rejection was more than twice as common in the rituximab-treated group (12 vs. 5). There were a similar number of episodes of AMR and mixed rejections between the two study groups. Mean serum–creatinine values (Table 2) at 1 and 2 years post transplant were 1.10 and 1.07 mg/dl, respectively, in the rituximab-treated cohort and 1.26 and 1.28 mg/dl, respectively, in the non-rituximab cohort ($P = 0.21$ and $P = 0.10$).

Analysis of patient and graft survival was augmented by linking patients to the Scientific Registry of Transplant Recipients (SRTR) data, and the Kaplan–Meier method was used to estimate patient and death-censored graft survival at 1 and 5 years post transplantation (Figure 4). Between-group differences were evaluated using the log-rank test. One and 5-year patient survival was 96.0 and 81.7% in the rituximab-treated group and 96.0 and 83.7% in the non-rituximab-treated group, respectively ($P = 0.6$; Figure 4a). One and 5-year death-censored graft survival was 100.0 and 94.7% in the rituximab-treated group and 95.8 and 77.5% in the non-rituximab-treated group, rspectively ($P = 0.23$; Figure 4b).

These transplants occurred during a period when we were randomizing patients to receive T-cell–induction therapy with either anti-interleukin 2 receptor (anti-CD25) or thymoglobulin. Analysis of post-transplant HLA antibody rebound, infection, and acute rejection did not correlate with the type of T-cell induction the patients received.

Table 2 | Clinical outcomes and complications

	Rituximab $N = 25$	No rituximab $N = 25$	*P*-value
DSA rebound <2 weeks post transplant (no. of patients)	9 (36%)	11 (44%)	0.77
Strength of rebound			
FCXM+	4 (16%)	5 (20%)	
FCXM−, DSA+	5 (20%)	6 (24%)	
DSA persistence at 1 year (no. of patients)	13	10	0.57
Class I	4	6	
Class II	5	3	
Class I and II	4	1	
Serum creatinine (mean ± s.d.)			
1 Year	1.10 ± 0.30	1.26 ± 0.39	0.21
2 Year	1.07 ± 0.22	1.28 ± 0.53	0.10
Infections ⩽1 year (no. of patients)	11	7	0.38
Bacterial (no. of infections)	15	6	
Viral	10	4	
Fungal	2	0	
Total infections	27	10	0.08
Acute rejection[a] ⩽3 months (no. of patients)	19	14	0.23
CMR (no. of episodes)	8	2	
Subclinical CMR	4	3	
Total	12	5	
AMR	3	4	
Subclinical AMR	1	2	
Total	4	6	
Mixed CMR and AMR	2	2	
Subclinical mixed	5	2	
Total	7	4	
Transplant glomerulopathy[a], no. of patients (<2 years)	4	2	0.17

Abbreviations: AMR, antibody-mediated rejection; CMR, cell-mediated rejection; DSA, donor-specific antibodies; FCXM, flow cytometric crossmatch.
[a]Biopsy-proven CMR and/or AMR rejection, or transplant glomerulopathy using updated Banff '97 criteria.[53–56]

DISCUSSION

HLA-incompatible recipients selected to receive rituximab induction in this study were at a higher risk for anamnestic

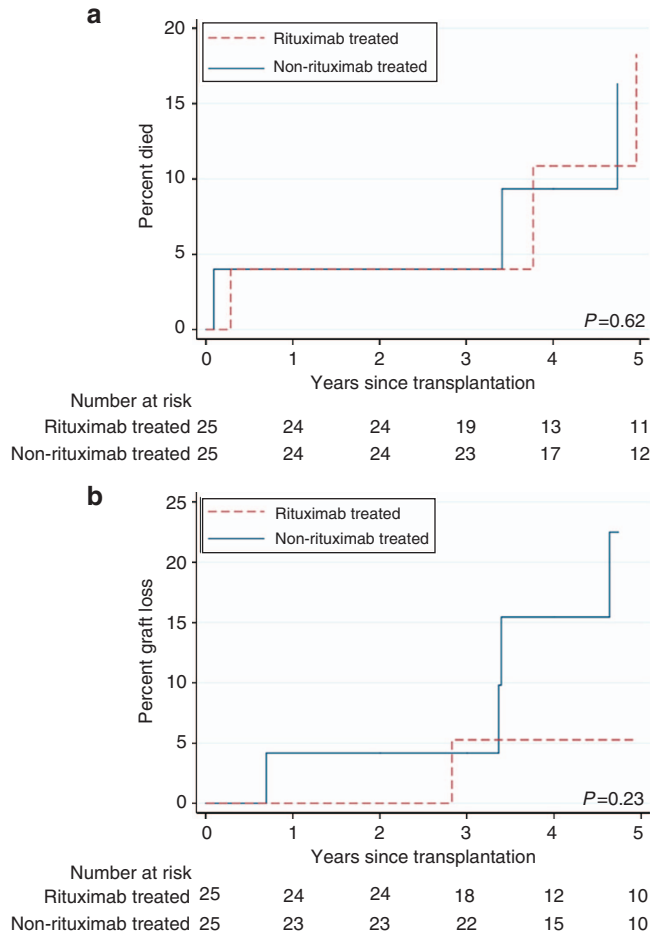

Figure 4 | Mortality and death-censored graft loss in the rituximab-treated versus non-rituximab–treated cohorts. Kaplan–Meier estimates of (**a**) the patients' 1- and 5-year survival were 96.0% (95% CI: 74.8–99.4%) and 81.7% (95% CI: 51.9–94.0%) in the rituximab-treated group and 96.0% (95% CI: 74.8–99.4%) and 83.7% (95% CI: 56.3–94.6%) in the non-rituximab-treated group ($P = 0.6$); (**b**) graft 1 and 5-year death-censored survival were 100.0 and 94.7% (95% CI: 68.0–99.0%) in the rituximab-treated group and 95.8% (95% CI: 73.9–99.4%) and 77.5% (95% CI: 49.7–91.1%) in the non-rituximab-treated group ($P = 0.23$). CI, confidence interval.

responses because of higher CPRAs, multiple transplants, and repeated HLA mismatches; yet, they experienced less HLA antibody rebound (DSA and non-DSA). This reduction in HLA humoral responses, however, did not translate into reduced rates of AMR when compared with patients transplanted without rituximab. Rituximab induction did not significantly affect the persistence of DSA at 1 year post transplantation, which was detected in 52% of the patients treated with rituximab vs. 40% in the non-rituximab-treated group. This incidence of DSA persistence following rituximab induction was higher in our study compared with reports by Loupy *et al.* (28.5%)[14] or Takagi *et al.* (14%)[25] at 1 year post transplantation and likely reflects the increased breadth and strength of HLA sensitization in our patients. No significant difference in renal function was observed at 1 and 2 years or graft survival at 5 years post transplantation between these cohorts. The number of cell-mediated rejections was higher in the rituximab-treated cohort, which may be due to increased allo-sensitization in this cohort or a consequence of deleting B-regulatory cells.[29,30]

Our study is the first to determine the impact of rituximab induction on DSA rebound in patient cohorts receiving the same desensitization regimen without additional B-cell–depleting therapy.[14,18,23,25] Gloor *et al.* reported decreased but persistent DSA in 10 of 12 HLA-incompatible renal recipients at 4 months post transplant. These recipients were desensitized using a protocol that included plasmapheresis, IVIg, and a single dose of rituximab (375 mg/m^2) as induction; however, all recipients also underwent splenectomy before transplant. Studies by Hirai *et al.*, Takagi *et al.*, and Loupy *et al.* compared post-transplant DSA levels in HLA-incompatible recipients transplanted following desensitization, which included plasmapheresis and rituximab induction, with retrospective cohorts transplanted across DSA with no desensitization, or with deceased donor transplants using a protocol involving high-dose IVIg alone.

HLA antibody rebounds observed at 1–3 months post transplantation (Figure 1) were transient and declined without intervention, suggesting that they were generated by newly formed short-lived plasma cells.[31,32] Analysis of both DSA and non-DSA levels post transplantation confirms that the trauma of surgery and antigenic stimulation of the immune system can elicit both antigen-specific and nonspecific antibody responses[33] and that the current B-cell depletion strategies can reduce or blunt but not prevent new HLA antibody production post transplant. Reducing B-cell numbers in sensitized patients before the transplant likely decreased the number of HLA-specific B cells that could be activated and transformed into antibody-producing plasma cells. Therefore, in the rituximab-treated recipients, the new plasma cells likely arose from memory B cells residing in secondary lymphoid niches and protected from depletion.[34–38] Examination of the HLA antibody specificities that increased post transplantation in our rituximab-treated patients showed that 34 of 39 were to immunogenic epitopes the patient had been previously exposed to in previous allografts or pregnancies and support the existence of these immune memory reservoirs. These findings suggest a benefit in avoiding repeated HLA antigen mismatches in patients with previous transplants or HLA antigens shared with spouses when transplanting multiparous women, to guard against reactivation of occult sensitization. In such cases, kidney paired donation may offer a mechanism to increase HLA matching between recipient–donor pairs and avoid repeated HLA mismatches.

The increase in some, but not all, HLA antibody specificities may reflect differences in the amount of B-cell memory that exists toward a particular HLA antigen. We have previously shown that the frequency of peripheral blood, HLA-specific B cells, measured using HLA tetramers, reflects prior sensitization to an HLA antigen and predicts

a post-transplantation anamnestic response to that antigen in patients with no detectable antibody to that HLA antigen.[39] In a subsequent study, we showed that rituximab induction abrogated this anamnestic response.[40] In this current study, we show that previous sensitization, and not the pretreatment antibody levels, is a better correlate of antibody rebound. This latter finding is also supported by data from Burns *et al.*[41]

Our findings differ from what has been reported in desensitization protocols that utilize B-cell depletion to reduce CPRA prior transplantation.[8–11] In those studies, rituximab in combination with high-dose IVIg produced only transient reductions in some HLA antibodies, primarily those of weaker levels. This current study showcases the impact of desensitization coupled with transplantation in reducing HLA antibodies (DSA and non-DSA) of all levels and, in this setting, the added benefit of B-cell depletion. The increased efficacy of rituximab administered at the time of transplantation may hinge on increased accessibility to HLA-specific B cells and plasma cells during times of inflammation.[42,43] Increased mobilization of cells from protective niches of the bone marrow occurs in response to inflammation due to reduced CXCL12 retention signals.[44] Once in the bloodstream, plasma cells lose necessary signals for long-term survival, whereas cells expressing CD20 become more vulnerable to rituximab depletion, and both events likely contribute to the subsequent decline in HLA antibody levels post transplantation.[42,45]

Similarly, the efficacy of rituximab in the treatment of AMR may also hinge on timing and accessibility to HLA-specific B cells. If rituximab is given early in the immune response, it can reduce the number of mobilized B cells specific for donor HLA antigens, thus minimizing the magnitude of the immune response. However, if rituximab is given after AMR is established, donor-specific B cells may have already transited from the periphery into protected niches within the spleen, where they become resistant to depletion and contribute to the generation of new plasma cells. This latter scenario may explain why splenectomy or bortezimib, and not rituximab, can be more effective in quelling an active alloimmune response after a significant rise in DSA.[46,47]

Our study compared patients desensitized under the same treatment protocol and confirmed the efficacy of rituximab induction in reducing DSA rebound and overall DSA levels post transplantation. Although it seems intuitive that this would translate into lower rates of AMR and better outcomes, this was not observed in this study. The limitations of the study include a nonrandomized methodology, limited clinical follow-up, and an intervention group that contained more retransplantations and higher baseline CPRA, which could offset the benefits of B-cell depletion. In HLA-sensitized cohorts, the impact of rituximab induction on transplantation outcomes may depend on individual factors such as the number of donor-specific memory B cells that are sequestered and resistant to depletion and the reserves of donor-specific plasma cells that are not effectively targeted. Therefore, further knowledge regarding the immunogenicity of HLA mismatches and the breadth of immune memory generated following HLA sensitizing events is still needed to assist in risk assessment and aid in donor selection before desensitization and transplantation.

MATERIALS AND METHODS

Patients and immunosuppression

This is a retrospective analysis of patients transplanted at the Johns Hopkins Hospital within the Incompatible Kidney Transplant Program. The Johns Hopkins Institutional Review Board approved the treatment protocol and use of the prospective clinical database as a research tool. Sequential HLA-incompatible live donor renal transplant recipients transplanted between 2006 and 2010 were evaluated. Inclusion criteria required that all plasmapheresis treatments occurred within the first 2 weeks post transplantation and that the patient received no additional rituximab treatments, splenectomy, or bortezomib. The desensitization protocol included alternate day, single-volume plasmapheresis, and 100 mg/kg IVIg (Cytogam, MedImmune, Gaithersberg, MD). The number of treatments was determined by the DSA strength prior to transplantation.[6] Mycophenolate mofetil (2 g/day) and tacrolimus (serum level of 8–10 ng/ml) were initiated with the start of plasmapheresis treatments. Intraoperative induction therapy included anti-IL2 receptor antibody (anti-CD25, daclizumab 2 mg/kg or thymoglobulin, 1.5 mg/kg per day for 5 days). Twenty-five patients with multiple previous transplants and/or repeat HLA mismatches received a single dose of anti-CD20 antibodies (Rituxan, 375 mg/m^2) on the day before transplant. Six of 26 recipients with previous transplants had allograft nephrectomies before the current transplant (four of 19 in the rituximab-treated and two of seven in non-treated recipients).

HLA antibody assays

Post-transplant monitoring protocol included alternate day testing for the first 2 weeks, monthly for 3 months, and quarterly thereafter. The specificity and level of HLA-specific antibodies were evaluated using HLA phenotype and single antigen bead assays (Lifecodes class I and II ID panels, Immucor Gen-Probe, San Diego, CA and Single Antigen Beads, One Lambda, Canoga Park, CA) performed on a Luminex platform. High titered sera were tested with and without dilution (1:8) and sera exhibiting high negative controls or a low positive control were treated with hypotonic dialysis before testing.[48] All HLA antibody specificities that could be clearly delineated on the phenotype panel were selected for year-long analysis. HLA antibody levels were assessed using phenotype bead panels, and changes in levels were further verified using single antigen beads. To control for test-to-test variation and the impact of interfering serum factors that can be removed by plasmapheresis, MFI values were normalized to the positive control bead, and the fold change from the pre-desensitization time point was calculated:

$$\text{Percent change} = [(\text{Aby}_n/\text{PC}_n - \text{Aby}_0/\text{PC}_0)/\text{Aby}_0/\text{PC}_0] * 100$$

Aby, test bead MFI; PC, positive control bead MFI; *n*, represents an individual time point (1, 3, 6, or 12 months post transplant); and 0 represents time zero before desensitization.

Crossmatch tests with donor T and B cells were performed using cytotoxicity and/or flow cytometry as previously described.[49,50] Our positive FCXM threshold correlates with DSA of moderate strength on a HLA phenotype panel (4000–9000 MFI), and a positive

CDC-XM correlates with stronger DSA (MFIs > 9000).[51] CPRA was determined for antibodies strong enough to yield a positive complement-dependent cytotoxicity crossmatch (CDC-XM), and/or flow cytometric crossmatch FCXM was calculated using the online UNOS CPRA calculator.[52]

Histopathology and rejection

Renal allograft biopsies were performed at 1, 3, 6, or 12 months post transplantation according to the protocol and additionally as indicated by dysfunction. C4d deposition was evaluated using indirect immunofluorescence. Presence of cell-mediated rejection, AMR, mixed, or subclinical rejection was determined using updated Banff '97 criteria.[53–56]

Statistical methods

The Kaplan–Meier method and the log-rank test were used to estimate survival in treatment groups and compare differences between groups, respectively. Patients were linked to the SRTR to allow death ascertainment from the Centers for Medicare and Medicaid Services. SRTR includes information on all donors, wait-listed transplant candidates, and transplant recipients in the U.S. provided by members of the Organ Procurement and Transplantation Network. The Health Resources and Services Administration, U.S. Department of Health and Human Services, provides oversight to the activities of the OPTN and SRTR contractors. Death-censored graft survival was defined as the time between transplant date and either graft failure date (marked by retransplantation, relisting, or a return to dialysis) or last follow-up date with a functioning graft, censoring for death, and administrative end of study. Survival analyses were performed using Stata 12.0. Summary statistics, including mean, median, and s.d., were calculated using Microsoft Excel. Statistical significance was determined using χ^2-and Student's *t*-test (two-tailed), with a *P*-value <0.05 considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

The data reported here have been supplied by the Minneapolis Medical Research Foundation (MMRF) as the contractor for the Scientific Registry of Transplant Recipients (SRTR). The interpretation and reporting of these data are the responsibility of the author(s) and in no way should be seen as an official policy of or interpretation by the SRTR or the U.S. Government. We also thank J Houp for assistance with CPRA calculations. This work was supported by a grant (R01 DK098431 and RC1 DK086731) from the National Institute of Diabetes and Digestive and Kidney Diseases and by the Charles T Bauer Foundation (RAM).

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