Surveillance of alloantibodies after transplantation identifies the risk of chronic rejection

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The monitoring of the levels of alloantibodies following transplantation might facilitate early diagnosis of chronic rejection (CR), the leading cause of renal allograft failure. Here, we used serial alloantibody surveillance to monitor patients with preoperative positive flow cytometric crossmatch (FCXM). Sixty-nine of 308 renal transplant patients in our center had preoperative positive FCXM. Blood was collected quarterly during the first postoperative year and tested by FCXM and single antigen bead luminometry, more sensitive techniques than complement-dependent cytotoxic crossmatching. Distinct post-transplant profiles emerged and were associated with different clinical outcomes. Two-thirds of patients showed complete elimination of FCXM and solid-phase assay reactions within 1 year, had few adverse events, and a 95% 3-year graft survival. In contrast, the remaining third failed to eliminate flow FCXM or solid-phase reactions directed against HLA class I or II antibodies. The inferior graft survival (67%) with loss in this latter group was primarily due to CR. Thus, systematic assessment of longitudinal changes in alloantibody levels, either by FCXM or solid-phase assay, can help identify patients at greater risk of developing CR.

RESULTS

Patients

Patients were first partitioned on the basis of whether preoperative FCXM was negative or positive. Patient demographics were similar (Table 1). Both groups were
primarily African Americans receiving first grafts from deceased donors. Age and gender distributions were equivalent. Both were poorly HLA matched with donors. The T and B panel-reactive antibodies were higher among the FCXM-positive group. In all, nine patients had positive FCXM because of non-HLA antibodies and were excluded from subsequent analysis. All remaining patients with positive FCXM showed donor-specific antibody (DSA) and non-DSA.

**Post-transplant alloantibody profiles.** Two post-transplant antibody profiles were observed. The majority of patients (group I, 65%) showed complete elimination of FCXM reactivity within the first postoperative year (Figure 1a–c). The T- and B-FCXM levels were equivalent preoperatively and at all time points (P = 0.4). Both T- and B-FCXM reactivity became negative in 71% patients by month 6 and 100% by month 12. Reductions in FCXM levels were mirrored using single antigen bead identification of DSA and non-DSA. Pretransplant DSA was exclusively directed against class I or II in 66 and 34% of patients. Pretransplant DSA levels were equivalent against class I and II (P = 0.06). DSA levels against class I and II declined 90% by month 6 and were undetectable by month 12. To estimate a total level of non-DSA, all individual mean fluorescent intensities were combined into a single value for each patient. Pretransplant non-DSA against class I or II were found in 60 and 50% of patients. Total pretransplant levels of non-DSA against class I and II were greater than DSA levels (P < 0.001) and predominantly directed against class I (P < 0.001). Total non-DSA mean fluorescent intensity levels against class I and II were reduced 50–70% by month 6 and 80–90% by month 12.

Three patients had profiles and clinical outcome similar to group I but failed to completely eliminate DSA against class I by month 12. Although they are probably a subgroup of group I in which antibody elimination proceeds more slowly, we eliminated them from subsequent analysis because of their small size and nonidentical profile. Post-transplant antibody profiles for the remaining patients (group II) differed markedly from group I (Figure 1d–f). Group II failed to eliminate or show significant reductions in FCXM, DSA, or non-DSA levels against class I or II during the first post-transplant year. Preoperative T- and B-FCXM levels were equivalent (P = 0.4). Despite transient fluctuations in T-FCXM levels, the levels subsequently rebounded. B-FCXM levels were unchanging throughout the first year. Solid-phase testing showed a similar pattern of alloantibody persistence. Preoperative DSA against class I or II were found in 60 and 40% of patients. DSA levels against both class I and II were unchanged throughout the year. Preoperative non-DSA against class I or II were found in 60 and 60% of patients and levels were significantly greater than DSA levels (P < 0.001). Non-DSA levels against class I and II declined 23 and 52% by month 6. However, by month 12, reactivity against class I and II was either unchanged or increased. Total non-DSA levels against class I and II were significantly greater among group II than group I by month 12 (P < 0.001).

**Comparison of pretransplant antibody characteristics** Once groups were identified based upon post-transplant alloantibody elimination profiles, we re-evaluated preoperative antibody characteristics hoping to identify features that would predict post-transplant antibody developments. Unfortunately, none were found. The group demographics were equivalent. The donor characteristics were equivalent (Table 2). Panel-reactive antibody distribution was equivalent with most patients showing sensitization (Table 3). T- and B-FCXM levels were equivalent between and within groups. All patients had DSA and non-DSA. DSA frequency (1 to 2 per patient) was equivalent between groups. DSA distribution was equivalent between groups and was directed against class I among 60–70% of patients or class II among 30–40% of patients. Few patients had DSA against both class I and II and most patients had only one DSA. DSA levels against class I and II were equivalent between groups. Although subjective, we consider DSA mean fluorescent intensity as low (< 1000), moderate (1000–5000), or high (> 5000) strength. Thus, patients had moderate to high alloantibody strengths in both groups. The frequency of non-DSA (7 ± 3) directed against class I or II was similar between groups. Total non-DSA levels against class I were equivalent between groups and greater than against class II (P < 0.001). The only overt difference was the elevated non-DSA levels against class II in group II relative to group I (P = 0.001).

**Clinical outcome** Patient and graft survival were compared between groups and patients with preoperatively negative FCXM. The 3-year actuarial patient survival was equivalent (P = 0.5) between FCXM-negative patients (99%) and groups I (100%) and II (100%). In contrast, the 3-year actuarial graft survival differed (Figure 2). Graft survival was equivalent between FCXM-negative patients (96%) and group I (95%, P = 0.5) but poorer among group II (67%, P < 0.001). Additional clinical outcome parameters were compared (Table 4). The frequency of acute cellular rejection was low.
Among FCXM-negative patients and group I but greater among group II (21%, P < 0.001). Antibody-mediated rejection (AMR) was minimal (≤ 3%) among FCXM-negative patients and group I but prevalent among group II (43%, P < 0.001) and responsible for 80% of graft failures versus 0% in other groups. Although mean serum creatinine at 1 year was greater among group II (P < 0.01), there was considerable overlap with the ranges seen among FCXM-negative patients and group I.

Despite alloantibody persistence, only half of group II experienced AMR and CR within the study interval. The remainder stayed AMR and CR free. A comparison of pretransplant alloantibody characteristics showed no obvious differences in FCXM, DSA, or non-DSA levels between the subgroups (Table 5). Acute cellular rejection occurred with equal frequency between the subgroups (25 vs 20%, P = 0.1). However, group II patients without AMR enjoyed the 3-year graft survival equivalent to group I (100 vs 97%, P = 0.9).

**DISCUSSION**

There is strong evidence that anti-HLA antibodies contribute to the development of CR, the leading cause of renal allograft failure.1,2,4,8,10,14,15,17–18,23–29 While it is hoped that post-transplant alloantibody surveillance might facilitate early diagnosis, there are few guidelines regarding how monitoring should be performed and results interpreted. This report

**Table 2 | Patient and donor characteristics between groups I and II**

|                          | Group I          | Group II         | P-value |
|--------------------------|------------------|------------------|---------|
| 1. Days on dialysis      | 1497 ± 1283      | 1738 ± 1510      | 0.6     |
| 2. Type of donor         |                  |                  |         |
| Living                   | 15%              | 33%              | 0.1     |
| Standard criteria        | 78%              | 47%              | 0.03    |
| Expanded criteria        | 2%               | 7%               | 0.4     |
| Donation after cardiac death | 5%         | 13%              | 0.3     |
| 3. Cold ischemia time (min) | 913.1 ± 617.1 | 728.9 ± 507.9 | 0.3     |

**Figure 1 | Longitudinal assessment of changes in flow cytometric crossmatch (FCXM), donor-specific antibody (DSA), and non-DSA levels during the first post-transplant year.** Blood collected quarterly among patients with preoperatively positive FCXM was tested by FCXM and single antigen bead luminometry. Nine patients with FCXM reactivity but lacking anti-HLA specificity and three patients with atypical group I profile were excluded from consideration. Group I (n = 33) showed complete elimination of FCXM, DSA, and non-DSA within 12 months. Group II (n = 15) maintained FCXM, DSA, and non-DSA levels against class I and II throughout the study interval. FCXM symbols: ○ T-FCXM channel shifts; ■ B-FCXM channel shifts. DSA and non-DSA symbols: ■ anti-class I mean fluorescent intensity (MFI); ▲ anti-class II MFI. Group I: (a) FCXM channel shifts ± s.d.; (b) DSA MFI ± s.d.; and (c) non-DSA MFI ± s.d. Group II: (d) FCXM channel shifts ± s.d. (e); DSA MFI ± s.d.; and (f) non-DSA MFI ± s.d.
excellent graft survival and minimal CR.6–7,12,16 We also focused our attention upon patients with preoperatively negative FCXM who typically experience first post-transplant year. We intentionally excluded patients FCXM and solid-phase assay reactivity occurring during the few patients with two DSAs against either class I or II, the MFI is the strength index of the immunodominant specificity instead of the cumulative strength. In contrast, total non-DSA antibody burden was calculated by combining all non-DSA MFIs into a single value for each patient. Unless otherwise indicated, data between groups were statistically equivalent (P>0.5). Preoperative DSA testing was performed upon patients with negative FCXM, but no DSAs were identified with preoperatively positive FCXM because of preformed anti-HLA antibody who, at this center, have poorer graft survival.6,27 Changes in specificity techniques throughout the years made original specificity data incomparable. To standardize the data, we reassayed patient serum for anti-HLA specificities using one lot of single antigen beads on a Luminex platform and also retested donor HLA for minor histocompatibility antigens not determined originally. Consistent with a recent report, the enhanced sensitivity of single antigen bead testing allowed identification of DSA and non-DSA in 100% of study patients.30

Table 3 | Pretransplant antibody comparison between groups

| Class       | Group I | Group II |
|-------------|---------|----------|
| 1 DSA       | 61%     | 47%      |
| > 1 DSA     | 6%      | 13%      |
| Class II    |         |          |
| 1 DSA       | 34%     | 27%      |
| > 1 DSA     | 0%      | 13%      |

Table 4 | Comparison of the 3-year clinical outcomes

| FCXM-neg | Group I | Group II |
|----------|---------|----------|
| n        | 239     | 33       |
| ACR      | 4%      | 5%       |
| AMR      | 1%      | 3%       |
| Banff I  | 66%     | 60%      |
| Banff II | 33%     | 30%      |
| Banff III| 0%      | 10%      |
| Months to AMR | 17 ± 13 | 14 ± 5 |
| CR       | 1%      | 0%       |
| Failure due |       |          |
| To CR    | 0%      | 0%       |
| Death    | 1%      | 0%       |
| 1-year sCr (mg/dl) | 1.4 ± 0.7 | 1.5 ± 0.5 |
| Range    | 0.7–8.6 | 0.8–2.9  | 0.6–9.5  |

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; CR, chronic rejection; FCXM, flow cytometric crossmatch; sCr, serum creatinine.

Figure 2 | Actuarial graft survival. Deaths with functioning grafts were censored. ■ Patients with preoperatively negative flow cytometric crossmatch (FCXM; n = 239); ▲ Group I; ▼ Group II. P<0.001, graft survival among group II versus group I.

Temporal changes in alloantibody levels were consistent whether measured using FCXM or solid-phase assay. Two post-transplant profiles emerged and showed different clinical outcomes. Patients showing complete elimination of alloantibody enjoyed excellent graft survival and few complications. In contrast, patients failing to eliminate alloantibody had poorer outcomes and higher incidence of
CR. Following delineation of specific groups, pretransplant antibody characteristics were compared with the hope that some distinguishing parameters would predict post-transplant outcome. However, similar to previous reports, pretransplant characteristics proved inadequate indicators of post-transplant developments.5,8,10,13 Before transplant, groups were equivalent in terms of demographics, donor characteristics, alloantibody levels, and specificities. Alloantibodies against class I predominated in all groups. However, group II did show a greater total amount of non-DSA against class II than group I.

It has always been puzzling why only a fraction of presensitized patients actually develop CR.6–10,16,19–20 Our surveillance study provides a partial explanation. The majority of patients (65%) showed rapid reduction of both T- and B-FCXM levels within 6 months of transplant and complete elimination within 12 months. These results were mimicked using solid-phase testing that showed that DSAs and non-DSAs against class I and II were simultaneously eliminated. Elimination of circulating alloantibody transformed this group into a FCXM-negative population that typically has excellent long-term graft survival and minimal CR. The cause of antibody elimination is unknown. However, one possible mechanism could be downregulation of antibody synthesis after transplantation. Considering the short half-life of immunoglobulin G, complete depletion of circulating alloantibody within 6–12 months is entirely possible if synthesis stopped soon after transplantation. Simultaneous depletion of DSA and non-DSA suggests that the effect is global and not specific to DSAs. Alternatively, alloantibody binding to the graft could artificially lower circulating levels below detection thresholds of assays. However, as this would not explain the simultaneous elimination of non-DSAs unless they also bound to the graft, we believe the first possibility is the more likely mechanism.

The second post-transplant profile identified a group that failed to eliminate FCXM, DSA, or non-DSA reactivity against class I and II within the first postoperative year. Persistence of circulating alloantibody predisposed this group for a higher risk of AMR and CR and ultimately poorer graft survival. The critical question is why is the alloantibody not eliminated among group II as occurred among group I? Unfortunately, we do not have an answer. Group II did not demonstrate any unique antibody characteristics before transplant. However, group II demonstrated several unique post-transplant differences that either individually or collectively may predispose this group for CR. First, only group II failed to eliminate alloantibody, which suggests that activation of downregulatory pathways are inhibited in this group. Second, only group II was unable to eliminate DSAs directed against class I and II. Numerous studies show that circulating DSA against either class I or II are deleterious to graft survival. Some studies have suggested that anti-class II DSAs may more aggressively promote CR than anti-class I.10,15 Thus, the perseverance of anti-class II among group II may enhance the chances of developing CR. Third, only group II was unable to eliminate non-DSA directed against class I and II and maintained significantly high levels of non-DSA against class I and II. The precise role of non-DSAs in allograft survival is unclear but there is growing evidence that they may contribute to allograft destruction by crossreactive recognition of shared epitopes on the allograft. If this occurred among group II, then the high cumulative burden of non-DSA levels combined with sustained DSA levels may escalate the probability of CR.

Interestingly, despite alloantibody persistence, nearly half of group II remained AMR and CR free and had excellent graft outcome. This is consistent with the historic inability to show 100% causal relationship between detection of circulating alloantibody and active disease. Because pretransplant alloantibody characteristics were equivalent between these subgroups (as well as group I), they were useless in forecasting post-transplant antibody profiles or clinical outcomes. Our data suggest that (1) alloantibody persistence is not an automatic trigger for AMR and (2) AMR without antibody persistence is not an automatic trigger for CR (group I patients with AMR have not yet demonstrated CR). Our interpretation is that alloantibody persistence during the first year heightens the risk of AMR. However, alloantibody alone is an insufficient trigger for AMR. The extended period of alloantibody persistence before AMR detection is similar to the lag reported in other studies between de novo alloantibody appearance and rejection. We suggest that perhaps certain minimum conditions must be met in order to initiate alloantibody-mediated tissue damage. Perhaps alloantibody must be composed of complement fixing isotypes at some minimal level in order to show lytic activity. Similarly, perhaps donor HLA expression must exist at some minimal density in order to be an adequate target.

### Table 5 | Pretransplant antibody profiles between group II patients who did or did not experience AMR

| Antibody Profile | Without AMR | With AMR | P-value |
|------------------|-------------|----------|---------|
| 1. T-FCXM (channels) | 94 ± 49 | 141 ± 48 | 0.5 |
| 2. B-FCXM (channels) | 193 ± 29 | 117 ± 25 | 0.07 |
| 3. T-PRA (%) | 56 ± 23 | 64 ± 13 | 0.7 |
| 4. B-PRA (%) | 11 ± 8 | 49 ± 14 | 0.1 |
| 5. DSA MFI ± s.d. | | | |
| Class I | 5601 ± 3601 | 5950 ± 1598 | 0.9 |
| Class II | 4376 ± 1376 | 4978 ± 938 | 0.8 |
| 6. Total non-DSA MFI ± s.d. | | | |
| Class I | 86,070 ± 84,080 | 63,720 ± 27,800 | 0.7 |
| Class II | 15,120 ± 12,870 | 39,250 ± 15,040 | 0.4 |

Abbreviations: AMR, antibody-mediated rejection; DSA, donor-specific antibody; FCXM, flow cytometric crossmatch; MFI, mean fluorescent intensity; PRA, panel-reactive antibody.
Confounding variables such as ongoing immune activating events (like cellular rejection or infection) or nonimmune-mediated allograft damage may boost inadequate alloantibody/donor antigen presentation and trigger rejection. We suggest that if certain minimal conditions are not met, then AMR does not occur, and maintaining high levels of DSA and non-DSA appears clinically irrelevant at least for the short term. On the other hand, AMR with persistent alloantibody must create a smoldering rejection that is difficult to completely eradicate and upon rebound leads to chronic disease. It is hoped that early detection would facilitate AMR reversal. As this study was a blinded protocol in which alloantibody tests were not reported, medical intervention was based upon clinical dysfunction and thus the earliest intervention was not possible.

The heightened sensitivity of the FCXM relative to cytotoxic crossmatching has always generated controversy concerning its relevance and concerns that some reactions are falsely positive. In this study, false-positive FCXMs are unlikely as we only included patients for analysis when the preoperatively positive FCXM reactions were validated by identifiable DSA. Similar to a recent report, we found that use of single antigen bead testing by luminometry detected DSA in virtually all patients with positive FCXM.30

Our goal was to develop a post-transplant monitoring protocol that was clinically informative and manageable from a laboratory perspective. We focused on the patient group most problematic to this center; the presensitized patients with preoperatively positive FCXM. We identified two distinct post-transplant alloantibody profiles that exhibited different clinical outcomes. Identification of patients at greater risk of CR should facilitate earlier diagnosis.

The study results allow us to consider strategies to alter the post-transplant course of patients with persistently positive FCXM and DSA. One strategy would be to avoid transplanting patients with positive FCXM and high levels of DSA. However, complete avoidance would deny transplants to the majority of patients who will develop a group I profile that unfortunately cannot be predicted pretransplant. If transplantation proceeds, preemptive immunomodulation (intravenous immunoglobulin, plasmapheresis, rituximab, Velcade) at the time of transplant might promote antibody depletion in patients who would develop a group II pattern. Perhaps drug tapering protocols should be reconsidered in patients with group II profile. We feel longitudinal monitoring is more effective than testing only when there is evidence of graft dysfunction. Implementing protocol biopsies with C4d staining would be informative. Antibody persistence or elevation coupled with rising serum creatinine or histological changes in the graft should be treated aggressively. Patients with persistent alloantibody who experience AMR would likely benefit from antibody-depleting therapies (perhaps Velcade and rituximab) to reduce or eliminate alloantibody burden that might minimize AMR recurrence. Last, alloantibody monitoring should continue beyond 1 year for patients with persistent antibody.

**patients and methods**

**Patients**

A total of 308 patients underwent renal transplantation. All had negative T and B cytotoxic crossmatches. FCXM was performed before transplant. Excluding patients who had undergone desensitization, 69 patients (22%) elaborated a positive preoperative FCXM. For these patients, blood was collected quarterly during the first post-transplant year. Serial specimens were unavailable for eight patients who were excluded from subsequent analysis. Clinical outcome for the remaining 61 patients was obtained from chart review. Immunosuppression consisted of thymoglobulin (1.5 mg/kg daily for 4 days), SoluMedrol (1 g at the day of transplant, 500 mg on day 1, 60 mg on day 2, tapered 5 mg/day until 20 mg/day, then tapered 5 mg each month to 10 mg/day), mycophenolate mofetil (1 g b.i.d.), and tacrolimus (0.1 mg/kg daily). Graft failure was defined as return to dialysis. Deaths with functioning graft were censored. Protocol biopsies were not performed. Biopsies were done for clinical indications (for example, serum creatinine increased >25% above baseline, nephrotic range proteinuria, delayed graft function). Rejections were biopsy proven with histological classification following Banff ’97 criteria with updates.29 AMR was based upon histological findings of peritubular capillaries filled with polymorphonuclear cells, C4d-positive stain in peritubular capillaries (staining available starting 2007), histological evidence of endothelialitis with positive FCXM, and/or finding of DSAs. CR was identified by interstitial fibrosis, fibrointimal arterial hyperplasia of arterioles, tubular atrophy and glomerulosclerosis, or membranoproliferative glomerular changes. AMR was treated with alternate day Cytogam and plasmapheresis. The study was conducted as a blinded investigation. The results were not reported and did not influence clinical management. All practices conformed to the institutional review board guidelines for the protection of human subjects.

**T- and B-FCXM**

Multicolor FCXM was performed as previously described.27,29 Channels shifts ≥50 were considered positive for T-FCXM and B-FCXM.

**Solid-phase testing**

Pretransplant panel-reactive antibody was determined using FlowBeads (One Lambda, Canoga Park, CA). Because various techniques for specificity determination were used throughout the years, data were not comparable. Therefore, to make data comparable and more sensitive, we (1) retested donor HLA using SSP (One Lambda) to identify minor histocompatibility antigens (Cw, DP) not determined originally and (2) retested sera collected pretransplant and 6 and 12 months post transplant using one lot of single antigen bead assay on Luminex platform. Specificities and mean fluorescent intensities were determined following the manufacturer’s instructions (GenProbe, San Diego, CA). DSAs are specificities against donor HLA antigens. Non-DSAs are specificities directed against HLA antigens other than the...
donor’s. Positive FCXM in the absence of anti-HLA specificities were considered non-HLA antibodies.

**Statistics**
Results were analyzed using Kaplan–Meier survival curve, two-way t-tests, and χ² test using Graphpad Prism Software (Graphpad Software, San Diego, CA). Significance was defined as $P \leq 0.05$.

**DISCLOSURE**
All the authors declared no competing interests.

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