Clinical Relevance of Anti-HLA Antibodies Detected by Flow-Cytometry Bead-Based Assays—Single-Center Experience

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ABSTRACT: The purpose of this study was to define the incidence, dynamics, and profiles of anti–human leukocyte antigen antibodies (HLA-Abs) produced after kidney transplantation and their impact on graft outcome. A total of 72 first cadaver donor kidney recipients were prospectively monitored for the development of HLA-Abs using bead-based flow-cytometry assays (One Lambda FlowPRA tests). Sixteen recipients (22.2%) developed HLA-Abs after transplantation (class I, n = 7; class I+II, n = 6; class II, n = 3), in most cases (81.25%) within the first 2 weeks post-transplantation. A strong association between alloantibody presence and delayed graft function (Chi-square = 7.659, \( p < 0.01 \)), acute rejection (Chi-square = 14.504, \( p < 0.001 \)), chronic rejection (Chi-square = 12.84, \( p < 0.001 \)), and graft loss (Chi-square = 20.283, \( p < 0.001 \)) was found. Patients with higher alloantibody titers experienced acute rejections and even early graft loss, compared with those with lower titers for whom chronic rejections were more common. Immunologic complications occurred in recipients with both donor-specific and cross-reacting groups or non–donor-specific antibodies alone. A positive correlation (Pearson correlation, 0.245; \( p < 0.05 \)) between HLA class I amino acid triplet incompatibility and alloantibody production was observed, mainly resulting from immunogenic triplotypes. Given the results obtained in this study, an alloantibody testing algorithm has been designed and implemented for routine monitoring and to define optimally the alloantibody reactivity in kidney transplant recipients. Human Immunology 67, 787–794 (2006). © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: Anti-HLA antibodies; kidney transplant; rejection

ABBREVIATIONS
CREG cross-reacting groups
DGF delayed graft function
DSA donor-specific antibodies
HLA human leukocyte antigen
HLA-Abs anti–human leukocyte antigen antibodies

INTRODUCTION
Renal transplantation is associated with multiple complications, some of which may cause irreversible loss of graft function. Despite reliable pretransplant screening methods and improvement of immunosuppression therapy, a considerable number of kidney allografts are still lost because of cellular and/or humoral mediated rejections. The presence of preformed human leukocyte antigen (HLA)—reactive antibodies in recipient serum before transplantation has long been recognized as a prominent risk factor for a generally worse graft outcome. Since the first description [1, 2] of an association between post-transplantation HLA antibodies and failures, there has been accumulating evidence for the role of de novo alloantibody production on the graft outcome (reviewed in [3, 4]). Over the past years many studies have been devoted to the relevance of alloantibodies detected after transplantation by more sensitive solid-phase techniques. In

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most publications, significant association of antibodies with acute rejection [5–11], number of rejection episodes [12, 13], chronic rejection [8, 9, 12], and reduced graft survival [14–16] have been reported. In addition, HLA class II–reactive alloantibodies, but not class I–reactive alloantibodies, have been described as significant risk factors for early (<1 year) graft loss [15] and for chronic allograft rejection [13]. Furthermore, donor-specific HLA antibodies (DSA) produced after transplantation have been shown to correlate with immunologic complications and graft failure [13, 16–18]. A strong association of non–donor-specific antibodies with rejection has also been noted [19–21]. Despite increasing recognition of the role of posttransplantation humoral alloreactivity in graft outcome, there is still debate regarding the clinical relevance of anti-HLA antibodies detected by sensitive solid-phase assays. In this study, we defined the incidence, dynamics, and profiles of HLA-directed antibodies produced after transplantation and their impact on graft outcome in kidney recipients using sensitive and specific flow-cytometry bead-based techniques. In addition, we propose a strategy for monitoring and assessment of humoral alloreactivity after transplantation.

**MATERIAL AND METHODS**

**Patients**

The study group consisted of 72 patients (47 male and 25 female; mean age at the time of transplantation 38.7 ± 11.8 years, range 18–57 years), who received their first cadaveric kidney transplant between October 1998 and January 2004. By design, all recipients had no detectable alloantibodies at the time of the routine alloantibody screening by both cytotoxicity and flow cytometry tests since they had been included in the waiting list. A history of sensitizing events (pregnancy, n = 4; transfusion, n = 28; pregnancy and transfusion n = 12) characterized 44 (61.1%) patients. Immediately before transplantation, donor-specific antibodies were excluded by both cytotoxicity and flow cytometry (T- and B-cell) crossmatching. The majority of transplantations (n = 53) were performed with 3 or 4 HLA-A, -B, -DRB1, and -DQB1 split mismatches. Cold ischemia time (CIT) of the transplanted kidney ranged from 12 to 24 hours. The follow-up time was from the day of transplantation till the graft was lost or 1–5 years after transplantation for the patients with functioning graft. A total of 696 serum samples were tested. Sera were collected at the early posttransplantation period (days 0, 7, 14, 21, 28), during routine posttransplantation evaluation, and during hospital admission for postransplantation complications. A total of 37 (51.4%) patients had delayed graft function (DGF). Eight recipients (11.1%) experienced acute and 11 (15.3 %) chronic biopsy-proven rejections. Graft failure, defined as loss of kidney function, was observed in 13 (18.1%) recipients. Nine patients (6.48%) died with a functioning graft. All patients received triple (cyclosporine A, prednisone, immuran/mycophenolate mofetil) immunosuppressive therapy. Mild to moderate rejections were treated with pulse steroids. Both severe and steroid-resistant rejections were treated with antithymocyte globulin.

**Flow-Cytometric Alloantibody Analysis**

Flow-cytometric detection of HLA-specific antibodies was performed by FlowPRA™ Screening Test (FL12-60; One Lambda, Canoga Park, CA, USA). FlowPRA™ Specific Tests (FL1SP, FL1SP44, FL1HD, FL2SP; One Lambda) were used for definition of HLA antibody specificity in the positive sera. The tests were performed according to the instruction of the manufacturer. In brief, 5 μl of FlowPRA microparticles were admixed with 20 μl of patient serum and incubated for 30 minutes at room temperature. Control non–MHC-coated beads were included in each sample to monitor the nonspecific interaction of the testing serum with the beads. After washing, the beads were stained with 100 μl of pretitered FITC-conjugated F(ab)’2 fragment of goat antihuman IgG for an additional 30 minutes. After a final wash, 500 μl of wash buffer was added per tube and analyzed on a FACSCalibur (Becton Dickinson, Immuno cytometry Systems, San Jose, CA, USA) flow cytometer. Screening results were recorded as positive when ≥5% of class I and/or class II beads exhibited fluorescence above the negative control serum and/or a significant change in the histogram architecture compared with the negative serum control. Specificity results were scored according to the scheme included in the kits. HLA specificities were determined by referring to the FlowPRA data sheets and software.

The positive sera were tested at serial dilutions from 1/2 to 1/1024 using FlowPRA Screening Test. The final dilution showing positive result for HLA antibody reactivity was determined as titer of antibodies.

**Statistical Analysis**

Comparisons across groups were performed by Chi-square and Fisher Exact tests where appropriate. Correlation between the presence of alloantibodies and HLA triplet mismatching was analyzed by Pearson correlation test. Association analysis of graft survival with alloantibodies was performed by Kaplan-Meier method and the statistical significance was assessed by log-rank test. A p-value <0.05 was accepted as statistically significant. These analyses were performed with SPSS version 10.0 software (SPSS Inc., Chicago, IL).
RESULTS

Anti-HLA antibody reactivity was detected in 16 of the 72 patients (22.2%) after transplantation. Of these, seven patients (9.7%) had alloantibodies that were HLA class I reactive only, three patients (4.2%) had class II–reactive alloantibodies only, and six patients (8.3%) had both HLA class I and class II reactive alloantibodies. In most cases (13 of 16; 81.25%), alloantibodies were observed in the early posttransplantation period (in four recipients at day 7 and in nine recipients at day 14 posttransplantation). Antibodies were detected 3 months after transplantation in one patient and 4 years posttransplantation in 2 patients. All but one of the antibody-positive subjects had a history of immunizing events (pregnancy, n = 1; transfusion, n = 11; pregnancy and transfusion n = 3). In 14 of the 37 recipients with delayed graft function, anti-HLA antibodies were identified and a significant, positive correlation was observed between alloantibody presence posttransplantation and DGF association (Chi-square = 7.659, p < 0.01). Recipients with posttransplantation HLA-reactive antibodies were more likely to have acute allograft rejection as compared with those with no detectable alloantibodies (37.5% vs 3.6%, Chi-square = 14.504, p < 0.001). A similar association was also observed with chronic rejection (43.75% vs 7.1%, Chi-square = 12.84, p < 0.001). Among patients who demonstrated alloantibody reactivity, nine of 16 (56.25%) lost their grafts because of immunologic causes, compared with only four of 56 (7.1%) in the antibody-negative group (Chi-square = 12.84, p < 0.001). A decline in graft survival rate after the third year posttransplantation was observed in antibody-positive recipients in comparison to those without detectable humoral alloimmunization, although this was statistically not significant (p > 0.05) (Figure 1).

When the persistence of HLA-directed antibodies was analyzed, it was noted that 10 patients were consistently positive throughout the observation period, compared with six patients who lost reactivity 7 months to 3 years after transplantation (Table 1). Longitudinal evaluation of sera from 13 positive recipients demonstrated stability in titers for eight individuals as long as antibodies were present. Among these eight subjects, six had lower levels (titer 1/4) of anti-HLA class I and/or class II antibodies, four of whom developed chronic rejection. The two remaining patients had anti-HLA class I antibody titers at a 1/8 sera dilution. Of these, one patient experienced an acute rejection but died of brain stroke with a functioning graft. The other one developed a chronic rejection.

Sera from five patients showed variations in alloantibody levels during posttransplantation monitoring (Table 1). In this subgroup, the peak-reactive sera from two recipients (patient 9 with HLA class I, and patient 10 with class II reactivity) had antibody titers greater than 1/1024 dilution. Both patients experienced severe irreversible acute rejections and lost their grafts. For patient No.11 an increase of anti-HLA class II antibody titer from 1/4 at day 7 to 1/16 at day 14 after transplantation was noted, which was associated with an acute rejection episode. In the remaining two patients, a progressive decrease of titers and eventual disappearance of antibodies were detected within the observation period. In one patient (patient 12), no immunologic complications were observed, as compared with another recipient (patient 13) in whom a chronic allograft nephropathy was diagnosed 3 years posttransplantation.

Using a variety of specific tests, including beads coated with single antigens, we determined donor-specific reactivity for 12/16 patients: seven recipients had anti-HLA class I, four had anti-HLA class II, and one had both class I and class II donor-specific HLA antibodies (Table 1). Alloreactivity spreading within the donor cross-reacting groups (CREG) was defined in three patients with antibodies against allograft mismatched HLA class I antigens. Similarly, among the individuals with anti-HLA class II DSA, additional reactivity against non–donor specificities was observed (n = 3). In the remaining four patients, antibodies reacting specifically with an HLA antigen of the donor could not be assigned. Donor-specific HLA antibodies most likely resulted in rejections leading to graft loss (Table 1). However, chronic rejections and graft failures were also observed in recipients with intra-donor and
extra-donor CREG alloreactivity alone. In consideration of these results, we performed an analysis of alloantibody production with respect to specific amino acid differences between HLA of donor and recipient using HLAMatchmaker algorithm [22, 23]. Details of donor-recipient compatibility at the structural level are shown in Table 2. A significant correlation between triplotype mismatches and HLA class I antibody appearance was observed (Pearson correlation, 0.245; \( p < 0.05 \)), mainly because of immunogenic class I amino acid triplets (Pearson correlation, 0.332; \( p < 0.05 \)). HLA class II triplet incompatibility did not correlate significantly (\( p > 0.05 \)) with alloantibody production in the posttransplantation period.

**DISCUSSION**

In the study described here, we summarize our experience with bead-based flow cytometry technology in the posttransplantation alloantibody monitoring of recipients of cadaveric kidneys. We have also extended our analysis on the clinical relevance of the humoral alloimmune response after transplantation. Post-transplant alloantibodies were detected in only 22.2% of our study population, which is in agreement with the data reported by others using the same methodology [13, 14]. In most cases (81.25%), anti-HLA antibodies were observed within the first 2 weeks posttransplantation and correlated with delayed graft function, immunologic complications, and reduced graft survival. Preformed HLA-reactive antibodies have been found to be associated with DGF [24]. A possible explanation of our findings in which a correlation was observed between DGF and posttransplantation alloantibody development is that reperfusion and prolonged cold ischemia time (a chief factor leading to DGF) induce activation of endothelium, impaired cytokine gene expression [25, 26], release of
proinflammatory cytokines [27, 28], and upregulation of HLA and adhesion molecules [29, 30, 31]. These events lead to stimulation of the immune response in the early posttransplantation period and, as a consequence, to alloantibody production. The antibodies could be truly de novo produced in response to the allograft. However, in some instances even in the absence of detectable pretransplantation sensitization reactivation of memory B cells from sensitizing events in the patient’s history may facilitate the alloantibody production in the early days posttransplantation. The influence of time of antibody appearance on graft outcome is a controversial issue. Our observations are not in agreement with those of Abe et al. [32] assessing the ineffectiveness of alloantibodies detected within the first month after transplantation in influencing graft survival. However, they are in agreement with other studies [8, 13] showing the detrimental role played by an early humoral immune response on graft outcome.

Our study of HLA antibody titers suggests that patients with higher titers experience severe acute rejections and even early graft loss, compared with patients with lower titers, for whom chronic rejections are more common. Similar associations have been noted for kidney recipients with alloantibodies detected before transplantation [33] and after allograft loss [34]. We speculate that low levels of anti-HLA antibodies probably do not lead to immediate and severe effects compared with high-titer antibodies. Patients with antibodies may survive for long periods with well-functioning grafts [4], which is partly supported by our data on graft survival rate. Lee et al. [19] also found that years could elapse between antibody appearance and graft rejection. However, the alloantibodies could bind to the endothelium, causing progressive injury and deterioration of graft function. In some instances the antibodies may even disappear, as observed in several of our study patients; but the damage is inevitable gradual, ultimately resulting in graft loss [4]. Our data suggest that the titer and the course of alloantibody response might be relevant to the level of risk for allograft loss. However, the impact of therapy on alloantibody decrease and/or disappearance could not be excluded.

Specificity analysis demonstrated that most posttransplantation anti-HLA antibodies are donor reactive and confirmed previous assertions for associations of both class I DSA [14, 15, 17, 35, 36] and class II DSA [14, 17, 37, 38] with rejections and generally worse graft outcomes. Immunologic complications occurred in our kidney recipients with CREG or non–donor-specific antibodies alone, which is consistent with other observations on alloantibody specificity pattern and graft outcome [19, 20]. For reasons still unknown, extra specificities are often produced upon immunization [4] and HLA antibodies directed toward non-donor-specific antigens are often observed in patients who have undergone transplantation. Several explanations have been proposed for these observations. Donor-specific antibodies could be absorbed by the kidney and therefore not present in the circulation [4]. The presence of soluble HLA antigens in the serum may complex with HLA antibodies, interfering with measurement of alloantibodies [39]. It could not be excluded that other sensitizing events that are not related to transplantation can account for the posttransplantation detection of non–donor-specific HLA antibodies. Although without detectable sensitization pretransplant, the majority of our antibody-positive patients had a history of alloimmunizing events. Non–donor-specific antibodies may come up as a consequence of nonspecific triggering of memory response by inflammation in some of these recipients. Nonetheless, when antibodies against non-donor HLA specificities are detected, they should be analyzed by the HLAMatchmaker algorithm. If these antibodies recognize amino acid triplets on donor-mismatched antigens, they should be defined as donor triplet–specific antibodies [19] and might be clinically relevant for posttransplantation immunologic complications.

Based on this assumption, we applied the triplet analysis to study the combined effect of all mismatched donor HLA antigens on alloantibody production. Thus, we confirmed previously reported positive correlations

### TABLE 2

<table>
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<th>Number of mismatched donor triplets</th>
<th>HLA-A Abs(+) n (%)</th>
<th>HLA-A Abs(−) n (%)</th>
<th>HLA-B Abs(+) n (%)</th>
<th>HLA-B Abs(−) n (%)</th>
<th>HLA-DR Abs(+) n (%)</th>
<th>HLA-DR Abs(−) n (%)</th>
<th>HLA-DQ Abs(+) n (%)</th>
<th>HLA-DQ Abs(−) n (%)</th>
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<td>11 (19.7)</td>
<td>2 (12.5)</td>
<td>9 (16.1)</td>
<td>2 (12.5)</td>
<td>24 (42.9)</td>
<td>5 (31.25)</td>
<td>26 (46.4)</td>
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<td>6 (37.5)</td>
<td>14 (25.0)</td>
<td>8 (50.0)</td>
<td>24 (42.9)</td>
<td>7 (43.75)</td>
<td>22 (39.3)</td>
<td>7 (43.75)</td>
<td>15 (26.8)</td>
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<tr>
<td>6–10</td>
<td>5 (31.25)</td>
<td>26 (46.4)</td>
<td>4 (25.0)</td>
<td>20 (35.7)</td>
<td>7 (43.75)</td>
<td>10 (17.8)</td>
<td>3 (18.75)</td>
<td>15 (26.8)</td>
</tr>
<tr>
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<td>4 (25.0)</td>
<td>5 (8.9)</td>
<td>2 (12.5)</td>
<td>3 (5.3)</td>
<td>—</td>
<td>—</td>
<td>1 (6.25)</td>
<td>—</td>
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**Abbreviations:** Abs = antibodies; HLA = human leukocyte antigen; n = number of cases.

* Defined by HLAMatchmaker program (http://spis.upmc.edu).
[20, 40] between the number of HLA class I triplet mismatches and the appearance of alloantibodies in the posttransplantation period. In addition, we demonstrated that this association was mainly caused by immunogenic triplet incompatibilities. Implementation of triplet sequence mismatches in kidney allograft allocation scheme has been proposed, particularly for highly sensitized patients [41] and those who are candidates for retransplantation [40]. However, if such an analysis is not performed pretransplantation, the immunogenic amino acid mismatches could be evaluated after transplantation to assess better the risk of antibody-mediated rejection.

Because humoral rejection is emerging as a leading cause of graft loss, HLA-directed antibodies produced after transplantation should be integrated into the patient assessment algorithm. Given the results obtained in this study, we have designed such an algorithm (Figure 2), which is based on sensitive methods for alloantibody detection and timely sample procurement; we have implemented this algorithm in the clinical practice for routine monitoring and to optimally define humoral alloreactivity in kidney recipients. Using alloantibody testing alone we cannot capture all of the subtleties encountered in an individual case. However, adoption of an alloantibody monitoring algorithm in the routine transplant recipient assessment appears to be justified, as it could improve early diagnosis of alloantibody-mediated complications and monitoring of kidney transplant development, resulting in more timely therapeutic intervention and ultimately in improved graft outcomes.

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