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# Flow Cytometric Crossmatching in Primary Renal Transplant Recipients with a Negative Anti-Human Globulin Enhanced Cytotoxicity Crossmatch

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*Abstract.* Flow cytometric crossmatching (FCXM) and panel reactive antibody (PRA) screening techniques are more sensitive than anti-human globulin enhanced cytotoxicity (AHG-CDC) techniques at detecting anti-HLA antibodies. The clinical significance of a positive FCXM in primary renal transplant recipients with a negative AHG-CDC crossmatch is unclear. We performed retrospective FCXM and flow cytometric panel reactive antibody (FlowPRA) determinations in primary renal transplant recipients with a negative T cell AHG-CDC crossmatch and a negative B cell CDC crossmatch pretransplant. Eighteen (13%) of 143 patients exhibited a positive retrospective T cell FCXM. Of these patients, six (33%) experienced early graft loss with explant histology, demonstrating antibody-mediated rejection in five of six cases. The 12 patients with positive T cell FCXM who maintained their grafts experienced more adverse events posttransplant, including more early, steroid-resistant, and recurrent rejection. Furthermore, in a subgroup of patients undergoing protocol biopsies, those with a positive T cell FCXM exhibited more subclinical rejection. Anti-HLA antibodies were detected by FlowPRA in all 18 patients with a positive T cell FCXM, whereas AHG-CDC PRA detected antibodies in only 8 of 18 patients. Therefore, flow cytometric techniques identify sensitized primary renal transplant recipients undetected by AHG-CDC techniques. In those patients, a positive T cell FCXM is associated with an increased risk of early graft loss due to antibody-mediated rejection and may represent a relative contraindication to transplantation. Moreover, those patients are also at increased risk of severe and recurrent rejection, which may carry implications for long-term graft outcomes.

Flow cytometric crossmatching (FCXM) and panel-reactive antibody (PRA) screening are capable of detecting anti-HLA antibodies undetected by both standard cytotoxicity (CDC) techniques and the more sensitive anti-human globulin enhanced cytotoxicity (AHG-CDC) technique (1,2). In studies that have examined AHG-CDC crossmatch negative renal regraft recipients, an association between a retrospective positive FCXM and poor graft outcomes has been consistently demonstrated (3–5). The clinical significance however, of anti-HLA antibodies detected by FCXM in primary renal transplant recipients with a negative AHG-CDC crossmatch is unclear (5–7).

The issue of FCXM in primary renal transplant recipients is clouded by the fact that many of the studies that have noted adverse events have studied retrospective FCXM in CDC crossmatch–negative recipients (8–11), rather than recipients screened by the more sensitive AHG-CDC method (12). The clinical relevance of such studies can justifiably be called into question by transplant centers that perform the more sensitive AHG-CDC technique. Only three studies have examined FCXM in AHG-CDC crossmatch–negative primary transplant recipients, and these have arrived at conflicting conclusions (5–7).

The goals of this study were to determine whether FCXM and flow-cytometric PRA screening techniques would identify alloimmunized primary transplant recipients undetected by AHG-CDC crossmatching and AHG-CDC PRA. Furthermore, we sought to determine whether these sensitized primary transplant recipients experienced more adverse immunologic events posttransplant.

## Materials and Methods

### *Study Population*

Between June 1992 and June 2000, 249 HLA nonidentical primary renal transplants were performed at our center. Retrospective FCXM and flow-cytometric PRA determinations were performed in all patients in whom donor cells and/or recipient sera were available. We were able to study 143 of 249 individuals with FCXM and a further 60 (total = 203) with flow-cytometric PRA. Baseline demographic characteristics for these two cohorts and for the entire patient population are presented in Table 1. Before transplantation, all patients were AHG-CDC T cell and CDC B cell crossmatch negative with current sera, and only one patient exhibited a positive AHG-CDC T

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*Table 1.* Baseline characteristics[a]

| | FCXM ($n$ = 143) | Flow PRA ($n$ = 203) | All Patients ($n$ = 249) | $P$ |
|---|---|---|---|---|
| Recipient age (yr) | 40.6 ± 1.2 | 39.2 ± 1.0 | 39.8 ± 0.9 | NS |
| Age group (<18 yr/≥18 yr) | 14/129 | 21/182 | 25/224 | NS |
| Male/female | 87/56 | 130/73 | 161/88 | NS |
| History of pregnancy | 35 of 56 | 44 of 73 | 56 of 88 | NS |
| Transfusion history | 99 (69%) | 132 (65%) | 155 (62%) | NS |
| Cadaveric donor | 129 (90%) | 155 (76%) | 192 (77%) | <0.001[b] |
| Donor age (yr) | 37.5 ± 1.4 | 38.1 ± 1.0 | 38.0 ± 1.0 | NS |
| CIT (min) | 952 ± 33 | 852 ± 31 | 847 ± 29 | 0.04[b] |
| HLA match (median [range]) | 2 (0 to 5) | 2 (0 to 5) | 2 (0 to 5) | NS |
| Peak AHG-CDC PRA ≥ 10% | 30 (21%) | 38 (19%) | 49 (20%) | NS |
| Current AHG-CDC PRA ≥ 10% | 6 (4%) | 9 (4%) | 14 (6%) | NS |

[a] FCXM, flow cytometric cross-matching; FlowPRA, flow cytometric panel reactive antibody; AHG-CDC PRA, anti-human globulin enhanced cytotoxicity panel reactive antibody.
[b] FCXM *versus* FlowPRA and all patients.

cell crossmatch and CDC B cell crossmatch with historical sera. During the study period, 26 recipients experienced early graft loss (≤2 wk posttransplant). We were able to perform a retrospective FCXM in 20 of these individuals. The remaining 123 of 143 FCXM study patients all maintained their grafts for a minimum of 3 mo.

Immunosuppressive protocols varied in the 143 FCXM study patients. All patients received cyclosporine and prednisone, as well as azathioprine ($n$ = 71), mycophenolate mofetil ($n$ = 42), basiliximab with mycophenolate mofetil ($n$ = 23), and sirolimus ($n$ = 7). Six patients who received cyclosporine, azathioprine, and prednisone also received induction therapy with OKT3 for either high PRA values ($n$ = 4) or as part of a pediatric protocol ($n$ = 2). The 20 patients who experienced early graft loss received cyclosporine, prednisone, and either azathioprine ($n$ = 10), mycophenolate mofetil ($n$ = 6), or basiliximab and mycophenolate mofetil ($n$ = 4). Only one individual with early graft loss received OKT3 induction.

Acute rejection episodes were diagnosed by core needle biopsy or when the serum creatinine increased by ≥10% in the absence of any other etiologies (*e.g.,* obstruction), returning to baseline with pulse corticosteroid ± OKT3 therapy. Protocol biopsies were performed at 1, 2, and 3 mo posttransplant in 69 of the FCXM patients. Biopsies were performed after obtaining informed patient consent and with the approval of the University of Manitoba Research Ethics Committee. Subclinical rejection was diagnosed when the biopsy displayed histologic rejection (Banff 97 ≥ Type Ia) and the serum creatinine was within 10% of baseline values. Other FCXM patients did not undergo protocol biopsies because they were transplanted in an era before protocol biopsies, participating in a randomized protocol biopsy study, or declined to consent to biopsies.

Explant histology was available for review in 23 of the 26 patients who experienced early graft loss during the study period, and we were able to perform a retrospective FCXM for 18 of these 23 cases. Biopsies were recut and examined with a Leder stain, to highlight polymorphonuclear leukocytes in allograft capillaries and by immunohistochemistry for the deposition of the C4d product of complement activation (13,14). C4d staining was performed on deparaffinized sections with a polyclonal anti-C4d antibody (15). An analysis of structure limited to non-necrotic areas of graft tissue was performed, and the cause of graft failure was categorized by a pathologist (S.D.) blinded to all clinical data. Antibody-mediated rejection was identified when a biopsy exhibited the following three features: arteriolar polymorphonuclear leukocytes aggregation; peritubular capillary polymorphonuclear leukocytes accumulation; and linear staining of C4d in small arteries, arterioles, and peritubular capillaries.

### *AHG-CDC Crossmatch and AHG-CDC PRA*

Since 1992, our center has performed AHG-CDC PRA screening and AHG-CDC T cell and extended incubation CDC B cell crossmatching. The following sera have been considered mandatory for final crossmatching: (*1*) a current sample within 1 mo of transplantation, (*2*) historical sera including the peak PRA sample and all historically high PRA sera, and (*3*) all posttransfusion sera. AHG-CDC and CDC crossmatch techniques were identical to those described elsewhere (16). A positive crossmatch was identified when cell lysis detected by uptake of eosin dye with phase contrast microscopy was ≥20% of that seen in negative control samples.

PRA screening for anti-HLA antibodies pretransplant was performed monthly and after any potential sensitizing event (*i.e.,* transfusion). PRA trays that contained peripheral blood T cells of known HLA specificity were generated from 50 local donors. The HLA class I antigen frequency was representative of the HLA allelic frequencies within our donor population. The percent PRA was determined by the proportion of wells exhibiting a positive AHG-CDC T cell crossmatch. An AHG-CDC PRA value ≥10% was deemed significant.

### *FCXM and FlowPRA*

Retrospective T and B cell FCXM was performed for all recipients in whom donor cells and recipient sera were available. Splenic lymphocytes were available from 129 cadaveric donors and peripheral blood lymphocytes were obtained from 14 living related donors (Table 1). As in the AHG-CDC crossmatch, the recipient sera used for FCXM included (*1*) a pretransplant sample, (*2*) historical high PRA samples and the peak PRA sample, and (*3*) all posttransfusion sera. All sera used in the FCXM had been represented in the pretransplant AHG-CDC crossmatch.

For the FCXM, 100 μl of a $2.5 \times 10^6$ cells/ml donor cell suspension was mixed with 20 μl of appropriate test and control sera. Samples were incubated for 20 min at 4°C then centrifuged and washed three times with cold phosphate-buffered saline. Fluores-

cence-labeled antibodies (3 μl anti-CD3 PerCP, 3 μl anti-CD19 phycoerythrin, and 20 μl of a working dilution of anti-human IgG F[ab]' FITC) were then added. After a 20-min dark incubation, two wash steps with phosphate-buffered saline were performed, and lymphocytes were resuspended in 500 μl phosphate-buffered saline with 0.05% sodium azide and transferred into tubes for analysis. Three-color flow cytometric analysis was performed with a FACSCalibur instrument (BD Biosciences, NJ). Lymphocytes were gated on the basis of their forward and side-scatter characteristics. With a scale that expressed staining intensity as a linear channel value (0 to 1024), median channel fluorescence for anti-human IgG F(ab)' FITC was quantified on $CD3^+$ T cells and $CD19^+$ B cells. A positive crossmatch was identified when the sample median fluorescence intensity exceeded that of negative control values by 3 SD. SD were derived by performing negative control FCXM with sera from 12 AB-negative nontransfused males and lymphocytes from 25 healthy donors (data not shown). A positive T cell FCXM and a positive B cell FCXM represented median channel shift values of ≥40 and ≥100, respectively.

Pretransplant serum samples were analyzed by both the AHG-CDC PRA technique and by a flow-cytometric technique that used microbeads coated with purified HLA antigens (FlowPRA, One Lambda, Canoga Park, CA) (2,17). These beads consist of a pool of 30 HLA class I– and 30 HLA class II–coated beads, representing the common HLA antigens. Five microliters of class I and II beads were incubated with 20 μl of recipient sera for 30 min. After two washes, 1 μl of anti-human IgG F(ab)' FITC was added and allowed to incubate for 30 min. After two further washes, the sample was analyzed by flow cytometry. Class I and II beads were gated on the basis of their differing light-scatter characteristics. Anti-HLA antibodies captured on the bead surfaces were identified by comparing fluorescence intensity in patient sera to positive and negative control sera.

### *Statistical Analyses*

Values reported are mean ± SEM or, where indicated, as medians and ranges. $P \leq 0.05$ was considered to be significant, and values ≥0.10 were reported as NS. All statistical analyses were performed by use of Statview 5.0 software (SAS Institute, Inc., Cary, NC). Tests of association ($\chi^2$ and Fisher's exact test) were used for comparison of categorical variables, whereas the *t* test was applied to comparisons of continuous variables.

## Results

Donor cells and recipient sera were available for retrospective FCXM in 143 of 249 AHG-CDC crossmatch–negative primary renal transplant recipients. Eighteen individuals (13%) were found to have a positive T cell FCXM. Eleven of these occurred with current (*i.e.*, pretransplant) sera, and seven were positive only with historical sera. The single patient with a historical positive AHG-CDC T cell crossmatch pretransplant exhibited both a current and a historical positive T cell FCXM. Significant demographic differences were observed between the positive and negative T cell FCXM individuals (Table 2). Patients with a positive T cell FCXM were more likely to be sensitized as detected by AHG-CDC PRA, were more frequently female, and tended to have received transfusions in the past. No significant differences were observed between the two groups in the frequency of induction immunosuppression use or the choice of maintenance immunosuppression agents.

*Table 2.* T cell FCXM patient characteristics

| | Positive T Cell FCXM ($n$ = 18) | Negative T Cell FCXM ($n$ = 125) | $P$ |
|---|---|---|---|
| Recipient age (yr) | 40.3 ± 3.1 | 40.6 ± 1.3 | NS |
| Male/female | 5/13 | 82/43 | 0.002 |
| History of pregnancy | 10 of 13 | 25 of 43 | NS |
| Transfusion history | 89% | 66% | 0.06 |
| Cadaveric donor | 89% | 90% | NS |
| Donor age (yr) | 37.6 ± 3.6 | 37.5 ± 1.5 | NS |
| CIT (min) | 937 ± 83 | 954 ± 36 | NS |
| HLA match (median [range]) | 2 (0 to 3) | 2 (0 to 5) | NS |
| Peak AHG-CDC PRA ≥ 10% | 8 (44%) | 22 (18%) | 0.02 |
| Current AHG-CDC PRA ≥ 10% | 3 (17%) | 3 (2%) | 0.03 |
| OKT3 induction | 2 (11%) | 4 (3%) | NS |
| Adjunctive immunosuppression[a] | | | |
| azathioprine | 12 (67%) | 59 (47%) | NS |
| mycophenolate mofetil | 4 (22%) | 38 (30%) | NS |
| basiliximab and mycophenolate mofetil | 2 (11%) | 21 (17%) | NS |
| sirolimus | 0 | 7 (6%) | NS |

[a] In addition to cyclosporine and prednisone.

### *A Positive T cell FCXM is Associated with Early Graft Loss Due to Antibody-Mediated Rejection*

Of the 18 positive T cell FCXM patients, 6 (33%) experienced graft loss within 2 wk of transplantation (Table 3). Five of these patients (three current positive T cell FCXM and two historical positive T cell FCXM) exhibited histologic features of antibody-mediated rejection (Table 4). The sixth patient experienced an extensive iliofemoral arterial thrombosis on the side of the transplant anastomosis that became clinically apparent upon recovery from anesthesia. The infarcted graft was

*Table 3.* T cell FCXM and adverse events post-transplant

| | Positive T Cell FCXM ($n$ = 18) | Negative T Cell FCXM ($n$ = 125) | $P$ |
|---|---|---|---|
| Early graft loss (≤2 wk) | 6 (33%) | 14 (11%) | 0.01 |
| No early graft loss | 12 (67%) | 11 (89%) | |
| Early rejection (0 to 2 wk) | 8 (67%) | 31 (28%) | 0.02 |
| OKT3 rescue | 3 (25%) | 6 (5%) | 0.04 |
| ≥2 rejections 0 to 3 mo | 8 (67%) | 34 (31%) | 0.02 |
| Number of rejections 0 to 3 mo median (range) | 2 (0 to 4) | 1 (0 to 4) | 0.007 |

*Table 4.* Early graft loss, T cell FCXM, and explant histology

| | Antibody-mediated Rejection | Non-immunologic Graft Loss | *P* |
|---|---|---|---|
| Positive T cell FCXM ($n = 6$) | 5 | 1 | 0.01 |
| Negative T cell FCXM ($n = 12$) | 1 | 11 | |
| FCXM not done ($n = 5$) | 4 | 1 | |

removed within 8 h of implantation, and no features of antibody-mediated rejection were seen.

Histology was available in 12 patients with early graft loss and a negative T cell FCXM. Only one of these individuals exhibited features of antibody-mediated rejection (Table 4). Histologic features of acute cellular rejection were also prominent in this case (*i.e.,* tubulitis, interstitial mononuclear cell infiltrates, and endothelialitis), and no anti-HLA antibodies were detected pretransplant with either AHG-CDC PRA or FlowPRA (*vide infra*). The remaining 11 patients with a negative T cell FCXM all appeared to have lost their grafts because of either a venous or an arterial thrombosis.

Explant histology was reviewed in an additional five patients who had a negative AHG-CDC crossmatch and early graft loss. Because of a lack of donor cells or recipient sera, however, we were unable to perform either a retrospective FCXM or FlowPRA in these cases. Four of these patients also exhibited features of antibody-mediated rejection (Table 4).

### *A Positive T Cell FCXM is Associated with Early, Severe, and Recurrent Rejection*

One hundred and twenty-three patients who underwent a retrospective FCXM kept their grafts beyond the early posttransplant period and could be evaluated for the incidence of rejection in the first 3 mo posttransplant. Of these, 12 individuals exhibited a positive retrospective T cell FCXM, and 111 were negative. The incidence of clinical events in these two groups is presented in Table 3. The 12 patients with a positive T cell FCXM experienced more adverse events, including early rejection episodes, steroid-resistant rejection that required OKT3 rescue, and multiple acute rejections. Indeed, all but 1 of the 12 positive T cell FCXM patients experienced at least one acute rejection during the first 3 mo posttransplant, and the majority experienced either multiple acute rejections, steroid-resistant rejection, or both. Maintenance immunosuppression in these 12 patients included cyclosporine, prednisone, and either azathioprine ($n = 9$) or mycophenolate mofetil ($n = 3$). Adverse events occurred with similar frequency in the azathioprine-treated patients and those treated with mycophenolate mofetil (data not shown).

### *Subclinical Rejection and a Positive T cell FCXM*

Sixty-nine FCXM study patients, including 9 of the 12 positive T cell FCXM patients who kept their grafts, underwent protocol biopsies at 1, 2, and 3 mo posttransplant (Table 5). The biopsy rates at these time periods were 91% (63/69), 94% (65/69), and 89% (62/69), respectively. A positive T cell FCXM was associated with a greater prevalence of subclinical rejection at 1 mo and with ≥2 subclinical rejections in the first 3 mo posttransplant.

A significant number of patients with subclinical rejection at 1 mo (6/17) had experienced an acute rejection during the preceding weeks. Notably, an acute rejection episode preceded a subclinical rejection at 1 mo in 4 of 6 positive T cell FCXM patients but only in 2 of 11 negative T cell FCXM patients ($P = 0.10$).

### *FlowPRA Identifies Sensitized Primary Renal Transplant Recipients Undetected by AHG-CDC PRA*

A current (*i.e.*, pretransplant) serum sample for AHG-CDC PRA and FlowPRA determinations was available in 203 of 249 primary transplant recipients. There was a marked difference in the sensitivity of anti-HLA antibody detection between the two techniques (Table 6). AHG-CDC PRA detected antibodies pretransplant in only 9 individuals (4%), whereas FlowPRA detected antibodies in 41 individuals (20%). Eighteen of the patients positive by FlowPRA exhibited isolated anti-HLA class I antibodies, 10 exhibited isolated anti-HLA class II antibodies, and 13 exhibited both class I and class II anti-HLA antibodies. As in patients with a positive T cell FCXM, patients with a positive FlowPRA pretransplant were more likely to be female, have a history of pregnancy, and were more likely to have received transfusions in the past (Table 7).

A positive AHG-CDC PRA did not correlate with clinical events posttransplant. In contrast, a positive FlowPRA (anti-HLA class I and/or II) was associated with an increased rate of early graft loss (27% *versus* 7%, $P = 0.0002$) and with acute rejection during the first 2 wk posttransplant (50% *versus* 30%, $P = 0.03$).

*Table 5.* T cell FCXM and subclinical rejection

| | Subclinical Rejection | | | ≥2 Subclinical Rejections Months 0 to 3 |
|---|---|---|---|---|
| | Month 1 | Month 2 | Month 3 | |
| Positive T cell FCXM ($n = 9$) | 6/9 (67%) | 4/9 (44%) | 1/9 (11%) | 4/9 (44%) |
| Negative T cell FCXM ($n = 60$) | 11/54 (20%) | 13/56 (23%) | 11/53 (21%) | 6/53 (11%) |
| *P* | 0.009 | NS | NS | 0.03 |

*Table 6.* Flow PRA *versus* AHG-CDC PRA

| | Flow PRA Negative | Flow PRA Positive |
|---|---|---|
| AHG-CDC PRA ≥10% | 2 | 7 |
| AHG-CDC PRA <10% | 160 | 34* |

* $P < 0.0001$.

There was a notable difference in anti-HLA antibody detection by the two PRA techniques in the 18 patients with a positive T cell FCXM. Examining both current and historical sera, AHG-CDC PRA identified anti-HLA antibodies in only 8 of 18 patients, whereas FlowPRA detected anti-HLA antibodies in all 18. When the significance of anti-HLA antibodies detected with FlowPRA was examined in patients that lacked donor-specific antibodies (*i.e.*, positive FlowPRA but negative T cell FCXM), a trend toward more early rejection was observed, but this did not reach statistical significance (Table 8). Therefore, the association of a positive FlowPRA with clinical events posttransplant was attributed to the presence of donor-specific antibodies in most patients.

### *An Isolated Positive B cell FCXM Did Not Correlate with Clinical Events*

Twenty-two of 143 (15%) patients were found to have an isolated positive B cell FCXM (*i.e.*, negative T cell FCXM).

*Table 7.* Flow PRA patient characteristics

| | Positive Flow PRA ($n = 41$) | Negative Flow PRA ($n = 162$) | *P* |
|---|---|---|---|
| Recipient age (yr) | 41.1 ± 1.8 | 38.8 ± 1.2 | NS |
| Male/female | 12/29 | 118/44 | <0.0001 |
| History of pregnancy | 23 of 29 | 21 of 44 | 0.006 |
| Transfusion history | 78% | 62% | 0.05 |
| Cadaveric donor | 78% | 76% | NS |
| Donor age (yr) | 38.8 ± 2.4 | 38.0 ± 1.2 | NS |
| CIT (minutes) | 849 ± 62 | 852 ± 36 | NS |
| HLA match (median [range]) | 2 (0 to 5) | 2 (0 to 5) | NS |
| Peak AHG-CDC PRA ≥ 10% | 17 (41%) | 21 (13%) | <0.0001 |
| Current AHG-CDC PRA ≥ 10% | 7 (17%) | 2 (1%) | <0.0001 |
| OKT3 induction | 3 (7%) | 3 (2%) | NS |
| Adjunctive immunosuppression[a] | | | |
| azathioprine | 26 (63%) | 83 (51%) | NS |
| mycophenolate mofetil | 10 (24%) | 52 (32%) | NS |
| basiliximab and mycophenolate mofetil | 4 (10%) | 19 (12%) | NS |
| sirolimus | 1 (2%) | 8 (5%) | NS |

[a] In addition to cyclosporine and prednisone.

*Table 8.* T cell FCXM, Flow PRA, and adverse events

| | Positive T Cell FCXM Flow PRA Positive ($n = 18$) | Negative T Cell FCXM Flow PRA Positive ($n = 14$) | Negative T Cell FCXM Flow PRA Negative ($n = 111$) |
|---|---|---|---|
| Early graft loss (≤2 wk) | 6 (33%)[a] | 3 (21%)[b] | 11 (10%) |
| No early graft loss | 12 | 11 | 100 |
| Early rejection (0 to 2 wk) | 8 (67%)[a] | 5 (45%)[b] | 26 (26%) |
| OKT3 rescue | 3 (25%)[a] | 1 (9%)[b] | 5 (5%) |
| ≥2 rejections 0 to 3 mo | 8 (67%)[a] | 4 (36%)[b] | 30 (30%) |

[a] $P < 0.05$ *versus* negative T cell FCXM FlowPRA negative.
[b] $P > 0.05$ *versus* negative T cell FCXM FlowPRA negative.

There was no correlation between an isolated positive B cell FCXM and adverse clinical events. FlowPRA was positive in only 5 of these 22 individuals, which suggests that, in the majority, non-HLA antibodies were detected with the B cell FCXM (*e.g.*, autoantibodies). Recipient cells for autologous FCXM were not available in these individuals. Four of the five sensitized patients with an isolated positive B cell FCXM possessed isolated anti-HLA class II antibodies detected with FlowPRA, whereas the fifth patient possessed both anti-HLA class I and class II antibodies. One of the four patients with an isolated anti-HLA class II antibody experienced steroid-resistant rejection on day 6 and required OKT3 rescue. The others had uneventful clinical courses.

## Discussion

Flow cytometric techniques are capable of detecting anti-HLA antibodies with greater sensitivity than standard and AHG-CDC techniques (1,2). Although an association between a positive retrospective FCXM and poor graft outcomes has been demonstrated in renal retransplant recipients with a negative AHG-CDC crossmatch (3–5), the clinical significance of a positive FCXM in primary renal transplants with a negative AHG-CDC crossmatch is a matter of debate (5–7). In the present study, 13% of primary transplant recipients were retrospectively found to have a positive T cell FCXM at the time of a negative AHG-CDC crossmatch. These previously undetected donor specific antibodies were associated with adverse immunologic events posttransplant, including early graft loss due to antibody-mediated rejection, multiple and severe acute rejections, and a greater prevalence of subclinical rejection.

A significant proportion (33%) of the positive T cell FCXM primary transplant recipients in this study experienced early graft loss (≤2 wk posttransplant). An analysis of explant histology revealed that all but one of these grafts was lost because of apparent antibody-mediated rejection. The flow-cytometric detection of donor-specific antibodies before trans-

plantation suggests that rejection in these cases was likely mediated by anti-HLA antibodies. In contrast, only 1 of 12 patients with a negative T cell FCXM and early graft loss exhibited histologic features of antibody-mediated rejection. No anti-HLA antibodies were detected before transplantation in this case, which suggests that either non-HLA antibodies were present (*e.g.*, antiendothelial cell antibodies) (18–20), *de novo* anti-HLA antibodies were formed posttransplant, or that anti-HLA antibodies were indeed present pretransplant but below the threshold of detection of FCXM or FlowPRA. Thus, in AHG-CDC crossmatch–negative primary renal transplant recipients, a positive T cell FCXM identified patients at substantial risk of early graft loss likely mediated by anti-HLA antibodies, whereas patients with a negative T cell FCXM rarely experienced antibody-mediated graft loss. Features of antibody-mediated rejection were also seen in four of five recipients for whom we were unable to perform a retrospective FCXM. It is possible, albeit a speculation, that these individuals also possessed undetected antidonor HLA antibodies pretransplant.

The corollary of the observed 33% graft loss rate is that the majority of positive T FCXM recipients maintained their grafts. These patients, however, experienced numerous adverse immunologic events during the first 3 mo posttransplant. All but one patient experienced at least a single acute rejection episode, and the majority experienced multiple acute rejections, steroid-resistant rejection, or both. These patients also exhibited a greater prevalence of subclinical rejection in protocol biopsies, and, notably, the 1-mo biopsy in these patients often exhibited subclinical rejection after the clinical resolution of an acute rejection, which suggests that treatment may have been inadequate.

Anti-HLA antibodies and a positive T cell FCXM are indicative of immunologic memory for donor HLA antigens. It has been demonstrated that individuals with anti-HLA antibodies possess primed T cells directed at these same alloantigens (21,22). A positive retrospective T cell FCXM in these patients with a negative AHG-CDC crossmatch pretransplant indicates that these recipients possessed undetected immunologic memory for donor HLA antigens at the time of transplantation. Both acute and subclinical rejection have been associated with the development of chronic rejection and allograft failure (23–25). Therefore, it is possible that primary renal transplant recipients with a negative AHG-CDC, crossmatch but a positive T cell FCXM, possess unrecognized immunologic memory for donor antigens that not only imparts a risk of early graft loss due to rejection but may also impart a risk of suboptimal long term outcomes as well (26). A future evaluation of this cohort of positive T cell FCXM patients will help address this question.

The finding that 67% (12/18) of positive T cell FCXM patients did not experience early graft loss highlights the principal quandary that physicians face allocating kidneys on the basis of FCXM results—there is an increased risk of early graft loss due to severe rejection, but in whom? Unfortunately, there exists to date no reliable method of predicting the clinical outcome in an individual with a positive T cell FCXM. Early reports suggested that a historical positive but current negative CDC crossmatch did not carry a significant risk of adverse events (27,28). However, several subsequent reports have contradicted this (29–31). Recently, Avlonitis *et al.* (31) reported a 1-yr graft survival of only 57% in renal transplant recipients with a current negative FCXM but a historical positive CDC crossmatch. Our experience mirrors this, in that a current negative, historical positive T cell FCXM imparted a significant risk of early graft loss due to rejection (3/11 current positive T cell FCXM *versus* 2/7 historical positive T cell FCXM).

To avoid early graft loss and severe rejection in crossmatch positive recipients, physicians have attempted to use more aggressive immunosuppressive regimens. Induction therapy with OKT3 or polyclonal antilymphocyte antibodies alone has met with limited success (32,33). Dafoe *et al.* (32) gave OKT3 induction to AHG-CDC crossmatch–positive recipients and observed fewer accelerated and recurrent rejections than in noninduced controls. These individuals, however, still experienced a high rate of primary nonfunction due to rejection, and 30% of those who experienced acute rejection developed steroid-resistant rejection. Other investigators have attempted to remove anti-HLA antibodies pretransplant with plasmapheresis or immunoabsorption in conjunction with induction and maintenance immunosuppressive regimens. Most have met with limited success, because considerable numbers of patients still experienced early immunologic graft loss, severe rejection, or suboptimal graft survival (34–37).

Recently, two groups have reported promising results by use of plasmapheresis, intravenous immune globulin (IVIG), and mycophenolate mofetil in a desensitization protocol for crossmatch–positive renal transplant recipients (38,39). Mycophenolate mofetil may possess more potent anti-B cell effects, whereas IVIG may contain anti-idiotypic antibodies and may also be capable of inhibiting alloantibody production (40–43). Montgomery *et al.* (38) treated four positive T cell FCXM patients preemptively with plasmapheresis, IVIG, tacrolimus, and mycophenolate mofetil, performing the transplants once the crossmatch had become negative. Although antibody-mediated rejection was encountered and further plasmapheresis and IVIG were required, short-term graft outcomes were satisfactory. Schweitzer *et al.* (39), with a similar protocol, were able to desensitize and successfully perform transplants in 11 of 15 recipients with positive AHG-CDC crossmatches against a living donor. No immunologic graft losses were seen, and only 4 of 11 individuals experienced acute rejection episodes, all of which were successfully treated. The positive T cell FCXM individuals in this study were treated with only standard immunosuppressive protocols (Table 2). We can therefore only speculate whether a different immunosuppressive regimen may have resulted in better graft survival or a lower rejection rate in our patients.

Two additional observations merit mention. First, we found 20% of primary transplant recipients to be allosensitized with FlowPRA, whereas AHG-CDC PRA identified only 4%. This degree of discrepancy between these two techniques is consistent with previous observations (2). All 18 individuals with a positive T cell FCXM had detectable anti-HLA antibodies by

FlowPRA, whereas the AHG-CDC PRA was positive in fewer than half (8/18). As suggested by Gebel and Bray (2), this finding highlights the importance of accurately determining sensitization in prospective transplant recipients and also demonstrates the necessity of using PRA screening and crossmatch techniques with similar sensitivities. Specifically, the accurate interpretation of a positive FCXM may be unclear when PRA screening, performed with an inferior technique (*e.g.,* AHG-CDC PRA), fails to detect anti-HLA antibodies that are indeed present. Second, a positive B cell crossmatch did not correlate with clinical events posttransplant, and the majority of these patients did not possess anti-HLA antibodies. A caveat is that within this group are patients who possess either low-titer anti-HLA class I or isolated anti-HLA class II antibodies detectable by FlowPRA, which may be causing the positive B cell FCXM (44,45). Some of these antibodies may be pathogenic to the graft, and we appeared to have observed one such case.

In conclusion, we have shown that a significant proportion of AHG-CDC crossmatch–negative primary renal transplant recipients possess donor-specific antibodies pretransplant detectable by FCXM. These individuals are at increased risk for early graft loss due to antibody-mediated rejection and experience an increased rate of adverse immunologic events posttransplant. These findings suggest that, in primary renal transplant recipients, a positive T cell FCXM in the face of a negative AHG-CDC crossmatch may represent a relative contraindication to transplantation. Whether immunosuppressive protocols that focus on alloantibody removal and address the associated B and T cell immunologic memory will lead to satisfactory long-term outcomes in these patients remains to be proved.

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