Alloreactivity in Renal Transplant Recipients with and without Chronic Allograft Nephropathy

EMILIO D. POGGIO,*† MICHAEL CLEMENTE,* JOCELYN RILEY,* MEAGAN RODDY,* NEIL S. GREENSPAN,‡ CORA DEJELO,§ NADER NAJAFIAN,‡ MOHAMED H. SAYEGH,‡ DONALD E. HRICIK,‡ and PETER S. HEEGER*†§

Departments of *Immunology and †Nephrology and Hypertension, The Cleveland Clinic Foundation, Cleveland, Ohio; Departments of ‡Medicine and §Pathology, Case Western Reserve University, Cleveland, Ohio; ‡Department of Pathology, University Hospitals of Cleveland, Cleveland, Ohio; and Transplantation Research Center, Brigham and Women’s Hospital and Children’s Hospital, Boston, Massachusetts

Abstract. The pathogenesis of chronic allograft nephropathy (CAN) involves both immunologic (antigen-dependent) and nonimmunologic (antigen-independent) mechanisms. In order to provide further insight into the immunologic basis of this disease, a cross-sectional analysis of cellular and humoral immunity in human renal allograft recipients with or without deteriorating renal function and biopsy proven CAN was performed. Interferon-γ enzyme-linked immunosorbent spot assays were used to assess cellular immunity to donor, or fully mismatched third-party stimulator cells (direct pathway), and to synthetic peptides derived from donor HLA molecules (indirect pathway). Anti-HLA antibodies were evaluated by flow cytometry using HLA-coated beads. Both the mean frequencies of donor-reactive peripheral blood lymphocytes and the number of individuals who responded to donor antigens per group were statistically higher in CAN patients versus control subjects (P < 0.02). Calculated ratios of donor/third-party enzyme-linked immunosorbent spot responses showed mean values of 2.61 ± 3.0 in the CAN group, with ratios of 0.50 to 0.72 ± 0.42 in control subjects (P < 0.001), confirming that direct, donor-specific cellular immunity predominated in patients with CAN. Fifty percent of CAN patients studied exhibited donor peptide reactivity compared with only 28.6% in control subjects. Finally, 33% of patients in the CAN group developed new posttransplantation anti-HLA antibodies compared with only 4% in the control group (P < 0.05). Overall, the results suggest that persistent cell-mediated and humoral alloimmunity contribute to the development of CAN and further demonstrate that anti-donor immunity in patients with CAN is heterogeneous. Immune monitoring to predict long-term outcome should include multiple measures of cellular and humoral immunity.

Significant improvements in 1-yr renal allograft and patient survival rates have been achieved over the last 10 yr, in part as a result of newer immunosuppressive regimens and the increasing use of living donors for transplantation (1). Despite these notable achievements in short-term outcome, long-term graft function and long-term graft survival rates remain less than optimal. Chronic allograft nephropathy (CAN) is one of the leading causes of late renal allograft loss and represents the most prevalent reason for patients to re-enter the already long waiting list for renal transplantation (1–4).

The pathogenesis of deteriorating renal function posttransplantation and CAN in particular is poorly understood but involves both immunologic and nonimmunologic mechanisms (3–5). Among immune factors, results from multiple animal studies performed by many laboratories suggest that both the humoral and cellular arms of the immune system can participate in the development of this pathologic entity (6–15). In human renal allograft recipients, pretransplantation anti-HLA antibodies as detected by high panel of reactive antibody (PRA) (16), as well as de novo posttransplantation anti-donor alloantibodies (17–19), have been correlated with poor long-term outcomes. Preformed or de novo posttransplantation alloantibodies can activate complement and/or macrophage-mediated effector mechanisms that are thought to participate in the development of transplant vasculopathy, glomerulopathy, and interstitial scarring, the pathologic hallmarks of CAN.

Donor-reactive T cell immunity, in particular CD4+ T cell immunity specific for self-restricted but donor-derived peptides presented through the indirect pathway, may also be important mediators of CAN in humans (12,20,21). Because indirect priming is dependent on antigen presentation by a recipient HLA molecule and can theoretically occur any time after the transplant, it has been postulated that CD4+ T cells responding through the indirect pathway actually play a dominant role in the development of CAN (20). Indeed, results from several laboratories have provided strong correlative evidence that indirectly primed CD4+ T
cells are associated with clinical/pathologic evidence of chronic allograft injury (22–27).

Alloreactive T cells also directly recognize allo-MHC molecules on the surface of donor antigen presenting cells (APC). This response involves a high frequency of T cells and has been hypothesized to mediate predominantly acute cellular rejection. Because the number of graft-derived donor APC is limited, direct priming is likely to occur only in the early period after the transplant. Nonetheless, early priming through the direct pathway will eventually result in a population of donor-reactive memory T cells that can become reactivated at later time points and participate in the development of CAN. Evidence supporting a role of directly primed T cells in CAN is inferential but includes the observation that recipients who experience acute cellular rejection episodes are at higher risk for CAN (28,29). In addition, donor-reactive immunity as assessed by proliferative mixed lymphocyte responses is less commonly detectable in patients with excellent renal function posttransplantation than in control subjects (30,31).

It remains unclear whether one form of donor-reactive immunity predominates over others in human allograft recipients with documented CAN. The high degree of polymorphism in human HLA molecules and the known complexity of the human alloimmune repertoire suggest that each patient may have a unique constellation of immunologic and nonimmunologic factors influencing graft outcome. It is unlikely that a single pathogenic mechanism is operative in each person with CAN, and no single study has examined direct and indirect cellular immunity along with humoral alloimmunity in the same patient population. Therefore, we sought to evaluate the correlation between these various immune parameters and the presence or absence of CAN in a single cohort of renal allograft recipients. Our findings confirm the complexity of human alloimmunity and demonstrate that direct and indirect cellular immunity as well as humoral immunity all are more prominently detectable in transplant recipients with CAN than in those with normal renal function. In addition to providing insight into the pathogenesis of CAN in humans, the results suggest that the use of immune monitoring to predict long-term outcomes should include multiple tests in an effort to assess optimally the alloimmune repertoire in each patient.

Materials and Methods

Patient Selection

All patients were enrolled under the approved guidelines of the Institutional Review Boards for Human Studies at the University Hospitals of Cleveland and the Cleveland Clinic Foundation. The study population consisted of 45 primary renal transplant recipients who received a transplant between June 1994 and September 2001. We identified 20 renal allograft recipients with serum creatinine (SCr) >2.0 mg/dl and a >0.5 mg/dl increase in SCr after the initial 6 mo posttransplantation (abnormal renal function group). CAN was confirmed histologically (Banff classification (32)), on the basis of an allograft biopsy, in nine of these 20 patients. The specific causes of deteriorating renal function in the remaining 11 patients could not be determined definitively but clinically were consistent with CAN. Twenty-five control patients who received a transplant during the same time period were identified on the basis of a SCr without change during the posttransplantation time period.

HLA Typing

Tissue typing of recipients and donors was performed by standard serologic (class I) and DNA-based (class II) techniques (33).

Peptides

Synthesized peptides corresponding to the polymorphic regions of selected HLA molecules were used to evaluate specific donor peptide responses (Table 1). The peptides were synthesized by Research Genetics (Huntsville, AL) or by Princeton Biomolecules (Langhorne, PA) and were >90% pure by HPLC. Selected peptides derived from HLA class I molecules were a gift of A. Jaramillo and T. Mohanakumar (Washington University, St. Louis, MO).

Table 1. Synthetic peptides used for ELISPOT analysis

<table>
<thead>
<tr>
<th>HLA Allele</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 0101</td>
<td>62–81</td>
</tr>
<tr>
<td>A 0201</td>
<td>75–89</td>
</tr>
<tr>
<td>A 0301</td>
<td>62–81</td>
</tr>
<tr>
<td>B 4402</td>
<td>62–77</td>
</tr>
<tr>
<td>B 0702</td>
<td>70–85</td>
</tr>
<tr>
<td>DRB1-0101</td>
<td>6–25</td>
</tr>
<tr>
<td>DRB1-02</td>
<td>1–20</td>
</tr>
<tr>
<td>DRB1-0301</td>
<td>6–21</td>
</tr>
<tr>
<td>DRB1-0401/02</td>
<td>1–18</td>
</tr>
<tr>
<td>DRB1-0701</td>
<td>1–18</td>
</tr>
<tr>
<td>DRB1-11041</td>
<td>6–21</td>
</tr>
<tr>
<td>DRB1-1201</td>
<td>6–21</td>
</tr>
<tr>
<td>DRB1-1301</td>
<td>6–21</td>
</tr>
<tr>
<td>DRB1-1401</td>
<td>6–21</td>
</tr>
</tbody>
</table>

a ELISPOT, enzyme-linked immunosorbert spot.
Preparation of Peripheral Blood Lymphocytes and Patient Plasma Samples

Donor splenocytes were obtained at the time of transplantation in recipients of cadaveric renal allografts, and peripheral blood lymphocytes (PBL) were obtained in heparinized tubes from the living donors. Donor stimulator cells were T cell depleted (RosetteSep; StemCell Technologies, Vancouver, BC, Canada) as described previously (34) and stored in aliquots at −70°C for later use as stimulator cells.

Recipient PBL samples were obtained in heparinized tubes from renal transplant recipients during their routine outpatient visits at University Hospitals of Cleveland and the Cleveland Clinic Foundation. PBL were isolated by standard Ficoll density gradient centrifugation, and live cell numbers were determined by counting with acridine orange/ethidium bromide staining using an immunofluorescence microscope (34). Aliquots of plasma were stored at −70°C and were tested for the presence of anti-HLA antibodies by flow cytometry (see below).

Enzyme-Linked Immunosorbent Spot Assays

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays were performed as described previously in detail (34,35). A total of 300,000 responder PBL, in 100 μl of T cell medium (90% RPMI 1640 medium [Sigma Chemical Co.] plus 10% human serum with l-glutamine plus penicillin/streptomycin [BioWhittaker, Walkersville, MD] and 50 mM 2-mercaptoethanol [Sigma]), were immediately placed in 96-well ELISPOT plates (Millipore, Bedford, MA) precoated with capture anti–IFN-γ antibody (Endogen, Woburn, MA). PBL were stimulated with media alone, T cell–depleted donor or complete mismatched third-party (3P) stimulator cells, mumps antigen (BioWhittaker), and a positive control, phytohemagglutinin (at 1 μg/ml medium [Murex Diagnostics, Dartford, UK]). Recipient PBL were also tested for reactivity to synthetic peptides derived from the polymorphic areas of HLA A, B, and DR antigens (1 to 30 μg/ml final concentration) when the relevant peptides were available. Plates were then incubated overnight at 37°C. After three washes with PBS and PBS-Tween, a biotinylated, anti–IFN-γ antibody (Endogen) was added to detect bound cytokine, and the plates were incubated overnight at 4°C. After an additional wash, streptavidin horseradish peroxidase conjugate (Dako, Glostrup, Denmark) was added for 1 h at room temperature.

After a final wash, the plates were developed with aminoethylcarbazole (10 mg/ml in N,N-dimethylformamide; Pierce Chemicals, Rockford, IL), prepared in 0.1 M sodium acetate buffer (pH 5.0) mixed with H2O2 (200 μl/well).

The resulting spots were counted with a Series 1 Immunospot computer-assisted ELISPOT image analyzer (Cellular Technology, Cleveland, OH). Results were depicted as the mean number of IFN-γ spots per 300,000 recipient PBL based on triplicate measurements in a given assay. Previous work has demonstrated that <10 spots per 300,000 cells represent background reactivity. Greater than 15 spots per 300,000 cells was considered a positive test. The well-to-well and assay-to-assay variability is 20 to 30% (34). Control wells assessing cytokine production by donor or 3P stimulators alone were included in all assays (generally <20 spots per 300,000), and detected spots in these control wells were subtracted from the total number of spots in wells in which responders and stimulators were mixed. We additionally calculated the ratios of total donor to 3P responses (D/3P).

Flow Cytometry

Anti-HLA antibody in plasma or serum samples was determined by flow cytometry using HLA class I and class II antigen-coated latex beads (FlowPRA Screening Test, One Lambda, Inc., Canoga Park, CA). A new posttransplantation alloantibody was defined as a PRA of >15% in patients with no detectable pretransplantation anti-HLA antibodies by flow PRA.

Statistical Analyses

All analyses were performed using SPSS version 11.5 (SPSS, Chicago, IL). Values are shown as mean ± SD, median (range), or percentage. Categorical variables were compared using the χ2 test or Fisher exact test. Comparison of mean values was tested using the t test for independent samples (two-tailed) and ANOVA, and median comparison was done using the Mann-Whitney U test. P < 0.05 was considered statistically significant.

Results

Clinical Characteristics of the Study Group

The clinical characteristics of the patients in the study are shown in Table 2. Twenty-three of the 45 patients received...

Table 2. Population characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>All Abnormal Graft Function</th>
<th>P Value</th>
<th>Only Biopsy-Proven CAN</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 25)</td>
<td>(n = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>47 ± 11</td>
<td>41 ± 10</td>
<td>NS</td>
<td>41 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Female gender</td>
<td>10 (40)</td>
<td>11 (55)</td>
<td>NS</td>
<td>4 (44.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Nonblack race</td>
<td>15 (60)</td>
<td>7 (35)</td>
<td>NS</td>
<td>3 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td>HLA mismatches</td>
<td>3.4 ± 2.1</td>
<td>3.4 ± 2.2</td>
<td>NS</td>
<td>2.6 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cadaveric</td>
<td>14 (56)</td>
<td>9 (45)</td>
<td>NS</td>
<td>2 (22.2)</td>
<td>NS</td>
</tr>
<tr>
<td>living</td>
<td>11 (44)</td>
<td>11 (55)</td>
<td>NS</td>
<td>7 (77.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Posttransplantation follow-up (mo)</td>
<td>24 (17–68)</td>
<td>24 (15–147)</td>
<td>NS</td>
<td>36 (20–147)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.4 ± 0.6</td>
<td>4.9 ± 2.5</td>
<td>&lt;0.05</td>
<td>4.8 ± 2.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>History of ACR</td>
<td>0 (0)</td>
<td>11 (55)</td>
<td>&lt;0.05</td>
<td>4 (44.4)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*All results are in n (%), mean ± SD, or median (range). ACR, acute cellular rejection.

b This group includes all patients with abnormal graft function including the subgroup with biopsy-proven CAN.

c Versus control subjects.
allografts from deceased donors, and 22 received allografts from living donors. As anticipated by the design of the study, the mean SCr in the control group was significantly lower than the SCr in the abnormal graft function group (1.4 ± 0.6 versus 4.9 ± 2.5 mg/dl; P < 0.05). Similarly, the incidence of biopsy-confirmed acute cellular rejection was significantly lower in the control subjects versus the recipients with abnormal graft function (0 versus 55%). There was no significant difference in the number of donor:recipient HLA mismatches or in the median follow-up time between control subjects and the abnormal function group. The nine patients with biopsy-confirmed CAN also had a significantly higher SCr and a higher acute cellular rejection rate compared with the control subjects. The patients within the subgroup with biopsy-confirmed CAN were followed for longer than those in the control group (median follow-up time 36 mo for CAN versus 24 mo for control subjects).

In addition to the data shown in Table 2, there was no statistically significant difference in the mean donor age between groups (control 42 ± 12 versus abnormal graft function, 44 ± 13 yr), in the median cold ischemia time in cadaveric transplants between groups (control: 17 h, range 14 to 19; all patients with abnormal graft function: 19 h, range 16 to 26; not shown), or in the incidence of delayed graft function between groups (<37% for each group; not shown). The immunosuppressive regimens consisted of steroids, a calcineurin inhibitor, and mycophenolate mofetil or azathioprine with a small number of patients taking sirolimus. There was no statistically significant difference in the use of any class of immunosuppressants or in the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, between those with normal renal function and the subgroup of patients with biopsy-proven CAN (data not shown).

**Anti-Donor Cellular Immunity in Patients with Abnormal Renal Function versus Control Subjects**

To assess the frequency of donor-reactive immune cells in each patient, we tested recipient PBL against donor or completely mismatched 3P stimulator cells using the IFN-γ ELISPOT assay. We have previously demonstrated that this approach provides a measure of the number of donor-reactive effector/memory T cells (as opposed to naive T cells) in the peripheral blood of the recipient at the time point tested (34,35). As shown in Figure 1A, the mean frequency of IFN-γ-producing, donor-reactive PBL was statistically higher in patients from the abnormal graft function compared with the control subjects (33 ± 41 IFN-γ spots/300,000 PBL versus 9 ± 14 in control subjects; P < 0.02). Similarly, the mean frequency of IFN-γ-producing, donor-reactive PBL was statistically higher in the subgroup with biopsy-confirmed CAN than in control subjects. In contrast, all recipients responded equally well to 3P stimulator cells (no significant difference among groups; Figure 1A). Reactivity to mumps antigen and to the mitogen phytohemagglutinin was also not different among groups (data not shown). These results suggest that the anti-donor immune reactivity detected in those patients with abnormal renal function, or the subgroup with confirmed CAN, may be relatively specific for donor antigens.

Because the mean frequency of donor-reactive PBL in a given group could be overly influenced by a small number of “outliers,” we also analyzed the data to assess the percentage of individuals within each group who responded to donor antigens (as defined by a frequency of >15/300,000 IFN-γ ELISPOT). Figure 1B shows that the percentage of individuals who responded to donor antigens was significantly higher in the groups with abnormal renal function or biopsy-confirmed CAN compared with the control group. Twelve (60%) of 20 patients in the abnormal function renal group and six (66.6%) of nine patients in the CAN subgroup had anti-donor–positive ELISPOT responses compared with only five (20%) of 25 patients in the control group (P < 0.05 versus either the abnormal function group or CAN subgroup). The frequency of patients with positive 3P reactivity did not differ among groups.

![Figure 1. Peripheral blood lymphocytes (PBL) from transplant recipients with abnormal renal function respond more vigorously to donor antigens than PBL from control subjects. (A) Mean frequencies of IFN-γ enzyme-linked immunosorbent spot (ELISPOT)/300,000 PBL after stimulation with donor cells (■) or completely mismatched third-party (3P) cells (□). (B) The percentage of transplant recipients within each group who exhibited a positive anti-donor (■) or anti-3P (□) ELISPOT assay (defined as >15 ELISPOT per 300,000 PBL) is shown. P values comparing individual groups are shown. *P = NS versus control subjects.](image-url)
In an effort to account for possible patient-to-patient variability in immune responsiveness and to better assess the specificity of the detected responses, we also calculated the ratio of donor to 3P IFN-γ ELISPOT (D/3P ratio). As shown in Figure 2A, the mean D/3P ratio in the patients with abnormal renal function was 1.57 ± 2.18 compared with 0.5 ± 0.52 in the control subjects \((P < 0.05)\). The D/3P ratio in the subgroup with biopsy-confirmed CAN was 2.61 ± 3.0, again statistically higher than those in the control group. Seven of 20 patients in the abnormal renal function group and five of nine with CAN exhibited D/3P ratios >1.3, whereas only one of 25 control subjects exhibited this ratio \((P < 0.05\) versus either abnormal graft function group or CAN subgroup; Figure 2B).

**Indirect T Cell Reactivity**

In an effort to assess cellular immunity specific for indirectly presented donor peptides, we synthesized a panel of ~50 peptides derived from the polymorphic regions of selected, common HLA A, B, and DR alleles (Table 1). We tested for peptide responses in PBL of recipients for whom we had available peptides synthesized from the polymorphic regions of the donor HLA molecules not present in the recipient (mismatched donor HLA molecules). Peptides were chosen on the basis of donor HLA type \(\text{(e.g., DR4 versus DR2)}\) when available, but the high degree of HLA polymorphism precluded obtaining specific peptides matching donor molecular subtypes in each instance. Peptide reactivity was determined over a concentration range of 1 to 30 \(\mu\)g/ml, with most detectable responses occurring at 10 to 30 \(\mu\)g/ml. Notably, because of the highly polymorphic nature of human HLA alleles and that the patients were chosen on the basis of the presence or absence of CAN but without *a priori* knowledge of donor and recipient HLA types, we were able to test for peptide reactivity in only 17 of our 45 patients. This was true despite that we had >50 peptides synthesized from 14 different HLA alleles.

As shown in Figure 3, PBL from five (50%) of 10 patients tested with abnormal renal function exhibited donor peptide reactivity (three of six in the CAN group were positive) but PBL from only two (28.6%) of seven control subjects exhibited similar responses. Although this difference did not reach statistical significance because of the small numbers of available patients for study, the findings suggest a trend toward a higher frequency of donor peptide reactivity in individuals with abnormal graft function/CAN versus control subjects.

**Serum Anti-HLA Antibodies**

We also screened for the development of anti-HLA alloantibodies in the study groups using flow cytometric analysis of HLA-coated beads. As shown in Figure 4, the groups did not differ with regard to the frequency or amount of pretransplantation antibodies.
anti-HLA class I or II alloantibodies. In contrast, 37.5% of patients with abnormal graft function (43% of those with biopsy-confirmed CAN) developed new anti-HLA class I or class II alloantibodies posttransplantation. Only 5% of control subjects developed new posttransplantation anti-HLA antibodies (P = 0.03).

Combined Analysis of Alloimmunity in Transplant Recipients

Figure 5 depicts the overall frequency and type of immune reactivity for the study groups. The presence of a positive D/3P ratio (>1.3, direct cellular immunity), a positive donor peptide response (indirect cellular immunity), or a new anti-HLA antibody (humoral immunity) was detected in 12 (60%) of 20 of those with abnormal renal function, seven (78%) of nine patients with biopsy-confirmed CAN, but only four (16%) of 25 control subjects (P < 0.01). It is noteworthy that no alloimmune responses were detected in 84% of control subjects, 40% of patients with abnormal renal function, and 22% of those with biopsy-confirmed CAN.

Immune Reactivity in Those with and without Previous Acute Cellular Rejection

Because acute cellular rejection can be associated with elevated donor-reactive immunity (33,34,36), it was possible that the alloimmune responses detected in individuals with abnormal renal function segregated predominantly to the individuals with a history of acute rejection. As shown in Table 3, however, this was not the case. Within the group of 20 patients with abnormal graft function, we did not note a difference in the incidence or magnitude of detectable cellular or humoral alloimmune responses between those with and without a history of acute cellular rejection episodes. Although the group sizes are small, the data suggest that the higher detected frequency of alloimmunity in patients with abnormal renal function versus control subjects (Figure 5) is not due to differences in acute cellular rejection rates between groups.

Discussion

Slowly deteriorating allograft function is a common occurrence after kidney transplantation (37). Contributing factors include recurrent disease in the allograft, poor adherence to prescribed medical regimens, infectious complications (e.g., polyoma virus), obstruction, immunosuppressant drug toxicity, and anti-donor immune-mediated injury (3–5). It has become increasingly apparent that examination of graft histology can help to identify some of the specific factors operative in damaging the allograft in an individual recipient (38). Still, although protocol biopsies may provide some prognostic information, they are not routinely performed because the procedure carries a small, inherent morbidity, and it remains questionable as to whether therapies can be effectively altered once the detected pathologic processes are manifested in the biopsy. Delineating less invasive approaches capable of differentiating and prospectively identifying those individuals at risk for developing worsening graft function due to immune-mediated versus non-immune processes (specifically identifying those individuals likely to have stable graft function over time) would therefore have important clinical implications (33,39–41). Ultimately, such information could be used to guide changes in therapy (either increases or decreases), so as to optimize clinical outcome. The results of the present cross-sectional study provide new information regarding the incidence and type of peripheral alloimmune reactivity in renal allograft recipients with and without deteriorating renal function and thus represent a foundation on which to base future clinical research to reach this goal.

Our data suggest that the peripheral alloimmune responses in a heterogeneous group of allograft recipients with worsening renal function were significantly different from the responses detected in control patients with stable graft function. This was true despite that the two groups were similar in terms of age, gender, race, donor/recipient HLA mismatch, immunosuppressive therapy, and median follow-up time. We acknowledge, however, that the current study was not large enough to detect small differences in

Figure 4. Posttransplantation alloantibodies are more frequently detectable in patients with abnormal renal function versus control subjects. The percentage of transplant recipients in each group with a positive (>10%) pretransplantation panel of reactive antibody (PRA; □) and a newly positive posttransplantation PRA (■) is depicted. P values comparing individual groups are shown. *P = NS.

Figure 5. The frequency of detecting any alloimmune reactivity differs in transplant recipients with and without abnormal renal function. The percentage of patients within each group who tested positive for D/3P ratio (Figure 2), donor peptide (Figure 3), or new posttransplantation anti-HLA antibody (Figure 4) is depicted. P values comparing individual groups are shown.
these variables between the groups. Peripheral alloimmune reactivity in the subgroup of patients with biopsy-confirmed CAN was also markedly different from that found in control patients. The detected immune responses were heterogeneous in their manifestations; cellular and humoral immune responses were detected in the patients with abnormal renal function, and these immune responses were specific for both directly presented and indirectly presented alloantigens. Of those with abnormal renal function, some patients had only cellular immunity (six of 20), others only humoral immunity (two of 20), and still others exhibited a combination of cellular and humoral immunity (four of 20).

Overall, the preferential detection of any alloimmune reactivity in the patients with CAN provides correlative evidence that such immune responses may be partially responsible for the poor outcome in those particular cases. Nonetheless, an alternative interpretation of the results is that patients with stable renal function developed preferential hyporesponsiveness to donor antigens, suggesting a tolerant state. The retained ability to respond to 3P peptides indicates that the patient population and raises the possibility that in these patients, the pathway may preferentially reside and function in the graft and be expressed in a more hyporesponsive manner.

In contrast with previously reported cross-sectional analyses (22,23,27), we found that elevated frequencies of donor-specific alloreactive PBL (direct reactivity) were significantly associated with CAN. The previously reported preferential detection of indirectly primed T cells, rather than direct T cell reactivity in association with CAN, may in part be based on different methods used. Because we used a highly sensitive IFN-γ ELISPOT (versus proliferative responses and/or limiting dilution analyses) and because we studied responder PBL rather than purified CD4⁺ T cells, we could detect anti-donor immunity at high resolution from any responding T cell in the study population. In particular, previously activated CD8⁺ memory cells, potentially primed during the early phases of transplantation or during a previous acute rejection episode, could be detected by our ELISPOT and may be important mediators of chronic immune injury. Studies in animal models have confirmed that low frequencies of anti-donor CD8⁺ T cells can be pathogenic mediators of chronic immune injury (43).

In addition to detection of donor-reactive PBL responding to directly presented alloantigens, our results confirm a previously reported association between T cells responding through the indirect pathway and the presence of CAN (22,23,27). Both direct and indirect alloimmune responses were found in our cohort of patients, and either or both could be operative in mediating chronic graft injury. Moreover, it should be noted that humoral alloimmunity and isotype switching to IgG is dependent on indirect reactivity (44); thus, our detection of antibodies is actually a surrogate marker for CD4⁺ T cells responding through the indirect pathway.

Although it is clear that CD4⁺ T cells responding through the indirect pathway are associated with CAN (22,23,25,27), it may be difficult to use routinely assays that assess responses to donor-derived peptides in the peripheral blood as surrogate markers for outcome for several reasons. First, several studies have demonstrated that immune responses to donor-derived determinants are dynamic and not predictable, consistent with the phenomenon of epitope spreading (23,27). Second, as illustrated by our results, the huge number of human HLA polymorphisms (including the variability in molecular subtypes of each HLA type) suggest that peripheral blood samples may need to be tested against large libraries of synthesized peptides to be able to assess comprehensively peripheral immunity directed at any potential indirectly presented peptide. Finally, T cells responding through the indirect pathway may preferentially reside and function in the graft and be detectable only in the peripheral blood intermittently. Thus, from a practical standpoint of identifying reliable surrogate markers of outcome, repeatedly assessing indirect reactivity using as many donor peptides as is technically feasible should be attempted.

Our data also revealed that an elevated D/3P ratio of IFN-γ-producing PBL (specifically, a ratio of ≥1.3), rather than the absolute frequency of IFN-γ-producing PBL, better segregates
the control patients from those with CAN. The result provides further evidence for donor-specific immunity in those with CAN versus control subjects. It is notable that in a previous study of patients who were evaluated early posttransplantation, any alloimmune reactivity, rather than donor-specific immunity, correlated with 6- and 12-mo renal function (33). The difference in the two results may be due to the timing of the detected responses. At early times posttransplantation, there may be less specificity to detected alloantigens. The anti-donor immune response may evolve and become more specific over time, preferentially leaving a residual number of donor-specific effector memory cells by 1 to 2 yr posttransplantation. Moreover, from a methodology standpoint, the 3P stimulators chosen here were fully mismatched to the donors, whereas in the previous work, the 3P stimulators were chosen to have the same number of mismatches with the recipient as the donor did.

Finally, our data confirm that the development of de novo posttransplantation alloantibodies was significantly higher in the patients with abnormal graft function, including those with the diagnosis of CAN, versus control subjects (17,19,45). This result is similar to those reported by others and suggests that IgG alloantibody formation, in addition to being a surrogate marker for indirect T cell immunity, may independently function as a pathogenic factor in the development of CAN. It is important to acknowledge that we did not perform posttransplantation flow cytometry cross-match studies, so we cannot state whether the newly detected antibodies were donor reactive. Nonetheless, the detection of new posttransplantation alloantibodies by flow cytometric analysis of HLA-coated beads may be a useful surrogate marker for assessing subsequent risk of CAN when used in conjunction with other monitoring tests.

In sum, the results of our study show that immune reactivity in renal allograft recipients with poor renal function differs significantly from those with stable renal function and shows that the detected responses are heterogeneous in nature. The findings raise the possibility that measures of posttransplantation cellular and humoral immunity, in conjunction with clinical acumen and histologic analysis of the graft, may eventually be clinically useful tools for prognosticating future risk and guiding therapeutic decision making (46,47). As examples, patients who take nephrotoxic immunosuppressive drugs, without detectable peripheral immune reactivity but with slowly deteriorating renal function and minimal inflammation in an allograft biopsy, may be the best candidates for drug withdrawal. In contrast, patients with persistently detectable anti-donor immune reactivity, worsening renal function, and/or biopsy evidence of active inflammation may benefit from alterations in therapy directed at controlling activated T cells or B cells, depending on the type of immunity detected. Prospective studies specifically addressing these issues will be required before final conclusions can be made, but it would be prudent to include these serial, comprehensive assessments of anti-donor immunity in future clinical trials of immunosuppression minimization (48) or tolerance (49).

Acknowledgments
The work was supported by a National Institutes of Health contract N01-AI-05410. Emilio D. Poggio is a recipient of a fellowship grant from the National Kidney Foundation. Portions of the data from this manuscript were presented at the American Transplant Congress 2003.

References


