Clinical Rejection Is Distinguished from Subclinical Rejection by Increased Infiltration by a Population of Activated Macrophages

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Abstract. It has been reported previously that one-third of protocol renal biopsies in asymptomatic, biochemically stable renal transplant recipients in the first 6 mo show unsuspected subclinical graft rejection (both infiltrate and tubulitis) and that subclinical rejection is a risk factor for chronic renal dysfunction. This study was performed to determine whether differences in phenotype or activation status of graft-infiltrating cells underlie these different manifestations of acute rejection. Biopsies with normal histology (n = 10), subclinical rejection (n = 13), and clinical rejection (n = 9) were studied using immunohistochemistry and computerized image analysis. Subclinical and clinical rejections had similar histologic Banff scores. Univariate analysis showed a trend for a higher infiltration with CD8+ (P = 0.053) and CD68+ (P = 0.06) cells in clinical rejection. Of the activation markers studied (CD25, perforin, tumor necrosis factor-α), only allograft inflammatory factor-1+ -activated macrophages were significantly (P = 0.014) increased in the infiltrate of clinical rejection biopsies. These data suggest that activated macrophages or their products are responsible for acute renal dysfunction associated with clinical rejection episodes.

We have reported previously that one-third of protocol biopsies performed in stable renal allograft recipients in the first 3 mo posttransplantation satisfy the Banff criteria for graft rejection (1). The finding of “subclinical” rejection has subsequently been confirmed by other groups (2,3). It appears likely that subclinical rejection is pathogenic. We have recently reported that subclinical rejection in a 6-mo protocol biopsy is an independent predictor of long-term graft dysfunction (4). Moreover, a subsequent randomized study demonstrated that corticosteroid treatment of early subclinical rejection episodes in months 1 to 3 caused a decrease in early as well as late (months 7 to 12) clinical rejection episodes, a decrease in the chronic tubulointerstitial score at 6 mo, and a lower serum creatinine at 2 yr (5). Finally, the finding of subclinical rejection in a protocol biopsy at 6 mo correlated with the development of allograft dysfunction at 2 yr (3). Taken together, these data suggest that subclinical rejection is detrimental to long-term graft function.

Subclinical rejection is indistinguishable histopathologically from those rejections associated with symptoms or with allograft dysfunction. The current study was performed to determine whether the differences in presentation between clinical and subclinical rejection could be accounted for by differences in the composition and/or the activation status of the cells infiltrating the graft. Specifically, we sought to determine whether the lack of increase in the serum creatinine of patients with subclinical rejection might be due to a relative paucity of cells capable of causing acute injury or functional change.

Materials and Methods
Renal Biopsies

Protocol renal biopsies were obtained at 1, 2, 3, and 6 mo posttransplant from recipients of living donor or cadaveric renal transplants as part of ongoing studies (1,4,6). In addition, biopsies were obtained from patients with acute deterioration of allograft function. The histologic diagnosis was performed using the Banff schema (7) by a nephropathologist (J.G.) blinded to the clinical status of the patient. During the study period, all biopsies that met the following criteria were used: (1) adequate sample as defined by Banff criteria; (2) tissue remaining after clinical studies were complete was sufficient for experimental analysis; (3) a clear diagnostic category of acute rejection (clinical or subclinical) or normal. Specimens that were borderline, chronic rejection, or additional disease, i.e., de novo glomerulonephritis, were excluded. There were 10 normal biopsies and 13 with subclinical rejection. The criteria for the diagnosis of sub-
clinical rejection were stringent, with the requirement of <10% rise in serum creatinine in the 2 wk before the protocol biopsy. The use of 10% is similar to that used by other investigators (2). Clinical rejection was diagnosed if the serum creatinine had risen >10% from baseline. Biopsies were obtained from nine such patients. The Banff acute score between clinical and subclinical rejection was the same (Table 1). In particular, both the extent of interstitial inflammation and the degree of tubulitis were similar between subclinical and clinical rejection (not shown). All patients were on triple therapy with cyclosporine, prednisone, and azathioprine. Other clinical details of this cohort have been reported previously (1,4,6).

**Immunohistology**

A portion of the biopsy was snap-frozen in OCT in dry ice, or isopentane precooled in liquid nitrogen, and stored at −140°C. The material was cut at 4 μM, momentarily thaw-mounted, then fixed in −20°C acetone for 5 min. All immunohistochemical procedures were performed with the Microprobe (Fisher Scientific, Pittsburgh, PA) apparatus, using capillary gap technology to ensure consistent and reproducible staining.

**Phenotypic Analysis**

Primary antibodies (to CD3, CD4, CD8, CD20, and CD68) were obtained from Dako Scientific (Carpinteria, CA). Monoclonal anti-CD45RO was a gift from Dr. John Wilkins (University of Manitoba). Primary antibodies were used at optimal dilutions obtained from titration experiments, using lymph node and irreversibly rejected kidney tissue. All reagents included the detergent 0.1% BRIJ 35 (Sigma, St. Louis, MO) to facilitate movement of fluids into and out of the capillary gap. Primary antibodies were diluted in Universal Antibody Diluent (Bio-Can Scientific, Mississauga, Ontario, Canada) and applied for 30 min at room temperature. After extensive washing in phosphate-buffered saline (PBS), anti-mouse antibody covalently linked to a polymer backbone and multiple peroxidase molecules (Envision System®, Dako) was applied for 30 min. After extensive washing in PBS, staining was developed in aminothiol-carbazole for 10 min. Slides were counterstained in Meyer’s hematoxylin and mounted in Permount (Fisher Scientific).

**Activation Marker Analysis**

We studied a broad selection of markers of immune cell activation in the renal allograft biopsies. CD25 (the interleukin-2 receptor α-chain) is a well known cell surface molecule upregulated on a variety of activated cells, including T cells, macrophages, and B cells (8). Perforin is primarily a product of activated cytotoxic T cells (9). Tumor necrosis factor-α (TNF-α) is primarily derived from macrophages (10), but also from CD69+ T cells (11). Cells expressing these markers have been described in renal allografts undergoing acute clinical rejection (8,9,12). We also examined CD69, a very early activation antigen expressed on the surface of mitogen-stimulated T lymphocytes (13,14) that has been found in human cardiac allograft rejection (15). Finally, allograft inflammatory factor-1 (AIF-1), cloned from a chronically rejecting rat cardiac allograft, is a product of interferon-γ-activated macrophages (16) and is highly conserved between human and rat with 95% similarity at the amino acid level. It has been identified by reverse transcription-PCR and immunohistochemistry in rejecting human cardiac allografts on a subpopulation of CD68+ macrophages (17). Antibody to AIF-1 was a polyclonal rabbit antibody generated by one of the authors (M.R.). Monoclonal anti-TNF-α (18) was a gift from Dr. Sue Stephens (Celltech Therapeutics, Slough, United Kingdom). Monoclonal anti-perforin (clone deltaG9) (19) was purchased from T Cell Diagnostics (Endogen catalog no. AA1717; Woburn, MA). Anti-CD25 was obtained from Dako, and anti-CD69 was purchased from Becton-Dickinson (Sunnyvale, CA).

**Image Analysis**

Computerized image analysis of immunohistochemically positive staining cells in the infiltrate was used as done by other investigators (20). For phenotypic markers, computerized morphometry was used to increase the reliability (number of points counted) and decrease the time required by classical point-counting techniques (20). Computerized image analysis was performed with a Nikon microscope with a ×40 objective and a Panasonic Neuvacon charge-coupled device camera attached to a Macintosh 7600 Power PC computer. The image analysis software used was NIH Image (21) with a custom-written macro (available from the author, Dr. Paul C. Grimm). The number of pixels of red staining due to aminothiol-carmine was determined. For each antibody, images from 250 individual cells were acquired and used to determine the average number of pixels staining positive per cell. This number was then used to convert the data from pixels to counts of positive cells/mm² (22). The entire renal cortex in the biopsy was analyzed by an operator blinded to the clinical status of the patient. However, the glomeruli, large vessels, and normal subcapsular infiltrate were excluded. Since the number of cells that stained positively for the activation markers was low, these were counted by hand. The cortical surface area of the biopsy was determined using image analysis acquired using a ×2 objective.

**Statistical Analyses**

Univariate statistical analysis on log-transformed measurements was performed using factorial ANOVA. Significant differences were further analyzed using post hoc tests of significance (Scheffe and

**Table 1. Demographic data**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal Biopsy</th>
<th>Subclinical Rejection</th>
<th>Clinical Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>4/6</td>
<td>3/10</td>
<td>4/5</td>
</tr>
<tr>
<td>Time from Tx to Bx (months)</td>
<td>8.4 ± 2.4</td>
<td>4.1 ± 0.8</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>CsA level at Bx (ng/ml)</td>
<td>251 ± 32</td>
<td>266 ± 35</td>
<td>284 ± 32</td>
</tr>
<tr>
<td>Total Banff score</td>
<td>0.4 ± 0.16</td>
<td>4.2 ± 0.34</td>
<td>4.6 ± 0.37</td>
</tr>
<tr>
<td>Biopsy cortical surface area (mm²)</td>
<td>1.54 ± 0.21</td>
<td>1.42 ± 0.19</td>
<td>1.58 ± 0.30</td>
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</tbody>
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*Results are given as mean ± SEM. The total Banff score is the sum of the individual acute components (t, i, g, v). Tx, transplantation procedure; Bx, biopsy; CsA, cyclosporin A.*
Bonferroni/Dunn). All procedures were performed using the StatView 4.5 software package (Abacus Concepts, Berkeley, CA).

Results

Immunostaining of the biopsies revealed clear staining that was straightforward to analyze by image analysis (Figure 1). The analyzed cortical surface area in each group (Table 1) was similar ($P = 0.88$).

Cell Phenotype

Data are given in Figure 2. Initial analysis with factorial ANOVA demonstrated a significant relationship between the three diagnostic groups (normal, subclinical rejection, and clinical rejection) and the frequency of cells staining with CD3, CD8, and CD68. Subsequent analysis by post hoc testing demonstrated statistically significant differences between normal biopsies and clinical rejection for CD3 ($P = 0.004$), CD8 ($P = 0.018$), and CD68 ($P = 0.003$). The difference between normal biopsies and subclinical rejection was statistically significant only for CD3 ($P = 0.0046$). Although there was no statistically significant difference between subclinical rejection and clinical rejection, there was a clear trend for all of the T cell and macrophage markers to be most highly expressed in clinical rejection, immediately expressed in subclinical rejection, and expressed at the lowest level in normal biopsies (Figure 2). The markers that were closest to statistical significance in differentiating subclinical rejection from clinical rejection were CD8 ($P = 0.053$) and CD68 ($P = 0.06$). On inspection, the results for CD20 and CD45RO staining repeat this trend, with normal biopsies having the lowest, clinical rejection the highest, and subclinical rejection expressing intermediate levels. The initial ANOVA analysis did not demonstrate statistical significance, so further analysis was not pursued.

Activation Markers

Data are given in Figure 3. Initial analysis with factorial ANOVA indicated a significant relationship between the three diagnostic groups and AIF-1+ cell count ($P = 0.018$). Post hoc testing indicated that the AIF-1+ cell count in clinical rejection (Figure 4) was significantly higher than normal ($P = 0.0125$) and subclinical rejection ($P = 0.0135$). Once again, the other activation markers studied (CD25, CD69, TNF-α, and perforin) demonstrated a trend for normal biopsies to score lowest, clinical rejections highest, and subclinical rejections intermediate; however, no relationship was significant (Figure 3).

Discussion

The aim of this study was to elucidate whether the differences in presentation of clinical and subclinical rejection of human renal allografts were associated with differences in the phenotype and activation status of the graft-infiltrating cells. It confirms previous studies of the phenotype of infiltrating cells in biopsies from patients with clinical rejection. For example, Ibrahim et al. (23) found that biopsies from patients with acute rejection had higher counts of CD3-, CD4-, CD8-, CD45RA-,

and CD45RO-positive cells, compared with graft biopsies showing no rejection. The greatest difference between rejecting and nonrejecting grafts in that study was the increased frequency of CD8 and CD45RO cells in the former. In our study, the surface area with both CD8+ and CD3+-infiltrating cells was significantly different between these patient groups.

The quantity of CD3+ cellular infiltrate in subclinical rejection was intermediate between normal and clinical rejection. Moreover, there was a trend for clinical rejections to express a larger frequency of CD8+ and CD68+ cells than subclinical rejections ($P = 0.053$ and $P = 0.06$, respectively) (Figure 2). Only a small fraction of the infiltrating T cells in allograft rejection is thought to be allospecific (24). In our study, there was a trend toward fewer CD45RO+ cells in nonrejecting versus rejecting biopsies. On the other hand, we found that the number of CD45RO+ cells was the same in subclinical and clinical rejection. Because the CD45RO marker is thought to identify cells that have been recently activated by antigen (25), it would appear that allospecific infiltrates are present in biopsies with subclinical or clinical rejection in similar numbers.

The macrophage (CD68) population comprised approximately one-half of the total infiltrating cells. This is in agreement with previous studies, such as that of Hancock et al. (26), who found that macrophages comprised between 30 and 60% of the total infiltrating leukocytes in renal allograft rejection. Ibrahim et al. (23), on the other hand, reported that macrophages accounted for less than 10% of the infiltrating population. However, in this latter study, the Leu-M5 antibody was used to identify macrophages, which has been reported to underestimate interstitial macrophage infiltrates in human renal biopsies (27). In the present study, macrophage infiltration alone, as detected by CD68, came close to differentiating subclinical rejection from clinical rejection by univariate analysis ($P = 0.06$). A preponderance of macrophages has been associated with more severe forms of rejection (28,29). Macrophages are a heterogeneous cell population whose functional state is determined by "stimulatory or regulatory factors" in the microenvironment. It is therefore possible that macrophage infiltration of the graft by specific subsets of activated macrophages such as those expressing AIF-1 would be indicative of a more aggressive injury process.

In this study, the prevalence of CD25+ cells in acute clinical rejection ($1.8 \pm 1.08$ cells/mm$^2$) was lower than that reported in previous studies. For example, Serón et al. (30) found 65 ± 45 CD25+ cells/mm$^2$ and Noronha et al. (12) 31.2 ± 4.8 CD25+ cells/mm$^2$ during acute clinical rejection. In both of these studies, the cyclosporine levels were lower than those of the present study. An inverse relationship between the cyclosporine level at the time of biopsy and the number of CD25+-infiltrating cells has been reported (30). It is therefore possible that the higher blood levels of cyclosporine obtained in this study were responsible for reducing the expression of CD25+ cells, despite its inability to completely prevent either clinical or subclinical rejection.

The expression of perforin mRNA, when used in combination with other cytotoxic markers such as granzyme B and Fas
Figure 1. Infiltrating cell phenotype in allograft biopsies. Representative photomicrographs of a normal biopsy (a, d, g, j, and m), subclinical rejection (b, e, h, k, and n), and clinical rejection (c, f, i, l, and o) biopsy. The antibodies used were CD3 (a through c), CD4 (d through f), CD45RO (g through i), CD8 (j through l), and CD68 (m through o). Hematoxylin counterstain bar, 200 μM.
ligand, has been shown to discriminate between normal biopsies and those with clinical rejection early posttransplantation (31). Moreover, the detection of these markers may be indicative of the severity of clinical renal allograft rejection (32). We have recently reported that normal biopsies have the lowest, clinical rejections the highest, and subclinical rejections an intermediate amount of perforin mRNA expression (33). In the present study, immunohistochemical quantification of perforin expression in later (median 4 mo) biopsies showed no significant difference between normal biopsies, subclinical rejection, and clinical rejection, although there was a trend toward an increase in perforin detection in both forms of rejection compared with normal biopsies. The recent results of Sarwal et al. support our finding of only a small number of perforin-positive cells in clinical rejection biopsies. They studied granulysin (another cytotoxic primarily T cell product) and also found a very sparse infiltrate in biopsies from patients with clinical rejection while on maintenance immunosuppression (34). The

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**Figure 2.** Frequency of infiltrating cells expressing phenotypic markers. Box plots show the median, 1 SD, and 2 SD of the frequency of cells expressing specific markers. This demonstrates the trend of normal biopsies to express the least, clinical rejections to express the most, and subclinical rejections to express an intermediate level of these phenotypic markers. $^aP = 0.005$ versus normal; $^bP = 0.004$ versus normal; $^cP = 0.018$ versus normal; $^dP = 0.003$ versus normal.

**Figure 3.** Frequency of infiltrating cells expressing activation markers. Box plots show the mean $\pm$ SEM of the frequency of cells expressing activation markers. This demonstrates the trend of normal biopsies to express the least, clinical rejections to express the most, and subclinical rejections to express an intermediate level of these activation markers. $^aP = 0.005$ clinical versus subclinical.
Acute Clinical Rejection

Figure 4. Allograft inflammatory factor-1 (AIF-1) expression. Representative photomicrograph of a clinical rejection biopsy stained with an antibody to AIF-1. Hematoxylin counterstain bar, 100 μM.

number of cells expressing granulysin markedly increased in kidneys in which immunosuppression had been withdrawn before nephrectomy for irreversible rejection (M. Pavlakis, personal communication).

We were unable to show a significant difference between expression of TNF-α in normal versus rejection biopsies. The expression of TNF-α using reverse transcription-PCR in human renal allograft biopsies (31) has likewise not been predictive of rejection. The frequency of CD69+ cells was not significantly different between groups (P = 0.33 by ANOVA).

With the exception of AIF-1, none of the activation markers distinguished subclinical from clinical rejection. The expression of AIF-1+ cells was similar in normal biopsies (0.332 ± 0.621 cells/mm²) and subclinical rejection (0.655 ± 1.65 cells/mm²), respectively (P = 0.87). However, AIF-1 expression in clinical rejection was 5.9 ± 2.56 cells/mm² (P = 0.0125 versus normal biopsies and P = 0.0135 versus subclinical rejection). This strong expression of AIF-1 in clinical compared with subclinical rejection may indicate that products released by activated macrophages promote the graft dysfunction leading to elevation of serum creatinine. In renal allograft rejection, Ramos et al. reported that macrophage thromboxane synthase expression was a marker for more rapid deterioration of renal function, and a risk factor for impaired renal function 6 mo after the diagnostic renal biopsy (35). Thromboxane synthase catalyzes the conversion of prostaglandin H₂ to thromboxane A₂, which mediates reduction in GFR (36) and stimulates extracellular matrix gene expression (37). Increased urinary excretion of thromboxane A₂ has been reported before acute clinical rejection episodes (38). Thus, we speculate that this or a similar macrophage product could be responsible for the deterioration in allograft function in clinical compared with subclinical rejection.

Although serum creatinine is not acutely elevated in subclinical rejection (by definition), this does not indicate that the subclinical rejection is benign. We have reported that subclinical rejection at 6 mo is an independent risk factor for poor graft function at 2 yr (4). Similarly, diagnosis and treatment of subclinical graft rejection have been shown to lead to improved allograft function at 2 yr in our recently published randomized clinical study (5). The frequency of CD25+, CD69+, and perforin-positive cells was equivalent in subclinical and clinical rejection. Therefore, the long-term damage associated with both forms of cellular rejection may be the result of infiltration with activated immune cells. Acute clinical rejection, on the other hand, is associated with a marked reduction in renal plasma flow and GFR (39), which are reflected in a reversible increase in serum creatinine. We speculate that this study provides evidence for the role of distinct subsets of activated macrophages or their products in the genesis of these reversible perturbations in renal vascular regulation.

In conclusion, we have studied a unique sample of human renal allograft biopsies obtained from stable asymptomatic patients and from patients with clinical allograft rejection. There was an increasing frequency of infiltrating cells of all phenotypes studied, with normal biopsies having the lowest, subclinical an intermediate, and clinical rejection the highest amount of interstitial infiltrate. Expression of the marker of activated macrophages, AIF-1, was able to clearly distinguish subclinical from clinical rejection. Taken together, the acute deterioration in glomerular filtration that characterizes acute clinical rejection may be due to products of activated, AIF-1-positive macrophages causing vasospasm or other injury to the graft.

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References


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