ORIGINAL ARTICLE

Molecular Heterogeneity in Acute Renal Allograft Rejection Identified by DNA Microarray Profiling

Minnie Sarwal, M.D., Ph.D., Mei-Sze Chua, Ph.D., Neeraja Kambham, M.D., Szu-Chuan Hsieh, M.S., Thomas Satterwhite, B.S., Marilyn Masek, M.S., and Oscar Salvatierra, Jr., M.D.

ABSTRACT

BACKGROUND

The causes and clinical course of acute rejection vary, and it is not possible to predict graft outcome reliably on the basis of available clinical, pathological, and genetic markers. We hypothesized that previously unrecognized molecular heterogeneity might underlie some of the variability in the clinical course of acute renal allograft rejection and in its response to treatment.

METHODS

We used DNA microarrays in a systematic study of gene-expression patterns in biopsy samples from normal and dysfunctional renal allografts. A combination of exploratory and supervised bioinformatic methods was used to analyze these profiles.

RESULTS

We found consistent differences among the gene-expression patterns associated with acute rejection, nephrotoxic effects of drugs, chronic allograft nephropathy, and normal kidneys. The gene-expression patterns associated with acute rejection suggested at least three possible distinct subtypes of acute rejection that, although indistinguishable by light microscopy, were marked by differences in immune activation and cellular proliferation. Since the gene-expression patterns pointed to substantial variation in the composition of immune infiltrates, we used immunohistochemical staining to define these subtypes further. This analysis revealed a striking association between dense CD20+ B-cell infiltrates and both clinical glucocorticoid resistance ($P=0.01$) and graft loss ($P<0.001$).

CONCLUSIONS

Systematic analysis of gene-expression patterns provides a window on the biology and pathogenesis of renal allograft rejection. Biopsy samples from patients with acute rejection that are indistinguishable on conventional histologic analysis reveal extensive differences in gene expression, which are associated with differences in immunologic and cellular features and clinical course. The presence of dense clusters of B cells in a biopsy sample was strongly associated with severe graft rejection, suggesting a pivotal role of infiltrating B cells in acute rejection.

From the Departments of Pediatrics (M.S., M.-S.C., S.-C.H., T.S., O.S.), Pathology (N.K., M.M.), and Surgery (O.S.), Stanford University, Stanford, Calif. Address reprint requests to Dr. Sarwal at the Department of Pediatrics, G320, 300 Pasteur Dr., Stanford, CA 94305, or at msarwal@stanford.edu.

N Engl J Med 2003;349:125-38.

Acute rejection is a complex process of injury to the allograft caused by infiltrating cells of the host immune system. It leads to multiple responses within the graft and is a major risk factor for chronic rejection and loss of the graft.[1-3] Acute rejection typically develops soon after transplantation and is thought to be secondary to cell-mediated immune responses involving delayed mechanisms of hypersensitivity and cytotoxicity. Despite efforts at systematization,[4,5] clinical and pathological diagnosis and classification of acute rejection remain unreliable in predicting responses to therapy and graft outcomes.[6-10] Thus, there is a great need to improve risk stratification and modes of early treatment. We investigated the possibility that variations in gene-expression patterns in allograft-biopsy samples from patients with acute rejection and related disorders would permit the identification of molecularly distinct subtypes of acute rejection that may be related to differences in clinical behavior.

METHODS

PATIENT INFORMATION

We analyzed 67 allograft-biopsy samples from 50 patients (1.4 to 22 years of age). Immunosuppressive therapy consisted of glucocorticoids, a calcineurin inhibitor (tacrolimus or cyclosporine), an antimetabolite (azathioprine or mycophenolate mofetil), and induction therapy with daclizumab. Nine graft losses occurred between 1.5 and 8.0 years after transplantation, a mean of 10 months after a biopsy was performed because of acute rejection. Written informed consent was obtained from all study patients, and the study was approved by the institutional review board of Stanford University.

BIOPSY SAMPLES

A total of 52 biopsy samples were obtained between 1 month and 10 years after transplantation during acute allograft dysfunction (defined by an increase of more than 10 percent in the serum creatinine concentration from base line) or chronic allograft dysfunction (defined by a glomerular filtration rate[11] of 50 ml per minute per 1.73 m^2 of body-surface area); 8 biopsy samples were obtained at the time of engraftment; and 7 samples were obtained at times when graft function was stable (as defined by a glomerular filtration rate of more than 80 ml per minute per 1.73 m^2). All biopsy samples were snap-frozen. All but five biopsy samples were obtained before the intensification of treatment for rejection. Microscopical analyses were performed by investigators who were unaware of the clinical outcomes.[5,12,13] No biopsy sample contained evidence of post-transplantation lymphoproliferative disorder or viral inclusions.

MICROARRAY HYBRIDIZATION AND DATA ANALYSIS

Each microarray was a lymphochip[14] gridded at Stanford University, and each contained 28,032 DNA spots representing approximately 12,440 human genes. Total RNA was isolated from frozen biopsy samples (TRI Reagent, Molecular Research Center). A common reference pool of RNA[15] was used as an internal standard. Sample or reference RNA was subjected to two successive rounds of amplification[16] before undergoing hybridization to microarrays.

All 67 biopsy samples were used for initial unsupervised, hierarchical clustering (i.e., analysis without prior knowledge of sample identity).[17] For subsequent supervised analyses (i.e., comparative analyses between defined sample groups), with the use of significance analysis of microarrays,[18] the five samples from patients with partially treated acute rejection were excluded in order to eliminate possible bias due to the effects of drugs. The enrichment of specific functional groups of genes was assessed in our data set on the basis of the hypergeometric distribution,[19] with the use of 86 T-cell–specific genes,[20] 2610 T-cell–inducible transcripts,[21] and 874 cell-cycle–related genes.[22] Kaplan–Meier survival analyses, based on the Cox log-rank method, were used to determine the relation between graft survival or recovery of graft function (defined as the return of the serum creatinine concentration to the base-line level one month after the treatment of acute rejection) and the density of CD20+ cells.

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining for CD20, CD4, CD8, and proliferating-cell nuclear antigen (PCNA) was performed on samples from patients with untreated acute rejection. In addition, an independent set of 31 archived biopsy samples from patients with acute rejection was also analyzed by CD20 staining.

Entire cores were scanned in a blinded fashion by a single observer to determine the density of CD20+, CD4+, and CD8+ cells. Cell density per high-power field and the number of high-power fields counted per core were documented. For each spec-

imen, the single high-power field with the highest CD20+ cell count was identified, and cell counts of more than 275 and less than 100 were chosen arbitrarily as definitions of CD20+ and CD20− status, so that the high threshold was more than 2.5 times the low threshold.

SUPPLEMENTAL INFORMATION

Additional information on methods, immunohistochemical images, and analytic methods are available as supplementary appendixes at http://genome-www.stanford.edu/rejection/ or from the National Auxiliary Publications Service (NAPS).* Data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

RESULTS

CLUSTERING OF SAMPLES

The gene-expression profiles of 67 allograft-biopsy samples were compared by the hierarchical clustering of samples according to the correlation in their patterns of expression in 1340 selected complementary DNA (cDNA) fragments, representing approximately 912 genes (Fig. 1 and supplementary appendixes). In general, biopsy samples from patients with similar clinical diagnoses clustered together on the basis of corresponding similarities in gene expression, irrespective of the immunosuppressive regimen the patient was receiving. The possibility that differential sampling of the medullary and cortical regions might account for the observed molecular variation was addressed through the comparison of these data with those obtained from an examination of variation in gene expression in distinct regions of the kidney. Patterns of gene expression in normal cortex and medulla were characterized in samples obtained from the gross dissection of normal kidneys (supplementary appendixes). The exclusion of data from genes whose expression was highly correlated with the depth of the biopsy did not change the composition of the clusters of samples (supplementary appendixes).

CLUSTERING OF ALLOGRAFT BIOPSIES ACCORDING TO GENE-EXPRESSION PROFILE

We identified four clusters of expression patterns in the biopsy samples, which generally corresponded closely with clinicopathological categories (Fig. 1 and Table 1). Biopsy samples from patients with acute rejection were observed to have relative molecular heterogeneity. Unlike the samples from normal kidney or those from patients with chronic allograft nephropathy, toxic drug effects, or infection, the samples from patients with acute rejection were dispersed among three of the four major clusters. Although one distinct cluster (cluster A) consisted only of samples from patients with acute rejection, the remaining 14 of the 26 samples from such patients were dispersed in clusters B and C.

All samples from patients with a clinicopathological diagnosis of toxic drug effects or infection were grouped in cluster B. All samples from patients with chronic allograft nephropathy were grouped in cluster C, and all the samples in this cluster showed clinicopathological evidence of chronic allograft nephropathy (in some cases, with accompanying acute rejection). All biopsy samples from normal kidneys were grouped in cluster D, and only normal samples were found in this cluster. The division of the samples into these four clusters reflects only a fraction of the molecular variations among them: within each of these clusters, extensive residual variation in gene expression was observed (the gene-expression signatures of each cluster are available at http://genome-www.stanford.edu/rejection/ or from the National Auxiliary Publications Service (NAPS).* We focused our detailed analysis on the molecular characterization of the samples from patients with acute rejection.

MOLECULAR HETEROGENEITY OF ACUTE REJECTION

We examined the characteristic gene-expression patterns that distinguished the 26 biopsy samples from patients with acute rejection. There were significant differences between these samples and those from normal kidney in the expression of 586 genes, representing 64 percent of all 912 unique genes analyzed; the median false discovery rate was 12 percent, or 68 genes (the false discovery rate, or the percentage of genes identified by chance, is calculated as the median number [or 90th percentile] of falsely identified genes divided by the number of genes achieving significance levels for differential expression[18]). The varying levels of expression of many of these genes suggests a varying abundance of distinctive cell populations, such as T and B lymphocytes, natural killer cells, macrophages, and endothelial cells.

At least three different groups of biopsy sam-

*See NAPS document no. 05611 for 56 pages of supplementary material. To order, contact NAPS, c/o Microfiche Publications, 248 Hempstead Tpke., West Hempstead, NY 11552.

ples from patients with acute rejection, which were not differentiated by light microscopy, could be defined by unsupervised hierarchical clustering on the basis of pervasive differences in their gene-expression profiles: acute rejection type I, which we designated as AR-I (cluster A, accounting for 12 biopsy samples, with one repeated experiment), acute rejection type II (designated as AR-II, cluster B, accounting for 9 biopsy samples, 5 of which were from patients who had been partially treated at the time of biopsy), and acute rejection type III (designated as AR-III, cluster C, accounting for 5 biopsy samples). These differences in patterns of expression may reflect distinct mechanisms of molecular pathogenesis of rejection (Fig. 1).

Figure 1 (facing page). Hierarchical Clustering of 1340 Transcripts in 67 Biopsy Samples on the Basis of Similarity in Gene-Expression Patterns (Panel A) and a Dendrogram Showing the Degree of Relatedness of Samples (Panel B).

A total of 59 samples were from pediatric renal-allograft recipients, and 8 were from donors. In Panel A, the genes (rows) and samples (columns) were ordered on the basis of the overall similarity in the expression pattern by investigators who were unaware of the clinical diagnoses. We selected 1340 complementary DNA fragments after filtering (for nonflagged spots with a fluorescence intensity more than 2.5 times that of the background, genes with technically adequate measurements in more than 75 percent of all samples, and messenger RNA levels differing from the median by at least a factor of 2.9 in at least six samples). The colored bars on the right of the diagram indicate clusters (labeled A, B, C, and D) with high discrimination scores. The degree of relatedness of the expression patterns in biopsy samples is represented by the dendrogram at the top of the panel. The color in each cell reflects the level of expression of the corresponding gene in the corresponding sample, relative to its mean level of expression in the entire set of biopsy samples. Gray areas represent missing or excluded data. The scale (shown at bottom right) extends from transcript abundance ratios of 0.25 to 4 relative to the mean level for all samples. In Panel B, replicate samples from the same patient (2 and 3) clustered together, indicating that experimental noise and artifacts caused by the handling or processing of the tissue are negligible in this analysis. AR-I denotes acute rejection type I, AR-II acute rejection type II, and AR-III acute rejection type III.

DEFINING SUBTYPES OF ACUTE RENAL ALLOGRAFT REJECTION

A total of 385 genes (42 percent of all unique genes analyzed) were differentially expressed in the biopsy samples in the AR-I group and the other samples from patients with acute rejection (median false discovery rate, 24 percent, or 94 genes). The functional theme reflected in these genes suggests that there is greater apoptosis as well as infiltration and activation of lymphocytes, driven by NF-κB and interferon-γ in AR-I than in the other subtypes of acute rejection (Fig. 2A and supplementary appendixes). Also prominent in this subtype are increased transcripts from T cells (interleukin-2–receptor chains and T-cell–receptor chains), natural killer cells (natural-killer–cell transcript 4), and macrophages (matrix metalloproteinase-7 and macrophage receptor).

Responses occurring downstream of T-cell activation may be enhanced, as suggested by increased expression of cytotoxic T-lymphocyte–effector genes (granzyme A and RANTES [regulated upon activation normal T-cell expressed and secreted], which are important effectors of acute rejection[23-25]), adhesion molecules, cytokines, cytokine receptors, and growth factors (Fig. 2A). In support of this hypothesis, we found an enrichment[19] of two classes of T-cell transcripts within the gene cluster characteristic of AR-I: 15 of 23 T-cell–specific transcripts[20] ($P<0.001$ for the enrichment of T-cell–specific genes in AR-I as compared with the rest of the gene cluster) (supplementary appendixes), and 43 of 145 T-cell–inducible transcripts[21] ($P<0.001$ for the enrichment of T-cell–inducible genes in AR-I as compared with the rest of the gene cluster) (supplementary appendixes). Furthermore, all eight of eight genes that were noted to be both T-cell–inducible and T-cell–specific were characteristic of the AR-I signature ($P<0.001$, supporting increased T-cell infiltration and activation in AR-I as compared with the rest of the gene cluster). Unexpectedly, an overriding signature for B cells (CD20, CD74, immunoglobulin heavy and light chains, and other molecules associated with B-cell receptors) was found in AR-I, as compared with the other subtypes (Fig. 2 and supplementary appendixes).

Nine samples from patients with acute rejection (AR-II) shared features with biopsy samples from grafts with clinicopathological evidence of toxic drug effects or infection. Some similarities to the gene-expression profiles of the AR-I samples (Fig. 1 and 2) may reflect common features of immune activation by pathogens and alloantigens. Many features of the expression program of the innate immune response were prominent in these samples. Genes of the annexin family — specifically, annex-

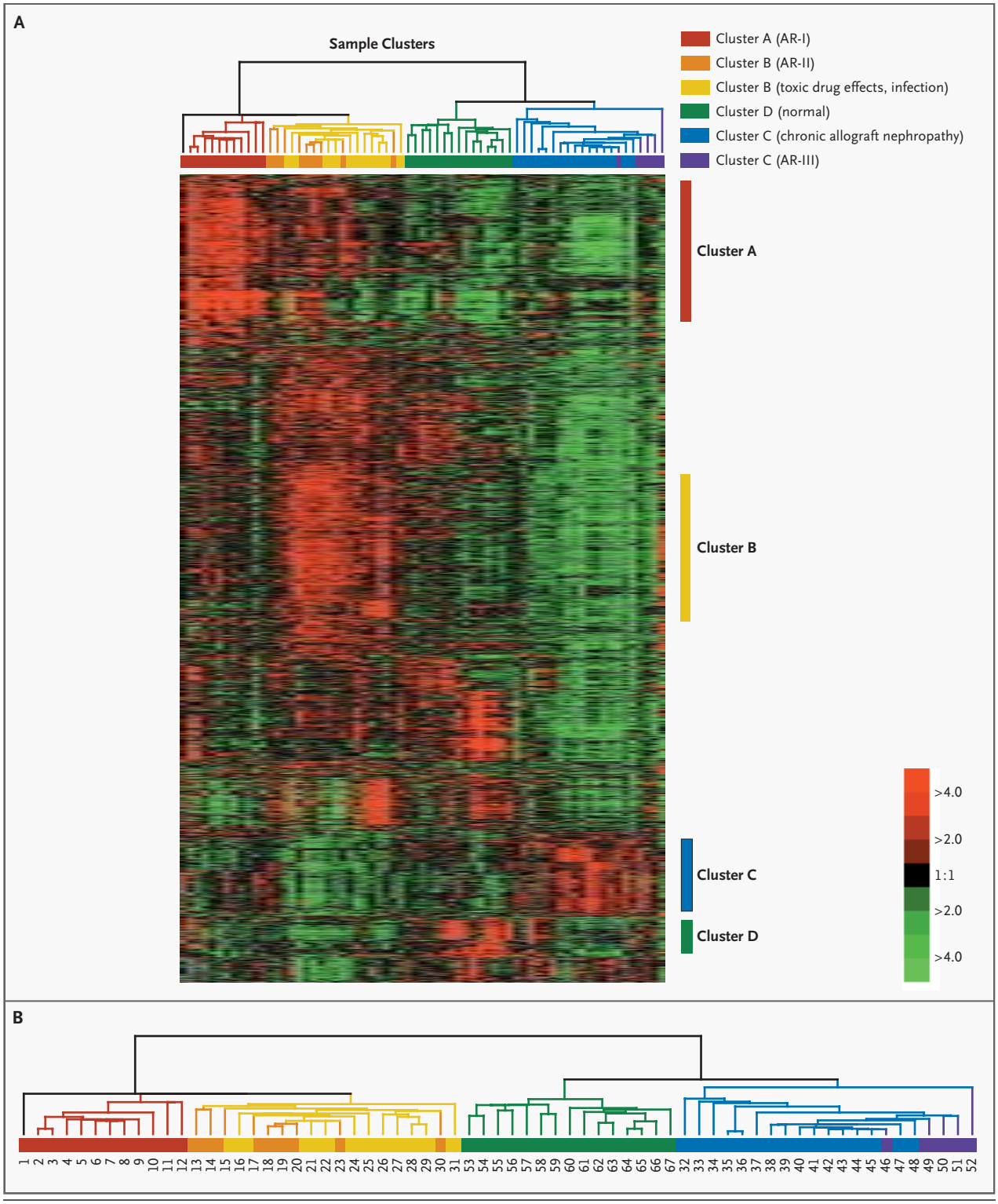


Table 1. Pathological and Clinical Characteristics of 52 Biopsy Samples from Dysfunctional Kidneys.*

Sample No.	Diagnosis	Banff Acute Rejection Grade	Infection	Treatment of Acute Rejection†	Recovery of Function after Acute Rejection	Graft Loss at Most Recent Follow-up	CD20 Staining	PCNA Staining
Cluster A								
1	Acute rejection	1A	Urinary tract infection	Glucocorticoid pulse	No	No	+	
2	Acute rejection	1A		Glucocorticoid pulse	Yes	No	–	
3	Acute rejection	1A		Glucocorticoid pulse	Yes	No	–	
4	Acute rejection	1A		Glucocorticoid pulse	No	Yes	+	
5	Acute rejection	1B		Glucocorticoid pulse, antibody therapy	No	Yes	+	
6	Acute rejection	1A		Glucocorticoid pulse	No	No	–	
7	Acute rejection	2A		Glucocorticoid pulse, antibody therapy	No	No	+	
8	Acute rejection	1B		Glucocorticoid pulse, antibody therapy	No	Yes	+	
9	Acute rejection	2A		Antibody therapy	No	Yes	+	
10	Acute rejection	1A		Glucocorticoid pulse	Yes	No	–	
11	Acute rejection	2A		Glucocorticoid pulse	No	Yes	+	
12	Acute rejection	1A		Glucocorticoid pulse	No	Yes	–	
Cluster B								
13	Acute rejection	1A		Glucocorticoid pulse, antibody therapy	No	No	–	
14	Acute rejection	1A		Glucocorticoid pulse	No	No	–	
15	Acute rejection, toxic drug effects, infection		Urinary tract infection	Glucocorticoids‡	Yes	No	–	
16	Toxic drug effects, infection		Urinary tract infection			No	–	
17	Acute rejection, toxic drug effects, infection		Septicemia	Glucocorticoids‡	Yes	No	–	
18	Acute rejection, toxic drug effects, infection		Urinary tract infection	Glucocorticoids‡	Yes	No	–	
19	Acute rejection	1A	Urinary tract infection	Glucocorticoid pulse	Yes	No	–	
20	Acute rejection, toxic drug effects, infection		Urinary tract infection	Glucocorticoids‡	Yes	No	–	
21	Toxic drug effects, infection		Urinary tract infection			No	–	

Table 1. (Continued.)

Sample No.	Diagnosis	Banff Acute Rejection Grade	Infection	Treatment of Acute Rejection*	Recovery of Function after Acute Rejection	Graft Loss at Most Recent Follow-up	CD20 Staining	PCNA Staining
22	Toxic drug effects, infection		Urinary tract infection			No	–	
23	Acute rejection	1A		Glucocorticoid pulse	No	Yes	+	
24	Toxic drug effects, infection		Septicemia			No	–	
25	Toxic drug effects					No	–	
26	Toxic drug effects, infection		Urinary tract infection			No	–	
27	Toxic drug effects, infection		Urinary tract infection			No	–	
28	Toxic drug effects					No	–	
29	Toxic drug effects, infection		Urinary tract infection			No	–	
30	Acute rejection, toxic drug effects, infection		Urinary tract infection	Glucocorticoids†	Yes	No	–	
31	Toxic drug effects, infection		Urinary tract infection			No	–	
Cluster C								
32	Chronic allograft nephropathy					No	–	–
33	Chronic allograft nephropathy					No	–	–
34	Chronic allograft nephropathy					No	–	–
35	Chronic allograft nephropathy					No	–	–
36	Chronic allograft nephropathy					No	–	–
37	Chronic allograft nephropathy					No	–	–
38	Chronic allograft nephropathy					No	–	–
39	Chronic allograft nephropathy					No	–	–
40	Chronic allograft nephropathy					No	–	–
41	Chronic allograft nephropathy					No	–	–
42	Chronic allograft nephropathy					No	–	–

Table 1. (Continued.)

Sample No.	Diagnosis	Banff Acute Rejection Grade	Infection	Treatment of Acute Rejection‡	Recovery of Function after Acute Rejection	Graft Loss at Most Recent Follow-up	CD20 Staining	PCNA Staining
43	Chronic allograft nephropathy					No	–	–
44	Chronic allograft nephropathy					No	–	–
45	Chronic allograft nephropathy					No	–	–
46	Chronic allograft nephropathy, acute rejection	2A		Glucocorticoid pulse	Yes	No	–	+
47	Chronic allograft nephropathy					No	–	–
48	Chronic allograft nephropathy					No	–	–
49	Chronic allograft nephropathy, acute rejection	1A		Glucocorticoid pulse	Yes	Yes	+	+
50	Acute rejection	1A		Glucocorticoid pulse	Yes	No	–	+
51	Acute rejection	1A		Glucocorticoid pulse	Yes	No	–	+
52	Chronic allograft nephropathy, acute rejection	1A		Glucocorticoid pulse	Yes	Yes	–	+

* Samples 2 and 3 are replicate samples from the same biopsy. The primary diagnosis associated with samples 37 and 42 was chronic allograft nephropathy, but the patients also had some toxic drug effects. The patient with sample 28 had recurrent diabetes, and the patient with sample 44 had recurrent Wegener's granulomatosis. Immunohistochemical staining for CD20 was performed on all 52 samples, including the 21 samples from patients with untreated acute rejection; a positive result was defined by more than 275 cells per high-power field. Staining for proliferating-cell nuclear antigen (PCNA) was performed on all samples in cluster C (chronic allograft nephropathy and acute rejection type III [AR-III]). Scattered plasma cells were seen in biopsy specimens 4, 5, 8, and 9 in acute rejection type I (AR-I), occupying between 5 and 10 percent of the core. A plus sign indicates a positive result, and a minus sign a negative result.

† Glucocorticoid pulse treatment consisted of three doses of 10 mg per kilogram of body weight. Treatment was given after biopsy in most patients but before biopsy in the patients with samples 15, 17, 18, 20, and 30. These samples were associated with a predominant histologic diagnosis of toxic drug effects or infections and were not given a Banff grade because they had already been treated. Antibody therapy consisted of muromonab-CD3 (OKT3) and was given to one patient for vascular rejection and to four patients for glucocorticoid resistance.

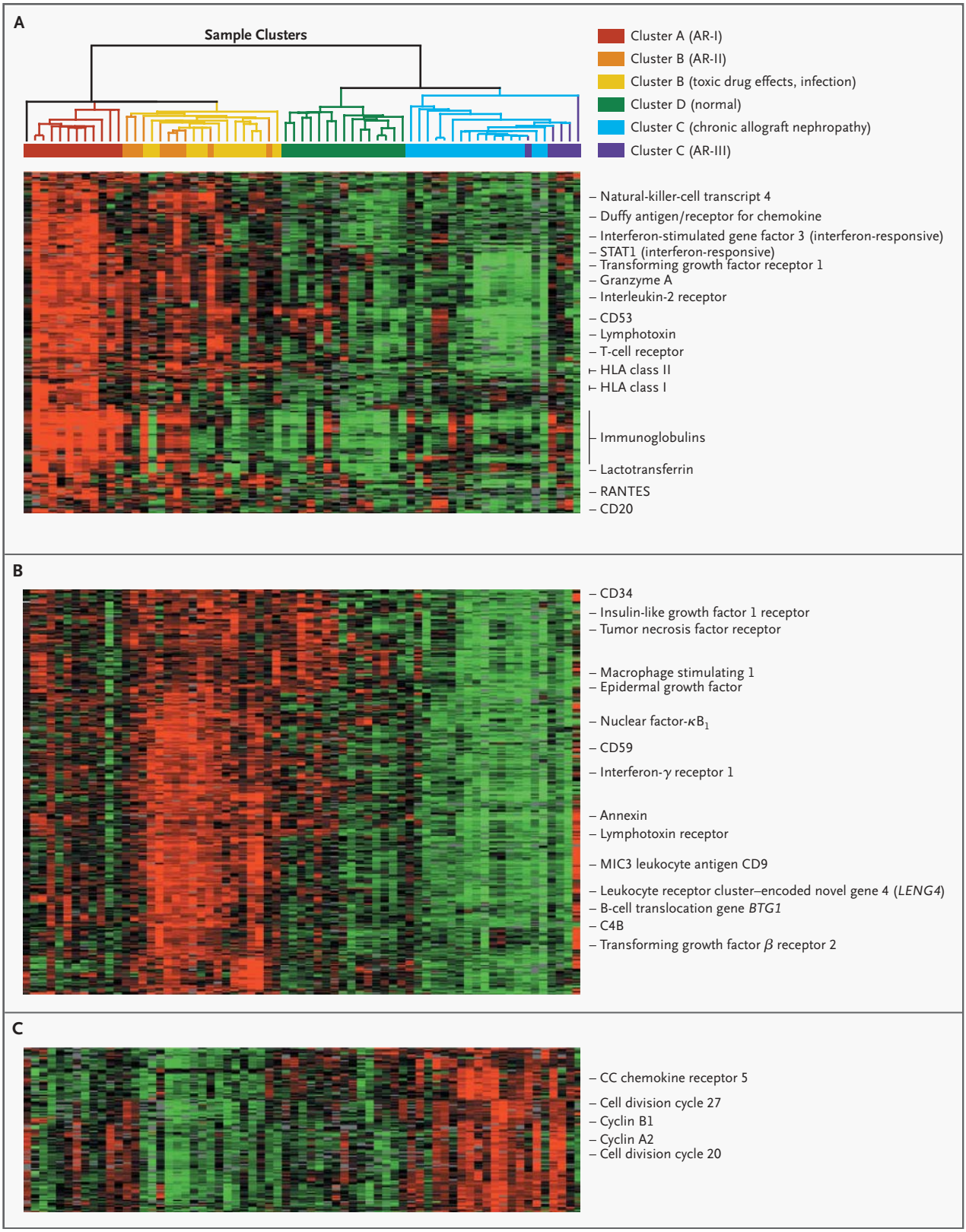
‡ Clinical graft dysfunction was treated with high-dose glucocorticoids before biopsy.

in V, a potential marker of acute rejection[26] — were expressed at a particularly high level in this group of biopsy samples. Expression of transforming growth factor β (induced by calcineurin-inhibitor drugs)[27] was relatively elevated, supporting the clustering of samples from patients with toxic drug effects in this group of biopsy samples.

Five AR-III samples clustered with samples from patients with chronic allograft nephropathy in cluster C, despite the fact that they met the Banff histologic criteria for acute rejection. Perhaps the most striking feature of these samples was the expression of genes involved in cellular proliferation and cell cycling (Fig. 2), suggesting active tissue repair and regeneration. Sixty of the 1340 transcripts in our data set were related to cell-cycle functions,[22] and 14 of these 60 genes were among the genes whose expression was significantly elevated in AR-III,

Figure 2 (facing page). Expanded View of the Gene Clusters, Showing Specific Features of the Gene-Expression Patterns within the Signatures in the Various Subtypes of Acute Rejection.

Panel A shows samples in the AR-I group, Panel B the AR-II group, and Panel C the AR-III group. RANTES denotes regulated upon activation normal T-cell expressed and secreted, AR-I acute rejection type I, AR-II acute rejection type II, and AR-III acute rejection type III.



representing a statistically significant enrichment[19] ($P<0.001$, for the enrichment of cell-cycle genes in AR-III as compared with the rest of the gene cluster). The molecular features of lymphocyte infiltration and activation were minimal in this subtype (Fig. 2 and supplementary appendixes), suggesting a relatively quiescent rejection process and ongoing recovery from previous or chronic tubulointerstitial inflammation or tubular necrosis.

IMMUNOHISTOCHEMICAL FEATURES OF SAMPLES FROM PATIENTS WITH ACUTE REJECTION

Because we observed not only a robust T-cell signature, but also a B-cell signature in the AR-I group, we used immunohistochemical analysis to investigate whether variation in the cellular composition of infiltrating lymphocytes in the 20 unique biopsy samples from patients with untreated acute rejection might account for some of the differences among groups in the observed gene-expression patterns; one sample in AR-I was examined twice by microarray analysis (Table 1). We were particularly interested in further study of B cells, since B cells have not historically been reported to be key players in acute rejection.[28] We chose CD20, a marker for B cells that is present in AR-I, to corroborate the observation of B-cell enrichment independently by immunohistochemical analysis.

On staining, we found that there was a greater abundance of CD8+ T lymphocytes than of CD4+ T lymphocytes in biopsy samples from patients with acute rejection. There were no overall quantitative differences in these patterns among the subtypes of acute rejection, although two biopsy samples with glucocorticoid resistance from patients in the AR-I group had a higher density of CD8+ cells (Fig. 3A and supplementary appendixes). The apparent absence of major differences in the density of CD4+ cells and CD8+ cells among the subtypes of acute rejection suggests that the relatively prominent T-cell signature in AR-I is largely attributable to an activated T-cell phenotype (evidenced by markers of early and late T-cell activation) rather than to increased numbers of infiltrating T cells and that, conversely, infiltrating T cells in AR-III are relatively quiescent.

CD20 staining revealed unexpected large aggregates of B cells without formation of follicles (Fig. 3A and supplementary appendixes) in 9 of 20 biopsy samples from patients with acute rejection: 7 of 11 in the AR-I group, 1 of 4 in the AR-II group, and 1 of 5 in the AR-III group (Table 1). This finding contrasts with a previous report of few B cells in samples from patients with acute rejection.[28] CD20 staining of 31 archived biopsy samples from patients with acute rejection that were not examined by microarray also revealed a similar proportion of CD20+ lymphocyte aggregates in 9 biopsy samples (supplementary appendixes). Immunofluorescence staining of biopsy samples for immunoglobulin and complement deposition was negative, despite the presence of the B-cell aggregates; in situ hybridization for Epstein–Barr virus and simian virus 40 was negative, ruling out an association between B-cell infiltrates and viral infection or post-transplantation lymphoproliferative disorder (data not shown).

The presence of proliferating-cell nuclear antigen, a marker of cell proliferation, was confirmed in all 5 AR-III samples but not in any of the samples from patients with chronic allograft nephropathy (Fig. 3B), distinguishing these groups of biopsy samples with otherwise similar expression profiles.

CLINICAL CORRELATES OF THE SUBTYPE OF ACUTE REJECTION AND CD20+ CELL DENSITY

Analysis of the recovery of graft function over time revealed that grafts that were clustered in the AR-I group had significantly poorer functional recovery than those classified as either AR-II or AR-III ($P=0.02$) (Table 2 and supplementary appendixes). When data from the five samples from partially treated patients in the AR-II group were removed from the data set, a trend toward a correlation remained, despite reduced numbers of samples ($P=0.06$) (Table 2 and supplementary appendixes). In addition, four of five samples from patients with

Figure 3 (facing page). Immunohistochemical Staining of Tissues.

Panel A shows staining with periodic acid–Schiff (PAS) and monoclonal antibodies against CD4, CD8, and CD20 in representative samples from patients with acute rejection of each subtype (AR-I, AR-II, and AR-III). The AR-I sample shows the presence of large B-cell clusters. Panel B shows staining for proliferating-cell nuclear antigen (PCNA) in cluster C revealing the presence of PCNA in tubular and interstitial cells in a representative AR-III biopsy sample but the absence of PCNA in a representative sample from a patient with chronic allograft nephropathy (CAN). AR-I denotes acute rejection type I, AR-II acute rejection type II, and AR-III acute rejection type III.

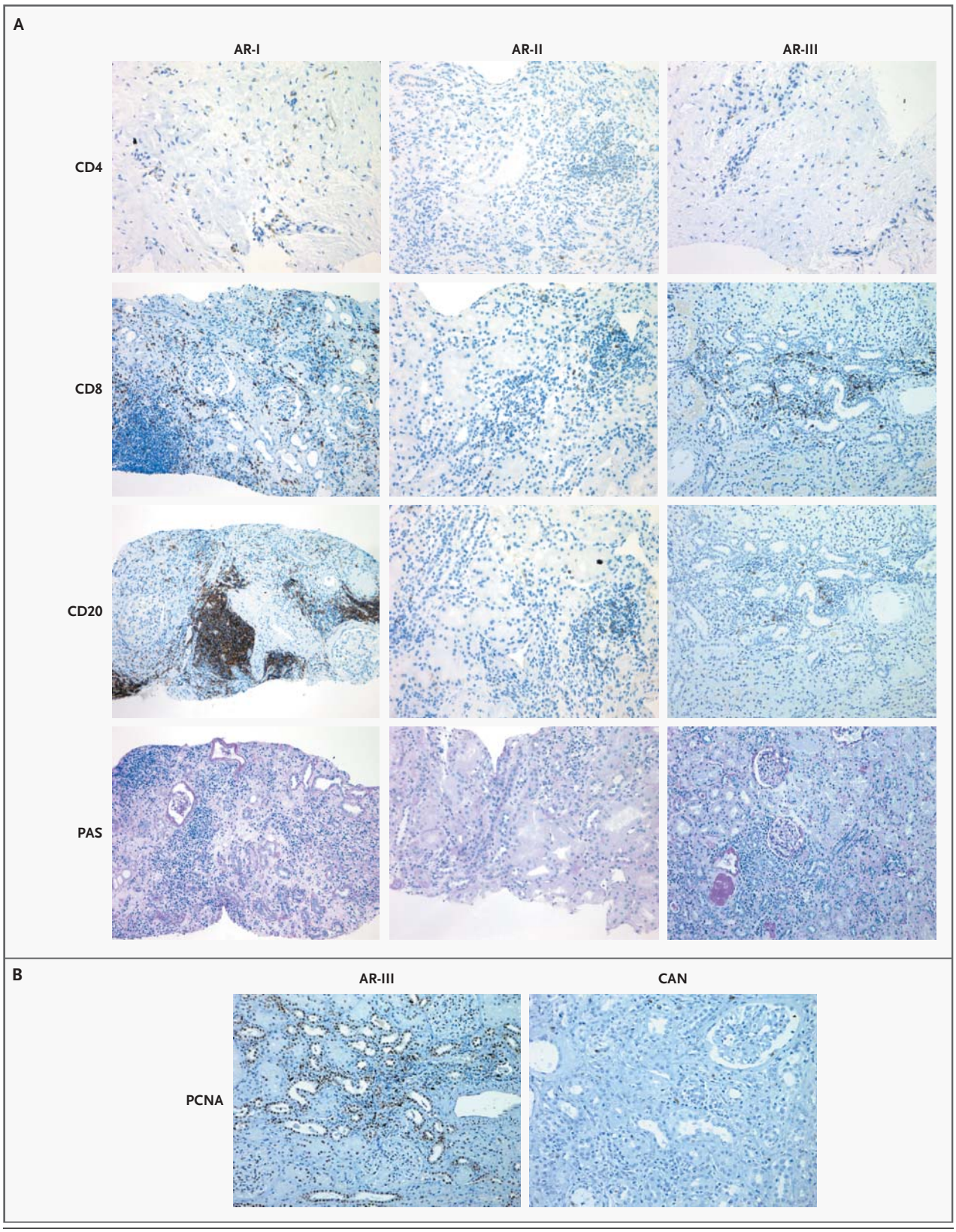


Table 2. Correlations between Acute Rejection (AR) Subtype or CD20 Status and Graft Outcome.

Variable	No. of Patients	Median Duration of Follow-Up	No. of Grafts Lost or with Incomplete Functional Recovery*	P Value†
		mo		
Subtype (25 samples from patients with acute rejection)				0.02
AR-I	11	11	9	
AR-II	9	13	3	
AR-III	5	13	0	
Subtype (20 samples from patients with untreated acute rejection)				0.06
AR-I	11	11	9	
AR-II	4	16	3	
AR-III	5	13	0	
CD20 status (20 samples from patients with untreated acute rejection)				<0.001
CD20+	9	13	8	
CD20–	11	10	1	

* Data are for grafts with incomplete functional recovery in the analyses according to subtype of acute rejection and for grafts lost in the analysis according to CD20 status.

† P values were calculated from Kaplan–Meier survival analyses.

glucocorticoid-resistant acute rejection (defined by the absence of a clinical response to glucocorticoid pulse treatment) clustered in the AR-I group.

A strong association between the density of CD20+ cells on immunostaining and the clinical phenotype of glucocorticoid resistance was observed among patients in the AR-I group: all four biopsy samples from patients in this group who had glucocorticoid-resistant acute rejection had a high density of CD20+ cells (one patient required antibody therapy with muromonab-CD3 [OKT3] at the outset for presumed vascular rejection, and the others required such therapy after the failure of glucocorticoid pulse therapy) (Table 1). The density of CD20+ cells was strongly correlated with graft loss when all samples from patients with acute rejection were considered together (P<0.001) (Table 2 and supplementary appendixes). To provide an independent test of the significance of this result, we examined the clinical correlates of the retrospective series of 31 biopsy samples from patients with acute rejection and confirmed that dense aggregates of CD20+ cells at the time of biopsy were strongly associated with glucocorticoid resistance (P<0.001) and poor graft outcomes (Table 3).

None of the other variables we studied correlated with either the density of CD20+ lymphocytes or the assignment of a subtype of acute rejection defined according to the pattern of gene expression. These variables were the weight of the donor or the recipient, the age of the recipient, the number of HLA mismatches, the use of a transplant from a living or cadaveric donor, whether or not there were repeated transplantations, the presence or absence of panel-reactive antibody before transplantation, the occurrence or nonoccurrence of delayed graft function, the interval since transplantation, the type of immunosuppression, the presence or absence of hypertension, the presence or absence of anemia, the type of immunosuppressive therapy, or the presence or absence of humoral rejection as determined by complement C4d staining.

DISCUSSION

We examined the global transcript profiles of kidney-biopsy samples to help us to understand and classify acute allograft rejection. Using DNA microarrays, we identified molecular variation suggesting the existence of distinct molecular and prognostic variants of acute rejection, which could not previously be clearly defined on the basis of clinical or pathological criteria. Many of the observed differences in gene-expression patterns among samples from patients with acute rejection appear to reflect differences in the composition and activation of infiltrating lymphocytes. Confounding influences of time may be involved in the ostensible disparities in gene expression that we report here, since there was residual heterogeneity within the subtypes of acute rejection that we defined by cluster analysis. A prospective and extensive longitudinal study of more samples by a variety of methods is needed to refine the classification of acute rejection, with clearer connections between patterns of gene expression, pathophysiology, and clinical course. Since our study is based largely on pediatric patients, similar analyses should be conducted in adult renal-transplant recipients.

The molecular and immunohistochemical evidence of B-cell infiltration in a subgroup of samples from patients with acute rejection was the most unexpected and important finding in this study. Dense CD20 staining was observed in approximately one third of the 52 biopsy samples from patients with

acute rejection that underwent immunohistochemical analysis and was significantly associated with glucocorticoid resistance and eventual graft failure. Staining for CD20 may make possible a rapid clinical test that will permit the definition of a high-risk group of patients with acute rejection who may warrant more aggressive and specific treatment. The association between CD20+ lymphocyte infiltration and graft loss was unexpected. We speculate that in patients who have such an infiltration, early treatment with a monoclonal antibody against CD20 (rituximab) may be beneficial.[29] The association of CD20 staining with glucocorticoid resistance does not suggest that these cases are necessarily humorally mediated despite the presence of C1s, C1r, and C4b in some biopsy samples: staining for complement C4d, which was used as a putative marker of humoral rejection in the biopsy samples from patients with acute rejection in our study, showed poor correlation with CD20 staining (P=1.00) (data not shown).

A preponderance of CD8+ T cells and plasma-cell infiltrates has been associated with glucocorticoid resistance and poor outcomes,[30-33] whereas B cells have been reported to be infrequent or absent in acute rejection. The pathophysiological role of B-cell infiltrates in this study requires further investigation; their presence does not seem to result in direct allograft injury, since immunofluorescence staining of biopsy samples for immunoglobulin and complement deposition was negative in out study (data not shown). As pharmacologic suppression of T cells has improved over the past decade (with the introduction of tacrolimus, mycophenolate mofetil, sirolimus, and monoclonal antibodies against the interleukin-2 receptor), B cells may have evolved as efficient antigen-presenting cells for indirect allorecognition, and their continued presence in the graft, unaffected by current immunosuppressive management, may be resulting in a large fraction of episodes of refractory rejection.[34,35]

In conclusion, molecular profiling of transplants in patients with acute rejection identified new subtypes of acute rejection and a correlation between CD20+ lymphoid aggregates and poorer graft outcomes; these findings may point toward improvements in the individualization of therapy. Gene-expression profiling thus opens a new door for the study of acute rejection and may provide a means to a better understanding of other categories of graft dysfunction.

Table 3. Clinical Correlates of CD20 Status in Renal-Biopsy Samples from Patients with Acute Rejection.

Variable	Retrospective Series of Biopsy Samples (N=31)	Biopsy Samples Included in the Microarray Analysis (N=20)
CD20+ on staining — no./total no. (%)	9/31 (29)	9/20 (45)
Graft loss		
In patients with CD20+ sample — no./total no. (%)	7/9 (78)	8/9 (89)
In patients with CD20– sample — no./total no. (%)	9/22 (41)	1/11 (9)
P value	0.11	<0.001
Glucocorticoid resistance		
In patients with CD20+ sample — no./total no. (%)	8/9 (89)	4/9 (44)*
In patients with CD20– sample — no./total no. (%)	1/22 (5)	1/11 (9)
P value	<0.001	0.01

* All four CD20+ samples from patients with glucocorticoid resistance were in the AR-I group.

Supported by grants from the National Institutes of Health (NIH5P3-05 and NIH3P3-05S1, to Dr. Sarwal), the Clinical Center for Immunological Studies at Stanford University (to Dr. Sarwal), the Packard Foundation, Roche Pharmaceuticals, and the Howard Hughes Medical Institute.

We are indebted to Dr. Patrick O. Brown for scientific direction and support, without which this work would not have been possible; to Drs. Xin Chen, Ash A. Alizadeh, and Maximilian Diehn for invaluable scientific assistance; to Addie Whitney for printing of the DNA microarrays and for helpful advice; to Stella Chang for assistance with RNA amplification; to Dr. John Higgins for the cortex and medulla samples; to the Pediatric Nephrology team for their assistance with sample collection; to Dr. Alan Krensky for his support; to Wijan Prapong for assistance with the supplementary material; to Dr. Robert Colvin at Harvard University for conducting C4d staining; to Jennifer Boldrick for helpful advice, discussions, and review of the manuscript; and to the staff at the Stanford Microarray Database for their support and assistance with Web-site maintenance.

REFERENCES

1. Meier-Kriesche HU, Ojo AO, Hanson JA, et al. Increased impact of acute rejection on chronic allograft failure in recent era. Transplantation 2000;70:1098-100.
2. Madden RL, Mulhern JG, Benedetto BJ, et al. Completely reversed acute rejection is not a significant risk factor for the development of chronic rejection in renal allograft recipients. Transpl Int 2000;13:344-50.
3. Guyot C, Nguyen JM, Cochat P, et al. Risk factors for chronic rejection in pediatric renal allograft recipients. Pediatr Nephrol 1996;10:723-7.
4. Furness PN, Taub N. International variation in the interpretation of renal transplant

biopsies: reports of the CERTPAP Project. Kidney Int 2001;60:1998-2012. [Erratum, Kidney Int 2001;60:2429.]
5. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999; 55:713-23.
6. Tullius SG, Nieminen M, Bechstein WO, et al. Prompt treatment of initial acute rejection episodes may improve long-term graft outcome. Transpl Int 1998;11:Suppl 1:S3-S4.
7. Humar A, Kerr S, Gillingham KJ, Matas AJ. Features of acute rejection that increase risk for chronic rejection. Transplantation 1999;68:1200-3.
8. Matas AJ, Gillingham KJ, Payne WD, Najarian JS. The impact of an acute rejection episode on long-term renal allograft survival (t1/2). Transplantation 1994;57:857-9.
9. Shinn C, Malhotra D, Chan L, Cosby RL, Shapiro JI. Time course of response to pulse methylprednisolone therapy in renal transplant recipients with acute allograft rejection. Am J Kidney Dis 1999;34:304-7.
10. Humar A, Payne WD, Sutherland DE, Matas AJ. Clinical determinants of multiple acute rejection episodes in kidney transplant recipients. Transplantation 2000;69: 2357-60.
11. Schwartz GJ, Haycock GB, Edelmann CM Jr, Spitzer A. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics 1976;58:259-63.
12. Colvin RB, Cohen AH, Saiontz C, et al. Evaluation of pathologic criteria for acute renal allograft rejection: reproducibility, sensitivity, and clinical correlation. J Am Soc Nephrol 1997;8:1930-41.
13. Randhawa PS, Shapiro R, Jordan ML, Starzl TE, Demetris AJ. The histopathological changes associated with allograft rejection and drug toxicity in renal transplant recipients maintained on FK506: clinical significance and comparison with cyclosporine. Am J Surg Pathol 1993;17:60-8.
14. Alizadeh A, Eisen M, Davis RE, et al. The lymphochip: a specialized cDNA microarray for the genomic-scale analysis of gene expression in normal and malignant lymphocytes. Cold Spring Harb Symp Quant Biol 1999;64:71-8.
15. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406:747-52.
16. Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM. High-fidelity mRNA amplification for gene profiling. Nat Biotechnol 2000;18:457-9.
17. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95:14863-8.
18. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116-21. [Erratum, Proc Natl Acad Sci U S A 2001;98:10515.]
19. Jakt LM, Cao L, Cheah KS, Smith DK. Assessing clusters and motifs from gene expression data. Genome Res 2001;11:112-23.
20. Su AI, Cooke MP, Ching KA, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci U S A 2002; 99:4465-70.
21. Diehn M, Alizadeh AA, Rando OJ, et al. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. Proc Natl Acad Sci U S A 2002;99:11796-801. [Erratum, Proc Natl Acad Sci U S A 2002;99:15245.]
22. Whitfield ML, Sherlock G, Saldanha AJ, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 2002; 13:1977-2000.
23. Kummer JA, Wever PC, Kamp AM, ten Berge IJ, Hack CE, Weening JJ. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. Kidney Int 1995;47:70-7.
24. Strehlau J, Pavlakis M, Lipman M, et al. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. Proc Natl Acad Sci U S A 1997;94:695-700.
25. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med 2001;344:947-54.
26. Ogura Y, Krams SM, Martinez OM, et al. Radiolabeled annexin V imaging: diagnosis of allograft rejection in an experimental rodent model of liver transplantation. Radiology 2000;214:795-800.
27. Olyaei AJ, de Mattos AM, Bennett WM. Nephrotoxicity of immunosuppressive drugs: new insight and preventive strategies. Curr Opin Crit Care 2001;7:384-9.
28. Platt JL, LeBien TW, Michael AF. Interstitial mononuclear cell populations in renal graft rejection: identification by monoclonal antibodies in tissue sections. J Exp Med 1982;155:17-30.
29. Aranda JM Jr, Scornik JC, Normann SJ, et al. Anti-CD20 monoclonal antibody (rituximab) therapy for acute cardiac humoral rejection: a case report. Transplantation 2002;73:907-10.
30. Meehan SM, Domer P, Josephson M, et al. The clinical and pathologic implications of plasmacytic infiltrates in percutaneous renal allograft biopsies. Hum Pathol 2001; 32:205-15.
31. Charney DA, Nadasdy T, Lo AW, Racusen LC. Plasma cell-rich acute renal allograft rejection. Transplantation 1999;68: 791-7.
32. Bishop GL, Hall BM, Duggin GG, Horvath JS, Sheil AG, Tiller DJ. Immunopathology of renal allograft rejection analyzed with monoclonal antibodies to mononuclear cell markers. Kidney Int 1986;29:708-17.
33. Hancock WW. Analysis of intragraft effector mechanisms associated with human renal allograft rejection: immunohistological studies with monoclonal antibodies. Immunol Rev 1984;77:61-84.
34. von Willebrand E, Hayry P. Composition and in vitro cytotoxicity of cellular infiltrates in rejecting human kidney allografts. Cell Immunol 1978;41:358-72.
35. Brandle D, Joergensen J, Zenke G, Burki K, Hof RP. Contribution of donor-specific antibodies to acute allograft rejection: evidence from B cell-deficient mice. Transplantation 1998;65:1489-93.