Intragraft T Cell Receptor Transcript Expression in Human Renal Allografts[1]

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ABSTRACT

Allograft rejection is a T cell–dependent process. It is not known whether rejection is mediated by a limited number of T cell clones or by a polyclonal population of T cells. Several studies attempting to answer this question using molecular techniques to analyze the T cell receptor (TCR) population have reached conflicting conclusions. Reverse transcription–assisted polymerase chain reaction (PCR) has been used to quantify T cell infiltration and examine TCR heterogeneity in kidney transplant biopsies from patients experiencing graft dysfunction. RNA from snap-frozen biopsies gathered on 23 transplant patients was reverse transcribed to cDNA and used as the template for PCR. The constant region gene of the TCR beta chain (Cβ), 22 different variable region genes of the TCR beta chain (Vβ) and the constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPD) gene were amplified. T cell infiltration, as estimated by the ratio of reverse-transcribed cDNA Cβ/glyceraldehyde phosphate dehydrogenase, was significantly higher in acute cellular rejection (ACR) (2.25) than in nonrejection (NR) (0.40, $P < 0.05$). The number of intragraft Vβ families was higher in chronic rejection and acute cellular rejection (18 and 16.4, respectively) than in nonrejection (8.7). Five serial biopsies from two patients progressing to immunologic graft loss showed an increase in the number of intragraft Vβ families. The finding of increased numbers of TCR Vβ families amplified from acutely and chronically rejecting grafts as compared with nonrejecting grafts supports the hypothesis that, at the time of clinically apparent rejection, there is a polyclonal infiltration of T cells. Analysis of reverse transcription–PCR amplification patterns of intragraft TCR Cβ and Vβ gene expression may facilitate the diagnosis in patients whose histopathologic analyses do not clearly indicate a specific diagnosis.

Key Words: *Rejection, kidney transplant, T cells*

Under most clinical circumstances, allograft rejection is a T cell–dependent process. Immunosuppressive agents that primarily target T cells have proved most useful in facilitating engraftment. Cyclosporine, which blocks that calcium-dependent pathway of antigen-stimulated T cell activation, is the linchpin of modern immunosuppressive protocols (1). The infusion of OKT3, a monoclonal antibody that binds to a T cell–specific protein, is a powerful treatment for aborting episodes of classic cellular rejection (2). Furthermore, immunohistologic examination of acutely rejecting renal allografts has revealed that T cells are the predominant cell type in the intragraft leukocyte infiltrate (3).

Functional studies of lymphocytes present in human renal allografts have also revealed that the infiltrate in rejecting grafts is enriched with host cytolytic T lymphocytes (CTL) targeted against the cells bearing donor major histocompatibility complex alloantigens (4,5). In keeping with this observation, we and others have determined that acutely rejecting, but not stable or chronically rejecting, human renal transplants contain high levels of CTL-selective granzyme B mRNA (6,7) (M. Suthanthiran, personal communications). Pattison *et al.* have reported that RANTES, a chemokine and important mediator of cytolytic T cells, is present in acute cellular transplant rejection but not in native renal biopsy specimens from patients with cyclosporine nephrotoxicity (8).

A limited pool of host T cells (1 to 5%) becomes activated by allogeneic cells in the mixed lymphocyte response (9). This raises the possibility that the infiltration and rejection of human renal allografts is orchestrated by a small number of T cell clones. In this case, a few clone-specific T cell receptors (TCR) would dominate the lymphocytic infiltrate. Targeting with select anti-TCR ligands could interrupt the sequence of events leading to clinical graft rejection. In order to begin to address whether this goal is attainable, we have studied the expression of TCR variable region gene expression in human renal allografts.

The TCR is an immunoglobulin-like heterodimer composed of two chains, α and β, that are covalently linked and expressed on the T cell surface alongside the CD3 complex (Figure 1). The antigen-binding portion of the TCR is molded by the structure of the variable region of both the α and the β chains (Vα and Vβ). Workers in many different fields of research have analyzed a limited or restricted expression of the Vβ gene as a first approximation for assessing the diver-

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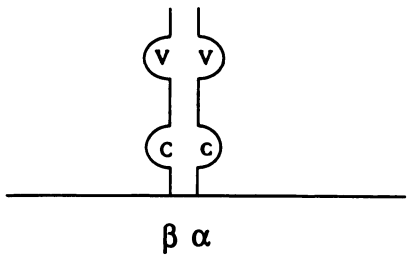

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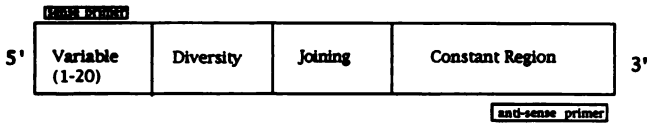

Figure 1. T cell receptor.

sity of T cell clones involved in an *in vivo* process. Studies have shown restriction of the TCR Vβ repertoire in lymphocytes from patients with toxic shock syndrome (10), graft-*versus*-host disease (11), autoimmune thyroid disease (12), rheumatoid arthritis (13), primary biliary cirrhosis (14), multiple sclerosis (15), and others. In striking contrast, other investigators find no evidence for B or T cell oligoclonality at the site of disease expression in several putative T cell–dependent disease states, including rheumatoid arthritis (16–18). Many different clonal T cell populations can use the same Vβ gene in the immune response while using different TCR Vα gene segments, as well as different β chain D and J genes. Therefore, the finding of Vβ gene restriction in disease states must be viewed as a clue to a limited clonal response, not as proof of such.

Studies of clinical renal intragraft TCR gene expression have produced conflicting interpretations. Some workers found a restricted repertoire of TCR Vβ genes predominately in acutely rejecting human renal allograft–derived cell lines by Southern blot analysis and polymerase chain reaction (PCR) (19,20). Similarly, others found a limited number of rearrangements of TCR β chain genes in graft-infiltrating cell lines from both cardiac and renal allograft biopsies (21). Bonneville *et al.* (22) found that the Vβ gene repertoire was not restricted in T cells derived from an irreversibly rejecting renal transplant. Krams *et al.* (23) found a heterogeneous population of Vα transcripts harvested directly from rejecting allograft tissue. Other studies of Vβ and Vα gene expression in allograft-derived cell lines have indicated that a heterogeneous T cell population is present in rejecting allografts (24,25). Our study is unique in that we have amplified both Cβ and the majority of known Vβ genes from rejecting and nonrejecting allograft snap-frozen tissue in an attempt to approximate the degree of clonal diversity of T cells present in rejecting renal allografts.

MATERIALS AND METHODS

Patients

Transplant biopsies were done solely to aid in the management decisions for patients with allograft dysfunction. All renal allograft recipients undergoing transplant biopsies were eligible for this study. Participation was voluntary, and informed consent was required. The study received appropriate approval from the Committee on Clinical Investigations. Two cores were obtained by percutaneous biopsy in adult patients. Open renal biopsy was performed in the four pediatric patients. One core was divided longitudinally, and half was immediately snap-frozen in liquid nitrogen and stored for further processing at −80°C.

Classification

Transplant biopsies were classified into these categories on the basis of the pathologist's analysis of the biopsy and their subsequent clinical course: acute cellular rejection (ACR), no rejection (NR), and chronic rejection (CHR). There were no cases of hyperacute/accelerated vascular rejection.

RNA Extraction and Reverse Transcription

Total RNA was extracted by the guanidium thiocyanate–cesium chloride method as has been described (6). RNA was quantified by spectrophotometry, and the 260/280 was consistently 1.6 to 2.0. We used 0.5 μg of RNA as the template for first-strand cDNA synthesis. The reaction was primed with 600 ng of oligo dT 12–18 and catalyzed with Maloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) in a final reaction volume of 20 μL under conditions suggested by the supplier. This final volume was diluted with 20 μL of water.

Amplification of cDNA by PCR

For each PCR, 1 μL of diluted cDNA was used. The PCR mix for each of the 22 Vβ genes included 0.2 mM dNTP, 2 mM MgCl, Mg-free Taq polymerase buffer (Promega, Madison, WI), 100 ng of sense primer from one of the 22 Vβ regions as has been described (26), and 100 ng of an antisense primer from the Cβ region (5'-CCTTTCTCTTGACCATGGCCAT-3'). Thus, for any specific Vβ family, the PCR product would also contain portions of the Cβ gene. Autoclaved water and Taq polymerase (0.4 U) were added for a final reaction volume of 50 μL. The mixture was overlaid with 75 μL of light mineral oil, and the PCR proceeded as follows: denature at 94°C for 1 min, anneal at 55°C for 1 min, and extend at 72°C for 1 min with a final 7 min of extension at 72°C after the last cycle. In control experiments, the amplification curves were determined for seven different tissue samples for TCR Vβ, glyceraldehyde phosphate dehydrogenase (GAPD), and TCR Cβ genes. We analyzed both coamplification and separate amplifications of the PCR products. In these control experiments, the results were analyzed with a variety of PCR cycle numbers ranging from 20 to 40 to determine the kinetics of product formation. Thirty-eight cycles of amplification consistently yielded a linearly increasing amount of product.

The specificity of the various amplified Vβ genes was confirmed by assessing the size of each product before and after digestion with various restriction endonucleases. For GAPD and Cβ gene amplification, the same PCR constituents were used except for the gene-specific oligonucleotides. A

9-μL aliquot of each sample mixture was size fractionated by electrophoresis on a 1.6% agarose gel and transferred to a Hybond N+ membrane (Amersham, Arlington Heights, IL). To probe the Vβ and Cβ PCR products, we used a cDNA probe that hybridizes to a sequence present in both the Cβ1 and the Cβ2 genes. These genes are nearly identical, and the use of one Cβ gene *versus* the other by any given T cell appears to be random. This cDNA probe was radiolabeled by a random priming with [^{32}P]dCTP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, MA). For GAPD mRNA, a nested nucleotide was similarly labeled with a TdT enzyme. The probes were added at 10^6 to hybridization solution for 24 h at 42°C. The autoradiographs were analyzed by scanning densitometry on a Beckman spectrophotometer (Beckman Instruments, Fullerton, CA).

Positive and Negative Controls

Positive control samples were obtained from total RNA extracted from phytohemagglutin A (5 μg/mL)- and phorbol myristate acetate (5 ng/mL)-stimulated peripheral blood mononuclear cells cultured for 12 h and pulsed with cyclohexamide (20 μL/mL) for an additional 4 h. The Cβ gene, GAPD gene, and each of the 22 Vβ gene families were amplified from the positive control sample, indicating that the oligonucleotides worked. Each PCR was run with a negative control specimen that had no DNA template. There were no false-positives. The reverse transcription-PCR experiments were repeated and found to be reproducible.

RESULTS

Cβ Amplification Correlates With Rejection

We chose to amplify GAPD and Cβ as a way to use PCR to assess the amount of tissue harvested at biopsy and T cell infiltration into that sample, respectively. GAPD is ubiquitously expressed. The amount of GAPD amplified by PCR should reflect the amount of RNA reverse transcribed. In our experiments, the GAPD gene was amplified in similar amounts in each of the samples. Because intragraft T cells are overwhelmingly of the TCR α/β phenotype, the quantity of Cβ amplified should reflect the degree of T cell infiltration. We chose to use the ratio of Cβ/GAPD to reflect the amount of T cell infiltration per renal tissue mass. For the 10 patients classified as having ACR, the average ratio was 2.25 as compared with 0.40 in the 10 patients classified as having NR ($P < 0.05$, as calculated by the Kruskal-Wallis method). The average Cβ/GAPD ratio for the six patients with CHR was 0.21 (Figure 2).

Number of Intragraft Vβ Families Increases With Rejection

The number of Vβ families that we amplified from biopsy specimens with ACR averaged 16.4 (Table 1). This was not statistically different from the average of 18 Vβ families amplified from the samples with CHR. In contrast, only 8.7 Vβ families on average were amplified from the patients with NR ($P < 0.01$) (Newman Keuls method).

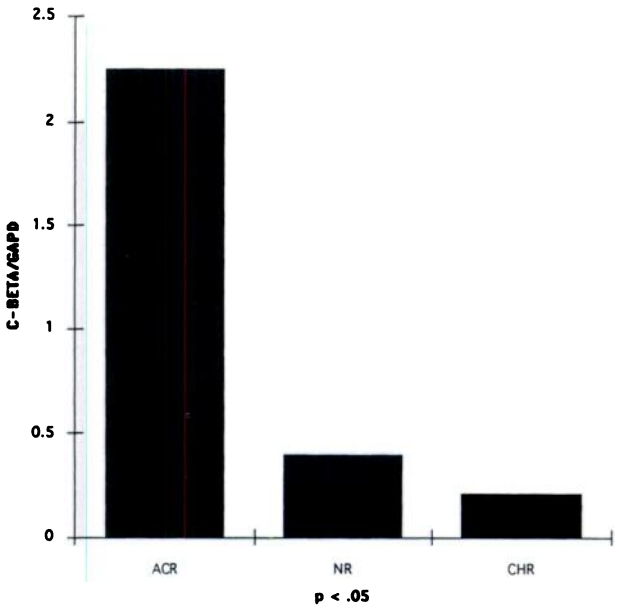

Figure 2. C-beta/GAPD is high in ACR.

TABLE 1. The number of Vβ families is highest in ACR and CHR compared with NR[a]

Diagnosis	Vβ Families	No. of Samples
ACR	16.4	10
NR	8.7	10
CHR	18	6

[a] $P < 0.05$.

HLA Matching Not Correlated With Vβ Expression

We analyzed the tissue typing data from all transplant recipients in this study (data not shown). The degree of HLA matching was not correlated to the number of Vβ families amplified by PCR in any of the diagnosis groups.

Sequential Biopsies on Two Patients

Two patients underwent sequential biopsies as they progressed to immunologic graft loss. The first patient had a biopsy 9 days posttransplantation and showed no evidence of rejection or inflammation. This sample had a Cβ/GAPD of 2.19 (well above the average of 0.40 for the NR group) and only one Vβ family, Vβ6. The second biopsy, done 2 months later, revealed ACR. This sample had a Cβ/GAPD of 0.188, and 16 Vβ families were amplified. The final biopsy from this patient was taken during transplant nephrectomy for irreversible ACR and had a Cβ/GAPD of 1.535 and 17 Vβ families.

The second patient underwent biopsy 2 months posttransplantation and had CHR (Figure 3). The Cβ/GAPD was 0.23, and 17 Vβ families were ampli-

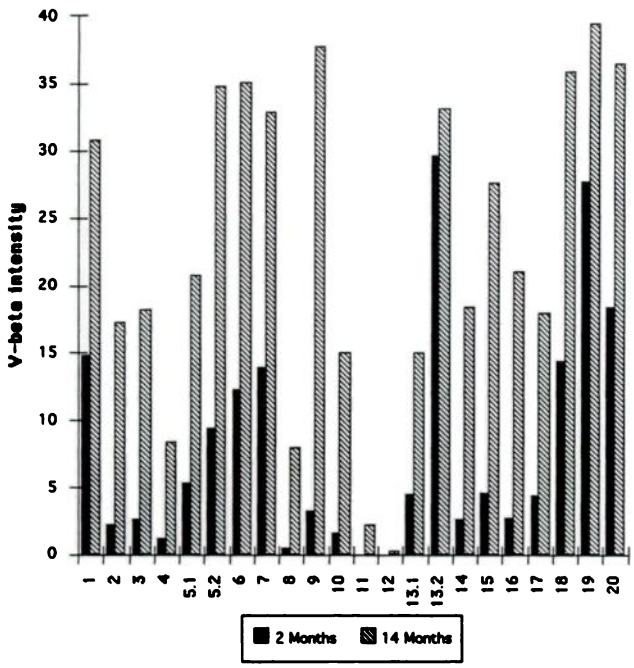

Figure 3. The number of V-beta families and amount of each V-beta increases over time in this patient with chronic rejection.

fied. The second biopsy was taken at the time of transplant nephrectomy 14 months later and also showed CHR. The Cβ/GAPD was 0.24, and 20 Vβ families were amplified. In both patients, the number of Vβ family genes expressed in the allograft increased as the allograft rejection progressed.

DISCUSSION

Virtually every human renal allograft biopsy shows some evidence of interstitial nephritis. Even grafts not undergoing clinically apparent rejection episodes can, on occasion, contain intense cellular infiltration (27). Insofar as intense T cell infiltration is a hallmark of rejection, we attempted to develop molecular correlates that could be linked to rejection. Our goal was to explore the degree of T cell clonal expansion or restriction in renal allograft rejection.

The novel features of this study are the combined use of TCR constant region gene amplification as a marker for rejection and the amplification of Vβ families directly from the graft (unbiased by the cultivation of infiltrating T cells in culture). Although others have analyzed the *in situ* Vα repertoire (23), this is the first report that analyzes the *in situ* TCR Vβ repertoire in human renal allografts. The important finding in these experiments is that the number of Vβ families is increased in renal transplants undergoing ACR or CHR. Also, the amount of Cβ amplified by these techniques correlates with the magnitude of T cell infiltration seen by light microscopy. The study lends further support to the application of PCR methods to renal transplant biopsies to analyze the intensity and heterogeneity of T cell infiltration. Krams *et al.* analyzed the *in situ* Vα repertoire and showed that there was no evidence of oligoclonality in renal transplants undergoing rejection. Taken together with our findings, it seems unlikely that a restricted TCR repertoire exists at the time of graft dysfunction in cellular rejection. The finding of a restricted TCR repertoire in cells cultured from rejecting allografts may arise from the selection process of stimulating a subgroup of T cells to grow out of proportion to their original numbers in the rejecting allograft.

This study is only a first step toward identifying the role and number of T cell clones that initiate allograft rejection. It is possible that an oligoclonal population of T cells initiates rejection episodes, but at the time of dysfunction, the T cell population is polyclonal. The role of PCR amplification of T cell–specific genes has yet to be defined in the diagnosis and treatment of human renal allograft rejection. It has been shown here to be a scientific tool of use in furthering our understanding of the clonality of T cells present in renal allograft rejection. The results of the PCR amplification of Cβ and the number of Vβ families show distinct patterns for ACR, CHR, and NR. We foresee two areas of possible clinical application of this technology. A program of routine monitoring of allograft tissue by PCR amplification could be used to further our understanding of T cell clonality before clinically apparent rejection and potentially allow therapy directed against an early-appearing T cell clonal population. Second, there could be a role for T cell–specific gene amplification by PCR to predict the response of rejection to certain forms of therapy. These and other applications of this technology will be the subject of future research.

REFERENCES

1. **Schreiber SL, Crabtree GR:** The mechanism of action of cyclosporin A and FK506. Immunol Today 1992;13:136–142.
2. **Cosimi AB, Burton RC, Colvin RB, *et al.*:** Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. Transplantation 1981;32:535–539.
3. **Braun W:** Allograft rejection: Clinical manifestations and diagnosis. In: Textbook of Nephrology. Baltimore: Williams & Wilkins; 1989:1477–1484.
4. **Strom TB, Tilney NL, Carpenter CB, Busch GJ:** Identity and cytotoxic capacity of cells infiltrating renal allografts. N Engl J Med 1975;292:1257–1263.
5. **Strom TB, Tilney NL, Paradysz JM, Bancewicz J, Carpenter CB:** Cellular components of allograft rejection: identity, specificity, and cytotoxic function of cells infiltrating acutely rejecting allografts. J Immunol 1977;118: 2020–2026.
6. **Lipman ML, Stevens AC, Bleakley RC, *et al.*:** The strong correlation of cytotoxic T lymphocyte-specific serine protease gene transcripts with renal allograft rejection. Transplantation 1992;53:73–79.
7. **Lipman ML, Stevens A, Strom TB:** Heightened intragraft CTL gene expression in acutely rejecting renal allografts. J Immunol 1994;152:5120–5127.
8. **Pattison J, Nelson P, Huie P, *et al.*:** RANTES chemokine expression in cell-mediated transplant rejection of the kidney. Lancet 1994;343:209–211.
9. **Wilson D, Bluthe J, Nowell P:** Quantitative studies in the

mixed lymphocyte reaction: III. Kinetics of the response. J Exp Med 1968;128:1157–1181.
10. **Choi YW, Lafferty JA, Clements JR, *et al.*:** Selective expansion of T cells expressing Vβ 2 in toxic shock syndrome. J Exp Med 1990;172:981–984.
11. **Yamanaka K, Kwok W, Mickelson E, Masewicz S, Smith F, Nepom GT:** Selective T-cell-receptor gene usage in allorecognition and graft-versus-host disease. Transplantation 1993;55:1167–1175.
12. **Davies T, Martin A, Concepcion ES, Graves P, Cohen L, Ben-Nun A:** Evidence of limited variability of antigen receptors on intrathyroidal T cells in autoimmune thyroid disease. N Engl J Med 1991;325:238–244.
13. **Paliard X, West SG, Lafferty JA, *et al.*:** Evidence for the effects of a superantigen in rheumatoid arthritis. Science 1991;253:325–329.
14. **Moebius U, Manns M, Hess G, Krober G, Meyer zum Buschenfelde KH, Meuer SC:** T-cell receptor gene rearrangements of T lymphocytes infiltrating the liver in chronic active hepatitis B and primary biliary cirrhosis (PBC): Oligoclonality of PBC-derived T-cell clones. Eur J Immunol 1990;20:889–896.
15. **Ben-Nun A, Liblau R, Cohen L, *et al.*:** Restricted T-cell receptor Vβ gene usage by myelin basic protein-specific T-cell clones in multiple sclerosis: Predominant genes vary in individuals. Proc Natl Acad Sci USA 1991;88: 2466–2470.
16. **Kono DH, Baccala R, Balderas RS, *et al.*:** Application of a multiprobe RNAse protection assay and junctional sequences to define Vβ gene diversity in Sezary syndrome. Am J Pathol 1992;140:823–830.
17. **Quayle AJ, Wilson KB, Li SG, *et al.*:** Peptide recognition, T-cell receptor usage and HLA restriction elements of human heat-shock protein (hsp) 60 and mycobacterial 65-KDa hsp-reactive T-cell clones from rheumatoid synovial fluid. Eur J Immunol 1992;22:1315–1322.
18. **Duby AD, Sinclair AK, Osborne-Lawrence SL, Zeldes W, Kan, Fox L:** Clonal heterogeneity of synovial fluid T lymphocytes from patients with rheumatoid arthritis. Proc Natl Acad Sci USA 1989;86:6206–6210.
19. **Miceli MC, Finn OJ:** T cell receptor β-chain selection in human allograft rejection. J Immunol 1989;142:81–86.
20. **Kirk AD, Ibraham MA, Bollinger RR, Dawson DV, Finn OJ:** Renal allograft-infiltrating lymphocytes. A prospective analysis of in vitro growth characteristics and clinical relevance. Transplantation 1992;53:329–338.
21. **Frisman D, Hurwitz A, Bennett W, *et al.*:** Clonal analysis of graft-infiltrating lymphocytes from renal and cariac biopsies. Dominant rearrangements of TcR beta genes and persistence of dominant rearrangements in serial biopsies. Hum Immunol 1990;28:208–215.
22. **Bonneville M, Moisan JP, Moreau JF, *et al.*:** TCR β and gamma gene rearrangements in human alloreactive T-cell clones extracted from a rejected kidney. Transplant Proc 1988;20:196–198.
23. **Krams SM, Falco DA, Villanueva JC, *et al.*:** Cytokine and T-cell receptor gene expression at the site of allograft rejection. Transplantation 1992;53:151–156.
24. **Datema G, Vaessen LM, Daane CR, *et al.*:** T-cell receptor V gene segment usage of graft-infiltrating T-lymphocytes after heart transplantation. Transplant Proc 1993;25: 77–79.
25. **Gecim IE, Christmas E, Flanagan BF, *et al.*:** Analysis of T-cell receptor Vβ gene usage in T cells infiltrating human renal allografts. Transplant Proc 1993;25:75–76.
26. **Choi YW, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J:** Interaction of staphylococcus aureus toxin "superantigens" with human T cells. Proc Natl Acad Sci USA 1989;86:8941–8945.
27. **Rush D, Henry S, Jeffrey J, Schroeder T, Gough J:** Histological findings in early routine biopsies of stable renal allograft recipients. Transplantation 1994;57:208–211.