Coronary Arteries from Human Cardiac Allografts with Chronic Rejection Contain Oligoclonal T Cells: Persistence of Identical Clonally Expanded TCR Transcripts from the Early Post-Transplantation Period (Endomyocardial Biopsies) to Chronic Rejection (Coronary Arteries)

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*average
Coronary Arteries from Human Cardiac Allografts with Chronic Rejection Contain Oligoclonal T Cells: Persistence of Identical Clonally Expanded TCR Transcripts from the Early Post-Transplantation Period (Endomyocardial Biopsies) to Chronic Rejection (Coronary Arteries)\textsuperscript{1,2}

C. A. Slachta,\textsuperscript{*} V. Jeevanandam,\textsuperscript{†} B. Goldman,\textsuperscript{‡} W. L. Lin,\textsuperscript{*} and C. D. Platsoucas\textsuperscript{3,*}

Chronic cardiac allograft rejection presents pathologically as graft arteriosclerosis (GA) characterized by recipient T cell and monocyte infiltration. To determine whether oligoclonal T cells are present in coronary arteries of cardiac allografts from patients with GA, we conducted sequencing analysis of \(\beta\)-chain TCR transcripts from these explanted coronary arteries using the nonpalindromic adaptor-PCR. Substantial proportions of identical \(\beta\)-chain TCR transcripts in three of five patients were observed, clearly demonstrating the presence of oligoclonal T cells. TCR transcripts from the arteries of two other patients were relative heterogeneous. High proportions of identical CDR3 \(\beta\)-chain TCR motifs were found in each patient. \textsc{genebank}/\textsc{embl}/\textsc{swiss prot} database comparison of all sequences revealed that these \(\beta\)-chain TCR transcripts were novel. Using \(\gamma\beta\)-specific PCR (independent amplification), we found in patient GA03 that the TCR transcript that was clonally expanded in the left anterior descending artery after nonpalindromic adaptor-PCR was also clonally expanded in the right coronary artery of the same allograft. These results demonstrate that this TCR transcript was clonally expanded at different anatomic sides of the cardiac allograft in a systemic manner. In two patients identical \(\beta\)-chain TCR transcripts that were found to be clonally expanded in the coronary arteries of their explanted cardiac allografts were also found to be clonally expanded in endomyocardial biopsies collected 17 and 21 mo earlier from each patient. The presence of oligoclonal populations of T cells in the rejected graft suggest that these T cells have undergone specific Ag-driven proliferation and clonal expansion early on within the graft and persist throughout the post-transplantation period. \textit{The Journal of Immunology}, 2000, 165: 3469–3483.

C hronic rejection of cardiac allografts is the major limitation of graft survival 1 year post-transplantation, and it is found in ~50% of the cardiac allograft recipients 5 years post-transplantation (1–5). Chronic rejection is associated with graft arteriosclerosis (GA)\textsuperscript{4} or transplantation-associated arteriosclerosis, which pathologically presents as diffuse, concentric, stenosing fibrointimal proliferation within the coronary arteries of allografts and eventually leads to obliterative arteriopathy and graft failure (1–7). The pathology of GA is different in several aspects from that of naturally occurring arteriosclerosis (6, 7).

Cell-mediated immune responses and perhaps injury of the arteries by an allogeneic immune response may be the primary cause of the development of GA (3–10). T cells may play a major role in the pathogenesis of GA. The development of GA can be enhanced in rats with cardiac allografts by prior sensitization of recipient animals to donor splenic lymphocytes (11). In contrast, rats transplanted with cardiac allografts from syngeneic animals did not develop GA (12). These results suggest the involvement of an alloantigen-driven T cell response in the pathogenesis of chronic rejection. Although this immune response appears to be the dominant factor, Ag-independent and nonimmunological factors, such as ischemia-reperfusion, viral infection, hyperlipidemia, insulin resistance, and hypertension, may also contribute to the development of GA (reviewed in Refs. 13–15).

Mononuclear cell infiltrates are commonly associated with GA and have been observed in the intima (16) or subendothelial space (17) of cardiac allograft arteries of patients with GA. These infiltrates are comprised predominantly of T cells and monocytes (6, 16–24) of recipient origin (24), which are believed to represent an immune response of the donor to the graft. These T cells may play a major role in the immunopathogenesis of chronic rejection. The majority of the infiltrating T cells appear to be CD8\textsuperscript{+}CD4\textsuperscript{−} (17, 20–23), although equal ratios of CD4\textsuperscript{+}CD8\textsuperscript{−} and CD4\textsuperscript{+}CD8\textsuperscript{+} lymphocytes have also been reported (18). Perforin-positive CTL have been identified in coronary arteries of patients with GA and may contribute to the development of disease (25). These CD8 cells may be responsible, directly or indirectly, for endothelial cell injury, which is an important step in the development of GA (17).
More than 90% of T cells in the peripheral blood express the αβ TCR, which is comprised of two highly polymorphic disulfide-linked peptide chains, the α-chain and the β-chain. Both are members of the Ig supergene family (26). T cells of the recipient recognize organ grafts by two different pathways of Ag presentation, the direct and the indirect (27–35). Antigenic specificity for the TCR is primarily associated with the sequences of the hypervariable, or CDR3, part of the β-chain TCR transcript (36–39).

To elucidate whether T lymphocytes infiltrating coronary arteries of cardiac allografts from patients with chronic rejection contain oligoclonal populations of T cells, we amplified β-chain TCR transcripts from the coronary arteries of five explanted cardiac allografts with GA by the nonpalindromic adaptor PCR (NPA-PCR) (40–43) and by Vβ-specific PCR. The NPA-PCR method is specifically designed for the amplification of transcripts with unknown 5' ends, such as TCRs and Igs (40–43). The amplified transcripts were cloned and sequenced. Sequence analysis revealed substantial proportions of identical β-chain TCR transcripts, strongly suggesting the presence of oligoclonal T cells infiltrating coronary arteries with GA. In one patient, identical TCR clones were found to be clonally expanded in the left anterior descending (LAD) and the right coronary artery (RCA) of the same explanted allograft with chronic rejection. Additionally, sequence analysis of archived endomyocardial biopsy material from two patients with GA demonstrated the presence of clonally expanded β-chain TCR transcripts identical with those found to be clonally expanded in the coronary arteries of these patients many months later.

Materials and Methods

Patients

Explanted cardiac allografts from five adult patients (Table I; mean age, 45.8) who had undergone primary, single-organ, orthotopic heart transplantation and developed chronic rejection were used in this study. Post-transplantation care in all cases was routine. These studies have been approved by the Temple University Hospital Institutional Review Board.

Cardiac explants

Coronary arteries from cardiectomy tissue obtained immediately before each patient’s retransplantation were used. Each patient was diagnosed with moderate (n = 1) to severe (n = 4) GA upon pathological examination, based on the observation of circumferential fibrointimal thickening, lumenal narrowing, and stenosis (20–90%; mean, 56%) in the main coronary arteries as well as the presence of stenosis in multiple small epicardial coronary branches. Samples of coronary arteries were taken from the proximal, middle, and distal thirds of each of two major epicardial arteries (RCA and LAD) and were chosen to represent the most abnormal portion of each segment. Tissue were snap-frozen within 1–2 h of procurement. The endomyocardium of the explants from patients GA05 and GA09 demonstrated focal moderate acute cellular rejection consistent with International Society of Heart and Lung Transplantation (ISHLT) rejection grade 3A/4. The remaining three explants demonstrated either no acute cellular rejection (GA02, GA03), or grade 1A (minimal) acute rejection (GA06).

Evidence of a Quilty effect (endocardial lymphoid infiltrate) (44) was present in all five explants studied.

Endomyocardial biopsies (EMBX)

EMBXs were obtained from patients GA05 and GA09 as part of routine post-transplantation care and monitoring. EMBX were snap-frozen and stored in liquid nitrogen. EMBXs used in this study were collected 13 and 16 mo post-transplantation (and 17 and 28 mo before retransplantation with a second cardiac allograft) from patients GA05 and GA09, respectively. These EMBXs were chosen on the basis of the pathology observed in each specimen. Rejection grade in each EMBX was diagnosed in accordance with the guidelines established by the ISHLT (45). EMBX from patient GA09 demonstrated grade 1A rejection (focal perivascular or interstitial infiltrate with no evidence of necrosis) as well as evidence of atherosclerosis. EMBX from patient GA05 exhibited grade 3A rejection (necrosis as well as evidence of diffuse inflammatory processes) and mild atherosclerosis.

Controls

Human PBMC were prepared as previously described (40) and were used as methodological controls. Control epicardial arteries, designated control arteries 1 and 2, respectively, were obtained from a nontransplanted normal adult male heart and from autopsy material of an adult female who died due to unrelated disease (diabetes mellitus).

Histology and immunohistochemical staining

Cardiectomy sections were embedded in paraffin. Serial 6-μm sections were stained using hematoxylin and eosin and were evaluated for the presence of GA by routine pathological examination. Immunohistochemistry was performed (46, 47) using the anti-CD3 mAb, clone NCL-CD3-P5 (Novocastra, Newcastle upon Tyne, U.K.) and an isotype-matched nonspecific mouse IgG as a negative control.

RNA isolation

RNA from sections of LAD or RCA, EMBX, and from PBMC were prepared using a guanidinium thiocyanate solution (Stratagene, La Jolla, CA) as recommended by the manufacturer.

cDNA synthesis

Double-stranded cDNA was synthesized from oligo(dT)-No1 (Promega, Madison, WI)-primed total RNA by use of the Superscript II (Life Technologies, Grand Island, NY) cDNA synthesis kit. Double-stranded cDNA was blunt ended (for efficient adaptor ligation) using T4 DNA polymerase.

Nonpalindromic adaptor-PCR (NPA-PCR)

NPA-PCR was conducted as previously described with minor modifications (40–43). Briefly, double-stranded blunt-ended cDNA was ligated with an equivalent molar concentration of nonpalindromic adaptor, which consisted of two complimentary oligonucleotides (Table II), EcoRI-XmnI and XmnI-G, that were preannealed to each other. cDNA and adaptor were incubated at 16°C overnight with T4 DNA ligase (1.2 U) and purified on a G-50 column (5 Prime 3 Prime, Boulder CO). NotI restriction endonuclease (20 U) treatment for 2 h at 37°C was then used to remove ligated

Table I. Demographic and clinical characteristics of patients with chronic cardiac allograft rejection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Race</th>
<th>Age</th>
<th>Years to Re-Txp</th>
<th>Primary Txp indication</th>
<th>Re-Txp indication</th>
<th>Stenosis (%)</th>
<th>Acute cellular rejection (ISHLT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA02</td>
<td>M</td>
<td>C</td>
<td>46</td>
<td>9</td>
<td>A RVD*</td>
<td>GA (sev.)</td>
<td>15–20</td>
<td>0 + Quilty</td>
</tr>
<tr>
<td>GA03</td>
<td>M</td>
<td>C</td>
<td>59</td>
<td>4</td>
<td>Ischemia</td>
<td>GA (sev.)</td>
<td>50–60</td>
<td>0 + Quilty</td>
</tr>
<tr>
<td>GA05</td>
<td>F</td>
<td>AA</td>
<td>31</td>
<td>2.5</td>
<td>Atherosclerosis</td>
<td>GA (mod.)</td>
<td>90</td>
<td>3A + Quilty</td>
</tr>
<tr>
<td>GA06</td>
<td>M</td>
<td>C</td>
<td>47</td>
<td>4.2</td>
<td>Ischemia</td>
<td>GA (sev.)</td>
<td>20–30</td>
<td>1A + Quilty</td>
</tr>
<tr>
<td>GA09</td>
<td>M</td>
<td>C</td>
<td>46</td>
<td>3.3</td>
<td>Atherosclerosis</td>
<td>GA (sev.)</td>
<td>70</td>
<td>3A + Quilty</td>
</tr>
</tbody>
</table>

* Percent stenosis in microscopic sections of coronary arteries were estimated as the ratio of (observed luminal cross sectional area)/(area within internal elastic lamella). When arterial profiles were not circular, areas were estimated as circles of equivalent circumference.

* Transplant.
adaptor from the 3′ end of the cDNA. The resultant cDNA was repurified on a G-50 column, and 0.5 vol of eluate was amplified using two rounds of PCR in a 100-μl reaction. In the first amplification 5′ amplification primer was the adaptor primer, EcoRI-XmnI, and a human HCB3 primer was used as the 3′ amplification primer (100 pmol each; Table II). In the second amplification, the adaptor primer was again used as the 5′ amplification primer, and the HCB2 primer, located 5′ to the HCB3 primer (nested design), served as the 3′ amplification primer. In addition to the primers and cDNA, each PCR contained the following: 2.5 mM MgCl2, 5 U Taq DNA polymerase with appropriate buffer (Promega), and 0.25 mM dNTPs (Phar- macia, Piscataway, NJ). PCR for the first amplification (30 cycles) included denaturation (94°C, 45 s), annealing (45°C, 45 s), and elongation (72°C, 45 s), followed by final incubation at 72°C for 10 min. PCR for the second amplification (35 cycles) included denaturation (94°C, 45 s), annealing (50°C, 45 s), elongation (72°C, 45 s), and a final incubation at 72°C for 10 min.

Table II. 

<table>
<thead>
<tr>
<th>Adaptor primer</th>
<th>DNA Sequence (5′ to 3′)</th>
<th>Primer Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI-XmnI</td>
<td>[d{ATTCGAAACCCTTCG}]</td>
<td></td>
</tr>
<tr>
<td>XmnI G (5′-phosphorylated)</td>
<td>[d{CGAAGGGGTTTCG}]</td>
<td></td>
</tr>
<tr>
<td>Non-palindromic adaptor (NPA)</td>
<td>5′-AACCCCTTCG-3′</td>
<td>3′-GGGTGGGAACG</td>
</tr>
</tbody>
</table>

TCR β-chain primers

| HCB3 | CAGGCAGTATCTGGAGTCATTGA | 203 bp 3′ of Jβ |
| HCB2 | ACCAGCTACGGTTGCTGCTCTG | 113 bp 3′ of Jβ |
| HCB1 | TTCTGATGGTCTAAAACACGGACCTC | 28 bp 3′ of Jβ |
| Vβ2.1 | ATACCTCTGAGGAGACACAGAGA | ~124 bp 5′ of N-Dβ-N |
| Vβ3.1 | GTCTCTAGAGGAGACACAGAGA | ~86 bp 5′ of N-Dβ-N |
| Vβ4.1 | TTCCCATGCGGCGCGACCTTAA | ~94 bp 5′ of N-Dβ-N |
| Vβ5.1 | ATCTCTCTGAGGAGACACAGAGA | ~148 bp 5′ of N-Dβ-N |
| Vβ9.1 | TTCTGAGCTGGTGGTGCTCAGCC | ~51 bp 5′ of N-Dβ-N |
| Vβ12.1 | GTCTGACGACTGGAGACACAGAC | ~214 bp 5′ of N-Dβ-N |

CD3 amplification primers

| Sense | 5′-CTGGAACCTGGGAAAACGCATC |
| Antisense | 3′-GACTCAGTACCATCTCGATC |

FIGURE 1. 
Histopathology and immunohistochemical staining of the left anterior descending (LAD) coronary artery of patient GA02 (A, B, C, and D) and a nontransplanted heart (E and F). A, Diffuse, concentric fibrointimal thickening with substantial mononuclear cell infiltrate within the intima, media, as well as the adventitia. Note damage to media near the large focus of infiltrating cells. Hematoxylin-eosin staining was used. Original magnification, ×40. B, Immunohistochemical staining for presence of T cells (anti-CD3 mAb) in serial section of tissue described in A. Staining patterns were consistent with the mononuclear cell infiltrate observed in hematoxylin-eosin-stained sections, indicating that a substantial proportion of these infiltrating cells are T lymphocytes. Original magnification, ×40. C, Mononuclear cell infiltrate within the intima along the luminal surface. Hematoxylin-eosin staining was used. Original magnification, ×400. D, Immunohistochemical staining for presence of T cells (anti-CD3 mAb) in serial section of tissue described in C. Staining patterns were consistent with mononuclear cell infiltrate observed in hematoxylin-eosin-stained sections, indicating that a substantial proportion of these infiltrating cells are T lymphocytes. Original magnification, ×400. E, LAD of nontransplanted heart. Slight thickening of the intima consistent with normal aging is evident. Rare mononuclear cells noted near the luminal surface. Hematoxylin-eosin staining was used. Original magnification, ×80. F, Immunohistochemical staining for presence of T cells (anti-CD3 mAb) in serial section of tissues described in C. Rare CD3+ cells, designated by arrows, were observed. Original magnification, ×80. Control sections were stained with an irrelevant mAb of the same isotype and were all negative (not shown).
<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0314</td>
<td>TGGCCGACAGC</td>
<td>TTTGATGACC</td>
<td>TGAACACTGAACT</td>
<td>Vβ3.1 D18.1 Jβ1.1</td>
</tr>
<tr>
<td>ga0339</td>
<td>TGGCCGACAGC</td>
<td>AGTGGAGAGGC</td>
<td>TGGATCCACTTAC</td>
<td>Vβ3.1 D18.1 Jβ1.2</td>
</tr>
<tr>
<td>ga0316</td>
<td>TGGCCGACAGC</td>
<td>GCCTCGCAGCTT</td>
<td>TGGATCCACTTAC</td>
<td>Vβ3.1 D18.1 Jβ1.3</td>
</tr>
</tbody>
</table>

**β-chain TCR transcripts in RCA following Vβ5.1-specific PCR**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0314</td>
<td>TGGCCGACAGC</td>
<td>TTTGATGACC</td>
<td>TGAACACTGAACT</td>
<td>Vβ3.1 D18.1 Jβ1.1</td>
</tr>
<tr>
<td>ga03543</td>
<td>TGGCCGACAGC</td>
<td>AGGCGCCCGAG</td>
<td>AGGCGCCCGAG</td>
<td>Vβ3.1 D18.1 Jβ1.2</td>
</tr>
<tr>
<td>ga03556</td>
<td>TGGCCGACAGC</td>
<td>PDRG</td>
<td>YNEQ</td>
<td>Vβ3.1 D18.1 Jβ1.3</td>
</tr>
<tr>
<td>ga03517</td>
<td>TGGCCGACAGC</td>
<td>ATCCTCGACGCG</td>
<td>ATCCTCGACGCG</td>
<td>Vβ3.1 D18.1 Jβ1.4</td>
</tr>
</tbody>
</table>

**β-chain TCR transcripts in RCA following Vβ3.1-specific PCR**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0314</td>
<td>TGGCCGACAGC</td>
<td>TTTGATGACC</td>
<td>TGAACACTGAACT</td>
<td>Vβ3.1 D18.1 Jβ1.1</td>
</tr>
<tr>
<td>ga03543</td>
<td>TGGCCGACAGC</td>
<td>AGGCGCCCGAG</td>
<td>AGGCGCCCGAG</td>
<td>Vβ3.1 D18.1 Jβ1.2</td>
</tr>
</tbody>
</table>

---

**Table III. β-chain TCR transcripts (CDR3 region) expressed in coronary arteries of patient GA03**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0314</td>
<td>TGGCCGACAGC</td>
<td>TTTGATGACC</td>
<td>TGAACACTGAACT</td>
<td>Vβ3.1 D18.1 Jβ1.1</td>
</tr>
<tr>
<td>ga03543</td>
<td>TGGCCGACAGC</td>
<td>AGGCGCCCGAG</td>
<td>AGGCGCCCGAG</td>
<td>Vβ3.1 D18.1 Jβ1.2</td>
</tr>
</tbody>
</table>

---

**β-chain-specific amplification**

β-chain specific amplifications of TCR transcripts were used to examine, in more detail, a single Vβ family or subfamily or for confirmation purposes if there was a reason to believe that β-chain TCR clonal expansion(s) was present in a particular Vβ family. On the basis of this criteria, the following Vβ families were amplified: Vβ2.1, Vβ3.1, Vβ4.1, Vβ5.1, Vβ9.1, and Vβ12.1.
DH5
the pCR2.1 plasmid vector (Invitrogen, San Diego, CA), transformed into
b
amplification primer (hC
based on the NPA-PCR results. 5
independent sample population is negligible. During transformation of
transcripts is
DNA sequencer (Applied Biosystems, Foster City, CA).

positive colonies using the Wizard Miniprep DNA Purification System

not immediately enter log phase, but the unlikely possibility for a few of

heat shock, however, the cells do

phase),

(42°C for 45 s) followed by incubation on ice for 2 min and growth for

yielded several examples of two identical copies of a single
E. coli

scripts were unique compared with each other, with the exception of one
families were chosen for amplification in each patient

hiJ
amplification primer (hCβ2) are shown in Table II. Template cDNA was syn-
thesized from RCA total RNA isolated from the same cardiectomies used for

computer analysis and comparison of sequences

remaining 12/35 TCR sequences were unique as compared to each other and are not shown here.

Table II). Different Vβ families were chosen for amplification in each patient
based on the NPA-PCR results. 5 amplification primers (Viβ) and the 3’ amplification primer (hiJβ) are shown in Table II. Template cDNA was syn-
thesized from RCA total RNA isolated from the same cardiectomies used for

on the basis of identity, diversity, and junctional segments. Surprisingly,

amplification

amplified using 36 PCR cycles (46, 47). Amplification primers are shown

b

results to determine whether fresh (not expanded in culture) mononuclear
cells infiltrating coronary arteries of cardiac allografts with GA

Table II

VA
NSGTTV
D
Q

Vβ3.1 DJβ2.1 DJβ2.3

SS

(2/38; 5.2%)

(2/38; 5.2%)

(2/38; 5.2%)

(1/38; 2.6%)

(1/38; 2.6%)

5

(2/35 cycles), included denaturation (94°C, 30 s), annealing (56°C, 45 s), elon-
gation (72°C, 1 min), and final elongation (72°C, 10 min).

CD3-β specific amplification
The presence of T cells in coronary arteries with GA was determined by
performing PCR analysis for the presence of CD3β (48). Transcripts were
amplified using 36 PCR cycles (46, 47). Amplification primers are shown in
Table II.

Cloning and sequencing of PCR products
PCR products from either NPA-PCR or Vβ-specific PCR were cloned into the pCR2.1 plasmid vector (Invitrogen, San Diego, CA), transformed into
dhβ5a-competent cells (Life Technologies) according to the manufactur-
er’s instructions, and subjected to blue-white screening. White colonies were screened for PCR cDNA by hybridization with 32P-labeled HCB1
(Table II). Plasmids were purified from randomly isolated hybridization-positive colonies using the Wizard Miniprep DNA Purification System
(Amersham) according to the manufacturer’s instructions. Plasmids were se-
quenced on a 6% polyacrylamide DNA sequencing gel using the ABI 373A
DNA sequencer (Applied Biosystems, Foster City, CA).
The maximum theoretical number of potentially unique β-chain TCR
transcripts is ~1012 (49). Theoretically, the probability of randomly finding
finding two identical copies of a single β-chain TCR transcript within a given
independent sample population is negligible. During transformation of
dhβ5a-competent cells, the plasmid/cell mix was subjected to heat shock
(at 42°C for 45 s) followed by incubation on ice for 2 min and growth for 1 h in SOC medium at 37°C before plating. Under ideal conditions (log
phase), Escherichia coli has a doubling time of 20 min, which would result in
two doublings after 60 min (50). After heat shock, however, the cells do
not immediately enter log phase, but the unlikely possibility for a few of
the transformed cells to double before plating does exist. Therefore,
identical TCR sequences from two different colonies (a doublet) may indicate
a clonal expansion or could be the result of a singly transfected E. coli

cell that doubled before plating. In the studies presented here we have se-

150 β-chain TCR transcripts from normal PBMC among which

The Table III

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga03318</td>
<td>CASSS</td>
<td>SS</td>
<td>TV</td>
<td>Vβ3.1 DJβ2.1 DJβ2.3 (2/38; 5.2%)</td>
</tr>
<tr>
<td></td>
<td>TGTGCCACAGTT</td>
<td>CTCCTCCCCGGAGCTGT</td>
<td>CACAGATACCCAG</td>
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<tr>
<td>ga03303</td>
<td>CASSL</td>
<td>PS</td>
<td>SYE</td>
<td>Vβ3.1 DJβ2.1 DJβ2.1 (2/38; 5.2%)</td>
</tr>
<tr>
<td></td>
<td>TGTGCCACAGTTA</td>
<td>CCAAGCTCTACAATAGAG</td>
<td>CTCTACATCGAG</td>
<td></td>
</tr>
<tr>
<td>ga03325</td>
<td>CASR</td>
<td>LDRAS</td>
<td>SYE</td>
<td>Vβ3.1 DJβ2.1 DJβ2.7 (1/38; 2.6%)</td>
</tr>
<tr>
<td></td>
<td>TGTGCCACAGCG</td>
<td>ACTCTGACGCGCTTCT</td>
<td>CTCTACATCGAG</td>
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</tr>
<tr>
<td>ga03326</td>
<td>CASR</td>
<td>IDRAS</td>
<td>SYE</td>
<td>Vβ3.1 DJβ2.1 DJβ2.7 (1/38; 2.6%)</td>
</tr>
<tr>
<td></td>
<td>TGTGCCACAGCG</td>
<td>GATAGACGCGCGCGG</td>
<td>CTCTACATCGAG</td>
<td></td>
</tr>
</tbody>
</table>

* Percentages expressed as number of identical β-chain TCR transcripts per total number of β-chain transcripts analyzed in each tissue specimen.

Results
Diagnosis of GA
All explanted cardiac allografts in this study were diagnosed with chronic rejection by pathologic examination. The mean graft sur-
vival was 4.6 years, and the range was 2.5–9 years (Table I). All
of these cardiac allografts were removed upon retransplantation of
these patients. Pathological examination of the explanted hearts
yielded appreciable arteriopathy with diffuse fibrointimal thickening
of proximal, distal, and small branch artery segments of both
LAD and RCA. Appreciable arterial stenosis was detected in both
LAD and RCA of patients GA03, GA05, and GA09 (Table I).

CD3+ T cell infiltrates in coronary arteries with chronic rejection
Representative pathology and immunohistochemical staining for the presence of CD3+ T cells is shown in Fig. 1. In study patients
hematoxylin and eosin staining revealed GA with chronic arteritis,
fibrointimal proliferation, thickening, stenosis, and evidence of
mononuclear infiltration within the intima and external area of the
media (Fig. 1A), consistent with chronic rejection of cardiac allo-
grafts. Immunohistochemical staining of 6-μm epidermal artery
tissue sections for the presence of CD3+ T cells demonstrated that
substantial proportions of CD3+ cells were present within the in-
tima and external layer of the media as well as in the adventitia
surrounding the chronically rejected artery (Fig. 1B). The intra-
mural and epicardial lymphoid nodules observed in arteries from
two of five patients contained large proportions of CD3+ cells
(Fig. 1A and B), Fig. 1, C and D, demonstrates representative high
powered fields (magnification, ×400) from Fig. 1, A and B, re-
spectively. In a nontransplanted epicardial coronary artery (Fig. 1, E
and F), no significant inflammation of the medium was seen;
however, slight fibrointimal hyperplasia commonly associated
with aging was seen. Rare CD3+ cells were observed in the non-
transplanted artery (Fig. 1F).

Oligoclonal T cell populations are infiltrating coronary arteries of human cardiac allografts with GA: identical β-chain TCR
transcripts are clonally expanded in the LAD and RCA of cardiac allografts with chronic rejection
To determine whether fresh (not expanded in culture) mononuclear
cells infiltrating coronary arteries of cardiac allografts with GA
contain oligoclonal T cells, we amplified, cloned, and sequenced
β-chain TCR transcripts from these coronary arteries. Sequence
analysis revealed substantial proportions of identical β-chain TCR

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transcripts in the coronary arteries of three of five patients, demon-
strating the presence of oligoclonal populations of T cells infiltr-
ating the coronary arteries of patients with GA. β-chain TCR trans-
scripts from a fourth patient exhibited some degree of oligo-
clonality, whereas these transcripts from the fifth patient were rel-
atively heterogeneous. Additionally, high proportions of identical
CDR3 β-chain TCR motifs were found in the coronary arteries of
all five patients.

In patient GA03, sequence analysis of β-chain TCR transcripts
from the LAD, after NPA-PCR amplification and cloning, revealed
substantial proportions of identical β-chain TCR transcripts (Table
III) comprised of the following: Vβ5.1 Dβ1.1 Jβ1.1 (clone ga0314;
CDR3: FDDNL), Vβ6.7 Dβ1.1 Jβ1.1 (clone ga03116; CDR3: AGT-
GQAAGG). Vβ3.1 Dβ1.1 Jβ1.5 (clone ga0339; CDR3: FS-
MAWD), and Vβ23 Dβ1.1 Jβ1.5 (clone ga03113; CDR3: WVT-
GEG). The first of these transcripts accounted for 20% (4 of 20)
and the remaining 15% (3 of 20), each, of all β-chain TCR tran-
scripts sequenced. Remaining TCR transcripts were unique compar-
ed with each other (Table III). The Vβ5.1 Dβ1.1 Jβ1.1 clonal expansion in the LAD of patient GA03 was confirmed by an in-
dependent amplification method (two-sided Vβ5.1-specific PCR),
using RNA from the RCA of this patient as starting material (Table
III). The TCR transcript (clone ga0314), initially found to be
clonally expanded in LAD by NPA-PCR, was also found to be
clonally expanded in RCA by Vβ5.1-specific PCR. Of 35 Vβ5.1-1
TCR transcripts sequenced from the RCA, 15 (42.8%) were
identical with clone ga0314 (Vβ5.1 Dβ1.1 Jβ1.1; Table III). The re-
mainning TCR clones were unique compared with each other, with
the exception of clone ga03543, which appeared in duplicate.
Seven of these clones are shown in Table III; the remaining 12
have been reported to GenBank. Amplification, cloning, and se-
quencing experiments using RNA from the LAD and RNA from
the RCA were conducted in 10 mo apart, making contamination
highly unlikely. β-chain TCR sequence analysis of RCA from the
same patient (GA03) following NPA-PCR amplification revealed
the presence of two TCR transcripts identical with clone ga0314 (2
of 28) and of one transcript identical with clone ga03116 (1 of 28),
which were observed in the LAD. These results demonstrate that
identical β-chain TCR transcripts are clonally expanded in the
LAD and the RCA of patient GA03. Analysis of Vβ3.1-1 TCR
transcripts in the RCA of patient GA03 after Vβ3.1-specific ampli-
fication (Table III) demonstrated substantial proportions of
identical TCR transcripts: clone ga03310, Vβ3.1 Dβ2.1 Jβ2.1, 10
of 38 (26%); clone ga03306, Vβ3.1 Dβ1.1 Jβ2.2, 7 of 38 (18%);
and clone ga03321, Vβ3.1Dβ1.2 Jβ2.1, 5 of 38 (13%).

In addition to the presence of clonally expanded TCR transcripts in
patient GA03, several conserved amino acid motifs were ob-
erved within the CDR3 of the transcripts sequenced. These in-
clude the Leu-Asn (LN) motif, which was observed in the clonally
expanded Vβ5.1 Dβ1.1 Jβ1.1 transcript (clone ga0314, 23 of 83
(28%) transcripts) and was also seen in 15 of 38 (39%) TCR tran-
scripts sequenced after Vβ3.1-specific amplification (Table III).
The LN motif was found in the CDR3 of 38 of 121 (31%) β-chain
TCR transcripts sequenced from patient GA03. PBMC from nor-
mal donors were negative for the FDDLN motif, and the LN motif
was observed only in 3 of 150 (2%) TCR transcripts sequenced.
The TG, RG, GG, and PG CDR3 motifs were observed, re-
spectively, in 18 of 121 (15%), 14 of 121 (12%), 18 of 121 (15%),
10 of 121 (8%), and 8 of 121 (7%) of the β-chain TCR transcripts
sequenced from patient GA03 (Table III).

Six of 33 (18%) β-chain TCR transcripts amplified by NPA-
PCR from the LAD of patient GA06 were identical (clone
06npa12, Vβ12.1Dβ1.1Jβ1.4; Table IV). The remaining 27 se-
quences were unique compared with each other. Sequence analysis
of β-chain TCR transcripts from the RCA of patient GA06 after
Vβ12.1-specific PCR amplification revealed 3 of 23 (13%) iden-
tical clones of the clone 6Vβ1208, Vβ12.1 Dβ2.1 Jβ2.2 (Table IV).
Four other transcripts were each present in duplicate (Table IV).
Vβ3.1-specific PCR amplification revealed unique sequences com-
pared with each other, with the exception of clones 09npa03 and
09npa64, which each appeared in duplicate (Table V). However, sequence analysis of TCR β-chain TCR tran-
scripts from patient GA09 after Vβ3.1-specific PCR amplification
revealed a very strong clonal expansion. Twenty-four of 38
63.2% of these transcripts were identical with clone 9vb913,
Vβ9.1Dβ1.1Jβ2.7, CDR3: QNPGTGH (Table V). These results
demonstrate that oligoclonal T cells infiltrate the coronary arteries
of patient GA09 with chronic rejection. Furthermore, 30 of 68
44% β-chain TCR transcripts used the CDR3 motif PG, 33 of 68
49% used the CDR3 motif TG, and 6 of 68 (9%) used the motif
RG. In four TCR clones the TG motif was followed by a hydro-
phobic amino acid (L, I, T, or V; Table V).

In patient GA02 sequence analysis of β-chain TCR transcripts from
the LAD after NPA-PCR and cloning revealed mostly unique tran-
scripts compared with each other, with the exception of three clones
ga203aa, ga222ib, and ga2037aa) that appeared in duplicate (Table
VI). Sequence analysis of TCR transcripts from the LAD of patient GA02 after Vβ4.1-specific amplification and cloning revealed 3 of 33
identical copies (9%) of clone ga02vβ413, Vβ4.1 Dβ1.1 Jβ2.1,
CDR3:VESDRGP, suggesting that this clone has been clonally ex-
panded (Table VI). Three other clones (ga02vβ417, ga02vβ426, and
ga02vβ414) each appeared in duplicate. A more heterogeneous pop-
ulation of TCR transcripts was found in the coronary arteries of this
patient compared with those discussed previously.

Sequence analysis of β-chain TCR transcripts from the LAD of
patient GA05 after NPA-PCR and cloning, revealed unique trans-
cripts compared with each other, with the exception of one clone
(ga0519) that appeared in duplicate (Table VII). However, 13 of
25 (52%) β-chain TCR transcripts amplified by NPA-PCR used
the GG motif in the CDR3 (Table VII). Sequence analysis of
Vβ3.1 TCR transcripts from the RCA of patient GA05 after
Vβ3.1-specific amplification and cloning revealed unique trans-
scripts compared with each other, with the exception of one clone
(05vβ03) that appeared in duplicate (Table VII). However, se-
quence analysis of β-chain TCR transcripts from the RCA of
patient GA05 after Vβ2.1-specific amplification and cloning revealed
even TCR clones (05vβ217, 05vβ201, 05vβ212, 05vβ203, and
05vβ208) that each appeared in duplicate (Table VII). The GG
CDR3 motif was used by a total of 24 of 88 (27%) transcripts
sequenced in patient GA05 (Table VII). The AG CDR3 motif was
used by 16 of 88 (18%) TCR transcripts sequenced (Table VII).
The PG motif was used by 10 of 88 (11%) TCR transcripts se-
quenced. The TG motif was used by 6 of 64 (9%) of the Vβ3.1
and Vβ2.1 TCR transcripts found in the RCA of patient GA05
(Table VII) as well as in 8 of 35 (23%) of Vβ5.1 clones obtained
from EMBX of the same patient (see below). The TG motif was
observed in only 2% of the PBMC from normal donors (data not
shown).

Normal donor PBMC were used as methodological control and
were subjected to either NPA-PCR or Vβ-specific (Vβ2.1, Vβ3.1,
Vβ4.1, Vβ5.1, Vβ9.1, and Vβ12.1) PCR amplification, followed by
cloning and sequencing. Sequences of TCR transcripts in
PBMC following amplification by NPA-PCR or Vβ-specific PCR
and cloning (Table VIII) were all unique compared with each other, in agreement with previous results (40–43), as anticipated for polyclonal populations of T cells, with the exception of one transcript (Vβ12.1 Dβ1.1 Jβ1.6) that was present in duplicate (of 15) in the Vβ12.1-specific PCR control. This sequence was not detected in any of the other specimens. These sequences are not shown here because of space limitations, but have been reported to the GenBank/EMBL database.

Control epicardial arteries from nontransplanted hearts were also subjected to analysis. As shown in Fig. 2, control artery 1 (nontransplanted heart from a normal adult male organ donor) did not exhibit CD3-δ transcripts compared with study arteries, indicating a lack of T cells in the sample. Analysis of control artery 2 (autopsy material from an adult female patient who died of end-stage diabetes mellitus; nonheart-related) for CD3-δ transcripts yielded a substantially diminished band compared with diseased arteries, demonstrating the presence of minimal T cell infiltrates, which have been associated with older diabetics (20). Neither artery expressed any of the Vβ gene segments (Vβ5, Vβ9) that were expressed in the study arteries (Fig 2); thus, no TCR sequences were obtained.

Comparison of the nucleic acid and the deduced amino acid sequences with those in the GenBank/EMBL/SWISS PROT database revealed that all β-chain TCR transcripts obtained as part of this study were novel and had not been previously reported. There were no incidences of identical TCR transcripts appearing in different patients. However, high proportions of identical CDR3 motifs were found in the coronary arteries of all five patients, and different patients. However, high proportions of identical CDR3 motifs were found in the coronary arteries of all five patients, and some of them are shown in Table IX. The CDR3 motif TG was particularly prominent in patients GA03, GA06, and GA09; the LN motif and the RG motif were prominent in patients GA03 and GA05 (Table IX).

Table IV. β-chain TCR transcripts (CDR3 region) expressed in coronary arteries of patient GA06

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>C A</td>
<td>S P G T G L P A T N E K L</td>
<td>Vβ12.1 Dβ1.1 Jβ1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGTGCC</td>
<td>C A S F</td>
<td>R R G</td>
<td>Y E Q Y F</td>
<td>Vβ6.3 Dβ1.1 Jβ1.2</td>
</tr>
<tr>
<td>TGTGCCAGCAGCTT</td>
<td>TGGCAGGG</td>
<td>CTACAGCAGTACCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGGCAGCAGCTTC</td>
<td>CAGAGGGCGGCCACGCTT</td>
<td>CTAACTAGGAGGTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C A S L G</td>
<td>L G R G P</td>
<td>T E A F F</td>
<td>Vβ6.5 Dβ1.1 Jβ1.2</td>
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</tr>
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<td>TGTCTAG</td>
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<td>CAACAGGAGGACCTT</td>
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<td>ATGGAGGAGGTCTT</td>
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<td></td>
</tr>
<tr>
<td>TGGCGCAGCAGCTTG</td>
<td>GAGGAGGGCGGCCAGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C A S S A</td>
<td>R A G</td>
<td>T E A F F</td>
<td>Vβ6.4 Dβ1.1 Jβ1.1</td>
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<td>TGGGCCGAGG</td>
<td>CACTAGGAGGACCTT</td>
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<tr>
<td>C S A R</td>
<td>T D D P G W A G A H</td>
<td>T D T Q Y</td>
<td>Vβ6.2 Dβ1.1 Jβ1.2</td>
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<td>TGGCGCAGCAGCTTCC</td>
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<td>TGGCAGCAGCTTG</td>
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<td>TGGCAGCAGCTTG</td>
<td>GAGGAGGGCGGCCAGCTT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G S F</td>
<td>S G G P R E D</td>
<td>P A P A F</td>
<td>Vβ2 Dβ1.1 Jβ1.2</td>
<td></td>
</tr>
<tr>
<td>C A S S</td>
<td>S G F</td>
<td>P A P A F</td>
<td>Vβ12.1 Dβ1.1 Jβ1.5</td>
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<tr>
<td>6Vβ1208</td>
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</tr>
<tr>
<td>TGGGCCAC</td>
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<td>TGGCCA</td>
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<tr>
<td>C A T S D</td>
<td>R T G G S E G</td>
<td>P Q H F G</td>
<td>Vβ2 Dβ1.1 Jβ1.5</td>
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<td>TGGCCATCAGTGA</td>
<td>GGGAGGCGGCCAGGAGGAGG</td>
<td>TGGCGCAGCTT</td>
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<tr>
<td>TGGCCACATCTAG</td>
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</tr>
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<td>CGGAGCAGGAGGCTACGGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G A R D S</td>
<td>W L H L R</td>
<td>Vβ12.1 Dβ1.1 Jβ1.2</td>
<td></td>
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<tr>
<td>6Vβ1223</td>
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<td>TGGCCACATCTAG</td>
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<tr>
<td>TGGCAGCAGCTTCC</td>
<td>GGGAGGCGGCCAGGAGGAGG</td>
<td>TGGCGCAGCTT</td>
<td></td>
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<tr>
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<tr>
<td>6Vβ1222</td>
<td>TGGCCATCC A</td>
<td>GGGAGGCGGCCAGGAGGAGG</td>
<td>TGGCGCAGCTT</td>
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</tr>
</tbody>
</table>

* Remaining 17/33 TCR sequences were unique as compared with each other and are not shown here.

* Percentages expressed as number of identical β-chain TCR transcripts per total number of β-chain transcripts analyzed in each tissue specimen.

* Remaining 10/23 TCR sequences were unique as compared with each other and are not shown here.

Views expressed as number of identical β-chain TCR transcripts per total number of β-chain transcripts analyzed in each tissue specimen.
Increased use of the Jβ2.7 gene segment (28.5%) was found in TCR transcripts from the RCA of patient GA03. Similarly, increased use (42%) of the Jβ2.1 gene segment was found in Vβ3.1 TCR transcripts from the RCA of the same patient (Table III). The frequencies of Jβ2.7 and Jβ2.1 in PBMC from normal donors were 17.9% (27 of 150) and 17.2% (26 of 150), respectively.

**Identical clonally expanded β-chain TCR transcripts are present in both previously archived EMBX and coronary arteries with GA from the same patient**

Two patients, GA09 and GA05, received their primary cardiac allografts at Temple University Hospital, and for this reason, archived (snap-frozen) EMBX were collected throughout the post-transplantation period and were available for analysis. One appropriately selected (on the basis of pathological examination) EMBX from each of these two patients was subjected to TCR transcript amplification, cloning, and sequencing. EMBX material from patient GA09 was collected 28 mo before retransplantation, and it was representative of grade 1A rejection (focal perivascular or interstitial infiltrates with no evidence of necrosis), in accordance with the ISHLT (45) guidelines.

EMBX from patient GA09 were analyzed for the presence of Vβ9.1 TCR transcripts, because the Vβ9.1 Dβ1.1 Jβ2.7 transcript, clone 09Vβ913, was clonally expanded in the RCA of this patient’s chronically rejected heart (24 of 38 transcripts were identical; 63.2%; Table V). The same Vβ9.1 Dβ1.1 Jβ2.7 transcript, clone 09Vβ913 (100% sequence identity), was found to be clonally expanded in the EMBX of patient GA09 (7 of 35 transcripts were identical; 20%; Table X). The same transcript, Vβ9.1 Dβ1.1 Jβ2.7, clone 09Vβ913, was present in duplicate in the RCA of patient GA05 with chronic rejection of cardiac allografts, and it was representative of grade 3A rejection (necrosis consistent with the ISHLT (45) guidelines, and also contained evidence of mild atherosclerosis. The Vβ5.1 Dβ1.1 Jβ2.7 transcript, clone 09Vβ913, was clonally expanded in the EMBX of patient GA05 (7 of 35 transcripts were identical; 20%; Table X). The same transcript, Vβ5.1 Dβ1.1 Jβ2.7, clone 09Vβ913, was present in duplicate in the RCA of patient GA05 with chronic rejection of cardiac allografts.
rejection (2 of 30 transcripts were identical; 6.6%; Table VII). Another transcript Vβ1.1 Dβ1.2 Jβ2.3, clone 05Vβ520, was also present in both the EMBX (Table X) and the RCA (Table VII) of this patient; however, in both it was present as a single copy. These results demonstrate that T cells in cardiac allografts are clonally expanded in response to Ag relatively early in the post-transplantation period in the myocardium, and that these clonal expansions persist in coronary arteries well after the development of chronic rejection. It is of interest that the pathology of the EMBXs from both patients demonstrated the onset of GA.

**Discussion**

We have determined that the coronary arteries of cardiac allografts with chronic rejection contain oligoclonal populations of T cells. β-chain TCR transcripts from these coronary arteries contained substantial proportions of identical TCR transcripts. These results were very prominent in three of five patients with chronic rejection. T cells infiltrating coronary arteries from a fourth patient exhibited some degree of oligoclonality, and those from the fifth patient were relatively heterogeneous. High proportions of identical CDR3 β-chain TCR motifs were found in the coronary arteries of all five patients. The β-chain TCR CDR3 motif TG was particularly prominent in patients GA03, GA06, and GA09; the LN motif was prominent in patient GA05; and the PG motif was prominent in patients GA09, GA02, and GA05 (Table IX). The CDR3 region of the TCR β-chain is presumed to be responsible for providing contact points for the binding of TCR to peptide/MHC complex (36 –39). The amino acid sequence of the CDR3 provides information regarding potential amino acid residues on the TCR that recognize peptide/MHC. Clonal expansions of β-chain TCR transcripts and/or conservation of amino acid motifs within the hypervariable CDR3

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0203aa</td>
<td>TGTCACGAGCAGG</td>
<td>CCGTTGAGGAGGGGAGTATT</td>
<td>NLPQHFF</td>
<td>Vβ5.1 Dβ1.1 Jβ1.5</td>
</tr>
<tr>
<td>ga0221b</td>
<td>TGGGCTAAGTGG</td>
<td>CGAG</td>
<td>AACTGAGCAGTACCTTC</td>
<td>(2/32; 6.25%)</td>
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<tr>
<td>ga0237a</td>
<td>TGGGATGGTTG</td>
<td>CAGG</td>
<td>ACATCAGGAGAAG</td>
<td>(2/32; 6.25%)</td>
</tr>
<tr>
<td>ga0210a</td>
<td>TGGCAGGCTG</td>
<td>GCAG</td>
<td>ACACATGAGCAGTATTTT</td>
<td>(2/32; 6.25%)</td>
</tr>
<tr>
<td>ga0278a</td>
<td>TGGCAGGAGGAGG</td>
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<td>NQPF</td>
<td>Vβ2.1 Dβ1.1 Jβ2.3</td>
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<td>ga02114</td>
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<td>TGATGAGGCTT</td>
<td>NQPF</td>
<td>Vβ2.1 Dβ1.1 Jβ1.5</td>
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<td>ga0214b</td>
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<td>Vβ2.1 Dβ1.1 Jβ2.2</td>
</tr>
</tbody>
</table>

* Remaining 20/32 TCR sequences were unique as compared to each other and are not shown here.
* Percentages expressed as number of identical β-chain TCR transcripts per total number of β-chain transcripts analyzed in each tissue specimen.
* Remaining 15/32 TCR sequences were unique as compared to each other and are not shown here.

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regions provides strong evidence for Ag-driven (MHC/peptide) T cell clonal expansions. In this study we identified 1) strong highly selective clonal expansions of β-chain TCR transcripts in the coronary arteries of cardiac allografts from patients with chronic rejection; and 2) conserved amino acid motifs within the CDR3 region of the β-chain TCR within individual patients and between different patients.

Identical β-chain TCR transcripts were clonally expanded in different anatomical sites (LAD, RCA, and EMBX) and at different times, demonstrating systemic clonal expansions of T cell clones. These T cells were very likely clonally expanded in situ in the allograft in response to as yet unidentified antigenic epitopes (peptide/MHC), very likely containing alloantigen, which appear to be constitutively expressed in these allografts. These clonally expanded T cells in the coronary arteries may play a significant role in the pathogenesis of GA. These results provide compelling evidence that chronic rejection is an Ag-driven T cell disease.

Only limited studies have been conducted on the TCR repertoire used by T cells infiltrating organ grafts with chronic rejection, and these have been focused on V region gene segment usage rather than on TCR transcript sequence analysis. Anti-HLA-DR3-reactive T cell clones, developed from renal allografts that used diverse TCR α and Vβ genes, possessed conserved CDR3 motifs (53). A restricted Vβ repertoire throughout episodes of acute and ongoing chronic rejection has been reported for 9 of 12 renal allograft recipients (54); however, it was not determined whether these T cells were clonally expanded. Others have reported heterogeneous populations of intragraft T cells in renal allografts undergoing rejection (55) and in some heart allografts (56) and have attributed their presence to nonspecific lymphocytes recruited to the site by

### Table VII. β-chain TCR transcripts (CDR3 region) expressed in coronary arteries of patient GA05

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-Dβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga0519</td>
<td>TGGCCACGACGTT</td>
<td>GGACGGCAATGCTCC</td>
<td>AGAGACGATAT</td>
<td>Vβ5.1 Jβ2.1 Jβ2.3 (2/5; 80%)</td>
</tr>
<tr>
<td>ga0551</td>
<td>TGCAAGCTTGA</td>
<td>ACAGAGGGGGG</td>
<td>CCAAGAAGCCCG</td>
<td>Vβ4.1 Jβ1.1 Jβ2.5</td>
</tr>
<tr>
<td>ga0513</td>
<td>TGCTGCAAGCTG</td>
<td>TTCTGAGGCAAGGTCACA</td>
<td>TGAGCACTTCTGG</td>
<td>Vβ2.1 Jβ1.1 Jβ2.5</td>
</tr>
<tr>
<td>ga0537</td>
<td>TGACAGCTG</td>
<td>AGCTTTGCAGCAG</td>
<td>CCAGACATGGA</td>
<td>Vβ2.1 Jβ1.1 Jβ2.3</td>
</tr>
<tr>
<td>ga0524</td>
<td>TGAGCTGC</td>
<td>TACTCCGAGACTGGGGGCG</td>
<td>CAAAGACCGAG</td>
<td>Vβ2.0 Jβ1.1 Jβ1.1</td>
</tr>
<tr>
<td>ga0502</td>
<td>TGACAGCTG</td>
<td>TAGGGCAGCAGGGGGGCG</td>
<td>CCTGGAGCAGTAC</td>
<td>Vβ2.2 Jβ1.1 Jβ2.1</td>
</tr>
<tr>
<td>ga0544</td>
<td>TGACAGCTG</td>
<td>TAGAGTCCACCGGCGGGGG</td>
<td>CCAAGAAGCCCG</td>
<td>Vβ2.1 Jβ1.1 Jβ1.3</td>
</tr>
<tr>
<td>ga0569</td>
<td>TGCGCCAGCAG</td>
<td>CTGCTTCTCTGGGGGCG</td>
<td>CCAAGAAGCCCG</td>
<td>Vβ6.2 Jβ1.1 Jβ1.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clones occurring in LAD following NPA-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>05v/β503</td>
</tr>
<tr>
<td>05v/β516</td>
</tr>
<tr>
<td>05v/β520</td>
</tr>
<tr>
<td>05v/β505</td>
</tr>
<tr>
<td>05v/β529</td>
</tr>
<tr>
<td>05v/β511</td>
</tr>
<tr>
<td>05v/β547</td>
</tr>
<tr>
<td>05v/β549</td>
</tr>
</tbody>
</table>

* Remaining 14/25 TCR sequences were unique as compared to each other and are not shown here.
* Percentages expressed as number of identical β-chain TCR transcripts per total number of β-chain transcripts analyzed in each tissue specimen.
* Remaining 21/30 TCR sequences were unique as compared to each other and are not shown here.
* Remaining 24/34 TCR sequences were unique as compared to each other and are not shown here.
locally produced cytokines. In studies performed using the LEW-ACI rat model of chronic cardiac graft rejection, limited heterogeneity of \( \beta \)-chain gene usage in association with a wide variety of CDR3 motifs and \( \gamma \) genes was observed, suggesting preferential use of particular \( \beta \)-chain transcripts (CDR3 region) expressed in normal donor PBMC before and during rejection episodes.

Two different mechanisms of Ag presentation, the direct and the indirect, are responsible for the recognition of organ drafts by T cells of the recipient (27–35). The direct recognition pathway involves recognition of allo-antigen-derived T cell lines from patients with human lung allografts (56). Oligoclonal expansions of T cells in patients who showed no evidence of rejection (58). DeBruyne et al. (59) reported restricted \( \beta \)-gene segment use by bronchoalveolar lavage-derived T cell lines from patients with human lung allografts and during rejection episodes.

Two different mechanisms of Ag presentation, the direct and the indirect, are responsible for the recognition of organ drafts by T cells of the recipient (27–35). The direct recognition pathway involves recognition of allo-antigen-derived T cell lines from patients who showed no evidence of rejection (58). DeBruyne et al. (59) reported restricted \( \beta \)-gene segment use by bronchoalveolar lavage-derived T cell lines from patients with human lung allografts and during rejection episodes.
arteries; therefore, they are very likely alloantigens. It is of interest that only a limited number of T cell clonotypes persist for so long that these clonally expanded TCR transcripts are clonally expanded in archived EMBX of patients with cardiac allografts subsequently diagnosed with chronic rejection. These results are in agreement with those of others that T cells responsible for autoimmune responses use highly restricted TCR V region gene segments (65, 66), suggesting that their TCR can be targeted by virtue of this selected TCR expression (66). The presence of TCR transcripts clonally expanded in EMBX that persist in the graft many months later and have also been found to be clonally expanded in coronary arteries with GA from the same patient may have certain clinical implications. It is likely that these TCR clonotypes are responsible for autoimmune responses that may be of use as in vivo therapeutics for treating or preventing chronic rejection.

The direct pathway of allorecognition is dominant during the early post-transplantation period, and the frequency of recipient T cells directly responding to donor APCs is at least 100-fold higher than that of recipient T cells recognizing donor allopeptides presented by APC (67) of the recipient. It has been suggested that the indirect recognition pathway may play a role in the pathogenesis of chronic rejection (31, 68). Although this is far from proven, several reports provide suggestive evidence that this may be the case (68–71). A strong correlation has been reported (27, 71) between donor-specific HLA-peptide alloreactivity of PBMC and the incidences of acute (27) and chronic rejection (71). The incidence of chronic rejection.

### Table IX. CDR3 epitopes conserved in the coronary arteries of patients with chronic rejection

<table>
<thead>
<tr>
<th>CDR3 Motif</th>
<th>GA03</th>
<th>GA06</th>
<th>GA09</th>
<th>GA02</th>
<th>GA05</th>
<th>Normal PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>18/121 (15%)</td>
<td>14/66 (21%)</td>
<td>33/68 (49%)</td>
<td>5/63 (8%)</td>
<td>8/88 (9%)</td>
<td>15/150 (10%)</td>
</tr>
<tr>
<td>LN</td>
<td>38/121 (31%)</td>
<td>0/66 (0%)</td>
<td>0/68 (0%)</td>
<td>1/63 (2%)</td>
<td>1/88 (1%)</td>
<td>3/150 (2%)</td>
</tr>
<tr>
<td>RG</td>
<td>14/121 (12%)</td>
<td>6/66 (9%)</td>
<td>6/68 (9%)</td>
<td>1/63 (2%)</td>
<td>8/88 (9%)</td>
<td>17/150 (11%)</td>
</tr>
<tr>
<td>AG</td>
<td>10/121 (8%)</td>
<td>5/66 (8%)</td>
<td>4/68 (6%)</td>
<td>1/63 (2%)</td>
<td>16/88 (18%)</td>
<td>20/150 (13%)</td>
</tr>
<tr>
<td>GG</td>
<td>18/121 (15%)</td>
<td>7/66 (11%)</td>
<td>7/68 (10%)</td>
<td>5/63 (8%)</td>
<td>24/88 (27%)</td>
<td>25/150 (17%)</td>
</tr>
<tr>
<td>PG</td>
<td>8/121 (7%)</td>
<td>6/66 (9%)</td>
<td>30/68 (44%)</td>
<td>7/63 (11%)</td>
<td>10/88 (11%)</td>
<td>8/150 (5%)</td>
</tr>
</tbody>
</table>

- β-chain TCR transcripts were amplified by either NPA-PCR or Vβ-specific PCR, and the amplified transcripts (see Table VIII) were cloned and sequenced.

### Table X. TCR transcripts (CDR3 region) expressed in archived EMBX of patients with cardiac allografts subsequently diagnosed with chronic rejection

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vβ</th>
<th>N-DJβ-N</th>
<th>Jβ</th>
<th>Transcript Frequency in Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ9.1 TCR transcripts (GA09)a,b,c,d,e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>09Vβ913</td>
<td>C A S S</td>
<td>N P G T G H</td>
<td>Y E Q X</td>
<td>Vβ9.1 Dβ1.1 Jβ2.7 (21/26; 80.1%)</td>
</tr>
<tr>
<td>09Vβ913</td>
<td>C A S S</td>
<td>P S G A G G</td>
<td>Q F F G</td>
<td>Vβ9.1 Dβ2.1 Jβ2.1 (5/26; 19.2%)</td>
</tr>
<tr>
<td>09Vβ913</td>
<td>C A S S</td>
<td>C C C C T C A G G G G G A G G G G</td>
<td>A G C G T T C T C T C G G G G</td>
<td></td>
</tr>
<tr>
<td>05Vβ503</td>
<td>T G G C C A C A G C G</td>
<td>W G T S G</td>
<td>G C T T C T C T G G A</td>
<td>Vβ5.1 Dβ1.1 Jβ1.1 (7/35; 20.1%)</td>
</tr>
<tr>
<td>05Vβ503</td>
<td>C A S S</td>
<td>R T S G N</td>
<td>T D T Q</td>
<td>Vβ5.1 Dβ2.1 Jβ2.3 (1/35; 2.8%)</td>
</tr>
<tr>
<td>05Vβ503</td>
<td>C A S S</td>
<td>P A R T G</td>
<td>S D T</td>
<td>Vβ5.1 Dβ1.1 Jβ2.3 (23/35; 5.7%)</td>
</tr>
<tr>
<td>05Vβ503</td>
<td>C A S S</td>
<td>C C G A T T A C G G G G</td>
<td>A G C C A G A T C G G</td>
<td></td>
</tr>
<tr>
<td>05Vβ503</td>
<td>C A S S</td>
<td>L G T A D</td>
<td>T D T Q</td>
<td>Vβ5.1 Dβ2.1 Jβ2.3 (2/35; 5.7%)</td>
</tr>
<tr>
<td>05Vβ503</td>
<td>C A S S</td>
<td>A C T C G G G A C T G C</td>
<td>C A C G A T A C G C</td>
<td></td>
</tr>
</tbody>
</table>

- a: EMBX procured 28 mo prior to retransplantation, ISHLT grade 1A with evidence of arteriolarisclerosis.
- b: Percentages expressed as number of identical β-chain TCR transcripts/total number β-chain transcripts analyzed. Sequences in bold represent those also observed in coronary artery of chronically rejected cardiac allograft.
- c: EMBX procured 17 mo prior to retransplantation, ISHLT grade 3A.
- d: Remaining 23/35 Vβ5.1 TCR sequences were unique as compared to each other.
rejection is much higher in patients with PBMC that continued to respond to donor HLA-DR peptides late after transplantation than in those without persistent PBMC alloreactivity (71).

Intermolecular and intramolecular epitope spreading has been demonstrated in the proliferative responses of PBMC to synthetic peptides of the hypervariable region of 32 HLA-DR alleles (71, 72). Epitope spreading has been originally described in autoimmune demyelinating diseases of the CNS (73–77), and it is defined as the generation of de novo immune responses to epitopes different from and noncross-reacting to those that initially induced the immune response. This definition (73–77) is extended now to the immunity against grafts (71, 72) and to the immunity against tumors (78). Progression to chronicity in demyelinating autoimmune disorders and in graft rejection is associated with broadening the T cell repertoire with time (79). The acquired neoactivity, that is, the epitope spreading, appears to be the result of endogenous priming to new self-determinants (79) during the chronic inflammation conditions that are associated with CNS demyelinating disease or graft rejection. These chronic inflammation conditions may clearly result in breaking tolerance to self-Ags and may be responsible for epitope spreading. Another possible explanation, particularly in the case of CNS demyelinating disease, is de novo priming of self-reactive T cells to sequestered (auto)Ag epitopes released as a result of the primary immune response. However, there is a significant difference between epitope spreading in chronic rejection (71, 72) and that in demyelinating diseases (73–77). To date, all epitope spreading responses in chronic rejection are recognizing allogeneic HLA-DR epitopes, dominant or cryptic, but do not recognize self-determinants (71, 72). At present we do not know whether the clonally expanded T cells that we observed in the coronary arteries of patients with chronic rejection are recognizing primary HLA-DR epitopes or represent immune responses to secondary epitopes due to epitope spreading. However, the fact that these clonal expansions have been observed in both EMX and coronary arteries (expanded 28 and 17 mo later) supports the view that they may represent primary immune responses. It should be noted that all the immune responses attributed to epitope spreading have been found in PBMC from patients with chronic rejection. The properties and the clonality of lymphocytes infiltrating coronary arteries from patients with chronic rejection have not been investigated. A statistically significant association has been described between intermolecular epitope spreading and the development of GA (p < 0.02), suggesting that the recruitment and response of T cells recognizing additional alloepitopes may be critical for the progression of GA (71).

Although primary and secondary responses of the recipient’s T cells in chronic rejection are directed against alloantigenic determinants, there is no information on whether other T cells infiltrating coronary arteries of cardiac allografts from patients with GA recognize self-Ags, at least in the late stages of the disease. Because of the extensive chronic inflammation in coronary arteries with GA, tolerance may be broken, and self-Ags may be recognized in an autoimmune fashion. T cell clones derived from classical atherosclerotic plaques responded by proliferation and cytokine production to oxidized low density lipoprotein (LDL) (80). This response was dependent on autologous APCs, and it was HLA-DR restricted (80). Local application of oxidized LDL to arteries promotes intimal thickening (81). Oxidized LDL is immunogenic and induces an Ab response (82). However, differences have been noted in the pathology of GA and atherosclerotic occlusive disease (6, 7). It is not known whether responses to oxidized LDL play a role in the late phase of the pathogenesis of chronic rejection.

Our observations demonstrate that the pathogenesis of chronic cardiac allograft rejection may involve clonal proliferation of T cells in response to persistent recognition of graft-derived antigenic epitopes. The fact that oligoclonal populations of T cells identified early in the post-transplantation period are also present in coronary arteries of allografts diagnosed with GA suggests a possible role for these T cells as targets of specific immunotherapy approaches as a means to prevent the development of GA.

References
Oligoclonal T cells and chronic rejection of cardiac allografts


