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Clonotype Analysis of Human Alloreactive T Cells: A Novel Approach to Studying Peripheral Tolerance in a Transplant Recipient

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The recognition of allo-MHC and associated peptides on the surface of graft-derived APC by host T cells (direct pathway allorecognition) plays an important role in acute rejection after organ transplantation. However, the status of the direct pathway T cells in stable long term transplants remains unclear. To detect alloreactive T cell clones in PBL and the allograft during the transplant tolerance, we utilized RT-PCR instead of functional assays, which tend to underestimate their in vivo frequencies. We established alloreactive CD4+ and CD8+ T cell clones from peripheral blood sampled during the stable tolerance phase of a patient whose graft maintained good function for 9 years, 7 without immunosuppression. We analyzed the sequence of TCR Vβ and Vα genes and made clonotype-specific probes that allowed us to detect each clone in peripheral blood or biopsy specimens obtained during a 1-year period before and after the rapid onset of chronic rejection. We found an unexpectedly high level of donor HLA-specific T cell clonotype mRNA in peripheral blood during the late tolerance phase. Strong signals for two CD4+ clonotypes were detected in association with focal T cell infiltrates in the biopsy. Chronic rejection was associated with a reduction in direct pathway T cell clonotype mRNA in peripheral blood and the graft. Our data are inconsistent with the hypothesis that direct pathway T cells are involved only in early acute rejection events and suggest the possibility that some such T cells may contribute to the maintenance of peripheral tolerance to an allograft. The Journal of Immunology, 2000, 164: 2240–2247.

I ncidences of sustained renal allograft function after withdrawal of all immunosuppressive drug therapy are rare; more commonly, withdrawal of maintenance immunosuppression results in rapid graft loss due to acute or chronic rejection. Occasionally, however, no rejection occurs, and the graft continues to function despite the presence of focal mononuclear cell infiltrates in the interstitium (1). This state of peripheral tolerance can occur in a patient with a history of early acute rejection episodes and persistent MLC reactivity to the donor (2). Such cases suggest that host donor-reactive T cells have not been deleted but are either nonfunctional (anergic), suppressive, or both. Because of the availability of APC from both host and donor, the transplanted organ elicits different types of alloreactive T cells that recognize alloantigen through two distinct pathways: 1) indirect pathway, whereby soluble HLA or other donor-derived Ags are reprocessed and presented as antigenic peptides to host T cells that are restricted by “self” HLA proteins; and 2) direct pathway, whereby donor APCs within the graft or in the peripheral lymphoid tissues present the intact cell surface-bound allogeneic HLA plus bound peptide to recipient T cells. The classical view of the direct pathway T cells is that these high frequency responders are important only in the early posttransplant period, when donor dendritic cells migrate from the graft to the regional lymph nodes and spleen of the host. The host alloreactive T cells then become activated by contact with donor passenger leukocytes and migrate to the graft from the regional lymph nodes, where they mediate cellular acute rejection. Once the early acute rejections have been reversed by immunosuppressive drugs, and as host APC replace donor APC within the graft direct pathway T cells are thought to be replaced by indirect pathway T cells as principal mediators of late, chronic rejection (3). An alternative view is that under circumstances in which acute rejection has resolved, these direct pathway T cells may develop immunoregulatory functions (4). Thus, direct pathway T cells could either 1) be eliminated, 2) remain, but in a quiescent state, or 3) become dominant in a suppressive role during transplant tolerance. In either case, functional assays that rely upon growth and/or long term culture in presence of IL-2 may be unable to detect the donor-specific T cells that predominate in vivo (5).

For this reason, we chose to use a molecular approach to tracking donor-specific T cells in vivo. To find a single clonotype with particular Ag specificity, PCR-based tracking of T cells using TCR junctional (V-D-Jβ and V-Jα) sequence-specific oligo primers or sequence-specific probes has been developed and utilized for the analysis in patients with heart transplants (6), multiple sclerosis (7), and HIV infection (8). Nixon et al. compared two clonotype analysis methods, one using mRNA and RT-PCR and the other using genomic DNA and direct PCR. They found that although the results were similar, they were not always identical; and the authors concluded that whereas DNA-based analysis reflected only the number of clones present in a sample, mRNA RT-PCR signals represent not only the number of clonotype+ T cells but also their activation state. Although mRNA signals for TCR are
not necessarily parallel to the frequency of the T cells, the sensitivity of the RT-PCR clonotype assays to detect Ag-specific T cells is much higher than that of traditional in vitro assays such as CTL precursor or Th lymphocyte precursor assay (7). In addition, unlike traditional functional tests, clonotype analysis can easily be applied to biopsy samples, so that graft-infiltrating alloreactive T cell frequency can be estimated without resorting to culture techniques that may select a new repertoire.

We analyzed mRNA samples from the graft and peripheral blood of a renal allotransplant recipient between 8 and 10 years post-transplant during the transition from functional tolerance to chronic rejection, and we measured the RT-PCR signals of direct pathway T cell clones specific for allo-HLA Ags expressed on donor cells. The data suggest that direct pathway CD8+ T cell clones may be readily detected by clonotype analysis even when such T cells cannot be detected by conventional precursor frequency analysis (2). The data also suggest that maintenance of peripheral tolerance in the absence of immunosuppressive drug therapy is compatible with high levels of direct pathway allospecific CD4+ T cells.

Materials and Methods

Clinical and histochemical analysis

The clinical status of the patient was monitored by serum creatinine level and by periodic biopsy studies. Biopsy sections were stained with hematoxylin and eosin and graded according the Banff criteria (9).

PBL and lymphoblastoid cell lines

PBL were obtained from patient JB (2), from his mother CL (the kidney donor), and from HLA-typed third-party individuals. B-lymphoblastoid cell lines (B-LCL)3 from patient, donor, and third parties were generated using EBV transformation of B cells. All PBL were initially typed for HLA-A, -B, -DR, and -DQ using microcytotoxicity (10). Subsequently, HLA-DR subtype assignments were made using PCR and sequence-specific oligonucleotide probes (11), and these assignments were confirmed by DNA sequencing.

Generation of CTL lines and clones

PBL obtained from the patient were cultured with gamma-irradiated donor PBL in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin for 7–10 days (primary MLC). Patient’s PBL were restimulated with gamma-irradiated donor PBL in the presence of absence of 10 U/ml rIL-2 for 7 days (secondary MLC). T cell clones were then generated by limiting dilution culture at 0.9–90 cells/well in the presence of rIL-2. In the experiment leading to the derivation of clone JD6, gamma-irradiated fresh autologous cells containing donor-derived veto cells (2) were used as a feeder layer in combination with gamma-irradiated donor (CL) stimulator cells. In all other experiments, clones and lines were derived by limiting dilution in the presence of gamma-irradiated donor PBL alone. To generate CD8+ T cell lines, CD4+ T cells were depleted by addition of anti-CD4 mAb (Becton Dickinson, Mountain View, CA) followed by goat anti-mouse IgG-coupled immunomagnetic bead (Dynal, Lake Success, NY) selection as described previously (2). Limiting dilution culture was the same as for CD8+ T cell cloning, except that, in addition to donor gamma-irradiated PBL stimulator cells, 5 μg/ml PHA-P (Sigma, St. Louis, MO) was added only at the initiation of culture, and expanded clones were maintained without PHA. From 4 to 6 wk after limiting dilution, both CD4+ and CD8+ clones were screened by CTL assay as described previously (2) using B-LCL targets.

TCR Vβ and Vα analyses by PCR

Total RNA was isolated from cell lines or clones (1 × 106 cells) by using the GLASSMAX RNA Microisolator Spin Cartridge System (Life Technologies, Gaithersburg, MD). RNA (1 μg) was reverse-transcribed into first-strand cDNA by using random hexamer primers (Life Technologies) according to the manufacturer’s instruction. The cDNA was amplified with 19 Vβ-specific (12) or 22 Vα-specific (Clontech Laboratories, Palo Alto, CA) oligonucleotides. The PCR conditions were denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, for 36 cycles on a thermal cycler (DNA Thermal Cycler, Perkin-Elmer, Foster City, CA). The PCR products were electrophoresed on 2% agarose gels, and the electrophoresed PCR products were transferred to a nylon Zeta-Probe membrane (Bio-Rad, Hercules, CA). After Southern blotting using radiolabeled either Cβ probe (5′-TTCTGATGGCTCTAACACGACGACCTGGG-3′) or Cα probe (5′-GTGTTACCAGTCGAGACTCTA-3′), bands were visualized by autoradiography on x-ray films.

DNA sequencing

TCR Vα and Vβ gene sequencing of each clone was performed using corresponding TCR Vα or Vβ primers with either Cα or Cβ sequencing primers (Cα primer = 5′-TCTCAAGCTGTTACAGCCC-3′; Cβ primer = 5′-CTCAAAACACAGCGACCTCT-3′). PCR products were purified using the Magic Prep PCR DNA Purification System (Promega, Madison, WI) according to the manufacturer’s instruction. The purified PCR products were sequenced by the Nucleic Acid Facility (Iowa State University, Ames, IA), using the sequencing primers.

Clonotype analysis of PBL and kidney biopsy

For semiquantitative clonotype analysis, we utilized modified methods developed by Hu et al. (6). Five hundred nanograms of RNA from PBL or kidney biopsy were reverse-transcribed into cDNA as described. The cDNA was amplified by PCR for 36–39 cycles using corresponding Vβ primer to each clonotype. Oligonucleotides complimentary to the clonotype-specific junctional region were used for Southern blotting (JD6 probe 5′-TCGGCAATTCCGAGAATAATG-3′, F3 probe 5′-CTGGGCTGACCCCTCT-3′, 7.22 probe 5′-CCAATGCTCGGGGTTCCCTTG-3′, probe 5′-TACTCGAGCGCGCCTG-3′). The sensitivity of the clonotype detection assay ranged from 0.0002% to 0.1% in most experiments. The density of bands on autoradiograms was linearly correlated with the clonotype number in the range between a clonal frequency of 10-3 and 10-5. In all experiments, the density of positive blots obtained from PBL was less than the density obtained at a frequency of 10-3 in the standard. To assure the quality of each cDNA and to control for the quantity of TCR β-chain expressed in each blood or tissue sample, control PCR was conducted using primers for GAPDH (5′-CCATGGAAGAGGCTGCGGG-3′, 5′-CAGGTTCGTCGGT GATGCC-3′) and Cβ (5′-GAGGACTGAAACAGAGGTTG-3′, 5′-CAT TCACCCACAGCTGACT-3′). The PCR products were stained with ethidium bromide after electrophoresis.

Table I. HLA haplotypes of individuals used in the study

<table>
<thead>
<tr>
<th>Initials</th>
<th>Cell Type</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-DR</th>
<th>HLA-DQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB</td>
<td>Patient</td>
<td>2</td>
<td>32</td>
<td>44</td>
<td>49</td>
</tr>
<tr>
<td>CL</td>
<td>Donor (mother)</td>
<td>2</td>
<td>3</td>
<td>44</td>
<td>62</td>
</tr>
<tr>
<td>BL</td>
<td>Half-brother of JB</td>
<td>31</td>
<td>3</td>
<td>13</td>
<td>62</td>
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<tr>
<td>LG</td>
<td>Third party</td>
<td>1</td>
<td>26</td>
<td>22</td>
<td>37</td>
</tr>
<tr>
<td>MO</td>
<td>Third party</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td>ED</td>
<td>Third party</td>
<td>3</td>
<td>2</td>
<td>51</td>
<td>62</td>
</tr>
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<td>TF</td>
<td>Third party</td>
<td>1</td>
<td>29</td>
<td>8</td>
<td>44</td>
</tr>
</tbody>
</table>

- NIMA are indicated by underline.
- NT, not tested.

3 Abbreviations used in this paper: B-LCL, B lymphoblastoid cells; CTLp, cytotoxic T lymphocyte precursor; DBT, donor-specific blood transfusion; HTlp, helper T lymphocyte precursor; NIMA, noninherited maternal Ag; DTH, delayed-type hypersensitivity.
Results

Clinical course of the patient

As we reported previously (2, 13), patient JB with end stage focal sclerosing glomerulonephritis received a kidney from his mother (CL) after 3 donor-specific blood transfusions (DST) under azathioprine coverage. Table I shows HLA haplotypes of the individuals used in the study, indicating the noninherited maternal Ag (NIMA) HLA with underline. Interestingly, the patient and maternal donor were serologically HLA-DR identical (DR4,5); on DNA typing, the NIMA HLA-DR5 was found to differ in subtype (DR5/11041) from the paternal inherited HLA-DR5/1102 allele, a difference of only 2 amino acids in the DRβ polymorphic domain (14). Fig. 1 shows the clinical course and serum creatinine levels of the patient. JB experienced two reversible acute rejection episodes within 1 month after the transplantation (2) and none thereafter. Of the patient’s own volition, cyclosporin A therapy was discontinued after 3 mo, and the remaining steroids and azathioprine were stopped 2 years posttransplantation at which time a biopsy (Biopsy 1) was performed. As we previously reported (13), the biopsy at yr 2.2 showed intense focal mononuclear cell infiltration adjacent to an area of fibrosis and arteriolar intimal hyperplasia, but glomeruli and tubuli were normal without cellular infiltrates. To resolve persistent hypertension and proteinuria, which had resulted in a rise in serum creatinine at yr 2.5, bilateral native nephrectomy was performed at 3.0 yr posttransplantation (Fig. 1). Thereafter the graft retained good function until 9.0 yr posttransplantation, as evidenced by serum creatinine < 2.0 mg/dl. Due to a slight rise in serum creatinine to 2.1 mg/dl, a second biopsy was performed at yr 9.1. No evidence of chronic rejection was present at this time. Instead, the biopsy histology showed focal mononuclear infiltrates (FMI) and interstitial fibrosis (Fig. 2B). Although the size of focal infiltrates was larger than that in the earlier biopsy (13), no cellular infiltration in either tubules (T) or glomerulus (G) was observed (Fig. 2A). A rise from 2.1 to 9.8 mg/dl in serum...
creatinine occurred between yr 9.2 and yr 10.7, and a third biopsy at yr 9.7 confirmed the diagnosis of chronic rejection as the cause of graft loss. Diffuse mononuclear infiltrates (DMI) instead of focal infiltrates were observed in the third biopsy specimen (Fig. 2D). Diffuse interstitial fibrosis, tubular atrophy, arterial obstruction, and glomerular edema/fibrosis were manifested (Fig. 2C). Banff criteria: chronic allograft nephropathy grade III).

**HLA-spectrum of donor-reactive CD4+ and CD8+ clones**

We have previously described, using conventional CTL precursor frequency analysis, a functional unresponsiveness toward donor HLA class I Ags in this patient during stable tolerance (yr 4–6). The unresponsive state was found to be associated with presence of rare donor-derived cells in peripheral blood capable of inhibiting CTL propagation in vitro (2). CTL anergy was profound, requiring at least two rounds of stimulation with donor cells and exogenous IL-2 to restore specific CTL function. Thus the use of CTL functional assays to monitor CD8+ donor-specific T cells during tolerance was not practical. Furthermore, functional assays of the donor-specific CD4+ T cells in the allograft was not practical, given the low yield of infiltrating cells and requirement for in vitro culture. Therefore, to investigate the role of direct pathway T cells specific for donor HLA in the change from stable tolerance to chronic rejection, we used a strategy based on analysis of anti-donor T cell clones established from limiting dilution culture during the tolerant phase (yr 6–7 posttransplant (Fig. 1)). Because each T cell clone manifested some degree of cytotoxicity toward donor target cells after prolonged in vitro culture, we used a panel of B-LCL targets that allowed us to dissect reactivity to individual donor mismatched HLA (NIMA) to determine the specificity of each clone. As shown in Table II, two representative CD4+ CD8− clones (F3, 7.22) and one CD4+ CD8α− CD8β− clone (JD6) (15) recognized only target that expressed the donor-subtype HLA-DR5/1104 alloantigen (HLA-DRB1*1104/1101) or the LG target which had HLA-DR5/1101 (DRB1*11011) and 1103 (DRB1*11033) Ags (see Tables I and II). The donor HLA-DRB1*1104 subtype differs from recipient DRB1*1102 in 2 amino acids at positions 67 and 71 adjacent to the peptide-binding groove (Fig. 3). The DRB1*1101 subtype shares the β67F/β71R motif with DRB1*1104 subtype but differs at position 86 from both DRB1*1104 and DRB1*1102, whereas the DRB1*1103 subtype shares β67F and β86V with donor DRB1*1104 but has the same β71E residue as the recipient 1102 Ag (Fig. 3). Interestingly, it has been reported that DR1101 and DR1104 had high cross-reactivity with each other in T cell recognition assays, less with DR1103 and the least or almost none with DR1102 (16, 17).

![FIGURE 3. Location of HLA-DRB1 polymorphism in four HLA-DR11 target cells. Polymorphism in four different HLA-DR11 types used in this study were compared. Positions of amino acid substitutions are indicated on the MHC class II model (modified based on the model by Brown et al. (24)). The thick lines show a potential Ag-binding groove.](http://www.jimmunol.org/)

We were also able to clone a CD4+ T cell (7.19) with the auto-cross-reactive phenotype previously found in primary low density anti-donor limiting dilutions cultures during the tolerant phase (2); interestingly, this clone failed to lyse the target LG (see Table II). Both of the CD8− clones, 9.21 and 10.7, recognized only target cells which expressed HLA-B62, suggesting that, like CD4+ clones F3, 7.22, and JD6, were also donor-HLA-specific, direct pathway T cells.

**TCR gene sequences of donor-reactive CD4+ and CD8+ clones**

We determined the Vβ and Vα assignments of the clones by RT-PCR using a panel of Vβ and Vα primers, followed by Southern blot analysis using a Cβ or Cα-specific probe. To determine whether T cell clones having similar allospecificity were characterized by a common sequence motif and junctional (complementarity-determining region 3) sequence homologies, we sequenced the expressed TCR β genes of CD4+ and CD8+ clones. The TCR α genes of the CD4+ clones were also sequenced. The nucleotide and predicted protein sequences spanning the junctional VDJ regions of the TCR β-chains of the clones are shown in Fig. 4. The sequence analysis revealed no common junctional sequence homologies in the expressed Vα and β of the HLA-DR5/1104-specific T cell clones. CD4+ clones JD6, F3, and 7.22 each used different Jβ families (BJ2S2, BJ1S5, and BJ1S3, respectively) and differed in the length of N+ Dβ + Jβ region (Fig. 4A); each clone also used different TCR Vα chains (AVS2, AVS3–5, and AVS8).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specific Target (% lysis)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD6 (CD4, CD4α)</td>
<td>-2 37</td>
<td>DR1104/1101/1103</td>
</tr>
<tr>
<td>F3 (CD4)</td>
<td>-5 60</td>
<td></td>
</tr>
<tr>
<td>7.22 (CD4)</td>
<td>0   62</td>
<td></td>
</tr>
<tr>
<td>9.21 (CD8αβ)</td>
<td>-3 60</td>
<td></td>
</tr>
<tr>
<td>10.7 (CD8αβ)</td>
<td>-1 26</td>
<td></td>
</tr>
<tr>
<td>7.19 (CD4)</td>
<td>63   54 61 68</td>
<td></td>
</tr>
</tbody>
</table>

* Target cells were B-LCL.

* All clones were assayed at a 20:1 E:T ratio except for 10.7 and 9.21 (at 10:1). Results are representative of 2–10 experiments per clone.

* Representative HLA types of third-party cells were as shown.

* Positive reaction (≥10% lysis) are indicated by underline.

* NT, not tested.

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**Table II. T cell cytotoxicity/specificity analysis of clones from patient JB isolated from peripheral blood during tolerance (yr 5–6)**
AV16S1, respectively) (Fig. 4A) and different Jα genes (data not shown). A similar high degree of TCR V gene diversity in alloreactive CD4+ T cell clones raised against a closely related HLA-DR Ag has been reported previously (18). The TCR β gene junctional sequence analysis of two direct pathway CD8+ CTL also indicated a diverse TCR repertoire in response to the donor HLA-B Ag (Fig. 4B). Although clones 9.21 and 10.7 both lysed only HLA-B62+ target cells, each used different Vβ (BV9S1 and BV13S2) and Jβ (BJ2S7 and BJ1S2) and had no common junctional VDJ amino acid sequences. Autoreactive CD4+ clone 7.19, which lysed both donor and autologous LCL (but not all LCL in the panel), expressed Vβ1, and, as expected, had no homology to either CD4+ or CD8+ direct pathway clones in its TCR gene sequences (Fig. 4C).

Clonotype analysis of anti-donor T cells in PBL before and after the onset of chronic rejection

The unique junctional sequences present in each clone (Fig. 4, underlined sequences) enabled us to develop specific oligonucleotide probes for clonotype detection analysis. Fig. 5A shows an example of assay sensitivity. A varying number of Vβ8+ JD6 clones were mixed into donor CL PBL (negative control) to a total of 10⁶ cells. Five hundred nanograms of extracted RNA from each sample were reverse transcribed and used as a template for PCR with Vβ8 primers, and clonotype was detected using radiolabeled JD6 junctional TCR β probe. Two positive cells in 10⁶ cells were detectable after a 39-cycle PCR (Fig. 5A).

Using this technique, we performed clonotype analysis of PBL collected between yr 8.8 (prerejection) and yr 9.8 (postrejection) to see whether the anti-donor direct pathway T cell clonotypes were present in the peripheral blood. As shown in Fig. 5B, densities of mRNA signals of TCR genes in the direct pathway CD4+ T cells (F3, JD6, and 7.22) showed a similar pattern: all three clonotypes were readily detected in samples obtained at yr 8.8 and yr 9.1 (pre-chronic rejection, late tolerance phase) with clonotypes F3 and 7.22 giving the strongest signals. The density of mRNA signals from all three CD4+ direct pathway clonotypes (JD6, 7.22, and F3) was decreased at 9.2 yr posttransplantation and around the time of biopsy-proved chronic rejection (9.7 and 9.8 yr). Although clonotype F3 remained detectable in PBL, clonotype 7.22 and JD6 mRNA signals fell to background level. Based on the standard curve for JD6, there was at least a 10-fold reduction in clonotype mRNA between yr 9.1 and yr 9.2, i.e., within a period of <2 mo after biopsy 2.

The intensity of one of the donor class I-specific CD8+ clonotypes, 9.21, changed in the same manner as the CD4+ anti-donor class II-specific clonotypes, 7.22 and F3, being strongly expressed at both prerejection time points, decreasing sharply with onset of chronic rejection. Like F3, the 9.21 mRNA signal in PBL persisted.

**FIGURE 4.** Nucleotide and amino acid sequence and alignment of TCR β-chain V(D)J regions. The names of the corresponding Vβ, Jβ, and associated Vα are indicated. Predicted amino acids are indicated by the single-letter code. Clonotype-specific probes were designed to be complimentary to the sequences as underlined. The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL Data Bank with accession numbers from U59674 to U59681, AF102880, and AF102881. N.T., not tested.

**FIGURE 5.** Detection of TCR mRNA signals of donor-reactive T cell clonotypes in PBL at chronic rejection. Mixtures of varying numbers (0–0.5%) of JD6 clones with negative control PBL (donor CL) were analyzed and shown as an example of assay sensitivity (A). Each clonotype in PBL was analyzed by RT-PCR, and subsequent Southern blot analysis was performed using clonotype-specific probes during transition from tolerance to chronic rejection (B). GAPDH and TCR Cβ signals showed that the quality and quantity of the each cDNA sample were comparable (B).
during chronic rejection but at a reduced level relative to yr 8.8 and 9.1 samples. This finding was in contrast to functional studies of anti-donor CTL, which showed no evidence of functional CTL in primary limiting dilution assay or bulk culture analysis during the early or late tolerance phase (Ref. 2; S. Kusaka, A. P. Grailer, and W. J. Burlingham, unpublished observations). This suggests that clonotype analysis, like HLA-tetramer analysis (19), can reveal the presence of anergic CD8<sup>+</sup> T cells. The other CD8<sup>+</sup> clonotype, 10.7, was barely detectable at all time points. The loss of mRNA signals for 4 of 5 anti-donor direct pathway clonotypes was well correlated with the increase of serum creatinine during the same period (Fig. 2). Interestingly, the signal of the one autoreactive CD4<sup>+</sup> T cell clonotype, 7.19, was detected only at yr 9.1.

To rule out the possibility that levels of clonotype detected using oligonucleotide probes for TCR <i>β</i> only might reflect non-donor-specific T cells due to pairing with irrelevant TCR <i>α</i>, we analyzed the expression of TCR <i>α</i> mRNA signals. A similar pattern of loss of the mRNA signals between yr 9.1 and 9.2 was confirmed by a similar method using primers for V<sub>α</sub>16 and V<sub>α</sub>2, and TCR <i>α</i> junctional gene sequence-specific probes for 7.22 and F3 (data not shown). As shown in Fig. 5B, the densities of both C<i>β</i> and GAPDH mRNA signals were equivalent between each time point, indicating that the quality of cDNA obtained at each time point was good and the number of T cells were comparable. This suggests that the observed loss of TCR V<i>β</i> mRNA signals from direct pathway clonotypes was not an artifact of general loss of T cells or T cell mRNA signals.

### Analysis of alloreactive clonotype-positive T cells in the graft

To determine whether the loss of mRNA signals of anti-donor direct pathway T cells from peripheral blood was the result of a redistribution of those clonotypes from the peripheral blood into the graft during chronic rejection, we performed clonotype analysis of biopsy specimens collected pre- (yr 9.1) and post- (yr 9.8) chronic rejection for all six direct pathway allospecific clonotypes (Fig. 6). Of the six clonotypes that were detected in the PBL at the prerejection time points, mRNA signals of only clonotypes 7.22 and F3 were readily detectable within the graft at yr 9.1, the prerejection time point. Clonotypes JD6 and 9.21 did not redistribute to the graft site after the onset of rejection, remaining undetectable in the biopsy mRNA (data not shown). Of the two clonotypes that were present in the graft at yr 9.1, the mRNA signal for the F3 clonotype was completely lost from the graft after chronic rejection, whereas 7.22 clonotype mRNA was still detectable but at a lower signal intensity (Fig. 6). Both C<i>β</i> and GAPDH mRNA signals in the biopsy samples were unchanged between the pre- and postrejection time points as measured by intensity of ethidium bromide staining of the appropriate RT-PCR products. Furthermore, analysis of RT-PCR products from the same mRNA samples using an HPLC semiquantitative method also indicated that similar amounts of TCR <i>β</i> mRNA were present in pre- and postrejection biopsy samples (20). Thus, the loss of mRNA signals for direct pathway T cell clonotype, F3, JD6, and 9.21 in the peripheral blood was not the result of a redistribution of these clonotypes to the graft. The loss of mRNA signals for clonotype 7.22 from the peripheral blood, in contrast, may have been due in part to such a redistribution, because it was detected weakly in the graft but not in the blood during chronic rejection.

### Discussion

Direct pathway alloreactive T cells may be defined by their requirement for a donor-derived APC. Such direct pathway alloreactivity plays an important role in acute rejection due to the abundance of donor passenger APCs in the early period after transplantation (21–23). The status of the direct pathway alloreactive T cells in long term transplant recipients, including functionally tolerant patients, is not clear. Furthermore, such T cells are often difficult to detect by functional assays, particularly when T cell anergy or active suppression is present. The purpose of this study was to determine whether clonotype analysis might reveal the presence of alloreactive direct pathway T cells in a case of long term functional tolerance and, if so, to determine whether the frequency of such T cells changes during the breakdown of tolerance. To favor selection of direct pathway clonotypic TCR, we established donor-reactive CD8<sup>+</sup> and CD4<sup>+</sup> clones in the presence of donor APC and tested the specificity of these clones using B-LCL lines without any addition of antigenic peptides. CD8<sup>+</sup> clones showed cytotoxicity only against target cells expressing donor-type HLA-B62 Ag. CD4<sup>+</sup> clones except 7.19 recognized the BL target, that expressed the donor-type DR1104, and the LG target, which had DR1101 and DR1103 but not recipient-type DR1102 (Table II). The cross-reactivity among these 4 DRB<sup>1</sup>*11 alleles was precisely analyzed using influenza HA306–320 peptide-specific (16) and tetanus toxin peptide p2-specific (17) DR11-restricted CD4<sup>+</sup> T cell clones. These studies agreed that most of the DR1101-restricted CD4<sup>+</sup> T cell clones recognized DR1104, and they had less reactivity with DR1103 and the least or almost none with DR1102, even though all four DR11 types had similar binding capacity of the same antigenic peptides. In other words, the β67F/β71R motif which is located in the middle of the Ag-binding groove (Fig. 3) (24) is the most critical in the Ag recognition by CD4<sup>+</sup> T cells. Whether those CD4<sup>+</sup> T cell clones recognized DR1101, DR1103, or both was not clear, but they failed to recognize recipient-type DR1102 expressed by autologous LCL. Although we cannot rule out the possibility that autologous LCL, when pulsed with the appropriate allopeptides, could express the ligand for these clones, the common recognition of DR1104- and DR1101/1103-positive targets argues that the epitope is donor not host DR restricted. It appears likely that all clones except autoreactive clone 7.19 recognized donor Ags through the direct allorecognition pathway.

We found a surprisingly high level of donor-specific CD4<sup>+</sup>-CD8<sup>+</sup>-T cell clonotypes in PBL and two CD4<sup>+</sup> clonotypes in the graft during the late tolerance, prerejection stage using clonotype analysis. By comparing the pattern of donor HLA-specific T cell clonotype mRNA expression in the graft and peripheral blood before and after the onset of chronic rejection, and by examining other parameters such as renal histopathology and soluble donor...
One is that direct pathway CD4+ T cell clonotypes isolated during the stable tolerance phase. This decrease in direct pathway T cell clonotype mRNA in PBL preceded increases in serum creatinine and in the level of soluble donor HLA Ag in serum (data not shown), indicating that changes in the alloreactive T cell repertoire may be a leading indicator of chronic rejection.

There are at least two possible interpretations of these findings. One is that direct pathway CD4+ and CD8+ T cells were quiescent during the stable tolerance phase (yr 2–8), but for unknown reasons they became activated just before the manifestation of chronic rejection (yr 8–9) and may themselves have triggered the rejection. Subsequent clonal exhaustion or activation-induced cell death, which are commonly seen in Th1 cells (25), could have resulted in a loss of clonotypes from the graft and the blood.

A second possibility is that the direct pathway CD4+ and CD8+ T cells that we cloned during the tolerance phase are ones that lack cytolytic function in vivo and may in fact inhibit proinflammatory Th1 cells. The unusual phenotype (CD4+ CD8α− β+) of many of the direct pathway CD4+ T cell clones (including JD6, for example) isolated from the peripheral blood during the tolerance phase is consistent with this interpretation (15). In this view the loss of these immunoregulatory T cells parallels the onset of chronic rejection and is related to the breakdown of tolerance.

The case for active immunoregulation of the anti-donor response in this patient is based on data from several sources. As noted previously, JB received three donor blood transfusions from his mother before the kidney transplant and displayed a relatively high level of microchimerism in peripheral blood and skin (2). It has been reported that graft passenger leukocytes, especially dendritic cells, may play an important role for graft acceptance in liver transplants (26) and DST-treated recipients (27). DST treatment has also long been known to induce suppressor cells (28, 29). Although the presence of such cells was controversial, recent reports have shown that they could be analogues to Tr1 cells (30, 31) and that DST treatment preferentially induces graft-infiltrating cells that produce TGF-β1, potential suppressor cells (32). Under conditions of microchimerism in which rare leukocytes of donor origin persist within the transplant recipient’s peripheral blood and tissues, donor APC may contribute both to the persistence of direct pathway T cells and to their control via immunoregulation (2).

Another possible driving force for the induction of such suppressive cells in this patient was a priming by noninherited maternal HLA. Recent studies of neonatal Ag exposure suggest that the common result of Ag priming in utero is a memory response that is antagonistic to Th1 function (33–36). By this interpretation, the loss of direct pathway CD4+ clonotype mRNA signals observed at the breakdown of tolerance might reflect a change in the long-standing (i.e., since in utero life) dominance of certain alloreactive memory T cell clonotypes, leading to reduced activation of direct pathway T cells that have immunoregulatory function (1). The reduced mRNA signals for clonotypic TCR between yr 9.1 and 9.7 could reflect a drop in clonal frequency or a decrease in activation, given that the synthesis of TCR mRNA may be linked to the cycling of the TCR after Ag-induced receptor modulation (8). Either way, the influence of the clonotypic T cells on events within the graft and the periphery would be diminished.

Our recent studies using the newly developed trans-vivo delayed-type hypersensitivity (DTH) assay (37) showed that this patient’s PBL from tolerant phase (yr 8.8) had no DTH reaction against a donor alloantigen sonicate preparation and that PBL after the rejection caused high DTH reaction against a donor Ag sonicate. These data suggest that host T cell-mediated reactivity to donor Ag through indirect pathway was only manifested after the breakdown of tolerance (W. J. Burlingham, E. Jankowska-Gan, A. M. VanBuskirk, and C. G. Orosz, manuscript in preparation).

The timing of this increase of DTH reaction was coincident with the sharp rise in serum creatinine and donor soluble HLA class I level between yr 9.2 and yr 9.7. Interestingly, the rapid decline in direct pathway T cell clonotype transcripts in PBL was seen 1 mo earlier than the rise of creatinine (between yr 9.1 and yr 9.2 (Fig. 5)). During this same 1-mo interval, the serum creatinine level remained low, suggesting that a decrease in the direct pathway T cells, like the increase in indirect pathway DTH reactivity, may have preceded the renal damage that gave rise to increased serum creatinine level.

In conclusion, T cell clonotype analysis of peripheral blood and graft biopsy appears to be a useful approach for the study of human allograft tolerance. Our data suggest that the direct pathway of allore cognition of donor HLA Ags, including both CD4+ and CD8+ T cell clones, persisted in the period of tolerance despite the absence of CTL function and the presence of inhibitory donor-derived leukocytes (2). The loss of these clonotypes occurred before a marked increase in serum creatinine and was associated with a change from a focal, interstitial to an invasive graft T cell infiltrate. Whether or not the direct pathway T cell clones derived from the peripheral blood during the stable phase of tolerance plays a positive role in the stabilization of the tolerant state remains to be determined. The clonotype analysis approach, as well as other direct approaches to quantitation of Ag-specific T cells in vivo including HLA dimer (38) and tetramer strategies (39) may therefore open a new avenue to analyzing donor-reactive T cells during peripheral tolerance, when conventional functional in vitro assays may fail to detect their presence.

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References


