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**Research Article**

To delineate how gene rearrangement influences the expressed human gamma delta T cell repertoire, we generated T cell receptor gamma (TCR gamma) V domain-specific cDNA libraries from the peripheral lymphocytes of eight donors and sequenced a total of 232 TCR gamma gene transcripts. The libraries consisted of both in-frame and out-of-frame rearranged TCR gamma genes. The in-frame TCR gamma gene transcripts were used to determine the diversity of functional T cells, whereas the out-of-frame transcripts, primarily derived from alpha beta T cells, were used to assess the frequencies of TCR V gamma-J gamma rearrangements in progenitor T lymphocytes. The results showed that both sets of transcripts exhibited strikingly restricted V gamma-J gamma combinations. Only 11 of 40 potential V gamma-J gamma rearrangements were common (> or = 3% of total). The pattern of gene usage in the functional and nonfunctional transcripts was similar and did not differ markedly among donors. The only exception was the predominance of V gamma 9-JP in potentially functional transcripts from seven of eight individuals. These results show that V gamma-J gamma rearrangement is nonrandom and suggest that the diversity of TCR gamma genes in the functional gamma delta T cell repertoire partly depends upon preferentially rearranged V gamma-J gamma gene combinations. However, the expansion of V gamma 9/V gamma 2 T cells in adult [...]

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# Regulation of the Mature Human T Cell Receptor $\gamma$ Repertoire by Biased V-J Gene Rearrangement

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## Abstract

To delineate how gene rearrangement influences the expressed human  $\gamma\delta$  T cell repertoire, we generated T cell receptor  $\gamma$  (TCR $\gamma$ ) V domain-specific cDNA libraries from the peripheral lymphocytes of eight donors and sequenced a total of 232 TCR $\gamma$  gene transcripts. The libraries consisted of both in-frame and out-of-frame rearranged TCR $\gamma$  genes. The in-frame TCR $\gamma$  gene transcripts were used to determine the diversity of functional T cells, whereas the out-of-frame transcripts, primarily derived from  $\alpha\beta$  T cells, were used to assess the frequencies of TCR V $\gamma$ -J $\gamma$  rearrangements in progenitor T lymphocytes. The results showed that both sets of transcripts exhibited strikingly restricted V $\gamma$ -J $\gamma$  combinations. Only 11 of 40 potential V $\gamma$ -J $\gamma$  rearrangements were common ( $\geq 3\%$  of total). The pattern of gene usage in the functional and nonfunctional transcripts was similar and did not differ markedly among donors. The only exception was the predominance of V $\gamma$ 9-JP in potentially functional transcripts from seven of eight individuals. These results show that V $\gamma$ -J $\gamma$  rearrangement is nonrandom and suggest that the diversity of TCR $\gamma$  genes in the functional  $\gamma\delta$  T cell repertoire partly depends upon preferentially rearranged V $\gamma$ -J $\gamma$  gene combinations. However, the expansion of V $\gamma$ 9/V $\delta$ 2 T cells in adult peripheral blood can only be explained by antigenic selection of relatively rare V $\gamma$ 9-JP recombinants. (*J. Clin. Invest.* 1993. 91:171-178.) **Key words:** gene recombination • adult peripheral T cell • anchored polymerase chain reaction • biased V-J gene usage • nonfunctional transcript

## Introduction

T lymphocytes express two mutually exclusive types of antigen receptor, either  $\alpha\beta$  or  $\gamma\delta$ , in association with the CD3 molecular complex. The smaller  $\gamma\delta$  subset of T cells has been found in all vertebrate species examined. Most  $\gamma\delta$  cells do not express CD4 or CD8 accessory molecules on their surface, and their recognition of antigen is not restricted by classical MHC molecules (1). Murine  $\gamma\delta$  T cells have a distinctive ontogeny and tissue distribution. The presence of two classes of murine  $\gamma\delta$  T

cells, i.e., intraepidermal lymphocytes with restricted V $\gamma$ /V $\delta$  receptors and secondary lymphoid organ lymphocytes with variable V $\gamma$ /V $\delta$  receptors, suggest that these two types of cells might fulfill special roles in the immune system (2). Human  $\gamma\delta$  T cells do not have the same tissue tropism. The physiological functions of murine and human  $\gamma\delta$  T cells are still unclear.

A small set of V, D, and J gene segments encode human  $\gamma\delta$  receptors. This limited diversity makes it possible to assess the factors that control development and expression of the mature  $\gamma\delta$  T cell repertoire. By analogy with  $\alpha\beta$  T cell receptor (TCR),<sup>1</sup> these could include preferential gene recombination, positive and negative selection by self-antigens in the thymus, and peripheral expansion of T cell clones by antigens. To date, the adult  $\gamma\delta$  T cell repertoire has been studied mainly with monoclonal antibodies. Two dominant subsets of human  $\gamma\delta$  T cells have been recognized in peripheral blood (3). Southern blot analyses of genomic DNA from cloned lymphocytes from each subset indicate that one class uses V $\gamma$ 9-JP-C $\gamma$ 1/V $\delta$ 2-J $\delta$ 1-C $\delta$ 1 to form the TCR heterodimer whereas the other uses V $\gamma$ 2 or V $\gamma$ 4-J2-C $\gamma$ 2/V $\delta$ 1-J $\delta$ 1-C $\delta$ 1 (4).

Evidence for regulation of TCR $\gamma$  gene rearrangement in early ontogeny has come from analyses of  $\gamma\delta$  transcripts in the fetal and neonatal lymphoid tissues (5, 6). However, whether selective rearrangement of TCR $\gamma$  genes influences the adult expressed human  $\gamma\delta$  T cell repertoire is not known. One way to determine the effect of intrinsic gene rearrangement on the expressed repertoire is to compare the frequencies of all potential V $\gamma$ -J $\gamma$  rearrangements in functional and nonfunctional TCR V $\gamma$  genes. This is possible because the TCR $\gamma$  cDNA in peripheral lymphocytes includes both functionally and nonfunctionally rearranged transcripts (7).

We, therefore, used the anchored polymerase chain reaction (PCR) method (8) to construct TCR $\gamma$  cDNA libraries from the peripheral blood lymphocytes of eight individuals, compared gene frequencies among the in-frame and out-of-frame TCR $\gamma$  transcripts, and sequenced a total of 232 V $\gamma$ -J $\gamma$  genes. Except for V $\gamma$ 9-JP-C $\gamma$ 1, both functional and nonfunctional genes exhibited a similarly restricted set of V $\gamma$ -J $\gamma$  combinations. We conclude that regulation of TCR V $\gamma$  gene rearrangement is a significant force in molding the mature functional  $\gamma\delta$  T cell repertoire. However, antigenic selection, as in the case of V $\gamma$ 9-JP-bearing cells, can potentially expand even very rare recombinants.

## Methods

**Blood samples.** PBMCs were obtained from eight donors. Their ages ranged from 39 to 65 yr. Mononuclear cells were isolated by density

1. **Abbreviations used in this paper:** PCR, polymerase chain reaction; TCR, T cell receptor.

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centrifugation and were stored in medium containing 10% DMSO at  $-80^{\circ}\text{C}$  until used.

**RNA isolation and first-strand cDNA synthesis.** Total RNA was isolated from  $5 \times 10^5$  PBMCs using RNazolB (Cinna/Biotech, Friendswoods, TX), which is based on the acid guanidium thiocyanate-phenol-chloroform method (9). First-strand cDNA was synthesized from total RNA using an oligo-dT primer and Superscript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The resulting cDNA/RNA hybrids were first size selected (0.5–3 kb) on agarose gels and extracted with phenol. The remaining RNA was hydrolyzed by incubation at  $60^{\circ}\text{C}$  in 0.3 M NaOH.

**dG tailing of first-strand cDNA and anchored PCR.** To amplify an entire TCR $\gamma$  V domain cDNA segment regardless of sequence, the first-strand cDNA was poly dG tailed with dGTP and terminal deoxytransferase. Subsequently free dGTP was removed by two sequential ammonium acetate precipitation steps. The G-tailed first-strand cDNA was subjected to primary anchored PCR amplification in 100  $\mu\text{l}$  of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl $_2$ , 0.001% gelatin, 200  $\mu\text{M}$  dNTPs, 1 U Perfect Match Polymerase Enhancer (Stratagene, Inc., La Jolla, CA), and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT). The primers consisted of a C $\gamma$ -specific antisense oligonucleotide (C $\gamma$ b: 5'-CACG-GTTCGACTCAAGAAGACAAAGGTATGT) and anchor primers (9:1 mixture of AN primer: 5'-ATTACGGCGGCCGCGGATCC and ANC primer: 5'-ATTACGGCGGCCGCGGATCCCCCCCCCCCC), at a concentration of 1  $\mu\text{M}$ . The ratio of ANI and ANIC was based on the report by Loh et al. (8). The anchor primers contained sequences that are recognized by restriction enzyme NotI (GCGGCCGC) and BamHI (GGATCC), respectively, whereas the C $\gamma$ b primer had a sequence recognized by SalI (GTCGAC) at its 5' end. These restriction sites facilitated the subsequent subcloning of the PCR products. The amplification included 20 cycles of  $95^{\circ}\text{C}$  for 30 s,  $42^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min followed by final extension of 7 min. The products were size selected (500–750 bp) on agarose gels and one third were removed for nested PCR. The reaction mixture was the same as that of the primary anchored PCR except for the primers, 1  $\mu\text{M}$  each of AN primer and antisense C $\gamma$ a primer (5'-ACGCGTCGACGG-AAGAAAATAGTGGGCTT, which contained a SalI recognition sequence at its 5' end, and primes upstream of the C $\gamma$ b primer sequence) and the concentration of MgCl $_2$  (1.5 mM). 20 cycles of nested PCR were carried out at an annealing temperature of  $55^{\circ}\text{C}$ , and the products were purified by phenol extraction.

**Construction of TCR $\gamma$ -specific cDNA libraries.** A short fragment of the multicloning site of pBluescript II SK $^+$  (Stratagene, Inc.) was released by enzymatic digestion with SacI and SalI restriction enzymes and inserted into the polylinker of the M13mp19 vector, to produce a M13 vector that includes SalI and NotI sites. The PCR products were digested with SalI and NotI to make cohesive ends, and fragments of the appropriate size were separated on agarose gels and extracted with phenol. The purified cDNA was ligated with the SalI/NotI-digested M13 vector and the ligated vector was electrotransformed into SURE *Escherichia coli* (Stratagene, Inc.).

**Screening and frequency analysis of TCR $\gamma$ -specific cDNA libraries.** M13 plates, with plaques containing amplified TCR $\gamma$  V domain cDNA, were lifted five times with Hybond-N $^+$  nylon membranes (Amersham Corp., Arlington Heights, IL). Both an internal C $\gamma$  probe (C $\gamma$ c: 5'-TAAACAACCTTGATGCAGATG, recognizing a region upstream of the C $\gamma$ a primer-priming sequence) and V $\gamma$  family-specific probes (V $\gamma$ I: 5'-GTCAGAAATCTTCCAACCTGGAAG, V $\gamma$ II: 5'-GACGGCACTGTGAGAAAGGAATCT, V $\gamma$ III: 5'-TCAGGCTTTG-GAGCACCTGATCT, and V $\gamma$ IV: 5'-CAAAGGCTTAGAATATT-TAT) were labeled with digoxigenin in 20  $\mu\text{l}$  of 300 pmol oligonucleotide, 200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 1.5 mM CoCl $_2$ , 0.25 mg/ml bovine serum albumin, 125  $\mu\text{M}$  digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5  $\mu\text{M}$  dATP, and 50 U terminal deoxytransferase. Five membranes were hybridized with the different probes at  $42^{\circ}\text{C}$ . After washing the mem-

branes, positive plaques were visualized using the Genius system (Boehringer Mannheim Biochemicals). Briefly, the membranes were blocked with blocking reagent and incubated with antidigoxigenin antibody conjugated with alkaline-phosphatase. Then they were soaked in an adamantyl 1,2-dioxetane phosphate (AMPPD; Tropix, Inc., Bedford, MA) solution and incubated at  $37^{\circ}\text{C}$  to activate the phosphatase. Positive signals were recorded on x-ray film and enumerated.

**Conventional PCR.** The ability of the V $\gamma$ IV PCR primer to amplify rearranged V $\gamma$ 11 genes (from the V $\gamma$ IV family) was confirmed by conventional PCR. Template DNA was isolated from PBMCs. Amplification was carried out using 100  $\mu\text{l}$  of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl $_2$ , 0.001% gelatin, 200  $\mu\text{M}$  dNTPs, 2.5 U AmpliTaq DNA polymerase, and 1  $\mu\text{M}$  of the V $\gamma$ IV family-specific sense oligonucleotide and either the C $\gamma$ -specific antisense oligonucleotides (C $\gamma$ b or C $\gamma$ c: 5'-ATCTGCATAAGTTGTTTA primer without restriction enzyme site) or a J1,J2-specific antisense oligonucleotide (J12:5'-GTGTTGTTCCACTGCCAAAG). The PCR products were visualized after separation by agarose gel electrophoresis.

**Enrichment of  $\alpha\beta$  T cell or  $\gamma\delta$  T cell populations.** PBMCs from one donor were incubated at  $2 \times 10^7$  cells for 30 min at  $4^{\circ}\text{C}$  with FITC-conjugated mouse anti-TCR $\delta$  chain antibody (TCR $\delta$ 1; T Cell Sciences, Inc., Cambridge, MA) at 0.8  $\mu\text{g}/\text{ml}$  in 0.8 ml of RPMI 1640 medium supplemented with 10% bovine serum albumin. Cells were washed twice and incubated for 30 min at  $4^{\circ}\text{C}$  with 0.3 mg of magnetic beads conjugated to sheep anti-mouse IgG (DYNAL, Inc., Great Neck, NY). After washing and magnetic removal of the beads, cell-coated beads were used directly for RNA extraction of cells enriched in  $\gamma\delta$  T lymphocytes. The remaining cells were further incubated with 3 mg of the beads to maximize the removal of  $\gamma\delta$  T cells and bound cells were removed by magnetic separation. The final cell suspension was used for RNA extraction of cells enriched in  $\alpha\beta$  T lymphocytes. Cytofluorometric analysis showed that the enriched  $\alpha\beta$  T cell population contained 0.3%  $\gamma\delta$  cells compared with 6% in unfractionated PBMCs.

**DNA sequencing.** Replica form double-strand DNA and/or single-strand phage DNA were extracted from bacterial cultures containing recombinant M13 and used as sequencing templates. Sequencing was carried out with the dideoxynucleotide chain-termination method (10).

## Results

**TCR $\gamma$  V domain gene repertoire of potentially functional and nonfunctional transcripts.** Of the 14 reported human V $\gamma$  genes, 8 are potentially functional, whereas 6 are pseudogenes. The eight potentially functional genes are divided into four families. The V $\gamma$ I family includes five functional genes (V $\gamma$ 2, V $\gamma$ 3, V $\gamma$ 4, V $\gamma$ 5, V $\gamma$ 8), whereas the three other V $\gamma$  families have only one functional member (V $\gamma$ 9, V $\gamma$ 10, and V $\gamma$ 11, respectively). Four oligonucleotides (V $\gamma$ I, V $\gamma$ II, V $\gamma$ III, and V $\gamma$ IV) were designed to specify members of each corresponding V $\gamma$  family. Poly G-tailed cDNAs were synthesized from mRNAs of PBMCs obtained from eight donors. Expressed V $\gamma$  genes were amplified from the cDNAs using primary anchored PCR and nested PCR. After TCR $\gamma$ -specific cDNA libraries were constructed in a modified M13 vector, they were screened with both a C $\gamma$  probe and V $\gamma$  family-specific probes. Gene frequencies were calculated by dividing the number of V $\gamma$  family positive clones by the number of total TCR $\gamma$  clones.

In the first set of experiments, the reproducibility and fidelity of the amplification method was validated. cDNA obtained from one donor was subjected to three different PCR amplifications. The calculated frequency data were conserved among different experiments and varied by  $< 5\%$ .

In eight separate TCR $\gamma$  gene-specific libraries from eight

donors, no clones were positive with the V $\gamma$ IV family-specific probe. Approximately 10 clones from the set of each V $\gamma$  family positive plaques were randomly selected and a total of 232 V $\gamma$ -J $\gamma$ -C $\gamma$  genes were sequenced. All genes could be classified according to the usage of J $\gamma$  chains. Since the V $\gamma$ I-family includes five functional genes, V $\gamma$ I subgroup positive clones were further classified by the usage of V $\gamma$  genes. Although the C $\gamma$ -specific primers used for amplification and probing were complimentary to both the C $\gamma$ 1 gene and the C $\gamma$ 2 gene, the C $\gamma$  chain usage was deduced from the known J $\gamma$  segment configurations (11).

The potential functionality of each clone was determined by examining the sequence at the V-J junction for rearrangements maintaining the correct reading frame. Representative

Table I. Representative Frequency Analyses of Individual Donors

Donor A	V $\gamma$ I		V $\gamma$ II (V $\gamma$ 9)		V $\gamma$ III (V $\gamma$ 10)	
	%		%		%	
Frequency in total transcripts	26.3		39.9		33.8	
Frequency in potentially functional transcripts	27.0		52.2		20.8	
Frequency in nonfunctional transcripts	25.6		28.3		46.1	
J C segment usage	V $\gamma$ I (n = 10)		V $\gamma$ II (n = 11)		V $\gamma$ III (n = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-C $\gamma$ 1	2*				2	2
JP-C $\gamma$ 1			6			
J1-C $\gamma$ 1		2 <sup>§</sup>				1
JP2-C $\gamma$ 2						
J2-C $\gamma$ 2	3 <sup>‡</sup>	3 <sup>  </sup>	1	4	1	4

\* V $\gamma$ 8(2). † V $\gamma$ 3, V $\gamma$ 5, V $\gamma$ 8. § V $\gamma$ 4, V $\gamma$ 5. || V $\gamma$ 2(2), V $\gamma$ 3.

Donor B	V $\gamma$ I		V $\gamma$ II (V $\gamma$ 9)		V $\gamma$ III (V $\gamma$ 10)	
	%		%		%	
Frequency in total transcripts	29.0		3.0		68.1	
Frequency in potentially functional transcripts	51.0		1.2		47.9	
Frequency in nonfunctional transcripts	0		5.4		94.6	
J C segment usage	V $\gamma$ I (n = 8)		V $\gamma$ II (n = 9)		V $\gamma$ III (n = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-C $\gamma$ 1	2*				2	5
JP-C $\gamma$ 1			1			
J1-C $\gamma$ 1					2	1
JP2-C $\gamma$ 2						
JP-C $\gamma$ 2	6 <sup>‡</sup>		1	7		

\* V $\gamma$ 2(2). † V $\gamma$ 2, V $\gamma$ 3, V $\gamma$ 8(4).

results from three donors (A, B, and C) are shown in Table I. Although the TCR $\gamma$  repertoires varied among donors, a similarly skewed pattern of V $\gamma$ -J $\gamma$  combinations was observed in all cases.

The V-J junctional sequences of amplified TCR $\gamma$  cDNA from the same donor are shown in Table II. An extensive number of different sequences at the V $\gamma$ -J $\gamma$  junction (N region) was observed. Only a single pair of identical sequences was found among 30 separate clones. These data show that the PCR amplification was performed without preferential amplification of particular sequences.

Average frequencies of transcripts classified by V $\gamma$  and J $\gamma$  chain usage. On the basis of the frequency and junctional sequence data of V $\gamma$  family genes, the frequency of each V $\gamma$ -J $\gamma$

Donor C	V $\gamma$ I		V $\gamma$ II (V $\gamma$ 9)		V $\gamma$ III (V $\gamma$ 10)	
	%		%		%	
Frequency in total transcripts	48.8		41.5		9.6	
Frequency in potentially functional transcripts	41.0		52.0		7.0	
Frequency in nonfunctional transcripts	63.4		20.0		16.6	
J C segment usage	V $\gamma$ I (n = 10)		V $\gamma$ II (n = 9)		V $\gamma$ III (n = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-C $\gamma$ 1	1*				1	2
JP-C $\gamma$ 1			6			
J1-C $\gamma$ 1						
JP2-C $\gamma$ 2						
J2-C $\gamma$ 2	6 <sup>‡</sup>	3 <sup>§</sup>	2	1	5	2

\* V $\gamma$ 4. † V $\gamma$ 2(3), V $\gamma$ 3, V $\gamma$ 4, V $\gamma$ 8; § V $\gamma$ 8(3).

The frequencies of expression of the V $\gamma$ I, V $\gamma$ II and V $\gamma$ III gene families in the libraries were calculated by enumerating positive plaques of each V $\gamma$  family, compared to the total C $\gamma$  positive plaques. Then, ~10 clones from each V $\gamma$  family were picked up randomly, sequenced, and were classified according to the usage of V $\gamma$  and J $\gamma$  gene segments. For each family, the numbers of in-frame (in) and out-of-frame (out) transcripts are shown. The individual V $\gamma$ I family gene members are indicated with footnote symbols. The use of C $\gamma$  gene segments was deduced from the known configuration of TCR $\gamma$  genes (11).

Table II. Junctional Sequences of TCR $\gamma$  Transcripts Derived from Lymphocytes of a Single Donor

Gene family	Clone	V $\gamma$ gene	V $\gamma$	N	J $\gamma$	J $\gamma$ chain	In frame
V $\gamma$ I	11	V $\gamma$ 3	GCCACCTGGGACAGCC	TCAT	TTATTATAAGAAAACCTCTTTGGC	J2	(+)
	12	V $\gamma$ 8	GCCACCTGGGATAGCC	GGTGG	TATTATAAGAAAACCTCTTTGGC	J2	(+)
	13	V $\gamma$ 5	GCCACCTGGG	CAGGCAAG	ATTATAAGAAAACCTCTTTGGC	J2	(+)
	14	V $\gamma$ 2	GCCACCTGGGACGGGC	CATCGT	TATAAGAAAACCTCTTTGGC	J2	(-)
	15	V $\gamma$ 4	GCCACC	CCGGGCT	GAACCTCTTTGGC	J1	(-)
	16	V $\gamma$ 5	GCCACCTGG	GGG	TTATTATAAGAAAACCTCTTTGGC	J1	(-)
	17	V $\gamma$ 8	GCCACCTGGGA	CCCC	GGTTGGTTCAAGATATTTGCTGAAGGG	JPI	(+)
	18	V $\gamma$ 8	GCCACCTGG	CGT	ACTGGTTGGTTCAAGATATTTGCTGAAGGG	JPI	(+)
	19	V $\gamma$ 3	GCCACCTGGGACAG	AACCCGTAITCTT	ATTATTATAAGAAAACCTCTTTGGC	J2	(-)
	110	V $\gamma$ 2	GCCACCTGGGACGGGC	TCACC	AGAAAACCTCTTTGGC	J2	(-)
	21	V $\gamma$ 9	CTTGTGGAGGTG	CATCGC	AGAAAACCTCTT	J2	(-)
	23	V $\gamma$ 9	CTTGTGGAGGT	CCCTGAACCTGGGGGAAA	GTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	24	V $\gamma$ 9	CTTGTGGGAG	TTTAC	TTATAAGAAAACCTCTT	J2	(+)
	25	V $\gamma$ 9	CTTGTG	TAGGGTA	AAGAAAACCTCTT	J2	(-)
	26	V $\gamma$ 9	CTTGTGGGA	CCCC	CAAGACTTGGCAAAAAAATCAAGGTATT	J2	(-)
	28	V $\gamma$ 9	CTTGTGGAGGTG	CCT	TTATAAGAAAACCTCTT	JP	(+)
	29	V $\gamma$ 9	CTTGTGGGA	A	CAAGACTTGGCAAAAAAATCAAGGTATT	JP	(+)
	210	V $\gamma$ 9	CTTGTGGGAG	CAAGACTTGGCAAAAAAATCAAGGTATT		JP	(+)
	221, 222	V $\gamma$ 9	CTTGTGGGAGG	GGCC	AGACTTGGCAAAAAAATCAAGGTATT	JP	(+)
223	V $\gamma$ 9	CTTGTGGGAGG	GCCGGT	AATTATAAGAAAACCTCTT	J2	(-)	
31	V $\gamma$ 10	TTACTACTGTGCTGGTGGGAT	CA	GATACCCTGGTTGGTTCAAGATA	JPI	(-)	
32	V $\gamma$ 10	TTACTACTGTGCTGGTGGGATTC	CCT	ATTATAAG	J2	(-)	
33	V $\gamma$ 10	TTACTACTGTGCTGGTGGGAT	TACCTCAAACCA	AATTATTATAAG	J2	(+)	
34	V $\gamma$ 10	TTACTACTGTGCTGGTGGGATTC	TACT	TTATAAG	J1	(-)	
35	V $\gamma$ 10	TTACTACTGTGCTGGTGGTGG	TCTATCTCCGACACAC	GTTGGTTCAAGATA	JPI	(+)	
36	V $\gamma$ 10	TTACTACTGTGCTGGTGGGAT	GGGGTAGGGTTGGT	TCAAGATA	JPI	(-)	
37	V $\gamma$ 10	TTACTACTGTGCTGGTGGTGG	AACGAAGACCACCT	TTGGTTCAAGATA	JPI	(+)	
38	V $\gamma$ 10	TTACTACTGTGCTG	GATATACATC	AATTATTATAAG	J2	(-)	
39	V $\gamma$ 10	TTGCTACTGTGCTGGCTGGT		TAAG	J2	(-)	
310	V $\gamma$ 10	TTACTACTGTGCTGGCTGGGAT	AAGGG	G	J2	(-)	

From the TCR $\gamma$  V domain-specific library shown in Table I, 10 clones from the V $\gamma$ I family transcripts, 11 clones from the V $\gamma$ II family transcripts, and 10 clones from the V $\gamma$ III family transcripts were sequenced. The clones V $\gamma$ II-221 and -222 share an identical sequence.

Table III. Average Frequencies of Transcripts for Each V $\gamma$ -J $\gamma$  Combination from Eight Donors

J $\gamma$ -V $\gamma$ combination	V $\gamma$ gene						
	V $\gamma$ 2	V $\gamma$ 3	V $\gamma$ 4	V $\gamma$ 5	V $\gamma$ 8	V $\gamma$ 9	V $\gamma$ 10
	%						
<b>Potentially functional transcripts</b>							
JP1-C $\gamma$ 1	1.5	0	1.0	0	1.1	0	5.3
JP-C $\gamma$ 1	0	0	0	0	0	25.3	0
J1-C $\gamma$ 1	0	7.0	0	0	0	0.4	4.1
JP2-C $\gamma$ 2	0	0	0.6	0	0	0	0
J2-C $\gamma$ 2	6.6	3.1	8.2	1.4	9.2	8.9	16.6
<b>Nonfunctional transcripts</b>							
JP1-C $\gamma$ 1	0	0	0	0	0	0	17.8
JP-C $\gamma$ 1	0	0	0	0	0	0.9	0
J1-C $\gamma$ 1	0	0	0.9	0.9	0	1.0	13.9
JP2-C $\gamma$ 2	0.6	1.1	0	0	0	0	0
J2-C $\gamma$ 2	9.7	3.5	4.1	4.6	5.8	13.1	22.1

Average frequencies of the potentially functional transcripts (upper) and nonfunctional transcripts (lower) for each V $\gamma$ -J $\gamma$ -C $\gamma$  combination are shown. The data are from eight individuals, including the donors shown in Table I. The frequency of each V $\gamma$  gene family was determined by plaque hybridization in all eight libraries; and then individual gene usage and gene functionality was assessed by sequence analysis of 10 clones from each V $\gamma$  family, in all eight libraries. Finally, the average frequencies for the V $\gamma$ -J $\gamma$  combinations were calculated.

combination was determined in the potentially functional and nonfunctional repertoire of each donor. Since the basic pattern of V $\gamma$ -J $\gamma$  combinations was similar among donors, the average frequencies of transcripts with each V $\gamma$ -J $\gamma$  combination were calculated (Table III). In both the in-frame transcripts and the out-of-frame transcripts, a biased increase of particular V $\gamma$  and J $\gamma$  pairs was observed. The J2 gene segment was used in 54% of the in-frame transcripts and in 63% of the out-of-frame transcripts. The V $\gamma$ 10-JP1, V $\gamma$ 10-J1, and V $\gamma$ 10-J2 gene combinations also occurred frequently in both the in-frame and out-of-frame transcripts. Among the 232 clones sequenced, the JP2 gene segment was used only three times (weighed frequency 2.3%). In all the donors except one, V $\gamma$ 9-JP gene rearrangements were dominant among the in-frame transcripts and were rare among the out-of-frame transcripts.

*No detectable transcription of the TCR V $\gamma$ 11 gene in adult peripheral lymphocytes.* As noted earlier, the V $\gamma$ IV family-specific probe did not hybridize to any clones in the TCR $\gamma$ -specific cDNA libraries. Conventional PCR reactions with a C $\gamma$ a primer and a V $\gamma$ IV family-specific primer also failed to amplify any TCR $\gamma$  gene product. To validate the ability of the chosen V $\gamma$ IV-specific oligonucleotide to prime the V $\gamma$ 11 gene and to verify the presence of a rearranged V $\gamma$ 11 gene in PBMC, genomic DNA from several donors was amplified with the V $\gamma$ IV primer and the J1,J2-specific primer (J12). In each case, PCR yielded the expected 230-bp product, under conditions where no detectable products were amplified in cDNA from PBMC using the same V $\gamma$ IV primer (results not shown). Collectively, these results indicate that TCR V $\gamma$ 11 transcription is minimal in human lymphocytes that have rearranged TCR V $\gamma$ 11 genes.

*The source of nonfunctional transcripts.* On the basis of the V $\gamma$  gene family frequencies in the total transcripts from several donors and the sequence data, we estimated that 28–54% of the transcripts were nonfunctional because of frame shifts or intro-

duced termination codons. To discern the source of the nonfunctional TCR $\gamma$  gene transcripts, PBMCs from one donor were sorted into  $\alpha\beta$  T cell-enriched and  $\gamma\delta$  T cell-enriched populations. RNA was extracted from each population or from total lymphocytes and TCR $\gamma$  transcripts were amplified by anchored PCR. After constructing TCR $\gamma$  libraries, randomly picked clones were sequenced and examined for their potential functionality. The results showed that unfractionated lymphocyte population had 73% potentially functional TCR $\gamma$  transcripts compared with 27% in the  $\alpha\beta$  T cell-enriched population and 90% in the  $\gamma\delta$  T cell-enriched population. Taken together, these data imply that most nonfunctional TCR $\gamma$  transcripts in peripheral blood lymphocytes come from  $\alpha\beta$  T cells.

## Discussion

These experiments show that the expressed potentially functional TCR $\gamma$  gene repertoire in adult peripheral lymphocytes derives from a limited set of V $\gamma$ -J $\gamma$  combinations. Only 11 of 40 potential V $\gamma$ -J $\gamma$  rearrangements were detected at a frequency of  $\geq 3\%$ . Unrelated subjects had the same basic pattern of V $\gamma$ -J $\gamma$  gene use. This restricted usage of particular V $\gamma$ -J $\gamma$  gene segments could be explained by three possible mechanisms. One is the presence of potent and common antigens in the periphery, which can stimulate and expand T cells bearing particular types of TCR $\gamma$  genes. Another is the preferential rearrangement of V $\gamma$  segments to J $\gamma$  segments at the progenitor T cell level. The last mechanism is positive or negative selection in the thymic environment, which is known to affect V $\beta$  gene usage in peripheral T cells of mice (12–15).

To delineate how the regulation of V $\gamma$ -J $\gamma$  gene recombination places limits on the mature  $\gamma\delta$  T cell repertoire, transcripts of potentially functional and nonfunctional rearranged TCR $\gamma$

genes were compared. The frequencies of out-of-frame V $\gamma$ -J $\gamma$  gene rearrangements cannot be controlled directly by antigen selection in the thymus or the periphery. The results showed that a similarly restricted set of V $\gamma$ -J $\gamma$ -C $\gamma$  genes was used in both functional and nonfunctional TCR $\gamma$  gene rearrangements. Most of the V $\gamma$ -J $\gamma$  combinations that appeared frequently in the in-frame transcripts were also found in the out-of-frame transcripts. As an example, the TCR J2 gene, rearranged to several different V $\gamma$  gene segments, was found frequently in both functional and nonfunctional transcripts.

The reputed locations of the V, J, and C gene segments and their frequencies of recombination in both functional and nonfunctional transcripts are shown in Fig. 1. The prevalence of V $\gamma$ 10 and J2 might be explained by positional effects, because the TCR V $\gamma$ 10 gene is the most downstream V $\gamma$  gene (except for V $\gamma$ 11) and J2 is the most downstream J $\gamma$  gene. In this regard, Triebel et al. (16) showed that the unexpressed TCR $\gamma$  alleles of eight cloned  $\gamma\delta$  T-cell lines had downstream V $\gamma$  genes joined to upstream J $\gamma$  segments. On the basis of analysis of four neonatal and three fetal thymocyte clones, Krangel and colleagues (5) proposed an ordered rearrangement of TCR $\gamma$  genes. McVay et al. (6) also described regulated expression of TCR $\gamma$  and TCR $\delta$  genes in fetal thymus and gut lymphocytes. However, the TCR $\gamma$  genes studied by these workers had minimal N region additions, as has been seen with both TCR and immunoglobulin genes that rearrange early in ontogeny (17-

21). In contrast, both the functional and nonfunctional TCR $\gamma$  genes in mature peripheral blood lymphocytes displayed extensive junctional diversity (Table II). Although the adult and fetal TCR $\gamma$  repertoires are not equivalent, the transcripts in mature peripheral blood  $\gamma\delta$  T cells still derived from a limited set of V-J combinations. Despite the fact that  $\gamma\delta$  T cell clones expressing V $\gamma$ 10 gene product have not been reported, we found many functionally rearranged V $\gamma$ 10 genes in peripheral blood lymphocytes. The discrepancy is probably due to the current lack of specific antibodies against the V $\gamma$ 10 region.

The TCR V $\gamma$ 11 gene is known to be potentially functional, but it has been unclear if it can express a TCR $\gamma$  gene product. In our experiments, no V $\gamma$ 11 gene transcription in peripheral blood lymphocytes was detected using either conventional PCR or anchored PCR, although we could readily amplify rearranged V $\gamma$ 11 genes from the same specimens. These results suggest that TCR V $\gamma$ 11 genes are rarely transcribed in mature peripheral blood lymphocytes, in accord with results recently reported by two other groups (21, 22). McVay et al. (6) used conventional PCR to demonstrate V $\gamma$ 11 gene expression in fetal tissues. However, the PCR primer that they used for V $\gamma$ 11 gene amplification (termed GVIV) is very homologous to the abundantly transcribed V $\gamma$ 10 gene, sharing 20 out of 22 bases, and these workers noted that expression of the V $\gamma$ 11 gene was always accompanied by expression of the V $\gamma$ 10 gene. Indeed, we found that the cloned V $\gamma$ 10 gene was readily amplified by

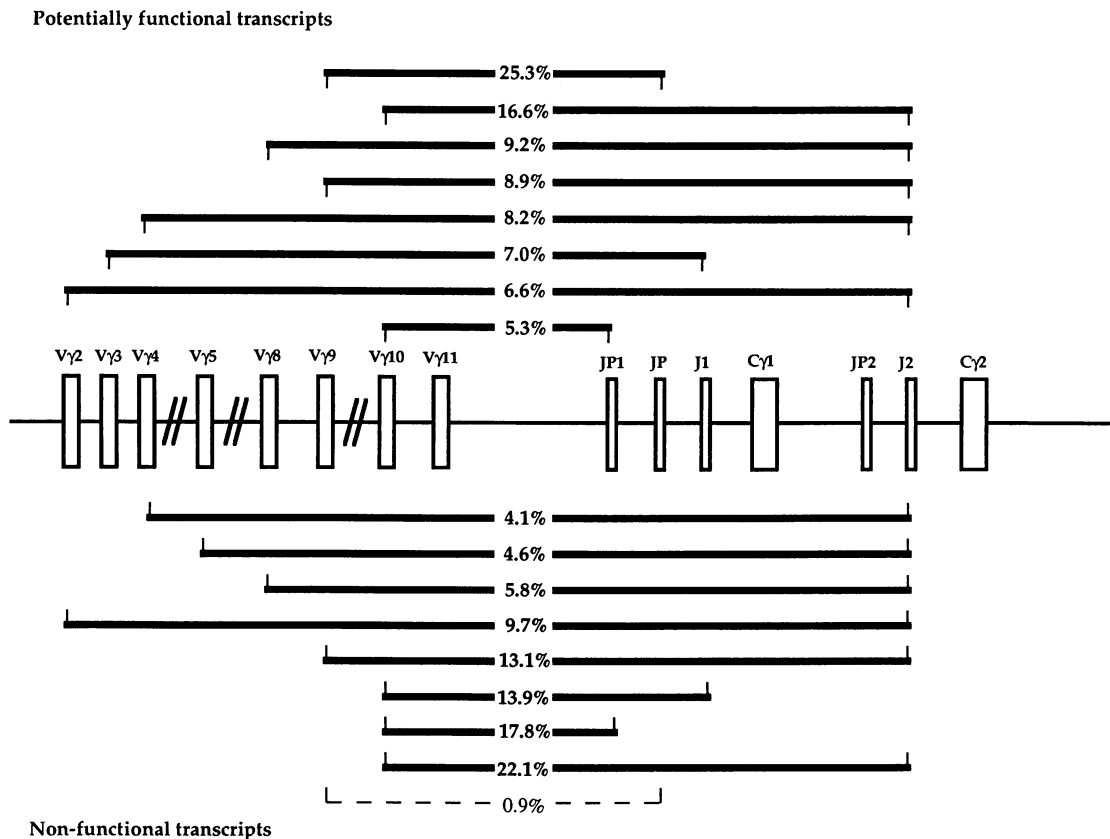


Figure 1. Genomic organization of potentially functional V $\gamma$ , J $\gamma$ , and C $\gamma$  genes on chromosome 7 and the recombination frequencies. The eight most frequent V $\gamma$ -J $\gamma$  combinations in potentially functional transcripts and in nonfunctional transcripts are shown. The frequency of V $\gamma$ 9-JP in nonfunctional transcripts is shown for reference.

PCR using the C $\gamma$ -specific primers with the GVIV primer of McVay, but not with the V $\gamma$ IV primer chosen for the present studies (data not shown).

Because antigenic stimulation can potentially expand even rare T cells in a population, the V $\gamma$ -J $\gamma$  pairs in the functional TCR $\gamma$  repertoire could be different, and more or less diverse, than those in the nonfunctional repertoire. However, the two gene repertoires were remarkably similar. The major discrepancy was in the V $\gamma$ 9-JP transcripts, that accounted for only 0.9% of the out-of-frame transcripts, but 25.3% (on the average) of the in-frame transcripts. This predominance of functional V $\gamma$ 9-JP transcripts was observed in seven of eight donors. The data indicate that lymphocytes expressing rearranged V $\gamma$ 9-JP-C $\gamma$ 1 genes were not preferentially generated during  $\gamma\delta$  T cell development but instead were expanded postthymically from small numbers of precursors. Consistent with this conclusion is the occurrence of two identical V $\gamma$ 9-JP joining sequences in randomly chosen transcripts from the same donor (Table II). The monoclonal antibody Ti $\gamma$ A recognizes a V $\gamma$ 9-associated epitope and stains the major population of peripheral  $\gamma\delta$  T cells in most adults (23). Triebel et al. (24) showed that all Ti $\gamma$ A<sup>+</sup> T cell lines transcribe V $\gamma$ 9-JP-C $\gamma$ 1 rearranged genes. Parker and colleagues (25) discovered that V $\gamma$ 9 cells are not predominant at birth but expand in the first 10 years of life. In this regard, T cells with V $\gamma$ 9/V $\delta$ 2 TCR have been shown to react with staphylococcal enterotoxin A, with the Daudi and Molt-4 lymphoblastoid cell lines, and with mycobacterial antigens (26–29).

Although it is still controversial if progenitor T cells only attempt  $\alpha$  and  $\beta$  rearrangements when  $\gamma$  and  $\delta$  rearrangements fail to produce functional transcripts, TCR $\gamma$  gene rearrangements are often found in  $\alpha\beta$  T cells (30, 31). Some  $\alpha\beta$  lymphoblastoid cell lines are known to transcribe TCR $\gamma$  genes (32). The results of our experiments with enriched  $\alpha\beta$  and  $\gamma\delta$  T cell populations suggest that the nonfunctional TCR $\gamma$  gene transcripts in peripheral blood lymphocytes also derive mainly from  $\alpha\beta$  T cells.

It is possible that some functionally rearranged TCR $\gamma$  transcripts come from  $\alpha\beta$  T cells and that different T cell clones transcribe V $\gamma$  genes at different rates. However, it is very unlikely that these transcripts strongly influenced the overall V $\gamma$ -J $\gamma$  frequencies in the entire T cell population. Thus, we found that V $\gamma$ 9-JP and V $\gamma$ 2- or V $\gamma$ 4-J2 expression occurred most frequently (average of 25.3 and 14.8%, respectively) in the pool of potentially functional transcripts. The prevalence of the two rearrangements agrees with previous enumeration analyses carried out with monoclonal antibodies (3, 4).

In summary, the random assortment of V and J gene segments in the TCR  $\gamma$  locus can produce 40 different recombinants. Among the 232 genes that were analyzed, only 11 combinations appeared frequently in both in-frame and out-of-frame transcripts. These data show that biased V $\gamma$ -J $\gamma$  rearrangement is an important force in molding the mature TCR $\gamma$  repertoire. (The nomenclature used for human TCR $\gamma$  gene segments is that of Lefranc et al. [33] and Forster et al. [34].)

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