Regulation of the Mature Human T Cell Receptor γ Repertoire by Biased V-J Gene Rearrangement

Hitoshi Kohsaka, Pojen P. Chen, Atsuo Taniguchi, William E. R. Ollier,* and Dennis A. Carson
*The Department of Medicine and the Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, La Jolla, California 92093; and *Arthritis and Rheumatism Council Epidemiology Research Unit, University of Manchester, Manchester, United Kingdom*

Abstract

To delineate how gene rearrangement influences the expressed human γδ T cell repertoire, we generated T cell receptor γ (TCRγ) V domain-specific cDNA libraries from the peripheral lymphocytes of eight donors and sequenced a total of 232 TCRγ gene transcripts. The libraries consisted of both in-frame and out-of-frame rearranged TCRγ genes. The in-frame TCRγ gene transcripts were used to determine the diversity of functional T cells, whereas the out-of-frame transcripts, primarily derived from αβ T cells, were used to assess the frequencies of TCR Vγ-Jγ rearrangements in progenitor T lymphocytes. The results showed that both sets of transcripts exhibited strikingly restricted Vγ-Jγ combinations. Only 11 of 40 potential Vγ-Jγ rearrangements were common (≥ 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 Vγ9-JP in potentially functional transcripts from seven of eight individuals. These results show that Vγ-Jγ rearrangement is nonrandom and suggest that the diversity of TCRγ genes in the functional γδ T cell repertoire partly depends upon preferentially rearranged Vγ-Jγ gene combinations. However, the expansion of Vγ9/Vδ2 T cells in adult peripheral blood can only be explained by antigenic selection of relatively rare Vγ9-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 αβ or γδ, in association with the CD3 molecular complex. The smaller γδ subset of T cells has been found in all vertebrate species examined. Most γδ 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 γδ T cells have a distinctive ontogeny and tissue distribution. The presence of two classes of murine γδ T cells, i.e., intraepidermal lymphocytes with restricted Vγ/Vδ receptors and secondary lymphoid organ lymphocytes with variable Vγ/Vδ receptors, suggest that these two types of cells might fulfill special roles in the immune system (2). Human γδ T cells do not have the same tissue tropism. The physiological functions of murine and human γδ T cells are still unclear.

A small set of V, D, and J gene segments encode human γδ receptors. This limited diversity makes it possible to assess the factors that control development and expression of the mature γδ T cell repertoire. By analogy with αβ T cell receptor (TCR),[1] 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 γδ T cell repertoire has been studied mainly with monoclonal antibodies. Two dominant subsets of human γδ 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 Vγ9-JP-Cγ1/Vδ2-Jδ1-Cδ1 to form the TCR heterodimer whereas the other uses Vγ2 or Vγ4-J2-Cγ2/Vδ1-Jδ1-Cδ1 (4).

Evidence for regulation of TCRγ gene rearrangement in early ontogeny has come from analyses of γδ transcripts in the fetal and neonatal lymphoid tissues (5, 6). However, whether selective rearrangement of TCRγ genes influences the adult expressed human γδ 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 Vγ-Jγ rearrangements in functional and nonfunctional TCR Vγ genes. This is possible because the TCRγ 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 TCRγ cDNA libraries from the peripheral blood lymphocytes of eight individuals, compared gene frequencies among the in-frame and out-of-frame TCRγ transcripts, and sequenced a total of 232 Vγ-Jγ genes. Except for Vγ9-JP-Cγ1, both functional and nonfunctional genes exhibited a similarly restricted set of Vγ-Jγ combinations. We conclude that regulation of TCR Vγ gene rearrangement is a significant force in molding the mature functional γδ T cell repertoire. However, antigenic selection, as in the case of Vγ9-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

Address correspondence to Hitoshi Kohsaka, M.D., Department of Medicine 0663, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663.

Received for publication 24 March 1992 and in revised form 13 August 1992.

J. Clin. Invest.

0021-9738/93/01/0171/08 $2.00
Volume 91, January 1993, 171–178

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

centrifugation and were stored in medium containing 10% DMSO at −80°C until used.

RNA isolation and first-strand cDNA synthesis. Total RNA was isolated from 5×10^5 PBMCs using RNAzolB (Cinna/Biotecx, 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°C in 0.3 M NaOH.

dG tailing of first-strand cDNA and anchored PCR. To amplify an entire TCRγ 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 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 200 μM 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 Cγ-specific antisense oligonucleotide (Cγb: 5′-CACGGTCGACTCAAGAAGACAAAGGTATGT) and anchor primers (9:1 mixture of AN primer: 5′-ATTACGGCGGCCGCGGATCC and ANC primer: 5′-ATTACGGCGGCCGCGGATCCCCCCCCCCCC-CC), at a concentration of 1 μM. The ratio of AN1 and AN1C 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 Cγb 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°C for 30 s, 42°C for 30 s, and 72°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 μM each of AN primer and antisense Cγa primer (5′-ACGCGTCGACGG-AAGAAAAATAGTGGGCTT, which contained a SalI recognition sequence at its 5′ end, and primes upstream of the Cγb 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°C, and the products were purified by phenol extraction.

Construction of TCRγ-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 TCRγ-specific cDNA libraries. M13 plates, with plaques containing amplified TCRγ V domain cDNA, were lifted five times with Hybond-N$^+$ nylon membranes (Amersham Corp., Arlington Heights, IL). Both an internal Cγ probe (Cγc: 5′-TAAACAACTTGATGCAGATG, recognizing a region upstream of the Cγa primer-priming sequence) and Vγ family–specific probes (VγI: 5′-GTCAGAAATCTTCCAACTTGGAAG, VγII: 5′-GACGGCACTGTCAGAAAGGAATCT, VγIII: 5′-TCAGGCTTTG-GAGCACCTGATCT, and VγIV: 5′-CAAAGGCTTAGAATATT-TAT) were labeled with digoxigenin in 20 μl 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 μM digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 μM dATP, and 50 U terminal deoxytransferase. Five membranes were hybridized with the different probes at 42°C. After washing the membranes, 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°C to activate the phosphatase. Positive signals were recorded on x-ray film and enumerated.

Conventional PCR. The ability of the VγIV PCR primer to amplify rearranged Vγ11 genes (from the VγIV family) was confirmed by conventional PCR. Template DNA was isolated from PBMCs. Amplification was carried out using 100 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 2.5 U AmpliTaq DNA polymerase, and 1 μM of the VγIV family-specific sense oligonucleotide and either the Cγ-specific antisense oligonucleotides (Cγb or Cγc: 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 αβ T cell or γδ T cell populations. PBMCs from one donor were incubated at 2×10^7 cells for 30 min at 4°C with FITC-conjugated mouse anti-TCRδ chain antibody (TCRδ1; T Cell Sciences, Inc., Cambridge, MA) at 0.8 μg/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°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 γδ T lymphocytes. The remaining cells were further incubated with 3 mg of the beads to maximize the removal of γδ T cells and bound cells were removed by magnetic separation. The final cell suspension was used for RNA extraction of cells enriched in αβ T lymphocytes. Cytofluorometric analysis showed that the enriched αβ T cell population contained 0.3% γδ 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

TCRγ V domain gene repertoire of potentially functional and nonfunctional transcripts. Of the 14 reported human Vγ genes, 8 are potentially functional, whereas 6 are pseudogenes. The eight potentially functional genes are divided into four families. The VγI family includes five functional genes (Vγ2, Vγ3, Vγ4, Vγ5, Vγ8), whereas the three other Vγ families have only one functional member (Vγ9, Vγ10, and Vγ11, respectively). Four oligonucleotides (VγI, VγII, VγIII, and VγIV) were designed to specify members of each corresponding Vγ family. Poly G–tailed cDNAs were synthesized from mRNAs of PBMCs obtained from eight donors. Expressed Vγ genes were amplified from the cDNAs using primary anchored PCR and nested PCR. After TCRγ-specific cDNA libraries were constructed in a modified M13 vector, they were screened with both a Cγ probe and Vγ family–specific probes. Gene frequencies were calculated by dividing the number of Vγ family positive clones by the number of total TCRγ 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 TCRγ gene–specific libraries from eight

donors, no clones were positive with the VγIV family–specific probe. Approximately 10 clones from the set of each Vγ family positive plaques were randomly selected and a total of 232 Vγ-Jγ-Cγ genes were sequenced. All genes could be classified according to the usage of Jγ chains. Since the VγI-family includes five functional genes, VγI subgroup positive clones were further classified by the usage of Vγ genes. Although the Cγ-specific primers used for amplification and probing were complimentary to both the Cγ1 gene and the Cγ2 gene, the Cγ chain usage was deduced from the known Jγ 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 results from three donors (A, B, and C) are shown in Table I. Although the TCRγ repertoires varied among donors, a similarly skewed pattern of Vγ-Jγ combinations was observed in all cases.

The V-J junctional sequences of amplified TCRγ cDNA from the same donor are shown in Table II. An extensive number of different sequences at the Vγ-Jγ 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 Vγ and Jγ chain usage. On the basis of the frequency and junctional sequence data of Vγ family genes, the frequency of each Vγ-Jγ

Table I. Representative Frequency Analyses of Individual Donors

Donor A	VγI	VγII (Vγ9)	VγIII (Vγ10)
	%	%	%
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	VγI (*n* = 10)		VγII (*n* = 11)		VγIII (*n* = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-Cγ1	2*				2	2
JP-Cγ1			6			
J1-Cγ1		2§				1
JP2-Cγ2						
J2-Cγ2	3‡	3‖	1	4	1	4

* Vγ8(2). ‡ Vγ3, Vγ5, Vγ8. § Vγ4, Vγ5. ‖ Vγ2(2), Vγ3.

Donor B	VγI	VγII (Vγ9)	VγIII (Vγ10)
	%	%	%
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	VγI (*n* = 8)		VγII (*n* = 9)		VγIII (*n* = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-Cγ1	2*				2	5
JP-Cγ1			1			
J1-Cγ1					2	1
JP2-Cγ2						
JP-Cγ2	6‡		1	7		

* Vγ2(2). ‡ Vγ2, Vγ3, Vγ8(4).

Donor C	VγI	VγII (Vγ9)	VγIII (Vγ10)
	%	%	%
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	VγI (*n* = 10)		VγII (*n* = 9)		VγIII (*n* = 10)	
Translational frame	In	Out	In	Out	In	Out
JP1-Cγ1	1*				1	2
JP-Cγ1			6			
J1-Cγ1						
JP2-Cγ2						
J2-Cγ2	6‡	3§	2	1	5	2

* Vγ4. ‡ Vγ2(3), Vγ3, Vγ4, Vγ8; § Vγ8(3).

The frequencies of expression of the VγI, VγII and VγIII gene families in the libraries were calculated by enumerating positive plaques of each Vγ family, compared to the total Cγ positive plaques. Then, ~10 clones from each Vγ family were picked up randomly, sequenced, and were classified according to the usage of Vγ and Jγ gene segments. For each family, the numbers of in-frame (in) and out-of-frame (out) transcripts are shown. The individual VγI family gene members are indicated with footnote symbols. The use of Cγ gene segments was deduced from the known configuration of TCRγ genes (11).

Table II. Junctional Sequences of TCRγ Transcripts Derived from Lymphocytes of a Single Donor

Gene family	Clone	Vγ gene	Vγ	N	Jγ	Jγ chain	In frame
VγI	11	Vγ3	GCCACCTGGGACAGGC	TCAT	TTATTATAAGAAACTCTTTGGC	J2	(+)
	12	Vγ8	GCCACCTGGGATAGGC	GGTGG	TATTATAAGAAACTCTTTGGC	J2	(+)
	13	Vγ5	GCCACCTGGGG	CAGGCAAG	ATTATAAGAAACTCTTTGGC	J2	(+)
	14	Vγ2	GCCACCTGGGACGGGC	CATCGT	TATAAGAAACTCTTTGGC	J2	(−)
	15	Vγ4	GCCACC	CCGGGCT	GAAACTCTTTGGC	J1	(−)
	16	Vγ5	GCCACCTGG	CGGG	TTATTATAAGAAACTCTTTGGC	J1	(−)
	17	Vγ8	GCCACCTGGGA	CCCC	GGTTGGTTCAAGATATTTGCTGAAGGG	JP1	(+)
	18	Vγ8	GCCACCTGG	CGT	ACTGGTTGGTTCAAGATATTTGCTGAAGGG	JP1	(+)
	19	Vγ3	GCCACCTGGGACAG	AACCCGTATTCTT	ATTATTATAAGAAACTCTTTGGC	J2	(−)
	110	Vγ2	GCCACCTGGGACGGGC	TCACC	AGAAACTCTTTGGC	J2	(−)
VγII	21	Vγ9	CTTGTGGGAGGTG	CATCGC	AGAAACTCTT	J2	(−)
	23	Vγ9	CTTGTGGGAGGT	CCCTGAACTGGGGGGAAA	GTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	24	Vγ9	CTTGTGGGAG	TTTAC	TTATAAGAAACTCTT	J2	(+)
	25	Vγ9	CTTGTG	TAGGGGTA	AAGAAACTCTT	J2	(−)
	26	Vγ9	CTTGTGGGA	CCCC	CAAGAGTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	28	Vγ9	CTTGTGGGAGGTG	CCT	TTATAAGAAACTCTT	J2	(−)
	29	Vγ9	CTTGTGGGA	A	CAAGAGTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	210	Vγ9	CTTGTGGGAG		CAAGAGTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	221, 222	Vγ9	CTTGTGGGAGG	GCGC	AGAGTTGGGCAAAAAAATCAAGGTATT	JP	(+)
	223	Vγ9	CTTGTGGGAGG	GCCCGGT	AATTATAAGAAACTCTT	J2	(−)
VγIII	31	Vγ10	TTACTACTGTGCTGCGTGGGAT	CA	GATACCACTGGTTGGTTCAAGATA	JP1	(−)
	32	Vγ10	TTACTACTGTGCTGCGTGGGATTC	CCT	ATTATAAG	J2	(−)
	33	Vγ10	TTACTACTGTGCTGCGTGGGAT	TACCTCAAACCA	AATTATTATAAG	J2	(+)
	34	Vγ10	TTACTACTGTGCTGCGTGGGATTC	TACT	TTATAAG	J1	(−)
	35	Vγ10	TTACTACTGTGCTGCGTGG	TCTATGTCCCGACAACAC	GTTGGTTCAAGATA	JP1	(+)
	36	Vγ10	TTACTACTGTGCTGCGTGGGATT	GGGGGTAGCGGTTGGT	TCAAGATA	JP1	(−)
	37	Vγ10	TTACTACTGTGCTGCGTGG	AACGAAGACCACT	TTGGTTCAAGATA	JP1	(+)
	38	Vγ10	TTACTACTGTGCTG	GATATACATC	ATTATTATAAG	J2	(−)
	39	Vγ10	TTGCTACTGTGCTGCGT		TAAG	J2	(−)
	310	Vγ10	TTACTACTGTGCTGCGTGGGATT	AAGGG	G	J2	(−)

From the TCRγ V domain–specific library shown in Table I, 10 clones from the VγI family transcripts, 11 clones from the VγII family transcripts, and 10 clones from the VγIII family transcripts were sequenced. The clones VγII-221 and -222 share an identical sequence.

Table III. Average Frequencies of Transcripts for Each Vγ-Jγ Combination from Eight Donors

Jγ-Vγ combination	Vγ gene						
	Vγ2	Vγ3	Vγ4	Vγ5	Vγ8	Vγ9	Vγ10
				%			
Potentially functional transcripts							
JP1-Cγ1	1.5	0	1.0	0	1.1	0	5.3
JP-Cγ1	0	0	0	0	0	25.3	0
J1-Cγ1	0	7.0	0	0	0	0.4	4.1
JP2-Cγ2	0	0	0.6	0	0	0	0
J2-Cγ2	6.6	3.1	8.2	1.4	9.2	8.9	16.6
Nonfunctional transcripts							
JP1-Cγ1	0	0	0	0	0	0	17.8
JP-Cγ1	0	0	0	0	0	0.9	0
J1-Cγ1	0	0	0.9	0.9	0	1.0	13.9
JP2-Cγ2	0.6	1.1	0	0	0	0	0
J2-Cγ2	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 Vγ-Jγ-Cγ combination are shown. The data are from eight individuals, including the donors shown in Table I. The frequency of each Vγ 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 Vγ family, in all eight libraries. Finally, the average frequencies for the Vγ-Jγ combinations were calculated.

combination was determined in the potentially functional and nonfunctional repertoire of each donor. Since the basic pattern of Vγ-Jγ combinations was similar among donors, the average frequencies of transcripts with each Vγ-Jγ combination were calculated (Table III). In both the in-frame transcripts and the out-of-frame transcripts, a biased increase of particular Vγ and Jγ 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 Vγ10-JP1, Vγ10-J1, and Vγ10-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, Vγ9-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 Vγ11 gene in adult peripheral lymphocytes. As noted earlier, the VγIV family–specific probe did not hybridize to any clones in the TCRγ-specific cDNA libraries. Conventional PCR reactions with a Cγa primer and a VγIV family–specific primer also failed to amplify any TCRγ gene product. To validate the ability of the chosen VγIV-specific oligonucleotide to prime the Vγ11 gene and to verify the presence of a rearranged Vγ11 gene in PBMC, genomic DNA from several donors was amplified with the VγIV 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 VγIV primer (results not shown). Collectively, these results indicate that TCR Vγ11 transcription is minimal in human lymphocytes that have rearranged TCR Vγ11 genes.

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

Discussion

These experiments show that the expressed potentially functional TCRγ gene repertoire in adult peripheral lymphocytes derives from a limited set of Vγ-Jγ combinations. Only 11 of 40 potential Vγ-Jγ rearrangements were detected at a frequency of ≥ 3%. Unrelated subjects had the same basic pattern of Vγ-Jγ gene use. This restricted usage of particular Vγ-Jγ 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 TCRγ genes. Another is the preferential rearrangement of Vγ segments to Jγ segments at the progenitor T cell level. The last mechanism is positive or negative selection in the thymic environment, which is known to affect Vβ gene usage in peripheral T cells of mice (12–15).

To delineate how the regulation of Vγ-Jγ gene recombination places limits on the mature γδ T cell repertoire, transcripts of potentially functional and nonfunctional rearranged TCRγ

genes were compared. The frequencies of out-of-frame Vγ-Jγ gene rearrangements cannot be controlled directly by antigen selection in the thymus or the periphery. The results showed that a similarly restricted set of Vγ-Jγ-Cγ genes was used in both functional and nonfunctional TCRγ gene rearrangements. Most of the Vγ-Jγ 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 Vγ 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 Vγ10 and J2 might be explained by positional effects, because the TCR Vγ10 gene is the most downstream Vγ gene (except for Vγ11) and J2 is the most downstream Jγ gene. In this regard, Triebel et al. (16) showed that the unexpressed TCRγ alleles of eight cloned γδ T-cell lines had downstream Vγ genes joined to upstream Jγ segments. On the basis of analysis of four neonatal and three fetal thymocyte clones, Krangel and colleagues (5) proposed an ordered rearrangement of TCRγ genes. McVay et al. (6) also described regulated expression of TCRγ and TCRδ genes in fetal thymus and gut lymphocytes. However, the TCRγ 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 TCRγ genes in mature peripheral blood lymphocytes displayed extensive junctional diversity (Table II). Although the adult and fetal TCRγ repertoires are not equivalent, the transcripts in mature peripheral blood γδ T cells still derived from a limited set of V-J combinations. Despite the fact that γδ T cell clones expressing Vγ10 gene product have not been reported, we found many functionally rearranged Vγ10 genes in peripheral blood lymphocytes. The discrepancy is probably due to the current lack of specific antibodies against the Vγ10 region.

The TCR Vγ11 gene is known to be potentially functional, but it has been unclear if it can express a TCRγ gene product. In our experiments, no Vγ11 gene transcription in peripheral blood lymphocytes was detected using either conventional PCR or anchored PCR, although we could readily amplify rearranged Vγ11 genes from the same specimens. These results suggest that TCR Vγ11 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 Vγ11 gene expression in fetal tissues. However, the PCR primer that they used for Vγ11 gene amplification (termed GVIV) is very homologous to the abundantly transcribed Vγ10 gene, sharing 20 out of 22 bases, and these workers noted that expression of the Vγ11 gene was always accompanied by expression of the Vγ10 gene. Indeed, we found that the cloned Vγ10 gene was readily amplified by

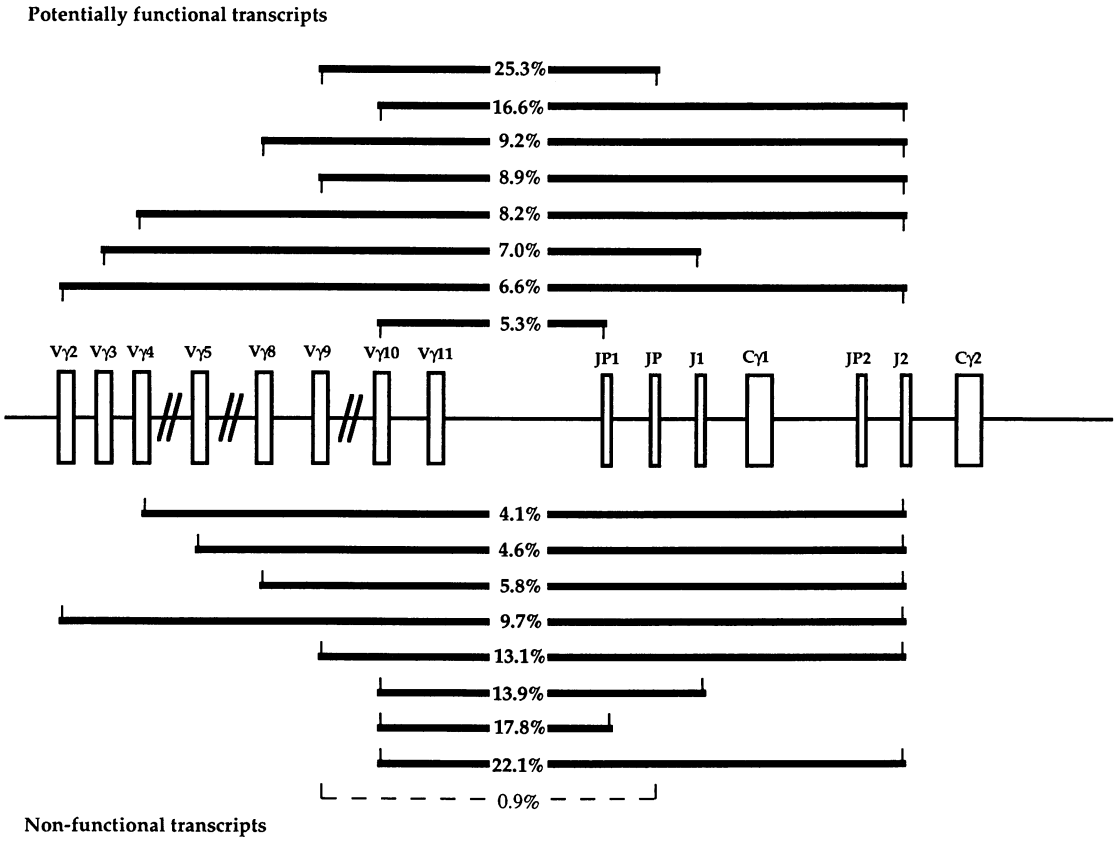

Figure 1. Genomic organization of potentially functional Vγ, Jγ, and Cγ genes on chromosome 7 and the recombination frequencies. The eight most frequent Vγ-Jγ combinations in potentially functional transcripts and in nonfunctional transcripts are shown. The frequency of Vγ9-JP in nonfunctional transcripts is shown for reference.

PCR using the Cγ-specific primers with the GVIV primer of McVay, but not with the VγIV primer chosen for the present studies (data not shown).

Because antigenic stimulation can potentially expand even rare T cells in a population, the Vγ-Jγ pairs in the functional TCRγ 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 Vγ9-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 Vγ9-JP transcripts was observed in seven of eight donors. The data indicate that lymphocytes expressing rearranged Vγ9-JP-Cγ1 genes were not preferentially generated during γδ T cell development but instead were expanded postthymically from small numbers of precursors. Consistent with this conclusion is the occurrence of two identical Vγ9-JP joining sequences in randomly chosen transcripts from the same donor (Table II). The monoclonal antibody TiγA recognizes a Vγ9-associated epitope and stains the major population of peripheral γδ T cells in most adults (23). Triebel et al. (24) showed that all TiγA$^+$ T cell lines transcribe Vγ9-JP-Cγ1 rearranged genes. Parker and colleagues (25) discovered that Vγ9 cells are not predominant at birth but expand in the first 10 years of life. In this regard, T cells with Vγ9/Vδ2 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 α and β rearrangements when γ and δ rearrangements fail to produce functional transcripts, TCRγ gene rearrangements are often found in αβ T cells (30, 31). Some αβ lymphoblastoid cell lines are known to transcribe TCRγ genes (32). The results of our experiments with enriched αβ and γδ T cell populations suggest that the nonfunctional TCRγ gene transcripts in peripheral blood lymphocytes also derive mainly from αβ T cells.

It is possible that some functionally rearranged TCRγ transcripts come from αβ T cells and that different T cell clones transcribe Vγ genes at different rates. However, it is very unlikely that these transcripts strongly influenced the overall Vγ-Jγ frequencies in the entire T cell population. Thus, we found that Vγ9-JP and Vγ2- or Vγ4-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 γ 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 Vγ-Jγ rearrangement is an important force in molding the mature TCRγ repertoire. (The nomenclature used for human TCRγ gene segments is that of Lefranc et al. [33] and Forster et al. [34].)

Acknowledgments

The authors acknowledge the secretarial assistance of Ms. Nancy Noon in the preparation of this manuscript.

This work was supported in part by grants AR25443 and AR40770 from the National Institutes of Health and by a grant from the Ciba-Geigy Corporation (Suncrest, NJ).

References

1. Brenner, M. B., J. L. Strominger, and M. S. Krangel. 1988. The γδ T cell receptor. *Adv. Immunol.* 43:133–192.

2. Allison, J. P., and W. L. Havran. 1991. The immunobiology of T cells with invariant γ/δ antigen receptors. *Annu. Rev. Immunol.* 9:679–705.

3. Falini, B., L. Flenghi, S. Pileri, P. Pelicci, M. Fagioli, M. F. Martelli, L. Moretta, and E. Ciccone. 1989. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. *J. Immunol.* 143:2480–2488.

4. Fagioli, M., E. Ciccone, C. Bottino, B. Falini, F. Grignani, A. Moretta, L. Moretta, and P. G. Pelicci. 1990. The Cγ1-encoded disulphide-linked and the Cγ2-encoded nondisulphide-linked forms of the γ/δ heterodimer use different γ and δ variable regions. *Blood.* 76:279–284.

5. Krangel, M. S., H. Yssel, C. Brocklehurst, and H. Spits. 1990. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J. Exp. Med.* 172:847–859.

6. McVay, L. D., S. R. Carding, K. Bottomly, and A. C. Hayday. 1991. Regulated expression and structure of T cell receptor γ/δ transcripts in human thymic ontogeny. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:83–91.

7. Yoshikai, Y., B. Toyonaga, Y. Koga, N. Kimura, H. Griesser, and T. W. Mak. 1987. Repertoire of the human T cell γ genes: high frequency of nonfunctional transcripts in thymus and mature T cells. *Eur. J. Immunol.* 17:119–126.

8. Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier, and M. M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. *Science (Wash. DC).* 243:217–220.

9. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

10. Sanger, F. 1981. Determination of nucleotide sequences in DNA. *Science (Wash. DC).* 214:1205–1210.

11. Quertermous, T., W. M. Strauss, J. J. Van Dongen, and J. G. Seidman. 1987. Human T cell gamma chain joining regions and T cell development. *J. Immunol.* 138:2687–2690.

12. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273–280.

13. Bill, J., O. Kanagawa, D. L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of Vβ11-bearing T cells. *J. Exp. Med.* 169:1405–1419.

14. Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73–76.

15. Liao, N. S., J. Maltzman, and D. H. Raulet. 1989. Positive selection determines T cell receptor Vβ14 gene usage by CD8$^+$ T cells. *J. Exp. Med.* 170:135–143.

16. Triebel, F., F. Faure, M. Graziani, S. Jitsukawa, M. P. Lefranc, and T. Hercend. 1988. A unique V-J-C-rearranged gene encodes a gamma protein expressed on the majority of CD3+ T cell receptor-α/β–circulating lymphocytes. *J. Exp. Med.* 167:694–699.

17. Elliott, J. F., E. P. Rock, P. A. Patten, M. M. Davis, and Y. H. Chien. 1988. The adult T cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. *Nature (Lond.).* 331:627–631.

18. Feeney, A. J. 1990. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J. Exp. Med.* 172:1377–1390.

19. Feeney, A. J. 1991. Junctional sequences of fetal T cell receptor β chains have few N regions. *J. Exp. Med.* 174:115–124.

20. George, J. F., Jr., and H. W. Schroeder, Jr. 1992. Developmental regulation of D β reading frame and junctional diversity in T cell receptor-beta transcripts from human thymus. *J. Immunol.* 148:1230–1239.

21. Olive, C., P. A. Gatenby, and S. W. Serjeantson. 1991. Variable gene usage of T cell receptor γ and δ chain transcripts expressed in synovia and peripheral blood of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* 87:172–177.

22. Doherty, P. J., S. X. Yang, R. M. Laxer, E. D. Silverman, R. Inman, and S. Pan. 1992. Evidence for clonal expression of T cell receptor VγII$^+$ T cells in the synovial fluid of patients with arthritis. *J. Immunol.* 149:295–299.

23. Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend. 1988. Characterization of human peripheral lymphocytes expressing the CD3-γ/δ complex with anti-receptor monoclonal antibodies. *J. Immunol.* 141:3357–3360.

24. Triebel, F., M. P. Lefranc, and T. Hercend. 1988. Further evidence for a sequentially ordered activation of T cell rearranging gamma genes during T lymphocyte differentiation. *Eur. J. Immunol.* 18:789–794.

25. Parker, C. M., V. Groh, H. Band, S. A. Porcelli, C. Morita, M. Fabbi, D. Glass, J. L. Strominger, and M. B. Brenner. 1990. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. *J. Exp. Med.* 171:1597–1612.

26. Fisch, P., M. Malkovsky, S. Kovats, E. Sturm, E. Braakman, B. S. Klein, S. D. Voss, L. W. Morrissey, R. DeMars, W. J. Welch, et al. 1990. Recognition by human Vγ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science (Wash. DC)*. 250:1269–1273.

27. Sturm, E., E. Braakman, P. Fisch, R. J. Vreugdenhil, P. Sondel, and R. L. Bolhuis. 1990. Human Vγ9-Vδ2 T cell receptor-gamma delta lymphocytes show specificity to Daudi Burkitt's lymphoma cells. *J. Immunol.* 145:3202–3208.

28. De Libero, G., G. Casorati, C. Giachino, C. Carbonara, N. Migone, P. Matzinger, and A. Lanzavecchia. 1991. Selection by two powerful antigens may account for the presence of the major population of human peripheral γ/δ T cells. *J. Exp. Med.* 173:1311–1322.

29. Rust, C. J., F. Verreck, H. Vietor, and F. Koning. 1990. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the Vγ9 region. *Nature (Lond.)*. 346:572–574.

30. Ishida, I., S. Verbeek, M. Bonneville, S. Itohara, A. Berns, and S. Tonegawa. 1990. T-cell receptor gamma delta and gamma transgenic mice suggest a role of a gamma gene silencer in the generation of alpha beta T cells. *Proc. Natl. Acad. Sci. USA.* 87:3067–3071.

31. Takeshita, S., M. Toda, and H. Yamagishi. 1989. Excision products of the T cell receptor gene support a progressive rearrangement model of the α/δ locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3261–3270.

32. Kimura, N., R. P. Du, and T. W. Mak. 1987. Rearrangement and organization of T cell receptor gamma chain genes in human leukemic T cell lines. *Eur. J. Immunol.* 17:1653–1656.

33. Lefranc, M. P., A. Forster, R. Bear, M. A. Stinson, and T. H. Rabbitts. 1986. Diversity and rearrangement of the human T cell rearranging gamma genes: nine germ-line variable genes belonging to two subgroups. *Cell.* 45:237–246.

34. Forster, A., S. Huck, N. Ghanem, M. P. Lefranc, and T. H. Rabbitts. 1987. New subgroups in the human T cell rearranging V gamma gene locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1945–1950.