DIVERSITY AND STRUCTURE OF HUMAN T CELL RECEPTOR δ CHAIN GENES IN PERIPHERAL BLOOD γ/δ-BEARING T LYMPHOCYTES

BY YOSHIHIRO TAKIHARA, JÖRG REIMANN, EFFIE MICHALOPOULOS, ERMANNO CICCONE,* LORENZO MORETTA,* AND TAK W. MAK

*From The Ontario Cancer Institute, Department of Medical Biophysics and Immunology, University of Toronto, Toronto, Canada M4X 1K9; and the *Instituto Nazionale per la Ricerca Sul Cancro, University of Genova, 16132, Italy*

The TCR can take either of two forms, an α/β heterodimer (1–5) or γ/δ heterodimer (6–9). Both these forms are associated with the CD3 complex, which participates in signal transduction across the cell membrane (10–12). The TCR-α/β is found on the majority of peripheral blood T cells (13) and has been shown to be responsible for the recognition of antigen in the context of cell surface proteins encoded by class I and class II genes of the MHC (14, 15). The TCR-γ/δ is found on a small percentage of peripheral T cells (13), but on a wide variety of different cell types (16–18), and its target structure remains to be identified. The TCR-γ/δ is expressed on $CD3^+$ thymocytes during fetal ontogeny, before the appearance of the TCR-α/β (19), and persists on $CD3^+$, $CD4^-$, $CD8^-$ adult thymocytes and on a subset (1–10%) of $CD3^+$ cells in adult peripheral lymphoid organs and the peripheral blood (13). The germline organization of the TCR loci consists of noncontiguous sequences that encode V, D, J, and C gene segments (20–30), which undergo somatic rearrangement in T cells during ontogeny, to produce a complete gene. However, the fine structures of the TCR loci differ considerably (31–39). The V gene repertoire can also differ in man and mouse. For example, the human V_β gene repertoire has been estimated to be four or five times higher than its murine counterpart (100 compared with 20–25) (40). In general, the repertoires of variable gene segments for both the α and β chains are much greater than that of the γ chain (41, 42) and recent evidence suggests that the number of V_δ in the mouse genes is also limited (42, 43). An estimation of the size of the V_δ repertoire is hampered by the fact that the δ locus is embedded in the α chain locus between the V_α and J_α genes (32, 38), resulting in no clear demarcation between V_α and V_δ genes. Preliminary data have suggested that the repertoire of human V_δ genes may be even more limited, as several groups have identified the same V_δ gene segments, but the repertoire and diversity of the human TCR δ chain gene is not known. Here, we describe the diversity and the structure of human TCR δ chain variable gene segments used in peripheral blood $CD4^-$ $CD8^-$ (double-negative) T lymphocytes. Although the function and

This work is supported by the Medical Research Council, Natural Sciences and Engineering Research Council of Canada, and National Cancer Institute of Canada, and a special research grant from the University of Toronto. Y. Takihara is a recipient of an award from the MRC of Canada, and J. Reimann is a recipient of a grant from the Mildred-School stifting für Krebsforschung.

specificity of γ/δ receptors are unknown, it is important to assess the functional diversity of these cells to address the question of the potential diversity of ligands that can be recognized by the γ/δ receptor. Furthermore, this information may be valuable in the evaluation of the mechanisms of both autoimmune disease and normal immune defense.

Materials and Methods

Isolation and Cloning of Peripheral Blood Double-negative T Lymphocytes. PBL from normal volunteers were isolated on Ficoll-Hypaque gradients and then E rosette-positive cells (T cells) were separated from E rosette-negative populations (non-T cells). E-rosetting cells were incubated with a mixture of anti-CD4 and anti-CD8 mAbs followed by treatment with rabbit C for 1 h at 37°C to isolate double-negative cells. These cells (5×10^4/flask) were cultured with PHA (0.5%, vol/vol) in the presence of autologous, irradiated, Percoll-enriched peripheral blood monocytes (2×10^4/flask). These bulk-cultured cells were then cultured under limiting dilution conditions in the presence of both autologous irradiated feeder cells and exongenous IL-2 derived from spleen cells stimulated with PHA as described before (44). The cell surface marker phenotype of these cells was confirmed by indirect immunofluorescence. The mAbs used in these experiments were OKT3 (anti-CD3), CK.79 (anti-CD4), B9.4 (anti-CD8), and WT31 (directed against a framework determinant of the TCR-α/β; Sanbio, Uden, The Netherlands).

Southern Blot Analyses and Northern Blot Analyses. DNA was extracted from double-negative T lymphocytes and peripheral polymorphocytes and digested with one of the restriction enzymes (Eco RI, Bam HI, or Hind III). Digested DNA (10 μg/lane) was subjected to electrophoresis on 0.8% agarose gels and transferred to Gene Screen Plus (New England Nuclear, Boston, MA) (45). RNA was extracted from double-negative T lymphocytes, thymocytes, and leukemic cell lines by the guanidine hydrochloride method (29). RNA (10 μg/lane) was size fractionated by electrophoresis on 1% agarose gels as described previously (29) and transferred to Gene Screen Plus according to the directions of the manufacturer. Hybridization and washing were performed as described previously (29).

cDNA Cloning. dscDNA was synthesized from total cellular RNA (20 μg), which had been derived from bulk-cultured double-negative T cells, by the procedure of Gubler and Hoffman (46). After treatment with Mung bean nuclease, DNA Polymerase I large fragment, and Eco RI methylase and subsequent size selection, the dscDNA was ligated into the Eco RI site of λgt 10 using Eco RI linkers. Recombinant phage were packaged using Gigapack cloning kits (Stratagene, San Diego, CA). In total, 2×10^5 recombinant λgt 10 phage were screened with ^{32}P-labeled TCR-δ cDNA probes (29).

DNA Sequencing. cDNA inserts were subcloned into the Eco RI site of M13mp9 and their nucleotide sequences were determined using the specific primer-directed dideoxynucleotide sequencing technique and the Sanger-dideoxy chain termination method (47).

Results

Characterization of Peripheral Double-negative T Cell Clones. To examine the δ chain diversity in human peripheral T cells, three CD4$^-$, CD8$^-$, WT31$^-$ T cell lines were established (Table I). These cell lines were analyzed by Southern blot hybridization using the restriction enzyme Bam HI, and were compared with bulk heterogeneous CD4$^-$ CD8$^-$ T cells. Southern blot analysis using Jδ1 probe (Fig. 1), revealed bands of 11, 10.5, and 9 kb with the same pattern (11 and 10.5 kb) in two lines and a 9-kb band in the third. Using a Jδ2 probe, however, patterns of rearranged bands were the same, suggesting that rearrangements involving the Jδ1 occurred in all three lines. Hybridization of a Jδ1 probe to the bulk-cultured DNA showed both 10.5- and 11-kb predominant bands, indicating that in peripheral double-negative T cells, two

TABLE I
Surface Phenotype of Cloned T Cell Lines and Bulk-cultured T Cells

Cell population	CD3	CD4	CD8	WT31
Clone 11	+	–	–	–
39	+	–	–	–
64	+	–	–	–
67	+	+	–	+
Bulk culture	ND	–	–	ND

Vδ gene segments are involved in the majority of Jδ1 rearrangements. These cell lines were also examined by Northern blot analysis. As can be seen in Fig. 2, four bands were detected. In thymocytes, the nonfunctional 2.0- and 1.3-kb bands were predominant but, in peripheral blood double-negative T cells and in a γ/δ-bearing leukemic T cell line (Peer), functional 2.2- and 1.5-kb bands were most evident. Neither a non-T cell line (EL-2K) nor an α/β-bearing leukemic T cell line (Jurkat) showed any TCR-δ transcription.

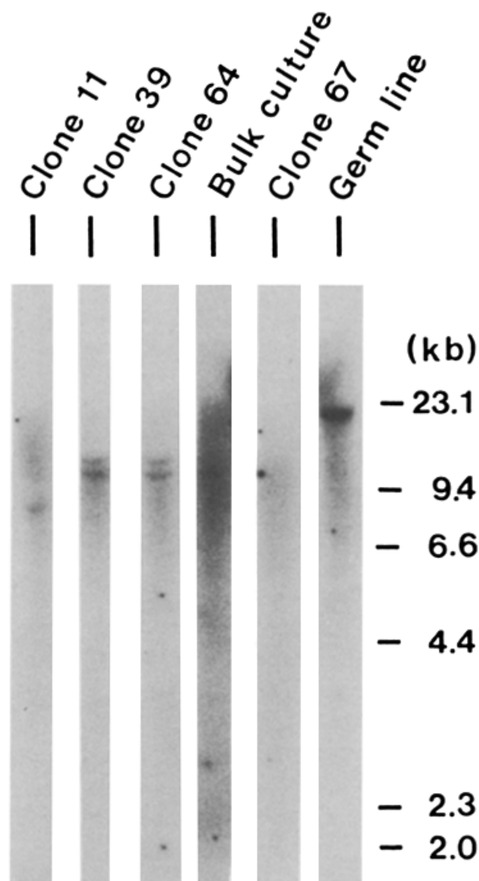

FIGURE 1. Southern blot analyses of peripheral blood double-negative T cells. DNA was extracted from cloned double-negative T cell lines and digested with Bam H1. DNA (10 μg/lane) was subjected to electrophoresis and transferred to Gene Screen Plus according to the directions of the manufacturer (New England Nuclear). Hybridization was performed using a nick-translated ^{32}P-labeled Jδ1 probe as described previously (33).

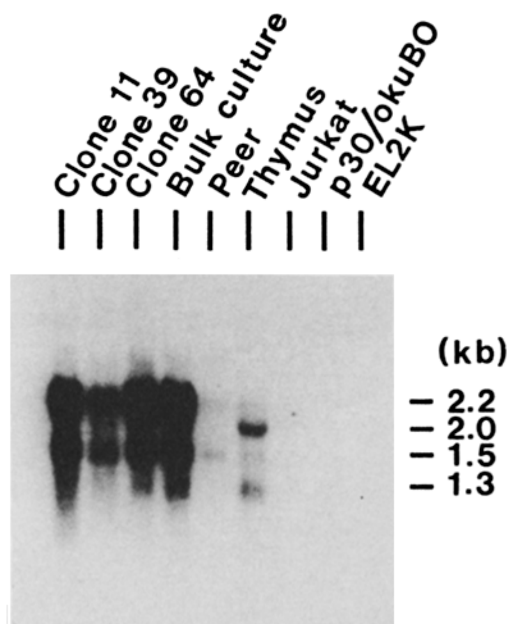

FIGURE 2. Northern blot analyses of peripheral blood double-negative T cells, thymocytes, and leukemic T cells lines. RNA was extracted by guanidine hydrochloride method and size fractionated by electrophoresis in a 1% agarose gel. Hybridization was carried out using ^{32}P-labeled nick-translated TCR δ chain cDNA probes, according to the protocol described previously (29).

Isolation and Structural Analyses of Human Vδ Genes. A complementary DNA library was constructed from total cellular RNA derived from bulk-cultured T cells and screened using a ^{32}P-labeled Cδ probe to give a frequency of 0.1% δ^+ cDNA clones. 20 clones were picked up using the ^{32}P-labeled DNA probe specific for the 5′ region of Cδ and analyzed in detail. These $V\delta^+$ cDNA inserts were subcloned into M13mp9 and their nucleotide sequences were determined. All 20 clones contained Vδ gene segments and most were shown to consist of leader (L), variable (V), diversity (D), and joining (J) regions by comparison with the published human germline-Dδ1, Dδ2, Jδ1, Jδ2, and Jδ3 sequences (33). 17 of the 20 cDNAs were found to encode functional messages, with the V, D, J, and C elements joined in-frame (Table II). Sequence analyses indicated that 7 of the 20 independently isolated Vδ gene segments were unique, as shown in Fig. 3. Of these 20 clones, nine cDNA clones were found to use the Vδ1 gene segment and seven cDNA clones were found to use the Vδ2 gene segment, indicating that these two Vδ gene segments are used in the majority of peripheral blood double-negative T cells. The nucleotide sequence of Vδ1 is identical to that of a Vδ gene published previously (27, 28, 30). Of the six Vδ2 clones, four were long enough to analyze. Two clones (KT19E and KT09E) had a C at position 229 (Vδ2), while in KT04E and KT14E, a G was substituted at this position (Vδ2′), resulting in a substitution of a methionine codon for an isoleucine codon, shown in Fig. 4. The finding of the same substitution in two clones argues against a cloning artifact being the source of the difference between Vδ2 and Vδ2′. Polymorphism between the alleles may result in this one nucleotide substitution, because Southern blot analysis using the Vδ2 gene segment as a probe indicated that the Vδ2 subfamily consists of a single member. Our results indicate that there

TABLE II

Human T Cell Receptor δ Chain Variable Gene Subfamilies and Frequencey of their Usage in Peripheral Double-negative T Cells

Subfamilies	Clones	Rearrangement	Subfamily size	Frequency of usage
Vδ1	KT003	F	1	9:20
	KT042	N		
	KT047	N		
	KT01A	F		
	KT16A	F		
	KT06E	F		
	KT10E	F		
	KT12E	N		
	KT18E	F		
Vδ2	KT12A	F	1	7:20
	KT43A	F		
	KT04E	F		
	KT09E	F		
	KT13E	F		
	KT14E	F		
	KT19E	F		
Vδ3	KT041	F	1	1:20
Vδ4	KT06A	F	2	1:20
Vδ5	KT08A	F	1	1:20
Vδ6	KT05E	F	1	1:20

The number of family size was estimated by the number of bands detected by Southern blot analyses shown in Fig. 5. Frequency of usage is given as the number of complete clones using a particular Vδ segment. Functional rearrangements are designated by F and nonfunctional rearrangements by N.

are at least six different Vδ genes used in human double-negative peripheral blood T cells.

The junctional diversity of cDNA clones is shown in Fig. 3 *a* and *b*. The cDNA sequences were aligned to maximize the contribution to diversity made by the two Dδ elements, which are listed above the cDNA sequences. As can be seen, there are N region sequences at the VD, DD, and DJ junctions, with addition of up to 18 nucleotides. In certain cases, exonuclease trimming of the gene segments at the junctions and subsequent replacement with N region sequences can also be seen. Comparison of the junctional sequences of the 20 clones reveals no significant similarity, indicating that the 20 clones were independently isolated.

Examination of the J gene sequences also indicated that 19 of 20 δ cDNA clones used the Jδ1 gene segment, while only one clone, KT041, utilized the Jδ3 gene segment, suggesting that Jδ1 gene segment is used preferentially in peripheral blood double-negative T cells. This finding is also compatible with the results of Southern blot analyses using the cloned double-negative T cell lines.

The deduced amino acid sequences of the different Vδ gene segments have been aligned for maximum homology to each other in Fig. 4.

Southern Analyses of Vδ Gene Segments in Human Germline DNA. To assess the multiplicity of germline Vδ genes, Southern blot analyses of Eco RI–, Bam HI–, or Hind III–digested germline DNA were performed using probes that included Dδ and Jδ

```
a
                      1     5' UT                                L                                              Vδ         120
Subfamilies(Clones)
Vδ3  ( KT041)                                 TTCTTACTGTGGGCTTTAGCTTTTTGTTTTTCTACAGGGGCACGCTGTGTGACAAAGTAACCCAGAGTTCCCCGGACCAGAC
Vδ1  ( KT10E)  ACAACTCACATTTGTGCAAAGAGCTACATGCCACATGCTGTTCTCCAGCCTGCTGTGTGTATTTGTGGCCTTCAGCTACTCTGGATCAAGTGTGGCCCAGAAGGTTACTCAAGCCCAGTC
Vδ2  ( KT19E)  GTGGTTGAGAGGCAGAGCTGCCCCTGAGTGAGCCATGCAGAGGATCTCCTCCCTCATCCATCTCTCTCTCTTCTGGGCAGGAGTCATGTCAGCCATTGAGTTGGTGCCTGAACACCAAAC
Vδ2' ( KT14E)  GTGGTTGAGAGGCAGAGCTGCCCCTGAGTGAGCCATGCAGAGGATCTCCTCCCTCATCCATCTCTCTCTCTTCTGGGCAGGAGTCATGTCAGCCATTGAGTTGGTGCCTGAACACCAAAC
Vδ5  ( KT08A)  CTGCATCTTCACAGGAGGGATGGCCATGCTCCTGGGGGCATCAGTGCTGATTCTGTGGCTTCAGCCAGACTGGGTAAACAGTCAACAGAAGAATGATGACCAGCAAGTTAAGCAAAATTC

                    121                                                Vδ                                               240
Subfamilies(Clones)
Vδ3  ( KT041)  GGTGGCGAGTGGCAGTGAGGTGGTACTGCTCTGCACTTACGACACTGTATATTCAAATCCAGATTTATTCTGGTACCGGATAAGGCCAGATTATTCCTTTCAGTTTGTCTTTTATGGGGA
Vδ1  ( KT10E)  ATCAGTATCCATGCCAGTGAGGAAAGCAGTCACCCTGAACTGCCTGTATGAAACAAGTTGGTGGTCATATTATATTTTTTGGTACAAGCAACTTCCCAGCAAAGAGATGATTTTCCTTAT
Vδ2  ( KT19E)  AGTGCCTGTGTCAATAGGGGTCCCTGCCACCCTCAGGTGCTCCATGAAAGGAGAAGCGATCGGTAACTACTATATCAACTGGTACAGGAAGACCCAAGGTAACACAATCACTTTCATATA
Vδ2' ( KT14E)  AGTGCCTGTGTCAATAGGGGTCCCTGCCACCCTCAGGTGCTCCATGAAAGGAGAAGCGATCGGTAACTACTATATCAACTGGTACAGGAAGACCCAAGGTAACACAATGACTTTCATATA
Vδ4  ( KT06A)                                                CATATGACACCAGTGATCCAAGTTATGGTCTATTCTGGTACAAGCAGCCCAGCAGTGGGGAAATCGATTTTTCTTATTTA
Vδ5  ( KT08A)  ACCATCCCTGAGCGTCCAGGAAGGAAGAATTTCTATTCTGAACTGTGACTATACTAACAGCATGTTTGATTATTTCCTATGGTACAAAAAATACCCTGCTGAAGGTCCTACATTCCTGAT

                    241                                                Vδ                                               360
Subfamilies(Clones)
Vδ3  ( KT041)  TAACAGCAGATCAGAAGGTGCAGATTTTACTCAAGGACGGTTTTCTGTGAAACACATTCTGACCCAGAAAGCCTTTCACTTGGTGATCTCTCCAGTAAGGACTGAAGACAGTGCCACTTA
Vδ1  ( KT10E)  TCGCCAGGGTTCTGATGAACAGAATGCAAAAAGTGGTCGCTATTCTGTCAACTTCAAGAAAGCAGCGAAATCCGTCGCCTTAACCATTTCAGCCTTACAGCTAGAAGATTCAGCAAAGTA
Vδ2  ( KT19E)  CCGAGAAAAGGACATCTATGGCCCTGGTTTCAAAGACAATTTCCAAGGTGACATTGATATTGCAAAGAACCTGGCTGTACTTAAGATACTTGCACCATCAGAGAGAGATGAAGGGTCTTA
Vδ2' ( KT14E)  CCGAGAAAAGGACATCTATGGCCCTGGTTTCAAAGACAATTTCCAAGGTGACATTGATATTGCAAAGAACCTGGCTGTACTTAAGATACTTGCACCATCAGAGAGAGATGAAGGGTCTTA
Vδ4  ( KT06A)  TCAGGGGTCTTATGACCAGCAAAATGCAACAGAAGGTCGCTACTCATTGAATTTCCAGAAGGCAAGAAAATCCGCCAACCTTGTCATCTCCGCTTCACAACTGGGGGACTCAGCAATGTA
Vδ5  ( KT08A)  ATCTATAAGTTCCATTAAGGATAAAAATGAAGATGGAAGATTCACTGTCTTCTTAAACAAAAGTGCCAAGCACCTCTCTCTGCACATTGTGCCCTCCCAGCCTGGAGACTCTGCAGTGTA
Vδ6  ( KT05E)                                                                                     TATCATGGATTCCCAGCCTGGAGACTCAGCCACCTA

                    361
                       Vδ                       N1        Dδ1            N2          Dδ2               N3                    480
Subfamilies(Clones)                                     CCTTCCTAC                  ACTGGGGATACG
Vδ3  ( KT041)  CTACTGTGCCT.................CCCTCAA...........CCTA............A...........ACTGGGG............TCTGAGGACGTTAG............
Vδ1  ( KT10E)  CTTTTGTGCTCTTGGGGA............GGCA........CCTTCC.............GC............CTGGGG...................AAAAC................
Vδ2  ( KT19E)  CTACTGTGCCTGTGACACCGTA......................TC..............GG.............TGGGGGATACG........CGACAGCCTCCGA............
Vδ2' ( KT14E)  CTACTGTGCCTGTGACACC.........................TT.............GCGT..........ACTGGGGGA...............CGACTGT...............
Vδ4  ( KT06A)  TTTCTGTGCAATGAGAGAGG..........AGA..............CTA........CGGAACT.........ACTGGGGGATACG...........CTTAAGA...............
Vδ5  ( KT08A)  CTTCTGTGCAGCAAG...........GCAAAGAA........CTTCCT........CCGGGGTCC...........GGGGGATACG.........AGGACCCGAA..............
Vδ6  ( KT05E)  CTTCTGTGCAGCAAG.................C........................................................ATA...........AGTCGCTGCGT.............

                                                      Jδ3                                            Cδ
                    481<-------------------------------------------------------------------><-------------------------------------------- 600
Subfamilies(Clones)  CTCCTGGGACACCCGACAGATGTTTTTCGGAACTGGCATCAAACTCTTCGTGGAGCCCCGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ3  ( KT041)  .CTCCTGGGACACCCGACAGATGTTTTTCGGAACTGGCATCAAACTCTTCGTGGAGCCCCGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ1  ( KT10E)  ..............ATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ2  ( KT19E)  .........ACACCGATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ2' ( KT14E)  .........ACACCGATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ4  ( KT06A)  .........ACACCGATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ5  ( KT08A)  ..............GATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
Vδ6  ( KT05E)  .........ACACCGATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAAGAAGTCAGCCTCATACCAAACCATCCGTTTTTGTCATGAAAAATGGAACAAATGTCGCTT
                        ACACCGATAAACTCATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCAA
                       <---------------------------------------------------->
                                              Jδ1
```

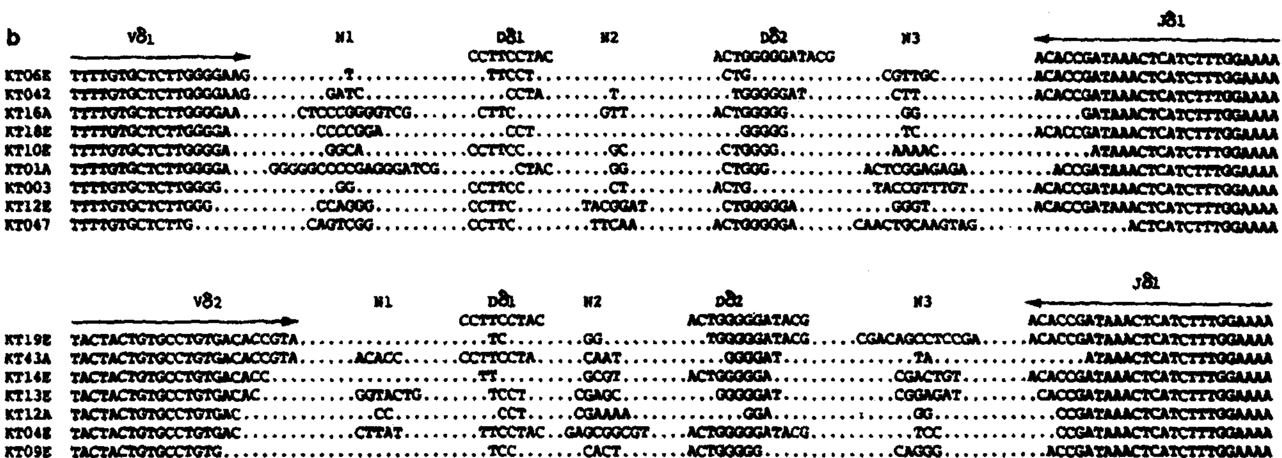

FIGURE 3. Sequences of the 20 TCR δ cDNA clones derived from human peripheral blood double-negative T cells. (*a*) Sequences of the seven unique V_δ gene segments. Sequence analyses of the 20 V_δ gene segments isolated revealed seven distinct sequences. These independent sequences have been aligned to obtain maximum homology to published germline $D\delta1$ and $D\delta2$ sequences and grouped on the basis of homology. The 5' untranslated (5'UT), leader (L), variable (V), diversity ($D\delta1$, $D\delta2$), joining ($J\delta1$, 3), and constant (C) segments are indicated by the appropriate lettering and potential N region sequences identified. The germline nucleotide sequences of D regions, ($D\delta1$ and $D\delta2$) and J regions ($J\delta3$ and $J\delta1$) are shown above and below the cDNA sequences respectively. (*b*) Junctional diversity of TCR δ cDNA clones containing $V\delta1$ and $V\delta2$ sequences. 9 of 20 V_δ cDNAs carried an identical V_δ gene segment disignated, $V\delta1$, while a further seven used another V_δ gene segment, $V\delta2$. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00793.

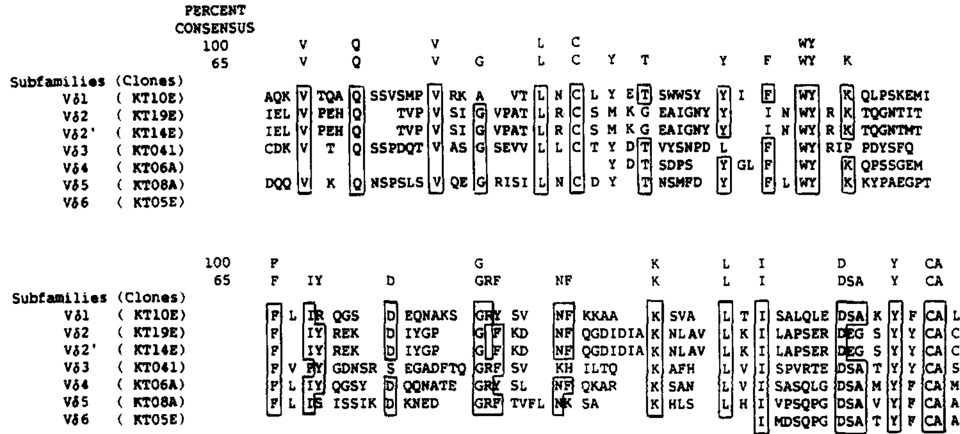

FIGURE 4. Deduced protein sequences of the unique seven Vδ gene segments. Protein sequences were derived from the cDNA sequences shown in Fig. 3 *a*. Spaces were added in the sequences to maximize homology. The conserved amino acids sequences are boxed. These sequence data have been submitted to th EMBL/GenBank Data Libraries under the accession number Y00793.

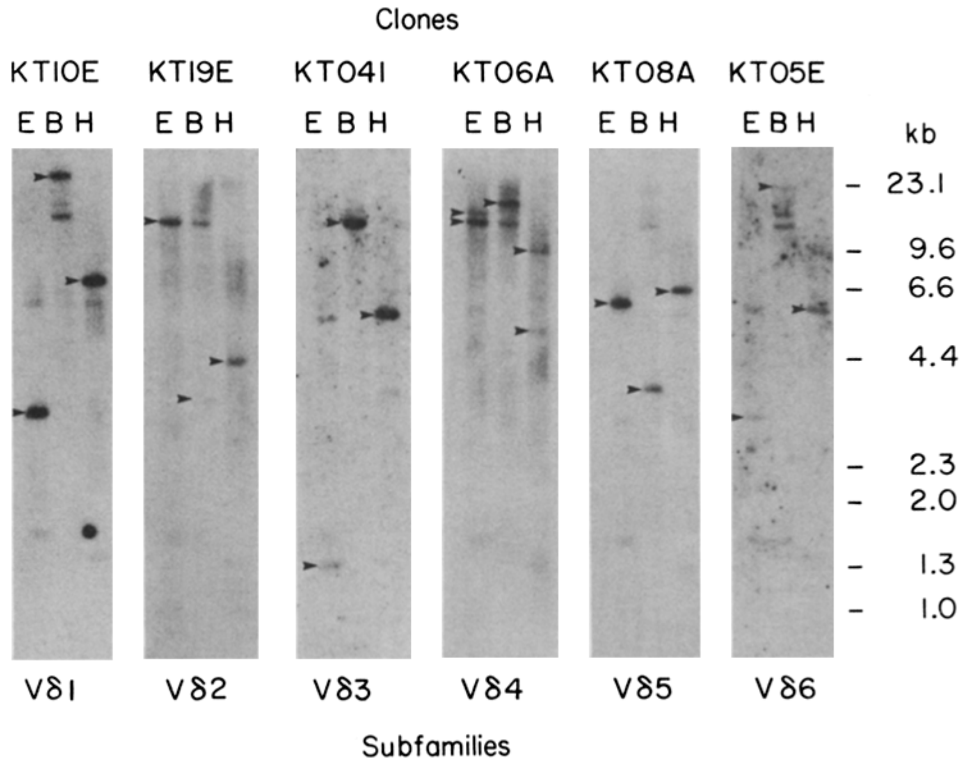

FIGURE 5. Southern blot analyses of germline DNA digested with Eco RI, Bam HI, or Hind III. Vδ gene segments were used as probes and the different Vδ gene subfamilies are indicated. Vδ gene fragments are indicated by closed arrowheads.

sequences as well as the 5′ region of Cδ. It was found that in contrast to the Vα genes, where large number of Vα subfamilies include multiple members, the majority of Vδ gene segments hybridize to a single band, using the three restriction enzymes. Two bands were found when the Vδ4 gene segment was used as a probe. The deduced number of subfamilies and their frequency of usage are summarized in Table II.

Discussion

Preliminary evidence suggested that there was only one human TCR δ chain V region (27, 28, 30). However our analysis of 20 TCR δ chain cDNA clones indicates that the germline Vδ gene repertoire may be larger. Sequence analysis of the 20 clones revealed seven unique sequences representing at least six subfamilies of Vδ genes (see Fig. 3 *a* and Table II). A high percentage of the cDNA clones (85%) encoded potentially functional messages, indicating that they may be used by functional double-negative T cells. A number of interesting findings have emerged in our studies. The cDNA clone, KT05E, included only 51 Vδ nucleotides, which, upon comparison to the sequence of Vδ5, showed an 80% homology on the nucleotide level. However, since the Vδ5 gene hybridizes to a single band on Southerns under stingent conditions, which is of a different size than the band to which KT05E hybridizes, we have classified these genes into two separate subfamilies (Vδ5 and Vδ6). In general, the 3′ portions of V genes are more similar to each other than the 5′ regions, so it is likely that these V genes differ greatly at their 5′ ends, although they are highly homologous at their 3′ ends. Comparison of the four Vδ2 clones showed that two of the four clones encoded an isoleucine at position 227–229, while the remaining two had a methionine codon at this position, suggesting that these genes are alleles of Vδ2. The possibility that the C→G substitution is a cloning artifact is remote, since the C→G change is the only change and this same substitution is found in both clones. These differences could be the result of polymorphism within the subfamily.

On a protein level, the structure of Vδ and Vα genes was found to be similar as a result of conservation of fundamental amino acid sequences (indicated in Fig. 4).

Computer comparison of the nucleotide sequence of these Vδ gene segments with the previously reported Vα gene segments showed no significant homology, except for the Vδ4 gene subfamily. Sequence comparison of Vδ4 gene segment to the Vα 6.1 (HAP01) gene (48) showed only 11 nucleotide differences between them, raising the possibility of an overlap in the V repertoire of the α and δ chains. Interestingly, the same overlap in the V repertoire of both chains has recently been reported (49).

Our results suggest that the germline repertoire of the human TCR δ chain genes consists of at least six Vδ gene segments, two Dδ gene segments, three Jδ segments, and suggest that this germline diversity is supplemented by the addition of N region sequences at each of the three junctions (VD, DD, and DJ). Although no Jδ2-containing clones were isolated, this segment is considered to be potentially functional, since genomic sequence analysis showed the presence of an available recombination signal and open reading frame with the characteristic J region amino acid sequences (phe Gly × Gly) in Jδ2 (33). The TCR δ chain seems to have a relatively limited germline V, D, and J gene repertoire, with the majority of the potential sequence diversity provided by junctional flexibility and the addition of N region sequences (shown in Fig. 3), suggesting that the VDJ junction is a very important

region in determining the specificity of binding. Using the calculation methods described previously (43), the amino acid diversity of the human TCR δ chain could consist of 2×10^{23} different sequences, with almost all diversity provided by junctional flexibility and the N region sequences.

Although the specific function of γδ-bearing cells has not been defined as of yet, recent evidence points to a role for these cells in autoimmunity and recognition of self. Alloreactive $CD3^+$, $CD4^-$, $CD8^-$ γ/δ-bearing T cell lines isolated from alloimmunized BALB/c *nu/nu* mice can be demonstrated to specifically recognize class I MHC molecules in both proliferative and cytotoxic assays (50). These cells can proliferate in response to allogenic cells in MLC and the resulting activated populations display a strong cytolytic activity against specific target cells (51). Furthermore, it is worth while noting that $CD3^+$ double-negative cytolytic clones have been isolated from lymphocytes infiltrating the joint fluid in juvenile rheumatoid arthritis (52) and from those infiltrating the thyroid tissue in Hashimoto thyroiditis (53, 54), as well as from the cerebrospinal fluid of a patient with subacute sclerosing panencephalitis (55).

It is hoped that the study of the repertoire and usage of the human δ chain V region genes will help shed light on function and specificity of γ/δ-bearing T cells and their contribution to autoimmune disease and immune defense mechanisms.

Summary

We have investigated the diversity and repertoire of human TCR δ chain variable gene segments in the human peripheral blood $CD4^-$ $CD8^-$ (double-negative) population, using rearrangement and expression studies and sequence analyses. 20 TCR δ DNA clones were derived from the RNA of bulk-cultured double-negative T cells and their nucleotide sequences determined. These clones can be classified into six different V_δ subfamilies. The distribution, however, was uneven in these cells, with 16 of 20 being derived from the $V_\delta 1$ (9) and $V_\delta 2$ (7) subfamilies. The remaining subfamilies, $V_\delta 3$, $V_\delta 4$, $V_\delta 5$, and $V\delta 6$, were only represented by one clone each. The majority of these subfamilies seem to consist of a single member, in contrast with the closely linked V_α subfamilies, which, in most cases, consist of multiple members. Our findings suggest that only a limited number of V_δ genes are used in human peripheral blood double-negative T cells and that two major V_δ subfamilies ($V_\delta 1$ and $V_\delta 2$) are used more frequently. Sequence comparison of our cDNA clones to V_α clones indicates that there is no overlap in usage of V_α and V_δ gene segments, except for the $V_\delta 4$ ($V_\alpha 6$) subfamily. Comparison of the different V_δ sequences suggests that the majority of the sequence diversity is concentrated in the junctions between V, D, and J segments and results from extensive N region diversity.

We thank John Cargill and David Ferrick for the excellent assistance on computer, Nicolette Caccia for comments on the manuscript, and Irene Ng for secretarial assistance.

Received for publication 3 October 1988.

References

1. Allison, J. P., B. W. MacIntyre, and D. J. Block. 1982. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. *J. Immunol.* 129:2293.

2. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157:1149.
3. Meuer, S. C., K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function: relationship to the T3 molecular complex. *J. Exp. Med.* 157:705.
4. Samelson, L. E., R. N. Germain, and R. H. Schwartz. 1983. Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. *Proc. Natl. Acad. Sci. USA.* 80:6972.
5. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C. A. Janeway, Jr. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med.* 158:836.
6. Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel. 1986. Identification of a putative second T-cell receptor. *Nature (Lond.).* 322:145.
7. Bank, I., R. A. Depindo, M. B. Brenner, J. Cassimeris, F. W. Alt, and L. Chess. 1986. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature (Lond.).* 322:179.
8. Pardoll, D. M., B. J. Fowlkes, J. A. Bluestone, A. Kruisbeek, W. L. Maloy, J. E. Coligan, and R. H. Schwartz. 1987. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature (Lond.).* 326:79.
9. Bluestone, J. A., D. M. Pardoll, S. O. Schanow, and B. J. Fowlkers. 1987. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature (Lond.).* 326:82.
10. Clevers, H., B. Alarcon, T. Wileman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629.
11. Weiss, A., J. Imborden, K. Harday, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of the T3/antigen receptor complex in T-cell activation. *Annu. Rev. Immunol.* 4:593.
12. Ohashi, P. S., T. W. Mak, P. Van den Elsen, Y. Yanagi, Y. Yoshikai, A. F. Calman, C. Terhorst, J. D. Stobo, and A. Weiss. 1985. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. *Nature (Lond.).* 316:606.
13. Lanier, L. L. and A. Weiss. 1986. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. *Nature (Lond.).* 324:268.
14. Dembić, Z., W. Haas, S. Weiss, J. McCubrey, H. Kiefer, H. Von Boehmer, and M. Steinmetz. 1986. Transfer of specificity by murine α and β T-cell receptor genes. *Nature (Lond.).* 320:232.
15. Saito, T., A. Weiss, J. Miller, M. A. Norcross, and R. N. Germain. 1987. Specific antigen Ia activation of transfected human T cells expressing murine Ti $\alpha\beta$ human T3 receptor complexes. *Nature (Lond.).* 325:125.
16. Koning, F., G. Stinge, W. M. Yokoyama, H. Yamada, W. Lee Maloy, E. Tschacher, E. M. Shevach, and J. E. Coligan. 1987. Identification of a T3-associated $\gamma\delta$ T cell receptor on Thy-1$^+$ dendritic epidermal cell lines. *Science (Wash. DC).* 236:834.
17. Kuziel, W. A., A. Takashima, M. Bonyhadi, P. R. Bergstresser, J. P. Allison, R. E. Tigelaar, and P. W. Tucker. 1987. Regulation of T-cell receptor γ chain RNA expression in murine Thy-1$^+$ dendritic epidermal cells. *Nature (Lond.).* 328:263.
18. Goodman, T., and L. Lefrançois. 1988. Expression of the γ-δ T-cell receptor on intestinal CD8$^+$ intraepithelial lymphocytes. *Nature (Lond.).* 333:855.
19. Chien, Y., M. Iwashima, K. B. Kaplan, J. F. Elliott, and M. M. Davis. 1987. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature (Lond.).* 327:677.
20. Yanagi, Y., Y. Yoshikai, K. Leggett, S. P. Clark, I. Aleksander, and T. W. Mak. 1984.

A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature (Lond.)*. 308:145.
21. Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature (Lond.)*. 308:149.
22. Chien, Y., D. Becker, T. Lindsten, M. Okamura, D. Cohen, and M. M. Davis. 1984. A third type of murine T-cell receptor gene. *Nature (Lond.)*. 312:31.
23. Saito, H., D. M. Kranz, Y. Takagaki, A. Hayday, H. N. Eisen, and S. Tonegawa. 1985. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature (Lond.)*. 309:757.
24. Saito, H., D. M. Kranz, Y. Takagaki, A. Hayday, H. Eisen, and S. Tonegawa. 1984. A third rearranged and expressed gene in a clone of cytolytic T lymphocytes. *Nature (Lond.)*. 312:36.
25. Sim, G. K., J. Yague, J. Nelson, P. Marrack, E. Palmer, A. Augustin, and J. Kappler. 1984. Primary structure of human T-cell receptor α-chain. *Nature (Lond.)*. 312:771.
26. Yanagi, Y., A. Chan, B. Chin, M. Minden, and T. W. Mak. 1985. Analysis of cDNA clones specific for human T cells and the α and β chains of the T-cell receptor heterodimer from a human T-cell line. *Proc. Natl. Acad. Sci. USA*. 82:3430.
27. Hata, S., M. B. Brenner, and M. S. Krangel. 1987. Identification of putative human T cell receptor δ chain complementary DNA clones. *Science (Wash. DC)*. 238:687.
28. Loh, E. Y., L. L. Lanier, C. W. Turck, D. R. Littman, M. M. Davis, Y. Chien, and A. Weiss. 1987. Identification and sequence of a fourth human T cell antigen receptor chain. *Nature (Lond.)*. 330:569.
29. Takihara, Y., E. Champagne, H. Griesser, N. Kimura, D. Tkachuk, J. Reimann, A. Okada, F. W. Alt, L. Chess, M. Minden, and T. W. Mak. 1988. Sequence and organization of the human T cell δ chain gene. *Eur. J. Immunol.* 18:283.
30. Okada, A., I. Bank, L. Rogozinski, Y. Takihara, T. W. Mak, L. Chess, and F. W. Alt. 1988. Structure of the γ/δ T cell receptor of a human thymocyte clone. *J. Exp. Med.* 168:1481.
31. Toyonaga, B., Y. Yoshikai, V. Vadasz, B. Chin, and T. W. Mak. 1985. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. *Proc. Natl. Acad. Sci. USA*. 82:8624.
32. Yoshikai, Y., S. P. Clark, S. Taylor, V. Sohn, B. Wilson, M. Minden, and T. W. Mak. 1985. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. *Nature (Lond.)*. 316:837.
33. Takihara, Y., D. Tkachuk, E. Michalopoulos, E. Champagne, J. Reimann, M. Minden, and T. W. Mak. 1988. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. *Proc. Natl. Acad. Sci. USA*. 85:6097.
34. Malissen, M., K. Minard, S. Mjolsness, M. Kronenberg, J. Goverman, T. Hunkapiller, M. B. Prystowsky, F. Fitch, Y. Yoshikai, T. W. Mak, and L. Hood. 1984. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the β polypeptide. *Cell.* 37:1101.
35. Gascoigne, N., Y. Chien, D. Becker, J. Kavaler, and M. M. Davis. 1984. Genomic organization and sequence of T-cell receptor β-chain constant and joining-region genes. *Nature (Lond.)*. 310:387.
36. Winoto, A., S. Mjolsness, and L. Hood. 1985. Genomic organization of the genes encoding mouse T-cell receptor α-chain. *Nature (Lond.)*. 316:832.
37. Hayday, A. C., D. J. Diamond, G. Tanigawa, J. S. Heilig, V. Folsom, H. Saito, and S. Tonegawa. 1985. Unusual organization and diversity of T-cell receptor α-chain gene. *Nature (Lond.)*. 316:828.
38. Toyonaga, B., and T. W. Mak. 1987. Genes of the T-cell antigen receptor in normal and malignant T cells. *Annu. Rev. Immunol.* 5:585.
39. Chien, Y., M. Iwashima, D. A. Wettstein, K. B. Kaplan, J. F. Elliot, W. Born, and M. M.

Davis. 1987. T-cell receptor δ gene rearrangements in early thymocytes. *Nature (Lond.).* 330:722.

40. Wilson, R. K., E. Lai, P. Concannon, R. K. Barth, and L. E. Hood. 1988. Structure, organization and polymorphism of murine and human T-cell receptor α and β gene families. *Immunol. Rev.* 101:149.
41. Kronenberg, M., G. Siu, L. E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.* 4:528.
42. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature (Lond.).* 334:395.
43. Elliott, J. F., E. P. Rock, P. A. Patten, M. M. Davis, and Y. Chien. 1988. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. *Nature (Lond.).* 331:627.
44. Ferrini, S., C. Bottino, R. Biassoni, A. Poggi, R. P. Sekaly, L. Moretta, and A. Moretta. 1987. Characterization of $CD3^+$, $CD4^-$, $CD8^-$ clones expressing the putative T cell receptor γ gene product. Analysis of the activation pathways leading to interleukin 2 production and triggering of the lytic machinery. *J. Exp. Med.* 166:277.
45. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
46. Gubler, U., and B. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene.* 25:263.
47. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.
48. Yoshikai, Y., N. Kimura, B. Toyonaga, and T. W. Mak. 1986. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. *J. Exp. Med.* 164:90.
49. Guglielmi, P., F. Davi, L. D'Auriol, J. Bories, J. Dausset, and A. Bensussan. 1988. Use of a variable α region to create a functional T-cell receptor δ chain. *Proc. Natl. Acad. Sci. USA.* 85:5634.
50. Matis, L. A., R. Cron, and J. Bluestone. 1987. Major histocompatibility complex-linked specificity of γδ receptor-bearing T lymphocytes. *Nature (Lond.).* 330:262.
51. Ciccone, E., O. Viale, C. Bottino, D. Pende, N. Migone, G. Casorati, G. Tambussi, A. Moretta, and L. Moretta. 1988. Antigen recognition by human T cell receptor γ-positive lymphocytes. Specific lysis of allogeneic cells after activation in mixed lymphocyte culture. *J. Exp. Med.* 167:1517.
52. De Maria, A., M. Malnati, A. Moretta, D. Pende, C. Bottino, G. Casorati, F. Cottafava, G. Melioli, M. C. Mingari, N. Migone, S. Romagnani, and L. Moretta. 1987. $CD3^+$ 4^- 8^- $WT31^-$ (T cell receptor γ^+) cells and other unusual phenotypes are frequently detected among spontaneously interleukin 2-responsive T lymphocytes present in the joint fluid in juvenile rheumatoid arthritis. A clonal analysis. *Eur. J. Immunol.* 17:1815.
53. Bagnasco, M., S. Ferrini, D. Venuti, I. Prigione, G. Torre, R. Biassoni, and G. W. Canonica. 1987. Clonal analysis of T lymphocytes infiltrating the thyroid gland in Hashimoto's throiditis. *Int. Arch. Allergy Appl. Immunol.* 82:141.
54. Del Prete, G. F., E. Maggi, S. Mariotti, A. Tiri, D. Vercelli, P. Parronchi, D. Macchia, A. Pinchera, M. Ricci, and S. Romagnani. 1986. Cytolytic T lymphocytes with natural killer activity thyroid infiltrate of patients with Hashimoto's thyroiditis: analysis at clonal level. *J. Clin. Endocrinol. & Metab.* 62:52.
55. Ang, S., J. G. Seidman, G. M. Peterman, A. D. Duby, D. Benjamin, S. J. Lee, and D. A. Hafler. 1987. Functional γ chain-associated T cell receptors on cerebrospinal fluid-derived natural killer-like T cell clones. *J. Exp. Med.* 165:1453.