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

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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 Vβ 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 Vδ in the mouse genes is also limited (42, 43). An estimation of the size of the Vδ repertoire is hampered by the fact that the δ locus is embedded in the α chain locus between the Vα and Jα genes (32, 38), resulting in no clear demarcation between Vα and Vδ genes. Preliminary data have suggested that the repertoire of human Vδ genes may be even more limited, as several groups have identified the same Vδ 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

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specificity of $\gamma/\delta$ 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 $\gamma/\delta$ 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 ($5 \times 10^4$/flask) were cultured with PHA (0.5%, vol/vol) in the presence of autologous, irradiated, Percoll-enriched peripheral blood monocytes ($2 \times 10^4$/flask). These bulk-cultured cells were then cultured under limiting dilution conditions in the presence of both autologous irradiated feeder cells and exogenous 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-$\alpha/\beta$; 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 $\mu$g/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 $\mu$g/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 $\mu$g), 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 $\lambda$gt 10 using Eco RI linkers. Recombinant phage were packaged using Gigapack cloning kits (Stratagene, San Diego, CA). In total, 2 $\times 10^5$ recombinant $\lambda$gt 10 phage were screened with $^{32}$P-labeled TCR-$\beta$ 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 dideoxy nucleotide sequencing technique and the Sanger-dideoxy chain termination method (47).

Results

Characterization of Peripheral Double-negative T Cell Clones. To examine the $\delta$ 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 $\lambda$s1 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 $\lambda$s2 probe, however, patterns of rearranged bands were the same, suggesting that rearrangements involving the $\lambda$s1 occurred in all three lines. Hybridization of a $\lambda$s1 probe to the bulk-cultured DNA showed both 10.5- and 11-kb predominant bands, indicating that in peripheral double-negative T cells, two
V\_8 gene segments are involved in the majority of J\_81 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 \gamma/\delta-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 \alpha/\beta-bearing leukemic T cell line (Jurkat) showed any TCR-\delta transcription.

Table I

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

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 HI. DNA (10 \mu g/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 J81 probe as described previously (33).
Isolation and Structural Analyses of Human \( V_\delta \) Genes. A complementary DNA library was constructed from total cellular RNA derived from bulk-cultured T cells and screened using a \( ^{32}\)P-labeled \( C_\delta \) probe to give a frequency of 0.1% \( \delta^+ \) cDNA clones. 20 clones were picked up using the \( ^{32}\)P-labeled DNA probe specific for the 5' region of \( C_\delta \) and analyzed in detail. These \( \delta^+ \) cDNA inserts were subcloned into M13mp9 and their nucleotide sequences were determined. All 20 clones contained \( \delta \) 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-D\( \delta_1 \), D\( \delta_2 \), J\( \delta_1 \), J\( \delta_2 \), and J\( \delta_3 \) 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 \( \delta_3 \) gene segments were unique, as shown in Fig. 3. Of these 20 clones, nine cDNA clones were found to use the \( \delta_1 \) gene segment and seven cDNA clones were found to use the \( \delta_2 \) gene segment, indicating that these two \( \delta \) gene segments are used in the majority of peripheral blood double-negative T cells. The nucleotide sequence of \( \delta_1 \) is identical to that of a \( \delta_3 \) gene published previously (27, 28, 30). Of the six \( \delta_2 \) clones, four were long enough to analyze. Two clones (KT19E and KT09E) had a C at position 229 (\( \delta_2 \)), while in KT04E and KT14E, a G was substituted at this position (\( \delta_2' \)), 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 \( \delta_2 \) and \( \delta_2' \). Polymorphism between the alleles may result in this one nucleotide substitution, because Southern blot analysis using the \( \delta_2 \) gene segment as a probe indicated that the \( \delta_2 \) subfamily consists of a single member. Our results indicate that there
TABLE II

Human T Cell Receptor δ Chain Variable Gene Subfamilies and
Frequency 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             |                |                   |
|             | KT01E    | F             |                |                   |
|             | KT12E    | N             |                |                   |
|             | KT18E    | F             |                |                   |
| Vδ2         | KT12A    | F             | 1              | 7:20              |
|             | KT43A    | F             |                |                   |
|             | KT04E    | F             |                |                   |
|             | KT09E    | F             |                |                   |
|             | KT13E    | F             |                |                   |
|             | KT14E    | F             |                |                   |
|             | KT19E    | F             |                |                   |
| Vδ3         | KT04I    | 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, KT04I, 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
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 Vδ gene segments. Sequence analyses of the 20 Vδ gene segments isolated revealed seven distinct sequences. These independent sequences have been aligned to obtain maximum homology to published germline Dδ1 and Dδ2 sequences and grouped on the basis of homology. The 5' untranslated (5'UT), leader (L), variable (V), diversity (D), and constant (C) segments are indicated by the appropriate lettering and potential N-region sequences identified. The germline nucleotide sequences of D regions, (Dδ1 and Dδ2) and J regions (Jδ3 and Jδ1) are shown above and below the cDNA sequences respectively. (b) Junctional diversity of TCR δ cDNA clones containing Vδ1 and Vδ2 sequences. 9 of 20 Vδ cDNAs carried an identical Vδ gene segment designated Vδ1, while a further seven used another Vδ gene segment, Vδ2. 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 the EMBL/GenBank Data Libraries under the accession number Y00793.

Clones

|        | KT10E | KT19E | KT041 | KT06A | KT08A | KT05E |
|--------|-------|-------|-------|-------|-------|-------|
| E      | B     | H     | E     | B     | H     | E     |
|       |       |       |       |       |       |       |

Subfamilies

|        | V81   | V82   | V83   | V84   | V85   | V86   |
|--------|-------|-------|-------|-------|-------|-------|
|        |       |       |       |       |       |       |

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. 3a 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. 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 Southern under stringent conditions, which is of a different size than the band to which KT05E hybridizes, we 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 $\delta$ chain could consist of $2 \times 10^{23}$ different sequences, with almost all diversity provided by junctional flexibility and the N region sequences.

Although the specific function of $\gamma\delta$-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$^-$ $\gamma/\delta$-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 $\delta$ chain V region genes will help shed light on function and specificity of $\gamma/\delta$-bearing T cells and their contribution to autoimmune disease and immune defense mechanisms.

Summary

We have investigated the diversity and repertoire of human TCR $\delta$ chain variable gene segments in the human peripheral blood CD4$^-$ CD8$^-$ (double-negative) population, using rearrangement and expression studies and sequence analyses. 20 TCR $\delta$ 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$\delta$ 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$6, were only represented by one clone each. The majority of these subfamilies seem to consist of a single member, in contrast with the closely linked V$\alpha$ subfamilies, which, in most cases, consist of multiple members. Our findings suggest that only a limited number of V$\delta$ genes are used in human peripheral blood double-negative T cells and that two major V$\delta$ subfamilies (V$\delta$1 and V$\delta$2) are used more frequently. Sequence comparison of our cDNA clones to V$\alpha$ clones indicates that there is no overlap in usage of V$\alpha$ and V$\delta$ gene segments, except for the V$\delta$4 (V$\alpha$6) subfamily. Comparison of the different V$\delta$ 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.

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