A Distinct Wave of Human T Cell Receptor γ/δ Lymphocytes in the Early Fetal Thymus: Evidence for Controlled Gene Rearrangement and Cytokine Production

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Summary

The rearrangement and expression of human T cell receptor (TCR)-γ and -δ gene segments in clonal and polyclonal populations of early fetal and postnatal human TCR-γ/δ thymocytes were examined. The data suggest that the TCR-γ and -δ loci rearrange in an ordered and coordinated fashion. Initial rearrangements at the TCR-δ locus join $V_\delta 2$ to $D_\delta 3$, and initial rearrangements at the TCR-γ locus join downstream V_γ gene segments ($V_\gamma 1.8$ and $V_\gamma 2$) to upstream J_γ gene segments associated with $C_\gamma 1$. These rearrangements are characterized by minimal junctional diversity. At later times there is a switch at the TCR-δ locus such that $V_\delta 1$ is joined to upstream D_δ gene segments, and a switch at the TCR-γ locus such that upstream V_γ gene segments are joined to downstream J_γ gene segments associated with $C_\gamma 2$. These rearrangements are characterized by extensive junctional diversity. Programmed rearrangement explains in part the origin of discrete subpopulations of peripheral blood TCR-γ/δ lymphocytes that have been defined in previous studies. In addition, cytokine production by early fetal and postnatal TCR-γ/δ thymocyte clones was examined. Fetal thymocyte clones produced significant levels of IL-4 and IL-5 following stimulation, whereas postnatal thymocyte clones did not produce these cytokines. Thus, these cell populations may represent functionally distinct subsets as well.

Previous studies of clonal and polyclonal human TCR-γ/δ lymphocytes derived from peripheral blood have revealed the presence of two major subpopulations based upon variable (V) and constant (C) gene segment usage (1–11). The majority of peripheral blood TCR $\gamma\delta$ lymphocytes bear receptors using both $V_\delta 2$ and $V_\gamma 2$ ($V_\gamma 9$ in the nomenclature of Lefranc et al. [12]).[1] The TCR γ and δ chains of these receptors are disulfide-linked, indicating that the TCR γ chains are encoded by $C_\gamma 1$ rather than $C_\gamma 2$ (13, 14). A smaller population of peripheral blood TCR– γ/δ lymphocytes bear receptors using $V_\delta 1$. The majority of these lymphocytes use members of the $V_\gamma 1$ gene family, rather than $V_\gamma 2$, and the TCR γ and δ chains of these receptors are frequently non-disulfide linked, indicating that the TCR γ chains are encoded by the $C_\gamma 2$ gene segment. The explanation for the presence of relatively discrete populations of TCR-γ/δ lymphocytes is unclear.

Analysis of the ontogeny of murine TCR-γ/δ lymphocytes indicates that rearrangements at the TCR-γ and -δ loci occur in an orderly fashion (15–19). The first detectable wave of rearrangements in early fetal thymocytes involves the $V_\gamma 3$[2] and $V_\delta 1$ gene segments. A distinct population of late fetal thymocytes displays rearrangements of the $V_\gamma 4$ and $V_\delta 1$ gene segments. These early rearrangements are also characterized by low junctional diversity, since there is minimal incorporation of template independent N region nucleotides, and only the 3′ D_δ segment ($D_\delta 2$) is used. Murine dendritic epidermal γ/δ cells apparently originate from the early $V_\gamma 3$-$V_\delta 1$ population of fetal thymocytes, and mucosal intraepithelial lymphocytes apparently originate from the subsequent $V_\gamma 4$-$V_\delta 1$ population (20–22). Adult thymocytes display rearrangements

[1] The nomenclature used for human Vγ and Jγ gene segments is that of Strauss et al. (66). The relationship between this scheme and that of Lefranc et al. (12, 67) is described in Ref. 63. The nomenclature for human Vδ gene segments is that of Hata et al. (8) and Takihara et al. (50), and for human Dδ and Jδ gene segments is that of Loh et al. (47).

[2] The nomenclature for murine Vγ gene segments is that of Garman et al. (15), and the relationship between this scheme and those of others is described in Ref. 69.

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involving distinct V_γ and V_δ gene segments, and display extensive junctional diversity (23, 24).

Based upon indirect evidence it has previously been suggested that rearrangements within the human TCR-γ locus may be ordered (25). It was proposed that the initial rearrangements at this locus join the downstream V_γ gene segments (i.e., $V_\gamma 2$, $V_\gamma 3$, $V_\gamma 4$) to the upstream J_γ gene segments (i.e., those associated with $C_\gamma 1$: $J_\gamma 1.1$, $J_\gamma 1.2$, $J_\gamma 1.3$), whereas subsequent rearrangements join the upstream V_γ gene segments (i.e., members of the $V_\gamma 1$ family) to the downstream J_γ gene segments (i.e., those associated with $C_\gamma 2$: $J_\gamma 2.1$, $J_\gamma 2.3$). To date, this has not been confirmed by the direct analysis of gene rearrangements in early human fetal thymocytes. Further, no information is available concerning the sequence of rearrangements at the human TCR-δ locus.

In this manuscript we have analyzed TCR-γ and TCR-δ gene rearrangement and expression in clonal and polyclonal early fetal and postnatal human TCR-γ/δ thymocytes. We show directly that early TCR-γ rearrangements join downstream V segments to upstream J segments, that these rearrangements occur coordinately with an early wave of $V_\delta 2$ rearrangements at the TCR-δ locus, and that these early TCR-γ and -δ gene rearrangements display limited junctional diversity.

Since our analysis showed the TCRs expressed by early fetal TCR-γ/δ thymocytes to be distinct from those of postnatal TCR-γ/δ thymocytes, we asked whether these cell populations represent functionally distinct subsets as well. We found that fetal TCR-γ/δ thymocyte clones were able to secrete significant levels of IL-4 and IL-5, whereas postnatal clones did not secrete these cytokines. Both sets of clones produce high levels of granulocyte/macrophage CSF (GM-CSF)[3] and IFN-γ.

Materials and Methods

Establishment of TCR-γ/δ Thymocyte Clones. Postnatal thymuses were obtained from children undergoing corrective heart surgery and were kindly provided by Dr. D. Regal, Centra de Transfusion Sanguine, Lyon, France. Fetal thymic material was obtained from J. L. Touraine, Hopital Eduard Herriot, Lyon, France. Thymocyte suspensions were prepared by gently teasing thymus tissue into a single cell suspension. An entire lobule was used to ensure that both cortical and medullary cells were represented. The fetal thymocyte samples and the samples from child thymus OM were cultured in a mixture of 20 U/ml IL-2 and 100 U/ml IL-4 for 10 d. This procedure increased the number of TCR-γ/δ cells from <1% to 10% (OM), 5% (Solo), 6% (BB), and 7% (CC). The cells were then stained with anti-TCR-δ1, which recognizes an epitope on the TCR δ chain, and were washed and incubated with goat anti-mouse-labeled human red blood cells. The TCR-γ/δ^+ cells were enriched as described previously (26). TCR-γ/δ cells of individual FH (4 mo old) were isolated from a fresh thymocyte sample using a combination of magnetic bead and FACS sorting as follows: thymocytes were incubated with anti-CD4 (RIV6; kindly provided by Dr. Kreeftenberg, RIV, Bilthoven, Netherlands) and anti-CD8 (WT-82; a kind gift from Dr. W. Tax, University of Nijmegen, Netherlands) mAbs for 30 min at 4°C, and were washed and then incubated with biotinylated goat anti-mouse antibody (Tago, Inc., Burlingame, CA) for 30 min. After washing, the cells were incubated for 5 min with FITC-labeled avidin (Becton Dickinson & Co., Mountain View, CA), and subsequently with biotinylated magnetic beads. $CD4^+$ $CD8^+$ cells were then removed on a MACS (27). The remaining FITC-labeled cells were then removed by sorting on a FACStar plus (Becton Dickinson). 50% of the depleted cell population stained with PE-labeled anti-CD3 (PE-Leu 4, Becton Dickinson) and <0.5% of the cells were $CD4^+$ $CD8^+$ as indicated by reanalysis of the sorted cells. The $CD4^-$ $CD8^-$ FH cells and the TCR-γ/δ enriched cultures of Solo, BB, CC, and OM were cloned by limiting dilution at 0.3 cell per well in a 96-well plate (Linbro, Flow Laboratories, McLean, VA) in the presence of a feeder cell mixture consisting of 5×10^5/ml irradiated (4,000 rad) PBL, 5×10^4/ml irradiated (5,000 rad) cells of the EBV-transformed B cell line JY, and 0.1 μg/ml purified PHA (Wellcome Diagnostics, Dartford, England). Yssel's medium (28) was used for all cell cultures. The clones were expanded by weekly stimulations with the feeder cell mixture. 3 d after each stimulation, the cultures were split and 20 U/ml rIL-2 was added. The feeder cells deteriorated between day 2 and 4 and at day 5 or 6 no feeder cells were visible or detectable with mAbs specific for B cells, monocytes, or TCR-α/β (data not shown). For molecular analysis of V_γ and V_δ usage, bulk cultures of the enriched BB and CC cells were maintained by weekly stimulations with feeder cells and PHA.

Monoclonal Antibodies and Immunofluorescence. The anti-TCR-γ/δ mAb TCR-δ1 (29) was a kind gift of Dr. M. Brenner (Dana Farber Cancer Institute, Boston, MA); δTCS1 (30) was purchased from T Cell Sciences Inc. (Boston, MA); and the mAbs BB3 (31) and TiγA (32) and were gifts from Drs. L. Moretta (Genova, Italy) and T. Hercend (Institute Gustave Roussy, Paris, France), respectively. Characterization of the TCR-γ/δ T cell clones was carried out as described previously (28). Two hundred thousand cells were incubated with mAb for 30 min at 4°C, washed, and then incubated with FITC labelled goat anti-mouse $F(ab)_2$ fragments (Tago Inc., Burlingame, CA) for 30 min at 4°C. Cells were washed and analyzed on a FACScan.

DNA Fragments and Oligonucleotides. The 5'D_δ probe is a 1.3-kb EcoRI-Bam HI fragment mapping 5' of $D_\delta 1$ that was isolated from the genomic clone BSKδ1 (a gift of S. Ang, Harvard Medical School, Cambridge, MA). All other DNA probes have been described previously (8, 33–35). Oligonucleotides used for the PCR were: $V_\delta 1$, 5'GGGGTCGACAAGTTGGTGGTCATATTA; $V_\delta 2$, 5'GGGGTCGACCCTCAGGTGCTCCATGAA; $V_\delta 3$, 5'GGGGTCGACACTGTATATTCAAATC; $V_\alpha 17$ (=$V_\delta 5$), 5'GGGGTCGACTATACTAACAGCATGTT; $V_\gamma 1$, 5'TACATCCACTGGTACCTACACCAGGA; $V_\gamma 2$, 5'GGGGTCGACCTGGTGAAGTCATACAGT; $J_\delta 1$, 5'GGGGAATTCCACAGTCACACGGGTTC; $J_\delta 3$, 5'GGGGAATTCCACGAAGAGTTTGATGCC; $J_\gamma 1.1$, 5'GGGGGATCCAGTTACTATGAGCTTAGTCC; $J_\gamma 1.3$, 5'GGGGGATCCTGTGACAACAAGTGTTGT.

Preparation and Blot Hybridization of Genomic DNA and RNA. Genomic DNA and RNA were prepared by established procedures (36, 37). Gel electrophoresis, blotting, hybridization with ^{32}P-labeled probes, and washing were as described previously (8).

Polymerase Chain Reaction. Genomic DNA (0.5 μg) was heated to 94°C for 7 min and was amplified under mineral oil for 35 cycles in a 25 μl reaction containing 0.2 mM each deoxyribonucleotide triphosphate, 50 mM KCl, 1.25 mM $MgCl_2$, 0.01% gelatin, 100 mM Tris-HCl (pH 8.3), 1 U Taq polymerase, and 20 pmol each oligonucleotide (38). Each cycle consisted of a 0.8 min denatura-

[3] *Abbreviation used in this paper:* GM-CSF, granulocyte/macrophage CSF.

tion step at 94°C, a 1.0-min annealing step at 51°C, and a 2.0-min. extension step at 72°C. The extension step following the last cycle was for 9.9 min. One-fifth of each reaction was analyzed by agarose gel electrophoresis. The remainder was extracted with chloroform, phenol/chloroform (1:1), and chloroform, and was ethanol precipitated. Pelleted DNA was resuspended, digested using appropriate restriction enzymes, and purified through low gelling temperature agarose. Fragments were then cloned into appropriately digested vectors prepared from Bluescript KS+ (Stratagene, La Jolla, CA), and plasmid minipreps were prepared. When DNA from a polyclonal cell source was amplified, secondary transformants and minipreps were prepared in order to obtain pure clones. Nucleotide sequences were determined on both strands by the dideoxy chain termination method using double-stranded templates (39) and modified T7 polymerase (40) (Sequenase; U.S. Biomedical Corp., Cleveland, OH).

Stimulation of T Cell Clones and Assays for Cytokine Production. Cells were stimulated on day 7 after the last stimulation with feeder cells, and were collected, spun, and then washed two times with medium. The cells were stimulated at a concentration of 10^6 cells/ml per well of a 24-well plate (Linbro). 24 h later the supernatant was removed, spun at 250 *g*, frozen, and kept at −20°C until testing. IL-4 (41), IL-5 (42), IFN-γ (43), and GM-CSF (42) were determined by ELISA as described. IL-2 levels were determined using the IL-2–dependent cell line CTLL-2 as described (44).

Results

Analysis of Cell Surface Expression of TCR-γ/δ on Thymocyte Clones. Thymocytes of three different fetal samples were obtained: Solo (8.5 wk of gestational age), BB (12 wk), and CC (15 wk). Postnatal thymus samples were obtained from two children: OM (6 mo old) and FH (4 mo old). The procedures to obtain the Solo, BB, CC, and OM clones were similar. Thymocytes were cultured in IL-2 and IL-4 for 10 d, and the $CD4^-$ $CD8^-$ cells were enriched by reversed rosetting and cloned by limiting dilution. The FH clones were obtained from freshly isolated purified $CD3^+$ $CD4^-$ $CD8^-$ cells as indicated in Materials and Methods. To begin to investigate the V_δ and V_γ usage of these clones, the cells were incubated with mAbs that recognize epitopes encoded by different V_γ and V_δ gene segments. 9 of 15 postnatal thymocyte clones from sample FH reacted with the δTCS1 mAb (Table 1), indicating that these clones expressed $V_\delta1$-$J_\delta1$ determinants (10, 45). None reacted with mAb BB3, which detects a $V_\delta2$ determinant (4, 9, 46). Most of the $V_\delta1^+$ clones were negative with the TiγA mAb, which detects a $V_\gamma2$ determinant (1), although two of the nine $V_\delta1^+$ clones did react with this antibody (Table 1). One of the FH clones (FH3) reacted with TiγA but with neither δTCS1 nor BB3, and the remaining FH clones were negative with all of these mAbs. Of the four OM clones, two (OM35 and OM64) reacted with the δTCS1 mAb, and one (OM35) reacted with the TiγA mAb. These observations confirm the findings of others that a large fraction of postnatal TCR-γ/δ thymocytes are $V_\delta1^+$, that some of these cells coexpress $V_\delta1$ and $V_\gamma2$, and that very few postnatal TCR-γ/δ thymocytes are $V_\delta2^+$ (5, 6, 10, 47).

In contrast to these results with postnatal thymocytes, all of the Solo, BB, and CC fetal thymus clones reacted with the BB3 mAb, and none reacted with δTCS1 (Table 1). Most of these were also $TiγA^+$, indicating coexpression of $V_\delta2$ and $V_\gamma2$, although two of the three Solo clones (Solo 6 and 15) were $TiγA^-$. In addition, at early stages in the expansion of the polyclonal BB and CC cultures a small fraction of the cells were $BB3^+$ and $TiγA^-$ (data not shown), although all of the clones obtained were $BB3^+$ $TiγA^+$. These data suggest that $V_\delta2$ is the predominant V_δ gene segment used in early fetal thymocytes, and that although $V_\gamma2$ is also

Table 1. *Reactivity of Fetal and Postnatal TCR-γ/δ Clones with mAbs Specific for Epitopes on the Constant and Variable Regions of the γ and δ Chains*

Donor	(Gestational) Age	Clones	Reactivity with mAbs			
			TCR-δ1	δTCS1	BB3	TiγA
Solo	(8.5 wk)	6,15	+	−	+	−
		19	+	−	+	+
BB	(12 wk)	2,4,5,8,10	+	−	+	+
CC	(15 wk)	6,7,15,27,33,40	+	−	+	+
FH	4 mo	1,6,9,20,26	+	−	−	−
		7,9,10,13,14,17,28	+	+	−	−
		16,31	+	+	−	+
		3	+	−	−	+
OM	6 mo	47,54	+	−	−	−
		64	+	+	−	−
		35	+	+	−	+

used with high frequency, $V_\delta 2$ usage is not strictly linked to $V_\gamma 2$ usage within this cell population, despite coordinate usage in peripheral blood (4, 8–10).

TCR-γ and -δ Rearrangements in Postnatal Thymocytes. To extend the above findings, we first analyzed the TCR-γ and TCR-δ gene rearrangements and transcripts in the OM postnatal thymocyte clones. By hybridization with a $J_\gamma 1.3/2.3$ probe in KpnI and EcoRI digests of genomic DNA, and with $V_\gamma 2$ and $V_\gamma 3$ probes in KpnI digests, we identified two rearrangements in each T cell clone. Some rearrangements were further characterized by sequence analysis of PCR products amplified from genomic DNA using V and J specific oligonucleotide primers. We found the array of rearranged V_γ gene segments to be heterogeneous, including multiple members of the $V_\gamma 1$ family, as well as $V_\gamma 2$ and $V_\gamma 3$ (Table 2). Based on these results and on surface staining with the TiγA mAb (Table 1), OM35 carries an in-frame $V_\gamma 2$ rearrangement, and OM54 and OM64 carry in-frame $V_\gamma 1.4$ and $V_\gamma 1.8$ rearrangements, respectively. OM47 displays predominantly $V_\gamma 1$ transcripts, and therefore is likely to carry an in-frame $V_\gamma 1.3$ rearrangement. Despite the observed heterogeneity in the rearranged V gene segments, it is striking that seven of the eight rearrangements in these cells involve J_γ segments upstream of $C_\gamma 2$, namely, $J_\gamma 2.1$ and $J_\gamma 2.3$.

Assignment of the TCR-δ gene rearrangements in these cells was accomplished by hybridization of $J_\delta 1$, $J_\delta 3$, $V_\delta 1$, $V_\delta 2$, $5'D_\delta$, and $V_\alpha 17$ probes to XbaI digests of genomic DNA. The $V_\alpha 17$ probe was included because this V segment has been previously mapped to the genomic region between the $V_\delta 1$ and $V_\delta 2$ gene segments (35). We identified $V_\delta 1$ rearrangements in OM35 and OM64, and a $V_\delta 3$ rearrangement in OM54. Based upon nucleotide sequence anal-

Table 2. *TCR-δ and -γ Gene Rearrangements in Fetal and Postnatal Thymocyte Clones*

		Southern							
		XbaI digest							
Cell line	Northern	$J_\delta 1$	$J_\delta 3$	$V_\delta 1$	$V_\alpha 17$	$V_\delta 2$	$5'D_\delta$	Rearrangements	
Solo 6	ND	–	4.4, 3.4	ND	ND	R,G	D,G	$V_\delta 2$ –$J_\delta 3$*	D–D–J_δ
Solo 15	ND	4.2	5.4, 3.4	ND	ND	R,R	D,D	$V_\delta 2$ –$J_\delta 1$*	$V_\delta 2$ –$J_\delta 3$*
Solo 19	$V_\delta 2$	1.7	5.4, 3.4	G,G	G,G	R,G	D,G	$V_\delta 2$ –$J_\delta 3$*	Germline
OM35	$V_\delta 1$	6.4	5.4	R,R	D,D	D,D	D,D	$V_\delta 1$ –$J_\delta 1$*	$V_\delta 1$ –$J_\delta 1$*
OM47	$V_\alpha 17$	2.9	5.4, 7.5	G,G	R,G	D,G	D,G	$V_\alpha 17$–$J_\delta 3$*	D–D–$J_\delta 1$
OM54	$V_\delta 3$	3.9, 1.9	5.4	G,G	G,G	D,G	D,G	$V_\delta 3$ –$J_\delta 1$*	–$J_\delta 1$
OM64	$V_\delta 1$	6.4, 3.2	5.4	R,G	D,G	D,G	D,D	$V_\delta 1$ –$J_\delta 1$*	–$J_\delta 1$

		Southern					
		KpnI digest			EcoRI digest		
		$J_\gamma 1.3/2.3$	$V_\gamma 2$	$V_\gamma 3$	$J_\gamma 1.3/2.3$		
Solo 6	ND	16, 14, 8.5	R,D	D,D	3.2, 1.5	$V_\gamma 1.8$–$J_\gamma 1.1$*	$V_\gamma 2$ –$J_\gamma 1.1$*
Solo 15	ND	16, 8.5	D,D	D,D	3.2, 1.5	$V_\gamma 1.8$–$J_\gamma 1.1$*	$V_\gamma 1.8$–$J_\gamma 1.1$*
Solo 19	$V_\gamma 2$	16, 9.0, 7.9	R,G	D,G	3.2, 2.3, 1.5	$V_\gamma 2$ –$J_\gamma 1.3$*	Germline
OM35	$V_\gamma 2$	7.6, 7.2	R,R	D,D	2.3, 1.8	$V_\gamma 2$ –$J_\gamma 2.3$	$V_\gamma 2$ –$J_\gamma 2.3$
OM47	$V_\gamma 1$	16, 5.0, 1.8	D,G	R,D	5.0, 3.2	$V_\gamma 1.3$–$J_\gamma 1.3$	$V_\gamma 3$ –$J_\gamma 2.1$
OM54	$V_\gamma 1$	7.6, 1.8	R,D	D,D	2.3, 0.9	$V_\gamma 1.4$–$J_\gamma 2.3$*	$V_\gamma 2$ –$J_\gamma 2.3$
OM64	$V_\gamma 1$	5.0, 1.8	D,D	D,D	4.0, 3.2	$V_\gamma 1.8$–$J_\gamma 2.3$	$V_\gamma 1.8$–$J_\gamma 2.1$*

Assignment of TCR-δ gene rearrangements is according to reference 8, and assignment of TCR-γ gene rearrangements is according to references 66 and 67. Known or presumed in-frame rearrangements are in the first column, except for Solo 15, which displays two in-frame rearrangements. Assignments marked by an asterisk were analyzed and confirmed by PCR and nucleotide sequence analysis. The second $J_\delta 1$ rearrangements in OM54 and OM64 are undefined. Sizes of the fragments detected by $J_\delta 1$, $J_\delta 3$, and $J_\gamma 1.3/2.3$ probes are in kilobases. Fragments detected by other probes are either G, germline; R, rearranged; D, deleted. Germline fragments are: $J_\delta 1$, 1.7 kb XbaI; $J_\delta 3$, 5.4 kb XbaI: $J_\gamma 1.3$, 9.0 kb KpnI and 1.5 kb EcoRI; $J_\gamma 2.3$, 16 kb KpnI and 3.2 kb EcoRI.

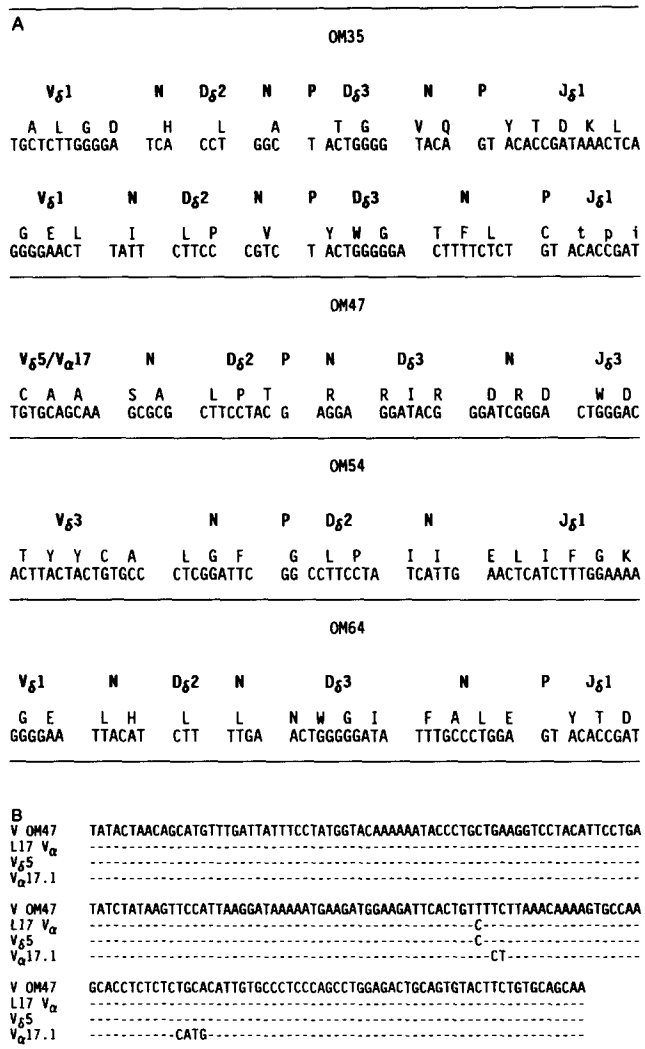

Figure 1. Nucleotide sequence analysis of TCR-δ gene rearrangements in postnatal thymocyte clones. (*A*) Junctional sequences were determined from PCR products amplified using appropriate pairs of primers. D elements were assigned with the requirement for a minimum of three contiguous matches to the germline sequence. P nucleotides were assigned according to reference 19. Deduced amino acid sequences are in the single letter code; a shift to lowercase letters indicates a shift to an inappropriate reading frame. (*B*) The nucleotide sequence of the amplified portion of the OM47 V segment is compared with the analogous portions of three previously reported V sequences: L17 V_α (48), $V_\delta 5$ (50), and $V_\alpha 17.1$ (49). The V-OM47 sequence was confirmed using multiple DNA templates. Differences between this sequence and the published sequences are noted.

ysis of V-D-J junctional regions amplified by PCR, one of the two $V_\delta 1$ rearrangements in OM35, as well as the $V_\delta 3$ and $V_\delta 1$ rearrangements in OM54 and OM64, respectively, are in-frame (Fig. 1 *A*).

Analysis of both Northern and Southern blots using the $V_\alpha 17$ probe revealed the rearrangement of $V_\alpha 17$ or a highly homologous V segment to $J_\delta 3$ in OM47. The nucleotide sequence of the V-D-J junction of this rearrangement following PCR amplification (Fig. 1 *A*) demonstrated it to be in-frame. The sequence of a portion of the rearranged V segment is compared with three other closely related sequences in Fig. 1 *B*. The V-OM47 sequence differs from the previously described L17 V_α (48) cDNA sequence by a single nucleotide, and from the previously described $V_\alpha 17.1$ (49) sequence by six nucleotides. Furthermore, a recently identified V_δ segment denoted $V_\delta 5$ (50) also differs from the V-OM47 sequence by a single nucleotide, and is identical to L17 V_α. Since an L17 V_α probe detected only a single hybridizing sequence on Southern blots (data not shown), the different sequences must represent polymorphic variants of the same V gene segment. Thus, this V segment, which maps to the proximal portion of the human TCR-α/δ locus, can rearrange to both D_δ and J_α segments, and thereby serves as both a V_δ and a V_α.

The analysis of a truncated cDNA clone representing the 3.9-kb $J_\delta 1$ rearrangement in OM54 revealed an out-of-frame rearrangement involving a previously undescribed V segment that maps between $V_\alpha 17$ (=$V_\delta 5$) and $V_\delta 2$ (data not shown). The 3.2-kb $J_\delta 1$ rearrangement in OM64 was not characterized.

TCR-γ and -δ Gene Rearrangements in Early Fetal Thymocytes. We next analyzed the TCR-γ and -δ gene rearrangements in the 8.5-wk fetal thymus clones Solo 6, 15, and 19, and found them to be strikingly different from those observed in the postnatal thymocyte clones (Table 2). Southern blots revealed the rearrangement of both $V_\gamma 2$ and $V_\gamma 1$ gene segments. Notably, all five TCR-γ rearrangements were found to involve the $J_\gamma 1$, rather than the $J_\gamma 2$ cluster, whereas one chromosome was in the germline configuration. To identify the rearranged $V_\gamma 1$ gene segments, each V-J rearrangement was amplified by PCR using an oligonucleotide that anneals to all $V_\gamma 1$ sequences as a 5′ primer, and a $J_\gamma 1.1$ oligonucleotide as a 3′ primer. Nucleotide sequence analysis indicated the rearrangement of $V_\gamma 1.8$ in every instance and showed that Solo 6 and Solo 15 each carry a single in-frame $V_\gamma 1.8$ rearrangement (Fig. 2). Similar PCR analysis revealed the $V_\gamma 2$ rearrangement in Solo 19 to be in-frame, and that in Solo 6 to be out-of-frame (Fig. 2), in accordance with staining results (Table 1).

Together with the staining analysis of the Solo, BB, and CC clonal and polyclonal lines (Table 1), the above results indicate that whereas $V_\gamma 2$ rearrangements are common in early fetal thymocytes, $V_\gamma 1$ rearrangements occur as well. Further, these results suggested that the rearrangement of $V_\gamma 1$ gene segments in early fetal thymocytes might not be random. To specifically address this issue, we analyzed DNA prepared from polyclonal BB and CC cultures by PCR using $V_\gamma 1$ and $J_\gamma 1.1$ primers. Six of six clones sequenced that were obtained following amplification of BB DNA used $V_\gamma 1.8$ (Fig. 3). Two of four clones obtained following amplification of CC DNA used $V_\gamma 1.8$, whereas one clone each used $V_\gamma 1.3$ and $V_\gamma 1.4$. All clones were independent based upon their V-J junction nucleotide sequences. Further, 8 of 10 were out-of-frame, in accordance with staining data indicating the BB and CC cultures to be composed primarily of TiγA^+ TCR-γ/δ lymphocytes and TCR-α/β lymphocytes. These results provide strong evidence that Vγ1 rearrangements in early fetal thymocytes involve $V_\gamma 1.8$ almost exclusively, at least

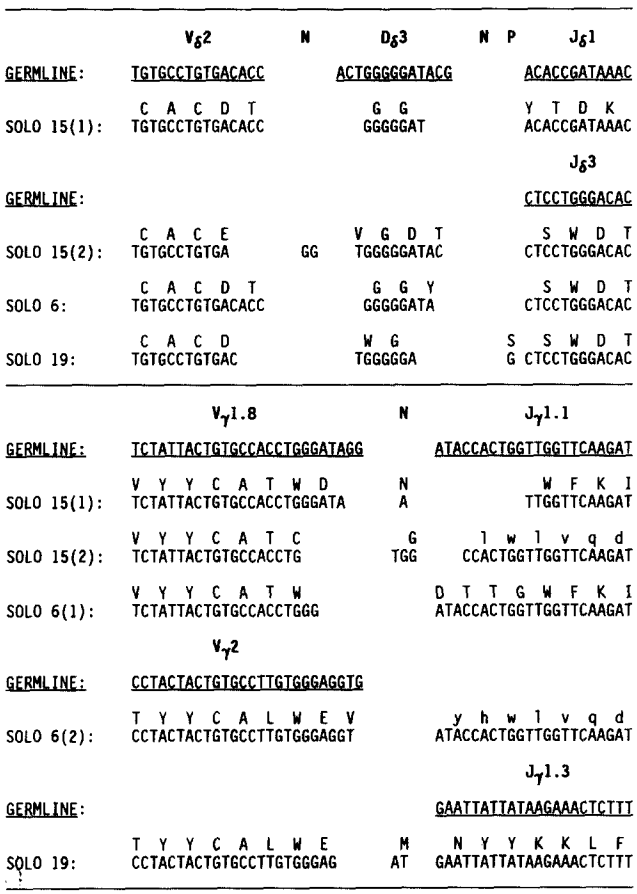

Figure 2. Nucleotide sequence analysis of TCR-δ TCR-γ gene rearrangements in fetal thymocyte clones. Junctional sequences were determined from PCR products amplified using appropriate pairs of primers. They are compared with the relevant germline gene segment sequences (*underlined*). All sequences were confirmed using multiple DNA templates. The V_γ segments involved in all three rearrangements amplified using $V_\gamma1$ and $J_\gamma1.1$ primers could be assigned as $V_\gamma1.8$ based on a comparison of the sequences determined 5′ of the junctional region with those reported in reference 12 (see for example Fig. 3).

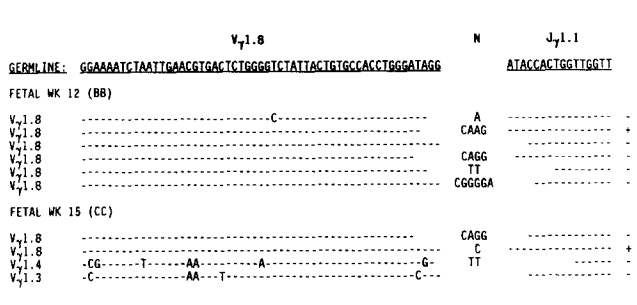

Figure 3. Nucleotide sequence analysis of $V_\gamma1$ rearrangements in polyclonal 12- and 15-wk fetal thymocytes. Junctional sequences were determined from PCR products amplified using $V_\gamma1$ and $J_\gamma1.1$ primers. They are compared with the germline $V_\gamma1.8$ and $J_\gamma1.1$ sequences (12, 67), with differences noted. V_γ assignments along the left border of the figure are based upon comparison to the reported $V_\gamma1.1$-$V_\gamma1.8$ sequences (12). The single nucleotide substitution in one putative $V_\gamma1.8$ sequence is most likely a mutation introduced during PCR amplification because this substitution is not found in any known $V_\gamma1$ sequence. Sequences are classified as either in-frame (+) or out-of-frame (−) along the right border.

through fetal week 12. This result is striking since $V_\gamma1.8$ lies at the end of the $V_\gamma1$ cluster that is proximal to $V_\gamma2$.

Analysis of TCR-δ rearrangements in the Solo clones revealed the $V_\delta2$ gene segment to be the only V_δ segment rearranged in these cells (Table 2). Solo 15 displays two $V_\delta2$ rearrangements and Solo 19 displays a $V_\delta2$ rearrangement on one chromosome and retains the germline configuration on the other chromosome. Solo 6 displays one $V_\delta2$ rearrangement and on the other chromosome displays a previously uncharacterized 4.4-kb rearrangement detected by the $J_\delta3$ probe in an XbaI digest. We conclude that this represents a partial rearrangement (D-J or D-D-J) involving $J_\delta2$ for the following reasons: First, the genomic region hybridizing with the 5′D_δ probe has been retained on one chromosome, arguing that on this chromosome rearrangement has not proceeded 5′ of $D_\delta1$. Second, the intensity of hybridization of the rearranged fragment is equivalent to germline as assessed with a $J_\delta3$ probe; since the probe straddles $J_\delta3$, hybridization is significantly diminished upon rearrangement of $J_\delta3$, but not $J_\delta2$. Finally, although the $V_\delta3$ gene segment maps 3′ of C_δ and would not delete the region hybridizing to the 5′D_δ probe upon rearrangement, the predicted size of a $V_\delta3$-$J_\delta2$ rearranged fragment is only 3.3 kb.

The V-D-J junctional regions of the $V_\delta2$ rearrangements in these cell lines were amplified by PCR and their nucleotide sequences were determined (Fig. 2). Notably, all four rearrangements were found to be functional. Solo 15 therefore displays two in-frame rearrangements, and possibly two γ/δ TCRs on its cell surface. This apparent violation of allelic exclusion could have arisen if the two rearrangements occurred nearly simultaneously. However, it is intriguing that other reports have suggested that allelic exclusion at the TCR α locus may not be complete (51–53). Nevertheless, these data, along with the results of surface staining (Table 1), argue that $V_\delta2$ rearrangements predominate in early fetal thymocytes, even though, as demonstrated in this and in other studies, they are rare in postnatal thymocytes (5, 6, 10, 47).

Minimal Diversity of Early Fetal Thymocyte TCR-γ and -δ V-(D)-J Junctions. A striking feature of the TCR-γ and -δ junctional sequences presented in Fig. 2 is the limited diversity they display relative to the junctions of rearrangements in postnatal thymocytes (Fig. 1 *A*) (47) and peripheral blood lymphocytes (7, 8, 34, 47). Two of the five TCR γ V-J junctions analyzed display no incorporation of template independent N-region nucleotides, and a total of only six N-region nucleotides are seen in the five junctions analyzed. The TCR-δ V-D-J junctions are even more striking. They reveal only two N-region nucleotides among the four sequences, with three of the junctions displaying no N-nucleotide incorporation. Further, the only D_δ segment used is $D_\delta3$. Hence, these V-D-J junctional sequences bear marked similarities to those described for TCR-δ gene rearrangements in very early murine fetal thymocytes: the use of a single V gene segment, the use of only the 3′ D gene segment, and minimal N nucleotide incorporation (16–19).

To confirm and extend these findings, we analyzed the junctions of $V_\delta2$ rearrangements in the polyclonal BB and CC fetal thymocyte cultures. Southern blot analysis of XbaI

digested BB and CC genomic DNA using a radiolabeled $V_\delta 2$ probe revealed that in both populations $V_\delta 2$ rearrangements to $J_\delta 3$ predominated over those to $J_\delta 1$ (data not shown). We then used PCR to amplify both types of junctions in each sample. The analysis of 13 junctions of 12-wk fetal thymocytes and 17 junctions of 15-wk fetal thymocytes is presented in Fig. 4. All 30 junctions display portions of the $D_\delta 3$ gene segment. By contrast, only three display possible contributions from $D_\delta 1$, and two from $D_\delta 2$. However, since in these instances the maximal contribution is only three nucleotides, apparent $D_\delta 1$ and $D_\delta 2$ contributions could have arisen by chance. We conclude that $D_\delta 1$ and $D_\delta 2$ rearrangements are rare events in early fetal thymocytes.

These junctions also display minimal incorporation of N-region nucleotides. Roughly one-third of the 12-wk junctions, and one-half of the 15-wk junctions, display no N nucleotides. The average number of N-nucleotides per junction increases from 0.5 at 9 wk to 2.4 at 12 wk and 2.7 at 15 wk. When only N-nucleotide positive junctions are considered, these numbers increase to 2.0 at 9 wk, 3.7 at 12 wk, and 5.4 at 15 wk. By contrast, the sequences of five different

V_δ	P	N	$D_\delta 1$	N	P	$D_\delta 2$	P	N	P	$D_\delta 3$	P	N	P	J_δ	
GERMLINE:															
1 GGAACT		GAAATAGT				CCTTCCTAC				ACTGGGGGATACG				ACACCGATAA	1
2 GACACC														CTCCTGGGAC	3
3 CTTTAG															
5 CAGCAA															
FETAL WK 8.5 (SOLO)															
2 GACACC										GGGGGAT				ACACCGATAA	1 +
2 GACACC										GGGGGATA				CTCCTGGGAC	3 +
2 GA				GG						TGGGGGATAC				CTCCTGGGAC	3 +
2 GAC										TGGGGGA			G	CTCCTGGGAC	3 +
FETAL WK 12 (BB)															
2 GAC										ACTGGGGGATACG				CCGATAA	1 +
2 G				TTGA						ACTGGGGGATAC				CGATAA	1 +
2 GACACC	G			TC						GGGGGAT		CGGTTG	G	CTCCTGGGAC	3 +
2 GACACC										TGGGGGA			G	CTCCTGGGAC	3 +
2 GACACC										TGGGGGATA				CCTGGGAC	3 -
2 GACACC	G			CGCA						GGGATAC			AG	CTCCTGGGAC	3 +
2 G						CCT	GTT	T	ACTGGGGGATA				G	CTCCTGGGAC	3 +
2 GACACC	G					CTA				GGGGATACG	CG		AG	CTCCTGGGAC	3 +
2 GAC	G	TAG		CT						GGGAT		TTT		CTCCTGGGAC	3 +
2 GACACC	G			C						CTGGGGGATACG				CTCCTGGGAC	3 +
2 GACA									T	ACTGGGGGATACG		ACT		TGGGAC	3 +
2 GACACC				T						GGGATACG		TA		CTGGGAC	3 +
2 GA									T	ACTGGGGGATACG		G		CTGGGAC	3 +
1 GGAA										GGGGGATA				CTCCTGGGAC	3 +
FETAL WK 15 (CC)															
2 GACAC		AAT							T	ACTGGGGGAT				ACACCGATAA	1 +
2 GAC										TGGGGGAT				ATAA	1 -
2 GACAC										TGGGGGAT				ACACCGATAA	1 +
2 GACAC				GGC						GGGATA				CGATAA	1 +
2 GAC				GCGCC					T	ACTGGGGGATACG	CG	AAAGGGG	G	CTCCTGGGAC	3 +
2 GACACC										GGGG		TC	AG	CTCCTGGGAC	3 +
2 GACACC	G			CA						GGGGG				CTCCTGGGAC	3 +
2 GACACC										ACTGGGGGATA				CTCCTGGGAC	3 +
2 GACACC										TGGGGG		TC		CTCCTGGGAC	3 +
2 GACA		AAA							T	ACTGGGGGATAC				CTGGGAC	3 +
2 GACAC										TGGGGGATA				CTGGGAC	3 +
2 GACACC	G			TAAG						TGGGGGATACG	C	TC		CCTGGGAC	3 +
2 GACACC				GA						GGGG		TCACGGTC		CCTGGGAC	3 -
2 GAC				CGG					GT	ACTGGGGGAT		CGG	AG	CTCCTGGGAC	3 +
2 GACACC										GGGGGATA				CTCCTGGGAC	3 +
2 GACA				GA					GT	ACTGGG		ACAA	AG	CTCCTGGGAC	3 +
2 GACACC	G									CTGGGGGATA				CTCCTGGGAC	3 +
1 GGAA									T	ACTGGGGGATACG				CTCCTGGGAC	3 +
POSTNATAL (OM)															
1 GGAACT				TATT		CTTCC		GTC	T	ACTGGGGGA		CTTTTCTCT	GT	ACACCGATAA	1 -
1 GGA				TCA		CCT		GGC	T	ACTGGGG		TACA	GT	ACACCGATAA	1 +
1 GGAA				TTACAT		CTT		TTGA		ACTGGGGGATA		TTTGCCCTGGA	GT	ACACCGATAA	1 +
3 C				CTCGGATT	GG	CCTTCCTA						TCATTG		A	1 +
5 CAGCAA				GCGCG		CTTCCTAC	G	AGGA		GGATACG		GGATCGGGA		CTGGGACA	3 +

Figure 4. Nucleotide sequence analysis of TCR-δ rearrangements in polyclonal 12- and 15-wk fetal thymocytes. Junctional sequences were determined from PCR products amplified from BB and CC DNA using appropriate pairs of primers. They are compared with germline sequences (*top*), fetal thymocyte clone sequences (Solo, Fig. 2), and postnatal thymocyte clone sequences (OM, Fig. 1 *A*). Numbers along the left border denote the V_δ segment used ($V_\delta 1,2,3,5$) and those near the right border denote the J_δ segment used ($J_\delta 1,3$). Sequences are classified as either in-frame (+) or out-of-frame (−) along the right border.

junctions from the OM postnatal thymocyte γ/δ T cell clones (using $V_\delta 1$, $V_\delta 3$, and $V_\delta 5$) reveal in most instances the usage of both $D_\delta 3$ and $D_\delta 2$, and display much more extensive N-nucleotide incorporation (15.8 per V-D-D-J junction) (Figs. 1 and 4).

To determine whether the differences noted between early fetal and postnatal thymocyte TCR-δ junctions are related to the age of the sample rather than the particular V gene segment rearranged, we amplified by PCR a single rare example of $V_\delta 1$ to $J_\delta 3$ rearrangement in each of the polyclonal fetal thymus samples (Fig. 4). Both junctions display the use of only the $D_\delta 3$ gene segment, and display no N-nucleotide incorporation. These data therefore argue that TCR-δ junctional diversity increases in an age-related fashion throughout early thymic development, and is only indirectly associated with V segment usage.

Cytokine Production by Fetal and Postnatal TCR-γ/δ Thymocyte Clones. In order to reveal possible functional differences between the $V_\delta 2^+$ fetal and $V_\delta 2^-$ postnatal thymocyte clones, we analyzed the capacity of the clones to produce cytokines upon stimulation. Therefore the clones were stimulated with 30 μg/ml ConA, a powerful polyclonal stimulant for human T cell clones (54), and the supernatants were assayed for IL-2, IL-4, IL-5, IFN-γ, and GM-CSF. Table 3 shows the cytokine production profiles of seven fetal TCR-γ/δ clones from samples Solo, BB and CC, and seven postnatal TCR-γ/δ clones from donors OM and FH. All fetal clones produced significant levels of IL-4 after activation with ConA. The fetal clones also produced IL-5 and high levels of GM-CSF and IFN-γ. Three additional fetal clones also produced IL-4 and IL-5 after activation, but one (CC40) consistently failed to produce IL-4 or IL-5, and secreted low levels of GM-CSF but significant amounts of IFN-γ (data not shown). In contrast to the fetal clones, the clones derived from postnatal thymic material did not produce IL-4 or IL-5 above background (Table 3). In general, the clones produced less GM-CSF than the fetal clones, although the levels of IL-2 and IFN-γ were comparable (Table 3). The same pattern of cytokine production was observed with five other postnatal TCR-γ/δ thymocyte clones (data not shown).

To determine the capacity of these clones to produce cytokines after activation via the TCR/CD3 complex or CD2, they were stimulated with a CD3 mAb in the presence or absence of the phorbol ester PMA or by a mitogenic combination of anti-CD2 mAbs. As a control, the clones were stimulated with PMA alone, a treatment that normally cannot activate mature T cells on its own (54). The cytokine production pattern of three representative fetal and three postnatal TCR-γ/δ clones is presented in Table 4. The combination of anti-CD2 mAbs was able to induce cytokine production in all clones. As expected, the anti-CD3 mAb SPV-T3b only induced cytokine production in combination with PMA. Interestingly, PMA alone activated the cells to produce cytokines. Whereas 1 ng/ml PMA was sufficient to trigger secretion of significant levels of cytokines (data not shown), 10 ng/ml was found to be optimal. The patterns of cytokine production after activation via CD2 or TCR/CD3 were the same as after activation via ConA, inasmuch as the fetal thymo-

Table 3. *Cytokine Production by Fetal and Postnatal Thymocyte TCR-γ/δ Clones Stimulated with 30 μg/ml ConA*

Clone	ConA	IL-2	IL-4	IL-5	IFN-γ	GM-GSF
		U/ml	*pg/ml*	*pg/ml*	*ng/ml*	*ng/ml*
Solo 6	−	<0.5	<50	ND	<0.3	<0.05
	+	4.0	2,397 ± 289	ND	125 ± 3	22 ± 3
Solo 15	−	<0.5	<50	<50	<0.3	<0.05
	+	10.0	200 ± 27	796 ± 102	25 ± 4	16 ± 1.3
Solo 19	−	<0.5	<50	<50	<0.3	<0.05
	+	6.7	496 ± 104	575 ± 43	89 ± 3	26 ± 9
BB2	−	<0.5	<50	<50	<0.3	<0.05
	+	6.5	1,658 ± 108	270 ± 39	220 ± 57	>20
BB5	−	<0.5	<50	<50	<0.3	<0.05
	+	5.0	502 ± 100	542 ± 88	110 ± 16	23 ± 7
CC6	−	<0.5	<50	<50	<0.3	<0.05
	+	9.6	1,096 ± 31	1,540 ± 500	81 ± 8	19 ± 3
CC7	−	<0.5	<50	<50	<0.3	<0.05
	+	8.0	286 ± 9	96 ± 20	121 ± 25	2 ± 0.2
OM35	−	<0.5	<50	<50	<0.3	<0.05
	+	4.3	<50	<50	18 ± 5	2.5 ± 0.8
OM47	−	<0.5	<50	<50	<0.3	<0.05
	+	3.9	<50	<50	10 ± 3	0.5 ± 0.02
OM64	−	<0.5	<50	<50	<0.3	55 ± 6
	+	5.0	<50	<50	50 ± 0.1	10 ± 0.4
FH3	−	<0.5	<50	<50	<0.3	<0.05
	+	9.3	<50	<50	83 ± 0.3	3 ± 0.5
FH13	−	<0.5	<50	<50	<0.3	<0.05
	+	4.6	<50	<50	37 ± 0.1	5 ± 0.9
FH26	−	<0.5	<50	<50	13 ± 0.1	<0.05
	+	9.6	<50	<50	68 ± 0.3	5 ± 0.5
FH31	−	<0.5	<50	<50	<0.3	<0.05
	+	7.3	<50	317 ± 72	21 ± 2.6	1 ± 0.03

Each value is the mean ± SD of duplicate determinations. One of three representative experiments is shown. Cytokine production varied quantitatively, but not qualitatively, between experiments.

cytes produced IL-4 and IL-5, whereas the postnatal thymocytes secreted at most only very low levels of these cytokines (Table 4).

Discussion

By analyzing the rearrangement and expression of the TCR-γ and -δ genes in early human fetal thymocytes we have obtained strong evidence that rearrangement at these loci occurs in an orderly and coordinated fashion, summarized schematically in Figure 5. Our data indicate that the initial rearrangement events at the TCR-δ locus involve the joining of the $V_\delta 2$ gene segment to the $D_\delta 3$ gene segment. The initial rearrangement events at the TCR-γ locus involve the joining of downstream V_γ gene segments, primarily $V_\gamma 1.8$ and $V_\gamma 2$, to J_γ gene segments of the $J_\gamma 1$ cluster. At later times there is a switch in the rearrangement pattern at the TCR-δ locus such that the $V_\delta 1$ gene segment (as well as some other V_δ gene segments), is joined primarily to the upstream D_δ gene segments, $D_\delta 1$ and $D_\delta 2$. Similarly, there is a switch in the rearrangement pattern at the TCR-γ locus such that upstream V_γ gene segments in the $V_\gamma 1$ family are joined to downstream J_γ gene segments of the $J_\gamma 2$ cluster. Superimposed on this highly ordered rearrangement pattern is a gradual increase in diversification at the junctions of the rearranged gene segments through the incorporation of template-independent N region nucleotides. Our results are almost certainly due to control at the level of gene rearrangement rather than to selection based upon surface TCR expression, since the out-of-frame rearrangements that were ana-

Table 4. *Cytokine Production by Fetal and Postnatal Thymocyte TCR-γ/δ Clones after Activation via CD3 or CD2*

Clone	Stimulus	IL-2	IL-4	IL-5	IFN-γ	GM-CSF
		U/ml	*pg/ml*	*pg/ml*	*ng/ml*	*ng/ml*
BB2	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	2.0	400 ± 3	389 ± 39	27 ± 4.4	3.4 ± 0.3
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	0.9	<50	550 ± 160	5 ± 0.06	2.1 ± 0.2
	αCD3 + PMA	5.5	538 ± 16	1,072 ± 180	93 ± 0.4	61 ± 14
BB8	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	7.6	1,814 ± 22	7,020 ± 2,670	39 ± 0.2	23 ± 6.9
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	0.9	<50	622 ± 218	6 ± 0.1	2.1 ± 0.1
	αCD3 + PMA	4.2	203 ± 10	3,034 ± 24	38 ± 8	5.4 ± 1.7
BB10	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	28.2	1,270 ± 50	5,104 ± 1,048	38 ± 3	27 ± 0.5
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	1.5	<50	4,452 ± 460	14 ± 6	1.8 ± 0.1
	αCD3 + PMA	4.0	678 ± 118	>10,000	38 ± 9	12.2 ± 0.3
FH3	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	4.5	<50	<50	93 ± 3.8	93 ± 0.6
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	<0.5	<50	<50	26 ± 0.6	2.4 ± 0.2
	αCD3 + PMA	<0.5	<50	<50	55 ± 3	1.2 ± 0.03
FH13	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	11.0	<50	176 ± 55	30 ± 0.2	2.4 ± 0.4
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	<0.5	<50	<50	<0.3	1.8 ± 0.02
	αCD3 + PMA	<0.5	<50	<50	40 ± 1.6	0.7 ± 0.1
FH26	–	<0.5	<50	<50	<0.3	<0.05
	αCD2	1.0	<50	<50	11 ± 0.5	1.2 ± 0.1
	αCD3	<0.5	<50	<50	<0.3	<0.05
	PMA	<0.5	<50	<50	52 ± 0.1	2.3 ± 0.1
	αCD3 + PMA	1.0	<50	<50	32 ± 11	1.2 ± 0.05

7 d after the previous stimulation, cells were washed twice and stimulated at a concentration of 10^6 cells/ml with a combination of 1 μg/ml purified SPV-T3b (70) and 1 ng/ml PMA, 10 ng/ml PMA, or a 1:2,500 dilution of ascites of the anti-CD2 mAbs X.11-1 and D66 (71). Each value is the mean ± SD of duplicate determinations. One of three representative experiments is shown.

lyzed displayed properties that were highly similar to the in-frame rearrangements.

Previous studies have shown that the predominant population of TCR-γ/δ lymphocytes in human peripheral blood displays cell surface TCRs that pair TCR δ chains using $V_\delta 2$ with TCR γ chains using $V_\gamma 2$-$C_\gamma 1$ (4, 8–10). We have previously demonstrated in transfection experiments that there is no physical barrier that prevents $V_\delta 2^+$ TCR δ chains from pairing with $V_\gamma 1$-$C_\gamma 2$ TCR γ chains (46). Consistent with this result, and the recent analysis of a large panel of postnatal thymocyte clones (10), we find that two of three early thymocyte clones display cell surface TCRs composed of $V_\delta 2^+$ TCR δ chains paired with $V_\gamma 1.8$-$C_\gamma 1$ TCR γ chains. We therefore suggest that the predominant $V_\delta 2/V_\gamma 2$-$C_\gamma 1$ peripheral blood population arises as the result of two distinct processes. Coordination between rearrangement events at the TCR-γ and -δ loci as described here dictates that TCR-γ/δ lymphocytes that use $V_\delta 2$ will also express TCR γ

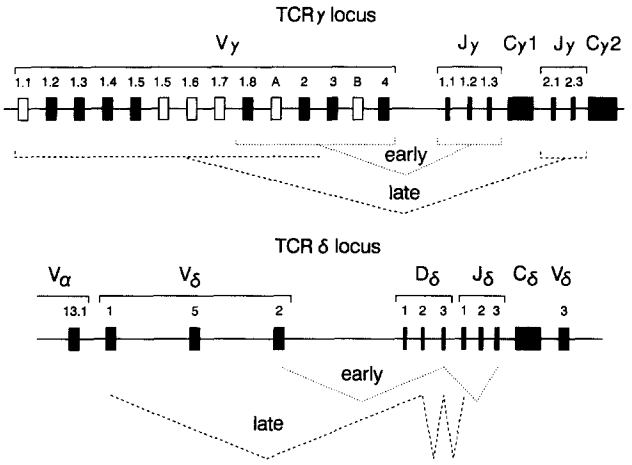

Figure 5. Controlled and coordinate TCR-γ and TCR-δ gene rearrangement. TCR-γ locus organization is according to references 66 and 72. Open boxes represent nonfunctional pseudogenes. TCR-δ locus organization is according to references 8, 35, and 47. The predominant early fetal (early) and postnatal (late) rearrangement patterns are depicted.

chains using downstream V_γ segments ($V_\gamma 1.8$, $V_\gamma 2$, etc.) along with $C_\gamma 1$. However, among these TCR γ chains, only those that use $V_\gamma 2$ are detected at significant levels in peripheral blood. Further, $V_\gamma 2$-$C_\gamma 1$ chains preferentially use $J_\gamma 1.2$ (1, 8, 10). These observations argue that selection must also play an important role in generating this specific cell population. In this regard, it is important to note that Parker et al. (55) have recently provided evidence for postnatal, extrathymic expansion of the $V_\delta 2/V_\gamma 2$-$C_\gamma 1$ subset.

Although we do not have formal evidence that the early fetal thymocytes analyzed in this study include the precursors of the predominant $V_\delta 2/V_\gamma 2$-$C_\gamma 1$ population in adult peripheral blood, a number of observations argue that this is probably the case. A comparison of our data on fetal and postnatal thymocyte TCR δ V-D-J junctions to similar data accumulated from peripheral blood TCR-γ/δ lymphocytes argues that the $V_\delta 1^+$ and $V_\delta 2^+$ subsets arise via rearrangements occurring at distinct phases of thymic ontogeny. Peripheral blood and postnatal thymocyte $V_\delta 1$ junctions typically display contributions from multiple D_δ segments, rather than solely from $D_\delta 3$, and display extensive N-nucleotide incorporation (this study; 34, 47, 50). By contrast, the peripheral blood $V_\delta 2$ junctions are generally less complex, often displaying a contribution from only $D_\delta 3$, and less extensive N-nucleotide incorporation (7, 8, 50). Further, $J_\delta 3$ usage, which apparently predominates in early fetal thymocytes, is much higher in the peripheral blood $V_\delta 2^+$ subset than in the peripheral blood $V_\delta 1^+$ subset (8, 10, 34). Taken together, these observations suggest that a significant fraction of the $V_\delta 2/V_\gamma 2$-$C_\gamma 1$ lymphocytes in peripheral blood are generated relatively early during thymic ontogeny. Whether a subset of the early fetal thymocytes also serves as the precursor of a population of human TCR-γ/δ lymphocytes with a distinct anatomical localization cannot be eliminated, however.

Our data demonstrate remarkable similarities between the earliest rearrangement events at the human and murine TCR-γ and -δ loci. In both systems the repertoire of rearrangeable V gene segments is initially highly restricted, and the usage of homologous D_δ gene segments ($D_\delta 3$ in the human, $D_\delta 2$ in the mouse) and J_δ gene segments ($J_\delta 3$ in the human, $J_\delta 2$ in the mouse) predominates (16, 18, 19). Further, junctional diversification by the incorporation of N-region nucleotides is minimal or absent in both systems at early times, presumably as the result of low levels of terminal transferase activity (56). However, the analysis of the V-(D)-J junctions of early murine fetal thymocytes that use specific pairs of V_γ and V_δ gene segments has also shown them to be highly homogeneous, and to encode the TCRs expressed on adult lymphocytes in certain epithelial locations (19–22). In marked contrast, the $V_\delta 2$-$D_\delta 3$-$J_\delta 3$ junctions analyzed here are quite heterogeneous. The junctions of the Solo clones were all distinct, and did not match any of the junctions obtained from the polyclonal BB and CC cultures. None of the CC junctions occurred more than twice among the sequences examined, and although one BB junction was detected at high frequency, it seems most plausible to attribute this result to oligoclonality as the result of expansion of the BB culture in vitro. These results would argue that early human TCR-γ/δ lymphocytes might not be subjected to the same types of intrathymic selection that limit the repertoire of certain intraepithelial lymphocytes in the mouse (19–22).

Striking differences were found in the capacities of fetal and postnatal TCR-γ/δ thymocytes to produce cytokines upon activation, suggesting that these two sets of TCR-γ/δ thymocyte clones represent functionally distinct subpopulations. In contrast to postnatal TCR-γ/δ thymocyte clones, the fetal clones produced significant levels of IL-4 and IL-5 after activation with either the lectin ConA, anti-CD3 mAb plus PMA, or a mitogenic pair of anti-CD2 mAbs. Both sets of clones were able to secrete GM-CSF and IFN-γ. The failure of postnatal TCR-γ/δ thymocyte clones to produce IL-4 is probably due to a lack of IL-4 gene transcription, because with a highly sensitive PCR technique no IL-4 mRNA could be detected after activation of the clones with ConA (De Waal Malefyt, R., and H. Spits, unpublished data). Since the postnatal and fetal clones were cultured under identical conditions, the inability of the postnatal clones to produce IL-4 and IL-5 is not acquired in vitro. Moreover, freshly isolated day 13–15 murine $CD3^+$ fetal thymocytes that contain only TCR-γ/δ cells produce IL-4 upon stimulation with anti-CD3 mAb (57). We believe, therefore, that the fetal TCR-γ/δ cells have the ability to produce IL-4 and IL-5 in vivo. The cloned TCR-γ/δ lines produced cytokines upon activation via the CD3/TCR-γ/δ complex or via CD2, but whether or not TCR-γ/δ cells are actually stimulated in vivo to produce IL-4 and IL-5 is unknown. In situ hybridization should be carried out to investigate this point.

TCR-γ/δ thymocytes might play a role in early TCR-α/β T cell development, and if so, cytokines are presumably involved. Our data could therefore be an indication that IL-4, IL-5, or both, are important regulators of early thymic development. In man, the main target cell of IL-5 is the eosino-

phil (58). In addition, some effects of IL-5 on human B cells have been documented (58, 59), but so far no effects of IL-5 on human T cells have been reported. It is therefore unclear what the biological significance is of IL-5 for thymic development. On the other hand, T cells can respond to IL-4. While activated mature human T cells proliferate in response to IL-4 (60), this factor can inhibit activation of resting human T cells under certain conditions (61). In the mouse, $CD4^-$ $CD8^-$ day 15 fetal thymocytes respond to IL-4 in the presence of PMA, although they do not acquire CD4 or CD8 under these conditions (62). In addition, mature $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes proliferate in response to IL-4 both in mouse and in man (60, 63). Fetal human thymocytes cultured for 14 d in IL-4 contain an elevated percentage of proliferating TCR-γ/δ T cells (data not shown), indicating that unstimulated TCR-γ/δ thymocytes can respond to IL-4. Not unexpectedly, the TCR-γ/δ fetal clones respond well to IL-4 (data not shown). Further, IL-4 can induce CD8 on mature $CD4^+$ T cells (64), which raises the possibility that IL-4 plays a role in the regulation of CD8 expression in the thymus. Lastly, it has been reported that IL-4 has the ability to induce class II MHC antigens on murine thymic macrophages, resulting in enhanced antigen presenting abilities in these cells (65). Thus, IL-4 has the potential to have pleiotrophic effects on cells in the thymus. Further research will be necessary to elucidate the possible regulatory role of IL-4 in early thymic development.

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References

1. Treibel, F., F. Faure, M. Graziana, S. Jitsukawa, M.-P. Lefranc, and T. Hercend. 1988. A unique V-J-C rearranged gene encodes a γ protein expressed on the majority of $CD3^+$ T cell receptor α/β^- circulating lymphocytes. *J. Exp. Med.* 167:694.
2. Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend. 1988. Characterization of human peripheral blood lymphocytes expressing the CD3-γ/δ complex with anti-receptor monoclonal antibodies. *J. Immunol.* 141:3357.
3. Jitsukawa, S., F. Triebel, F. Faure, C. Miossec, and T. Hercend. 1988. Cloned $CD3^+$ TCRα/β^-TiγA^- peripheral blood lymphocytes compared to the TiγA^+ counterparts: structural differences of the γ/δ receptor and functional heterogeneity. 1988. *Eur. J. Immunol.* 18:1671.
4. Treibel, F., F. Faure, F. Mami-Chouaib, S. Jitsukawa, A. Griscelli, C. Genevee, S. Roman-Roman, and T. Hercend. 1988. A novel human V_δ gene expressed predominantly in the TiγA fraction of γ/δ peripheral lymphocytes. *Eur. J. Immunol.* 18:2021.
5. Bottino, C., G. Tambussi, S. Ferrini, E. Ciccone, P. Varese, M.C. Mingari, L. Moretta, and A. Moretta. 1988. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. *J. Exp. Med.* 168:491.
6. Lanier, L.L., J. Ruitenberg, R.L.H. Bolhuis, J. Borst, J.H. Phillips, and R. Testi. 1988. Structural and serological heterogeneity of γ/δ T cell antigen receptor expression in thymus and peripheral blood. *Eur. J. Immunol.* 18:1985.
7. Loh, E.Y., J.F. Elliot, 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.
8. Hata, S., M. Clabby, P. Devlin, H. Spits, and J.E. de Vries, and M.S. Krangel. 1989. Diversity and organization of human T cell receptor δ variable gene segments. *J. Exp. Med.* 169:41.
9. Sturm, E., E. Braakman, R.E. Bontrop, P. Chuchana, R.J. Van de Griend, F. Koning, M.-P. Lefranc, and R. Bolhuis. 1989. Coordinated V_γ and V_δ gene segment rearrangements in human T cell receptor γ/δ^+ lymphocytes. *Eur. J. Immunol.* 19:1261.
10. Casorati, G., G. de Libero, A. Lanzavecchia, and N. Migone. 1989. Molecular analysis of human γ/δ^+ clones from thymus and peripheral blood. *J. Exp. Med.* 170:1521.
11. Seki, H., M. Nanno, P.-F. Chen, K. Itoh, C. Ioannides, R.A. Good, and C.D. Platsoucas. 1989. Molecular heterogeneity of $\gamma\delta$ T-cell clones form normal donors: both disulfide- and non-disulfide-linked receptors are δTCS1$^+$. *Proc. Natl. Acad. Sci. USA.* 86:2326.
12. Lefranc, M.-P., A. Forster, R. Baer, M.A. Stinson, and T.H. Rabbitts. 1986. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. *Cell.* 45:237.
13. Krangel, M.S., H. Band, S. Hata, J. McLean, and M.B. Brenner. 1987. Structurally divergent human T cell receptor γ proteins encoded by distinct C_γ genes. *Science (Wash. DC).* 237:64.
14. Band, H., F. Hochstenbach, C.M. Parker, J. McLean, M.S. Krangel, and M.B. Brenner. 1989. Expression of human T cell receptor-$\gamma\delta$ structural forms. *J. Immunol.* 142:3627.
15. Garman, R.D., P.J. Doherty, and D.H. Raulet. 1986. Diversity, rearrangement and expression of murine T cell gamma genes. *Cell.* 45:733.
16. Chien, Y.-H., 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.
17. Havran, W.L., and J.P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature (Lond.).* 335:443.
18. Ito, K., M. Bonneville, Y. Takagaki, N. Nakanishi, O. Kanagawa, E.G. Krecko, and S. Tonegawa. 1989. Different γδ T-cell receptors are expressed on thymocytes at different stages of development. *Proc. Natl. Acad. Sci. USA.* 86:631.
19. Lafaille, J.J., A. DeCloux, M. Bonneville, Y. Takagaki, and S. Tonegawa. 1989. Junctional sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell.* 59:859.
20. Asarnow, D.M., W.A. Kuziel, M. Bonyhadi, R.E. Tigelaar, P.W. Tucker, and J.P. Allison. 1988. Limited diversity of γδ antigen receptor genes of thy-1$^+$ dendritic epidermal cells. *Cell.* 55:837.
21. Itohara, S., A.G. Farr, J.J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa. 1990. Homing of a γδ thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature (Lond.).* 343:754.
22. Havran, W.L., and J.P. Allison. 1990. Origin of thy-1$^+$ dendritic epidermal cells of adult mice from fetal thymic precursors. *Nature (Lond.).* 344:68.
23. 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.
24. Korman, A.J., S.M. Galesic, D. Spencer, A.M. Kruisbeek, and D. Raulet. 1988. Predominant variable region gene usage by γ/δ T cell receptor-bearing cells in the adult thymus. *J. Exp. Med.* 168:1021.
25. Treibel, 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.
26. Yssel, H., J.E. de Vries, J. Borst, and H. Spits. 1987. Distribution and functional analysis of a 120- to 130-kDa T cell surface antigen. *Cell. Immunol.* 105:161.
27. Abst, H., M. Emmerich, S. Mitlenyi, A. Radbruch, and H. Tesch. 1989. CD20 positive human B lymphocytes separated with the magnetic cell sorter (MACS) can be induced to proliferation and antibody secretion in vitro. *J. Immunol. Methods.* 125:19.
28. Yssel, H., J.E. de Vries, M. Koken, W.V. Blitterswijk, and H. Spits. 1984. A serum free method for the generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
29. Band, H., F. Hochstenbach, J. McLean, S. Hata, M.S. Krangel, and M.B. Brenner. 1987. Immunochemical proof that a novel rearranging gene encodes the T cell receptor δ subunit. *Science (Wash. DC).* 238:682.
30. Wu, Y.-J., W.-T. Tian, R.M. Snider, C. Rittershaus, P. Rogers, L. LaManna, and S.H. Ip. 1988. Signal transduction of γ/δ T cell antigen receptor with a novel mitogenic anti-δ antibody. *J. Immunol.* 141:1476.
31. Ciccone, E., S. Ferrini, C. Bottino, O. Viale, I. Prigione, G. Panteleo, G. Tambussi, A. Moretta, and L. Moretta. 1988. A monoclonal antibody specific for a common determinant of the human T cell receptor γ/δ directly activates CD3$^+$ WT31$^-$ lymphocytes to express their functional program(s). *J. Exp. Med.* 168:1.
32. Jitsukawa, S., F. Faure, M. Lipinski, F. Treibel, and T. Hercend. 1987. A novel subset of human lymphocytes with a TCR γ complex. *J. Exp. Med.* 166:1192.
33. Hata, S., M.B. Brenner, and M.S. Krangel. 1987. Identification of putative human T-cell receptor δ complementary DNA clones. *Science (Wash. DC).* 238:678.
34. Hata, S., K. Satyanarayana, P. Devlin, H. Band, J. McLean, J.L. Strominger, M.B. Brenner, and M.S. Krangel. 1988. Extensive junctional diversity of rearranged human T cell receptor δ genes. *Science (Wash. DC).* 240:1541.
35. Satyanarayana, K., S. Hata, P. Devlin, M.G. Roncarolo, J.E. de Vries, H. Spits, J.L. Strominger, and M.S. Krangel. 1988. Genomic organization of the human T-cell antigen-receptor α/δ locus. *Proc. Natl. Acad. Sci. USA.* 85:8166.
36. Wigler, M., R. Sweet, G.K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. *Cell.* 16:777.
37. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303.
38. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487.
39. Chen, E.Y., and P.H. Seeburg. 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA (NY).* 4:165.
40. Tabor, S., and C.C. Richardson. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA.* 84:4767.
41. Chretien, I., A. Van Kimmenade, M.K. Pearce, J. Banchereau, and J.S. Abrams. 1989. Development of polyclonal and monoclonal antibodies for immunoassay and neutralization of human IL-4. *J. Immunol. Methods.* 117:67.
42. Bacchetta, R., R. de Waal Malefyt, H. Yssel, J. Abrams, J.E. de Vries, H. Spits, and M.G. Roncarolo. 1989. Hostreactive CD4$^+$ and CD8$^+$ T cell clones isolated from a human chimera produce IL-5, IL-2, IFN-γ and GM-CSF but not IL-4. *J. Immunol.* 144:902.
43. Favre, C., J. Wijdenes, H. Cabrillat, O. Djossou, J. Banchereau, and J.E. de Vries. 1989. Epitope mapping of recombinant human gamma interferon using monoclonal antibodies. *Mol. Immunol.* 26:17.
44. Gillis, S., W. Ferm, W. Ou, and K.A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
45. Miossec, C., F. Faure, L. Ferradini, S. Roman-Roman, S. Jitsukawa, S. Ferrini, A. Moretta, F. Treibel, and T. Hercend. 1990. Further analysis of the T cell receptor γ/δ$^+$ peripheral lymphocyte subset. The $V_δ1$ gene segment is expressed with either $C_α$ or $C_δ$. *J. Exp. Med.* 171:1171.
46. Solomon, K.R., M.S. Krangel, J. McLean, M.B. Brenner, and H. Band. 1990. Human T cell receptor-γ and -δ chain pairing analyzed by transfection of a T cell receptor-δ negative mutant cell line. *J. Immunol.* 144:1120.
47. Loh, E.Y., S. Cwirla, A.T. Serafini, J.H. Phillips, and L.L. Lanier. 1988. Human T-cell-receptor δ chain: Genomic organization, diversity, and expression in populations of cells. *Proc. Natl. Acad. Sci. USA.* 85:9714.
48. Leiden, J.M., J.D. Fraser, and J.L. Strominger. 1986. The complete primary structure of the T-cell receptor genes from an alloreactive cytotoxic human T-lymphocyte clone. *Immunogenetics.* 24:17.
49. Klein, M.H., P. Concannon, M. Everett, L.D.H. Kim, T.

Hunkapiller, and L. Hood. 1987. Diversity and structure of human T-cell receptor α-chain variable region genes. *Proc. Natl. Acad. Sci. USA.* 84:6884.

50. Takihara, Y., J. Reimann, E. Michalopoulos, E. Ciccone, L. Moretta, and T.W. Mak. 1989. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. *J. Exp. Med.* 169:393.
51. Bluthmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. T-cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous α- and β-genes. *Nature (Lond.).* 334:156.
52. Malissen, M., J. Trucy, F. Letourneur, N. Rebai, D.E. Dunn, F.W. Fitch, L. Hood, and B. Malissen. 1988. A T cell clone expresses two T cell receptor α genes but uses one αβ heterodimer for allorecognition and self MHC-restricted antigen recognition. *Cell.* 55:49.
53. Marolleau, J.-P., J.D. Fondell, M. Malissen, J. Trucy, E. Barbier, K.B. Marcu, P.-A. Cazenave, and D. Primi. 1988. The joining of germ-line V_α to J_α genes replaces the preexisting V_α-J_α complexes in a T cell receptor α,β positive T cell line. *Cell.* 55:291.
54. Paliard, X., R. de Waal Malefyt, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J.E. de Vries, and H. Spits. 1988. Simultaneous production of IL-2, IL-4, and IFN-γ by activated human $CD4^+$ and $CD8^+$ T cell clones. *J. Immunol.* 141:849.
55. 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.
56. Landau, N.R., D.G. Schatz, M. Rosa, and D. Baltimore. 1987. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. *Mol. Cell. Biol.* 7:3237.
57. Tentori, L., D.M. Pardoll, J.C. Zuniga, J. Hu-Li, W.E. Paul, J.A. Bluestone, and A.M. Kruisbeek. 1988. Proliferation and production of IL-2 and B cell stimulatory factor 1/IL-4 in early fetal thymocytes by activation through Thy-1 and CD3. *J. Immunol.* 140:1089.
58. Sanderson, C.J., H.D. Campbell, and I.G. Young. 1988. Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. *Immunol. Rev.* 6:29.
59. Yokota, T., N. Arai, J.E. de Vries, H. Spits, J. Banchereau, A. Zlotnik, D. Rennick, M. Howard, Y. Takebe, S. Miyatake, F. Lee, and K. Arai. 1988. Molecular biology of interleukin 4 and interleukin 5 genes and biology of their products that stimulate B cells, T cells, and hemopoietic cells. *Immunol. Rev.* 102:137.
60. Spits, H., H. Yssel, Y. Takabe, N. Arai, T. Yokota, F. Lee, K. Arai, J. Banchereau, and J.E. de Vries. 1987. Recombinant interleukin 4 promotes the growth of human T cells. *J. Immunol.* 139:1142.
61. Martinez, O.M., R.S. Gibbons, M.R. Garovoy, and F.R. Aronson. 1990. IL-4 inhibits IL-2 receptor expression and IL-2 dependent proliferation of human T cells. *J. Immunol.* 144:2211.
62. Lowenthal, J.W., J. Ransom, M. Howard, and A. Zlotnik. 1988. Upregulation of interleukin 4 receptor expression on immature (Lyt-2/L3T4$^-$) thymocytes. *J. Immunol.* 140:474.
63. Zlotnik, A., J. Ransom, G. Frank, M. Fischer, and M. Howard. 1987. Interleukin 4 is a growth factor for activated thymocytes: possible role in T cell ontogeny. *Proc. Natl. Acad. Sci. USA.* 84:3856.
64. Paliard, X., R. de Waal Malefyt, J.E. de Vries, and H. Spits. 1988. IL-4 mediates CD8 induction on human $CD4^+$ T cell clones. *Nature (Lond.).* 335:642.
65. Ransom, J., M. Fischer, L. Mercer, and A. Zlotnik. 1987. Lymphokine-mediated induction of antigen presenting ability in thymic stromal cells. *J. Immunol.* 139:2620.
66. Strauss, W.M., T. Quertermous, and J.G. Seidman. 1987. Measuring the human T cell receptor γ-chain locus. *Science (Wash. DC).* 237:1217.
67. Huck, S., and M.-P. Lefranc. 1987. Rearrangements to the JP1, JP and JP2 segments in the human T-cell rearranging gamma gene (TRGγ) locus. *FEBS (Fed. Exp. Biol. Soc.) Lett.* 224:291.
68. Brenner, M.B., J.L. Strominger, and M.S. Krangel. 1988. The γδ T cell receptor. *Adv. Immunol.* 43:133.
69. Raulet, D.H. 1989. The structure, function, and molecular genetics of the γ/δ T cell receptor. *Annu. Rev. Immunol.* 7:175.
70. Spits, H., G. Keizer, J. Borst, C. Terhorst, A. Heleman, and J.E. de Vries. 1984. Characterization of monoclonal antibodies against cell surface molecules associated with cytotoxic activity of natural and activated killer cells and cloned CTL lines. *Hybridoma.* 2:423.
71. Brottier, P., L. Boumsell, C. Gelin, and A. Bernard. 1985. T cell activation via CD2 (T, gp50) molecules: accessory cells are required to trigger T cell activation via CD2-D66 plus CD2-9.6/$T11_1$ epitopes. *J. Immunol.* 135:1624.
72. Lefranc, M.-P., P. Chuchana, P. Dariavach, C. Nguyen, S. Huck, F. Brockly, B. Jordan, and G. Lefranc. 1989. Molecular mapping of the human T cell receptor gamma (TRG) genes and linkage of the variable and constant regions. *Eur. J. Immunol.* 19:989.