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T Cell Receptor δ Gene Mutant Mice: Independent Generation of αβ T Cells and Programmed Rearrangements of γδ TCR Genes

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Summary

T cells bearing T cell receptor (TCR) γ and δ chain heterodimers are first generated early in ontogeny. They form distinct subsets that differ in their TCR repertoires and tissue distribution. Disruption of the mouse TCR Cδ gene segment by a gene targeting method caused the complete loss of T cells bearing TCR γδ chains, but had little or no effect on the development of T cells bearing TCR αβ chains. The analyses of TCR γ and δ genes in the mutant mice suggest that intracellular mechanisms acting at the level of DNA rearrangement play key roles in the differential γ and δ gene rearrangements and in the generation of the highly restricted junctional sequences during fetal thymic development.

Introduction

Two types of T cells exist; one utilizes the T cell receptor (TCR) αβ heterodimer and the other the TCR γδ heterodimer. During thymic ontogeny, rearrangement and cell surface expression of TCR γ and TCR δ chains precede that of TCR α and TCR β chains (Raulet et al., 1985; Pardoll et al., 1987; Itohara et al., 1989). An analysis of γδ TCR transgenic mice has suggested that αβ and γδ T cell lineages segregate prior to the expression of either TCR on the cell surface (Bonneville et al., 1989; Ishida et al., 1990). We and others have observed normal development of γδ T cells in αβ T cell–deficient mice (Mombaerts et al., 1992a; Philpott et al., 1992). However, it is not known whether the prior development of γδ T cells somehow influences the subsequent development of the αβ T cell lineage through a γδ TCR–mediated mechanism.

There are several γδ T cell subsets that express distinct TCRs and differ with regard to their ontological appearance and tissue distribution. The two γδ T cell subsets that appear first in thymic ontogeny are unusual in that their TCRs show virtually no diversity (Lafaille et al., 1989), even though they are encoded by rearranged TCR γ and δ genes. One expresses a canonical Vγ5 chain, and the other expresses a canonical Vγ6 chain. Both share a particular Vδ1-Jδ2 chain. These so-called Vγ5-Vδ1 and Vγ6-Vδ1 subsets appear in two consecutive waves in the fetal and perinatal thymus (Havran and Allison, 1988; Ito et al., 1989) and home to the epidermis (Asarnow et al., 1988; Havran and Allison, 1990) and to the mucosal epithelia of the uterus, vagina, and tongue (Itohara et al., 1990), respectively. Subsets that bear highly diversified Vγ1, Vγ4, or Vγ7 chains in combination with multiple Vδ chains are generated later in the neonatal and adult thymus (Elliott et al., 1988; Korman et al., 1988; Takagaki et al., 1989) and home to the blood and peripheral lymphoid organs.

The molecular mechanisms underlying the appearance of distinct γδ T cell subsets are obscure. One possible explanation is the selection of particular cells via TCR–ligand interactions provided by a particular thymic microenvironment. However, it is also possible that a development-associated intracellular machinery determines both the V gene usage and the degree of TCR diversity. To address these issues, it is desirable to design an experimental system in which TCR gene rearrangement takes place without TCR-mediated cellular selection. In the present study we accomplished this by generating γδ T cell–deficient mice by gene targeting (Capecchi, 1989). The results show that apparently normal αβ T cell development occurs without γδ T cells and that an intracellular mechanism acting at the level of DNA rearrangement in the thymus, rather than a cellular selection mechanism, plays a key role in the differential generation of γδ T cell subsets and in the generation of highly restricted junctional sequences among fetal γδ T cell subsets. The results also suggest that cell surface expression of the γδ TCR aids homing to and/or expansion of the γδ T cells in the epidermis.

Results

Generation of the Mutation in the TCR δ Locus

To generate a defect in the TCR δ locus that would prevent the expression of any complete TCR δ chain, we disrupted the TCR δ constant gene segment (Cδ) by gene targeting in embryonic stem (ES) cells. The targeting vectors (pPMKO-20 and pPMKO-21) utilize 12.5 kb homologous DNA sequences of AKR/J origin and the *pgk–neo* and *MC1–tk* gene cassettes as positive and negative selection markers, respectively (Figure 1A). These vectors were designed to replace most of the coding sequences of Cδ, but

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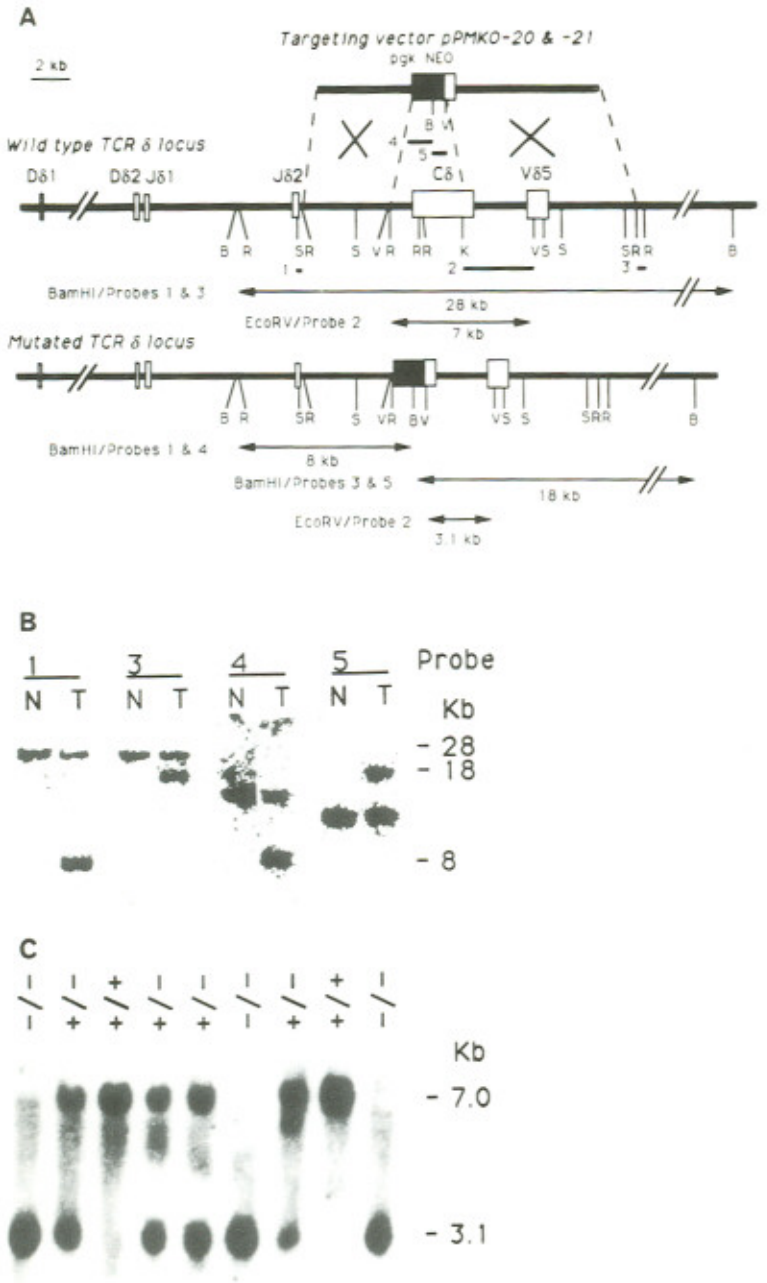

Figure 1. Targeting of TCR Cδ and Southern Analysis of Targeted Cells and Mutant Mice

(A) (Top) TCR δ targeting vectors pPMKO-20 and pPMKO-21, which carry one and two copies of the *tk* gene at the ends of the sequences, respectively. The crosses indicate the hypothetical crossovers between the targeting construct and the TCR Cδ gene segment. (Middle) Genomic structure of the wild-type TCR D-J-Cδ locus. (Bottom) Structure of the disrupted TCR Cδ gene segment. Probes used are probes 1, 3, 4, and 5 to characterize the ES clones and probe 2 to genotype the mice. The sizes of the DNA fragments hybridizing to these probes are indicated under the double-arrowed lines. Abbreviations for restriction enzyme sites: B, BamHI; R, EcoRI; S, SacI; V, EcoRV; K, KpnI.

(B) Southern blots of a representative targeted ES cell clone. BamHI digests of DNA from a targeted clone (T) and wild-type E14 cells (N) were hybridized with probes indicated at the top of the figure. The bands from TCR Cδ locus were indicated at right. Probes 4 and 5 hybridize also to the endogenous *pgk* gene.

(C) Southern blot analysis of tail DNA of littermates from a cross between TCR δ^- heterozygotes. The DNA was cut with EcoRV, and the Southern blot was hybridized with probe 2. The wild-type allele is at 7.0 kb and the mutant allele at 3.1 kb. +/+, wild-type mice; +/−, δ^- heterozygotes; −/−, δ^- homozygotes.

retain all other segments of the TCR δ locus. Of the 384 ES cell clones analyzed, 11 were found to have the targeted mutation. Extensive analysis by Southern blot hybridization using internal and external probes revealed the correct integration of a single copy of the *pgk–neo* gene at the expected site in 9 out of 11 clones. Results obtained with one representative clone are shown in Figure 1B. Two clones showed an aberrant integration event at the 5′ or 3′ side of the marker gene (data not shown). Although the vectors were designed to use positive and negative selection (Mansour et al., 1988), the negative selection gave little enrichment for ES cell clones with a homologous recombination event: targeted clones were obtained at similar frequency from cultures maintained either in the presence or absence of thymidine analogs. In parallel experiments using another targeting vector (pPMKO-27), which contains fewer homologous sequences (7.5 kb), 4 out of 260 clones were targeted (data not shown).

Cells of five clones, A148 and B36 derived from the E14 cell line (Hooper et al., 1987) and A287, A621, and A713 derived from the D3 cell line (Gossler et al., 1986), were injected into C57BL/6 or BALB/c blastocysts. All clones gave rise to germline chimeras. Intercrosses between δ^- heterozygotes gave rise to δ^- homozygotes at the ex-

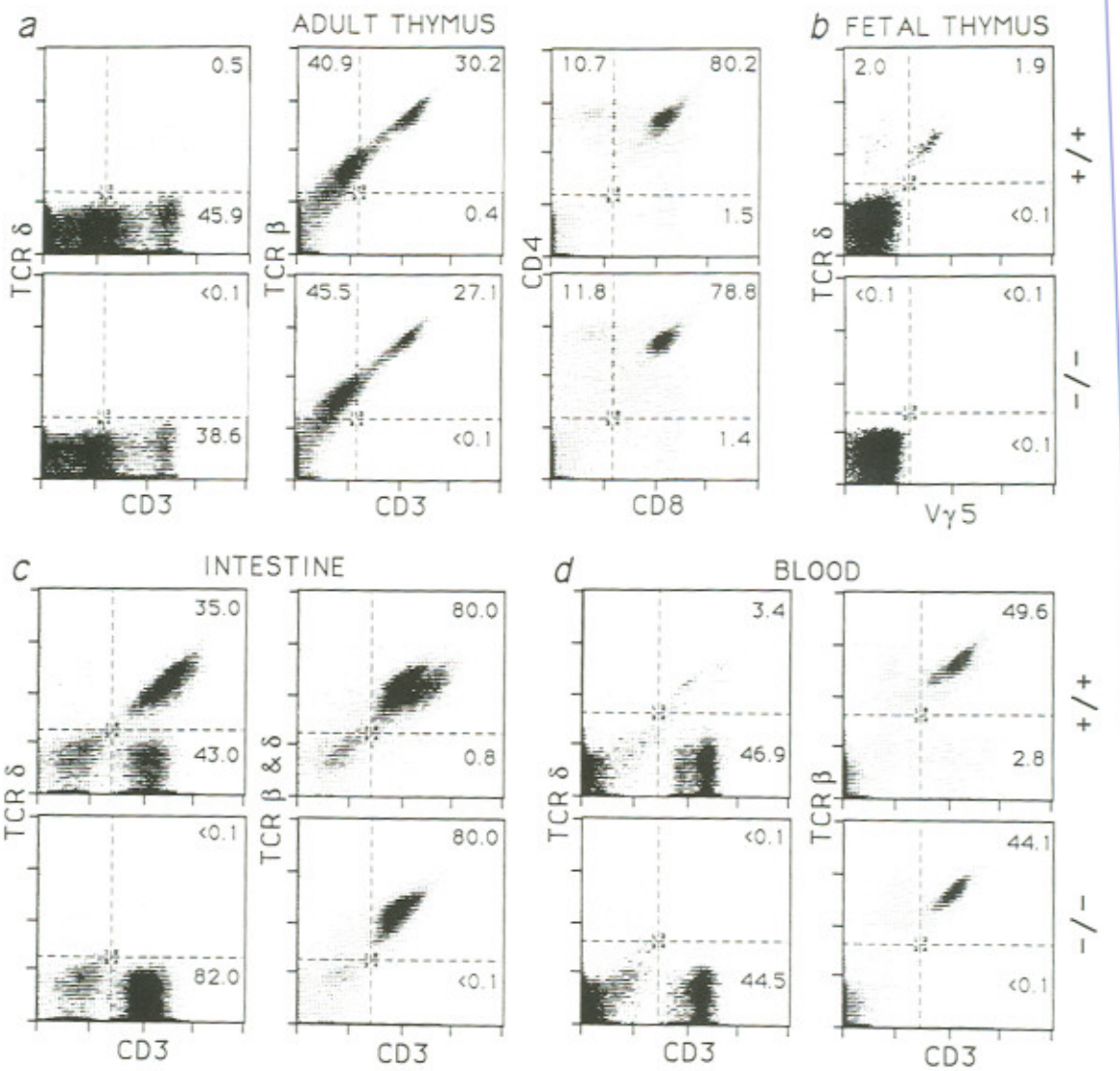

Figure 2. Flow Cytometric Analysis of TCR δ Mutant Mice

(a) Thymocytes from 4-week-old normal (+/+; top) and mutant (−/−; bottom) mice.

(b) Thymocytes from 16-day-old fetuses.

(c) Intestinal intraepithelial lymphocytes from 4-week-old normal (+/+; top) and mutant (−/−; bottom) mice.

(d) Peripheral blood lymphocytes from 4-week-old mice. The percentages of cells in some of the quadrants are shown. Antibodies used are 2C11 for CD3 ε, 3A10 for pan-TCR δ, H57-597 for pan-TCR β, 53-6.7 for CD8, GK1.5 for CD4, and F536 for Vγ5.

pected frequency of 1:4. An example of Southern analysis data is shown in Figure 1C. δ^- homozygotes that were kept under specific pathogen-free conditions remained healthy for at least 17 months. δ^- male and female homozygotes proved to be fertile.

Lack of T Cells Bearing TCR γδ Chains

To analyze the effect of the mutation, we first examined the cellular composition of various organs by flow cytometry (Figure 2). As expected, we did not find any cells reactive with an anti-pan TCR δ monoclonal antibody (MAb 3A10) in any of the lymphoid or epithelial organs from the mutant mice. Furthermore, fetal thymocytes obtained at day 16 of gestation were not stained with an anti-Vγ5-specific MAb (Figure 2b). All CD3 ε positive cells were stained with an anti-pan TCR β MAb (H57-597) (Figures 2a, 2c, and 2d), indicating the absence of cells expressing γδ, αγ, or γ TCR chains associated with CD3 ε.

We also analyzed the expression of TCR γδ by immunoprecipitation using an anti-TCR γ MAb, KN365, reactive to the denatured TCR Cγ1 domain (Maeda et al., 1987). In the δ^- homozygotes, we found no material reactive with this MAb in the lysate of day 16.5 fetal thymocytes that had been surface radioiodinated (Figure 3). These results confirm that no γδ TCR is expressed on the surface of mutant thymocytes.

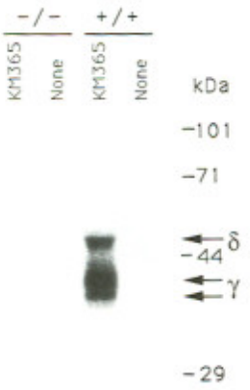

Figure 3. Immunoprecipitation Analysis of Fetal Thymocytes

Fetal thymocytes at day 16.5 of gestation were radioiodinated and subjected to immunoprecipitation analysis under nonreducing conditions. The lysates were precipitated either with or without MAb KN365, which reacts to the denatured Cγ1 domain (Maeda et al., 1987).

Normal Development of αβ T Cells

The development of the αβ T cell lineage in the γδ T cell–deficient mice was examined at various time points between day 15 of gestation and 5 months after birth. No major differences in total thymocyte numbers were observed among the δ⁻ homozygotes, δ⁻ heterozygotes, and wild-type mice obtained as littermates of crosses between a pair of δ⁻ heterozygotes. Moreover, we did not detect substantial differences in CD4, CD8, and TCR αβ expression patterns in thymic ontogeny between the mutant and the control mice (see Figure 2a; Table 1; data not shown). The same was true for the expression of other cell surface molecules such as LFA1, CD2, Ly-1, J11δ, CD69, and interleukin 2R by thymocytes (data not shown). These results suggest that αβ thymocytes develop independently of γδ thymocytes. Furthermore, we did not observe any differences between the mutant and control mice with respect to peripheral αβ T cells and B cells. For instance, in δ⁻ homozygotes the immunoglobulin G_1 response is not significantly altered upon immunization with ovalbumin (Figure 4), suggesting that the αβ T helper function in these mutant mice is not impaired.

Differential Rearrangement of TCR γ and TCR δ Genes in the Developing Thymus

Rearrangement of TCR γ and TCR δ genes was examined by a quantitative polymerase chain reaction (PCR) method followed by Southern blot hybridization. The mutation did not inhibit the rearrangement of TCR γ or TCR δ genes (Figure 5). In both normal and δ⁻ homozygotes, different variable region gene segments were preferentially rearranged in fetal (days 17 and 19 in gestation) and adult (6-week-old) mice in the absence of surface γδ TCR expression: rearrangements of Vγ5-Jγ1, Vγ6-Jγ1, and Vδ1 to Jδ1 or Jδ2 were frequent in the fetal but rare in the adult thymus in both the mutant and wild-type mice. Rearrangements of Vγ4, Vγ7, and Vγ1 were predominant in the adult thymus. Vδ4-Jδ1 and Vδ5-Jδ1 rearrangements occurred both in the fetal and adult thymuses, with slightly higher frequency in the latter. These findings demonstrate that rearrangement of the various Vγ and Vδ gene segments is programmed in development by an intracellular machinery.

An Intracellular Mechanism for the Junctional Sequence Homogeneity

The developmentally programmed rearrangement mentioned above contributes to the TCR homogeneity of fetal γδ T cell subsets with respect to the choice of γ and δ gene segments. We then investigated whether the junctional sequence homogeneity of these T cell subsets results from an intracellular mechanism or from TCR-mediated extracellular selection. Since no γδ TCR is expressed on the surface of cells in the TCR δ mutant mice, any effect of γδ TCR–mediated cellular selection should be absent during T cell development in these mice. As shown in Figure 6, the junctional sequences of Vγ5-Jγ1, Vγ6-Jγ1, and Vδ1-D-Jδ2 were as restricted in the fetal thymus of the mutant mice as in the wild-type mice, providing evidence for an intracellular mechanism. The number of sequences that were generated in fetal γδ progenitor cells is much smaller than the number of sequences that could be generated if the process was as random as in adult thymocyte progenitors. A role of an intracellular mechanism is also indicated by the recurrence of some out-of-frame sequences at the Vγ5-Jγ1 and Vγ6-Jγ1 junctions of both the wild-type and mutant mice (Figures 6A and 6B) and by the recurrence of a few sequences at the Vδ1-D2 junction of Vδ1-D-Jδ1 products as well as of Vδ1-D-Jδ2 products (Figures 6C and 6D).

A Possible Role of P Nucleotides in the Generation of Homogeneous Junctional Sequences

We have examined all junctional sequences in the Vγ5-Vδ1 and Vγ6-Vδ1 subsets that we determined in the pres-

Table 1. αβ Thymocytes in Mutant Mice

Animal	Genotype	Total Thymocytes ($\times 10^{-6}$)	Frequency (%)				
			β High	β Low	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
Day 16.5 fetuses[a]	+/+ or +/−	1.21 ± 0.41	0.5 ± 0.1	28.5 ± 0.9	23.9 ± 3.6	25.9 ± 0.6	3.9 ± 0.5
	−/−	1.36 ± 0.46	0.6 ± 0.1	25.9 ± 5.1	25.7 ± 2.6	26.0 ± 3.0	4.0 ± 0.1
Adults[b]	+/+	278 ± 94	14.6 ± 2.0	69.3 ± 2.0	83.4 ± 2.5	10.9 ± 2.1	2.1 ± 0.4
	−/−	257 ± 33	15.3 ± 1.7	71.1 ± 1.7	85.6 ± 1.1	10.1 ± 1.1	1.8 ± 0.3

[a] Examined are 11 and 6 samples for the total cell number counting, and 6 and 2 samples for the cell subpopulation study, respectively, for +/+ or +/− and −/− mouse groups. The data of the cell subpopulation study were obtained with littermates generated by crossing a pair of +/− animals.
[b] Data with 3- to 4-week-old mice (+/+, n = 12; −/−, n = 7) for total cell numbers, and with 5-month-old mice (+/+, n = 11; −/−, n = 9) for cell population.

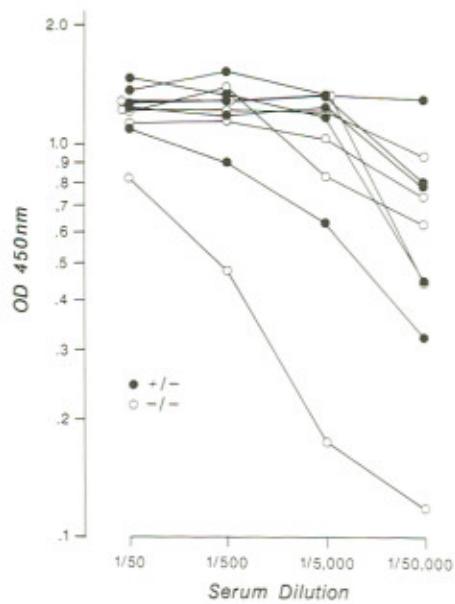

Figure 4. Serum Antibody Response of TCR δ Mutant Mice Immunized with the Thymus-Dependent Antigen Ovalbumin

Mice were immunized intraperitoneally with 100 mg of ovalbumin in complete Freund's adjuvant and boosted on day 15 with 100 mg of ovalbumin in normal saline. The level of ovalbumin-specific immunoglobulin G_1 serum antibodies was determined by enzyme-linked immunosorbent assay on day 30. Results are expressed as a binding curve by plotting absorbance at 490 nm (Y axis) versus serum antibody dilution (X axis). Closed circles (+/−), δ^- heterozygous littermates; open circles (−/−), δ^- homozygotes.

ent study and in our earlier studies (Lafaille et al., 1989; Itohara and Tonegawa, 1990). As shown in Table 2, just a few sequences, in-frame or out-of-frame, compose over 70% of each of the Vγ5-Jγ1, Vγ6-Jγ1, Vδ1-D2, and D2-Jδ2 junctions detected in the fetal thymuses. A number is assigned to each of these recurring junctional sequences in Figure 6 and Table 2. These junctional sequences can be grouped into two classes: in the class I sequences (sequences 1, 2, 4, 5, 7, and 8), a 1–3 nt overlap of germline sequences occurs at the breakpoints, and in the class II sequences (sequences 3, 6, and 9–12) no such overlap exists. However, if we take P nucleotides (Lafaille et al., 1989) into consideration, not only a substantial overlap (2–6 bp) emerges at the breakpoints of the class II junctional sequences, but also the overlap of some of the class I junctional sequences (sequences 2, 5, and 7) is enlarged from 1–2 bases to 3–5 bases. (In the original paper, a P sequence was proposed to be made up of 2 nt [Lafaille et al., 1989], but subsequent studies indicated that they can be longer [Ferrier et al., 1990; Kienker et al., 1991]. We arbitrarily used 5 nt for the present purpose.) We will discuss below a possible role of the overlap and of P nucleotides in the generation of the highly homogeneous junctions in the TCR of the Vγ5-Vδ1 and Vγ6-Vδ1 subsets.

TCR Expression Is Required for the γδ Dendritic Epidermal Cell Migration into and/or Maintenance in the Epidermis

Previous studies with TCR transgenic mice indicated that homing specificities of γδ T cell subsets are independent of the TCR specificities (Bonneville et al., 1990). In this study, we asked whether γδ TCR expression per se is required for homing, maintenance, or both of γδ dendritic epidermal cells in epidermis by analyzing the intraepidermal T cells of the TCR δ mutant mice by two-color immunocytochemistry. The results are summarized in Table 3, and some representative pictures are shown in Figure 7.

As expected, we observed large numbers of γδ T cells and very few αβ T cells in epidermal sheets prepared from δ^- heterozygotes and wild-type littermates (Figures 7a and 7b). Only data from the δ^- heterozygotes are presented here, since epidermal sheets prepared from these two groups of mice were phenotypically indistinguishable (data not shown). Virtually all (>98%) of the Thy-1^+ cells in these preparations expressed either αβ or γδ TCR (Figure 7c). In contrast, no γδ T cells were detected in epidermal samples from the TCR δ^- homozygotes (Figure 7d), and all of the CD3 ε^+ cells expressed αβ TCR (Figures 7d and 7e). It was also noted that in the δ^- homozygotes neither the Thy-1^+TCR$^-$ nor the Thy-1^+TCR αβ$^+$ dendritic epidermal cells displayed the dendritic morphology characteristic of γδ dendritic epidermal cells in δ^- heterozy-

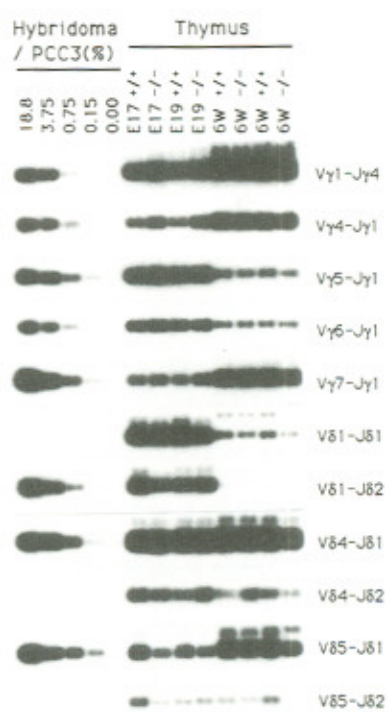

Figure 5. Rearrangement of TCR γ and TCR δ Genes Examined by Quantitative PCR–Southern Blot Analysis

The DNA from thymocytes of fetuses at day 17 (E17) and day 19 (E19) of gestation and of 6-week-old adult mice was amplified by PCR reaction, and the Southern blots of the products were probed with labeled oligonucleotide probes (see Experimental Procedures). To show the quantitative nature of the reaction, DNAs of hybridomas with known TCR gene rearrangement were mixed with PCC3 DNA that carries no rearrangement of TCR γ and δ genes in the indicated proportions and were analyzed side by side with the thymus DNA samples. Combinations of primers used are shown at right. +/+, wild-type mice; −/−, δ^- homozygotes.

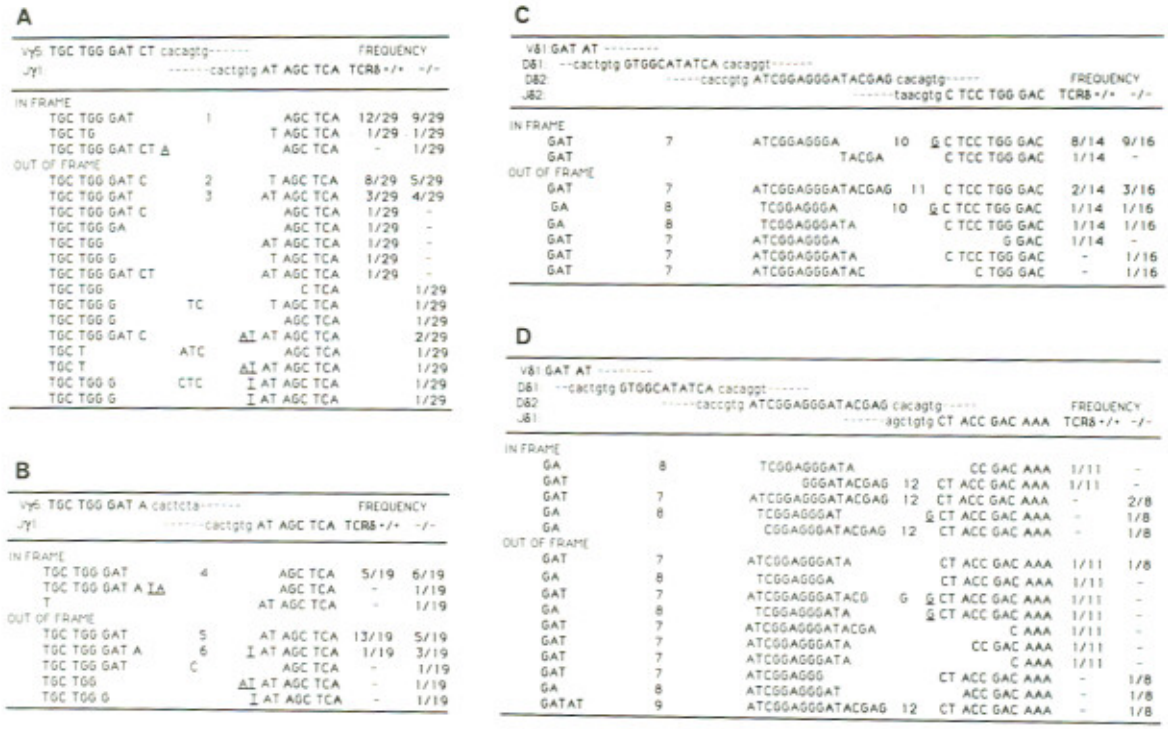

Figure 6. Nucleotide Sequences of Rearranged TCR γ and TCR δ Genes

Junctional sequences of Vγ5-Jγ1 (A), Vγ6-Jγ1 (B), Vδ1-Jδ2 (C), and Vδ1-Jδ1 (D) joints present in the fetal thymocytes from wild-type mice (+/+) and δ⁻ homozygotes (−/−). The sequences are aligned with germline sequences of TCR γ (Garman et al., 1986; Lafaille et al., 1989) and TCR δ (Chien et al., 1987) gene segments. The recombination signal sequences are shown in lowercase letters. P nucleotide(s) is underlined. The frequency of the clones with a particular sequence among the total number of clones randomly chosen and sequenced is listed in the last column. Numerical names (1–12) were assigned to junctional sequences that appeared multiple times among the clones sequenced in this and previous studies (Lafaille et al., 1989; Itohara and Tonegawa, 1990).

gotes (compare Figure 7f with Figure 7a). As shown in Table 3, the density of epidermal αβ T cells observed in δ⁻ homozygotes represented an approximately 17-fold increase compared with αβ T cells in epidermal sheets from δ⁻ heterozygotes. Although the density of αβ T cells was increased in the δ⁻ homozygotes, the density of epidermal CD3 ε⁺ T cells in these mice was still significantly reduced (about 20% of the densities observed in epidermal samples from δ⁻ heterozygotes). In all groups of mice, the bulk of the αβ T cells expressed a $CD4^-CD8^-$ phenotype (data not shown). The reduction of epidermal T cells in the δ⁻ homozygotes was also apparent when the density of Thy-1⁺ cells was examined and found to be about a third of the values obtained with samples from δ⁻ heterozygotes. Furthermore, in contrast with this latter group of mice, where >98% of the Thy-1⁺ cells expressed either αβ or γδ TCR, almost 40% of the Thy-1⁺ cells in the δ⁻ homozygotes expressed neither αβ nor γδ TCR. While this represented an approximately 8-fold increase versus the wild type in the density of this population of cells in the δ⁻ homozygotes, their density was still about 13% of the Thy-1⁺ γδ cells found in the δ⁻ heterozygous and wild-type littermates.

Thus, surface expression of TCR γδ seems to be required for the full representation of γδ T cells in epidermis. If the low level of $Thy\text{-}1^+TCR^-$ cells found in the epidermis of the δ⁻ homozygotes represents γδ lineage cells, this could mean that fetal γδ thymocytes can home to epidermis without TCR expression but cannot expand or be positively selected at that site. Alternatively, the $Thy\text{-}1^+TCR^-$ cells may represent a non-γδ T cell lineage as previously suggested for similar cells in nude mice (Nixon-Fulton et al., 1988).

Discussion

In this study, we examined the effects of a mutation in the constant region of the mouse TCR δ chain on the generation of γδ and αβ T cells, on the rearrangement of TCR γ and δ genes, on the generation of the highly restricted junctional sequences of the γδ TCR of the fetal T cell subsets, and on the homing of the γδ fetal thymocytes to epidermis. Below, we will discuss some of these issues separately.

Generation of γδ and αβ T Cells

As expected, we detected no sign of T cells bearing γδ TCR in the thymus or in the periphery of δ⁻ homozgotes using an anti-pan TCR δ MAb (3A10), and no protein was precipitated from the surface of mutant thymocytes with the anti-TCR γ MAb (KN365). In addition, since all CD3 ε⁺ cells were also stained with the anti-pan TCR β MAb

Table 2. Short Sequence Overlap at the V(D)J Junctions

Class	Type	Sequences	Frequency: Experiments A	B	C	Subtotal (%)	Total (%)
		Vγ5: TGCTGGG **AT** CTAGATC					
I	1	Jγ1: GCTAT **AT** AGCTCAG	21	26	45	92 (38)	183 (76) (Types 1–3)
		TGCTGGG **AT** AGCTCAG	58	45	139	242	242
		Vγ5: TGCTGGGATC **TAG** ATC					
I*	2	Jγ1: GCTATA **TAG** CTCAG	13	6	32	51 (21)	
		TGCTGGGATC **TAG** CTCAG	58	45	139	242	
		Vγ5: TGCTGGG **AT** CTAGATC					
II	3	Jγ1: GCT **AT** ATAGCTCAG	7	4	29	40 (17)	
		TGCTGGG **AT** ATAGCTCAG	58	45	139	242	
		Vγ6: TGCTGGG **ATA** TATCC					
I	4	Jγ1: GCTAT **ATA** GCTCAG	11	23	34	68 (31)	173 (80) (Types 4–6)
		TGCTGGG **ATA** GCTCAG	38	39	140	217	217
		Vγ6: TGCTGGG **ATATA** TCC					
I*	5	Jγ1: GCT **ATATA** GCTCAG	18	12	54	84 (39)	
		TGCTGGG **ATATA** GCTCAG	38	39	140	217	
		Vγ6: TGCTGGGA **TATAT** CC					
II	6	Jγ1: GC **TATAT** AGCTCAG	4	1	16	21 (10)	
		TGCTGGGA **TATAT** AGCTCAG	38	39	140	217	
		Vδ1: TCA **GATAT** ATATC					
I*	7	Dδ2: CC **GATAT** CGGAGGGA	34	33	67	119 (61)	142 (72) (Types 7–9)
		TCA **GATAT** CGGAGGGA	49	49	98	196	196
		Vδ1: TCAG **AT** ATATATC					
I	8	Dδ2: CCGAT **AT** CGGAGGGA	8	4	1	13 (7)	
		TCAG **AT** CGGAGGGA	49	49	98	196	
		Vδ1: TCAGAT **ATAT** ATC					
II	9	Dδ2: CCG **ATAT** CGGAGGGA	1	4	5	10 (5)	
		TCAGAT **ATAT** CGGAGGGA	49	49	98	196	
		Dδ2: GGAG **GGA** TACGAGCTCGT					
II	10	Jδ2: A **GGA** GCTCCTGG	19	13	58	90 (57)	118 (75) (Types 10–11)
		GGAG **GGA** GCTCCTGG	30	29	98	157	157
		Dδ2: GGAGGGATAC **GAGCTC** GT					
II	11	Jδ2: AG **GAGCTC** CTGG	5	7	16	28 (18)	
		GGAGGGATAC **GAGCTC** CTGG	30	29	98	157	
		Dδ2: GGAGGGATACG **AGCT** CGT					
II	12	Jδ1: GGT **AGCT** ACCGA	5	6	ND	11 (28)	11 (28) (Type 12)
		GGAGGGATACG**AGCT** ACCGA	19	21		40	40

The type numbers correspond to those of Figures 6A–6D. P nucleotides are underlined. The junctional sequences of a pair of germline gene segments and their recombinants are aligned on the basis of maximal sequence homology. The overlapping nucleotides around the junctions are shown in bold. The frequency shown indicates the number of clones with the particular junctional sequence among the total number of clones representing rearrangement between the indicated pair of germline gene segments (Experiment A, this study; Experiment B, Lafaille et al., 1989; Experiment C, Itohara and Tonegawa, 1990). The data from Experiment C include unpublished out-of-frame sequences.

ND, not determined.

(H57-597), it is highly unlikely that cells in the mutant mice express CD3 ε–associated TCR composed of γδ heterodimers, γ chains only, or a γ chain to which a truncated δ chain (i.e., Vδ domain) is bound. The possibility that the latter type of abnormal TCR is expressed without associated CD3 ε on the surface of some mutant cells has not been excluded. However, it is unlikely that such molecules would transduce any physiologically meaningful signal.

Table 3. Quantitation of Two-Color Immunofluorescence of DEC Cells

	TCR β or δ versus CD3		THY-1 versus TCR β and δ	
Genotype	β^+CD3^+/mm^2	δ^+CD3^+/mm^2	$THY\text{-}1^+/mm^2$	$THY\text{-}1^+TCR^-(\%)$
−/−	57.7(16.2)[a,b]	0	97.5(6.4)[b,c]	38.5(9.6)[c]
+/−	3.4(1.0)[a]	280.8(30.4)	293.0(53.1)[c]	1.6(1.0)[c]

Presented above is the mean (standard error) of those averages. Number of animals examined per group: TCR/CD3 −/−, n = 7; +/−, n = 9; THY1/TCR −/−, n = 5; +/−, n = 7.
Probability of oneway t statistic for comparison of means: [a] p = .001, [b] p = .0005, [c] p = .029, [d] p = .006.

Since γδ T cells appear prior to αβ T cells in thymic ontogeny, it has been proposed that γδ T cells may play a regulatory role in the generation of αβ T cells. For instance, a model incorporating this concept was proposed based on the phenotype of TCR V1-C4γ gene transgenic mice (Ferrick et al., 1989). However, our analysis of the TCR δ mutant mice revealed no effect of the mutation on the total number of αβ T cells generated in thymus or periphery, on the expression of several cell surface markers on these T cells, or on their T helper function. While further study is necessary to rule out a more subtle effect, our data suggest strongly that the development of αβ T cells is independent of γδ T cells. A reciprocal conclusion was previously drawn on the basis of the analysis of TCR α (Mombaerts et al., 1992a; Philpott et al., 1992) and TCR β mutant mice (Mombaerts et al., 1992a). Thus, all of these TCR mutant mice provide tools for the identification of functions unique to αβ T cells or γδ T cells.

Developmentally Programmed Rearrangement of TCR γ and TCR δ Genes

The issue of whether the differential generation of γδ T cell subsets is mediated by programmed TCR gene rearrangement or cellular selection has been addressed in the past. One approach has been to analyze nonproductive rearrangements in particular γδ T cell subsets (Asarnow et al., 1988; Takagaki et al., 1989; Raulet et al., 1991). Another was to follow the pattern of rearrangement in developing fetal thymus in vivo (Lafaille et al., 1989, 1990; Iwasato and Yamagishi, 1992) or ex vivo (Ikuta et al., 1990; Itohara and Tonegawa, 1990). These studies, however, have been inconclusive because of the paucity of data or the inability to dissociate the intrinsic rearrangement regulation from cellular selection. By contrast, our mutant mice circumvent both of these difficulties and demonstrate the existence of temporally programmed rearrangement of TCR γ and TCR δ genes. The mechanism of such differ-

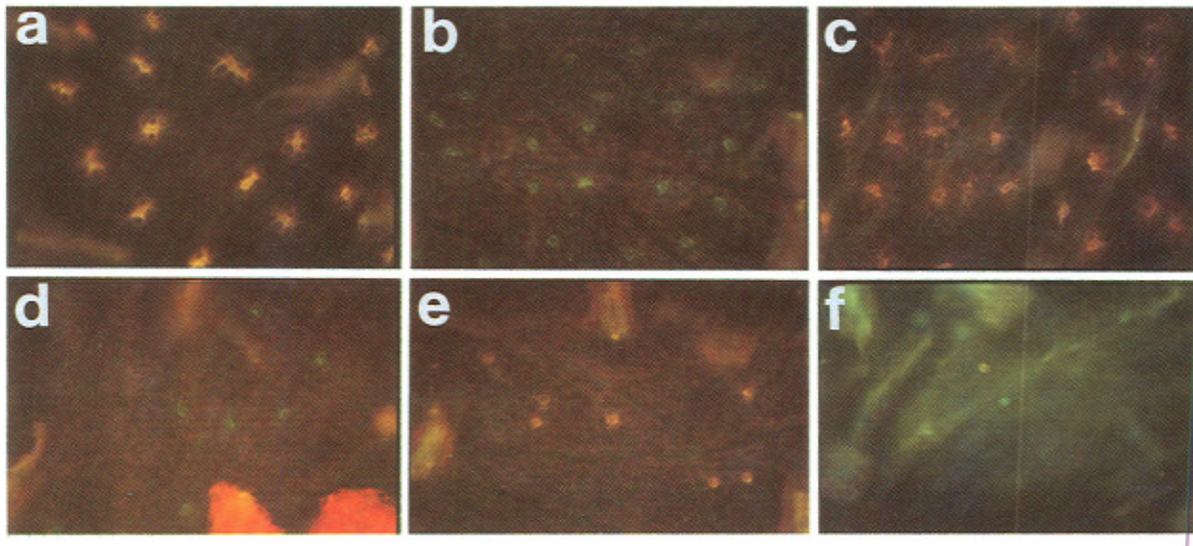

Figure 7. Immunofluorescent Labeling of Epidermal Sheets

Epidermal sheets prepared from δ^- heterozygotes ([a]–[c]) and δ^- homozygotes ([d]–[e]) were processed for the demonstration of simultaneous expression of CD3 ε and TCR δ chains ([a] and [d]), CD3 ε and TCR β chains ([b] and [e]), and Thy-1 and TCR δ and β chains ([c] and [f]). Fluoresceinated anti-CD3 ε ([a], [b], [d], and [e]) antibodies were used in conjunction with biotinylated anti-pan TCR δ chain antibodies ([a] and [d]) or anti-pan TCR β chain antibodies ([b] and [e]). Fluoresceinated anti-Thy-1 antibodies ([c] and [e]) were used in conjunction with a mixture of biotinylated anti-pan TCR δ chain and anti-pan TCR β chain antibodies ([d] and [f]). Biotinylated antibodies were detected with streptavidin–Texas red conjugates. Approximate magnification, 400×.

ential rearrangement is unknown, but an obvious possibility is that a set of cis-acting regulatory elements associated with the TCR γ and TCR δ gene loci play a key role by rendering individual gene segments differentially accessible for V(D)J joining. The potential for differential development of γδ T progenitor cells in fetal liver and adult bone marrow has previously been observed (Ikuta et al., 1990). The program directing rearrangement may be an important component of the differentiation potential of the progenitor cells.

Short Sequence Homology Helps Generate Canonical γδ TCR

Programmed rearrangement (this study), the lack of N-region additions (Asarnow et al., 1988; Lafaille et al., 1989) resulting from a reduced level of terminal deoxynucleotidyl transferase activity (Rothenberg and Triglia, 1983), and the reduced trimming by the putative exonuclease (Lafaille et al., 1989) all contribute to the generation of canonical TCR for the Vγ5-Vδ1 and Vγ6-Vδ1 T cell subsets. However, the issue of whether site-restricted recombination (molecular constraint model) or TCR-mediated cellular selection (cellular selection model) play a major role in the production of the canonical TCR has remained unsettled. The finding that the junctional sequences of rearranged Vγ5 and Vδ1 genes in fetal thymocytes and epidermal γδ T cells showed a limited diversity in nonproductive rearrangements but almost none in productive rearrangements argued for the cellular selection model (Lafaille et al., 1990). In contrast, the finding that the Vδ1-Dδ2 and Vγ6-Jγ1 junction show an unusually high degree of precision even in nonproductive rearrangements (Chien et al., 1987; Lafaille et al., 1989; Allison and Havran, 1991) was consistent with the molecular constraint model.

In an effort to block cellular selection, Itohara and Tonegawa (1990) added the MAb 3A10 (specific for TCR δ) to fetal thymus organ culture, in which the monospecific fetal γδ T cell subsets are normally generated. This led to an increase in the frequency of productive Vγ5, Vγ6, and Vδ1 rearrangements with noncanonical junctions, supporting the cellular selection model. However, as shown here, a similar increase in the frequency of noncanonical junctions was not observed in the thymus of the TCR δ mutant mice in which the putative TCR-mediated positive selection must have been eliminated. This latter finding strongly supports the molecular constraint model. It also suggests that the effect of the anti-TCR δ MAb in the earlier fetal organ culture experiment was not simply inhibition of positive selection. Perhaps the antibody treatment led to the expansion of rare cells expressing noncanonical TCRs, with little effect on cells expressing the canonical TCR, which presumably encountered endogenous ligands in the culture.

Thus, the unusual homogeneity of the TCR of Vγ5-Vδ1 and Vγ6-Vδ1 subsets appears to result from multiple processes, namely rearrangement of specific V segments (i.e., programmed rearrangements), absence of N-region additions, limited trimming of coding ends, and site-restricted recombination guided by short homologous regions at the breakpoints (molecular constraint model; see below) and by TCR-mediated positive selection (cellular selection model).

The hypothesis that relatively homogeneous junctions are formed in V(D)J joining using a short homology at the breakpoint has previously been proposed for neonatal B cells (Ichihara et al., 1989; Gu et al., 1990; Feeney, 1990). In those studies, only the homology provided by germline gene segments was considered. In a more recent study, Feeney (1992) included P nucleotides for the homology survey, but found that their contribution is minimal: P nucleotides were involved in the junctional homology in only 5 out of 25 junctional sequences studied, and in each of these cases only one P nucleotide contributed to the homology. By contrast, P nucleotides seem to play a much more pronounced role in the homology-mediated V(D)J joining of fetal TCR γ and δ genes. For instance, the highly homogeneous D2-Jδ2 junction (type 10, Figure 6C and Table 2) seems to be mediated by a 3 nt homology that is provided on the Jδ2 side entirely by P nucleotides. Likewise, most of the productively rearranged fetal TCR δ genes contain the type 7 Vδ1-D2 junction that seems to be mediated by a 5 nt homology, 3 nt of those on the D2 side being provided by P nucleotides (Figure 6C; Table 2).

Neither this nor previous studies provide direct evidence for the guiding or promoting role of the short homology in V(D)J joining. However, as pointed out previously (Ichihara et al., 1989; Gu et al., 1990; Feeney, 1990) and as confirmed here, there is a strong correlation between the existence of such homology and its employment as a recombination breakpoint. For instance, among the 25 Vγ5-Jγ1 junctional sequences that appear only once (Table 2; unpublished data), none had an overlap of three or more

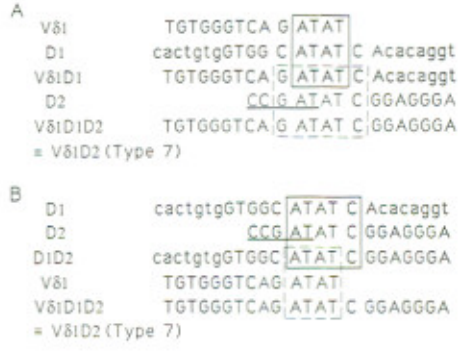

Figure 8. Possible Vδ1-D1-D2 Joining in Fetal Thymuses Mediated by Short Homology

(A) Vδ1-D1 joining is mediated by a tetranucleotide homology ATAT. Recombination between the joined Vδ1-D1 and D2 is mediated by a hexanucleotide homology GATATC.

(B) D1-D2 joining is mediated by a pentanucleotide homology ATATC. Recombination between the joined D1-D2 and Vδ1 is mediated by a tetranucleotide homology ATAT.

Note that the final Vδ1-D1-D2 recombinants of (A) and (B) are identical and are equivalent to the type 7 Vδ1-D2 recombinant. In both (A) and (B), the homologies in the first and the second recombination are indicated by solid line and dotted line boxes, respectively. P nucleotides of D2 are underlined, and recombination signal sequences of D1 are indicated in lowercase letters.

nucleotides, five had only a 1 nt overlap, and two had a 2 nt overlap, even after P nucleotides were considered. Likewise, among 19 Vγ6-Jγ1 junctional sequences that appeared only once (Table 2; unpublished data), none had an overlap of three or more nucleotides, and only one each had a 1 or 2 nt overlap. We conclude that the joining ends of the fetal TCR γ and TCR δ gene segments have evolved to be particularly well suited for the short homology-mediated recombination.

Lack of D1 Segment in Fetal TCR δ Genes

The TCR δ gene preferentially expressed in the fetal thymus is almost always composed of Vδ1-D2-Jδ2 and Cδ gene segments with no apparent participation of the D1 gene segment (Chien et al., 1987; Lafaille et al., 1989). This is in contrast with the TCR δ genes utilized by adult thymocytes that are composed of one of several different Vδ gene segments joined to D1, D2, and Jδ1 or Jδ2 gene segments (Elliott et al., 1988; Takagaki et al., 1989; Lacy et al., 1989). The lack of participation of the D1 gene segment in the generation of fetal TCR δ genes could be a part of the developmental program that controls the accessibility of TCR gene segments. However, the homology-mediated recombination discussed above offers an alternative explanation.

The only substantial homology that could be used for a homology-mediated Vδ1-D1 joining is provided by the tetranucleotides ATAT present at the end of the Vδ1 segment and at the sixth nucleotide position of the D1 gene segment (Figure 8A). However, since the tetranucleotide ATAT is a part of a hexanucleotide GATATC that is likely to be utilized in a subsequent homology-mediated joining between the joined Vδ1-D1 and the D2 gene segments (Figure 8A), the end result of the Vδ1-D1-D2 joining becomes indistinguishable from the type 7 Vδ1-D2 joining (Figure 8A). The same outcome is reached when a D1-D2 joining occurs first and is followed by a Vδ1-D1-D2 joining (Figure 8B). Thus, in fetal thymuses, the D1 gene segment may in fact participate in the generation of TCR δ genes, but because of the particular distribution of short homology among the Vδ1, D1, and D2 gene segments, the homology-mediated recombination excludes any D1-specific sequence from the final product. Alternatively, the Vδ1 gene segment may directly join with the D2 gene segment through the pentanucleotide GATAT homology (type 7, Table 2). In any case, the apparent lack of the D1-coded sequence in the fetal TCR δ genes seems to be a result of a strategy developed during evolution that utilizes homology-mediated recombination to minimize junctional diversity. In the TCR δ genes produced in adult thymuses where junctional diversity is desired, the D1-coded sequence appears more frequently because the homology-mediated recombination plays a greatly reduced role.

Experimental Procedures

Construction of Targeting Vectors

The targeting vectors (pPMKO-20 and pPMKO-21) were constructed from cosmid clone TA9 isolated from AKR/J mice (Winoto and Baltimore, 1989). The fragments used were a 4 kb EcoRI fragment between Jδ2 and the first exon of Cδ; an 8.5 kb KpnI–EcoRI fragment, downstream of Cδ; a 1.8 kb *pgk–neo* gene derived from pKJ1 (McBurney et al., 1991; a gift from Michael A. Rudnicki); and a 1.9 kb *MC1–tk* gene cassette derived from pMC1-tk (Mansour et al., 1988; a gift from Mario Capecchi).

Targeting Experiment

The ES cell lines used were D3 (Gossler et al., 1986) and E14 (Hooper et al., 1987). Cell culture and the targeting experiment were carried out as described previously (Mombaerts et al., 1992b). In brief, 5 × 10^7 ES cells were electroporated with a Bio-Rad Gene Pulser (800 V, 3 mF; electrode distance, 0.4 cm), using 20–75 mg of DNA. Prior to electroporation, pPMKO-20 and pPMKO-21 were linearized by digestion with NotI and SfiI, respectively. The electroporated cells were plated in ten 10 cm dishes coated with mitotically inactivated embryonic fibroblasts derived from *neo*-transgenic mice (Gossler et al., 1986). G418 (GIBCO) at 125–150 mg/ml was added 24 hr after electroporation, and ganciclovir (a gift from Syntex, Palo Alto, California) at 2 mM was added after 4–7 days to most of the plates. Colonies were picked at day 7–8 of selection into wells of 24-well plates, and one half of the cells was stored in liquid nitrogen until the completion of screening. The other half of the cells was screened by digestion with BamHI and hybridization with a 5′ external probe (probe 1), a 0.2 kb SacI–EcoRI fragment. The homologous recombination event was confirmed by hybridization with a 3′ external probe (probe 3), a 0.35 kb AluI fragment, a 5′ *pgk–neo* probe (probe 4; EcoRI–BamHI), and a 3′ *pgk–neo* probe (probe 5; BamHI–HindIII). Southern blots were analyzed using a Fujix Bio-Image Analyzer BAS2000.

Generation of Chimeric Mice and Screening of Mutant Mice

Chimeras were generated as described (Bradley, 1987). In brief, approximately 15 ES cells were injected into C57BL/6J or BALB/cJ blastocysts at 3.5 days postcoitum. After injection, the embryos were transferred into the uteri of pseudopregnant (C57BL/6 × DBA/2)F1 mice. δ^- heterozygotes were obtained by crossing the chimeras to either C57BL/6J or BALB/cJ mice. To determine the genotype of the mice, DNA was prepared from tails at the time of weaning, and Southern blots of EcoRV digests were hybridized with probe 4 (a 2.8 kb EcoRV–KpnI fragment containing the sequences between Cδ and Vδ5).

Epidermal Sheet Preparation and Immunocytochemistry

Epidermal sheets were prepared for two-color immunofluorescence as described by Boorsma et al. (1986) with minor modifications. In brief, after sacrificing by cervical dislocation, mice were shaved and depilated with Nair (Carter–Wallace, New York, New York). The skin was dissected, mounted on dermatome tape (Padgett, Kansas City, Kansas) epidermal side down, cut into pieces, and gently pressed onto glass slides. After incubation at 37°C in Hanks' balanced salt solution containing 20 mM EDTA, the dermis was gently peeled away, leaving the epidermis attached to the slide. Slides with mounted epidermal sheets were stored at 4°C and then processed the next day for two-color immunofluorescence.

Two-color immunofluorescence was performed as previously described (Farr et al., 1991). Fluorescein isothiocyanate (FITC) conjugates of anti-CD3 (clone 500A2), anti-CD4 (clone GK1.5), and anti-CD8 (clone 53-6.7, Becton-Dickinson, Mountain View, California) were used in conjunction with biotinylated anti-pan TCR δ (clone 3A10; Itohara et al., 1989) and anti-pan TCR β (H57-597; Kubo et al., 1989) along with streptavidin–Texas red (Molecular Probes, Eugene, Oregon) as a secondary reagent. Tissue samples were coverslipped with fluoromount G (Southern Biotechnology Associates, Birmingham, Alabama). For quantitation of cell densities, 25 randomly chosen graticule fields of 0.16 mm^2 each were scored and averaged per animal.

Flow Cytometric Analysis

Flow cytometric analysis was carried out as described previously (Itohara et al., 1989; Mombaerts et al., 1992b) using a FACScan (Becton-Dickinson). The data were analyzed with the FACScan Research Software. Reagents used were 2C11–FITC conjugate (anti-CD3 ε; Leo et al., 1987), H57-597–biotin conjugate (anti-pan TCR β; Kubo et al., 1989), 3A10–biotin conjugate (anti-pan TCR δ; Itohara et al., 1989), 536–FITC conjugate (anti-TCR Vγ5; Havran and Allison, 1988), GK1.5–phycoerythrin conjugate (anti-CD4; Becton-Dickinson), 53-

6.7–FITC or 53-6.7–biotin conjugates (anti-CD8; Becton-Dickinson), 30-H12 (anti-Thy1.2; Becton-Dickinson), and streptavidin–phycoerythrin conjugate (Becton-Dickinson).

Cell preparation from organs was carried out as described previously (Bonneville et al., 1990).

PCR Analysis

Total cellular DNA was prepared from thymocytes and several γδ T cell hybridomas (Takagaki et al., 1989; Ito et al., 1989) by an SDS–proteinase K–phenol method and recovered by ethanol precipitation. Hybridomas used were KN12 (for Vδ4-Jδ1, Vγ1-Jγ4, and Vγ4-Jγ1 rearrangements), KN106 (for Vδ5-Jδ1 and Vγ7-Jγ1 rearrangements), KI21 (for Vδ1-Jδ2 and Vγ6-Jγ1 rearrangements), and V17 (for Vγ5-Jγ1 rearrangement). The DNA was digested with EcoRI, and the concentration was determined by a spectrophotometer prior to the PCR reaction (Saiki et al., 1988). To prepare the standard curves, the hybridoma DNAs were serially diluted with a DNA solution derived from the PCC3 embryonal carcinoma cell line (Avner et al., 1978) lacking any TCR γ and δ gene rearrangement, and the mixture was subjected to PCR reaction that was performed using the GeneAmp kit (Perkin–Elmer–Cetus) and a thermal cycler (Perkin–Elmer–Cetus). Each cycle consists of incubation at 94°C for 45 s, followed by 2 min annealing (at 50°C for γ genes, at 60°C for Vδ1, and at 58°C for Vδ4 and Vδ5 genes) and by extension for 1 min at 72°C. Before the first cycle, a 1 min 94°C denaturation step was included, and after the 30 cycles the extension at 72°C was prolonged to 7 min. The reaction mixture (50 ml) included 400 ng of EcoRI-digested DNA, 50 pmol of each primer, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.0l% gelatin, and 1.25 U of Taq polymerase. Samples (5 μl) were electrophoresed in 1.5% agarose gel, blotted onto the GeneScreen Plus filters (New England Nuclear), and probed with end-labeled oligonucleotides shown below. Primers used were STJL037 for Vγ1, 5′-CCGGCAAAAAGCAAAAAAGTT-3′; STP073 for Vγ4, 5′-TGTCCTTGCAACCCCTACCC-3′; STPO94 for Vγ5, 5′-TGTGCACTGGTACCAACTGA-3′; STSI002 for Vγ6, 5′-AGTGTTCAGAAGCCCGATGCA-3′; STSI007 for Vγ7, 5′-ACAACTGTGGTGGATTCCAGA-3′; STPI00 for Jγ1, 5′-CAGAGGGAATTACTATGAGC-3′; STSI005 for Jγ4, 5′-ACTACGAGCTTTGTCCCTTTG-3′; STSI12 for Vδ1, 5′-GGGATCCTGCCTCCTTCTACTG-3′; STP075 for Vδ4, 5′-CCGCTTCTCTGTGAACTTCC-3′; STP082 for Vδ5, 5′-CAGATCCTTCCAGTTCATCC-3′; STSI15 for Jδ1, 5′-CAGTCACTTGGGTTCCTTGTCC-3′; STSI18 for Jδ2, 5′-CAAAGAGCTCTATGCCAGTTCC-3′. Probes used were STSI006 for Jγ1, 5′-TGAATTCCTTCTGCAAATACCTTG-3′; STSI009 for Jγ4, 5′-AAATATCTTGACCCATGATGTG-3′; STSI10 for Jδ1, 5′-GTTCCTTGTCCAAAGACGAGTT-3′; STSI11 for Jδ2, 5′-GTTCCAAAAAACATCTGTCGGG-3′.

DNA Sequencing

The PCR products were fractionated by electrophoresis in 1.5% agarose gel, and the appropriate fragments were purified by using a glass bead method (Mermaid, BIO101). The fragments were subcloned into the SmaI site of pUC13 and sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) with Sequenase Version 2.0 (U. S. Biochemicals), following the instructions of the manufacturer.

Cell Surface Labeling and Immunoprecipitation

Cell surface radioiodination and immunoprecipitation under nonreducing conditions were carried out as described previously (Maeda et al., 1987). In brief, 1.5 × 10^7 thymocytes from fetuses at day 16.5 of gestation were labeled with ^{125}I by a lactoperoxidase method, and the cells were lysed with the lysis buffer (10 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The lysates were denatured by treatment with 1% SDS at 68°C for 5 min, and then 4 vol of 1.5% Triton X-100 in 10 mM Tris-buffered saline (pH 8.0) were added. After repeated clearing with affinity-purified anti-mouse immunoglobulins (TAGO) and protein G–Sepharose 4B (Pharmacia), the denatured lysates were immunoprecipitated with protein G–Sepharose 4B beads precoated with a MAb (KN365) specific to Cγ1 (Maeda et al., 1987). Precipitates solubilized with SDS and 2-mercaptoethanol were electrophoresed in a 10%–20% linear gradient polyacrylamide gel. Radioactivity of the gel was analyzed using the Fujix Bio-Image Analyzer BAS2000.

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