Rearrangement and Junctional-Site Sequence Analyses of T-Cell Receptor Gamma Genes in Intestinal Intraepithelial Lymphocytes from Murine Athymic Chimeras

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The molecular organization of rearranged T-cell receptor (TCR) γ genes in intraepithelial lymphocytes (IEL) was studied in athymic radiation chimeras and was compared with the organization of γ gene rearrangements in IEL from thymus-bearing animals by polymerase chain reaction and by sequence analyses of DNA spanning the junction of the variable (V) and joining (J) genes. In both thymus-bearing mice and athymic chimeras, IEL V-J γ -gene rearrangements occurred for V γ 1.2, V γ 2, and V γ 5 but not for V γ 3 or V γ 4. Sequence analyses of cloned V-J polymerase chain reaction-amplified products indicated that in both thymus-bearing mice and athymic chimeras, rearrangement of V γ 1.2 and V γ 5 resulted in in-frame as well as out-of-frame genes, whereas nearly all V γ 2 rearrangements were out of frame from either type of animal. V-segment nucleotide removal occurred in most V γ 1.2, V γ 2, and V γ 5 rearrangements; J-segment nucleotide removal was common in V γ 1.2 but not in V γ 2 or V γ 5 rearrangements. N-segment nucleotide insertions were present in V γ 1.2, V γ 2, and V γ 5 IEL rearrangements in both thymus-bearing mice and athymic chimeras, resulting in a predominant in-frame sequence for V γ 5 and a predominant out-of-frame sequence for V γ 2 genes. These findings demonstrate that (i) TCR γ -gene rearrangement occurs extrathymically in IEL, (ii) rearrangements of TCR γ genes involve the same V genes regardless of thymus influence; and (iii) the thymus does not determine the degree to which functional or nonfunctional rearrangements occur in IEL.

In peripheral lymphoid tissues, most T cells are critically dependent on the thymus for maturation and development. Those events involve complex developmental processes which include the acquisition of T-cell-specific membrane markers and the T-cell receptor (TCR)/CD3 molecular complex (41). Also occurring within the thymus is rearrangement of TCR genes for both TCR- $\alpha\beta$ and TCR- $\gamma\delta$ T cells (9, 41), as well as events which shape and define the peripheral T-cell repertoire within that host. The latter process includes steps in the "education" of T cells which impart upon the emerging T-cell repertoire the ability to recognize foreign antigens in the context of host-derived major histocompatibility complex molecules (positive selection) (4, 27, 28, 51) and the ability to effect the elimination of potentially destructive autoreactive T-cell specificities from that repertoire (negative selection) (5, 13, 19, 47). Thus, education of precursor T cells during passage through the thymus is considered to be an important and essential stage in the development of mature peripheral T cells.

T cells can be broadly categorized according to the type of TCR utilized, i.e., either an $\alpha\beta$ or a $\gamma\delta$ TCR heterodimer. Additionally, $\gamma\delta$ T cells display several differences from $\alpha\beta$ T cells, such as the limited array of γ and δ variable (V)-region genes compared with the considerably greater number of V-region genes available to $\alpha\beta$ T cells (33). However, despite the limited number of V γ and V δ genes available for generating combinatorial diversity, some though not necessarily all $\gamma\delta$ T cells exhibit considerable potential for additional diversity resulting from junctional-site modifications through exonuclease-mediated nucleotide

removal from V and joining (J) regions and through N-segment nucleotide insertions between V and J for the γ chain (26). Thus, it has been calculated that the overall potential TCR diversity of $\gamma\delta$ T cells exceeds that of $\alpha\beta$ T cells (9). A second distinctive feature of peripheral $\gamma\delta$ T cells is the site-specific anatomical distribution of some $\gamma\delta$ T cells as determined on the basis of V γ gene utilization. In mice, for example, $\gamma\delta$ T cells located in the purlieu are largely restricted in V γ gene utilization. Thus, $\gamma\delta$ T cells in the skin epidermis utilize almost exclusively the V γ 3 gene (1, 2, 12, 16) (nomenclature of R. D. Garman, et al. [reviewed in reference 33]). By contrast, TCR- $\gamma\delta$ intestinal intraepithelial lymphocytes (IEL) preferentially use V-region genes encoded by V γ 5 (1, 25, 43), and possibly V γ 1.2 (43), V γ 2 (1, 25, 43), and/or V γ 4 (1, 25), but virtually no genes encoded by V γ 3 (1, 25, 43).

Although thymus-dependent maturation of T cells has been regarded as an essential stage in the development of most peripheral T cells, as discussed above, there is longstanding evidence suggesting that the IEL differ from other T cells in that regard. Over 20 years ago, Fichtelius predicted that lymphocytes of the gut epithelium (then called theliolymphocytes) are non-thymus-derived hematopoietic cells (11, 12). Subsequent studies using various experimental athymic systems have, to one degree or another, led to similar conclusions (10, 15, 22, 29, 46), and our laboratory recently demonstrated that functionally mature IEL expressing either TCR- $\alpha\beta$ or TCR- $\gamma\delta$ develop from bone marrow precursors in athymic chimeras (30), thus confirming extrathymic maturation of IEL. As yet no studies have examined the molecular organization of TCR gene arrangements in IEL generated in an athymic environment. In this study, we have addressed three fundamental questions that bear

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directly on issues of IEL development and have important implications regarding the mammalian immune system overall. We sought to determine first, to what extent TCR γ -gene rearrangements occur in IEL in the absence of thymus influence; second, whether differences exist in V γ gene utilization in IEL arising in athymic versus thymus-bearing mice; and third, whether TCR γ -gene rearrangement occurs in IEL in athymic chimeras and whether differences exist in the nature of diversity of IEL $\gamma\delta$ T cells between thymus-bearing and athymic animals.

MATERIALS AND METHODS

Mice. C57BL/6 and CB6F1 [(BALB/c $\times$ C57BL/6) F_1] mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were housed at the University of Tulsa vivarium.

Construction of athymic radiation chimeras. Procedures for preparing F_1 parent athymic radiation chimeras have been described elsewhere (30). Briefly, C57BL/6 (H-2^b) mice were thymectomized at 8 to 10 weeks of age by aspiration of thymus tissue through a transsternal incision. The incision was closed with an autoclip, and mice were allowed to recover for 2 to 3 weeks. Thymectomized mice were irradiated with 950 rads from a ¹³⁷Cs source and reconstituted within 4 h with 10×10^6 CB6F1 (H-2^d $\times$ H-2^b) F_1 bone marrow (BM) stem cells treated with two cycles of anti-Thy-1, anti-CD4, and anti-CD8 monoclonal antibodies (see below) plus complement (Cedarlane Laboratories, Westbury, N.Y.) to remove mature T cells. Control mice consisted of sham-thymectomized animals. IEL from athymic chimeras were used 3 to 6 months after chimera construction.

Isolation of lymphocytes. Methods of extracting murine IEL from small intestine epithelia by Percoll-gradient fractionation have been described elsewhere (22, 23, 30, 31). Briefly, mice were sacrificed by cervical dislocation and the small intestine was removed. After removal of intestine segments containing Peyer's patches, tissues were cut into 1- to 1.5-cm pieces, flushed of fecal material, and opened longitudinally to expose the epithelium. Tissues were minced into 3- to 4-mm pieces and stirred for 30 min at 37°C in phosphate-buffered saline containing 1 mM EDTA. Extracted cells were filtered rapidly through nylon wool, pelleted by centrifugation, suspended in 12 ml of 40% isotonic Percoll (Sigma Chemical, St. Louis, Mo.), and centrifuged for 20 min at $600 \times g$. IEL were recovered from the pelleted portion, and erythrocytes were lysed by hypotonic shock. Spleen cell suspensions were prepared by pressing tissues through a 60-mesh stainless steel screen.

Antibodies and flow cytometry. Antibodies used in this study were anti-leukocyte common antigen (T200) (M1/89.18.7.HK) (42), anti-CD3- ϵ (KT3) (45), anti-Thy-1.2 (J1j.10) (7), anti-CD4 (RL172.4) (8), anti-CD8 (3.155) (39), anti-CD4 (phycoerythrin [PE]-coupled GK1.5) (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.), anti-CD8 (fluorescein isothiocyanate [FITC]-coupled anti-Lyt-2) (Becton Dickinson), biotinylated anti-TCR- $\alpha\beta$ (H57.597) (PharMingen, San Diego, Calif.), biotinylated anti-TCR- $\gamma\delta$ (GL3) (PharMingen), biotinylated anti-H-2D^d (PharMingen), and PE-coupled streptavidin and PE-coupled anti-rat immunoglobulin G (Jackson ImmunoResearch, West Grove, Pa.). Normal rat and normal hamster immunoglobulin was used for control staining. For one-color analyses, cells were reacted with a control antibody or with anti-CD3 or anti-TCR monoclonal antibody, washed, and reacted with

PE-coupled streptavidin or PE-coupled anti-rat immunoglobulin G. For two-color analyses, cells were reacted with control antibody or with PE-coupled anti-CD4 and FITC-coupled anti-CD8 monoclonal antibody and washed. Cells were fixed in formalin and analyzed on an EPICS 751 flow cytometer interfaced to an MDADS II computer (Coulter Electronics, Hialeah, Fla.). Data were collected on 5,000 cells (one-color analyses) or 25,000 cells (two-color analyses), and percentages of cells expressing antigenic markers were determined relative to control staining. In all experiments, IEL were discriminated from nonhematopoietic enterocytes according to forward-angle and right-angle light scatter and based on expression of the T200 leukocyte common antigen as previously described (23, 30, 31).

PCR, cloning, and sequence analyses. IEL were isolated individually from four athymic chimeras and three thymus-bearing mice and were used to prepare genomic DNA by a standard protocol (37). DNAs were studied separately from individual mice (three thymus-bearing and four athymic mice) rather than as pooled preparations, to permit evaluation of similarities or differences in γ -gene use and diversity between mice. Isolated DNA sequences spanning the V-J junction were amplified by polymerase chain reaction (PCR), using V γ -specific primers and a consensus J γ primer described by others (1) (Midland Certified Reagents, Midland, Tex.). Note that the V γ 1.2 primer used here hybridizes to V γ 1.1 and V γ 1.2 (1). Primer sequences were as follows:

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V $\gamma$ 1.2 5'-ACATTGGTACCGGCAAAAAAC-3'
V $\gamma$ 2 5'-GGGGGAATTCCTCCCTACCCATATTTTCTT-3'
V $\gamma$ 3 5'-GCACTGGATCCAAGTAAAGAAG-3'
V $\gamma$ 4 5'-CCAAAGAATTCTGTGTAGTTC-3'
V $\gamma$ 5 5'-TCCACTGGTACCGATTCCAAGAA-3'
J $\gamma$  5'-GGGAAGCTTACCAGAGGGAATTACTATGAG-3'
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PCR amplification of DNA was done in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) with a Gene-Amp *Taq* (35) polymerase kit (Perkin-Elmer Cetus), using a 33-cycle program of 1 min at 94°C, 2 min at 60°C, and 2 min at 72°C. An additional incubation of 7 min at 72°C was included at the end of the program to complete the product extension. PCR products were analyzed on 1.5% agarose gels to determine whether gene rearrangement had occurred. PCR products of rearranged genes were digested with *Eco*RI and *Hind*III for V γ 2-J γ 1 products and with *Hind*III and *Kpn*I for V γ 1.2-J γ 2 and V γ 5-J γ 1 products. Digested products were cloned by ligation into an appropriately cut pUC19 vector and transformed into competent DH5 α cells (Bethesda Research Laboratories, Gaithersburg, Md.). Recombinant clones were picked from an average of 150 clones per ligation, identified by a lack of color (Blue-gal; Bethesda Research Laboratories); clones were confirmed by PCR amplification of inserts. Clones containing inserts were sequenced by the procedure of Sanger et al. (38), using a fluorescent dye-labeled universal M13 sequencing primer (Applied Biosystems, Foster City, Calif.) with *Taq* polymerase (20, 32). The primer was extended by linear PCR, using 10 cycles of 1 min at 94°C, 1 min at 65°C, and 4 min at 72°C. Reaction products were analyzed on a Biosystems 370A automated DNA sequencer (Applied Biosystems) (48).

RESULTS

Characterization of IEL from athymic chimeras. We recently demonstrated that IEL from athymic chimeras are nearly identical to IEL from thymus-bearing mice with regard to the presence and distribution of T-cell subsets and

FIG. 1. One-color (CD3, TCR-αβ, and TCR-γδ) and two-color (CD4 and CD8) log fluorescence flow cytometric analyses of spleen cells and IEL from athymic chimeras 4 months after chimera construction. Numbers within the histograms indicate percentage reactivity relative to background staining due to normal rat or normal hamster immunoglobulin.

with regard to the expression of TCR and CD3 (30). In the present study, IEL from athymic chimeras were characterized phenotypically as a preliminary indicator that extrathymic maturation had occurred. As shown in Fig. 1, IEL isolated from athymic chimeras consisted of 85 to 87% CD3⁺ CD8⁺ T cells; CD4⁺ IEL included both the CD4⁺CD8⁻ and the CD4⁺CD8⁺ IEL subsets (30, 31). IEL consisted of TCR-αβ⁺ and TCR-γδ⁺ T cells (Fig. 1), as reported for IEL in general (3, 6, 10, 15, 18, 46) and for IEL in athymic chimeras (30). Unlike IEL, spleen cells from athymic chimeras were notably depleted of T cells. Approximately 90% of spleen cells were CD3⁻ CD4⁻ CD8⁻ cells (Fig. 1) of which 89% expressed surface immunoglobulin (data not

shown). Cells expressing surface TCR were minimally present among spleen cells from athymic chimeras (Fig. 1). As reported previously (30), repopulation of IEL in athymic chimeras by donor BM was carefully monitored on the basis of expression of the H-2D^d class I major histocompatibility antigen. Note that 97% of IEL from chimera mice expressed H-2D^d (Fig. 2C), whereas IEL from C57BL/6 (H-2^b) mice, the host strain in F₁ parent chimeras, did not express H-2^d (Fig. 2B), thereby confirming that virtually all IEL in athymic chimeras were of donor BM origin and were not residual host T cells.

TCR γ-gene rearrangement in peripheral T cells from athymic chimeras and thymus-bearing mice. Genomic DNAs from spleen cells and IEL were isolated from thymus-bearing control mice and athymic chimeras and were examined for evidence of TCR γ-gene rearrangement in PCR-amplified material. Rearranged TCR γ genes were identified as a V-J product of 200 to 300 bp, using the primer sequences described above. V and J segments of unrearranged γ genes span a distance of several thousand bases (33) and would not be detected under the conditions of this study. As shown in Fig. 3, rearrangement of Vγ1.2, Vγ2, Vγ3, and Vγ5, but not Vγ4, genes had occurred in spleen cells from thymus-bearing mice. In contrast, γ-gene rearrangements in spleen cells from athymic chimeras occurred for Vγ5 and possibly for Vγ2, though not for Vγ1.2, Vγ3, or Vγ4.

The pattern of TCR γ-gene rearrangement was identical for IEL from thymus-bearing mice and athymic chimeras. In both types of animals, rearrangement of γ genes occurred for Vγ1.2, Vγ2, and Vγ5 (Fig. 3). PCR products were not obtained for Vγ3 or Vγ4 from either thymus-bearing or athymic mice. It should be noted that in these sets of experiments, no PCR products were obtained from control DNA from liver or by PCR amplification using Vγ and Jγ primers in the absence of template (data not shown). Collectively, these findings are generally consistent with the limited number of studies which have examined TCR γ-gene utilization in thymus-bearing mice (1, 25, 43) and demonstrate that rearrangement of TCR γ genes in IEL is independent of thymus influence.

Molecular organization of rearranged TCR γ genes in IEL from athymic chimeras and thymus-bearing mice. Rearrangement of TCR genes does not imply that a functional gene has been constructed, given that rearrangement may lead to construction of a gene that is not in the proper translational reading frame. That outcome is the consequence of diversity-generating mechanisms involving nucleotide removal from the 3' end of the V segment and the 5' end of the J

FIG. 2. Expression of H-2D^d. IEL from athymic chimeras (C) expressed H-2D^d as a result of F₁ BM repopulation of C57BL/6 mice. IEL from C57BL/6 mice (B), the host strain of BM radiation chimeras, did not express H-2D^d. (A) Staining with isotype-matched control antibodies. Numbers within the histograms indicate percentage reactivity.

FIG. 3. Evaluation of TCR γ -gene rearrangement in splenic (A and B) and IEL (C and D) lymphocytes from thymus-bearing mice (A and C) and athymic chimeras (B and D) as determined by migration of PCR-amplified γ genes spanning V and J on 1.5% agarose gels. Numbers below the lanes indicate the V γ primer used; 1 refers to V γ 1.2. Molecular sizes (base pairs) are indicated between panels.

segment and insertions of N-segment nucleotides between V and J. To evaluate the molecular organization and TCR diversity of IEL γ -gene rearrangements in athymic chimeras, PCR products for V γ 1.2, V γ 2, and V γ 5 from athymic and thymus-bearing mice were cloned and sequenced (Fig. 4). Sequence analyses of clones from V γ 1.2-J γ 1 PCR products (Fig. 4A) indicated that in both thymus-bearing and athymic mice, rearrangements had taken place between the V γ 1.2 and the J γ 2 genes (36). (Note that V γ 1.2 rearranges to J γ 2 [33] and that the J γ 1 primer used in this study is a consensus primer for all J γ genes [1].) Of interest was the finding that productive rearrangements had occurred in three of nine clones from thymus-bearing mice and in three of eight clones from athymic chimeras. N-segment insertions had occurred in all V γ 1.2-J γ 2 clones, and V- and/or J-segment nucleotide removal occurred in most, though not all, rearranged genes (Fig. 4A).

Most V γ 2-J γ 1 sequences consisted of out-of-frame genes as previously reported for V γ 2 rearrangements in IEL from thymus-bearing mice (1, 43); only 1 of 20 V γ 2 rearrangements yielded an in-frame gene (Fig. 4B). In all clones sequenced, nucleotide removal occurred at the 3' end of the V segment, although nucleotide removal at the 5' end of J γ 1 was rare. Moreover, 17 of 19 out-of-frame genes shared a common sequence, a phenomenon reported by others for some out-of-frame IEL V γ gene rearrangements (1).

With regard to the V γ 5 gene, there were notable similarities in γ -gene rearrangements in IEL from thymus-bearing and athymic mice. In both types of animals, the majority of clones contained in-frame sequences for V γ 5-J γ 1 (8 of 9 for thymus-bearing mice and 8 of 11 for athymic mice) (Fig. 4C). Nucleotide removal occurred at the 3' end of the V gene for all clones, whereas no nucleotide removal occurred in 13 of 16 total J segments. There was an 11-nucleotide in-frame insertion between the V and J segments in both types of animals, using IEL DNA from different mice studied separately rather than as pooled material (Fig. 4C).

Finally, evaluation of predicted amino acid sequences of in-frame rearranged γ genes for V γ 1.2, V γ 2, and V γ 5 indicated that in both thymus-bearing and athymic mice, a high proportion of clones with N-segment nucleotide insertions resulted in codons for hydrophilic residues, including charged residues (Fig. 5). Moreover, a codon for tyrosine was present in the in-frame N segment of V γ 5 and in in-frame N segments for all three V-region rearrangements, similar to what has been reported by others for IEL (1, 25, 43), and was noted because of its potential use in recognition of antigen or a restricting element (1). Of all three rearranged V genes in IEL, only one in-frame rearrangement, that occurring in V γ 1.2 in athymic mice (Fig. 5), resulted in a stop codon.

The 11-nucleotide insertion in V γ 5-J γ 1 rearrangements was not a cloning or PCR artifact but was present in IEL genomic DNA. Control experiments were done to ensure that ge-

nomeric DNAs had not been contaminated with plasmid DNA from previous clonings (e.g., via equipment) and to ensure that there had been no contamination of V or J primers with genomic DNAs. No PCR product was obtained with use of V γ 5 or J γ 1 primers in the absence of a DNA template (Fig. 6, lane 4), indicating that primers had not been contaminated. Lane 5 is a positive control showing amplification of genomic DNA with the V γ 5 and J γ 1 primers. Lane 6 is a PCR-amplified product of V γ 5-J γ 1 cloned plasmid DNA obtained by using M13 primers, demonstrating that contamination of genomic DNA by plasmid DNA, if it occurred, would be detected by using that approach; the additional size of the V γ 5-J γ 1 PCR product is contributed by the M13 primers. Lanes 1 to 3 are genomic DNAs from IEL of three separate athymic chimeras amplified with M13 primers; note that no PCR products were obtained, demonstrating that genomic DNAs had not been contaminated with plasmid DNA.

A second set of experiments addressed the possibility that the 11-nucleotide insertion in V γ 5-J γ 1 clones was a PCR or cloning artifact. A computer search of the sequence spanning the distance of the V γ 5-J γ 1 PCR product identified an *Apa*I restriction site associated with the 11-nucleotide insertion (Fig. 7A); no other *Apa*I sites were present in the DNA between the positions of the V γ 5 and J γ 1 primers. Because of that, it was possible to directly address the question of whether the sequence containing the 11-nucleotide insertion had been preferentially cloned over other sequences (i.e., were a cloning artifact) or whether that sequence was abundant in the PCR product itself. To determine this, V γ 5-J γ 1 PCR products were treated with *Apa*I prior to agarose gel analyses. Shown in Fig. 7B are two V γ 5-J γ 1 PCR products not digested with *Apa*I (lanes 2 and 4). In contrast, V γ 5-J γ 1 PCR products digested with *Apa*I prior to agarose gel analyses resulted in two discrete bands of the size expected if the sequence was cut in the region between V γ 5 and J γ 1. These data demonstrate that a substantial proportion of the V γ 5-J γ 1 PCR product contained an N-region insertion with an *Apa*I site and thus ruled out the possibility that the identical sequence obtained in cloned material was due to preferential cloning of that sequence out of many.

Next we sought to determine whether the sequence containing the 11-nucleotide insertion was an artifact of PCR amplification, i.e., whether that sequence was selectively amplified over other sequences, or whether there was an abundance of that sequence in the genomic DNA. For this purpose, genomic DNAs and 10-fold dilutions were subjected to *Apa*I digestion or reacted with buffer in the absence of *Apa*I prior to V γ 5-J γ 1 amplification. A lesser amount of PCR product was obtained in *Apa*I-treated DNA (Fig. 8A, lanes 1 to 3) than in untreated DNA (lanes 4 to 6), demonstrating that sequences containing an *Apa*I site within the N region were heavily represented in IEL DNA before PCR

A (V γ 1.2 - J γ 2)

Thymus-Bearing:

V						N	J								
germline:	TAC	TGT	GCA	GTC	TGG	A		AT	AGC	TCG	GGC	TTT			
clone:															
1t.22	TAC						CGGG			GC	TCG	GGC	TTT	+	(1t)
1t.57	TAC	TGT	GCA	GTC	TGG	A	GAT	AT	AGC	TCG	GGC	TTT	+	(3t)	
1t.60	TAC	TGT	GCA	GTC	TGG	A	G	T	AGC	TCG	GGC	TTT	+	(3t)	
1t.4	TAC	TGT	GCA	GTC	TGG	A	TGACGGGGGGG			GC	TCG	GGC	TTT	-	(1t)
1t.14	TAC	TGT	GCA	GTC	TGG		TCGTCT			G	GGC	TTT	-	(1t)	
1t.43	TAC	TGT	GCA	GTC	TGG		GGGGGT			GC	TCG	GGC	TTT	-	(2t)
1t.50	TAC	TGT	GCA	GTC	TGG	A	TGAGGGAG			GC	TCG	GGC	TTT	-	(2t)
1t.51	TAC	TGT	GCA	GTC	TGG		CTC	T	AGC	TCG	GGC	TTT	-	(3t)	
1t.58	TAC	TGT	GCA	GTC			CTCAT	AT	AGC	TCG	GGC	TTT	-	(3t)	

Athymic:

	V					N	J								
germline:	TAC	TGT	GCA	GTC	TGG	A	AT	AGC	TCG	GGC	TTT				
clone:															
1a.4	TAC	TGT	GCA	GTC	TGG				AGC	TCG	GGC	TTT	+	(1a)	
1a.9	TAC	TGT	GCA	GTC	TGG	A				G	GGC	TTT	+	(1a)	
1a.27	TAC	TGT	GCA	GTC	TGG					G	GGC	TTT	+	(2a)	
1a.6	TAC	TGT	GCA	GTC	TGG	A			AT	AGC	TCG	GGC	TTT	-	(1a)
1a.21	TAC	TGT	GCA	GTC	TGG					AGC	TCG	GGC	TTT	-	(2a)
1a.22	TAC	TGT	GCA	GTC	TGG				AT	AGC	TCG	GGC	TTT	-	(2a)
1a.26	TAC	TGT	GCA	G					AT	AGC	TCG	GGC	TTT	-	(2a)
1a.28	TAC	TGT	GCA	GTC	TGG	A			AT	AGC	TCG	GGC	TTT	-	(2a)

B (V γ 2 - J γ 1)

Thymus-Bearing:

V					N	J							
germline:	TCC	TAC	GGC	TAA	AG		AT	AGC	TCA	GGT	TTT		
clone:													
2t.23	TCC	TAC	GGC	T		CAT	AT	AGC	TCA	GGT	TTT	+	(1t)
2t.1	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.2	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.3	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.5	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.6	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.14	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.21	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.22	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(1t)
2t.24	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(3t)
2t.31	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	-	(3t)

Athymic:

	V					N	J					
germline:	TCC	TAC	GGC	TAA	AG		AT	AGC	TCA	GGT	TTT	
clone:												
2a.2	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (1a)
2a.21	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (1a)
2a.22	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (2a)
2a.23	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (1a)
2a.24	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (1a)
2a.24	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (2a)
2a.26	TCC	TAC	GGC	T		GAAT	AT	AGC	TCA	GGT	TTT	- (2a)
2a.27	TCC	TAC	GGC			CCTCA			CA	GGT	TTT	- (2a)
2a.28	TCC	TAC	GGC			CCTCA			CA	GGT	TTT	- (2a)

C (V γ 5 - J γ 1)

Thymus-Bearing:

V					N		J						
germline:	GCC	TCC	TGG	GCT	GGA		AT	AGC	TCA	GGT	TTT		
clone:													
5t.12	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(2t)
5t.14	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(2t)
5t.18	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(2t)
5t.25	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(3t)
5t.27	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(3t)
5t.1	GCC	TCC	TGG	GCT	GG	GGGGG	AT	AGC	TCA	GGT	TTT	+	(1t)
5t.2	GCC	TCC	TGG	G			AT	AGC	TCA	GGT	TTT	+	(1t)
5t.3	GCC	TCC	TGG	GCT		CGAT	AT	AGC	TCA	GGT	TTT	+	(2t)
5t.4	GCC	TCC	TGG	GC		AGAGC	T	AGC	TCA	GGT	TTT	-	(1t)

Athymic:

	V					N	J						
germline:	GCC	TCC	TGG	GCT	GGA		AT	AGC	TCA	GGT	TTT		
clone:													
5a.2	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(1a)
5a.5	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(1a)
5a.6	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(2a)
5a.8	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(4a)
5a.9	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(4a)
5a.10	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(4a)
5a.19	GCC	TCC	TGG	GC		CCAGGGGGGTAT	AT	AGC	TCA	GGT	TTT	+	(4a)
5a.3	GCC	TCC	CGG	GCT	GG	CCGGG	AT	AGC	TCA	GGT	TTT	+	(1a)
5a.15	GCC	TCC	TGG	GC		AGAGC	T	AGC	TCA	GGT	TTT	-	(2a)
5a.22	GCC	TCC	TGG	GC		AGAGC	T	AGC	TCA	GGT	TTT	-	(2a)
5a.1	GCC	TCC	TGG	GCT	G	CCGG	AT	AGC	TCA	GGT	TTT	-	(1a)

FIG. 4. IEL TCR γ -gene sequences of cloned PCR-amplified DNAs spanning the V-J segments of V γ 1.2 (A), V γ 2 (B), and V γ 5 (C) rearranged genes from thymus-bearing mice and athymic chimeras. Data represent cloned V-J PCR products from three to four mice per group studied separately. Symbols at the right refer to in-frame (+) or out-of-frame (-) gene status; numbers in parentheses designate individual mice used as a source of IEL DNA.

	V	N	J
Vγ1.2-Jγ2:			
clone:			
1t.22	Tyr	Arg Gly	Ser Gly Phe
1t.57	Tyr Cys Ala Val Trp	Arg Tyr	Ser Ser Gly Phe
1t.60	Tyr Cys Ala Val Trp	Ser	Ser Ser Gly Phe
1a.4	Tyr Cys Ala Val Trp	Leu Leu	Ser Ser Gly Phe
1a.9	Tyr Cys Ala Val Trp	Ile Gly Gly	Gly Phe
1a.27	Tyr Cys Ala Val Trp	Val STOP Leu	Gly Phe
Vγ2-Jγ1:			
clone:			
2t.23	Ser Tyr Gly	Ser Tyr	Ser Ser Gly Phe
Vγ5-Jγ1:			
clones:			
5t.12, 5t.14, 5t.18, 5t.25, 5t.27,			
5a.2, 5a.5, 5a.6, 5a.8, 5a.9,			
5a.10, 5a.19:			
5t.1	Ala Ser Trp Ala	Gln Gly Val Tyr	Ser Ser Gly Phe
5t.2	Ala Ser Trp Ala	Gly Gly Asp	Ser Ser Gly Phe
5t.3	Ala Ser Trp	Asp	Ser Ser Gly Phe
5t.3	Ala Ser Trp Ala	Arg Tyr	Ser Ser Gly Phe
5a.3	Ala Ser Trp Ala	Gly Arg Asp	Ser Ser Gly Phe

FIG. 5. Predicted amino acid sequences of V-(N)-J regions for in-frame V γ 1.2, V γ , and V γ 5 genes in IEL from thymus-bearing and athymic mice.

amplification. To further confirm this finding, some of the PCR-amplified material from *Apa*I-digested DNA was recut with *Apa*I and analyzed on an agarose gel. In that experiment, only a single band of the size expected for intact V γ 5-J γ 1 product was obtained (Fig. 8B, lane 1), indicating that sequences with an *Apa*I site in the N region had been removed prior to PCR amplification. However, when PCR-amplified material from uncut genomic DNA was cut with *Apa*I, bands characteristic of those observed in Fig. 7B were obtained (Fig. 8B, lane 2); unmodified product due to sequences not containing N-segment *Apa*I sites was also present. Taken together, these data provide strong evidence that IEL DNA contained an abundance of sequences with an *Apa*I site in the position of the N region and that sequences with the 11-nucleotide insertion in cloned PCR product were in fact present in the genome. It should be noted that although we cannot rule out the possibility that there were different rearrangements also containing N segments with *Apa*I sites, an examination of 49 V γ 5-J γ 1 rearrangements (1, 25, 43) showed that only 2 resulted in *Apa*I sites, suggesting that this possibility is unlikely.

DISCUSSION

This study has compared the utilization and molecular organization of TCR γ genes in murine intestinal IEL that

FIG. 6. V γ 5-J γ 1 PCR product of IEL DNA from athymic chimera amplified with V γ 5 and J γ 1 primers (lane 5) and PCR product of V γ 5-J γ 1 cloned plasmid DNA amplified with M13 primers (lane 6). No PCR products were obtained with use of primers in the absence of DNA template (lane 4), demonstrating that primers had not been contaminated with DNA, or with use of three separate IEL DNAs amplified with M13 primers (lanes 1 to 3), demonstrating that genomic DNAs were not contaminated with plasmid DNA. Lane s, molecular size markers.

FIG. 7. (A) Location of the *Apa*I restriction site within the 11-nucleotide N segment of the multiple identical sequence observed in V γ 5-J γ 1 rearrangements; (B) agarose gel analysis of V γ 5-J γ 1 PCR-amplified products of IEL DNAs from two athymic chimeras without *Apa*I digestion (lanes 2 and 4). *Apa*I digestion of V γ 5-J γ 1 PCR products prior to agarose gel analysis resulted in two bands due to the cut within the N segment (lanes 1 and 3). Lane s, molecular size markers.

have developed in the presence or absence of a thymus. The data reported here provide formal evidence that (i) extrathymic TCR γ -gene rearrangements occur in IEL, (ii) rearrangements of TCR γ genes are restricted to the same V γ genes regardless of thymus influence, and (iii) there are remarkable similarities between V γ 1.2, V γ 2, and V γ 5 rearrangements leading to construction of either functional or nonfunctional genes in both thymus-bearing and athymic mice. In-frame rearrangements occurred in IEL from athymic mice for V γ 1.2 and V γ 5 and were rare for V γ 2. The presence of productively rearranged V γ 5 genes in IEL from mice, regardless of the presence or absence of thymus, further reinforces the notion (1, 25, 43) that V γ 5 is an important component of murine IEL $\gamma\delta$ T cells. Our finding that V γ 1.2 genes were rearranged in both thymus-bearing and athymic mice is interesting in that, at present, the extent to which V γ 1.2 is used by IEL T cells is unclear. One study (43) reported V γ 1.2 transcripts in murine IEL (note difference in nomenclature), though sequence analyses of that material

FIG. 8. (A) IEL genomic DNAs from athymic chimeras were diluted 10-fold, digested overnight with *Apa*I (lanes 1 to 3) or with buffer in the absence of *Apa*I (lanes 4 to 6), PCR amplified with V γ 5 and J γ 1 primers, and analyzed on agarose gels; note reduction in the PCR product in *Apa*I-digested genomic DNA. (B) Genomic DNAs (as used in panel A, lane 1) were digested with *Apa*I, amplified with V γ 5 and J γ 1 primers, redigested with *Apa*I, and analyzed on an agarose gel (lane 1); the results demonstrate that genomic DNA sequences containing N segments with *Apa*I sites had been removed during the initial *Apa*I digestion of genomic DNA. Undigested V γ 5-J γ 1 PCR products (as used in panel A, lane 4) were digested with *Apa*I and analyzed on an agarose gel (lane 2); the results demonstrate that N segments containing an *Apa*I site were present in IEL DNA from athymic chimeras. Lanes s, molecular size markers.

were not done. In other studies, no PCR-amplified products were obtained from IEL for V γ 1.2 (1) and were not appreciably detected by *in situ* hybridization (25). However, productive rearrangement of V γ 1.2 in IEL from both athymic and thymus-bearing mice as shown here demonstrates that V γ gene rearrangements occur extrathymically in those IEL and suggests, though does not formally prove, that some IEL utilize the V γ 1.2 gene. However, it is interesting that V γ 1.2 rearrangements have been demonstrated in activated, CD8⁺ TCR- $\alpha\beta$ ⁺ T cells (14, 17, 24, 34), both of which are characteristics of gut IEL (Fig. 1 [3, 6, 10, 15, 18, 30, 46]). Thus, the possibility exists that some IEL with rearranged V γ 1.2 express TCR- $\alpha\beta$. Further work is needed to explore those possibilities. Finally, the predominance of out-of-frame IEL rearrangements for V γ 2 in both thymus-bearing and athymic mice is consistent with findings obtained by others for IEL (1, 43).

The presence of multiple identical in-frame sequences with N-segment insertions in cloned, rearranged TCR γ genes has been documented for murine intestinal IEL (1), as also found in this study. A potential explanation for such sequences is that after rearrangement has occurred, those IEL have been clonally expanded within the gut epithelium, possibly as a result of intestinal antigens. In that vein, it is interesting that although multiple identical in-frame and out-of-frame γ -gene rearrangements have been reported in IEL studies from different laboratories (1; this study), rearrangements involving identical sequences are uncommon between laboratories, further implying that specific microenvironmental factors may be involved in the acquisition of cells with given TCR rearrangements. "Preferential expansion" of $\gamma\delta$ T cells has been proposed for resident pulmonary lymphocytes based on selection according to peptide motifs of expressed TCR (40). This also may be true for IEL. Although selection of IEL would not occur according to nucleotide sequences, it is possible that identical sequences have been generated in different mice, followed by selection and expansion. It should be noted that there is no direct evidence that the 11 nucleotides spanning the V-J junctional site of V γ 5 rearrangements were derived by N-segment insertion. The possibility must be considered that this portion was contributed from the genome, perhaps as a diversity (D)-like segment associated with V γ 5. In that case, only minor gene modifications would be needed to obtain the sequence in question (and the corresponding TCR γ -chain polypeptide) in different animals, i.e., removal of four nucleotides from the 3' end of V γ 5 and insertion of a D gene (Fig. 4C). Although no D genes have been identified for γ genes, several large portions of the genome between V γ 5 and J γ 1, where a D segment would likely reside, remain uncharted (33). This possibility warrants consideration given that the V γ 5-J γ 1 rearrangements bearing the 11-nucleotide insertion were not artifacts of PCR amplification or of cloning and were abundant in IEL genomic DNA.

Out-of-frame rearrangements bearing identical sequences with N-nucleotide insertions, as reported here, are not without precedent for IEL. For example, 11 of 29 V γ 4 IEL clones had the same out-of-frame rearrangement, and 16 of the total 29 sequences were identical to at least one other out-of-frame sequence (1). Although it is not yet evident what might account for the presence of identical out-of-frame sequences, including those obtained from different mice, it is known that factors exist which could result in the presence of identical sequences generated independently. Thus, AT and T palindrome (P) nucleotide insertions associated with N segments (21, 26) are frequently present at the

N-J junction of rearranged IEL γ genes that have unmodified J segments (1, 25, 43), as also found in this study. Given that P nucleotides are probably derived from the genome (26), there is a greater likelihood of obtaining identical sequences in short N segments containing P nucleotides than of obtaining sequences generated by random nucleotide insertion. For example, the number of potential combinations in a four-nucleotide N segment containing an AT palindrome is reduced from 256 to 16. In that light, it is interesting that the rearrangement of the identical out-of-frame V γ 2 sequence described here also has been reported in another laboratory (44) for one of five out-of-frame V γ 2 sequences in $\gamma\delta$ T-cell hybridomas.

In addition to rearrangements of γ genes in IEL in athymic mice, some γ -gene rearrangements were evident in spleen cells from those animals, most notably for V γ 5 and possibly V γ 2, though not for other γ genes (Fig. 3B). While those data indicate that in athymic mice, T cells with γ -gene rearrangements can be found outside the gut epithelium, as reported by others for γ (50) and δ (49) genes, they do not address the question of where extrathymic rearrangement had occurred. On the one hand, it is possible that rearrangement of V γ 5 and V γ 2 in athymic mice occurred in multiple peripheral sites (spleen, gut epithelium, blood, etc.). Alternatively, rearrangements may have occurred in a single site followed by seeding of some cells to other compartments. Thus, if extrathymic TCR γ -gene rearrangement occurred within the gut epithelium itself, the presence elsewhere of such T cells would depend on the extent of extraintestinal trafficking of those cells. Since nothing is currently known about the trafficking potential of IEL $\gamma\delta$ T cells, further study will be needed to delineate those and other important questions regarding extrathymic T-cell maturation.

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REFERENCES

- Asarnow, D. M., T. Goodman, L. LeFrancois, and J. P. Allison. 1989. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. *Nature (London)* **341**:60-62.
- Asarnow, D. M., W. A. Kuziel, M. Bonyhadi, R. E. Tigelaar, P. W. Tucker, and J. P. Allison. 1988. Limited diversity of $\gamma\delta$ antigen receptor genes of Thy-1⁺ dendritic epidermal cells. *Cell* **55**:837-847.
- Bandeira, A., T. Mota-Santos, S. Itohara, S. Degermann, C. Heusser, S. Tonegawa, and A. Coutinho. 1990. Localization of $\gamma\delta$ T cells in the intestinal epithelium is independent of normal microbial colonization. *J. Exp. Med.* **172**:239-244.
- Bevan, M. J. 1977. In a radiation chimera host H-2 antigens determine the immune responsiveness of donor cytotoxic cells. *Nature (London)* **269**:417-418.
- Blackman, M., J. Kappler, and P. Marrack. 1990. The role of the T cell receptor in positive and negative selection of developing T cells. *Science* **248**:1335-1341.
- Bonneville, M., S. Itohara, E. G. Krecko, P. Mombaerts, I. Ishida, M. Katsuti, A. Berns, A. G. Farr, C. A. Janeway, and S. Tonegawa. 1990. Transgenic mice demonstrate that epithelial homing of $\gamma\delta$ T cells is determined by cell lineages independent of T cell receptor specificity. *J. Exp. Med.* **171**:1015-1026.
- Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* **127**:2496-2501.
- Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. MacDonald.

- ald. 1985. Expression of interleukin 2 as a differentiation marker on intrathymic stem cells. *Nature (London)* 314:98-100.
9. Davis, M. 1990. T cell receptor gene diversity and selection. *Annu. Rev. Biochem.* 59:475-496.
10. De Geus, B., M. Van den Enden, C. Coolen, L. Nagelkerken, P. Van der Heijden, and J. Rozing. 1990. Phenotype of intraepithelial lymphocytes in euthymic mice: implications for differentiation of cells bearing a CD3-associated $\gamma\delta$ T cell receptor. *Eur. J. Immunol.* 20:291-298.
11. Fichtelius, K. E. 1967. The gut epithelium—a first level lymphoid organ? *Exp. Cell. Res.* 49:87-92.
12. Fichtelius, K. E., E. J. Yunis, and R. A. Good. 1968. Occurrence of lymphocytes within the gut epithelium of normal and neonatally thymectomized mice. *Proc. Soc. Exp. Biol. Med.* 128:185-188.
13. Fry, A. M., L. A. Jones, A. M. Kruisbeek, and L. A. Matis. 1989. Thymic requirement for clonal deletion during T cell development. *Science* 246:1044-1046.
14. Garman, R. D., P. J. Doherty, and D. H. Raulet. 1986. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell* 45:733-742.
15. Guy-Grand, D., N. Cerf-Bensussan, B. Malissen, M. Malassis-Seris, C. Briottet, and P. Vassalli. 1991. Two gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. *J. Exp. Med.* 173:471-481.
16. Havran, W. L., and J. P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature (London)* 335:443-445.
17. Heilig, J. S., and S. Tonegawa. 1987. T-cell γ gene is allelically but not isotypically excluded and is not required in known functional T-cell subsets. *Proc. Natl. Acad. Sci. USA* 84:8070-8074.
18. Hershberg, R., P. Eghtesady, B. Sydora, K. Brorson, H. Cheroutre, R. Modlin, and M. Kronenberg. 1990. Expression of the thymus leukemia antigen in mouse intestinal epithelium. *Proc. Natl. Acad. Sci. USA* 87:9727-9731.
19. Hodes, R. J., S. O. Sharrow, and A. Solomon. 1989. Failure of T cell receptor V β negative selection in an athymic environment. *Science* 246:1041-1044.
20. Innis, M. A., K. Muambo, D. H. Gelfand, and M. A. Brow. 1988. DNA sequencing with Thermus aquaticus DNA polymerase and direct polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. USA* 85:9436-9440.
21. Itoharu, S., and S. Tonegawa. 1990. Selection of $\gamma\delta$ T cells with canonical T-cell antigen receptors in fetal thymus. *Proc. Natl. Acad. Sci. USA* 87:7935-7938.
22. Klein, J. R. 1986. Ontogeny of the Thy-1-, Lyt-2+ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. *J. Exp. Med.* 164:306-314.
23. Klein, J. R., R. L. Mosley, and D. Kaiserlian. 1990. Expression of the asialo GM1 determinant on murine intestinal epithelia. *Proc. Soc. Exp. Biol. Med.* 195:329-334.
24. Kranz, D. M., H. Saito, M. Heller, Y. Takagaki, W. Haas, H. N. Eisen, and S. Tonegawa. 1985. Limited diversity of the rearranged T-cell γ gene. *Nature (London)* 313:752-754.
25. Kyes, S., E. Carew, S. R. Carding, C. A. Janeway, and A. Hayday. 1989. Diversity in T-cell receptor γ gene usage in intestinal epithelium. *Proc. Natl. Acad. Sci. USA* 86:5227-5531.
26. Lafaille, J. J., A. DeCloux, M. Bonneville, Y. Takagaki, and S. Tonegawa. 1989. Junctional sequences of the T cell receptor $\gamma\delta$ genes: implications for $\gamma\delta$ T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell* 59:859-870.
27. Lafaille, J. J., W. Haas, A. Coutinho, and S. Tonegawa. 1990. Positive selection of $\gamma\delta$ T cells. *Immunol. Today* 11:75-78.
28. MacDonald, H. R., R. K. Lees, R. Schneider, R. M. Zinkernagel, and H. Hengartner. 1988. *Nature (London)* 336:471-473.
29. Mayerhofer, G. 1980. Thymus-dependent and thymus-independent subpopulations of intestinal intraepithelial lymphocytes: a granulated subpopulation of probable bone marrow origin. *Blood* 55:532-535.
30. Mosley, R. L., D. Styre, and J. R. Klein. 1990. Differentiation and functional maturation of bone marrow derived intestinal epithelial T cells expressing membrane T cell receptor in athymic radiation chimeras. *J. Immunol.* 145:1369-1375.
31. Mosley, R. L., D. Styre, and J. R. Klein. 1990. CD4+CD8+ murine intestinal intraepithelial lymphocytes. *Int. Immunol.* 2:361-365.
32. Murray, V. 1989. Improved double-stranded DNA sequencing using linear polymerase chain reaction. *Nucleic Acids. Res.* 17:8889-8891.
33. Raulet, D. H. 1989. Structure, function, and molecular genetics of the $\gamma\delta$ T cell receptor. *Annu. Rev. Immunol.* 7:175-207.
34. Reilly, E. B., D. M. Kranz, S. Tonegawa, and H. N. Eisen. 1986. A functional γ gene formed from γ -gene segments is not necessary for antigen-specific responses of murine cytotoxic T lymphocytes. *Nature (London)* 321:878-880.
35. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487.
36. Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa. 1984. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature (London)* 309:787-762.
37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 916-917. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
39. Sarmiento, M., A. L. Glasebrook, and F. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytotoxicity in the absence of complement. *J. Immunol.* 125:2665-2672.
40. Sim, G. K., and A. Augustin. 1991. Extrathymic positive selection of $\gamma\delta$ T cells. V γ 4J γ 1 rearrangements with "GxYS" junctions. *J. Immunol.* 146:2439-2445.
41. Sprent, J., and S. Webb. 1987. Function and specificity of T cell subsets in the mouse. *Adv. Immunol.* 41:39-133.
42. Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1978. Monoclonal antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539-546.
43. Takagaki, Y., A. DeCloux, M. Bonneville, and S. Tonegawa. 1988. Diversity of $\gamma\delta$ T-cell receptors on murine intestinal intraepithelial lymphocytes. *Nature (London)* 339:712-714.
44. Takagaki, Y., N. Nakanishi, I. Ishida, O. Kanagawa, and S. Tonegawa. 1989. T cell receptor- γ and - δ genes preferentially utilized by adult thymocytes for the surface expression. *J. Immunol.* 142:2112.
45. Tomonari, K. 1988. Rat antibody against a structure functionally related to the mouse T cell receptor-T3 complex. *Immunogenetics* 28:445-458.
46. Viney, J. L., T. T. MacDonald, and P. J. Kilshaw. 1989. T-cell receptor expression in intestinal intra-epithelial lymphocyte subpopulations of normal and athymic mice. *Immunology* 66:583-587.
47. von Boehmer, H., and H. Kiselow. 1990. Self-nonself discrimination by T cells. *Science* 248:1369-1373.
48. Wilson, R. K., C. Chen, and L. Hood. 1990. Optimization of asymmetric polymerase chain reaction for rapid fluorescent DNA sequencing. *BioTechniques* 8:184-189.
49. Yoshikai, Y., G. Matsuzaki, Y. Takeda, S. Ohga, K. Kishihara, H. Yuuki, and K. Nomoto. 1988. Functional T cell receptor delta chain gene messages in athymic nude mice. *Eur. J. Immunol.* 18:1039-1043.
50. Yoshikai, Y., M. D. Reis, and T. W. Mak. 1986. Athymic mice express a high level of functional γ -chain but greatly reduced levels of α - and β -chain T-cell receptor messages. *Nature (London)* 324:482-485.
51. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J. Exp. Med.* 147:882-895.