Extrathymic Development of Vα14-positive T Cells

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Summary

It is known that rearrangement of the T cell antigen receptor (TCR) gene occurs in the thymus during T cell development and consequently results both in the deletion of DNA between the variable (V) and diversity/joining segments and in the formation of a circular DNA with recombination signal sequences. Here, we provide evidence that Vα14$^+$ TCR gene rearrangements take place in extrathymic sites, such as bone marrow, liver, and intestine, but not in spleen, because we were able to detect frequent productive and nonproductive Vα14$^+$ coding and signal sequences as a result of TCR rearrangements in extrathymic sites. Similar findings were also detected in athymic mice. Quantitative analysis shows that the relative amounts of Vα14 gene–mediated signal sequences in extrathymic tissues are higher than those in thymus. On the contrary, TCR rearrangements of Vα1.1 T cells, which are known to develop in the thymus, were mainly detected in the thymus, Peyer's patch, and spleen, but not in other extrathymic tissues, showing patterns distinct from Vα14 TCR rearrangements. These findings are evidence of extrathymic development of Vα14$^+$ T cells. Differential characteristic TCR rearrangement patterns also indicate that distinct TCR repertoires are generated in different lymphoid tissues.

Diversity of the TCR gene is generated by rearrangement of the V and J gene segments during T cell development in the thymus. The TCR V and J gene segments, like Ig genes, possess recombination signals in which heptamer and nonamer sequences, separated by a 12/23-bp spacer, are flanked by germline V and J gene segments. Several mechanisms of Ig/TCR gene rearrangement have been proposed: intramolecular deletion, unequal sister chromatid exchange, or chromosomal inversion (1–3). In fact, the presence of reciprocal recombination products of Ig/TCR genes has been demonstrated in extrachromosomal circular DNA isolated from mouse lymphocyte nuclei (4–6); linear deletion products also have been observed recently (7). This indicates that a model of a looping-out and excision of chromosomal DNA represents a proven molecular mechanism of the VJ joining event during lymphocyte development.

In our previous studies, Vα14$^+$ T cells were found to dominate in the periphery at an extremely high frequency (~2–3% in spleen). In those studies, >90% of the Vα14$^+$ T cells used a homogenous TCR α chain encoded by Vα14 and Jα281 genes with a one-nucleotide N region (8, 9; also see Figs. 1 and 2). Because the N region corresponds to the third base of the triplet code for glycine, the amino acid in the VJ junction always becomes glycine. This is a general phenomenon that is found in all laboratory mice and in some subspecies of wild mice (10). Furthermore, the majority of Vα14$^+$ TCR associated with Jα are those other than Jα281 at the neonatal stage, and the frequency of Vα14Jα281 expression increases with time after birth (9). These results indicate that Vα14Jα281 T cells are positively selected in the periphery and that the VJ junction is important for the selection.

Another intriguing finding is that athymic mice also show the dominant expression of Vα14Jα281 in the spleen (10). Therefore, the homogenous V14J281 TCR α chain could be a useful probe for analyzing extrathymic selection of the TCR repertoire, which includes, for example, several TCR Vα/β genes or the invariant TCR γ/δ (i.e., BALB/c invariant δ [BID]) reported by other investigators (11–15). In addition, the signal sequences composed of the flanking regions of germline V or D and J segments of TCR α/β genes in the episomal circular DNA could also be a beneficial index to investigate T cell generation in extrathymic tissues. In this paper, we demonstrate strong evidence that certain TCR gene rearrangements preferentially take place in extrathymic tissues and discuss the role of these extrathymic tissues in T cell development.

Materials and Methods

Animals. Specific pathogen-free BALB/c and BALB/c *nu/nu*

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mice (12 wk old) were purchased from Shizuoka Experimental Animal Co. (Hamamatsu, Japan).

RNase Protection Assay. First, total cellular RNA was isolated from spleen, lung, bone marrow (BM)[2] and liver of BALB/c by guanidine/CsCl method. Next, an RNase protection assay was performed, as previously described (9). ^{32}P-labeled antisense RNA probes were prepared by in vitro translation of SP6 polymerase using cDNA (pTsαVJC of C57BL/6 origin) encompassing a part of the 5′ untranslated region, Vα14.1Jα281 region, and the 5′ side of Cα region, as described (9; also see Fig. 2). Predicted protection bands are indicated in Fig. 2.

Polymerase Chain Reaction. The PCR was carried out using 0.1–10 μg of total RNA from three to four different batches of various tissues (Peyer's patch [PP], intestinal intraepithelium [IEL], BM, liver, thymus, spleen, PBL) of BALB/c and/or BALB/c *nu/nu* with primers specific for Vα14 and Cα or for recombination activating gene (RAG) as described (9). The amplification cycles consisted of denaturation for 2 min at 94°C, annealing for 2 min at 63°C, and extension for 2 min at 73°C. Oligonucleotide primers used for PCR amplifications were Vα14, 5′-TCGAATTCTAAGCACAGCACGCTGCACA-3′ and Cα, 5′-TCGAATTCTGTCCTGAGACCGAGGATC-3′; RAG-1, 5′-CCAAGCTGCAGACATTCTAGCACTC-3′ and 5′-CAACATCTGCCTTCACGTCGATCC-3′; RAG-2, 5′-CACATCCACAAGCAGGAAGTACAC-3′ and 5′-GGTTCAGGGACATCTCCTACTAAG-3′; and mouse β-actin, 5′-GAGAGGGAAATCGTGCGTGAC-3′ and 5′-ACATCTGCTGGAAGGTGGACA-3′.

Preparation of Nuclear DNA. Cells (5×10^7) were suspended in 3 ml of 0.5% NP-40 buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, homogenized, and centrifuged at 10,000 rpm for 10 min. The pellet containing nuclei was resuspended in 3 ml of buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% SDS), and was subsequently treated with 8 U proteinase K. The mixture was gently shaken overnight at room temperature. Next, the mixture was treated with phenol and chloroform isoamylalcohol. The supernatant was dialyzed against buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and then used for experiments. Four batches of DNA samples were prepared independently. For measurement of DNA amounts, quantitative PCR was carried out with RAG-2 primers on the genomic DNA in different sample materials. The amounts of DNA in samples were normalized on the basis of copy numbers of RAG-2 genes. The PCR products, which had been amplified in varied concentrations of the standard RAG-2 cDNA, were used as a standard. The RAG-2 cDNA (MR2) was kindly obtained from H. Shiku (Nagasaki University). Oligonucleotides used in the amplifications were 5′-CACAGTCTTGCCAGGAGGAAT-3′ and 5′-GGGGGTTTCTTTTGGGAGTTT-3′ for 5′ and 3′ of RAG-2 coding regions, respectively. The *Pst*-1 fragments (852 bp) of RAG-2 cDNA were used as the specific probe for DNA blots.

Detection of Signal Sequences in the Circular DNA. The double-step PCR was carried out using 0.1–1.5 μg of nuclear DNA (equivalent to 0.2–3 $\times$ 10^7 copies of RAG-2 genes) obtained from NP-40 lysates of lymphocytes from various tissues of 10–20 BALB/c or BALB/c *nu/nu* mice, using 0.33 μM of the first primers in the first 20 cycles and 3.3 μM of the second primers in the second 20-cycle amplifications. The amplification cycle consisted of denaturation for 2 min at 94°C; annealing for 2 min, usually at 55°C (but for Vα14 Jα281 only, at 60°C); and extension for 2 min, at 72°C. For amplification of signal sequences, the following combinations of primers were used: VA1/VA2 and JA1/JA2 for Vα14-Jα281 rearrangements, VA3/VA4 and JA1/JA2 for Vα1.1-Jα281, VA1/VA2 and JA3/JA4 for Vα14-JαTA57, VA3/VA4 and JA3/JA4 for Vα1.1-JαTA57, VB1/VB2 and DB1/DB2 for Vβ10.1-Dβ1, VB3 and DB1/DB2 for Vβ8.1-Dβ1, VB1/VB2 and DB3/DB4 for Vβ10.1-Dβ2, and VB3 and DB3/DB4 for Vβ8.1-Dβ2. Oligonucleotide primers used for circular TCR α DNA amplification were VA1, 5′-CTTTGTCACCTATGTCTGGAA-3′; VA2, 5′-TCACCTATGTCTGGAAGCCTC-3′; VA3, 5′-AGTAGTGCCTTCCCTGAGATG-3′; VA4, 5′-GCACACAGATAGAAACAGAAT-3′, JA1, 5′-GAACAAAGGAAGTGGGGTGAC-3′; JA2, 5′;-CTGGCGGTGGAAAGACTATTG-3′; JA3, 5′-CCTCTTTCTTTCCCACCACAC-3′; and JA4, 5′-TTCTCTTTATGCCTCATTTTC-3′. Oligonucleotide primers used for circular TCR β DNA amplification were VB1, 5′-GACTGAAATACCACTGATGCT-3′; VB2, 5′-CTTGGCTTGCACTGCTTCCTC-3′; VB3, 5′-ACACAGAGAGCTAGTTTTCCC-3′; DB1, 5′-AACAGGGGGTAAAGAGGAAAA-3′; DB2, 5′-CCTTCCTTATCTTCAACTCCC-3′; DB3 5′-GACTTTTCCCAGCCCCTCTCA-3′; and DB4, 5′-AACCTCTCTGCCACCTGTCTC-3′.

Sequencing. The PCR products amplified from three independent samples were blunt ended with *Escherichia coli* polymerase I and T4 kinase and cloned into the HincII site of pBluescript SK II(+) (Stratagene, La Jolla, CA). Nucleotide sequences were determined by the dideoxy chain termination method with a Taq dye primer cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) in an automated DNA sequencer (370A; Applied Biosystems, Inc.) as described previously (10).

Southern Blotting. The PCR products amplified from PP, IEL, thymus, BM, liver, spleen, and kidney of BALB/c or BALB/c *nu/nu* mice, herring sperm DNA (hsDNA), and control (water) were subjected to electrophoresis on a 2% agarose gel. For detection of reciprocal signal joints, PCR products were hybridized with the following probes: the oligomer, 5′-GATTTGTGTAAAGGGGGCTGGCACTGTGCACCATGCTCCCCAGAAACTTACAGCCA-3′ or, alternatively, a 288-bp Vα14-Jα281 circular DNA PCR product for Vα14-Jα281, Vα1.1-Jα281, and Vα14-JαTA57 signal sequences; 330-bp PCR product of Vα1.1-Jα281 for Vα1.1-JαTA57 signal sequences; the oligomer, 5′-GAGCAGCTTATCTGGTGGTTTCTTCCAGCCCTCAAGGGGT-3′ for Vβ10.1-Dβ1 and Vβ8.1-Dβ1; and the oligomer, 5′-CCCCTGCCAGGCTCTGGGGTAGGCACCTGTGGGGAAGAAA-3′ for Vβ10.1-Dβ2 and Vβ8.1-Dβ2 signal sequences. For detection of RAG-2, PCR products were hybridized with ^{32}P-labeled PstI fragments of RAG-2 cDNA. The signals were detected by the Bio Image Analyzer (Fujix BAS2000; Fuji Film, Tokyo, Japan). The general procedures were described previously (9, 10, 16).

Quantitative PCR Analysis. The frequencies of signal sequences in genomic DNA samples were determined by a double-step quantitative PCR using two sets of primers. The first-step amplification was performed by a PCR of 20 cycles using the first primers. In the first step of PCR, we confirmed that the amplifications did not reach plateau level. Next, the first PCR products were quantitated in the second-step PCR of 20 cycles (30 cycles for nude tissues) using nested primers. The PCR primers used were VA1,VA2/JA1,JA2 and VA3,VA4/JA1,JA2 for Vα14-Jα281 and Vα1.1-Jα281 rearrangements, respectively. Varied concentrations of 288- and 330-bp purified PCR fragments, of Vα14-Jα281 and Vα1.1-Jα281 signal sequences, were amplified to make standard curves. The PCR products were subjected to electrophoresis on 2% agarose gel and were detected by DNA blot hybridization with ^{32}P-labeled 288-bp PCR products containing the Vα14-Jα281 signal sequence. The radioactivities were measured by the Bio Image Analyzer. To determine the

[2] *Abbreviations used in this paper:* AU, arbitrary units; BM, bone marrow; IEL, intestinal intraepithelium; PP, Peyer's patch; RT, reverse transcriptase.

quantities of target amplicons in different tissues, photo-stimulated luminescences (PSLs) of the PCR products were compared with those of the standard curves.

FACS® Analysis and Separation of B220⁻ Cell Fractions. Cells ($3–8 \times 10^7$) were stained with PE-labeled anti-B220 and FITC-labeled anti-Thy-1 mAbs. B220⁻ cells were separated by FACStar® (Becton Dickinson & Co., Mountain View, CA). For detection of Vα14 T cells in extrathymic tissues, cells were stained with PE-labeled anti-Vα14 (17) and FITC-labeled anti-CD3 (2C11) and/or anti-TCR-β (H57-597). They were then analyzed by FACScan® with logarithmic amplifier.

Results

Expansion of T Cells Bearing Homogenous Vα14Jα281 TCR in Extrathymic Tissues. Our previous experiments have demonstrated that T cells bearing homogenous TCR, composed of Vα14Jα281 with a one-base N region, dominate in spleen (~2% of total T cells) (9, 10). Therefore, we further investigated the homogenous Vα14⁺ TCR expression in other peripheral tissues, such as BM and liver. As shown in Fig. 1, the significant numbers of CD3⁺ T cells were stained with anti-Vα14 mAb. The percentage of Vα14⁺ T cells in CD3⁺ populations is 5.8% in BM, 9.1% in liver, and 2.0% in spleen.

The expression of Vα14Jα281 TCR was investigated by RNase protection assay. The results were shown in Fig. 2. The protected bands with a 401-bp length corresponding to the Vα14Jα281 TCR were detected in these tissues. The intensities of the protected bands were calculated to be 4.2% of total α chains in BM and 12.3% in liver. The results are comparable to those obtained by FACS®. Thus, compared with homogenous Vα14⁺ TCR expression in spleen (1.9%), the frequency of Vα14Jα281 expression in other extrathymic sites is significantly higher (9, 10). In addition, the results of our previous PCR experiments have strongly suggested that the selection of Vα14⁺ T cells occurs at extrathymic sites. We reached this conclusion because the frequency of homogenous Vα14Jα281 TCR expression was demonstrated to be >95% of total Vα14⁺ α chains in nude and athymic mice that had been irradiated, thymectomized, and reconstituted with BM (T×B), the same frequency as for euthymic mice (10). Therefore, it is likely that T cells expressing the homogenous Vα14Jα281 TCR develop without thymic influence.

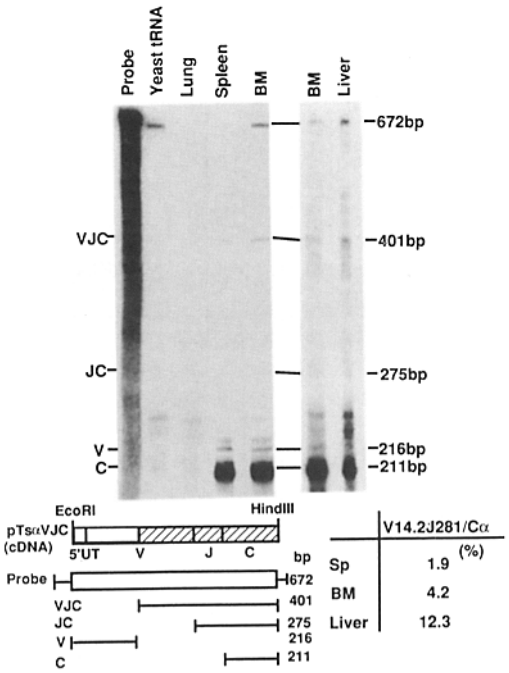

Figure 2. RNase protection analysis of V14J281 TCR α chain mRNA levels in various tissues of BALB/c. Total RNA (50 μg) from several batches of samples were analyzed. The probe used was pTsαVJC (Vα14.1Jα281Cα), obtained from C57BL/6 and diagrammed on the bottom left. Regions of the probes corresponding to Vα14.2Jα281Cα (401 bp), Jα281Cα (275 bp), Vα14.2 (216 bp), and Cα (211 bp) sequences are indicated. As there were three amino acid differences at positions 50–52 between Vα14.1 (C57BL/6 type) and Vα14.2 (BALB/c type), full protection bands (*hatched*) of 401 bp were detected in BALB/c. The ratios of the VJC (401 bp) to Cα (211 bp) transcripts were calculated by the automated densitometer and summarized on the bottom right.

Next, we investigated possible extrathymic sites for Vα14⁺ T cell differentiation by PCR, using the coding sequences of Vα14 and Jα281 genes as primers. In the first experiment, lymphocytes from thymus, PP, BM, IEL, liver, PBL, and spleen were prepared as described (9, 18, 19), and their RNA and DNA were analyzed by reverse transcriptase (RT)-PCR using Vα14- and Cα-specific primers for RT-PCR

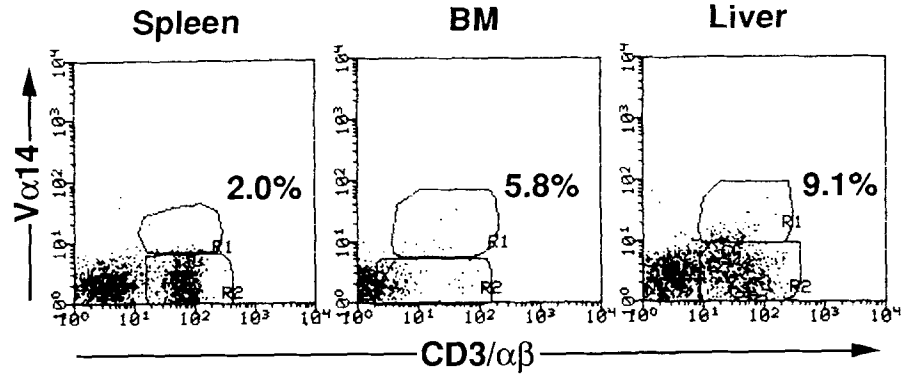

Figure 1. FACS® profiles of Vα14⁺ T cells in extrathymic tissues. Cells were stained with PE-labeled anti-Vα14 and FITC-labeled anti-CD3 (2C11) and anti-TCR-β (H57-597). The numbers of Vα14-positive (R1) and Vα14-negative (R2) T cell populations (CD3/TCR α/β^+) were counted, and the percentage of Vα14⁺ in T cells was calculated by the following formula: percent = 100× [R1/(R1 + R2)].

A

	Vα14						Jα281		
Germline Vα14	TGT	GTG	GTG	GG	CGcaccatgctc				
Germline Jα281			tggcactgtgTA		GAT	AGA	GGT	TCA	
	C	V	V	G	D	R	G	S	
Vα14 (Type A)	TGT	GTG	GTG	GG	N	GAT	AGA	GGT	TCA
Vα14 (Type B)	TGT	GTG	GTG	GG	N	- - -	AGA	GGT	TCA

B

Vα14 sequences Frame	Type	Frequencies of V14 TCR sequences in PP	IEL	BM	Liver	Thymus	Spleen	PBL
Euthymic mice								
In-frame	A	13	0	11	12	19	39	8
	B	2	7	0	0	0	0	0
	other Jα	6	9	2	0	4	2	1
	subtotal	21/33	16/29	13/20	12/17	23/28	41/43	9/9
out-of-frame		12/33 (37.4)	13/29 (44.9)	7/20 (35)	5/17 (29.5)	5/28 (17.9)	2/43 (4.7)	0/9 (0)
Athymic nude								
In-frame	A	3	0	2				
	B	2	6	0				
	other Jα	0	1	0				
	subtotal	5/7	7/9	2/8				
out-of-frame		2/7 (28.6)	2/9 (22.2)	6/8 (75)				

Figure 3. Frequency of in-frame and out-of-frame Vα14⁺ TCR sequences in various tissues. (*A*) Two major types of homogenous Vα14⁺ TCR with Jα281 sequences were shown and compared with germline Vα14 and Jα281 sequences: type A and type B, which is identical to type A but lacking one amino acid (D) in the VJ junctional region. The deduced amino acids are indicated as a one-letter code above the nucleotide sequences. The flanking sequences of Vα14 and Jα281 are illustrated by small letters, in which heptamer sequences are underlined. (*B*) The frequency of in-frame and out-of-frame Vα14⁺ sequences analyzed in various tissues of euthymic and athymic mice. The in-frame sequences are divided into three subgroups: type A, type B, and other Jα, which were associated with Jα other than Jα281. The percentages of Vα14⁺ out-of-frame sequences in the total Vα14⁺ clones examined are shown in parentheses.

and Vα14 and Jα281 primers for genomic PCR. The results of both RT- and DNA-PCR analyses were similar, and Fig. 3 shows the data of RT-PCR. Two types of Vα14Jα281 sequences were preferentially detected: type A is the major Vα14⁺ sequence dominant (60–100%) in all tissues, except for IEL; and type B is detected mainly in IEL (44% type B and 0% type A) and is almost identical to type A except it lacks one amino acid (D, aspartic acid) at the VJ junctional region. The occurrence of two types of homogenous Vα14⁺ TCR most likely suggests the presence of distinct ligands in different extrathymic tissues. These findings were also observed in athymic nude mice, confirming that both types of Vα14⁺ T cells are selected for in particular tissues without thymic influence (Fig. 3). This may imply that selection of Vα14⁺ T cells effectively occurs in extrathymic tissues rather than in thymus.

A High Frequency of Out-of-Frame Vα14⁺ TCR Gene Sequences Is Detected in Extrathymic Tissues. The central question we asked is whether homogenous Vα14⁺ T cells are generated in the extrathymic sites where they are positively selected. To find the answer, we investigated the frequency of nonproductive Vα14⁺ TCR. Sequencing analysis revealed that out-of-frame sequences were detected in PP, IEL, BM, and liver as well as thymus at a high frequency (Fig. 3); 12/33 (37.4%) of Vα14⁺ cDNA clones in PP, 13/29 (44.9%) in IEL, 7/20 (35%) in BM, and 5/17 (29.5%) in liver were nonproductive. Even in nude mice, the V14⁺ out-of-frame TCR were frequently detected in PP (28.6%), IEL (22.2%), and BM (75%) (Fig. 3). These results imply that the frequent nonproductive TCR sequences detected represent the level of gene rearrangements occurring in extrathymic sites. Since the random nature of VJ joining leads to a considerable proportion of rearrangements being out-of-frame at the site of T cell differentiation, these results strongly suggest that organs with a high frequency of nonproductive TCR could be the sites for extrathymic T cell development.

Rearrangement of Vα14⁺ TCR Gene and Detection of Signal Sequence with Reciprocal Heptamers in Extrathymic Tissues. To confirm the presence of extrathymic Vα14 T cell differentiation, we attempted to detect recombination signal sequences in circular DNA that resulted from the Vα14-Jα281 gene rearrangement event. For this purpose, DNA from the nuclei of extrathymic tissues was used for the PCR to amplify potential signal joints of the circular DNA. The experimental strategy is diagrammed in Fig. 4 *A*. The two sets of primers are prepared for a double-step PCR in opposite outward orientations at the unrearranged germline locus in such a way that no DNA amplification is possible. When circular episomal products are created by the formation of a signal joint, the PCR primers will amplify fragments with a signal sequence. This sequence includes two signal heptamers, two nonamers, and 12/23 spacers in flanking sequences of Vαβ and Jα/Dβ genes.

DNA blot analysis of PCR products was carried out using specific probes for the Vα14-Jα281 signal joints (Fig. 4 *B*). We detected a band of ~300 bp in size in most extrathymic tissue samples, except in spleen and kidney. Significant amplifications were observed in PP, BM, IEL, and liver. In fact, by quantitative PCR (Fig. 5 *A* and Table 1), the relative amounts of Vα14-Jα281 signal sequences per DNA in PP, liver, BM, and IEL were calculated to be 1.7, 2.5, 0.7, and 0.7, respectively, assuming that those in thymus were 1.0. Arbitrary units (AU) of amounts of signal sequences per T cells in each tissue were 5.6 in PP, 16.8 in liver, 17.5 in BM, and 3.5 in IEL, compared with those in thymus (Table 1). As the amounts of signal sequences in those tissues are greater than those in thymus, these extrathymic tissues are likely to be sites for generation of Vα14 T cells. On the other hand, in spleen, Vα14-Jα281 signal sequences were hardly detected, and their frequencies were calculated to be <0.01 (Table 1). Thus, it is likely that spleen is not the site, or it is less efficient for the generation of Vα14⁺ T cells. Since circular DNA is formed during T cell development, these results imply that significant numbers of the cells in BM, liver, and intestine are progenitors for Vα14⁺ T cells and have been undergoing rearrangement and creating the episomal DNA.

To confirm the formation of the reciprocal heptamer/heptamer joining, we subcloned and sequenced the PCR products shown in Fig. 6 *A*. If the rearrangement occurred by the mechanism of an intrachromosomal deletion mediated through signal sequences, the circular DNA was expected to contain the reciprocal heptamer repeats in the flanking regions of Vα14 and Jα281 with or without insertion or

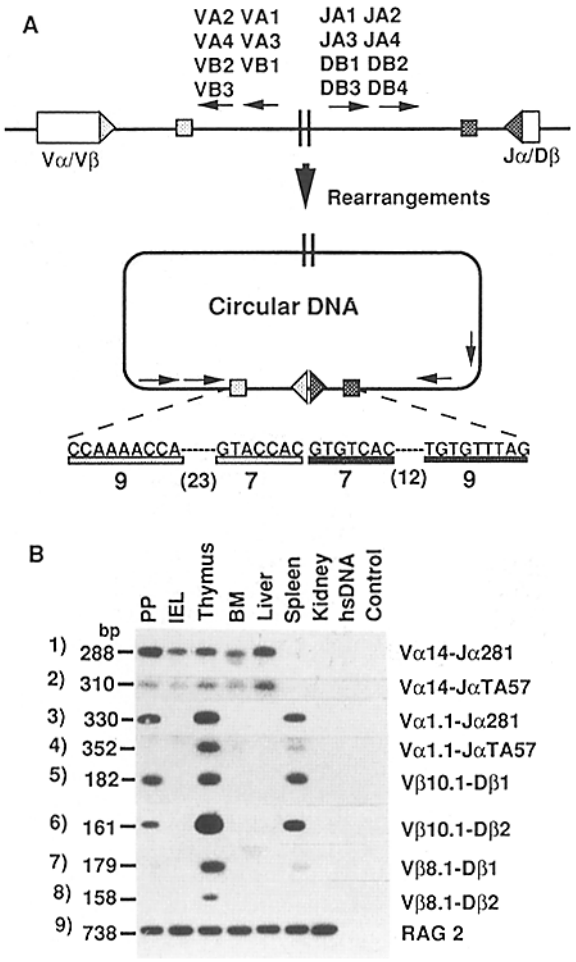

Figure 4. Signal joints in the circular DNA created by Vα-Jα and Vβ-Dβ rearrangements. (*A*) Schematic representation of the experimental strategy used to amplify the signal joints from DNA amplified by double-step PCR. Typical signal sequence heptamers and nonamers flanking the Vα/Jα and Vβ/Dβ gene segments are represented by triangles and squares, respectively. The position and orientation of PCR primers (VA1, VA2, VA3, VA4, VB1, VB2, VB3/JA1, JA2, JA3, JA4, DB1, DB2, DB3, DB4) are indicated by arrows. (*B*) DNA blots of PCR products of BALB/c containing signal sequences of: (*1*) Vα14-Jα281, (*2*) Vα14-JαTA57, (*3*) Vα1.1-Jα281, (*4*) Vα1.1-JαTA57, (*5*) Vβ10.1-Dβ1, (*6*) Vβ10.1-Dβ2, (*7*) Vβ8.1-Dβ1, (*8*) Vβ8.1-Dβ2, and (*9*) DNA blots of RAG-2 PCR products (relative amounts of DNA used for PCR). The copy numbers of DNA in samples (PP, IEL, thymus, BM, liver, spleen, kidney) were normalized by those of RAG-2 genes, and 10^7 (0.5 μg) were used for PCR. Herring sperm DNA (*hsDNA*) (1.5 μg) and control (water) were also amplified as controls. The PCR products (3 μl) were subjected to electrophoresis and hybridized with ^{32}P-labeled probes. The expected sizes of PCR products are shown on the left. Two other experiments using different concentrations of sample DNA (0.1 and 1.5 μg) gave similar results. The quantitative curves on the PCR products shown here were illustrated in Fig. 5.

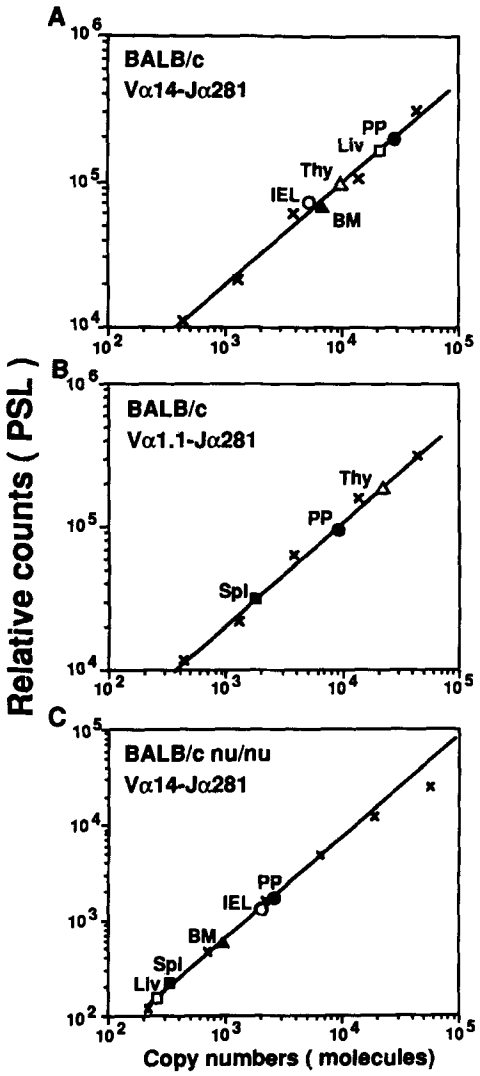

Figure 5. Quantitative PCR analysis of signal sequences. Amounts of nuclear DNA isolated from thymus (Δ), spleen (■), PP (●), IEL (○), liver (□), and BM (▲) measured by quantitative PCR with RAG-2 PCR primers were normalized. They were subsequently used for PCR to measure the frequency of signal sequences generated by Vα14-Jα281 (*A*), Vα1.1-Jα281 rearrangement of BALB/c (*B*), and by Vα14-Jα281 rearrangement of BALB/c *nu/nu* (*C*). Varied concentrations of the standard DNA (×) were amplified. Their radioactivities are expressed as AU of photostimulated luminescence (*PSL*) and plotted against template concentrations.

deletion of nucleotides at recombination sites. All clones obtained from various extrathymic tissues (PP, IEL, BM, and liver) shown in Fig. 4 revealed a sequence pattern typical of circular DNA. Two recombination heptamers were joined together in a head-to-head fashion, followed by 12- or 23-bp spacers and nonamers identical to the flanking sequences of germline Vα14 and Jα281.

We also investigated other TCR-α gene rearrangements in various tissues, i.e., Vα1.1-Jα281, Vα14-JαTA57, and Vα1.1-JαTA57. Distinct from the Vα14-Jα281 rearrangement, the Vα1.1-Jα281 and Vα1.1-JαTA57 signal sequences were detected only in thymus, spleen, and PP, but not in other extrathymic sites (Fig. 4 *B*). These results were confirmed at a nucleotide level (Fig. 6 *B*) and also by quantitative PCR analysis (Fig. 5 *B* and Table 1). The relative amounts of Vα1.1-Jα281 signal sequences were 0.4 (1.3 AU) in PP and 0.2 (0.5 AU) in spleen as compared with those in thymus. Similarly, those of Vα1.1-JαTA57 were 0.2 (0.5 AU) in spleen and negligible in all other tissues. However, the relative frequencies of Vα14-JαTA57 signals were 2.0 (13.4 AU) in liver,

Table 1. *Quantitative Analysis of Signal Sequences in Various Tissues*

Tissues	T cells	Relative ratios of the amounts of signal joints detected: Vα14-Jα281 per DNA	Vα14-Jα281 per T cell	Vα1.1–Jα281 per DNA	Vα1.1–Jα281 per T cell
	%				
Thymus	>90	1	1	1	1
Spleen	40	<0.01	(<0.01)	0.2	(0.5)
PP	30	1.7	(5.6)	0.4	(1.3)
IEL	20	0.7	(3.5)	UD*	UD
BM	4.0	0.7	(17.5)	UD	UD
Liver	15	2.5	(16.8)	UD	UD

The amounts of signal sequences in samples were measured by quantitative PCR according to the standard curves of specific Vα14-Jα281 and Vα1.1-Jα281 signal sequences of known copy numbers. The relative ratios (R) of amounts of signal sequences per DNA were calculated by the following formula, assuming that 1.0 was their relative amount in thymus: R = [sample C (SJ)/thymus C (SJ)] × [thymus C (RAG)/sample C (RAG)]; where *SJ* is signal joint, *RAG* is RAG-2, and C is copy number of genes (SJ or RAG) in tissues. The relative ratios were then adjusted to the numbers of Thy-1$^+$ T cells and expressed as AU per T cells in parenthesis. The percentage of Thy-1$^+$ T cells in various tissues was determined by FACS® analysis. Two independent experiments using different sample batches produced similar results.
* UD, undetectable (<0.001).

0.9 (22.5 AU) in BM, 0.8 (2.6 AU) in PP, and 0.5 (2.5 AU) in IEL, but negligible in spleen, relative frequencies that were similar to those of Vα14-Jα281. Therefore, the Vα1.1-Jα281 and Vα1.1-JαTA57 TCR rearrangement patterns occurring in the periphery were distinct from those of Vα14-Jα281 and Vα14-JαTA57, suggesting that Vα1.1$^+$ T cells do not develop in BM, liver, and IEL, but that Vα14$^+$ T cells do develop in these tissues. The differential TCR rearrangement patterns observed in extrathymic organs suggest that certain subsets of T cells are generated in extrathymic tissues.

In athymic nude mice, the Vα14-Jα281 signal sequences were significantly detected, whereas Vα1.1-Jα281 signal sequences were undetectable (<0.001) (Fig. 7). Although the frequencies of signal sequences per DNA were much lower (~10 times less; see Fig. 5 *C*) than those in euthymic mice

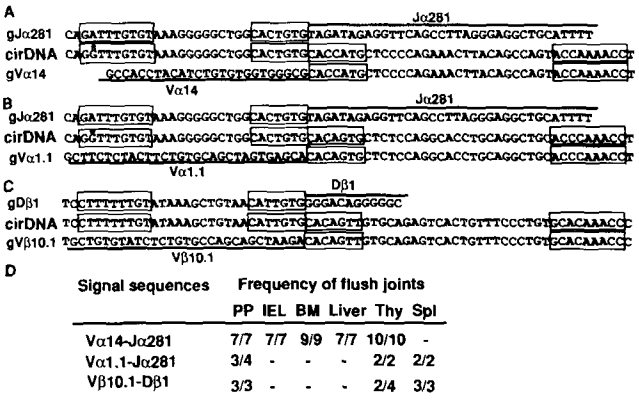

Figure 6. Representative nucleotide sequences and frequencies of the reciprocal signal joints detected in extrathymic and thymic tissues. (*A*) The Vα14-Jα281 recombinant sequence (*cirDNA*) detected was compared with its corresponding germline sequences of Vα14 (gVα14) and Jα281 (gJα281). (*B*) Comparison of Vα1.1-Jα281 reciprocal sequence with corresponding germline sequences (gVα1.1 and gJα281). (*C*) Vβ10.1-Dβ1 signal sequence compared with corresponding germline sequences (gVβ10.1 and gDβ1). (*D*) Frequency of signal sequences of the circular DNA with flush joint is expressed as numbers in PCR clones with reciprocal joints examined. The nucleotide mutation is indicated by asterisks. The Vα14, Vα1.1, Vβ10.1, Jα281, and Dβ1 coding sequence are marked with a thick line. The heptamer and nonamer sequences are boxed. Note that no sequences were amplified in any of the kidney, herring sperm DNA, and control material (water) tested.

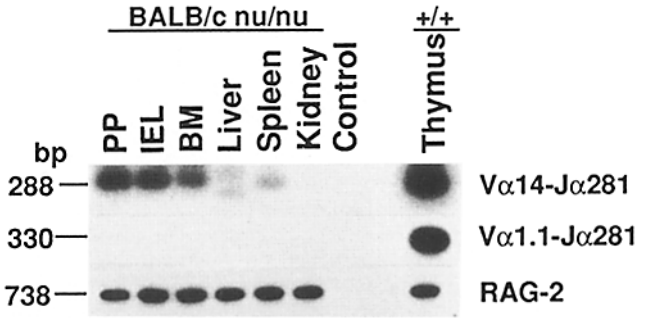

Figure 7. Detection of Vα14 signal sequences in athymic mice. PCR was carried out using nuclear DNA from various tissues of BALB/c *nu/nu* mice under the same conditions as described in Fig. 4, except for 30-cycle amplification in the second step. The copy numbers of DNA in samples were normalized by those of RAG-2 genes, and 3 × 10^7 (1.5 μg) were used for PCR. For comparison of amounts of signal sequences in *nu/nu* mice, the first-step PCR products of thymus used in Fig. 4 were amplified under the same conditions and used as control. For electrophoresis, one-fourth of thymus PCR products were applied.

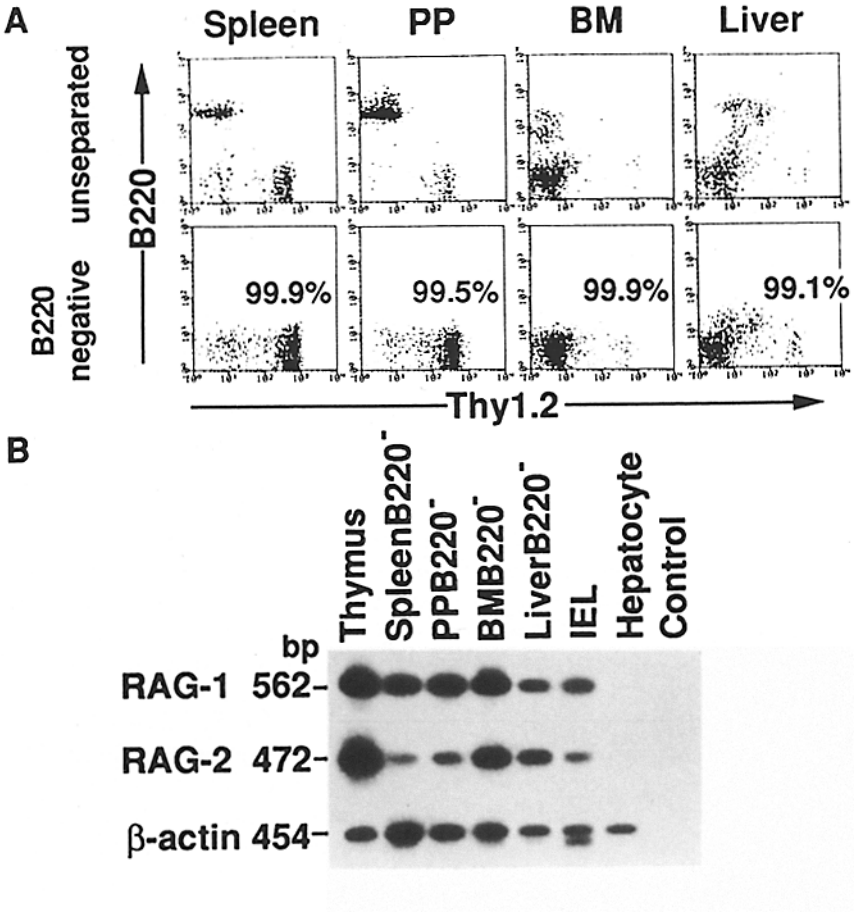

Figure 8. Detection of RAG-1/RAG-2 transcripts. (*A*) FACS® profiles of unseparated and separated ($B220^-$) populations. Their RNA was extracted by the standard method as described. (*B*) The first-strand cDNA was synthesized with random hexanucleotide primers using total RNA (0.1–1.0 μg). PCR was carried out with mouse β-actin primer to normalize sample materials and then with RAG-1 and RAG-2 primers under the conditions described previously (29). PCR products were hybridized with ^{32}P-labeled probes for 566-bp HincII fragment of RAG-1 cDNA, 852-bp PstI fragment of RAG-2 cDNA, and 250-bp XbaI/KpnI fragments of mouse β-actin cDNA. The expected sizes of RAG-1 and RAG-2 PCR products are 562 and 472 bp, respectively.

due to the small numbers of lymphocytes in *nu/nu* mice, AU of signals per T cells are equivalent to or much higher in PP and IEL than those in the thymus. It is thus clear that Vα14 T cell development occurs in extrathymic tissues without thymus.

Rearrangement of TCR β Chain Genes in Extrathymic Tissues. We investigated signal sequences generated by TCR-β gene rearrangements in extrathymic tissues. For this purpose, we designed two sets of primers for amplification of the signal sequences of the circular DNA only when the Vβ and Dβ gene segments were rearranged as described in Fig. 4 *A*. Some sequences and PCR bands detected were illustrated and summarized in Figs. 4 and 6 *C*. Vβ-Dβ rearrangement patterns were different from $V\alpha14^+$ TCR patterns but similar to the $V\alpha1.1^+$ TCR patterns. Thymus appeared to have all Vβ-Dβ rearrangement patterns.

However, nonthymic organs showed differential TCR patterns. The frequencies of signal sequences in PP and spleen were extremely low, that is, ~1/7–15 (Vβ8.1-Dβ1), negligible (Vβ8.1-Dβ2), and 1/4–5 (Vβ10-Dβ1/Vβ10-Dβ2) of those in thymus, respectively. Thus, no Vβ-Dβ signal sequences have so far been detected in other extrathymic tissues. All signal sequences detected so far contain two reciprocal hepatamers joined in a head-to-head fashion, followed by 12/23-bp spacers and nonamer sequences identical to the flanking sequences of either germline Vβ or Dβ.

Detection of RAG-1 and RAG-2 Messages in the $B220^-$ Cell Fractions in Extrathymic Tissues. $B220^-$ cell fractions were isolated by FACS® (Fig. 8 *A*). The purity was >99% in all preparations. Then, RT-PCR was carried out on isolated mRNA from those fractions using RAG-1- and RAG-2-specific primers. As shown in Fig. 8 *B*, $B220^-$ cell fractions in extrathymic tissues all expressed RAG-1 and RAG-2 messages at a relatively high amount.

Discussion

In this paper we have presented the first molecular evidence for extrathymic development of some T cell populations. The following results demonstrate this type of T cell development. (*a*) The Vα14Jα281 TCR sequences are discernible in extrathymic organs of athymic mice at frequencies similar to those in euthymic mice. (*b*) Signal sequences created by the Vα14-Jα281 and Vα14-JαTA57 TCR rearrangements are detected in most extrathymic tissues, including BM, PP, IEL, and liver, but are not found in spleen. However, other TCR rearrangements, such as Vα1.1-Jα and Vβ-Dβ, are observed in spleen and PP but not in the majority of extrathymic tissues, indicating that only a certain subpopulation, but not all T cells, develops at extrathymic sites. (*c*) Vα14 TCR rearrangements are observed in athymic mice, whereas Vα1.1 TCR rearrangements are not detected, sug-

gesting that Vα14 T cells develop without thymus. Finally, (*d*) quantitative analysis revealed that the relative amounts of certain TCR signal sequences in some extrathymic tissues are higher than those in thymus, implying that certain TCR gene rearrangements do occur in extrathymic tissues.

The detection of circular DNA in extrathymic tissues may be viewed as evidence in favor of the argument that thymic T cells bearing circular DNA migrate to the extrathymic tissues. This is because we have detected these persisting signal sequences only when generated from thymus but not from extrathymic sites. However, we believe this possibility is unlikely, because signal sequences created by the Vα14-Jα281 rearrangement are indeed found in athymic mice (Fig. 7).

Furthermore, Vα14-Jα281 signal sequences are not found in spleen, the recirculating tissue of thymic T cells (Fig. 4 and Table 1). If all peripheral T cells were derived from thymus and were carrying the circular DNA that had been created in thymus, spleen should have signal sequences generated by Vα14-Jα281 recombinations at a similar frequency as those in other extrathymic tissues. However, they are not present in spleen. In addition, the relative amounts of Vα14-Jα281 signal sequences in extrathymic tissues are higher than those in thymus (Table 1). Particularly, the frequencies of Vα14-Jα281 signal sequences in extrathymic sites were higher than those in thymus. Similar observations are found in Vα14-JαTA57 rearrangements where their frequencies are almost equal to those of Vα14-Jα281. It seems difficult to explain the increase of relative amounts of circular DNA in extrathymic tissues by the notion that the signal sequences detected are derived from thymus migrants, because circular DNA do not autonomously propagate by themselves, but have been found at diluted concentrations in tissues peripheral to the thymus, as demonstrated by Takeshita et al. (20). Furthermore, it is unlikely that the Vα14 circular DNA detected are created in mature cells by rearrangement events that occurred in the circular DNA after excision from genomic DNA. This is because the Vα14 gene is known to be located far upstream of the TCR Vα gene clusters (21). Therefore, it is apparent that Vα14$^+$ T cells actually create their circular DNA and develop in nonthymic sites.

Interestingly, additional unique characteristics of Vα14-Jα281$^+$ T cell development are found by analysis of other TCR-α rearrangements. For example, the Vα1.1 gene rearrangement patterns, such as Vα1.1-Jα281 and Vα1.1-JαTA57 rearrangement patterns, are entirely different from those of Vα14$^+$ TCR (Fig. 4). Quantitative PCR analysis also supports this finding (Fig. 5 and Table 1). The frequencies of Vα1.1-Jα281 and Vα1.1-JαTA57 rearrangements in PP and spleen are equal to or less than those in the thymus, and both are negligible in other extrathymic tissues. Thus, it is possible that PP and spleen could be the organs for the recirculating pools of T cells of thymus origin. As the Vα1.1-Jα281 but not Vα14 circular DNA were hardly detectable in athymic mice (Fig. 7 and Table 1), they are likely to be preferentially generated in thymus and detected as a persisting circular DNA from thymus migrants in extrathymic tissues.

Our recent studies on Vα14Jα281 transgenic mice showed a unique characteristic of Vα14 T cell development. About 13% Vα14$^+$ T cells were detected in the spleen by anti-Vα14 mAb, whereas only 1% were found in the thymus. RNase protection assay also support the FACS® data, showing that ~50% of TCR α chains in the spleen are the transgenic Vα14Jα281, while 5% are in the thymus. These results indicate that Vα14$^+$ T cells are preferentially developed in extrathymic tissues rather than in the thymus.

Most TCR-β gene rearrangement patterns detected in extrathymic tissues are basically similar to Vα1.1$^+$ TCR rearrangements, but not similar to those of Vα14$^+$ TCR (Fig. 4). Vβ10-Dβ1 patterns are detected in thymus, spleen, and PP (the frequency of Vβ10.1-Dβ1 in PP and spleen is ~1/4–5 of that in thymus), whereas Vβ8.1-Dβ1 and Vβ8.1-Dβ2 are found mainly in thymus and faintly in spleen (1/10 of thymus) but not in other peripheral tissues. Again it is possible that Vβ circular DNA detected in PP and spleen are the thymus migrants, because the patterns are basically similar to Vα1.1 rearrangements. However, it is equally possible that they are generated in these extrathymic tissues. In any event, the differential patterns observed in TCR α/β rearrangements indicate that certain subsets of TCR repertoires are generated in different extrathymic sites.

RAG-1/RAG-2 genes are known to be expressed in immature T cells and are necessary for gene rearrangement (22, 23). It is therefore of interest to detect whether extrathymic tissues contain RAG-1/RAG-2 transcripts. Both RAG-1/RAG-2 transcripts were detected in the T cell fractions in PP, IEL, BM, and liver, as well as thymus (Fig. 8). The results, in part, confirm the data by Guy-Grand et al. (24), who also detected RAG-1 mRNA in the intestine. The results strongly support the notion that T cells do develop in extrathymic tissues.

Speiser et al. (25) have recently demonstrated that T cells or stem cells of nude mice can be positively selected for self-MHC restriction extrathymically. Our findings, in part, support their functional results at the molecular level. T cells developed and selected extrathymically might represent only a minority of the whole T cell population, but might be accumulated at higher levels in certain tissues. For example, among Vα14Jα281$^+$ T cells, the type B Vα14Jα281$^+$ T cells are primarily expanded in IEL, whereas the type A Vα14-Jα281$^+$ T cells dominate in BM, PP, and liver, but not in IEL. It is thus likely that distinct TCR repertoires are formed in different extrathymic tissues. Similarly, there is other evidence for extrathymic T cell maturation, for example, IEL (26, 27). Furthermore, several of the IEL subpopulations could be reconstituted in the irradiated, thymectomized mice given T cell–depleted BM (28). Therefore, certain fractions of functional TCR repertoire actually develop in extrathymic sites per se without thymus.

At present we do not know whether certain V gene segments, like Vα14, are preferentially rearranged and selected outside the thymus or whether extrathymic rearrangement can randomly include all V genes. The detection of differential TCR gene rearrangement patterns supports the former possibility. As extrathymic TCR rearrangement is a non-

random event, it can be hypothesized that these extrathymically developed T cells play a special role in the immune system that might be distinct from that of thymically selected T cells. Experiments to test the above possibilities are now in progress.

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