The Junctional Modifications of a T Cell Receptor γ Chain Are Determined at the Level of Thymic Precursors

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Summary

T precursors from fetal liver and adult bone marrow were compared for their ability to give rise to $V\gamma4^+$ T cell development. Fetal thymic lobes were repopulated with fetal liver or adult bone marrow cells, and the organ-cultured thymocytes were analyzed for their T cell receptor expression by the polymerase chain reaction (PCR). Both day 14 fetal liver and adult bone marrow cells gave rise to thymocytes with $V\gamma4$-$J\gamma1$ transcripts. However, the average size of the PCR products derived from adult precursors was slightly larger than that from fetal precursors. DNA sequence analysis of the $V\gamma4$-$J\gamma1$ transcripts showed that early fetal liver precursors predominantly gave rise to thymocytes with the $V\gamma4$-$J\gamma1$ transcripts without N nucleotide insertion, while late fetal liver and adult marrow precursors predominantly gave rise to thymocytes with modified $V\gamma4$-$J\gamma1$ junctions. These results suggest the possibility that the level of the N nucleotide insertion is programmed at the level of thymic precursors. This study also supported the model presented previously that the developmental potential of hematopoietic stem cells may change during ontogeny.

T cell development in the mouse thymus occurs in several successive waves during fetal life. The first thymocyte wave is largely made up of T cells that have rearranged and express the $V\gamma3$ TCR; these cells are later distributed in the skin (1). The second wave of thymic T cell development largely includes T cells with the $V\gamma4$ TCR; they are later distributed in the epithelium of the tongue and of the reproductive organs (2). These two epithelial T cell subsets express homogeneous γ/δ TCRs with a single species of $V\gamma$-$J\gamma1$ junctional sequence (canonical sequence), representing in-frame rearrangement of the germline $V\gamma$-$J\gamma$ elements, without addition or deletion of nucleotides at the junction (3). It appears that fetal but not adult hematopoietic stem cells (HSCs) are required to achieve detectable $V\gamma3^+$ T cell development (4, 5). Here, we demonstrate that in the fetal thymic microenvironment early fetal liver precursors predominantly give rise to thymocytes with canonical $V\gamma4$-$J\gamma1$ transcripts, while late fetal liver and adult marrow precursors predominantly give rise to thymocytes with modified $V\gamma4$-$J\gamma1$ junctions. This study supports the idea that the developmental potential of HSCs may change during ontogeny (4).

Materials and Methods

Hanging Drop and Organ Culture of Fetal Thymic Lobes. Thymic lobes from BALB/c fetuses at day 14 of gestation were treated with dGuo in vitro to deplete host thymocytes, and repopulated with 3×10^5 BALB/c fetal liver or adult bone marrow cells by hanging drop culture as described (4). The lobes were then organ cultured for 14 d.

mAbs and Immunofluorescence Staining. Organ-cultured thymocytes were stained either with biotin-anti-γ/δ (UC7-13D5) (6) or biotin-anti-$V\gamma2$ (UC3-10A6) (6) (both from PharMingen, San Diego, CA), or biotin-anti-$V\gamma3$ (M181.1) (4) (provided by Dr. I. MacNeil, DNAX Research Institute, Palo Alto, CA) antibodies, followed by Texas red-avidin, anti-CD3 (500A2), and FITC-anti-hamster IgG (Caltag Laboratories, South San Francisco, CA) antibodies as described (4). The labeled cells were analyzed as described (4).

PCR and DNA Sequencing. Poly(A)$^+$ mRNA was isolated and reverse PCR was carried out for 40 cycles as described (4). PCR primers used were as follows. $V\gamma4$-1, 5'-AGTGACGAAAGATATGAGGC-3'; $J\gamma1$-1,5'-AGAGGGAATTACTATGAGCT-3'; $V\gamma4$-2, 5'-CCGAATTCAGTCCTCACCAT-3'; $J\gamma1$-2, 5'-GCAAGCTTAGTTCCTTCT-3'; H-2K-5',5'-CGATTACATCGCCCTGAACG-3'; H-2K-3',5'-GCTCCAAGGACAACCAGAAC-3'. The H-2K PCR primer pair encompasses two introns on genome (7). Amplified cDNA was purified, digested with EcoRI and HindIII, and cloned. Colonies were screened with labeled $V\gamma4$ probe (800-bp SphI fragment of clone 11) (8) (provided by Dr. D. Raulet, Massachusetts Institute of Technology, Cambridge, MA). Plasmid DNA from positive colonies was sequenced by the dideoxy method.

Results and Discussion

Differentiation of γ/δ T Cells from Thymic Precursors in Fetal Liver. Fetal thymic lobes were repopulated with fetal liver cells, and the organ-cultured thymocytes were analyzed for

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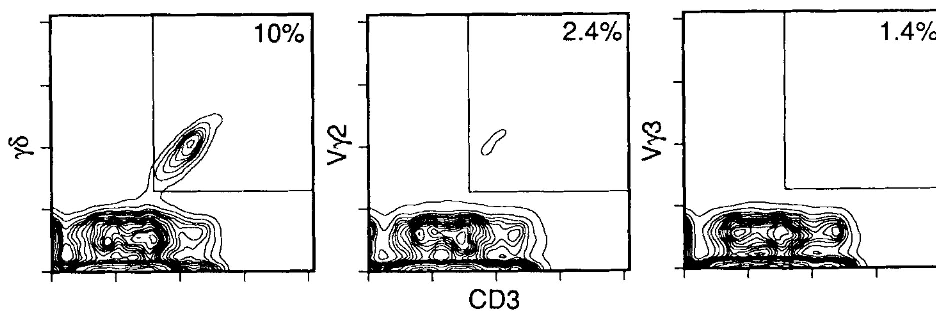

Figure 1. Differentiation of γ/δ T cells from fetal liver precursors. Organ-cultured thymocytes were stained with anti-γ/δ (*left*), anti-Vγ2 (*middle*), or anti-Vγ3 (*right*) antibodies, along with an anti-CD3 antibody. Although the $CD3^+$ $V\gamma3^+$ T cells do not show up in this plot, they make up a distinct population in a logarithmic plot (4).

their TCR expression by flow cytometry. Fetal thymic lobes from day 14 fetuses were treated with 2'-deoxyguanosine (dGuo) for 5 d to deplete host thymocytes. The lobes were then cocultured with day 14 fetal liver cells in a hanging drop for 2 d and organ cultured for 14 d. Thymocytes were stained either with anti-γ/δ, anti-Vγ2, or anti-Vγ3 antibodies, followed by anti-CD3 (a TCR complex protein) antibody as shown in Fig. 1. $CD3^+$ T cells made up 35% of these thymocytes, while $CD3^+$ γ/δ^+ T cells made up 10%. Therefore, 25% of the thymocytes were considered to be $CD3^+$ α/β^+ T cells. Because $CD3^+$ $V\gamma2^+$ and $CD3^+$ $V\gamma3^+$ T cells constituted 2.4% and 1.4% of the cells, respectively, ~6% of the thymocytes were likely to be non-Vγ2 and non-Vγ3 γ/δ T cells, suggesting the possibility that this population may contain some $V\gamma4^+$, $V\gamma1^+$, and/or $V\gamma5^+$ T cells.

Expression of Vγ4 Transcripts in Organ-cultured Thymocytes. PCR amplification of cDNA was carried out to detect Vγ4-Jγ1 transcripts in organ-cultured thymocytes. $Poly(A)^+$ RNA preparations from the organ-cultured thymocytes and freshly isolated fetal liver cells were reverse transcribed, and their cDNAs were amplified by PCR with Vγ4 and Jγ1 primers as shown in Fig. 2 *a*. Thymocytes derived from day 14 fetal liver cells (Fig. 2 *a*, lanes *1* and *3*) and adult bone marrow cells (lanes *2* and *4*) gave PCR products of approximately the predicted size (168 bp). The average size of the PCR products derived from adult precursors was slightly larger than that from fetal precursors. SphI digestion of the PCR products gave DNA fragments with the sizes of 102 and 66 bp long, suggesting that the DNA fragments detected are the correct Vγ4-Jγ1 PCR products (Fig. 2 *b*). The size of the 66-bp fragments from adult precursors, which contain V-J junctions, was slightly larger than that from fetal precursors. This observation suggested the possibility that the Vγ4-Jγ1 PCR products derived from adult precursors contain more N nucleotide insertions at the V-J junction than those from fetal precursors. Neither unreconstituted thymic lobes (Fig. 2 *a*, lane *5*) nor day 14 fetal liver cells (lane *6*) showed Vγ4-Jγ1 PCR products of the size predicted for successful rearrangement. These data suggest that the Vγ4-Jγ1 PCR products are derived from organ-cultured thymocytes, but not from host thymocytes that may have survived dGuo treatment, or any $V\gamma4^+$ T cells in fetal liver cells. The PCR amplification with H-2K primers gave a 522-bp fragment in all samples except for the unreconstituted thymic lobes (Fig. 2 *c*), suggesting that $poly(A)^+$ RNA was similarly prepared.

Vγ4-Jγ1 Junctional Sequences from Organ-cultured Thymocytes. To check whether the transcripts detected by PCR contain functional Vγ4-Jγ1 sequences, the PCR products were cloned and their nucleotide sequences were determined (Fig. 3 and Table 1). Of the 29 DNA clones obtained from thymocytes derived from day 14 fetal liver precursors, 21 had in-frame V-J joints. Of the 21 in-frame clones, 19 (90%) had the canonical sequence. Therefore, the Vγ4-Jγ1 transcripts in thymocytes derived from early fetal precursors had highly homogeneous junctions. On the other hand, of 26 clones

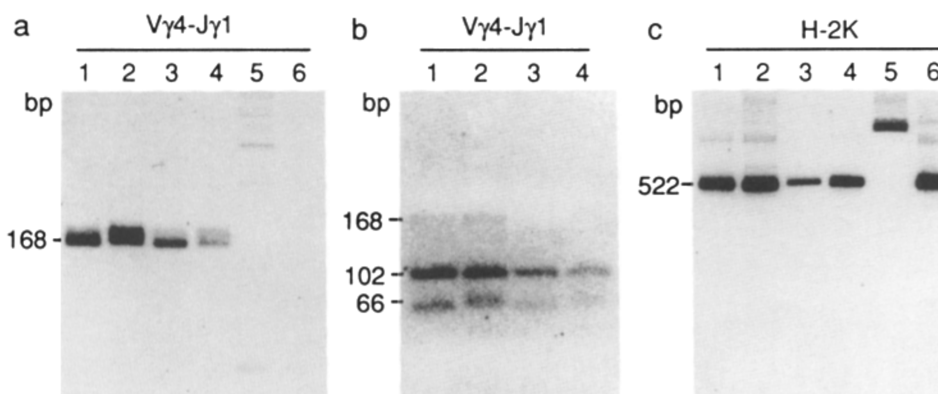

Figure 2. Expression of Vγ4 transcripts in organ-cultured thymocytes detected by PCR. (*a*) Expression of Vγ4 transcripts in the thymocytes derived from fetal and adult precursors. Lanes *1–5*, organ-cultured thymocytes from the thymic lobes repopulated with day 14 fetal liver (lanes *1* and *3*), adult bone marrow (lanes *2* and *4*), or no stem cells (lane *5*); lane *6*, day 14 fetal liver cells. Reverse PCR was done with Vγ4-1 and Jγ1-1 primers. The predicted size of the DNA fragment of the canonical Vγ4-Jγ1 sequence is 168 bp long. Other faints bands are produced by nonspecific binding of primers. (*b*) Restriction enzyme analysis of Vγ4 cDNA fragment generated by PCR. Lanes *1–4* correspond to those in *a*. The Vγ4-Jγ1 PCR products were digested with SphI. The digested DNA fragments of predicted size are 102 and 66 bp long. (*c*) Expression of H-2K transcripts. Lanes *1–6* correspond to those in *a*. The DNA fragment of predicted size is 522 bp long.

Germline Sequences

Vγ4: TGT GCA TGC TGG GAT A cactcta..........

Jγ1: ....cactgtg AT AGC TCA GGT TTT

Stem Cells		Vγ4	N	Jγ1	Frequency
E14 Fetal Liver in frame	*	TGT GCA TGC TGG GA		T AGC TCA GGT TTT	19/21
		TGT GCA TGC TGG GAT A	GGGGAGG	T AGC TCA GGT TTT	1/21
		TGT GCA TGC TGG GAT	G	AT AGC TCA GGT TTT	1/21
out of frame		TGT GCA TGC TGG GA	AAT	AT AGC TCA GGT TTT	2/8
		TGT GCA TGC TGG GAT A		T AGC TCA GGT TTT	3/8
		TGT GCA TGC TGG GAT A	G	AT AGC TCA GGT TTT	1/8
		TGT GCA TGC TGG G	TATGTTTTCC	AT AGC TCA GGT TTT	1/8
		TGT GCA TGC TGG GAT A	T	AT AGC TCA GGT TTT	1/8
Adult Bone Marrow in frame	*	TGT GCA TGC TGG GA		T AGC TCA GGT TTT	3/17
		TGT GCA TGC TGG GA	TGCCA	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A	CAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A		AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A	TGAAG	AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GA	AT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A	CGGG	C TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A	AAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG G	TCGTAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GAT A	AAA	GC TCA GGT TTT	1/17
		TGT GCA TGC TGG G	TAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG G	GAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG	CTCCCTG	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GA	CCGAT	AT AGC TCA GGT TTT	1/17
		TGT GCA TGC TGG GA	AGAGGGAT	AT AGC TCA GGT TTT	1/17
out of frame		TGT GCA TGC TGG G	C	AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT	GAG	AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT A	CGGG	AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT	CGG	AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT	CCCCGT	AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT A	GG	T AGC TCA GGT TTT	1/9
		TGT GCA TGC		AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG G	TT	AT AGC TCA GGT TTT	1/9
		TGT GCA TGC TGG GAT A	C	AGC TCA GGT TTT	1/9

Figure 3. Vγ4-Jγ1 junctional sequences from organ-cultured thymocytes. The V-J junctional DNA sequences are aligned with published germline sequences (3, 8). N regions due to nucleotide additions are shown. The frequency with which a particular sequence was found among DNA clones is listed in the last column. Asterisks indicate the canonical sequence (2, 3). Reverse PCR was done with Vγ4-2 and Jγ1-2 primers.

from the thymocytes derived from adult bone marrow cells, 17 had in-frame V-J joints, and only three out of them (18%) had the canonical sequences. The percentage of Vγ4-Jγ1 canonical sequence obtained from thymocytes derived from precursors from late-stage fetal livers (day 16 and day 18) was at an intermediate level between day 14 fetal liver and adult bone marrow. In these cases, N nucleotide insertion is more frequent in thymocytes derived from late fetal liver and adult marrow than early fetal liver precursors. No significant differences in base deletion at the V-J junctions was observed.

The Junctional Modifications of TCR Vγ4 Chain Are Determined at the Level of Thymic Precursors. Because it has been shown that T precursor activity in fetal liver and adult bone marrow is predominantly found in a HSC population (4, 9), these results suggested that fetal HSCs can give rise to T cells with the canonical Vγ4-Jγ1 sequence more efficiently than adult HSCs. It also suggests the possibility that one difference in the development of Vγ4$^+$ T cells is due to a determinative event that is expressed at the level of HSCs: the predilection for N nucleotide insertion at Vγ4-Jγ1 joints. It has been postulated that N nucleotide insertions are caused by the activity of terminal nucleotidyl transferase (TdT) (10). Therefore, it is reasonable to propose that the level of TdT activity might be programmed at the stage of T cell precursors. However, our results do not exclude the possibility that the efficiency of Vγ4-Jγ1 recombination events might be different between fetal and adult precursors.

Because the capacity for N nucleotide insertion is different between fetal and adult progenitors, it is possible that thymocyte populations derived from fetal and adult HSCs might have different V-J junctional diversity. It has been reported that the entry of T cell progenitors into the mouse thymus occurs in two waves, the first wave at day 10–13 of gestation, and the other wave at late fetal to neonatal stages (11). It is possible that the precursors in the first wave are cells with low N nucleotide insertion capacity, and those of the second wave with high N nucleotide insertion capacity. In B cells, it has been shown that N nucleotide insertion is more prevalent in adult than in fetal B cells (12, 13). Clonal analyses should help determine whether clones that have a given N nucleotide insertion capacity for Vγ4-Jγ1 joints "breed true" for TCR α/β joining diversity. These and related (4, 5) studies can be interpreted that HSCs are the locus of a complicated but precise developmental clock that may determine both the time-dependent closure of some gene loci (e.g., Vγ3, Vγ4 TCR, and perhaps embryonic and fetal globin), and the activation of others (e.g., the N nucleotide insertion machinery). If this interpretation is correct, it shall be important next to determine the mechanisms by which the clock(s) operate, and the genes that control its initiation and progression.

Table 1. *Vγ4-Jγ1 Junctional Sequences from Organ-cultured Thymocytes*

Source of stem cells	In frame	Canonical	Out of frame	Total	Percent canonical sequence	Base addition per transcript	Base deletion per transcript
E14FL	21	19	8	29	90	0.90	2.4
E16FL	16	7	9	25	44	2.5	2.6
E18FL	9	5	8	17	56	2.1	1.7
BM	17	3	9	26	18	3.0	2.4

Fetal thymic lobes were repopulated with fetal liver cells from day 14, day 16, and day 18 fetus or adult bone marrow cells. The Vγ4-Jγ1 junctional DNA sequences of PCR products were determined as described. The percentage of the canonical Vγ4-Jγ1 sequence is calculated in in-frame sequences. The average of base addition and deletion is calculated in total sequences. The difference of base addition between day 14 fetal liver and adult bone marrow is $p < 0.001$.

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