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 Vγ4+ 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 Vγ4-Jγ1 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 Vγ4-Jγ1 transcripts showed that early fetal liver precursors predominantly gave rise to thymocytes with the Vγ4-Jγ1 transcripts without N nucleotide insertion, while late fetal liver and adult marrow precursors predominantly gave rise to thymocytes with modified Vγ4-Jγ1 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.

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 x 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-Vγ2 (UC3-10A6) (6) (both from PharMingen, San Diego, CA), or biotin-anti-Vγ3 (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. Vγ4-1, 5'-AGTGACGAAGATAGAGGAGGC-3'; Jγ1-1, 5'-AGAGGGATTCTATGCT-3'; Vγ4-2, 5'-CCGAATTCAGTCCTCACCAT-3'; Jγ1-2, 5'-GCAAGCTTGGTTTCTCAAGCT-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 Vγ4 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
I

10% 2.4%

CD3

1.4%

Figure 1. Differentiation of \( \gamma/\delta \) T cells from fetal liver precursors. Organ-cultured thymocytes were stained with anti-\( \gamma/\delta \) (left), anti-V\( \gamma2 \) (middle), or anti-V\( \gamma3 \) (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).

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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-\( \gamma/\delta \) anti-V\( \gamma2 \), or anti-V\( \gamma3 \) 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\(^+\) \( \gamma/\delta \) T cells made up 10%. Therefore, 25% of the thymocytes were considered to be CD3\(^+\) \( \alpha/\beta \) T cells. Because CD3\(^+\) V\( \gamma2 \) and CD3\(^+\) V\( \gamma3 \) T cells constituted 2.4% and 1.4% of the cells, respectively, \( \sim6\% \) of the thymocytes were likely to be non-V\( \gamma2 \) and non-V\( \gamma3 \) \( \gamma/\delta \) T cells, suggesting the possibility that this population may contain some V\( \gamma4^+ \), V\( \gamma1^+ \), and/or V\( \gamma5^+ \) T cells.

Expression of V\( \gamma4 \) Transcripts in Organ-cultured Thymocytes. PCR amplification of cDNA was carried out to detect V\( \gamma4 \)-J\( \gamma1 \) 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 V\( \gamma4 \) and J\( \gamma1 \) 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 V\( \gamma4 \)-J\( \gamma1 \) PCR products (Fig. 2 b). The size of the 66-bp fragments from adult precursors, which contain VJ junctions, was slightly larger than that from fetal precursors. This observation suggested the possibility that the V\( \gamma4 \)-J\( \gamma1 \) PCR products derived from adult precursors contain more N nucleotide insertions at the VJ junction than those from fetal precursors. Neither unreconstituted thymic lobes (Fig. 2 a, lane 5) nor day 14 fetal liver cells (lane 6) showed V\( \gamma4 \)-J\( \gamma1 \) PCR products of the size predicted for successful rearrangement. These data suggest that the V\( \gamma4 \)-J\( \gamma1 \) 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.

V\( \gamma4 \)-J\( \gamma1 \) Functional Sequences from Organ-cultured Thymocytes. To check whether the transcripts detected by PCR contain functional V\( \gamma4 \)-J\( \gamma1 \) 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 V\( \gamma4 \)-J\( \gamma1 \) transcripts in thymocytes derived from early fetal precursors had highly homogeneous junctions. On the other hand, of 26 clones
The frequency with which a particular sequence was found among DNA clones is listed in the last column. Asterisks indicate the canonical sequence obtained from thymocytes derived from adult bone marrow, from the thymocytes derived from adult bone marrow cells, and from the fetal liver cells from day 14, day 16, and day 18 fetus. From these results, 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. 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-Jγ4 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 should be important next to determine the mechanisms by which the clock(s) operate, and the genes that control its initiation and progression.

![Figure 3](image-url)

**Figure 3.** Vγ4-Jγ4 junctional sequences from organ-cultured thymocytes. The V-J junctional DNA sequences are aligned with published germ-line sequences (2, 3). Reverse PCR was done with V3-4-2 and J3-1-2 primers. The frequency with which a particular sequence was found among DNA clones is listed in the last column. Asterisks indicate the canonical sequence obtained from thymocytes derived from adult bone marrow cells, and from the fetal liver cells from day 14, day 16, and day 18 fetus. From these results, 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. 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-Jγ4 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-Jγ4 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                   | 13       | 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-Jγ4 junctional DNA sequences of PCR products were determined as described. The percentage of the canonical Vγ4-Jγ4 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.
We thank Ms. L. Jerabek, L. Hu, and M. Hurlbut for their excellent technical assistance, and Mr. L. Hidalgo and the animal facility for maintaining mice. We thank Drs. I. MacNeil, D. Raulet, C. Okada, and Y. Chien for the antibody and probe. We also thank Dr. E. Lagasse for critical reading of the manuscript.

This work was supported by the Howard Hughes Medical Institute, and in part by the National Institutes of Health (CA-42551 and AI-09072) (I. L. Weissman). K. Ikuta was supported by the Howard Hughes Medical Institute.

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Received for publication 3 July 1991.

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