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Development of Vα14$^+$ NK T cells in the early stages of embryogenesis

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ABSTRACT **The majority of T lymphocytes start to develop at around day 15 of gestation (d15)–d17 in the thymus and comprise the peripheral repertoire characterized by the expression of polymorphic T-cell antigen receptors (TCRs). Contrary to these conventional T cells, a subset of T cells, called natural killer (NK) T cells (most of them expressing an invariant TCR encoded by the Vα14Jα281 gene with a 1-nt N-region), preferentially differentiates extrathymically and dominates the peripheral T-cell population at a high frequency (5% in splenic T cells and 40% in bone marrow T cells). Here, we investigated the development of NK T cells and found that the invariant Vα14$^+$ TCR transcripts and the circular DNA created by Vα14 and Jα281 gene rearrangements can be detected in the embryo body at d9.5 of gestation and in the yolk sac and the fetal liver at d11.5–d13.5 of gestation, but not in the thymus, whereas T cells with Vα1$^+$ TCR expression, a major population in the thymus, were not observed at these early stages of gestation. Fluorescence-activated cell sorter analysis also demonstrated that there exist CD3$^+$ αβ$^+$ T cells, almost all of which are Vα14/Vβ8$^+$ NK$^+$ T cells, during early embryogenesis. To our knowledge, this demonstrates for the first time that a T lymphocyte subset develops in extrathymic tissues during the early stages of embryogenesis.**

Mature T lymphocytes express T-cell antigen receptors (TCRs) composed of heterodimers of either αβ or γδ chains that are associated with the CD3 complex (1). The lymphoid precursor cells are believed to arise from the yolk sac or the fetal liver and migrate into the thymus (2). During lymphocyte development, the TCR genes then become functional by rearrangements of variable (V), diverse (D), and joining (J) gene segments, which take place primarily in the thymus (3). The first productive TCR gene rearrangement occurs in the TCRγ genes and is detected in the thymus at day 13 of gestation (d13) just after the thymus formation (4). The TCRβ gene is transcribed before the TCRα genes, with full-length TCRβ mRNA observable at around d15, before the detection of full-length TCRα mRNA at d17 of embryogenesis (5). Thus, conventional αβ T cells with functional TCR expression appear only during the later stage (d17) of gestation.

Previous experiments have shown that a subset of T cells expresses both NK1.1 and TCRαβ on the cell surface and preferentially uses an invariant TCRα encoded by Vα14 and Jα281 gene segments with a 1-nt N-region (6, 7). Moreover, Vα14$^+$ natural killer (NK) T cells, unlike conventional αβ T cells, are selected by non-major histocompatibility complex-encoded class I-like molecules expressed on bone marrow-derived cells but not by thymic epithelial cells (8–10) and predominate in extrathymic tissues, even in athymic mice (11). Therefore, it is of interest to know whether Vα14$^+$ NK T cells develop during the early stages of embryogenesis without thymic influence. Here, we describe how Vα14$^+$ TCR transcripts and their rearrangements are detected in the embryo body but not in the yolk sac at d9.5 of gestation and also successfully demonstrate that Vα14$^+$ NK T cells are indeed present mainly in the fetal liver while other conventional T cells are undetectable at the stages. The physiological role of Vα14$^+$ NK T cells is also discussed.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c and C57BL/6 mice were purchased from Shizuoka Animal Center (Hamamatsu, Japan). Recombination-activating gene (*RAG*)-1-deficient mice were provided by P. Mombaerts (12). Timed pregnancies were prepared as described (4). The day of the detection of a vaginal plug was designated d0.

Detection of TCR Transcripts. Total RNA was isolated from fetal tissues, including yolk sac, fetal liver, aorta-gonad-mesonephros (AGM), and thymus of d9.5–d13.5 embryos of (*RAG*-1$^{-/-}$ × BALB/c)F1 and (BALB/c × C57BL/6)F1 mice using Trizol Reagent (Life Technologies, Gaithersburg, MD). To amplify limited amounts of TCR transcripts in the early fetal tissues, we performed nested reverse transcriptase-PCR (RT-PCR) with 70–80 cycles of amplification according to the results on the single cell PCR reported by Brady *et al.* (13). First strand cDNA was synthesized by incubation of oligo(dT) primers with 0.1 μg of RNA as described (4). To detect TCR transcripts of (*RAG*-1$^{-/-}$ × BALB/c)F1 samples (see Fig. 1*A*), the cDNAs were amplified by nested PCR with primer pairs specific for Vα/Cα sequences: VC1/VC5 and VC2/VC6 for the detection of Vα14 mRNA, and VC4/VC5 and VC3/VC6 for Vα1 mRNA. The first 20 cycles of amplification were carried out with denaturation at 94°C for 1 min, annealing at 62°C for Vα14 or at 57°C for Vα1 for 2 min, and extension at 72°C for 2 min, followed by the next 55 cycles under the same conditions except for annealing at 58°C for Vα14. Oligonucleotide primers were as follows: VC1, 5′-CCGAATTC-CCAAGTGGAGCAGAGTCCT-3′; VC2, 5′-GGAAT-TCAAACAGGACACAGGCAAAGG-3′ for Vα14; VC3, 5′-TAGAATTCCCTGCACATCAAAGACTCCCA-3′; VC4, 5′-GGAATTCAATCCCTCATTGTCCCAGAG-3′ for Vα1; VC5, 5′-CAGGAGGATTCGGAGTCCCAT-3′; and VC6, 5′-GGAATTCAGTCGGTGAACAGGCAGAGG-3′ for Cα.

To detect CD3ε, *RAG*-1, and β-actin expression, RT-PCR was performed with specific primers under the following conditions: 40 cycles of denaturation at 94°C for 1 min, annealing at 54°C for CD3ε and β-actin or at 57°C for *RAG*-1 for 1 min, and extension at 72°C for 1 min. Oligonucleotide primers were as follows: for CD3ε, 5′-AACACTTTCTGGG-GCATCCTG-3′ and 5′-TGATGATTATGGCTACTGCTG-3′. Those for *RAG*-1 and β-actin have been published (11).

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Abbreviations: d, day of gestation; AGM, aorta-gonad-mesonephros; NK, natural killer; *RAG*, recombination-activating gene; RT-PCR, reverse transcriptase-PCR; TCR, T-cell antigen receptor; TdT, terminal deoxynucleotidyl transferase; UT, untranslated.

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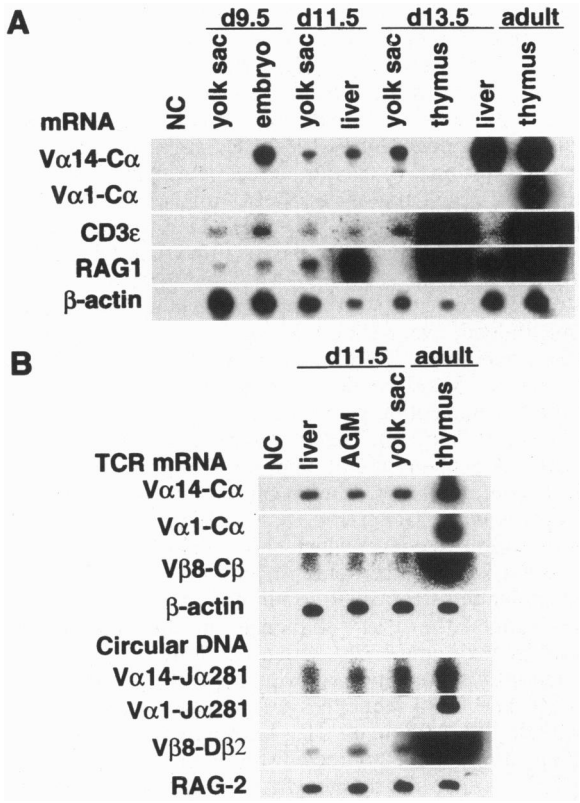

FIG. 1. Detection of TCR transcripts and signal sequences in early embryogenesis. PCR was performed on nuclear DNA and RNA isolated from fetal tissues of d9.5–d13.5 embryos of (*RAG*-1$^{-/-}$ × BALB/c)F1 and (BALB/c × C57BL/6)F1 mice. (*A*) DNA blot analysis of products amplified by RT-PCR using RNA from d9.5–d13.5 (*RAG*-1$^{-/-}$ × BALB/c)F1 embryos. CD3ε and *RAG*-1 expressions were also assayed. (*B*) DNA blot analysis of PCR products of TCR transcripts and signal sequences in the circular DNA from a d11.5 (BALB/c × C57BL/6)F1 embryo.

To detect terminal deoxynucleotidyl transferase (TdT) expression (see Fig. 4), nested RT-PCR was carried out with primers: TDT1/TDT2 and TDT1/TDT3 under conditions of the first 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, followed by the next 40 cycles under the same conditions. Oligonucleotide primers were: TDT1, 5′-AAAGAAGATGGGAACAACT-CG-3′; TDT2, 5′-AATCCCCTCTGTGTCTTTCAT-3′; and TDT3, 5′-GGGCATCCGTGAATAGTTGGT-3′.

To detect TCR transcripts of (BALB/c × C57BL/6)F1 samples (see Fig. 1*B*), RT-PCR was performed with primers specific for Vα14 (VC1)/Cα (CU1), Vα1 (VC3)/Cα (CU1), and Vβ8/Cβ. To avoid detecting the transcripts of the maternal origin, as illustrated in Fig. 2*A*, the primer CU1 was designed to include the 3′-half of the 18-nt insertion sequences specific for the 3′-untranslated (UT) region in the Cα gene of C57BL/6 origin (14). The primer sets of VC1/CU1 and VC3/CU1 exclusively amplify the TCR transcripts of paternal origin. The PCR conditions consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 63°C for Vα14Cα or 55°C for Vα1Cα and Vβ8Cβ for 1 min, and extension at 72°C for 1 min. Oligonucleotide primers were as follows: VC1, VC3, CU1, 5′-TCGAATTCCCAGACAGCCGTCTTGACGA-3′; Vβ8, 5′-CATATGGTGCTGGCAGCACT-3′; and Cβ (Cβ3), 5′-CCGAATTCGGTAGCCTTTTGTTTGTTTGC-3′. The *Eco*RI linker sequences are underlined.

PCR products were fractionated by electrophoresis on 2% agarose gel and were analyzed by Southern blot hybridization with ^{32}P-labeled probes. Probes used for detection of RT-PCR products were as follows: Vα14, 5′-CCACACAGATGTAG-GTGGCA-3′; Vα1, 5′-TGCACAGAGATAGAGAGCAG-3′; Cβ, 5′-TCAAACAAGGAGACCTTGGGT-3′; CD3ε, 5′-CCATCTCAGGAACCAGTGTAG-3′; 566-bp *Hin*cII fragments of *RAG*-1 cDNA; 250-bp *Xba*I/*Kpn*I fragments of mouse β-actin cDNA; and 520-bp cloned PCR products of TdT cDNA. The signals were detected by the Bio Imaging Analyzer (Fujix BAS2000; Fuji, Tokyo, Japan).

Sequencing. The PCR products of (*RAG*-1$^{-/-}$ × BALB/c)F1 and (BALB/c × C57BL/6)F1 were cloned into pGEM-T Vector (Promega) and were sequenced by the dideoxy chain termination method with a *Taq* dye primer cycle sequencing kit (Applied Biosystems/Perkin–Elmer) in an automated DNA sequencer (model 370A, Applied Biosystems; ref. 11).

Detection of A Signal Sequence in Circular DNA. Nuclear DNA was isolated from fetal tissues of d11.5 (BALB/c × C57BL/6)F1 embryos and was investigated for the detection of signal sequences as described (11). In brief, the amounts of nuclear DNA were first measured by a quantitative PCR using *RAG*-2 primers and normalized. Nuclear DNA was then amplified by nested PCR with primer sets of VA1/JA1 and VA2/JA2 for Vα14-Jα281, VA3/JA1 and VA4/JA2 for Vα1-Jα281, and VB4/DB3 and VB4/DB4 for Vβ8-Dβ2 rearrangements. Oligonucleotides for PCR primers were as follows: *RAG*-2, 5′-CACAGTCTTGCCAGGAGGAAT-3′; 5′-GGG-GGTTTTCTTTTGGGAGTTT-3′; VA1, 5′-CTTTGTCAC-CTATGTCTGGAA-3′; JA1, 5′-GAACAAAGGAAGTGGG-GTGAC-3′; VA2, 5′-TCACCTATGTCTGGAAGCCTC-3′; JA2, 5′-CTGGCGGTGGAAAGACTATTG-3′; VA3, 5′-AGT-

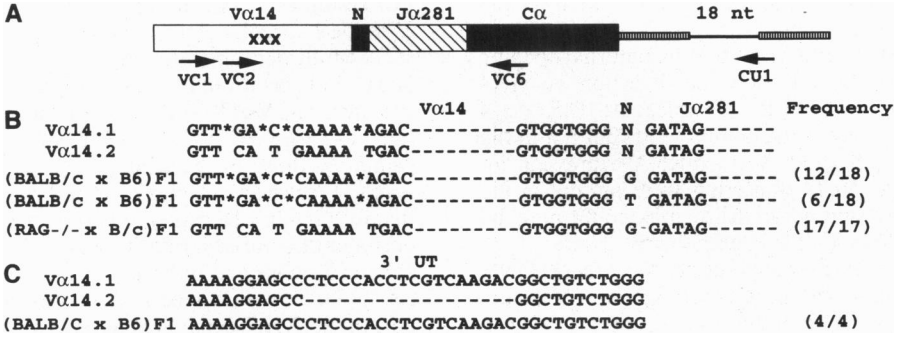

FIG. 2. Nucleotide sequences of Vα14$^+$ TCR transcripts detected in the early stages of embryogenesis. (*A*) A schematic illustration of RT-PCR primers designed for detection of Vα14 mRNA and its strain polymorphism. VC2/VC6 for detection of Vα14$^+$ mRNA in (*RAG*-1$^{-/-}$ × BALB/c)F1 and VC1/CU1 for amplification of Vα14.1 of C57BL/6 type bearing the 18-nt insertion in the 3′-UT region in (BALB/c × C57BL/6)F1. (*B*) Nucleotide sequences with polymorphism in the V region and the VJ junction. Vα14.1 is C57BL/6 type, whereas Vα14.2 is BALB/c type. The frequencies are illustrated in the right margin. DNA blots shown in Fig. 1*A* were confirmed by the sequences from (*RAG*-1$^{-/-}$ × BALB/c)F1 mice. (*C*) Nucleotide sequences with genetic polymorphism in the 3′-UT region of Cα.Vα14.1$^+$ mRNA of C57BL/6 type possesses the 18-nt insertion at nucleotide position 837, whereas the insertion is missing in BALB/c type Vα14.2$^+$ mRNA.

AGTGCCTTCCCTGAGATG-3′; VA4, 5′-GCACACAGATAGAAACAGAAT-3′; VB4, 5′-GGCACTTTGTTCTCCTTATTT-3′; DB3, 5′-GACTTTTCCCAGCCCCTCTCA-3′; and DB4, 5′-AACCTCTCTGCCACCTGTCTC-3′. PCR was carried out for 20 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1min in the first step, and subsequently for 40 cycles under the same conditions as the first step. PCR products were hybridized with ^{32}P-labeled probes. Probes for the detection of signal sequences were as follows: 288-bp PCR product of Vα14-Jα281 signal sequence for the Vα14-Jα281 and Vα1-Jα281 signal sequences; oligonucleotides of the 5′-flanking region of Dβ2 (5′-CCCCTGCCAGGCTCTGGGGTAGGCACCTGTGGGGAAGAAA-3′) for Vβ8-Dβ2 signal sequences; and 852-bp *Pst*I fragments of *RAG*-2 cDNA. The signals were detected by the Bio Imaging Analyzer.

Antibodies and Flow Cytometry. Mononuclear cells in the d13.5 fetal liver of C57BL/6 mice were separated on a Ficoll/Hypaque density gradient, incubated with anti-FcR antibody (2.4G2) to block FcR-mediated binding, and stained with monoclonal antibodies. Anti-Vα14 was raised in our laboratory (15). Other antibodies used were obtained by PharMingen. Phycoerythrin-conjugated antibodies were as follows: anti-Vα14, anti-Vα3 (RR3–16), and anti-TCRδ (GL3). Fluorescein isothiocyanate-labeled antibodies were as follows: anti-TCRβ (H57–597), anti-Vβ8 (MR5–2), anti-Vβ2 (B20.6), anti-Vβ11 (RR3–15), anti-Vβ13 (MR12–4), anti-CD3 (145–2C11), anti-CD4 (RM-4–5), and anti-CD8 (53–6.7). Biotinylated anti-NK1.1 (PK136) was used with Cy-chrome-streptavidin. Dead cells were gated out with propidium iodide staining, and 1–2 × 10^5 cells were then analyzed by EPICS-XL (Coulter) with a logarithmic amplifier.

RESULTS AND DISCUSSION

Detection of Vα14$^+$ TCR Transcripts and Signal Sequences Generated by Vα14 and Jα281 Gene Segments. Because Vα14$^+$ T cells can develop extrathymically, we investigated Vα14$^+$ transcripts and Vα14 gene-mediated circular DNA in the early fetus at d9.5 (somites 21–25)–d13.5 of gestation before the thymus formation. For this purpose, we prepared nuclear DNA and cDNA from fetal liver, yolk sac, AGM, and thymus at different gestation times and carried out PCR to amplify Vα14 gene products and the signal sequences composed of inverted heptamer repeats and nonamer sequences in the circular DNA (11), both of which are created only by rearrangements of the Vα14 and Jα281 gene segments. Furthermore, to exclude the possibility of maternal contamination in the fetal tissues, we used two systems: one to elucidate the paternal type of Vα14$^+$ TCR in (*RAG*-1-deficient female × conventional male)F1 mice, where no lymphoid cells of maternal origin are expected (12), and a second to identify the polymorphism (16) of the paternal type Vα14$^+$ TCR in (BALB/c × C57BL/6)F1 fetus amplified by paternal-specific primers. The results in Fig. 1*A* show the detection of Vα14 transcripts in (*RAG*-1$^{-/-}$ × BALB/c)F1 mice. At d9.5, Vα14 transcripts were positive only in the embryo body but not in the yolk sac, whereas at d11.5–d13.5 Vα14 mRNA expression increased significantly, and Vα14 transcripts were present in the yolk sac and the fetal liver but not in the thymus, confirming the fetal development of Vα14 T cells in extrathymic tissues.

As shown in Fig. 1*B*, the signal sequence of the circular DNA generated by Vα14 and Jα281 gene rearrangement was successfully demonstrated at d11.5 in the yolk sac and the embryo body, including the fetal liver and AGM. These are known sites for the generation of hematopoietic precursors (2, 17). In contrast to Vα14 gene expression, neither Vα1$^+$ transcripts nor the Vα1 gene-mediated circular DNA were found in the same sample materials. Since Vα1-bearing T cells are known to be a major repertoire in the adult and differentiated only in the thymus (11), it is clear that Vα1$^+$ TCR of the thymic type cannot be functional at this early stage of gestation or that Vα1$^+$ T cells cannot proliferate, resulting in being undetectable of Vα1 TCR transcripts. The results suggest that Vα14$^+$ TCR is expressed in the extrathymic sites at an early stage of gestation before conventional αβ TCR appear in the thymus.

Molecular Evidence for Vα14$^+$ TCR Expression in the Early Stage of Gestation. We tried to detect paternal polymorphism in the V and 3′-UT regions of Vα14$^+$ mRNA in (BALB/c × C57BL/6)F1 samples with VC1/CU1 PCR primer set. The CU1 primer is specific for the TCRα mRNA of C57BL/6 but not BALB/c origin (see Fig. 2*A*). Therefore, only paternal Vα14$^+$ TCR transcripts are supposed to be amplified by the system. Indeed, we defined Vα14.1$^+$ TCR transcripts of C57BL/6 type with ^{154}GACCAAAAA^{163}G sequences in the V region whose 3′-UT region possessed the 18-nt insertion beginning at nucleotide position 837 only found in C57BL/6 (ref. 14; Fig. 2 *B* and *C*). These results suggest that the Vα14$^+$ TCR mRNA detected are of embryonic origin, not a result of maternal contamination. In addition, we confirmed the Vα14$^+$ TCR expression in (*RAG*-1$^{-/-}$ × BALB/c)F1 mice shown in Fig. 1 at a sequence level which should be of paternal origin, because *RAG*-1$^{-/-}$ mice demonstrated no Vα14$^+$ TCR transcripts under the same PCR conditions (data not shown).

Interestingly, the Vα14 sequences in the embryos were encoded by Vα14 and Jα281 with a 1-nt N-region, in which all VJ junctions were substituted by guanine (G) or thymine (T) (Fig. 2*B*). The Vα14 sequences are identical to the one found in the adult (18). Although TdT is known to be essential for the N-region addition (19, 20), recent studies by Gilfillan *et al.* (21) using TdT$^{-/-}$ mice clearly show that a short N-region addition occurs even in the absence of TdT. As we have successfully detected TdT mRNA in the early fetal tissues (see Fig. 4), the N-region of Vα14Jα281 TCR is likely to be generated by TdT. It is also quite surprising that Vα14$^+$ TCR sequences detected were exclusively invariant. Therefore, it seems that invariant Vα14$^+$ TCR is due to the selection in the fetal tissues.

Detection of Vα14$^+$ NK T cells in the Fetal Liver at the Early Stage of Gestation. Fluorescence-activated cell sorter analysis on the d13.5 fetal liver revealed that TCRαβ$^+$ T cells were in fact present (Fig. 3). Interestingly, the majority of αβ T cells belong to CD4$^-$/CD8$^-$ population and expressed NK1.1 and Vα14$^+$ TCR, which is apparently associated with the CD3 complex. Because no anti-Vα3 staining was observed in this population and Vα14 staining was completely blocked by excess amounts of cold anti-Vα14, anti-Vα14 staining seems to be specific. Moreover, as almost all Vα14 T cells were stained by anti-Vβ8 but not by other anti-Vβ antibodies, Vβ8 was preferentially used as TCRβ by Vα14$^+$ T cells at this stage. TCRβ expression in the fetal Vα14$^+$ T cells was also confirmed at a molecular level. We investigated rearrangements and expression of Vβ8 gene in fetal tissues. As shown in Fig. 1*B*, Vβ8-Dβ2 circular DNA as well as Vβ8 transcripts were successfully demonstrated in the fetal liver, AGM, and yolk sac at d11.5 of gestation. Because peripheral NK T cells expressing the invariant Vα14 on the cell surface in the adults preferentially use Vβ8 (6), Vα14$^+$ NK T cell repertoire in the fetus is similar to those in the adult.

NK T cells are originally found as a minor subset in the thymus (22–26). Recently, it is reported that NK T cells in the periphery comprise a relatively large fraction in spleen (5% of splenic T cells) and in bone marrow (40% of bone marrow T cells) (7). However, several discrepancies between thymic and peripheral NK T cells, such as cell surface phenotypes including Vα14 TCR expression, are demonstrated; most thymic NK T cells are negative for invariant Vα14 TCR expression on the cell surface, whereas the majority of peripheral NK T cells are positive (7). In addition, the invariant Vα14$^+$ NK T cells are detected in athymic nude mice, indicating their extrathymic origin (11). Taken together with the present data, peripheral Vα14$^+$ NK T cells in the adult may be the same as those in the

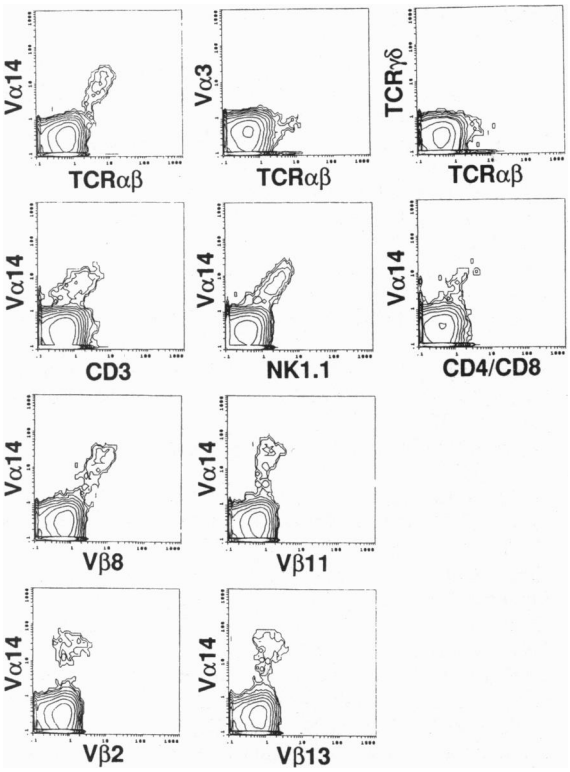

FIG. 3. Fluorescence-activated cell sorter profiles of d13.5 fetal liver. Mononuclear cells in the d13.5 fetal liver of C57BL/6 mice were separated and stained with antibodies. They ($1–2 \times 10^5$) were analyzed with EPICS-XL. The majority of $\alpha\beta$ T cells (0.5% of total mononuclear cells separated) expressed $V\alpha14^+$ TCR which was associated with CD3, NK1.1, and Vβ8. Specificity of staining was confirmed by cold antibody blocking. All monoclonal antibodies used stained 0.1% Vα3, 0.9% Vβ2, 0.5% Vβ11, 0.4% Vβ13, and 0.4% NK1.1 in adult (8-week-old) total thymocytes, 26% Vβ8, >95% CD3ε, and >95% TCRβ in $TCR^{high/med}$ fraction of thymocytes, and 37% TCRδ in intestinal intraepithelial lymphocytes.

fetus. Based on these findings, it is important to determine whether $V\alpha14^+$ or $V\alpha14^-$ NK T cells in the adult are of the same lineage in the future.

Origin of Vα14 T Cells. *RAG* gene expression is known to be essential for the rearrangement of TCR genes (27) and is demonstrated at d9 of gestation (28). Fig. 1*A* illustrates that by d9.5 *RAG*-1 and CD3ε mRNAs are readily seen in all fetal tissues in which Vα14/Vβ8 expression is detectable except for the d9.5 yolk sac and is rather diminished from the yolk sac at d13.5 of gestation. Thus, the results suggest that lymphoid precursor cells might be present in the yolk sac at d9.5, but migrate from the yolk sac at d13.5 of gestation. Palacios and Imhof (29) have reported that the yolk sac contains lymphoid precursors at d8.0–d8.5 of mouse embryogenesis; the above results on *RAG*-1 expression in the d9.5 yolk sac in part support their results. However, the d9.5 yolk sac does not seem to contain $V\alpha14^+$ T-cell precursors, because $V\alpha14^+$ TCR mRNA is detected only in the embryo body but not in the yolk sac at d9.5 (Fig. 1*A*). Because B1a cell progenitors are demonstrated to originate from AGM at d8.5–d9.0 of embryogenesis (30), it is likely that Vα14 precursor cells are derived from the embryo body, particularly from AGM, although the possibility that the precursor cells are originated from yolk sac cannot be ruled out in the experiments.

The findings of the successful detection of $V\alpha14^+$ T cells as well as the machinery necessary for TCR expression in non-thymic tissues at an early embryogenesis are intriguing and interesting, because rearrangements and transcription of conventional TCR are known to start at around d15 of gestation

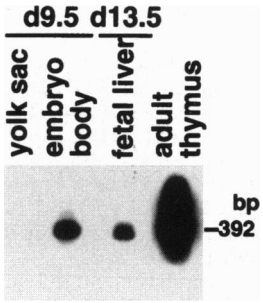

FIG. 4. DNA blot analysis on RT-PCR products with TdT primers in the early fetal tissues. PCR was performed with RNA from (*RAG*-$1^{-/-}$ × BALB/c)F1 embryos as well as 8-week-old adult thymus of C57BL/6 as a control.

in the thymus (5). These findings might be explained by the several possibilities. First, as the machinery for the TCR rearrangement and expression, such as *RAG*, TdT, and CD3 (see Fig. 1 and 4), is already prepared in the early fetal tissues, it is possible that any of TCR can be transcribed. However, only NK T cells, but not conventional T cells of thymic type such as $V\alpha1^+$ TCR, are of great advantage of the selection and proliferation in the early fetal tissues. Thus, NK T cells were only detected. However, after thymus formation, T cells bearing conventional TCR are rapidly increased in number so that NK T cells bearing invariant $V\alpha14^+$ TCR become a minor population until 2 weeks after birth (18). This assumption is likely because the invariant $V\alpha14^+$ TCR sequence was indeed detected in the neonatal thymus at a relatively low frequency as shown (18). The second possibility is that selective rearrangement of Vα14/Jα281 TCR in the NK T cells is the mechanism. In fact, B1a cells, unlike conventional B cells, develop earlier and preferentially use a rather unique receptor composed of VH11/JH1 gene segments (31). It is also true that TCRγ genes show ordered rearrangements whose expression patterns are dependent on the cell types (2, 32). Thirdly, TCR rearrangements and transcripts were mainly investigated so far in the thymus but not in the non-thymic fetal tissues under the usual conditions with low cycles of PCR amplifications, resulting in the failure to detect TCR transcripts with limited amounts. In fact, Brady *et al.* (13) used 80 cycles of PCR amplifications for detection of transcripts from the single cell.

The function of NK^+ $\alpha\beta$ T cells has not yet been well defined. However, several investigators have shown that NK T cells play a role in bone marrow allograft rejection (33), the inhibition of metastasis of lymphoid cell tumor (34), and the control of abnormal T cell development in *lpr* mice (35). In fact, a decrease in $V\alpha14^+$ T cells is tightly correlated with the development of autoimmune diseases in *lpr* or *gld* mice (36). Because the target cells of $V\alpha14^+$ T cells seem to be bone marrow-derived cells (8–10), $V\alpha14^+$ NK T cells in the early embryogenesis may control hematopoietic systems. In any event, Vα14 gene-deficient mice will provide a model in which to identify the functional role of $V\alpha14^+$ NK T cells in the early embryogenesis.

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