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Extrathymic differentiation of T lymphocytes and natural killer cells from human embryonic liver precursors

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ABSTRACT **Liver cells were isolated on Ficoll/Hypaque gradients from embryos or fetuses at 6–10 weeks of gestation; 2–20% of the cells expressed CD45 or HLA class I surface antigens and 2–6% expressed CD7. Other T- or natural-killer (NK)-cell-lineage-specific markers were undetectable. Liver-cell suspensions cultured in the presence of phytohemagglutinin and recombinant interleukin 2 gave rise to large proportions of $CD3^+$ lymphocytes expressing either α/β or γ/δ T-cell receptors. This occurred not only in bulk cultures but also when cells were cloned under limiting dilution conditions. Importantly, these figures were obtained also in embryos at 6–8 weeks of gestation, which is before colonization of the thymic rudiment by T-cell precursors. When the same liver-cell suspensions were cultured in the presence of irradiated H9 cells and recombinant interleukin 2 (either in bulk cultures or under cloning conditions), large proportions of cells (or clones) expressed surface CD16 and CD56 antigens and displayed a strong cytolytic activity against both NK-sensitive (K562) and NK-resistant (M14) target cells. In addition, liver-derived T or NK cells expressed functional receptor molecules since they could be activated via either CD3/T-cell receptor or CD16 surface antigens, respectively. Further fractionation of liver cells on the basis of CD45 antigen expression indicated that only $CD45^+$ cells could give rise to T or NK cells in culture. Thus, CD45 can be used as a marker for identification of an early liver-cell population containing T- and NK-cell precursors. That T or NK cells were derived from male embryos and not from the mother was shown by PCR amplification of X and Y chromosomal sequences. Our present data may offer an *in vitro* model for extrathymic embryonic T-cell maturation that can be used to examine fundamental aspects of human T-cell development and function.**

The embryonic/fetal liver contains precursors of both the myeloid (1) and lymphoid (2–5) cell lineages. However, it is commonly accepted that major histocompatibility complex-restricted antigen-reactive T lymphocytes further require the thymus for their maturation. Thus, mature T lymphocytes develop from fetal-liver-derived progenitors that colonize the human fetal thymus between 8 and 9 weeks of gestation (2, 3). No mature T lymphocytes have been detected before this gestational age. Recently, $CD3^-CD4^-CD8^-CD16^-$ thymic precursors were shown to give rise *in vitro* to either $CD3^-CD16^+$ natural killer (NK) cells or $CD3^+$ T-cell-receptor-positive (TCR^+) T lymphocytes, depending on the culture conditions (i.e., type of feeder cells and/or stimulus) (6, 7). Here, we analyzed liver cells from virtually intact embryos or fetuses at 6–10 weeks of gestation (8). We show that fetal liver cells were induced to undergo both proliferation and maturation toward either T lymphocytes expressing a functional CD3/TCR complex (α/β or γ/δ) or $CD3^-CD16^+$ NK cells, the differentiation pathway depending on the culture conditions. It is of note that these results could be obtained also with liver cells from embryos at 6–8 weeks of gestation, which is before colonization of the thymic rudiment by lymphoid precursors. Finally, all fetal-liver-derived T- and NK-cell precursors were confined to a small subset expressing CD45 surface antigen.

MATERIALS AND METHODS

Antibodies and Reagents. Monoclonal antibodies (mAbs) Hle-1 (anti-CD45), Leu9 (anti-CD7), Leu5b (anti-CD2), Leu4 (anti-CD3), Leu2a (anti-CD8), Leu11a,b,c (anti-CD16), anti-HPCA-1 (anti-CD34), anti-My9 (anti-CD33), anti-TCR1 (WT31), and anti-interleukin 2 (IL-2) receptor (anti-CD25) were purchased from Becton Dickinson. Anti-HLA class I mAb (W6/32) was from Dakopatts (Glostrup, Denmark). VD4 (anti-CD16) (9), KD1 (anti-CD16) (9), A13 (anti-Vδ-1) (10), BB3 (anti-Vδ-2) (10), and JT3A (anti-CD3) (11) mAbs were obtained in this laboratory. D1-12 (anti-DR) and UCHT-1 (anti-CD3) mAbs were generous gifts of R. Accolla (Istituto di Scienze Immunologiche, Verona, Italy) and P. C. L. Beverley (Imperial Cancer Research Fund, London), respectively. Anti-TCRδ1 and anti-TCRγ/δ1 mAbs, which recognize all $TCR\gamma/\delta^+$ cells, were kindly provided by M. B. Brenner (Dana–Farber Cancer Institute, Boston) and R. L. H. Bolhuis (Radio-Therapeutic Institute, Rotterdam, The Netherlands), respectively. Fluorescein isothiocyanate-conjugated anti-isotype-specific goat anti-mouse immunoglobulin was purchased from Southern Biotechnology Associates (Birmingham, AL). Phytohemagglutinin (PHA), RPMI 1640 medium, L-glutamine, and penicillin/streptomycin were purchased from Biochrom (Berlin). Recombinant (r) IL-2 was kindly provided by Cetus.

Isolation of Cells from Liver or the Upper Part of Thorax of Embryos or Fetuses. The liver and the upper part of thorax from 6- to 8-week embryos and 9- to 10-week fetuses were obtained during legal curettage abortions at 6–10 weeks of gestation. All the embryos and fetuses analyzed were virtually intact. Gestational age was evaluated on the basis of morphological staging according to multiple criteria including age vs. crown–rump length plots. The integrity of the examined embryos and fetuses gave a dating error of as little as ±2 days (8). Cells from the liver or the upper part of thorax were obtained by mincing the tissue and then perfusing the tissue pieces with RPMI 1640 medium. The number of cells obtained from each specimen varied from 6×10^5 to 1×10^7

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Abbreviations: NK, natural killer; PHA, phytohemagglutinin; TCR, T-cell receptor; rIL-2; recombinant interleukin 2; mAb, monoclonal antibody.

cells, depending on the age. More than 90% of cells recovered were viable as judged by trypan blue exclusion test. Cell suspensions were analyzed for the expression of various surface markers (see Table 1) either before or after separation by Ficoll/Hypaque gradient centrifugation and no appreciable phenotypic differences were observed for all the markers studied. $CD45^+$ liver cells (1–20% of total cell suspension) were obtained by positive selection using magnetic beads coated with goat anti-mouse IgG (Unipath, Milan) after staining with anti-CD45 mAb Hle-1. Further phenotypic analysis, by direct immunofluorescence, of freshly isolated $CD45^+$ liver cells indicated that no $CD3^+$ or $CD16^+$ cells were present in all the specimens analyzed (6–10 weeks of gestation).

Generation of $CD3^+TCR^+$ or $CD3^-CD16^+$ Cells from Liver-Cell Suspensions. Cell suspensions isolated from the liver (unfractionated or $CD45^+$ cell population) or the upper part of thorax at various ages of gestation (6–10 weeks) were cultured in 200 μl of RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (Biochrom), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete RPMI 1640 medium) in 96-well U-bottomed microplates (Greiner, Nürtingen, F.R.G.) containing 10^5 irradiated peripheral blood lymphocytes either in the presence or in the absence of 2×10^4 irradiated H9 cells (human tumor T-cell line). In some experiments, PHA (0.5%) was added to the culture. In all cultures, 100 μl of rIL-2 (100 units/ml) was added at the beginning of the culture. No cell proliferation could be detected in cell cultures derived from the upper part of thorax. In contrast, in liver cell cultures, cell growth was evident after 10–14 days. Cell proliferation, in the presence of rIL-2, could be detected for at least 4–6 weeks.

Cloning of Liver Cells. Cloning of liver cells isolated from embryos (6–8 weeks of gestation) or fetuses (9–10 weeks of gestation) was performed under limiting dilution conditions as described (12) in complete RPMI 1640 medium with rIL-2 (100 units/ml) in 200 μl in 96-well U-bottomed microplates (Greiner). The number of cells plated per well and the calculation of the cloning efficiencies (≈1%) were based on $CD45^+$ cells present in a given cell suspension. Microcultures contained 10^5 irradiated peripheral blood lymphocytes with or without 2×10^4 irradiated H9 cells per well, in the presence or in the absence of 0.5% PHA, respectively. Cell growth could be detected after 20–25 days of culture. Clones were further maintained in culture for 2–4 weeks.

Analysis of Cytolytic Activity. Cytolytic activity of liver-derived clones against NK-sensitive (erythroleukemia cell line K562) and NK-resistant (melanoma cell line M14) target cells was tested in a 4-h ^{51}Cr release assay, and the percent of specific ^{51}Cr release was determined as described (13). In the redirected killing assay, we used P815 (positive for Fc receptors for mouse IgG, $Fc\gamma R^+$, murine mastocitoma) as target cells and anti-CD3 (JT3A or UCHT-1), anti-CD16 (KD1), or anti-CD7 (Leu9) mAbs at 20 ng/ml as described (9).

Sex Assessment of Lymphoid Populations Derived from Embryonic or Fetal Liver. Genomic DNA was extracted as described (14) from $CD3^+$ or $CD3^-CD16^+$ polyclonal or clonal populations derived from human embryonic or fetal liver at 7–9 weeks of gestation. Polymerase chain reaction (PCR) was performed on 500 ng from these samples by using a pair of oligodeoxynucleotide primers derived from homologous sequences of the X and Y chromosomes (15). The amplified DNA was loaded and size-fractionated on a 1% agarose gel as described (14). By using this technique, we could simultaneously amplify the X and Y sequences in our samples. As male and female controls, we used DNA derived from two Epstein–Barr virus-infected B-cell lines. Control female DNA showed only one 970-bp band, whereas control male DNA gave an additional band that was 177 bp shorter and specific for the Y chromosome.

RESULTS AND DISCUSSION

Surface Markers of Liver Cells Isolated from Embryos or Fetuses. Liver-cell suspensions isolated from 15 specimens (embryos or fetuses at 6–10 weeks of gestation) were analyzed for a number of informative surface markers. As shown in Table 1, 2–20% of cells expressed the common leukocyte antigen CD45 or HLA class I molecules and 2–6% of cells expressed CD7, whereas cells expressing CD2, CD3, CD56, and CD16 were virtually undetectable. In addition, no cells expressed CD4, CD8, or CD25 antigens (data not shown). These data are in keeping with the demonstration that mature T cells are not detectable in the liver before the 13th week of gestation (16). It is noteworthy that parallel analysis of cell suspensions obtained from the upper part of thorax of two embryos at 6 and 8 weeks of gestation, respectively, showed no cells expressing the above markers, with the exception of CD56 (present on ≈40% of cells) and most likely representative of a nonlymphoid population. These results indicate that both liver and the upper part of thorax do not contain mature T cells before the 9th week of gestation.

Proliferation of $CD3^+TCR^+$ or $CD3^-CD16^+$ Cells from Liver Precursors. In all instances fetal liver cells underwent proliferation when cultured in the presence of rIL-2 and PHA; liver-cell fractionation on the basis of CD45 antigen expression indicated that the proliferating population was derived from the small $CD45^+$ cell subset. Indeed, up to a 200-fold increase of the original input of $CD45^+$ cells could be detected after 3–4 weeks of culture. In contrast, the $CD45^-$ subset did not proliferate under the same conditions. More importantly, the population of $CD45^+$ cells undergoing pro-

Table 1. Surface phenotype of cell suspensions isolated from liver or the upper part of thorax of human embryos (or fetuses)

Tissue	Gestational age, weeks	% with surface antigen phenotype									
		CD45	HLA class I	CD7	CD2	CD3	CD16	CD56	HLA class II	CD34	CD33
Fetal liver	6	3.2	3.7	1.7	0.0	0.0	0.0	0.0	0.4	0.2	0.4
	7	14.3	28.2	5.6	0.0	0.0	0.1	0.0	0.2	0.1	0.4
	8	9.9	11.1	0.0	0.0	0.0	0.0	0.1	0.2	0.2	1.2
	9	1.9	2.1	0.5	0.0	0.0	0.0	0.0	0.0	0.3	2.6
	10	6.8	5.7	2.7	0.2	0.1	0.0	0.1	0.5	1.8	2.9
Upper part of thorax	6	0.0	0.1	0.0	0.0	0.1	0.0	28.5	0.1	0.1	0.2
	8	0.0	0.0	0.0	0.0	0.0	0.0	34.7	0.0	0.4	0.8

Cell suspensions were derived from the liver or the upper part of thorax of embryos (or fetuses) obtained from legal curretage abortions at 6–10 weeks of gestation. For each gestational age, a representative sample is shown. Cell suspensions, further purified over Ficoll/Hypaque gradients, were stained with mAbs recognizing the corresponding antigen, followed by fluorescein-conjugated isotype-specific goat anti-mouse immunoglobulin, and analyzed on a FACstar (Becton Dickinson). Control samples were stained with the fluorescein-conjugated second reagent alone. Gestational age of the embryos or fetuses was evaluated on the basis of morphological staging according to multiple criteria including age vs. crown–rump length plots: the integrity of the examined embryos and fetuses gave a dating error of as little as ±2 days. Data indicate the percent of positive cells after subtraction of background values. Background values did not exceed 1.9%.

liferation was characterized by the surface expression of the CD3/TCR complex, as assessed by staining with anti-CD3 (Fig. 1, row C) or WT31 and TCRδ1 mAbs (refs. 10, 17, and 18; data not shown). These results were obtained also in cell cultures derived from embryos at 6–8 weeks of gestation, which is before the lymphoid colonization of the thymic rudiment (2, 3). This would imply that, at this stage, these cells could not be thymic emigrants and that T-cell maturation (i.e., expression of the CD3/TCR complex) from liver precursors can occur in an extrathymic (*in vitro*) environment. This concept was further supported by the finding that no proliferation occurred when cell suspensions were derived from the upper part of thorax of two embryos at 6 and 8 weeks of gestation.

The same fetal liver cell populations (i.e., the unfractionated or the $CD45^+$ subpopulation) were cultured in the presence of irradiated H9 feeder cells and rIL-2. Under these culture conditions, the proliferating cells after 2–3 weeks of culture were mainly represented by $CD3^-CD16^+CD56^+$ populations (Fig. 1, row B).

Cloning of Liver Cells Under Limiting Dilution. Liver cells isolated from five embryos or fetuses at 6–9 weeks of gestation were cultured under limiting dilution conditions in the presence of either PHA and rIL-2 or H9 cells and rIL-2, and the resulting clones were analyzed for their phenotypic and functional characteristics (Fig. 1, rows D–F). The maximal clonal efficiency in both culture conditions was 1% of purified $CD45^+$ cells (calculated on the basis of $CD45^+$ cells present in a given cell suspension). In the presence of rIL-2 and PHA, out of 141 clones analyzed, 133 (94%) were $CD3^+$ and only 8 (6%) were $CD3^-CD16^+$. Among these $CD3^+$ clones 12 of 133 (9%) were $CD4^-CD8^-$ $TCR\gamma/\delta^+$. Interestingly, none of the 12 $TCR\gamma/\delta^+$ clones reacted with BB3 or A13 mAbs, recognizing the two most commonly represented Vδ variable regions of adult $TCR\gamma/\delta^+$ cells (data not shown) (10). Of the remaining $CD3^+$ clones, 99 (82%) expressed CD4 and 22 (18%) expressed CD8 surface antigens.

Of the clones obtained in the presence of irradiated H9 feeder cells and rIL-2, 90 were randomly selected. Forty-five clones (50%) were $CD3^-CD16^+CD56^+$, 9 clones (10%) were $CD3^+$, and the remaining 36 clones (40%) expressed neither CD3 nor CD16 surface molecules but did express CD45 and CD56 antigens.

Liver-Derived $CD3^+TCR^+$ or $CD3^-CD16^+$ Clones Express Functional Receptors. We next investigated whether liver-derived $CD3^-CD16^+$ or $CD3^+TCR^+$ clones, isolated from 6- to 8-week embryos, expressed functional receptor molecules. To this end, we applied a redirected killing assay using mAbs directed to CD16, CD3 (Table 2), or $TCR\gamma/\delta$ (data not shown) molecules and, as target cells, the $Fc\gamma R^+$ P815 murine tumor cell line. As shown in Table 2, appropriate mAbs (anti-CD16 or anti-CD3) triggered target-cell lysis by representative NK- or T-cell clones. Interestingly, the majority (>80%) of $CD4^+$ clones analyzed displayed cytolytic activity after triggering with anti-CD3 mAbs (Table 2).

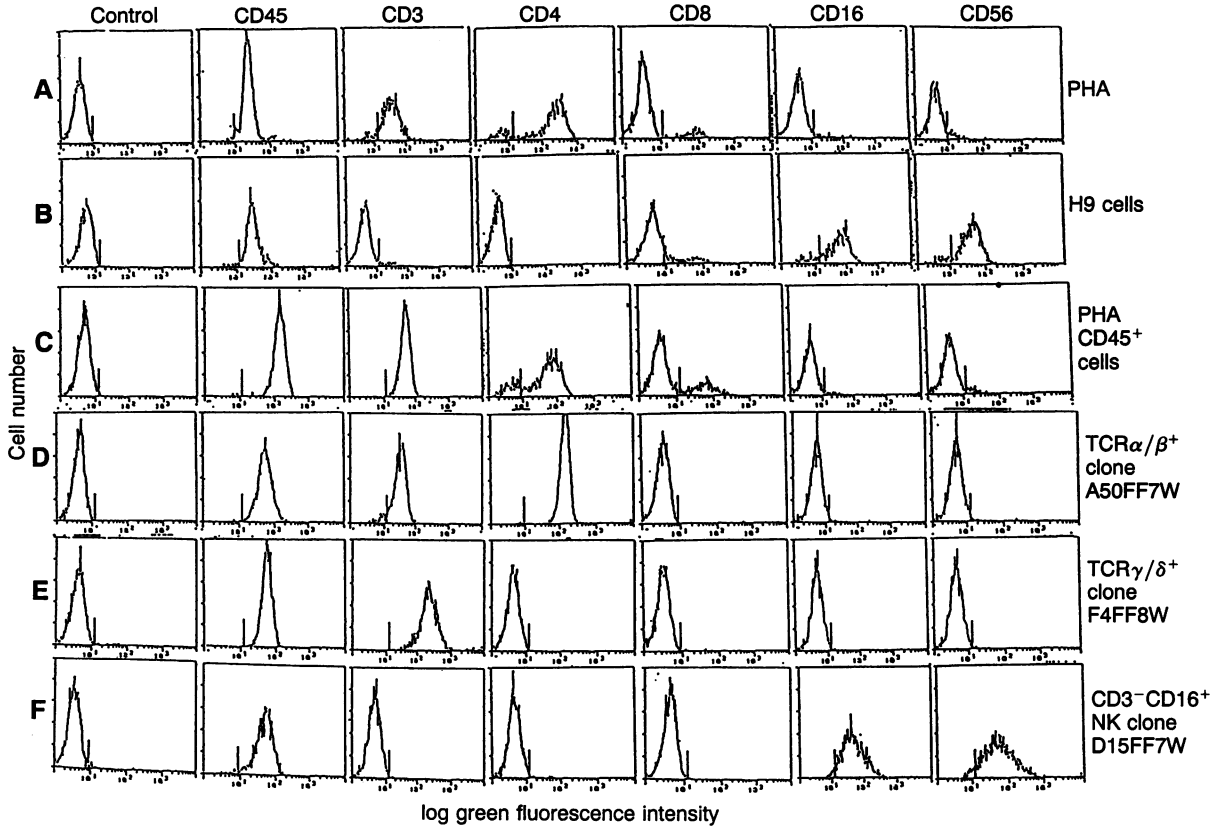

FIG. 1. Surface phenotype of cell lines or clones derived from embryonic liver. Liver cells derived from embryos or fetuses at 6–10 weeks of gestation were isolated and cultured as indicated. Representative polyclonal cell lines derived from an 8-week embryo (shown in rows A and B) were cultured for 3 weeks in the presence of either PHA and rIL-2 (with irradiated peripheral blood lymphocytes) (row A) or irradiated H9 cells, irradiated peripheral blood lymphocytes, and rIL-2 (row B). Row C shows a polyclonal population derived from the purified $CD45^+$ fraction of 8-week liver cells cultured as in row A. In this experiment, $CD45^+$ cells represented 3% of the total fetal liver cell suspension and were purified by magnetic bead separation. Clones derived from embryos at 7 or 8 weeks of gestation and expressing $TCR\alpha/\beta$, $TCR\gamma/\delta$, or the $CD3^-CD16^+CD56^+$ (NK cell) phenotype are shown in rows D–F, respectively. These clones are representative of 133 $CD3^+TCR^+$ clones and 45 $CD3^-CD16^+CD56^+$ NK clones. Cells were stained with one of the following mAbs: Hle-1 (anti-CD45), Leu4 (anti-CD3), Leu3a (anti-CD4), Leu2a (anti-CD8), KD1 (anti-CD16) (19), or Leu19 (anti-CD56). Fluorescein-conjugated isotype-specific goat anti-mouse immunoglobulin was used as second reagent. The *y* axis indicates the number of cells, and the *x* axis indicates the log green fluorescence intensity (arbitrary units). Cells were analyzed on a FACStar (Becton Dickinson).

Table 2. Cytolytic activity of T and NK clones derived from embryonic liver: evidence for the expression of functional receptors

Clone (phenotype)	Gestational age, weeks	Cytolytic activity, % specific lysis									
		K562			M14			P815 at an E/T ratio of 1:1			
		20:1	10:1	5:1	20:1	10:1	5:1	None	Anti-CD3	Anti-CD16	Anti-CD7
C6FF ($CD3^-CD16^+$)	8	100	100	85	100	55	45	20	21	75	20
C1/2.FF ($CD3^-CD16^+$)	7	90	90	55	75	70	70	8	7	85	6
A8FF ($CD3^+TCR\gamma/\delta^+$)	8	20	15	10	15	10	5	0	93	0	0
B12FF ($CD8^+TCR\alpha/\beta^+$)	7	25	20	15	20	15	10	0	69	2	1
B1FF ($CD4^+TCR\alpha/\beta^+$)	8	30	15	10	20	10	5	0	45	0	2
C1FF ($CD4^+TCR\alpha/\beta^+$)	6	20	15	10	10	5	5	0	50	3	2

Clones were derived under limiting dilution conditions, in the presence of either PHA and IL-2 (T-cell clones) or irradiated H9 cells and IL-2 (NK clones). The cytolytic activity of representative clones was tested in a 4-h ^{51}Cr release assay. The effector/target (E/T) ratios used in the test are indicated for the target cells identified. The mAbs used in the redirected killing assay are the following: JT3A and UCHT-1 (anti-CD3), KD1 (anti-CD16), and Leu9 (anti-CD7). These mAbs were stimuli added to the cytolytic test. Note that clone B12FF displayed a male phenotype (see also *Results and Discussion* and Fig. 2).

Further analysis of the cytolytic activity of liver-derived T- or NK- cell clones indicated that $CD3^-CD16^+$ clones efficiently lysed both K562 (NK sensitive) and M14 (NK resistant) target cells. On the other hand, $CD3^+$ clones displayed a variable, but usually low, cytolytic activity against these targets (Table 2).

Proliferating $CD3^+TCR^+$ or $CD3^-CD16^+$ Lymphocytes Are Derived from the Embryo but Not from the Mother. To exclude possible contamination of fetal male liver cells with mother-derived lymphocytes, we performed PCR amplification using the X–Y homologous primer technique (15) with DNA extracted from cultured $CD3^+TCR^+$ or $CD3^-CD16^+$ lymphocyte populations or clones derived from two embryos at 7 and 8 weeks and a fetus at 9 weeks of gestation. As shown in Fig. 2, two of the three samples analyzed (the B12FF $CD3/TCR^+$ clone derived from a 7-week embryo and a polyclonal population of 9 weeks) showed two bands (one specific for X sequences and the other specific for Y sequences), typical of the male phenotype. The third sample, a polyclonal population from an 8-week embryo, resulted in a single band (specific for an X sequence) typical of the female phenotype (data not shown). These data clearly indicate (at least in two of three cases analyzed) that the lymphocyte populations or clones undergoing *in vitro* expansion were derived from the male embryo and not from the mother.

Our data demonstrate that $CD3^+$ lymphocytes equipped with functional TCRs can originate *in vitro* from fetal liver cells lacking all detectable T-cell lineage markers. In addition, in agreement with previous reports, we show that

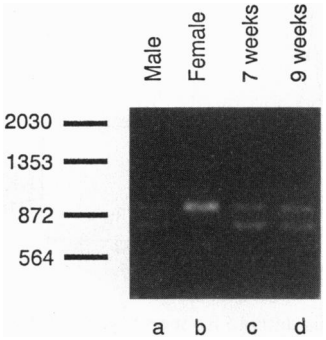

FIG. 2. Male-specific sequences in lymphoid populations derived from embryonic or fetal liver. Genomic DNA was extracted from lymphocyte populations or clones derived from human embryonic and fetal liver at 7 and 9 weeks of gestation, respectively. As male and female controls, we used DNA derived from two Epstein–Barr virus-infected B-cell lines (lanes a and b, respectively). The 7-week (the B12FF $CD3/TCR^+$ clone) and 9-week (a polyclonal population) (in lanes c and d, respectively) samples showed the two bands typical of the male phenotype. Molecular sizes in bp are shown.

$CD3^-CD16^+$ NK cells may be derived from fetal liver under appropriate culture conditions (20).

Previous studies indicated that $CD3^+$ cells were obtained from rIL-2-cultured liver cells isolated from fetuses at 12.5 weeks of gestation (2). Our studies are in line with these data. More importantly, they show that mature T cells equipped with functional TCRs can be derived also from embryos at 6–8 weeks of gestation, which is before the earliest colonization of the epithelial thymus with lymphoid precursors (2, 3). In addition, $CD3^+TCR^+$ cells could not be generated from cells isolated from the upper part of thorax of embryos of the same gestational age. These data clearly indicate that liver-derived proliferating lymphocytes are not thymic emigrants, but they are rather derived from liver precursors.

Further dissection of embryonic liver cells was possible on the basis of CD45 antigen expression. It is noteworthy that small proportions of $CD45^+$ cells had been described in 7-week fetal liver (2, 19). Although $CD45^+$ cells represented only a minor fraction of liver cells, also in our studies, the precursors of both T lymphocytes and NK cells were uniquely contained in this phenotypically defined subset. Our present data are consistent with the concept that the fetal or embryonic liver contains precursors that are committed to the T-cell (or to the NK-cell) lineage. If this is the case, appropriate maturational signals would be sufficient for these committed precursors to undergo maturation into $CD3^+TCR^+$ cells (or NK cells). An alternative interpretation, equally compatible with our data, is that the liver precursors of T and NK cells are represented by uncommitted lymphoid precursors or pluripotent stem cells. If this is the case, the culture conditions used in our experiments would mimic the microenvironment (e.g., thymus) needed to induce differentiation of lymphoid precursors into mature T or NK cells. Along the same line, recent data in the mouse have shown that an early fetal Fc receptor *II/III*-positive lymphoid population matures *in vivo* into functional T lymphocytes or NK cells, depending on the type of microenvironment provided (21). These data, and our present experiments, do not clarify whether T and NK cells are derived from the same or different cell precursors. It is evident that the solution to this problem would be offered only by the development of culture conditions that allow, in single-cell assays, optimal maturation toward different hemopoietic cell lineages (including T and NK cells). Along this line, fetal liver cells have been shown to include myeloid precursors that give rise to mixed colonies including both erythroid [erythroid burst-forming units (BFU-E), and colony-forming units (CFU-E)] and granulo-monocytic [granulocyte–macrophage colony-forming units (CFU-GM)] cells. It is perhaps worth noting that, under physiological *in vivo* conditions (i.e., erythropoietin release), fetal liver cells almost exclusively undergo differentiation/maturation to BFU-E/CFU-E, whereas *in vitro* addition of

appropriate growth factors induces differentiation/maturation of not only BFU-E/CFU-E but also CFU-GM (1). As we show here, a subset of fetal liver cells characterized by CD45 antigen expression could also undergo maturation/differentiation toward lymphoid lineages when cultured with appropriate stimuli and growth factors.

Whatever the interpretation of our present results, the possibility of inducing T-cell maturation from liver precursors may provide insight in the mechanism of T-cell maturation and selection. For example, this model system can be applied (*i*) to determine whether $CD3^+/TCR^+$ cells derived from fetal liver *in vitro* utilize a large or, rather, a limited number of TCR variable segments (22) and (*ii*) to define whether autoreactive clones exist among T (and NK)-cell clones derived from fetal liver (23–25).

Note Added in Proof. Sanchez et al. (26), after this article was submitted, reported the phenotype and molecular characterization of putative prethymic T-cell precursors in the early human embryonic liver and proposed that primitive fetal liver hematopoietic $CD3/TCR^-$ progenitors may differentiate into $CD3/TCR^+$ lymphocytes when cocultured with thymus-derived epithelial cells.

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