EARLY EVENTS IN HUMAN T CELL ONTOGENY
Phenotypic Characterization and Immunohistologic Localization of T Cell Precursors in Early Human Fetal Tissues

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During early fetal ontogeny in birds, the epithelial thymic rudiment is colonized by T cell precursors at specific developmentally regulated time intervals (1, 2). Avian yolk sac, bone marrow, and to a lesser degree, spleen and bursa of fabricius contain hematopoietic cell precursors that are able to migrate to thymic rudiments and give rise to T lymphocytes (2, 3).

In vitro studies using embryonic mouse thymic cultures have similarly demonstrated that hematopoietic cells in fetal yolk sac, bone marrow, and fetal liver can migrate to and colonize 10-d thymic epithelial rudiments (4, 5). Using avian thymic rudiment cultures and bone marrow cells, thymic epithelial cell-derived chemotactic peptides have been defined that are secreted during the time that the thymic microenvironment is colonized by T cell precursors (6). Moreover, the phenomenon of avian thymic rudiment colonization by sequential cohorts of T cell precursors in waves has been shown to be due to developmentally regulated periods of thymic epithelial cell production of chemotactic peptides (2, 3, 6).

While it is known that hematopoietic cell precursors are present in embryonic tissues before periods of thymic rudiment colonization by T cell precursors (1, 7), little is known regarding the identification via cell marker expression of T cell precursors in avian (8) mouse (5, 9, 10) or human (11, 12) fetal tissues before precursor cell entry into the thymus.

In our previous studies of one human fetal tissue before hematopoietic cell colonization of the epithelial thymic rudiment, we identified CD7+, CD45+ (T200+) cells in perithymic mesenchyme, and demonstrated that the CD7 T antigen (13-15) is the only known T lineage marker expressed on fetal hematopoietic cells before lymphoid cell colonization of the thymus (15). Moreover, we demonstrated that in man, the critical window of time for thymic development is between 7 and 14 wk of fetal gestation (16). This early time of colonization of the human thymus has hampered both the identification of fetal T cell precursors and the study of early differentiation events of human T cells.

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Since these initial studies, we have been able to study a large number of early (7-12 wk) human fetal tissues. The present study was undertaken to identify the human precursor of the T lymphocyte lineage in fetal tissue using immunohistologic studies on tissue sections with the CD7 monoclonal antibody, and using functional studies of isolated CD7+ fetal cells in vitro. Moreover, we sought evidence to determine whether a discrete time period of influx of T cell precursors into the thymic rudiment was present in human fetal ontogeny, as has been demonstrated in avian systems. We found that CD7+ cells could be identified in fetal liver and yolk sac from as early as 7 wk of fetal gestation. Moreover, at 7-8.5 wk of gestation, CD7+ cells were concentrated in mesenchymal areas of upper fetal thorax regions. Functional studies of CD7+ cells from fetal liver and thoracic mesenchyme of early fetal tissues demonstrated the ability of CD7+ cells to proliferate in the presence of T cell-conditioned media (TCM)1 and rIL-2, to form T cell colonies (CFU-T) in vitro, and to be driven in vitro to express markers of mature T cells.

Materials and Methods

Fetal Tissues. Fetal tissues were obtained as discarded tissue from the pathology department, Duke University Medical Center, from either elective first trimester abortions or at the time of surgery for ectopic pregnancy. Gestational age, determined by crown-rump length, menstrual records, and fetal part morphology, ranged from 7 to 12.75 wk (17). In all experiments the region of the body from which the tissue was obtained was identified using a dissecting microscope. In number 50 (7 wk), the neck, thorax, and abdomen were serial sectioned at 4 μm intervals from the liver through the oral cavity. Hematoxylin and eosin stains were performed on sections every 80 μm (18). In this tissue, the 7-wk epithelial thymic rudiments were identified by their ventral positions relative to the carotid artery and by their reactivity with thymic epithelial markers TE-4 and A2B5 (18). In tissues 24 (8 wk) and 20 (8.5 wk), upper thorax was compared with upper extremity for the presence of hematopoietic cells. In tissues 20 (8.5 wk), 48 (9.5 wk), 2 (10 wk), 22 (10 wk), and 40 (12.75 wk), upper thorax tissue was compared, in the studies described below, with thymic tissue. Yolk sac was available from one tissue (number 19, 7 wk of gestation).

Fetal tissues were divided into 5 mm cubes or smaller, were snap frozen in a dry ice/ethanol slurry, and were stored in liquid nitrogen. Tissue cubes were embedded in OCT Compound (Lab Tek Products, Naperville, IL), were cut into 4 μM sections, air-dried, and fixed in cold acetone (-20°C) for 5 min (18). Tissue sections were either used immediately in indirect or direct immunofluorescence assays or were stored at -70°C.

Monoclonal Antibodies. The specificities of the monoclonal antibodies used in this study are listed in Table I (13-15, 19-31). Each antibody was used at an optimal saturating concentration as determined from titration experiments using IF assays on tissue sections or IF assays on cell suspensions using cytofluorography.

IF Assays. Indirect IF assays were performed on acetone-fixed 4 μm tissue sections as described (18) using goat anti-mouse Ig (Kirkegaard and Perry, Gaithersburg, MD). In preliminary experiments it was found that acetone fixation of tissue sections either did not decrease or slightly increased the reactivity of the monoclonal antibodies in Table I to human tissues except for antibodies WT31 (24) and BMA031 (25). We found the reactivity of these two latter antibodies (against the T cell receptor for antigen [T]α and β chains) (24, 25) with human tissues was markedly decreased by fixation of tissues with acetone. Consequently all experiments using WT31 or BMA031 were performed using nonfixed 4 μm tissue sec-

1 Abbreviations used in this paper: BFU-E, Burst forming unit-erythroid; BM, Bone marrow; rEPO, recombinant erythropoietin; GEMM, granulocyte, erythroid, megakaryocyte/macrophage; GM, granulocyte/macrophage; IF, immunofluorescence; PLA, placental conditioned media; TCM, T cell conditioned media; Ti, T cell receptor for antigen.
A recent study has suggested that when high concentrations of antibody WT31 are used, or after neuraminidase treatment of T cells, WT31 can weakly bind to framework determinants of the γ, δ forms of T cell receptors (32). To validate the specificity of WT31 in this study we demonstrated that, under the conditions used here, antibody WT31 did not react with Ti8⁺ cells. The saturating binding titer of antibody WT31 was determined on postnatalthymic tissue sections and on peripheral blood mononuclear cells in suspension using indirect IF assays. Antibodies WT31 and TCR81 (anti-Ti6) (26) were next compared with regard to their reactivity patterns on a series of tissues and cell lines. We found that TCR81 did not react with WT31⁺ mature T cell lines, and that antibody WT31 did not react with CD3⁺, Ti6⁺ normal immature thymocytes in in vitro culture. Postnatal thymus that contained WT31⁺ medullary zones were essentially all TCR81⁺ using IF assays on frozen tissue sections. Finally, two 8-wk fetal thorax tissue were found that were WT31⁺ and contained clusters of TCR81⁺ cells. Thus, under the conditions used in this study, antibodies WT31 and TCR81 reacted with mutually exclusive patterns on tissue sections and cell suspensions. Sections stained in IF assays were viewed on a Nikon Optiphot fluorescent microscope. Double IF assays were performed as described previously (18). In several experiments cell populations grown from thoracic fetal tissue, or cells isolated or grown from fetal liver tissue were analyzed in indirect IF assays either by staining cytocentrifuge preparations of cells or by performing indirect IF assays on live cells in suspensions and assaying for antibody reactivity using a Coulter 753 cytofluorograph.

**Cell Culture Methods.** Fetal liver or thorax cells were cultured in medium (standard medium) consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% horse serum and 10% heat-inactivated FCS (both from Gibco), nonessential amino acids, 1 mM pyruvate, 2 mM glutamine, and penicillin/streptomycin antibiotics in 5% CO₂ in air at 37°C. Thawing medium consisted of RPMI 1640 containing 30% heat-inactivated FCS, penicillin/streptomycin antibiotics, heparin 20U/ml, 0.01 mg/ml DNase, and 1,000 U/ml Urokinase.
Isolation of CD7⁺ and CD7⁻ Fetal Liver Cell Subsets. CD7⁺ and CD7⁻ subsets of fetal liver cells were obtained by panning techniques as previously described (33) using fetal liver cells coated with the 3A1, CD7 monoclonal antibody (13) and plastic petrie dishes coated with goat anti-mouse Ig (Kirkegaard and Perry).

Growth Supplements. Media conditioned by human peripheral blood T cells (TCM) (obtained by E-rosette separation) for 72 h in the presence of 1% PHA (34), and placental conditioned medium (PLA) (35) were prepared as described. 5637-conditioned medium (CM) (36-38) was harvested from a culture of the 5637 human bladder carcinoma cell line at 60% confluence. Conditioned media supernatants were centrifuged at 3,000 g for 30 min, aspirated, and filtered through a 0.22 μm filter, and stored at −70°C until use. Human recombinant GM-CSF and IL-3 were kindly provided by Dr. Steven Clark, Genetics Institute, Cambridge, MA. Human recombinant IL-2 and erythropoietin (rEPO) were purchased from E. I. Dupont de Nemours and Co., (Glenolden, PA) and Amgen Biologicals, (Thousand Oaks, CA), respectively.

Morphologic Characterization. Standard 200-cell differential counts were performed on cytocentrifuge (Shandon II; Shandon Southern Instruments, Inc., Sewickley, PA) preparations of cells stained with the Diff-Quik modification of the Wright's/Giemsa technique (39).

Differentiation of Fetal Cells in Culture. Fetal cells were obtained as described and initiated into suspension culture at a density of 10⁶ cell/ml in a 24-well tissue culture plate (Costar, Cambridge, MA) in a total volume of 2 ml of standard medium supplemented with either: 10% PLA as a source of GM-CSF (35) or human recombinant GM-CSF (5 U/ml); 10% 5637 CM as a source of G-CSF, GM-CSF, or crude IL-3 (36-38) or human recombinant IL-3 (5 U/ml), plus rEPO (2 U/ml); 10% TCM plus rIL-2 (80 U/ml); or without additions. 100 μl aliquots of each culture were removed weekly, cytocentrifuged, stained with Diff-Quik, and evaluated microscopically. All cultures were expanded as needed with standard medium plus growth supplements to maintain cell density at 10⁶ cells/ml. Fetal cells were also cultured in semisolid medium to assess the potential of these cells to give rise to committed clonal hematopoietic progenitors (CFU-GM-C, colony forming unit of granulocyte/macrophage; CFU-GEMM, CFU of granulocyte/erythrocyte/monocyte/megakaryocyte; BFU-E, burst forming unit of erythroid cells; CFU-T, CFU of T cell). For these assays 10⁵, 5 × 10⁵, and 10⁶ cells were cultured in a base mixture of 0.9% methyl cellulose (1,500 centipoises; Sigma Chemical Co., St. Louis, MO) containing equal amounts of α-MEM and Iscove's Modified Dulbecco's Medium (both from Gibco), 12.5% horse serum (Gibco) 12.5% heat-inactivated FCS (Gibco), and penicillin/streptomycin antibiotics in a total volume of 1 ml in a gridded plate (Lux, Miles Laboratories, Naperville, IL). To assay GM-CFU-C, 10% placental conditioned medium was added to each culture plate. To assay CFU-GEMM and BFU-E, 1% deionized BSA (Sigma Chemical Co.), 10⁻³ 2-ME, 10⁻² human recombinant erythropoietin were added to each plate. To assay CFU-T, 10% TCM 80 U/ml of rIL-2, and 10⁻³ M 2-ME were added to each plate. All samples were cultured in duplicate and maintained at 37°C in a humidified 5% CO₂ in air atmosphere. Colonies were scored on an inverted microscope (Olympus) after 7, 14, and 21 d of culture. Representative colonies from each plate were picked, washed, cytocentrifuged and stained with Wright's-Giemsa and examined microscopically for morphologic conformation of colony composition.

Results

Phenotype of Hematopoietic Cells in Human Fetal Yolk Sac, Neck, and Thorax. Using expression of the CD45 and CD7 antigens as markers of T cell precursors, we studied 11 fetal tissues for the distribution of CD45⁺, CD7⁺ cells, and determined the phenotype of these cells with regard to expression of other markers of the T cell lineage (Table II). We have previously reported in 7-wk fetal tissue, using double indirect IF assays, that only a subset of fetal CD45⁺ cells are CD7⁺, whereas all CD7⁺ cells are CD45⁺ (16).

At 7 wk of gestation, before the time of hematopoietic cell colonization of the thymus
### Table II

**Phenotype of Hematopoietic Cells in Human Fetal Yolk Sac, Neck, and Thorax**

| Tissue number | Gestational age | Source | CD45 | CD7 | CD2 | CD3 | TCR81* | WT31† | CD8 | CD1 | CD4§ |
|---------------|-----------------|--------|------|-----|-----|-----|--------|--------|-----|-----|------|
| 17            | 7 wk            | Yolk sac | +    | +   | -   | -   | -      | -      | -   | -   | +    |
| 46            | 7               | Upper thorax | +    | +   | -   | -   | -      | -      | -   | -   | +    |
| 50            | 7               | Neck     | +    | -   | -   | -   | -      | -      | -   | -   | +    |
| 50            | 7               | Upper thorax | +    | +   | -   | -   | -      | -      | -   | -   | +    |
| 50            | 7               | Lower thorax | +    | -   | -   | -   | -      | -      | -   | -   | +    |
| 35            | 7               | Upper thorax | +    | +   | +   | +   | +/-    | -      | +    | -   | -    |
| 35            | 7               | Neck     | +    | +   | +   | +   | +/-    | -      | +    | -   | -    |
| 32            | 8               | Thorax   | +    | +   | +   | +   | +/-    | -      | +    | -   | -    |
| 24            | 8               | Upper thorax | +    | +   | +   | +   | +/-    | -      | +    | -   | -    |
| 20            | 8.5             | Upper thorax | +    | +   | +   | +   | +/-    | -      | +    | -   | -    |
| 48            | 9.5             | Upper thorax | +    | -   | -   | -   | -      | -      | -   | -   | +    |
| 22            | 10.6            | Neck     | +    | -   | -   | -   | -      | -      | -   | -   | +/-  |
| 22            | 10.6            | Upper thorax | +    | -   | -   | -   | -      | -      | -   | -   | -    |
| 40            | 12.75           | Upper thorax | +    | -   | -   | -   | -      | -      | -   | -   | +    |

- , no cells positive in IF assay.
+/-, <10 positive cells per tissue sections.
+, >10 positive cells per tissue sections.
+ +, >5 positive cells per 400× field.

* TCR81 is a monoclonal antibody reactive with the δ chain of the T cell receptor for antigen (26).
† WT31 is a monoclonal antibody reactive with the α and β chains of the T cell receptor for antigen (24).
Antibody BMA031, also against the T cell receptor α and β chains, was also assayed for reactivity against tissues, with identical results to those shown using WT31 (not shown).
§ Reactivity of anti-CD4 antibodies with tissue in indirect IF assays identified T200⁺, Leu M3⁺, CD7⁻ pigment-containing tissue macrophages.

(18, 40), CD7⁺ cells were present in yolk sac (Fig. 1A and B), upper thorax (Fig. 1C) and fetal neck areas. In fetus number 50, we were able to demonstrate that CD7⁺ cells were more prevalent in the neck and upper thorax by serial sectioning through the thymic region (neck and upper thorax) where there were >10 CD7⁺ cells per tissue section. In many neck and upper thorax areas, there were foci of 10–20 CD7⁺ cells per 400× field. 200 tissue sections caudal to the thymic region (800 μM), the lung buds were located along with the distal esophagus. At this level of the lower thorax, no CD7⁺ cells were present. Only scattered CD45⁺, CD14⁺, CD7⁻ tissue macrophages were present at the level of the primordial lungs (Fig. 1D). At 7 wk, in the heart and aorta of number 50, only E5⁺ (glycoporphin β⁺) cells of erythroid lineage (31) were present (Fig. 1E), with no CD45⁺, CD14⁺, or CD7⁺ cells seen (Fig. 1E).

In one (number 35) of four 7-wk thoracic tissues tested, large numbers of CD7⁺ cells were present throughout mesenchyme (>20 positive cells per 400× field). Unlike tissues number 19, 46, and 40, where no CD3⁺, CD8⁺, or CD2⁺ cells were seen, in number 35 upper thorax, rare (<5% of CD7⁺ cells) CD2⁺, CD3⁺, and CD8⁺ cells were present (Table II).

At 8 and 8.5 wk of gestation, just at the time of hematopoietic cell colonization of the human thymus (40) (tissue numbers 32, 24, and 20), CD7⁺ cells were seen in every field of every neck and upper thorax tissue section (Fig. 2). As with 7 wk
**FIGURE 1.** Phenotype of hematopoietic cells in 7-wk human fetal tissues. (A) Hematoxylin and eosin-stained section of yolk sac from number 19. (B) A sequential 4-μm section reacted with the anti-CD7 antibody, 3A1A, in indirect IF assay. Long arrows point out endoderm of yolk sac that was weakly autofluorescent in B. Short arrow points out CD7* cell in B and likely mononuclear cell counterpart in A. Open arrows point out mature granulocytes in A. (C) Cluster of CD7* cells (arrows) in upper thorax of 7-wk tissue from number 46. While no CD7* cells were present at the level of the lung bud in tissue number 50, clusters of CD45* macrophages (arrows) were present (D). (E) All cells present within aorta at 7 wk were glycophorin B* erythroid precursors (arrows) while D shows that no intravascular cells were Leu-M3+. Arrows point to Leu-M3* cells. 400x.

**FIGURE 2.** CD7* cells are present in mesenchyme of the upper thorax at 8 wk of fetal gestation. (A) Keratin-positive skin (as defined by antibody AE-1) of 8-wk (number 24) fetal thorax; B and C show sequential 4-μm sections following that seen in A reacted with anti-CD7 antibody (B) and anti-CD45 antibody (C). (D and E) Sections of extremity of number 24, with keratin-positive skin shown in D. In contrast to upper thorax, no CD7* cells were seen in number 24 extremity, as shown in E. (F) Whereas many CD7* and CD45* cells were present in upper thorax and neck of 8-wk tissue, no CD7* cells (not shown) and rare (arrow) CD45* cells were present in the 10-wk neck tissue of number 2. (G) 8-wk number 24 thorax tissue that is nonreactive with WT31 (anti-T1a,b). (H and I) Intrathorax small vessels (V) in tissue number 24 containing rare CD8* cells (arrows) (H) and CD3* (I) cells (arrows). 400x.
tissue number 35, rare CD2+, CD3+, and CD8+ cells were present in all 8- and 8.5-wk thorax tissues, with cells frequently centered in and around vessels (Fig. 2, H and I).

To determine if the presence of CD7+ cells in the upper thorax of 8- and 8.5-wk tissue represented regional localization of CD7+ cells in the fetus at this stage of gestation, other fetal tissues distant from upper thorax (i.e. upper extremity) from numbers 24 and 20 were sectioned and assayed for the presence of CD7+ cells. Whereas numbers 24 and 20 upper thoracic regions contained >50 CD7+ cells per 400× field (Fig. 2 B) there were ≤5 CD7+ cells per tissue section of upper extremity from these same fetuses (Fig. 2 E). Thus, as with the 7-wk fetus tested (number 50), in 8-8.5-wk fetal tissues, CD7+ cells were concentrated in the upper thorax.

By 9.5 wk (number 48), after hematopoietic cells had colonized the thymus, and in the other fetal tissues up to 12.75 wk (numbers 2, 22, and 40), no CD7+ cells were present in upper thorax or neck regions (Fig. 2 F and Table II).

To study T cell receptor expression at the protein level, CD7+ cells in 7- and 8-wk upper thorax areas were assayed for their reactivity with monoclonal antibodies specific for T cell receptor α chain (TCRα1) (26), and T cell receptor α and β chains (WT31, BMA031) (24, 25). We found that, in all but two (numbers 24 and 32) tissues studied, no Tiα+ or Tiα, β+ cells were present (Fig. 2 G and Table II). In 24 and 32 rare Tiα+ cells were seen that were also CD3+ (Fig. 3). However, most CD3+ cells in 7-8.5-wk tissues were both TCRα1− and WT31−.

Phenotype of Hematopoietic Cells in Fetal Thymus. In our study we were able to evaluate the thymus in six fetuses (numbers 50, 20, 48, 2, 22, and 40) (Table II). As previously reported (19, 41), at 7 wk the epithelial thymus of number 50 contained no lymphoid or monocytoid cells, and therefore was at a stage prior to the time of any hematopoietic cell colonization. At 8.5 wk, thymus number 20 contained CD45+, CD4+, CD14+, macrophages as well as CD7+ thymocytes. Using double IF assays with directly fluoresceinated CD7 antibody and CD4 or CD8 antibodies, we demonstrated that 8.5-wk CD7+ cells were CD4− and CD14− (not shown). ~20% of CD7+ cells in thymus at 8.5 wk were CD2+ (41). Thus, the first T cell antigen acquired by CD7+ cells after their entry into the epithelial thymic rudiment is CD2. Rare (<5 cells per tissue section) CD3+ cells were also seen in 8.5-wk thymic tissue. As previously reported, by 9.5–10 wk all T cell antigens listed in Table III were expressed as determined by indirect IF assays (16, 41).

Phenotype of Hematopoietic Cells in Fetal Liver. Many investigators have documented the presence of T cell precursors in the fetal liver of mice, dogs, and men (4, 5, 9, 10, 42–44). Thus, if the CD7+ cell population in fetal tissues contained T cell precursors, then CD7+ cells should be present in fetal liver. We found (Table IV) that CD7+ cells were present in all four fetal livers available for study, with the phenotype of CD7+ fetal liver cells being CD3−, CD8−, CD1−, CD2+, and CD45+. As with the majority of upper thorax and neck CD7+ cells, CD7+ cells in fetal liver from 7 through 12.75 wk were Ti δ− and Ti α, β− (Table IV). To determine the number of CD7+ cells in fetal liver tissue, the number of CD7+ cells per tissue section was counted and expressed per volume of tissue (i.e., section length × width × width × 4 μm thick). The number of CD7+ cells present in fetal liver was at 7 wk (number 50), 40 cells/mm3; 9.5 wk (number 48), 2,708 cells/mm3; 10 wk (number 22), 1,550 cells/mm3; and 12.75 wk (number 40) 2,650 cells/mm3.

CD7+ Fetal Liver Cells Give Rise to CFU-T and Phenotypically Mature T Cells. Fresh
FIGURE 3. 8-wk fetal tissue contained scattered TCR^+ cells that were CD45^+ and CD3^+.
(A) CD45^+ cells in thorax of 8-wk tissue number 24. (B) Sequential 4-μm tissue section in which the CD45^+ cell indicated by arrow in A is TCR^+ as well. Arrowheads show CD45^+ cells that were TCR^-.
(C) An autofluorescent rim of epithelium.
(D) A rare cluster of CD3^+ cells of 8-wk thorax tissue number 24 beneath skin epithelium.
(E) A sequential 4-μm section of the identical area in which one of the CD3^+ cells (arrow) is TCR^+. 400×.

sterile fetal liver cells from 9.5, 10, and 12.75 wk of gestation were available for study in vitro. First, we assayed unfractionated fetal liver cells for their ability to give rise to myeloid (GM-CFU-C, CFU-GEMM), erythroid (BFU-E), and lymphoid (CFU-T) colonies in vitro. Fetal liver cells from these gestational ages all contained cells capable of giving rise to lymphoid (CFU-T) colonies, and as others have shown (43, 44), erythroid and myeloid colonies (Table V).
CD7+ HUMAN T CELL PRECURSORS

**Table III**

*Phenotype of Thymocytes in Fetal Thymus*

| Tissue number | Gestational age (wk) | Marker |
|---------------|----------------------|--------|
|               | CD45                 | CD7    | CD2 | CD3 | CD8 | CD1 | CD4* |
| 50            | 7                    | -      | -   | -   | -   | -   | -    |
| 20            | 8.5                  | +      | +   | +/– | –   | –   | +    |
| 48            | 9.5                  | +      | +   | +   | +   | +   | +    |
| 2             | 10                   | +/–    | +   | +   | +   | +   | +    |
| 22            | 10                   | +      | +   | +   | +   | +   | +    |
| 40            | 12.75                | +      | +   | +   | +   | +   | +    |

- , no cells positive in IF assay.
+ /– , <10 positive cells per 400 x field.
+ , 10-40 positive cells per 400 x field.
+ + , >50% of all thymocytes positive.

* Reactivity of anti-CD4 antibodies with 8.5-wk thymus in double indirect IF assay identified T200+, Leu-M3+, CD7+ macrophages. In 9.5-, 10-, 12.75-, and 14-wk thymuses, anti-CD4 antibodies reacted with thymocytes as well as with scattered thymic macrophages.

**Table IV**

*Phenotype of Hematopoietic Cells in Fetal Liver*

| Tissue number | Gestational age (wk) | Marker |
|---------------|----------------------|--------|
|               | CD45                 | CD7    | CD2 | CD3 | TCR81 WT311 CD8 | CD1 | CD4* |
| 50            | 7                    | +      | +   | –   | –   | –   | –   |
| 48            | 9.5                  | +      | +   | –   | –   | –   | –   |
| 22            | 10                   | +      | +   | –   | –   | –   | –   |
| 40            | 12.75                | +      | +   | –   | –   | –   | –   |

- , no cells seen positive in indirect IF assay.
+ /– , <10 positive cells seen per tissue section.
+ , >10 positive cells seen per tissue section.

* In tissues no. 48, 22, and 40, rare (0–2 cells/section) Leu4+, OKT3+ cells were seen.
† Identical results were obtained with the anti-Ti a, b reagent BMA031 (not shown).
‡ In the 7-, 10-, and 12.5-wk fetal liver tissues, all CD4+ cells were large pigment-containing cells. Double IF experiments using fetal liver number 22 confirmed that CD7+ cells were CD4+, and that CD4+ cells were both CD7+ and Leu-M3+ (i.e., tissue macrophages and Kupffer cells).

**Table V**

*Growth of Hematopoietic Progenitors from Human Fetal Liver*

| Fetal liver number | Gestational age (wk) | Cell suspension | GM-CFU-C | CFU-GEMM | BFU-E | CFU-T |
|--------------------|----------------------|-----------------|----------|----------|-------|-------|
| 48                 | 9.5                  | Fetal liver     | 1,360    | 620      | 1,700 | 320   |
| 22                 | 10                   | Fetal liver     | 380      | 15       | 110   | ND    |
| 40*                | 12.75                | Fetal liver     | 103      | 18       | 6     | 6     |
| 40                 | 12.75                | CD7+ fetal liver| 145      | 35       | 0     | 130   |
| 40                 | 12.75                | CD7- fetal liver| 90       | 125      | 100   | 0     |

* Fetal liver number 40 was either cultured in vitro after Ficoll-Hypaque purification of mononuclear cells, or after panning separations performed using anti-Ig-coated plates for CD7+ and CD7- populations, as described in Materials and Methods. Data for CD7+ and CD7- fetal liver number 40 cells represent the mean of two separate experiments.
To determine the phenotype of CFU-T from CD7⁺ fetal liver cells that proliferated in response to TCM and rIL-2, unfractionated fetal liver (cells number 48, 9.5 wk), were stimulated for 15 d with TCM and 80 U rIL-2, cyt centrifuge slide preparations were made, and the cells were assayed in indirect IF assay for reactivity with the monoclonal antibodies listed in Table VI.

Unfractionated fresh fetal liver cells (number 48) at the time of culture were 3% blast forms, and 92% erythroid, 1% lymphoid, 2% monocytoid, and 2% megakaryocytoid lineages, as determined on Wright's-stained cyt centrifuge preparations. After culture for 15 d in TCM plus rIL-2, fetal liver cells (number 48) were 81% blast morphology, with the phenotype shown in Table VI. Whereas none of the number 48 fetal liver cells were reactive with anti-CD8, WT31, or CD2 antibodies before culture, 13–19% of cells were reactive with these T lineage-specific reagents after 15 d.

Although these data demonstrated that cells within 9.5-wk fetal liver can give rise to cells of the T cell lineage, they did not address the question of whether CD7⁺ or CD7⁻ fetal cells could give rise to T-lineage cells. To address this question, purified CD7⁺ and CD7⁻ populations were obtained from number 40 12.75-wk fetal liver tissue using panning techniques (Tables V and VII). We found that freshly isolated CD7⁺ fetal liver cells were 71% blast forms, 27% erythroid, and 11% monocytoid forms, (Fig. 4 A). CD7⁺ fetal liver cells were enriched for CFU-T 20-fold compared with fresh fetal liver cells (Table V), whereas CD7⁻ fresh fetal liver cells [containing 11% blasts, 88% erythroid, and 1% monocytoid forms (Fig. 4 B)] contained no CFU-T (Table V). Importantly, both CD7⁺ and CD7⁻ fetal liver cells contained cells capable of giving rise to CFU-GM and CFU-GEMM colonies under the appropriate culture conditions (Table V) (Fig. 4, C and D).

Next, to determine the phenotype of cells in CFU-T from CD7⁺ fetal cells, CD7⁺ and CD7⁻ fetal liver cells from number 40 were cultured in the presence of TCM plus 80 U rIL-2 for 10 d, cyt centrifuge preparations were made, and the cells were assayed for reactivity with the monoclonal antibodies listed in Table VII.

We found that ~40–50% of CD7⁺, 12.75-wk fetal liver cells expressed CD2, CD3,

### Table VI

**Phenotype of 9.5-wk Unfractionated Fetal Liver Cells Stimulated for 5 d with T Cell-conditioned Media and rIL-2**

| Marker | Positive cells |
|--------|----------------|
| CD7    | 24             |
| CD2    | 19             |
| CD3    | 15             |
| CD4    | 6              |
| CD8    | 14             |
| CD1    | 0              |
| WT31   | 13             |
| TCR61  | 1              |
| CD25 (TAC) | 16           |

The percentage of cells positive for each marker was determined by preparing acetone-fixed cyt centrifuge preparations and using these in indirect IF assays with the indicated monoclonal antibodies.
CD7', CD4', or CD8', and WT31 antigens after 10 d in culture with TCM plus IL-2 (Fig. 4, E and F), whereas few CD7' fetal liver cells expressed these antigens under the same culture conditions (Table VII and Fig. 4). At the end of 10 d in TCM plus IL-2, CD7' cells were 59% lymphoblasts, and 5% myeloid, 33% mast cell or monocytoid, and 6% megakaryocytoid morphologies. In contrast, CD7' fetal liver cells, cultured under the same conditions, were 5% lymphoblasts, and 18% myeloid, 70% mast cell or monocytoid, and 3% megakaryocytoid morphologies (Fig. 4).

**CD7’ Cells from the 7-wk Upper Thorax Tissue Gave Rise to T-lineage Cells.** To address the question of whether fetal thorax CD7’ cells could also give rise to cells of the T cell lineage, fresh tissue from the thorax region of 7-wk fetal tissue from number 46 was divided and half was preserved for frozen tissue sections and analysis in indirect IF assay (Table II). The other half was teased apart by mechanical dissociation and cultured in TCM with rIL-2. After 4 d, clumps of proliferating cells were seen, and by 14 d sufficient cells were present for analysis in IF assays on a cytofluorograph. As seen in Fig. 5, whereas all CD7’ cells in situ in the thorax before culture were CD3’, WT31’, CD8’, and CD2’ (Table II), the CD7’ cells that grew out from number 46 thorax were 57–94% positive for these T cell markers. In double-label experiments using cytofluorography and directly labeled CD4 and CD8 monoclonal antibodies, we found 6% of cells from number 46 cultured in TCM with rIL-2 were both CD4’ and CD8’ on day 14, while the remainder of the cells reciprocally expressed either CD4 or CD8 (not shown).

**Discussion**

In this study, we show that CD7’, CD45’, CD2’, CD3’, CD8’, WT31’, TCRα1’, cells are present in fetal 7-wk yolk sac, thorax/neck, and liver before hematopoietic cell colonization of the epithelial thymic rudiment. Moreover, between 7 and 8.5 wk of gestation, CD7’ cells are concentrated in neck and upper thorax areas. Finally, we have shown that CD7’ cells from thorax and fetal liver give rise to T-lineage cells expressing CD8, CD4, CD2, CD3, and WT31 antigens.

**TABLE VII**

Phenotype of 12.5-wk Fetal Liver Cells Stimulated for 10 d with T Cell-conditioned Media and rIL-2

| Marker     | CD7’ fetal liver cells | CD7’ fetal liver cells |
|------------|------------------------|------------------------|
| CD7        | 64                     | 7                      |
| CD2        | 47                     | 6                      |
| CD3        | 39                     | 13                     |
| CD4 + CD8  | 35                     | 9                      |
| CD1        | 0                      | 0                      |
| WT31       | 48                     | 3                      |

The percentage of cells positive for each marker was determined by preparing acetone-fixed cytocentrifuge preparations and using these in indirect IF assays with the indicated monoclonal antibodies. Data are the means of two separate experiments using cells from fetal liver number 40. Anti-CD4 and anti-CD8 antibodies were only used together in these experiments to identify cells either CD4’ or CD8’ due to limited numbers of cells.
Figure 4. Hematopoietic cells isolated from human fetal liver. A. Blast morphology of freshly isolated CD7+ cells from 12.75-wk (number 40) fetal liver. B. Erythroid morphology of CD7- fetal liver number 40 cells. B, basophilic normoblast; P, pronormoblast; N, polychromatophilic normoblast; R, fragmented red blood cell. C and D. CFU-GEMM colony derived from CD7+ fetal liver cells (C) and CD7- fetal liver cells (D). E. Representative T cell colony grown in TCM/IL-2 from 10-wk fetal liver number 22. F. CD3+ cells (arrows) from GFU-T arising in cultured purified CD7+ fetal liver number 40 cells.
LeDourain et al. (1-3) have demonstrated that T cell precursors first home to the avian epithelial thymic rudiment during early embryonic development. Using the quail-chick hybrid system, it was shown that migration of T cell precursors from yolk sac and bone marrow to the epithelial thymus occurred in waves at defined periods of time in thymic ontogeny (1-3). Champion et al. (6) have gone on to show that during the receptive period of the avian thymus for homing T cell precursors, thymic epithelial cells produce peptides of 1 and 4 kD that are chemotactic for T cell precursors. Moore and Owen (7) in classic early studies on the development of the avian and murine thymus observed lymphoid cells in and around the epithelial thymic rudiment just before and during the time of hematopoietic cell colonization of the thymus. The likely explanation of localization of CD7+ cells to the neck and upper thorax at a defined point (7-8.5 wk) in human fetal gestation is that thorax CD7+ cells represent a wave (or waves) of migration of T cell precursors to the human thymic rudiment. Thus, in man, there appears to be a developmentally regulated window of time at 7-8.5 wk gestation during which the migration of T cell precursors to the epithelial thymus occurs.

In contrast to the work of Le Dourain et al. in the avian model, in which multiple waves of precursors were observed entering the thymus at defined intervals, we have only observed one possible wave of precursor cells at 7-8 wk of gestation. In this regard it is important to note that at 7-8 wk the human thymus is poorly vascularized, with only a few thin-walled capillaries present (41, 45). Moreover, we found in the present study no CD7+ cells in heart, aorta, or smaller thorax vessels in 7- or 8-wk tissue. Thus, a potential reason for observing only one time period in which CD7+ cells are concentrated in thoracic mesenchyme may relate to initial colonization of the thymus occurring via migration of T cell precursors through tissue rather than via thymic blood vessels. Le Douarin and Jotereau (1) found that lymphoid precursors were present in thymic rudiments of avian interspecific chimeras.
before vascularization of the thymic rudiment. Once full vascularization of the thymus occurs at 8–9 wk, then ingress of T cell precursors into the thymus likely occurs via thymic vessels (42, 46).

In addition to demonstrating time- and location-dependent appearance of CD7+ cells in human fetal tissues, our studies have directly demonstrated that CD7+ cells within fetal liver contain cells capable of CFU-T formation (Table V), and that CD7+ CFU-T contain cells of the T lineage (Table VII). A large number of studies have investigated the phenotype of human and murine T cell precursors from a variety of sources (16, 47–57). Murine and human postnatal bone marrow (BM) T cell precursors have been reported to be TdT+ (47, 52). Human postnatal BM T cell precursors have been postulated to reside in the RFB-1+, CD7+, TdT+ population of cells (47, 52). RFB-1+, TdT+ cells in human fetal liver have also been postulated to contain T cell precursors (54, 55). In postnatal human thymus, a population of CD7+, CD2−, CD3−, CD4+, CD8−, WT31− cells have been identified that are germline in configuration for Tβ genes and express cytoplasmic but not surface CD3 molecules (58). From these latter observations, Furley et al. (58) concluded that the earliest stages of human intrathymic maturation are the expression of CD3 genes in CD7+, CD2− cells, followed by Tβ gene rearrangement and expression. In our present studies, the observation of many fetal CD3+ cells that were neither WT31+ (Tiα, β+) nor TCRδ1+ (Tiδ+) in several 7- and 8-wk tissues suggested that an analogous pathway of T cell maturation to that proposed by Furley et al. (58) occurs during early human fetal ontogeny.

Whether CD3 expression can occur in T cell precursors in vivo before migration to the thymus is at present unknown. In tissues number 35, 24, and 32, where scattered CD3+ cells that were Tiα, β− and Tiδ− were present in thorax, thymic tissue was not available for study to determine if thymic colonization by hematopoietic cells had occurred. In tissue number 20 (8.5 wk) we were able to show that thymic colonization had occurred, and both the thymus and thorax contained scattered rare CD3+ cells. In number 50 (7 wk) thymic colonization had not occurred, and no CD3+ cells were seen in neck and upper thorax areas. Thus, based on these observations one would infer that CD3 expression by T cell precursors only occurs after CD7+ T cell precursors have migrated through the thymus. However, the observation that 7-wk thorax cells and 9.5- and 12.75-wk fetal liver cells could be stimulated to acquire reactivity with CD3 monoclonal antibodies without contact with thymic epithelium suggests that CD3 expression may occur in CD7+ T cells before their migration to the thymus. Thus, at the critical time of T cell precursor migration to the thymus, there is either spillover of CD7+, CD3+ cells into thorax mesenchyme from thymus, or the simultaneous onset in fetal thymus and in extrathymic tissues of expression of CD3 genes in CD7+ cells.

It is of particular interest that expression of the CD7 antigen is the earliest marker of the T lineage in man. While the function of the CD7 molecule is not yet known, Jung et al. (59) have described a case of severe combined immunodeficiency disease in which the T cells present were deficient in CD7 expression. In this patient, T cell proliferative responses to mitogens were abnormal and IL-2 expression on T cells was deficient. The authors proposed that CD7 deficiency in this patient was related to a defect in T cell precursors, and that the CD7 molecule plays an important role in early lymphoid development (59).
The nature of the full differentiating capacity of the CD7+ fetal liver cell remains to be fully explored. The data in Table V suggest that in addition to containing T cell precursors, the CD7+ subset contains cells capable of forming CFU-GM and CFU-GEMM colonies. Data from two other studies support the notion that the multipotent nature of CD7+ fetal liver cells may indeed reflect the expression of the CD7 molecule on pluripotent stem cells. First, we have recently shown that CD7+, CD4−, CD8− cells from normal human postnatal thymus contain cells that are pluripotent, in that they give rise to CFU-GM and GFU-GEMM colonies in vitro with appropriate stimuli, yet proliferate and remain CD4− and CD8− in the presence of thymic epithelial cells and IL-2 (60, 61). Second, it has been shown that malignancies of clonal CD7+, CD4−, CD8−, CD3− cells occur, and are capable of pluripotent differentiation both in vitro and in vivo (60, 62, 63).

The ability to identify T cell precursors in fetal tissues using antibodies against the CD7 surface antigen has important ramifications. First, the presence of comparatively large numbers of CD7+ cells in the thorax of 7-8 wk fetal tissues now provides a source of these important cells for growth and isolation of clonal populations of T cell precursors and their progeny. Second, the demonstration of large numbers of CD7+ cells in fetal thorax at 7-8 wk suggests that fetal thorax tissue may be a useful source of T cell precursors for use in therapeutic transplantation into patients with forms of human cellular immunodeficiency diseases.

Summary

During early fetal development, T cell precursors home from fetal yolk sac and liver to the epithelial thymic rudiment. From cells that initially colonize the thymus arise mature T cells that populate T cell zones of the peripheral lymphoid system. Whereas colonization of the thymus occurs late in the final third of gestation in the mouse, in birds and humans the thymus is colonized by hematopoietic stem cell precursors during the first third of gestation.

Using a large series of early human fetal tissues and a panel of monoclonal antibodies that includes markers of early T cells (CD7, CD45), we have studied the immunohistologic location and differentiation capacity of CD45+, CD7+ cells in human fetal tissues. We found that before T cell precursor colonization of the thymus (7-8 wk of gestation), CD7+ cells were present in yolk sac, neck, upper thorax, and fetal liver, and were concentrated in mesenchyme throughout the upper thorax and neck areas. By 9.5 wk of gestation, CD7+ cells were no longer present in upper thorax mesenchyme but rather were localized in the lymphoid thymus and scattered throughout fetal liver. CD7+, CD2+, CD3+, CD8+, CD4+, CD4−, WT31− cells in thorax and fetal liver, when stimulated for 10-15 d with T cell-conditioned media and rIL-2, expressed CD2, CD3, CD4, CD8, and WT31 markers of the T cell lineage. Moreover, CD7+ cells isolated from fetal liver contained all cells in this tissue capable of forming CFU-T colonies in vitro. These data demonstrate that T cell precursors in early human fetal tissues can be identified using a mAb against the CD7 antigen. Moreover, the localization of CD7+ T cell precursors to fetal upper thorax and neck areas at 7-8.5 wk of fetal gestation provides strong evidence for a developmentally regulated period in man in which T cell precursors migrate to the epithelial thymic rudiment.
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