The Ontogeny of Terminal Deoxynucleotidyl Transferase Positive Cells in the Human Fetus

By Michael P. Bodger, George Janossy, Fred J. Bollum, Graham D. Burford, and A. Victor Hoffbrand

The ontogeny of cells containing the enzyme terminal deoxynucleotidyl transferase (TdT) in human fetal liver, bone marrow, and thymus has been studied using a highly specific antiserum to TdT together with monoclonal anti-precursor cell antibodies in double and triple marker immunofluorescence. TdT+ cells were first observed in fetal liver at 12 wk of gestation and accounted for 55% of the lymphoid-like cells isolated after Ficoll-Hypaque separation. TdT+ cells were first observed in the bone marrow 16 wk after gestation. Like TdT+ cells in normal infant bone marrow, the majority of TdT+ cells in fetal liver and bone marrow expressed both BA-1 and RFB-1 antigens. This suggests that fetal TdT+ cells include progenitors of the B lineage (BA-1) and perhaps of thymocytes (RFB-1). Nevertheless, TdT was not observed in fetal thymocytes until after 20 wk of gestation, although thymic blasts and the majority of thymocytes were strongly RFB-1+ from 12 wk of gestation. These results clearly show that fetal thymus is first populated by TdT+, RFB-1+, BA-1 cells, but does not exclude the fact that a second "wave" of TdT+ prothymocytes, possibly bone marrow derived, also exists.

In man, cells containing the enzyme terminal deoxynucleotidyl transferase (TdT) are confined to the bone marrow and cortical areas of the thymus and are not observed in peripheral lymphoid organs such as lymph nodes, blood, and tonsils. TdT is also found in the blast cells of most patients with acute lymphoblastic leukemia (ALL) and in lymphoid blast crisis of chronic granulocytic leukemia. There is evidence in rodents that TdT+ cells in the bone marrow may contain precursors of thymic TdT+ cells. However, it has also been shown that rare human TdT+ cells have cytoplasmic IgM and that common ALL lymphoblasts have rearrangement of the IgM chain genes. These TdT+ cells are therefore regarded as early pre-B-cells.

Preparation of Fetal Cells

The samples were removed within 2–3 hr after delivery and placed into RPMI medium containing 10% fetal calf serum (FCS). Thymus tissue was gently teased apart and a single cell suspension of thymocytes was prepared. Liver samples were homogenized through a 16-gauge hypodermic needle. The clumps were discarded, and the supernatant was layered over Ficoll-Hypaque (1.077 g/ml) and centrifuged for 20 min at 1,000 g. Mononuclear cells were recovered from the interface. Bone marrow cells were collected by flushing out the long bones with 10% FCS/RPMI, and mononuclear cells were isolated after centrifugation on Ficoll-Hypaque. All cell samples were washed twice in phosphate-buffered saline (PBS) and finally resuspended in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide (PBSA).

Monoclonal Antibodies

The mouse anti-human monoclonal antibodies used to label TdT+ cells were as follows.

1. RFB-1, an IgG1 class antibody that labels cortical thymocytes as well as TdT+ cells and early myeloid cells, but not pre-B (cytoplasmic IgM) or B cells in the bone marrow; RFB-1 is also unreactive with medullary thymocytes.

2. BA-1, an IgM class antibody that labels TdT+ cells, pre-B and...
B cells in the bone marrow, and B cells in the peripheral lymphoid tissues. 

(3) RFB-HLA-DR, an IgM class antibody that reacts with HLA-DR (Ia-like) antigens on TdT+ cells in bone marrow, B cells, and myeloid cells.

(4) J5, an IgG2 class antibody that detects the common ALL antigen, was purchased from Coulter Electronic Inc., Hialeah, FL.

(5) NA1/34, an IgG2 class antibody that detects human thymocyte antigen (HTA-1) on cortical thymocytes.

Reagents

Each monoclonal antibody was used in double combination with rabbit antisera to calf TdT (R-anti-TdT). The fluorochrome-conjugated second layers were goat anti-mouse Ig labeled with tetraethylrhodamine isothiocyanate (G-anti-M-Ig-TRITC) and goat anti-rabbit Ig coupled to fluorescein isothiocyanate (G-anti-R-Ig-FITC). Similarly, monoclonal antibodies were used in combination with goat anti-human IgM-FITC in order to analyze the reactivity of B cells. Finally, RFB-1 (IgG1) was used in combination with BA-1 or RFB-HLA-DR (both IgM) using goat anti-mouse subclass-specific second layer antisera (G-anti-M-IgG-FITC and G-anti-M-IgM-FITC, respectively). It was observed that this double staining for membrane antigens could be combined with staining for nuclear TdT (see Fig. 2 for details).

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described. Briefly, 50-μl cell samples were incubated with antisera (1:20 dilution) or monomonal antibodies (1:100–1:500 dilution of ascites), washed twice in PBSA, and stained with second layers coupled to FITC or TRITC (1:25–1:50 dilution; see below). After staining the cells in suspension, cytocentrifuge preparations were made and fixed in cold methanol and restained for nuclear TdT. Smears were examined under a Standard 14 Zeiss microscope with 63-phase oil objective and IV/F epifluorescence condenser with selective filters for FITC (green) and TRITC (red).

RESULTS

Morphology of Liver and Bone Marrow Cells

Cytospin preparations of Ficoll-Hypaque-separated liver and bone marrow cells were examined by phase contrast. In 12–14-wk fetal liver samples, 10%–15% of cells had lymphoid-like and "undifferentiated" cell morphology, and 80%–85% of the cells were identifiable erythroid cells (Table 1). In samples of older fetuses, the percentage of lymphoid-like and "undifferentiated" cells remained relatively constant at between 10% and 20%, but a progressive reduction in the percentage of erythroblasts and an increase in myeloid (granulocytic) cells was observed. Ficoll-Hypaque-separated bone marrow samples from the long bones of fetuses 16, 18, and 21 wk of age contained high percentages of lymphoid-like (35%–50%) and myeloid cells (45%–60%) and lower proportions of erythroid cells (5%–15%).

Table 1. Morphology of Fetal Liver and Bone Marrow Cells

<table>
<thead>
<tr>
<th>Fetal Age (wk)</th>
<th>Liver</th>
<th>Bone Marrow</th>
<th>Myeloid</th>
<th>Lymphoid/Like &quot;Undifferentiated&quot;</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>6</td>
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<td>0</td>
<td>3</td>
</tr>
<tr>
<td>23</td>
<td>&lt;0.5</td>
<td>8</td>
<td>82</td>
<td>1</td>
</tr>
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The percentage of TdT+ cells is given within the total cell population isolated after Ficoll-Hypaque separation and within the lymphoid-like and "undifferentiated" cell population. *TdT+ cells are undetectable in the bone marrow of fetuses aged 12–15 wk and in the lymphus of fetuses aged 12–20 wk.

TdT+ Cells in Fetal Tissue

The percentage of TdT+ cells in liver and bone marrow was analyzed within the lymphoid-like and "undifferentiated" cell population and within the total cell population isolated after Ficoll-Hypaque separation (Table 2; Fig. 1). In 12–14-wk-old fetuses, 50%–60% of the lymphoid-like cell population from the liver were TdT+. By 15–16 wk of gestation, the proportion of TdT+ cells had decreased to approximately 30%, while less than 10% TdT+ cells were observed in 18–23-wk-old liver samples. The percentage of TdT+ cells within the total cell population isolated from liver ranged from 4% to 6% in 12–14-wk fetuses down to less than 1% in 21–23-wk fetuses.

Samples of the bone marrow from 12–15-wk-old fetuses were virtually acellular and contained only a few erythroid and myeloid cells. TdT+ cells were first observed in fetal bone marrow during the 16th week, when more than 40% of the lymphoid-like cells expressed TdT. There was a slight reduction in the percentage of TdT+ cells in samples of bone marrow...
TdT+ Cells in Human Fetal Tissue

Fig. 1. The development of TdT+ cells in human fetal tissue. The values represent TdT+ cells in the thymus (□) and TdT+ cells within the lymphoid-like and "undifferentiated" cell population from liver (●) and bone marrow (▲).

from 18–23-wk-old fetuses (25%–35%). When the results were expressed as the proportion of TdT+ cells within the total population of cells, values of 13%–16% (16 wk) down to 8%–9% (21 and 23 wk) TdT+ cells were observed.

Although thymocytes could be recovered from the fetal thymus from 12 wk onwards, TdT+ thymocytes were first observed in 1 of 2 thymuses from 21-wk-old fetuses. The TdT staining was relatively weak when compared to that observed in normal infant thymocyte controls and to the brilliant staining of TdT+ cells in the fetal bone marrow.

The time course of TdT expression is summarized in Fig. 1. At 12–15 wk, TdT+ was only observed in the liver. At 16–20 wk, TdT+ was observed in the liver and bone marrow, and at 21–23 wk, TdT+ was observed in the liver, bone marrow, and thymus.

Membrane Antigens on TdT+ Cells in the Liver and Bone Marrow

TdT+ cells in the liver, bone marrow, and thymus were further characterized by monoclonal antibodies against membrane antigens. In triple marker studies, staining for TdT (nuclear-TRITC) was combined with BA-1 (membrane-FITC) and RFB-1 (membrane-TRITC). Approximately 80% of TdT+ cells in 13-wk fetal liver were RFB-1+, BA-1+ (Table 3). The staining for BA-1 in this TdT+ population was variable (Fig. 2), and indeed, the remaining 20% of TdT+ cells were BA-1−, RFB-1+. The triple-labeled population of TdT+, BA-1+, RFB-1+ cells was observed in all samples of liver and bone marrow (Table 3) but could not be identified in the fetal thymus (see below). It was also observed that TdT+ cells in the liver and bone marrow were J5+ but HTA-1− (cortical thymocyte antigen negative).

The next phase of the study was focused on BA-1+ cells. In the 12–15-wk-old fetal liver samples, BA-1+ cells were more abundant than TdT+ cells. BA-1+ cells were heterogeneous in respect of TdT expression, since only 30%–40% of BA-1+ cells were also TdT+ (Figs. 2 and 3). In these samples, approximately 50%–60% of lymphocyte-like cells expressed surface IgM (sIg+). These corresponded to the BA-1+, TdT+ population. The relative expression of TdT and BA-1 in liver samples varied with age of the fetus. In early fetal liver samples (at weeks 13 and 14) a few TdT+ cells were BA-1−, and many showed only a weak BA-1 expression, while TdT−, sIg− cells showed particularly strong BA-1 expression (Fig. 2). In the fetal liver at later gestational age (16th week), the BA-1 expression on TdT+ cells was somewhat stronger and therefore slightly more uniform (Fig. 3). Similarly, strong BA-1 expression was observed on TdT+ cells from the bone marrow at 16 and 21 wk of gestation.

RFB-1+ cells were also more numerous than TdT+ cells. In the 13-wk-old liver, approximately 55%–60% of RFB-1+ cells were TdT+. In the liver and bone marrow, the percentage of TdT+ cells within the RFB-1+ population was reduced to 30%–40%. The RFB-1+, TdT− cells did not include sIg+ B cells (Fig. 3) and were predominantly larger blasts. These blast cells appeared to represent both myeloblasts and erythroblasts (Fig. 3). In parallel samples, the same population of TdT− blasts was shown to be strongly HLA-DR−.

Membrane Antigens on Fetal Thymocytes

The histogenesis of cortical and medullary areas in the fetal thymus occurs at an earlier stage (at 12–13 wk of gestation) than the appearance of TdT+ cells (20–21 wk; Fig. 1). We have reinvestigated membrane antigens in thymocytes at these early (TdT+) and later (TdT−) stages. At the 13th and 16th weeks, the majority of TdT+ cells (70%–75%) were RFB-1+, BA-1+, corresponding to the previously described HTA-1+ (cortical thymocyte antigen positive) population.5,6 The rest (25%–30%) were RFB-1−, BA-1−, corresponding to the strongly OKT-3+ medullary population.18 No BA-1+ cells were detected in these samples.

In the 21-wk fetal thymus sample, the majority of TdT+ cells showed the RFB-1+, BA-1+, HTA-1+ cortical thymocyte phenotype (Fig. 4) and were therefore different from the majority of TdT− cell types in the liver and bone marrow (Figs. 2 and 3), which are not only BA-1+ but also HTA-1− (data not shown).

DISCUSSION

We have studied the appearance of TdT+ cells in the liver, bone marrow, and thymus of human fetuses.
between 12 and 23 wk of age and analyzed the phenotypic characteristics of these cells using antipre-cursor cell monoclonal antibodies. In the earliest fetuses examined (12–14 wk), TdT+ cells were found exclusively in the liver. TdT+ cells first appeared in fetal bone marrow at 15–16 wk of gestation, when fetal bone marrow became cellular. Interestingly, the expression of TdT in fetal thymocytes was a relatively late event, occurring after TdT expression in the liver and bone marrow.

TdT+ cells in rodents13 and chicken,14 are first observed exclusively in fetal thymus and do not appear in the bone marrow until a few days after birth. Transient populations of TdT+ cells are also observed in the liver, spleen, and blood of the rat 1–4 days after birth, but disappear by the onset of puberty. These observations have suggested that TdT+ cells in the bone marrow and spleen might be of thymic origin.20 However, normal percentages of TdT+ cells are observed in the bone marrow and spleen of neonatally thymectomized rats and congenitally athymic mice, indicating that at least some of these TdT+ cells are unlikely to derive from the thymus.13,21 Previous studies show that TdT+ cells in human bone marrow have a different antigenic phenotype from those in the thymus and may therefore arise independently of the thymus.1 The present observations clearly support this view, since TdT+ cells appear in the bone marrow before the thymus, and the thymic TdT+ cells show a phenotype (BA-1+) different from marrow TdT+ cells (BA-1+).

Table 3. Expression of TdT and Surface Antigens in Human Fetal Tissue

<table>
<thead>
<tr>
<th>Fetal Age (wk)</th>
<th>Within TdT+ Cells*</th>
<th>Within RFB-1+ Cells</th>
<th>Within BA-1+ Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TdT+</td>
<td>RFB-1+</td>
<td>BA-1+</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>&gt;99</td>
<td>78</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>&gt;99</td>
<td>98</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>&gt;99</td>
<td>85</td>
</tr>
<tr>
<td>21</td>
<td>13</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>NA†</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>21</td>
<td>80</td>
<td>&gt;99</td>
<td>&lt;1</td>
</tr>
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</table>

*The values represent the percentage of RFB-1+ and BA-1+ cells within the TdT+ population (column 1), the percentage of TdT+ and HLA-DR+ cells within the RFB-1+ cell population (column 2), and the percentage of TdT+ and IgM+ cells within the BA-1+ cell population (column 3).
†The thymocytes in the cortical area are RFB-1+, BA-1- and lack TdT. The RFB-1+ cells represent 80% of total thymocytes at weeks 13, 16, and 21.
NA, not applicable; TdT+ cells are undetectable in these tissues. ND, not determined.

Fig. 2. Analysis of the phenotypic characteristics of TdT+ cells in 12-wk-old fetal liver. Figures A to C depict the same fields photographed by phase and immunofluorescence. (A) Phase; (B) RFB-1-membrane-TRITC, TdT-nuclear-TRITC; (C) BA-1-FITC, TdT+. RFB-1+, BA-1+ cells are indicated by small arrows; some of these cells are only weakly BA-1+. An RFB-1+, TdT+, BA-1- cell of myeloblast morphology is present (asterisk). In addition, 6 cells react only with BA-1+ and are TdT+, RFB-1+. These cells include B lymphocytes, many of which are strongly BA-1+ (see large arrow and Fig. 3).
During the purification of mononuclear cells from fetal liver, considerable enrichment of cell types, including immature erythroid cells (erythroblasts and normoblasts) and lymphoid-like cells, was achieved. The latter cells are likely to correspond to the lymphocytes and "unclassified" blast cells, representing 1.5%-6% of the hemopoietic cells in the fetal liver during 12–23 wk of gestation. We have shown that these cells include large proportions of immature cells that contain nuclear TdT and express the RFB-1', BA-1' or, less frequently, the RFB-1', BA-1' antigenic phenotypes. In addition, these cells are also J5', thus carrying the common ALL antigen. B lymphocytes (sIg', BA-1', TdT', RFB-1') were also present in this cell population. This finding, together with the demonstration that a similar cell population appears in the bone marrow (but not in the thymus) at 15–16 wk of gestation, strongly indicates that we have identified a progenitor cell population that appears first in the liver and then seeds the bone marrow. These findings

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**Fig. 3.** Bone marrow from a 21-wk-old fetus labeled with antisera as in Fig. 2. Figures A to C and figures D to F depict the same areas, respectively. (A) Phase; (B) IgM-membrane-FITC, TdT-nuclear-FITC; (C) BA-1-TRITC. TdT', BA-1' cells are IgM' (asterisks). IgM' B cells are TdT' (arrows). (D) Phase; (E) IgM-membrane-FITC, TdT-nuclear-FITC; (F) RFB-1-TRITC. TdT', RFB-1' cells are IgM' (asterisks) and a single IgM' B cell is also present (arrow).

**Fig. 4.** TdT expression in thymocytes from a 21-wk-old fetus. (A) Phase; (B) TdT-FITC; (C) RFB-1-TRITC. A few thymocytes are RFB-1', TdT' (asterisk).
are consistent with other experimental observations showing the migration of stem cells from the liver to the bone marrow. Thus, TdT+ cells probably give rise to B lineage cells at these sites. These TdT+ cells may, however, also include prothymocytes or common progenitors of B- and T-cell lineages.

An interesting observation was that thymic blasts and the majority of thymocytes were strongly RFB-1 but BA-1 from 12 wk of gestation. Similar observations showing the expression of thymocyte membrane antigens (e.g., NA1/34, OKT-6) on fetal thymocytes before the expression of TdT have also been reported. These results therefore indicate that fetal thymus is first populated by TdT+, RFB-1+, BA-1 cells, which might arise independently of the liver and bone marrow. Fetal thymocytes either continue to express RFB-1 after the local appearance of TdT, or at later stages, the thymus is repopulated with TdT+, RFB-1 progenitors. Although the origin of these progenitor cells is unknown, evidence from rodent and human studies indicates that they might also come from the bone marrow.

Based on our observations using TdT, RFB-1, and BA-1, the various stages of lymphoid development in human fetal tissue are schematically represented in Fig. 5. The common lymphoid progenitor cell population (TdT+, RFB-1+, BA-1+) generates B cells first in the liver and then in the bone marrow. In 12–20-wk-old fetuses, the thymus is populated by TdT+, RFB-1+ cells (pathway 1). In later fetuses, the thymus may be repopulated by TdT+, RFB-1+ cells, which derive from the TdT+ common lymphoid progenitor cell population in the bone marrow (pathway 2). The present studies do not establish the phenotype of pluripotent hematopoietic stem cells or the origin of those RFB-1+, TdT- cells that first seed into the thymus. Nevertheless, our study suggests the independent emergence of two lymphoid lineages in the fetal liver and thymus.

ACKNOWLEDGMENT

We thank Wendy Lake for TdT staining and Dr. Tucker LeBien (University of Minnesota, Minneapolis, MN) for a gift of BA-1 monoclonal antibody. We also thank Professor I. Craft, and members of the academic departments of Obstetrics and Gynecology, and staff of the Gynecological Wards of the Royal Free Hospital for their help.

REFERENCES


![Figure 5: Stages of lymphoid development in fetal tissue. The broken line separates population of the thymus by TdT cells (pathway 1) and TdT' cells (pathway 2).](image-url)
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