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Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells

JOANNE KURTZBERG*, STEPHEN M. DENNING, LYNN M. NYCUM, KAY H. SINGER, AND BARTON F. HAYNES

Departments of Pediatrics and Medicine, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **The signals and cellular interactions required for hematopoietic stem-cell commitment to the T lineage are unknown, yet are central to understanding the early stages of normal T-cell development. To study the differentiative capacity of T-cell precursors, we isolated CD4⁻, CD8⁻, surface (s) CD3⁻ thymocytes from postnatal human thymuses and determined their capacity to differentiate into lymphoid and nonlymphoid lineages *in vitro*. We found that CD4⁻, CD8⁻, sCD3⁻ thymocytes, which differentiated in the presence of T-cell conditioned medium plus interleukin 2 into T cells expressing the $\gamma\delta$ receptor for antigen, were capable of differentiating into myeloid or erythroid lineages in the presence of either 5637 bladder carcinoma cell line conditioned medium plus recombinant human erythropoietin or human thymic epithelial cell conditioned medium. Thymic epithelial cell conditioned medium was as effective as 5637 supernatant plus erythropoietin in inducing myeloerythroid differentiation in the CD4⁻, CD8⁻, sCD3⁻ thymocytes. Sixty-eight ± 14% of CD4⁻, CD8⁻, sCD3⁻ thymocytes underwent nonlymphoid differentiation within 4 days in culture with 5637 supernatant plus erythropoietin. Twenty-six ± 4% of freshly isolated CD4⁻, CD8⁻, sCD3⁻ cells were CD34⁺, and clonal granulocyte/macrophage, granulocyte/erythrocyte/monocyte/megakaryocyte, and T-cell progenitors were found in both CD34⁺ and CD34⁻ subsets of CD4⁻, CD8⁻, sCD3⁻ thymocytes. Thus, cells within the human CD4⁻, CD8⁻, sCD3⁻ thymocyte subset can give rise to $\gamma\delta^+$ T cells as well as to cells of myeloerythroid lineages. Moreover, CD34⁺, CD4⁻, CD8⁻, sCD3⁻ cells can give rise to clonal T-cell progenitors as well as to clonal myeloid progenitors.**

While progress has been made in the identification of precursors of the human T-cell lineage (1–3), a major question that remains is whether cells committed to the T lineage home to the thymic microenvironment, or whether multipotent stem cells home to the thymus and intrathymically receive signals necessary for terminal commitment to the T lineage. Human CD4⁻, CD8⁻, surface (s) CD3⁻ lymphocytes in fetal liver and thorax at 7 weeks gestational age express the CD7 T-cell antigen prior to entry into the epithelial thymic rudiment and are precursors of mature T cells (4). Critical to the hypothesis that multipotent stem cells home to the thymic microenvironment would be the demonstration in postnatal thymus of CD7⁺, CD4⁻, CD8⁻, sCD3⁻ cells that have retained the capacity to differentiate into nonlymphoid lineages. In this study, we show that human CD7⁺, CD4⁻, CD8⁻, sCD3⁻ T-cell precursors isolated from postnatal thymus can be driven to differentiate into nonlymphoid lineages by interleukin 3 (IL-3), erythropoietin (Epo), and granulocyte/macrophage-colony-stimulating factor (GM-CSF), as well as by factors produced by thymic epithelial (TE) cells.

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MATERIALS AND METHODS

Cell Preparation. Thymic tissue was obtained from normal children (aged 1 day to 8 years) undergoing median sternotomy incision and corrective cardiovascular surgery. TE-cell cultures were established by an explant technique and were subcultured as described (5). Thymocytes were separated and purified as described (6). CD4⁻, CD8⁻, sCD3⁻ thymocytes were prepared by "panning" (7, 8) followed by immunomagnetic bead separation or preparative cell sorting.

Antibodies. Monoclonal antibodies 3A1 (CD7) (9) and E5 and E6 (10) were used as described. Antibodies WT31 (11), TCRδ1 (12) and βF1 (13), TiγA (14), and 35.1 (CD2) (15) were gifts of Wil Tax, Michael Brenner, Thierry Hercend, and John Hansen, respectively. Monoclonal antibodies anti-Leu-2a (CD8), anti-Leu-3a plus anti-Leu-3b (CD4), anti-Leu-4 (CD3), and anti-Leu-5 (CD2), fluorescein isothiocyanate (FITC)-conjugated anti-Leu-9 (CD7), avidin-FITC, and phycoerythrin-conjugated goat anti-mouse immunoglobulins were purchased from Becton Dickinson. Monoclonal antibodies Mo1 (CD11), B1 (CD20), B2 (CD21), B4 (CD19), MY7 (CD13), MY9 (CD33), J5 and J5-FITC [common acute lymphoblastic leukemia antigen, CALLA (CD10)], and MY10 (CD34) were purchased from Coulter Immunology. P3X63/Ag8 ascites was used as a negative control. FITC-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used at a 1:50 dilution in indirect immunofluorescence assays.

Cell Culture. Cells were routinely cultured in standard medium (16) under an atmosphere of 5% CO_2 in air at 37°C. Cells were counted on a Coulter Counter ZM. Medium conditioned by human peripheral blood T cells [obtained by erythrocyte (E)-rosette separation] for 72 hr in the presence of phytohemagglutinin (TCM) was prepared as described (16). Conditioned medium (CM) from the human bladder carcinoma cell line 5637 (17) was prepared as described (16). Recombinant human GM-CSF and IL-3 were kindly provided by Steven Clark (Genetics Institute, Cambridge, MA). Recombinant human IL-2 and Epo were purchased from DuPont and Amgen Biologicals, respectively.

Nonlymphoid Differentiation of Cultured CD4⁻, CD8⁻, sCD3⁻ Thymocytes. CD4⁻, CD8⁻, sCD3⁻ thymocytes were cultured in the presence of 5637 CM (10%, vol/vol) plus Epo (2 units/ml) for 7 days. Aliquots were removed for characterization with cell counts, morphologic (200 cell count differentials) examination of Wright-stained cytocentrifuge preparations, benzidine staining, and surface immunophenotypic examination on indicated days as described (16).

Abbreviations: TE cell, thymic epithelial cell; Epo, erythropoietin; GM-CSF, granulocyte/macrophage-colony-stimulating factor; CFU-GM, granulocyte/macrophage-colony-forming unit(s); CFU-GEMM, granulocyte/erythrocyte/monocyte/megakaryocyte-CFU; CFU-T, T-cell-CFU; BFU-E, erythroid burst-forming unit; IL-*n*, interleukin *n*; sCD3, surface CD3; TCR, T-cell antigen receptor; CM, conditioned medium; TCM, T-cell CM.

*To whom reprint requests should be addressed.

Thymocytes were assayed for the presence of clonal hematopoietic progenitor colonies in semisolid medium by methods described for hematopoietic precursors derived from bone marrow (16). In brief, 10^5–10^6 cells were cultured in a base mixture of 0.9% methyl cellulose (1500 centipoises; Sigma) containing equal amounts of minimum essential medium α and Iscove's modified Dulbecco's medium (both from GIBCO), 12.5% horse serum, 12.5% heat-inactivated fetal bovine serum, and antibiotics (penicillin and streptomycin) in a total volume of 1 ml in a 35-mm gridded plate (Lux; Miles). To assay colonies derived from granulocyte/macrophage- or granulocyte/erythrocyte/monocyte/megakaryocyte-colony-forming units (CFU-GM or CFU-GEMM) or from erythroid burst-forming units (BFU-E), 1% deionized bovine serum albumin (Sigma), 10 μM 2-mercaptoethanol, 10% 5637 CM, and 2 units of Epo were added to each plate. To assay CFU-T lymphocyte (CFU-T), 10% TCM and 8 units of IL-2 were added to each plate. Colonies were scored on an inverted microscope (Olympus) on day 14. Assay cultures were plated in duplicate and one plate from each condition was stained *in situ* with benzidine to confirm the presence of early myeloid precursors in the CFU-GM colonies and of hemoglobinized cells in CFU-GEMM colonies. Representative colonies were plucked, washed, cytocentrifuged, and Wright-stained for morphologic confirmation of colony composition.

TE/Thymocyte Cocultures. $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes were cocultured in standard medium with TE cells in double-well chambers (Transwell; Costar) separated by 0.4-μm-pore polycarbonate membranes to prevent TE cell–thymocyte contact but to allow TE cell-derived cytokines to come in contact with $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes.

RESULTS

In both humans and rodents, the $CD4^-$, $CD8^-$, $sCD3^-$ intrathymic lymphocyte subset contains the earliest precursors of the T-cell lineage (reviewed in refs. 2, 18, and 19). To study the ability of $CD4^-$, $CD8^-$, $sCD3^-$ cells to differentiate into nonlymphoid lineages, we have isolated purified populations of these cells from postnatal thymus by depleting thymus cell suspensions of cells expressing CD4, CD8, CD3, and CD14 surface antigens (20). As previously reported, 78.7 ± 3% of freshly isolated $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes expressed cytoplasmic ε chains of the CD3 complex but expressed neither surface nor cytoplasmic T-cell antigen receptor (TCR) $\alpha\beta$ and δ molecules (20). We previously showed (20) that human $CD7^+$, $CD4^-$, $CD8^-$, $sCD3^-$ postnatal thymocytes were precursors of the T lineage, as they rapidly expressed surface CD3 and TCR δ chain after 36 hr *in vitro* when cultured under conditions that supported the growth of CFU-T. Hence we tested whether this thymocyte subset contained precursors of nonlymphoid lineages.

Human $CD4^-$, $CD8^-$, $sCD3^-$ Thymocyte Subset Contains Clonal Hematopoietic Progenitor Cells of T-Lymphoid as Well as Nonlymphoid Lineages. We tested for the ability of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes, as well as other thymocyte populations, to be driven *in vitro* to form either clonal T-cell progenitor colonies (CFU-T) or clonal nonlymphoid hematopoietic progenitor colonies (CFU-GM or CFU-GEMM). We found that myeloid and erythroid progenitor cells were present in the unfractionated thymocyte population, and when unfractionated thymocytes were subdivided into various subsets, all CFU-GM and CFU-GEMM progenitor cells were contained in the $CD4^-$, $CD8^-$, $sCD3^-$ subset (Table 1). Compared to unfractionated thymocytes, $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes were enriched 20-fold for CFU-GM, 9-fold for CFU-GEMM, and 4-fold for CFU-T (Table 1). BFU-E, the committed erythroid progenitors arising from CFU-GEMM, were found in greater frequency in the $CD4^-$, $CD8^-$ subset than in the $CD4^-$, $CD8^-$, $sCD3^-$ subset. Since the CD7 molecule is expressed on normal T-cell precursors (reviewed in refs. 2 and 4) and on pluripotent leukemic stem cells (16, 21), we directly determined CD7 expression by $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes capable of giving rise to both T-lymphoid and myeloerythroid clonal hematopoietic progenitor cells. Seventy-two ± 2% of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes were $CD7^+$. We found that complement-mediated lysis of $CD7^+$ cells in the $CD4^-$, $CD8^-$, $sCD3^-$ subset completely eliminated CFU-T, CFU-GM, and CFU-GEMM (Table 1). Thus, as with leukemic $CD7^+$, $CD4^-$, $CD8^-$ stem cells (16, 21), 100% of the $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes that gave rise to either myeloerythroid or T-cell colonies were $CD7^+$.

Time Course of Nonlymphoid Differentiation of $CD4^-$, $CD8^-$, $sCD3^-$ Thymocytes. After 4 days in the presence of CM from the human bladder carcinoma cell line 5637 (17) plus recombinant human Epo (a time at which total cell numbers were unchanged from those at culture initiation and >95% of cells remained viable), $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes had converted from 100% lymphoid morphology to 68.0 ± 14% (mean of three experiments; range 40–85%) myeloid or erythroid lineages. Fig. 1*A* shows an experiment in which after 4 days *in vitro* in the presence of 5637 CM plus Epo, 22% of $CD4^-$, $CD8^-$, $sCD3^-$ cells stained positively when incubated with benzidine, a vital stain taken up by hemoglobinized cells (evidence for erythroid differentiation), and 63% of cells had myeloid morphologies.

Human $CD4^-$, $CD8^-$, $sCD3^-$ Thymocyte Subset Is Heterogeneous. Previous investigators have demonstrated the heterogeneity of the $CD4^-$, $CD8^-$, $sCD3^-$ population of murine thymocytes (reviewed in ref. 19). We examined the immunophenotype of freshly isolated human $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes (Table 2). Although negative for mature T, B, myeloid, or erythroid antigens, 31.7 ± 5% of freshly isolated

Table 1. Growth of clonal hematopoietic progenitors from thymocyte subsets

		Colonies per 10^6 thymocytes			
Subset	n	CFU-GM	CFU-GEMM	BFU-E	CFU-T
Unfractionated	21	23.4 ± 7.0	7.9 ± 2.0	44.7 ± 13.6	240 ± 69.8
$CD4^+$, $CD8^+$	11	0.7 ± 0.6	0.0	1.2 ± 1.2	264.8 ± 101.6
$CD4^-$, $CD8^-$	11	337.1 ± 136.2	50.4 ± 19.8	216.3 ± 79.2	572.0 ± 203.4
$CD4^-$, $CD8^-$, $sCD3^-$	4	550 ± 198.4	73.0 ± 27.2	47.5 ± 44.3	867.7 ± 266.2
$CD4^-$, $CD8^-$, $sCD3^-$ depleted of $CD7^+$ cells*	2	0	0	0	0

Unfractionated thymocytes or the indicated thymocyte subsets were isolated, washed and diluted to 10^5–10^6 cells per ml in RPMI 1640 with 10% horse serum and 10% fetal bovine serum, and plated in methyl cellulose for clonal progenitor assays. Values are means ± SEM the indicated number (n) $CD4^-$, $CD8^-$ subset contained 20–50% $sCD3^+$ thymocytes. $CD4^-$, $CD8^-$, $sCD3^-$ subset contained <1% $sCD3^+$ thymocytes. $CD4^+$, $CD8^+$ subset contained $CD4^+$, $CD8^+$ thymocytes, $CD4^+$, $CD8^-$ thymocytes, and $CD4^-$, $CD8^+$ thymocytes.

*$CD4^-$, $CD8^-$, $sCD3^-$ thymocytes were incubated with 3A1 (anti-CD7) ascites (1:500 dilution) plus baby rabbit complement (1:8 dilution) for 2 hr at 37°C. Cells were washed twice and plated for progenitor assays. Incubations with P3X63/Ag8 ascites (negative controls) did not eliminate colony growth (data not shown).

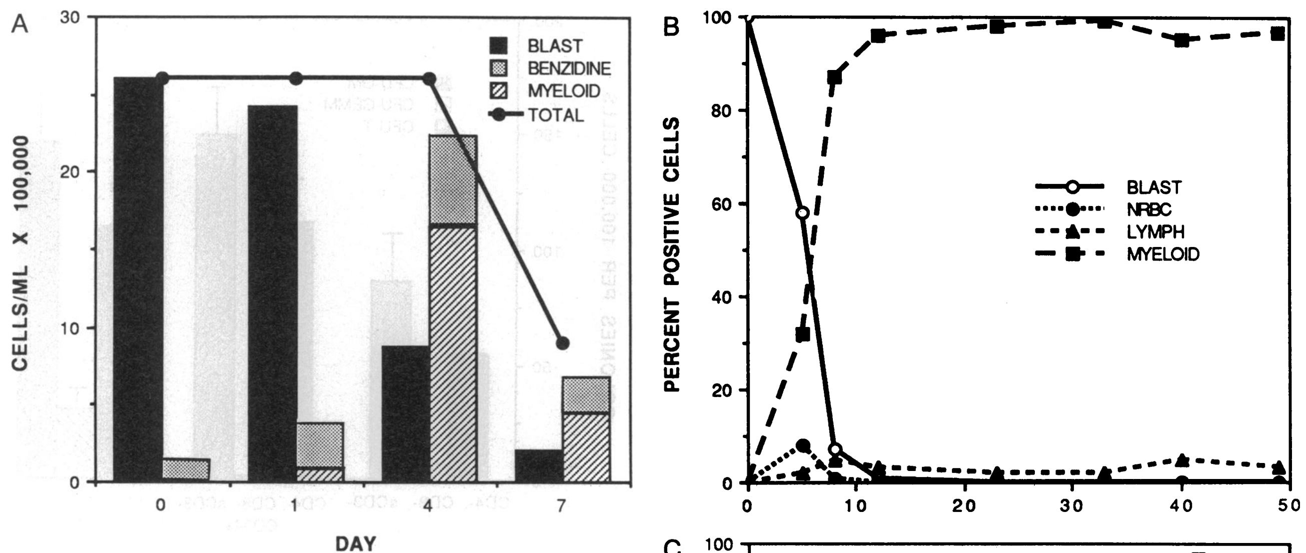

FIG. 1. Nonlymphoid differentiation of CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes cultured under standard conditions to support the growth of myeloid or erythroid cells (*A*) or cocultured with TE cells in double-chambered wells allowing contact with TE-secreted factors but preventing direct contact with TE cells (*B* and *C*). In all experiments freshly isolated CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes were 98% lymphoblastoid morphology, 3–5% benzidine-positive, 72 ± 2% CD7$^+$, 65.6 ± 3% CD2$^+$, 2.1 ± 1% E5$^+$, 26.4 ± 4% CD34$^+$, 31.7 ± 5% CD10$^+$, and negative for myeloid (CD13, CD33), B-lymphoid (CD20, CD21), and mature T-lymphoid (CD4, CD8) surface markers. (*A*) Morphologic and phenotypic data from an experiment performed during the first week in culture under standard conditions. During days 1–4, while the total cell count remained stable, nonlymphoid (myeloerythroid) differentiation, as determined by morphology and histochemical staining, occurred in 85% of CD4$^-$, CD8$^-$, sCD3$^-$ cells. In 3 experiments the mean percentage of myeloid or erythroid cells was 68 ± 14% (range 40–85%). Additional experiments (data not plotted) detailing morphologic and histochemical changes were performed under standard conditions without quantitation of total cell numbers. In 11 experiments, cells were examined on day 3 or 4, and in 9 experiments, cells were examined on day 6. On day 3 or 4, 43 ± 4% and on day 6, 83 ± 4% of CD4$^-$, CD8$^-$, sCD3$^-$ cells were of myeloid or erythroid morphologies. Cells of myeloid morphology included neutrophils, segmented band forms, metamyelocytes, myelocytes, monocytes, and mast cells. Criterion for erythroid lineage differentiation was either surface reactivity with erythroid-specific anti-glycophorin monoclonal antibodies (E5, E6; ref. 10) (data not shown in *A*) or the uptake of benzidine by live cells. Benzidine positivity was defined as cytoplasmic uptake without nuclear staining to exclude the identification of promyelocytes, which could also metabolize benzidine via primary granules containing myeloperoxidase. (*B*) Differentiation of CD4$^-$, CD8$^-$, sCD3$^-$ cells cultured with TE cells in double wells to prevent TE cell–thymocyte contact but to allow TE cell-derived cytokines to come in contact with the thymocytes. Under these conditions, CD4$^-$, CD8$^-$, sCD3$^-$ cells were completely nonlymphoid within 10 days. In these cultures, the growth of terminally differentiated mast cells was sustained for >50 days. (*C*) Time course of TE cytokine-induced nonlymphoid differentiation of CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes over 10 days in culture. Note that 60% of cells on day 10 stained positive with benzidine. The data in *B* and *C* are representative of five separate experiments.

human CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes expressed surface CD10 (CALLA) and 26.4 ± 4% expressed surface CD34, an antigen expressed on early hematopoietic progenitor cells (CFU-GEMM) (23). In double-labeling experiments using flow cytometry (24), 98% of the CD4$^-$, CD8$^-$, sCD3$^-$, CD34$^+$ thymocytes also expressed the CD7 antigen (data not shown). We reasoned that a population of CD34$^+$ cells residing within CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes contained myeloerythroid progenitor cells whereas CD34$^-$ cells contained precursors of the T-cell lineage. However, when assayed for the presence of lymphoid and myeloerythroid clonal hematopoietic progenitors, CFU-T and CFU-GM were found in both CD34$^+$ and CD34$^-$ subsets of CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes, while CFU-GEMM were enriched in the CD34$^-$ subset (Fig. 2). Thus, both CD34$^-$ and CD34$^+$ subsets of CD7$^+$, CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes each contained progenitors of both T-lymphoid and nonlymphoid hematopoietic lineages.

TE-Cell-Derived Cytokines Induce Nonlymphoid Differentiation of Immature Thymocytes. Human TE cells produce IL-1α and -1β (25), granulocyte (G)-CSF (26), macrophage (M)-CSF (26, 27), and GM-CSF (28). To determine the effect of TE-cell-derived factors on CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes, highly purified populations of CD4$^-$, CD8$^-$, sCD3$^-$ cells were cultured in the presence of TE cell culture supernatant. When added directly (10%, vol/vol) to CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes, TE supernatant induced proliferation that peaked at 5 days (29). When CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes were cultured in direct contact with TE cells, only clonal expansion of CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes was seen (30). However, when CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes were cocultured with TE cells in double-chambered tissue culture plates (to prevent direct TE cell–thymocyte contact but allow diffusion of TE cytokines), nonlymphoid differentiation of CD4$^-$, CD8$^-$, sCD3$^-$ thymocytes occurred, with 98% of cells after 10 days in culture expressing myeloid or erythroid morphologies or benzidine positivity (Fig. 1 *B* and *C* and Fig. 3 *F* and *H*).

***In Situ* Evidence for Nonlymphoid Differentiation in Normal Thymus.** Finally, to localize CD34$^+$ cells within human

Table 2. Phenotype of fresh CD4⁻, CD8⁻, sCD3⁻ thymocytes

Antibody	CD	Specificity	% positive
Surface phenotype			
3A1	CD7	Pan T cell	61.8
35.1	CD2	E-rosette receptor	45.8
Anti-Leu-4	CD3	CD3 ε chain	1.2
WT31		TCR $\alpha\beta$	0.7
TCRδ1		TCR δ chain	1.0
Anti-Leu-3a+3b	CD4	T helper cells	2.8
Anti-Leu-2a	CD8	T suppressor cells	2.9
MY10	CD34	CFU-GEMM	21.5
J5	CD10	CALLA	28.6
E5		NRBC*	2.7
Mo1	CD11b	Monocytes	8.4
MY7	CD13	Myeloid cells	4.5
MY9	CD33	Myeloid progenitors	5.5
Cytoplasmic phenotype			
3A1	CD7	Pan T cell	75.8
Anti-Leu-4	CD3	CD3 ε chain	78.7
βF1		TCR β chain	1.4
WT31		TCR $\alpha\beta$	0.7
TCRδ1		TCR δ chain	0.8
TiγA		TCR γ chain ($V_\gamma 2$)	3.0

Values are means from 3–10 separate thymocyte separations.
*Nucleated red blood cell precursors.

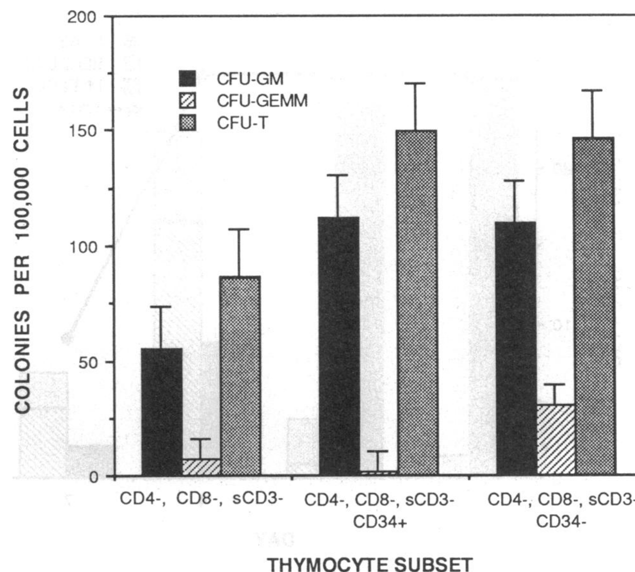

FIG. 2. Growth of clonal hematopoietic progenitor cells from $CD34^+$ and $CD34^-$ subsets of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes. CFU-GEMM were enriched in the $CD34^-$, $CD4^-$, $CD8^-$, $sCD3^-$ subset. CFU-GM and CFU-T were present in both the $CD34^+$ and the $CD34^-$ subset.

thymus and to document nonlymphoid differentiation within normal thymus, normal thymus tissue sections were assayed for the presence and location of $CD34^+$ cells and for glycophorin α-positive (erythroid) cells (10). $CD34^+$, $cCD3^+$ mononuclear cells were identified around the outer walls of vessels and scattered throughout the cortex of the normal thymus (Fig. 3 *A–D*). Interestingly, areas of erythroid differentiation in normal thymus were documented by the presence of clusters of glycophorin α-positive cells scattered throughout areas of normal cortex (Fig. 3*E*) (31). In addition, a previous report (21) showed that myeloid differentiation occurred on a massive scale in the thymus of a patient with $CD7^+$, $CD4^-$, $CD8^-$ leukemia of stem-cell origin.

DISCUSSION

In this paper, we have shown that highly purified populations of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes were enriched for both

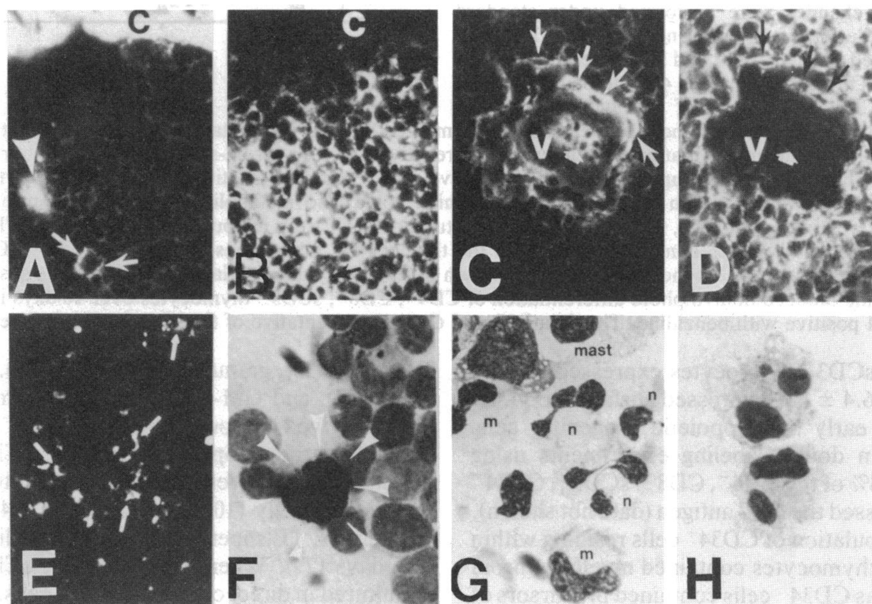

FIG. 3. Location of $CD34^+$ and glycophorin α-positive cells in thymus and response of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes to TE cytokines. (*A* and *B*) Sequential 4-μm sections from a normal thymus from a 6-month-old infant. *A* shows a single cell (arrows) staining positive for CD34 (MY10); in *B* double indirect immunofluorescence (30) shows that the same $CD34^+$ cell is also $CD3^+$ (anti-Leu-4) (arrows). In *A*, CD34 antibody MY10 also bound to the fibrous capsule (c) and vessels (arrowhead) of the thymic cortex. (*C* and *D*) The same 4-μm sections from normal 6-month postnatal human thymus. *C* shows three $CD34^+$ cells (long arrows) around a thymic vessel (v); in *D*, double indirect immunofluorescence shows that these cells are $CD3^+$ (long arrows). CD34 antibody MY10 also reacted with fibroblasts around the vessel wall and with endothelial cells in the vessel lumen (short arrow). (*E*) A cluster of glycophorin α-positive (E3 antibody) cells (arrows); such cells were found throughout the thymic cortex. (*F*) Myeloperoxidase-positive promyelocyte (arrowheads) derived from $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes cultured for 24 hr with TE cytokines as described in Fig. 1. (*G* and *H*) Terminally differentiated myeloid cells in Wright-stained cytocentrifuge preparations of $CD4^-$, $CD8^-$, $sCD3^-$ cells cultured with 5637 CM plus Epo for 22 days (*G*) or with TE cytokines as in Fig. 1 for 21 days (*H*). In *G*: n, neutrophil; m, myelocyte; mast, mast cell. The cells in *H* are both neutrophils. (*A–E*, acetone-fixed, ×280; *F*, *G*, and *H*, ×700.)

CFU-T and clonal myeloerythroid progenitor cells. Moreover, both $CD34^+$ and $CD34^-$ subsets of $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes gave rise to both T-cell and myeloid progeny in bulk cultures *in vitro*. Finally, in the presence of TE supernatant, $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes differentiated nearly exclusively into nonlymphoid lineages.

While we have yet to prove at a single-cell level that the same $CD7^+$, $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes that are capable of T-lymphoid differentiation are also capable of myeloid or erythroid lineage maturation, we have shown that, after 4 days in culture with myeloid differentiation factors, without a change in total cell number, $68 \pm 14\%$ of the cells in the $CD4^-$, $CD8^-$, $sCD3^-$ thymocyte subset gave rise to nonlymphoid progeny (Figs. 1*A* and 3*G*). There are two possible explanations for this phenomenon. The first is that the majority of cells within $CD4^-$, $CD8^-$, $sCD3^-$ thymocyte population at day 0 were multipotent and thus were capable of undergoing simultaneous differentiation with little or no proliferation during the 4-day culture period. Alternatively, the possibility exists that overgrowth of a small percentage of committed myeloid and erythroid precursor cells within the $CD4^-$, $CD8^-$, $sCD3^-$ population occurred over 96 hr *in vitro*. In that case, if 1% of the $CD4^-$, $CD8^-$, $sCD3^-$ cells were committed myeloid or erythroid precursors they would have had to double at least every 12 hr while terminally differentiating, while 80% of the other $CD4^-$, $CD8^-$, $sCD3^-$ cells died over the same time period, to explain the results obtained. In further support of the first explanation, previous reports described a syndrome of leukemic stem cells characterized by $CD7^+$, $CD4^-$, $CD8^-$ leukemic blasts capable of multilineage (lymphomyeloerythroid) differentiation both *in vivo* (21) and *in vitro* (16, 32). Three patients with $CD7^+$ stem-cell leukemias had clonal chromosome 14 translocations such that myeloid and lymphoid progeny of $CD7^+$, $CD4^-$, $CD8^-$ leukemic cells could be demonstrated to be derived from a single clone (16). In addition, $CD7^+$, $CD4^-$, $CD8^-$, $sCD3^-$ cells purified from human fetal liver have been shown to contain CFU-T as well as CFU-GEMM and CFU-GM (4). Taken together, these data suggest that at least subsets of human $CD7^+$, $CD4^-$, $CD8^-$, $sCD3^-$ cells in fetal liver (before emigrating to the thymus) and in postnatal thymus (after thymic colonization) are not terminally committed to the T-cell lineage but rather that events subsequent to thymic seeding by these $CD7^+$ cells determine terminal commitment to the T-lymphoid lineage. This hypothesis is consistent with previous work in murine systems that suggested that mouse peripheral lymphoid organs can be seeded by multipotent progenitor cells (33–37) and that the lineage program undertaken by stem cells is a function of the microenvironment in which the stem cell develops (33, 34). Micromanipulation studies of normal $CD7^+$, $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes at a single-cell and clonal level will be required to show their cloning efficiency and pluripotent differentiating potential.

Our observation that normal human $CD34^+$, $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes contain precursors of the T-cell lineage (as well as myeloerythroid lineages) is supported by the recent report by Thiel *et al.* (38) in which 64% of blasts from patients with pre-T ($CD7^+$, $CD4^-$, CD8, $sCD3^-$) acute lymphoblastic leukemia were $CD34^+$.

Fetal $CD4^-$, $CD8^-$, $sCD3^-$ T-cell precursors mature to TCR $\alpha\beta$-positive cells (2, 4), whereas postnatal $CD4^-$, $CD8^-$, $sCD3^-$ T-cell precursors mature nearly exclusively to TCR $\gamma\delta$-positive cells (20). The signals or cellular interactions necessary for the generation of TCR $\alpha\beta$-positive cells from postnatal $CD4^-$, $CD8^-$, $sCD3^-$ thymocytes remain unknown. Given that the same stimuli (TCM plus IL-2) can drive fetal T-cell precursors to express TCR $\alpha\beta$ and postnatal T-cell precursors to express TCR $\gamma\delta$, it seems likely that intrinsic differentiating capacities exist in fetal versus postnatal $CD4^-$, $CD8^-$, $sCD3^-$ cells.

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