T Lymphocyte Differentiation In Vitro from Adult Human Prethymic CD34+ Bone Marrow Cells

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Summary

Pluripotent lymphohematopoietic stem cells are probably confined to bone marrow cells expressing CD34 surface molecules. To investigate the capacity of adult human CD34+ bone marrow cells to differentiate along the T lymphoid lineage, we plated purified CD34+ cells from healthy adults in liquid culture on adherent thymic stromal cells prepared from HLA- or blood group–mismatched postnatal thymic tissue. We show that purified CD34+CD3-CD4-CD8- bone marrow cells contained progenitors with the ability to differentiate into CD4+ and CD8+ T lymphocytes expressing surface (s)CD3 and T cell receptor α/β in vitro. These progenitors were found in the CD34+CD2+sCD3-CD4-CD8-, CD34+CD7+sCD3-CD4-CD8-, and CD34+CD2+CD7+sCD3-CD4-CD8-, as well as in the CD34+CD2-sCD3-CD4-CD8-, CD34+CD7-sCD3-CD4-CD8-, and CD34+CD2-CD7-sCD3-CD4-CD8- subsets, indicating that T lymphocyte progenitors sensitive to signals mediated by thymic stroma in vitro are not restricted to CD34+ cells already coexpressing early T lymphocyte–associated markers. Finally, we show that T lymphopoiesis was enhanced by c-*kit* ligand.

Bone marrow cells expressing CD34 surface molecules constitute ~1% of low density nucleated bone marrow cells (1). There are several observations to suggest that CD34+ cells include pluripotent stem cells (2–12). In mice, T lymphopoiesis can be demonstrated by transfer of bone marrow cells into irradiated hosts, and differentiation to mature human T lymphocytes from liver-derived fetal hematopoietic progenitors is observed in the Thy SCID-human (hu)[1] mouse (13–16). Recently, Péault et al. (17) have succeeded in reconstituting T lymphopoiesis in fetal thymus in SCID-hu mice with fetal human CD34+ cells. In these experiments T lymphocyte differentiation was accomplished in thymus in vivo. Maintenance of the thymic microenvironment has been claimed to be a prerequisite for differentiation of stem cells into mature T lymphocytes (17).

The aim of this study was to investigate whether postnatal human CD34+ bone marrow cells can give rise to T lymphocyte differentiation in vitro, and whether full differentiation to mature T lymphocytes can be accomplished. This study shows that when cultured on thymic stroma, a population of human CD34+ bone marrow cells devoid of the surface (s) molecules CD3, CD4, and CD8 can be directed into T lymphopoiesis in vitro, as ascertained by the appearance of cells expressing CD3, CD4, CD8, and TCR α/β surface molecules after culture. We also present data showing that the T lymphocyte progenitors are present among CD34+ cells expressing the T lymphocyte–associated markers CD2 and/or CD7, as well as among CD34+ cells devoid of these markers. Furthermore, we show that the growth ability of CD34+ cells is significantly enhanced by *c-kit* ligand without changing the phenotypic profile of the progeny.

[1] *Abbreviations used in this paper:* CM, complete medium; hu, human; rm, recombinant murine; s, surface.

Materials and Methods

Cell Donors and Cell Preparation. Bone marrow samples were obtained by aspiration from consenting healthy adults. Mononuclear cells were prepared by density gradient centrifugation (1.077 g/ml, Lymphoprep; Nycomed Pharma, Oslo, Norway), washed twice, and resuspended in IMDM (Flow Laboratories, Irvine, Scotland) with 10% (vol/vol) FCS (Gibco, Paisley, Scotland), glutamine (2 mmol/liter; Gibco), penicillin (60 μg/ml), and streptomycin (100 μg/ml), referred to as complete medium (CM). CD34+ cells were isolated with Dynabeads M-450 coupled with a mAb against CD34 (BI-3C5) (Dynal, Oslo, Norway) as previously described (18, 19). Contaminating mature B lymphocytes (CD34-CD19+CD37+) but not mature T lymphocytes (CD34-sCD3+CD4+ or CD34-sCD3+CD8+) can be demonstrated among isolated CD34+ bone marrow cells (19). To increase the purity of the isolated CD34+ cells to >95% to minimize the possibility of outgrowth of contaminating T lymphocytes to account for the appearance of CD4+ and CD8+ cells after culture, immunomagnetic depletion with Dynabeads M-450 coupled with mAbs against CD4, CD8, and CD19 (Dynal) was performed (20).

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Thymic tissue specimens were obtained from two children <6 mo of age undergoing median sternotomy and corrective cardiovascular surgery (kindly provided by H. Lindberg, Surgical Department A, The National Hospital, Oslo, Norway). One donor was HLA-A2$^+$,ARh$^+$, and the other was HLA-A2$^-$,ORh$^+$. The thymic tissue was transported to the laboratory in HBSS (Gibco) with penicillin and streptomycin. The thymic tissue was minced into pieces, and single cells were eluted in HBSS with collagenase (0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO) and DNase I (1 μg/ml) (Sigma Chemical Co.) at 37°C under continuous shaking for 1.5 h. After six to eight repeated cycles of elution the thymic tissue was totally disintegrated. Single cell suspensions from the last three to four eluates were washed twice and resuspended in RPMI 1640 (Gibco) with penicillin and streptomycin before cryopreservation.

Antibodies. The following mAbs were used for flow cytometry: FITC-labeled anti-Leu-5b (CD2), anti-Leu-4 (CD3), anti-Leu-9 (CD7), and anti-HPCA-2 (8G12, CD34), and PE-labeled anti-HPCA-2, anti-Leu-M7 (CD13), Simultest CD4/CD8 (anti-Leu-3a FITC + anti-Leu-2a PE), Simultest LeukoGATE (anti-HLe-1 FITC + anti-Leu-M3 PE), and Simultest control (IgG1 FITC + IgG2a PE) (Becton Dickinson Immunocytometry Systems [BDIS], Mountain View, CA). PE-labeled anti-CD2, and anti-CD34 (ICH3) were purchased from Caltag Laboratories, Inc. (South San Francisco, CA), anti-TCR α/β (BMA031) from Behringwerke AG (Marburg, Germany), and anti-CD3 (101.1) from Novo Research Institute (Copenhagen, Denmark). Anti-HLA-A2 (ITI-8G12) was developed in our own laboratory by G. Gaudernack. Anti-A (blood group A) was purchased from Lorne Laboratories Ltd. (England). FITC-labeled anti-Leu-5b and anti-Leu-9 and PE-labeled anti-HPCA-2 from BDIS were used for cell sorting by flow cytometry. The following mAbs were used for immunofluorescence: TE-7, reactive with nonkeratinized, mesodermal-derived thymic fibrous stroma, and TE-15, reactive with Hassell's bodies (a kind gift from K. H. Singer, Duke University School of Medicine, Durham, NC) (21). Fluoresceinated goat anti-mouse isotype- and subclass-specific polyclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Culture Assays. After thawing, eluted thymic cells were washed twice, resuspended in CM, and plated, with 2 × 10^6 cells/well in 24-well tissue culture plates (Costar, Cambridge, MA). The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C for 6–8 d to establish a confluent layer of adherent cells. Supernatants were collected, filtered, and frozen for later use. Nonadherent cells were removed from the wells by repeated washing.

CD34$^+$ cells were plated on the adherent thymic stromal cells, with 10^5 cells/well in 1 ml CM with 10% (vol/vol) thymic stromal cell supernatant, and with four to five wells per series. The cells were cultured in the absence or presence of recombinant murine (rm) c-*kit* ligand (2 U/ml) (a kind gift from S. C. Clark, Genetics Institutes, Cambridge, MA). Media were half changed with fresh supernatant media twice weekly. rmc-*kit* ligand was added according to protocol. Nonadherent cells were harvested after 14 and 28 d, enumerated in a Bürker chamber, and analyzed by flow cytometry.

To more closely identify prethymic T lymphocyte progenitors, flow cytometry–sorted CD34$^+$CD2$^+$, CD34$^+$CD7$^+$, CD34$^+$CD2$^+$CD7$^+$, CD34$^+$CD2$^-$, CD34$^+$CD7$^-$, and CD34$^+$CD2$^-$CD7$^-$ cells (2,500–5,000 cells/well) were cultured on thymic stromal cells in the presence of rmc-*kit* ligand, with two to three wells per series. Nonadherent cells were harvested, enumerated, and phenotyped by flow cytometry after 21 d.

Bone marrow cells from the nonCD34$^+$ fraction (i.e., bone marrow cells not rosetting with BI-3C5-coated Dynabeads), CD4$^+$ T lymphocytes, or CD8$^+$ T lymphocytes (10^5 cells/well) were also cultured under the same conditions. To study the necessity of thymic stroma to support T lymphopoiesis, adherent cells prepared from MRC-5 (a fibroblast cell line), Hep-2 (a hepatoma cell line), and allogeneic peripheral blood monocytes, and nonadherent allogeneic Epstein-Barr blasts were substituted for thymic stromal cells. Furthermore, CD34$^+$ cells were cultured in 96-well U-bottomed tissue culture plates (Costar), with 1,000 cells/well in 200 μl CM, in the presence of irradiated allogeneic PBMC, PHA (1 μg/ml) (Welcome, Dartford, England), and rIL-2 (10 U/ml) (Genzyme, Cambridge, MA). The cultures were restimulated with irradiated allogeneic PBMC, PHA, and rIL-2 after 7 d and cultured for an additional 8 d. The cells were cultured for the last 18 h in the presence of 10 μM [^{3}H]thymidine (2 Ci/mmol), harvested on filter mats, and counted using a scintillation counter (LKB Instruments, Turku, Finland). Non-CD34$^+$ bone marrow cells and PBMC served as positive controls, and irradiated PBMC served as negative control.

Flow Cytometry and Cell Sorting. Cell staining was performed according to the manufacturer's instructions and the cells were resuspended in HBSS with 1% paraformaldehyde. Flow cytometry analyses were performed on a FACScan® with the FACScan® Research Software (BDIS). 50,000 events were acquired and stored in list mode files.

Cell sorting was performed on an Epics Elite (Coulter Electronics, Hialeah, FL). Cells were sorted at a rate of 800–1,000 cells/s. A sort gate within a dual parameter cytogram of forward light scatter against 90° side scatter was drawn. A second amorphous gate was drawn on the two-color cytograms shown (Fig. 6). Sort equations were set to positively sort cells satisfying both gates.

Immunofluorescence Microscopy. To investigate the identity of the adherent cell layer, the adherent cells were fixed in ethanol, incubated with the mAbs for 30 min at room temperature, washed twice, and incubated with a goat anti–mouse fluoresceinated antibody.

Limiting Dilution Assays. To examine the T lymphocyte precursor frequency of CD34$^+$ cells expressing the CD2 and CD7 markers and CD34$^+$ cells devoid of these markers, we performed experiments in limiting dilution assays. Allogeneic adherent thymic stromal cells were established in 96-well flat-bottomed tissue cultures plates (Costar) as described above. CD34$^+$CD2$^+$, CD34$^+$CD7$^+$, CD34$^+$CD2$^-$, and CD34$^+$CD7$^-$ cells were plated with CM supplemented with rmc-*kit* ligand (2 U/ml) and 10% (vol/vol) thymic stromal cell supernatant at 15–240 cells/well with 20 replicates per concentration. Media were doubled at day 5 and then half changed with fresh rmc-*kit* ligand and supernatant media twice weekly until week 3. Wells were harvested and the cells phenotyped by an immunomagnetic method (22). Dynabeads M-450 (Dynal) coupled with a mAb against CD3 (38,1; kindly provided by J. A. Hansen, Fred Hutchinson's Cancer Research Center, Seattle, WA) were selected for detection of T lymphocytes in the cultures. Wells were determined positive for T lymphocytes when >5% of the cells made rosettes with more than five beads and the rosetting cells were small lymphoid cells. Based on Taswell's equation, the frequency of T lymphocyte progenitors was calculated (23).

Statistics. The growth abilities of CD34$^+$ cells in the absence and presence of rmc-*kit* ligand were compared using the Student's *t* test for paired observations.

Results

Characterization of Isolated CD34$^+$ Cells. The immunomagnetically isolated cells contained >90% CD34$^+$ cells in

all experiments and >95% in most experiments. The flow cytometry–sorted cells contained >98% $CD34^+$ cells in all experiments. Cells expressing CD3 surface molecules could not be detected by flow cytometry (Fig. 1), and neither could cells expressing CD4 or CD8 molecules (not shown).

Preparation of an Adherent Layer of Thymic Stromal Cells. The eluted cells were enriched for thymic stromal cells as judged by the presence of thymus rosette–forming cells. During culture of the eluted cells for 6–8 d, they developed a confluent, adherent cell layer. The adherent cell layer contained ~20% cells of epitheloid morphology reactive with the TE-15 mAb and ~80% cells of fibroblastoid morphology reactive with the TE-7 mAb.

Growth Ability. The growth ability of $CD34^+$ cells on adherent thymic stromal cells in the absence and presence of rmc-*kit* ligand are shown in Fig. 2. Cell multiplication was significantly enhanced by rmc-*kit* ligand: from an 8- to 42-fold increase in cell number ($p < 0.03$) after 28 d in culture. The interexperimental variations were large. A decrease in number of nonadherent cells was observed when non-$CD34^+$ bone marrow cells, $CD4^+$ T lymphocytes, or $CD8^+$ T lymphocytes were cultured for 28 d on thymic stromal cells. This was also the case when $CD34^+$ cells were cultured on adherent cells prepared from the MRC-5 cell line, the Hep-2 cell line, or allogeneic peripheral blood monocytes, or together with allogeneic EB blasts. Opposed to non-$CD34^+$ bone marrow cells and PBMC, no proliferative response was demonstrated when $CD34^+$ cells were cultured in the presence of PHA, rIL-2, and irradiated allogeneic PBMC (data not shown).

Characterization of Cultured Cells. The progeny of the purified $CD34^+sCD3^-CD4^-CD8^-$ cells were analyzed in flow cytometry for cell size, granularity, and expression of

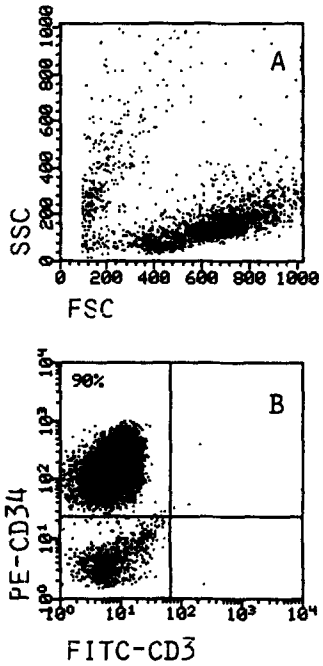

Figure 1. Characteristics of isolated $CD34^+$ cells. The light scatter profile (*A*) defines two populations: a "lymphocyte" gate and another gate comprising cells with a wide range of cell size and granularity. The two-color cytogram (*B*) illustrates that no detectable mature T lymphocytes ($sCD3^+$) were detected among isolated $CD34^+$ cells.

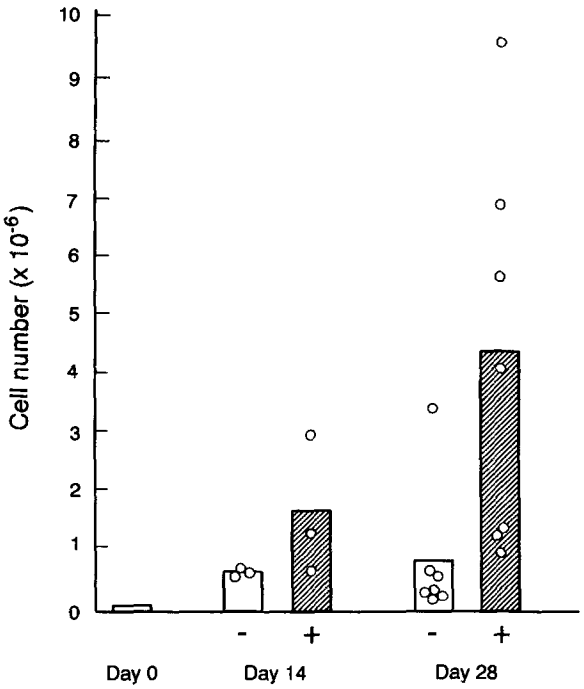

Figure 2. The growth ability of $CD34^+$ cells. The cells were cultured on thymic stromal cells in the absence (−) or presence (+) of rmc-*kit* ligand. Cell multiplication is presented as results of individual experiments (*circles*) and as mean values of all experiments (*bars*). At day 28 the enhancement of cell multiplication accomplished by rmc-*kit* ligand reached statistical significance ($p < 0.03$, Students *t* test, paired observations).

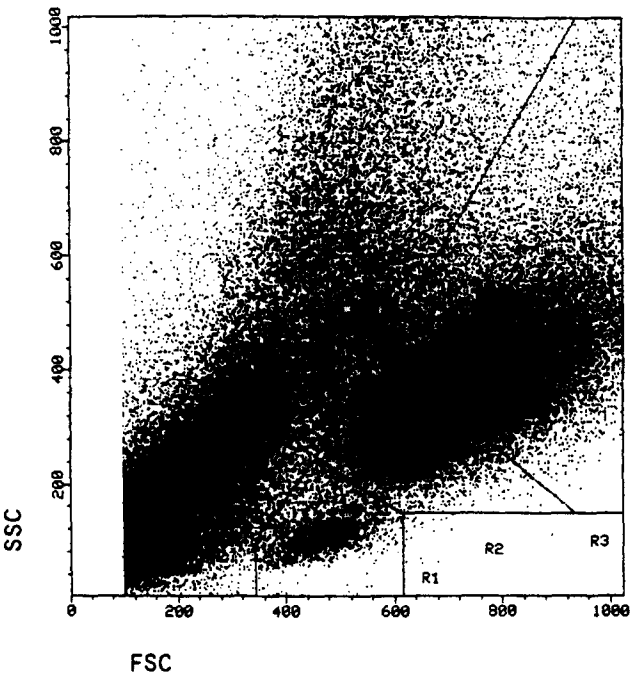

Figure 3. Characteristics of cultured cells. A representative light scatter profile of cultured cells where R1 defines small, agranular cells (the "lymphocyte" gate), R2 defines cells with intermediate size containing no or a few granules, and R3 defines large cells, heavily granulated.

CD34- and T lymphocyte–associated surface molecules after 14 and 28 d in culture. Fig. 3 shows the results of a representative analysis of size and granularity of the cells. A population of cells (R1) is discernible with relatively low light scatter signals characteristic of agranular lymphoid cells, and two larger populations are discernible (R2 and R3) with intermediate and high light scatter signals compatible with cells of intermediate size with no or a few cytoplasmic granules and large cells heavily granulated, respectively.

Table 1 shows the relative number of cells expressing CD34- and T lymphocyte–associated surface molecules of the progeny of the $CD34^+sCD3^-CD4^-CD8^-$ cells after 28 d in culture. Apart from a slightly higher relative number of $CD34^+$ cells and $CD4^+CD8^+$ cells after 14 d than after 28 d, there were no differences between the relative numbers of cells expressing the different surface molecules analyzed after 14 and 28 d. CD34 expression was mainly confined to cells in the R2 fraction (Fig. 3 and Table 1). On the other hand, cells expressing the CD2, CD7, CD3, CD4, and CD8 molecules were mainly confined to the R1 fraction (Fig. 4 and Table 1). The majority of $sCD3^+$ cells in R1 fraction coexpressed TCR α/β (Fig. 4). The low numbers of $CD4^+$ $CD8^+$ cells cannot account for the high numbers of $sCD3^+$ TCR α/β^+ cells demonstrated after culture (Fig. 5, *B*, *C*, and *E*). Although not demonstrated in a triple-color cytogram, most if not all $CD4^+$ and $CD8^+$ cells had to be

Table 1. *Relative Number of Cells Expressing Different Cell Surface Markers after 28 d in Culture*

		Characteristics of plated cells					
		CD34-pos. cells		$CD34^+$ cells depleted for CD4,8,19		c-*kit* ligand added to $CD34^+$ cells	
Exp.	Marker	R1*	R2*	R1	R2	R1	R2
				%			
1	CD34	1	3	1	4	1	4
2	CD34	1	3	1	2	1	2
3	CD34	1	7	nt‡	nt	1	9
4	CD34	nt	nt	2	5	nt	nt
1	CD2	21	2	12	1	6	1
2	CD2	56	1	54	1	48	4
3	CD2	22	4	nt	nt	18	4
4	CD2	nt	nt	38	3	nt	nt
1	CD7	15	2	8	2	6	1
2	CD7	61	2	40	1	62	4
3	CD7	20	5	nt	nt	17	5
4	CD7	nt	nt	37	3	nt	nt
1	CD3	20	3	12	2	10	2
2	CD3	61	1	52	3	56	4
3	CD3	29	5	nt	nt	26	6
1	CD4	9	1	9	1	9	<1
2	CD4	16	1	13	1	20	4
3	CD4	4	1	nt	nt	10	1
4	CD4	nt	nt	7	<1	nt	nt
1	CD8	13	2	9	1	2	<1
2	CD8	22	1	24	1	20	1
3	CD8	15	2	nt	nt	10	2
4	CD8	nt	nt	33	3	nt	nt

* R1 and R2 refer to regions defined in Fig. 3.
‡ nt, not tested.

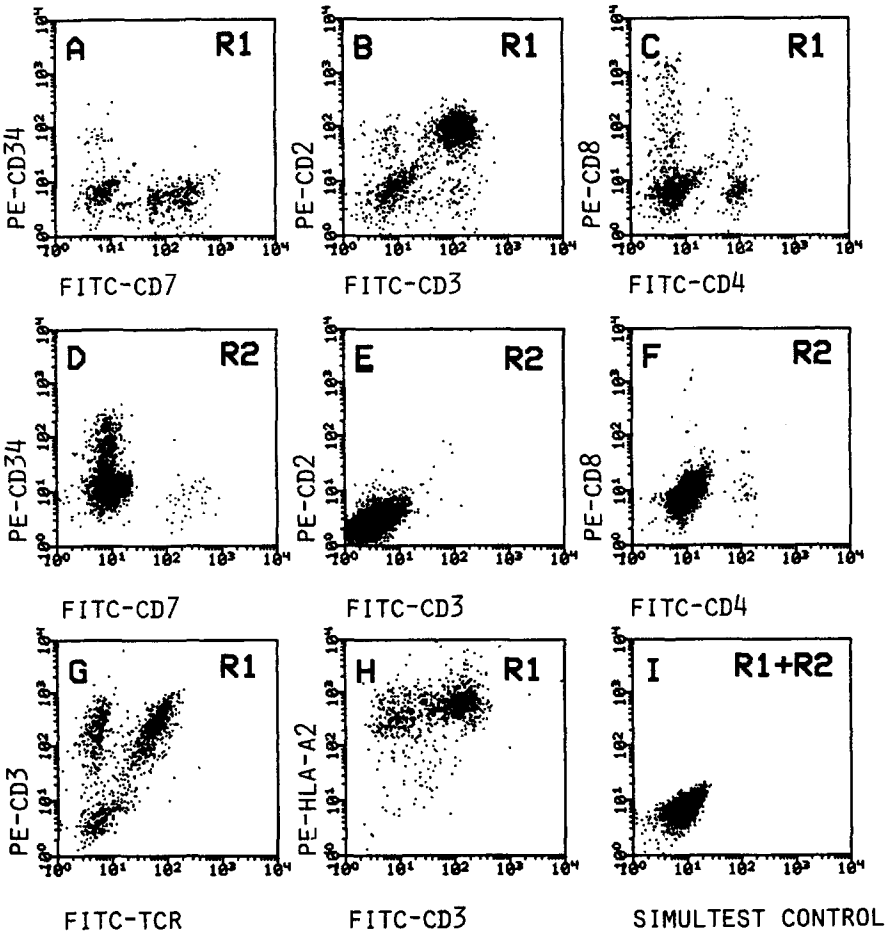

Figure 4. Phenotypic characterization of cultured cells. Representative two-color cytograms with a negative control (*I*). Cells expressing the T lymphocyte–associated CD7, CD2, and CD3 surfaces molecules were mainly located in the lymphocyte gate, R1, whereas cells expressing CD34 surface molecules were confined to R2 (*A–F*). A major part of the $CD3^+$ cells expressed TCR α/β (*G*). All cells in the lymphocyte gate expressed HLA-$A2^+$, i.e., they are of bone marrow donor HLA type (bone marrow donor HLA-$A2^+$ and thymus donor HLA-$A2^-$) (*H*).

$sCD3^+TCR\ \alpha/\beta^+$. A certain number of cells coexpressed the CD4 and CD8 molecules. Data illustrated in Fig. 5 suggest that the relative number of $CD4^+CD8^+$ cells decreased from days 14 to 28. To exclude that the few $CD4^+CD8^+$ cells observed were actually doublets of single-positive cells, light scatter properties (i.e., size) of the double-positive cells were compared with single-positive cells. $CD4^+CD8^+$ cells were exclusively confined to R1, and no differences in size between single- and double-positive cells were demonstrated, excluding the $CD4^+CD8^+$ cells to be doublets. Furthermore, dots compatible with $CD4^+CD4^+$ or $CD8^+CD8^+$ doublets could not be demonstrated. A major part of the cells in R2 and R3 fractions could be shown to belong to the myeloid lineage by detection of cells expressing CD13 and CD14 molecules (data not shown).

There were no differences in distribution of T lymphocyte–associated markers whether the progeny were derived from $CD34^+$ cells, $CD34^+$ cells after CD19, CD4, and CD8 depletion, or $CD34^+$ cells cultured in the absence or presence of rmc-*kit* ligand.

The T lymphocytes emerging after culture could be demonstrated to be of bone marrow donor origin by coexpression of bone marrow donor-specific HLA or blood group molecules and sCD3 (Fig. 4).

T Lymphocyte Progenitors. A minor proportion of $CD34^+sCD3^-CD4^-CD8^-$ cells was found to coexpress the CD2 and/or CD7 molecule (1–2%) (Fig. 6). When cultured in bulk, these subsets gave rise to lymphoid cells of T lymphocyte lineage just like unfractionated $CD34^+$ cells (Figs. 4 and 5). To examine whether T lymphocyte precursors were exclusively confined to the $CD34^+CD2^+$, $CD34^+CD7^+$, and $CD34^+CD2^+CD7^+$ subsets, $CD34^+CD2^-$, $CD34^+CD7^-$, and $CD34^+CD2^-CD7^-$ cells were cultured under the same conditions. The results of these experiments clearly demonstrate that even $CD34^+$ cells devoid of the T lymphoid markers CD2 and/or CD7 contained T lymphocyte progenitors with the ability to proliferate and differentiate into T lymphocytes when cultured on thymic stroma in vitro. However, apart from giving rise to T lymphocyte differentiation, both $CD34^+CD2^+$ and $CD34^+CD7^+$ cells (and the other subsets studied) also contained progenitors able to differentiate into the myeloid lineage, as ascertained by detection of cells expressing CD13 and CD14 after culture (data not shown).

To examine whether any of the subsets of $CD34^+$ cells studied in bulk cultures were enriched for T lymphocyte precursors, limiting dilution analyses were performed. The precursor frequency of $CD34^+CD2^+$ cells was found to be

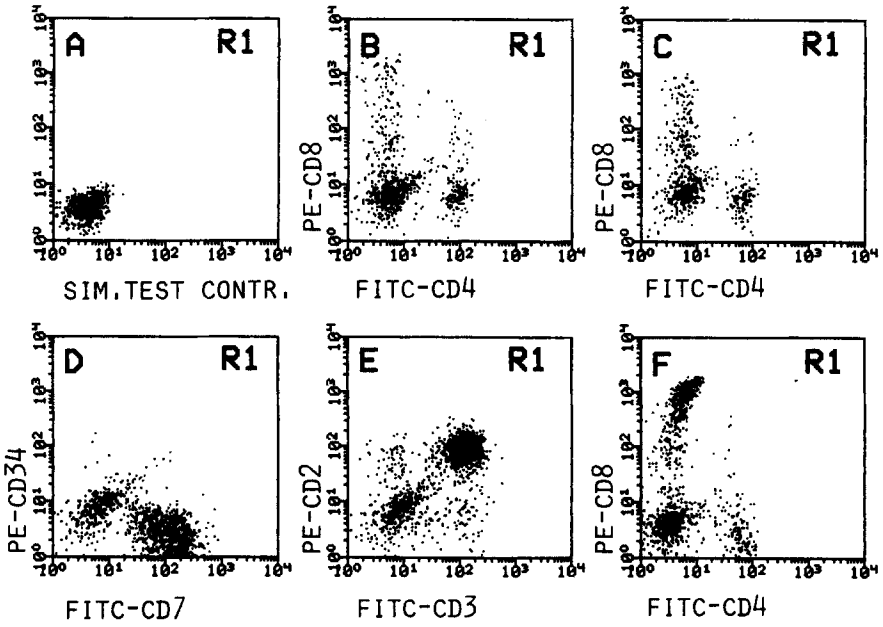

Figure 5. Phenotypic characterization of cultured CD34+ cells and subsets of CD34+ cells. (*B* and *C*) Representative two-color cytograms of cultured CD34+ cells. At day 14 (*B*) the presence of CD4+CD8+ cells are clearly detectable, whereas at day 28 (*C*) double-positive cells are hardly detectable. (*D–F*) Representative two-color cytograms of cultured CD34+CD7+ cells. Similar cytograms were obtained from cultured CD34+CD2+, CD34+CD2+CD7+, CD34+CD7−, CD34+CD2−, and CD34+CD2−CD7− cells (not shown). (*A*) Negative control.

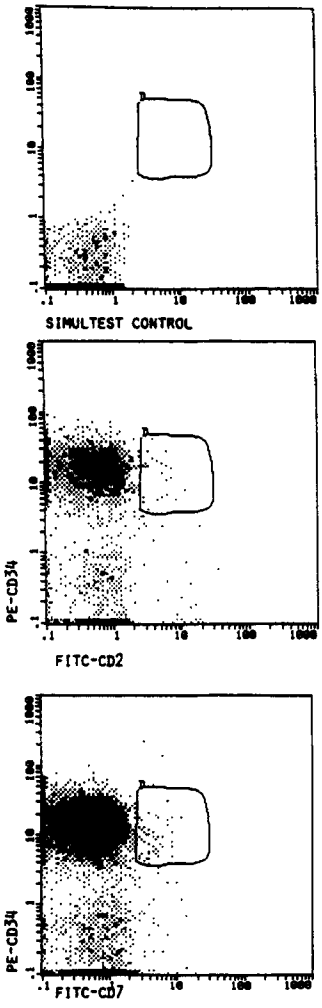

Figure 6. Characteristics of CD34+CD2+ and CD34+CD7+ cells. Representative two-color cytograms illustrating the presence of CD34+ cells coexpressing CD2 or CD7 (1–2%). The sort gates are also indicated.

1:13; CD34+CD2− cells, 1:18; CD34+CD7+ cells, 1:9; and CD34+CD7− cells, 1:8. Accordingly, none of the subset investigated were found to be enriched for T lymphocyte precursors. However, given the level of progenitors revealed by the limiting dilution analyses, contaminating mature T lymphocytes, <1% according to flow cytometry, cannot account for appearance of T lymphocytes after culture.

Discussion

T lymphopoiesis from human fetal hematopoietic stem cells has been demonstrated in vivo in the Thy SCID-hu mouse model (15–17). Furthermore, T lymphopoiesis from fetal human CD10+ bone marrow cells and from mouse hematopoietic stem cells has been achieved in vitro (24, 25). T lymphopoiesis from adult human prethymic CD34+ bone marrow cells has been demonstrated neither in vivo nor in vitro. The present study demonstrates that T lymphopoiesis from adult human CD34+ bone marrow cells can be accomplished in vitro. After culture of CD34+sCD3−CD4−CD8− cells for 14 and 28 d on allogeneic thymic stromal cells, cells of bone marrow donor origin with characteristics of lymphoid cells expressing the phenotype of mature T lymphocytes were demonstrated.

It was necessary to exclude the possibility that the purified CD34+ cells contained mature T lymphocytes, the expansion of which could account for the T lymphocytes demonstrated. We are confident that this was not the case. First, no mature T lymphocytes (sCD3+ cells) were detected by flow cytometric analyses of purified CD34+ cells. Contaminating cells were almost exclusively small agranular cells with the phenotype of mature B lymphocytes (CD34−CD19+CD37+) (19, 26). Second, culture of non-CD34+

bone marrow cells, which contained large numbers of mature T lymphocytes, did not result in any cell multiplication, nor did cultures of $CD4^+$ nor $CD8^+$ T lymphocytes. Third, $CD34^+$ cells did not proliferate after stimulation with irradiated allogeneic PBMC, PHA, and rIL-2. In addition, we have previously demonstrated lack of generation of cells expressing T lymphocyte–restricted markers after stimulation of purified $CD34^+$ cells with PHA and rIL-2 (27). This is in accordance with the finding that $CD7^+CD2^-CD3^-$ human thymic and bone marrow cells do not respond to PHA and/or rIL-2 (28, 29). Fourth, after depletion with mAbs against CD4CD8 and $CD19CD34^+$ cells still gave rise to T lymphocytes. Fifth, the finding of $CD4^+CD8^+$ cells cannot be explained by contamination of mature T lymphocytes among the purified $CD34^+$ cells. Last, limiting dilution analyses demonstrated T lymphocyte precursor frequency of 1:8–18 in sorted $CD34^+$ cells. Thus, outgrowth of rare contaminating mature T lymphocytes, <1% according to flow cytometry, cannot account for the appearance of $CD4^+$ and $CD8^+$ cells after culture.

We have presented evidence that when cultured on thymic stroma, $CD34^+$ human bone marrow cells proliferate and differentiate along the T lymphoid lineage. It might be argued that the single-positive $CD4^+$ and $CD8^+$ cells observed after culture could correspond to immature single-positive (i.e., $preCD4^+CD8^+$) cells and not mature $CD4^+$ and $CD8^+$ cells. In the presence of IL-7, highly purified human triple-negative thymocytes can proliferate and differentiate to express CD4 and/or $CD8(\alpha^+/\beta^-)$. These cells, which remain $CD3^-$, are thought to precede the common cortical double-positive thymocytes that are $CD3^{low}CD4^+$ $CD8\alpha^+/\beta^+$ (29). In our study, the demonstration of $CD3^{bright}$TCR α/β^+ cells cannot be accounted for by the low number of $CD4^+CD8^+$ cells detected. Accordingly, most, if not all, $CD4^+$ and $CD8^+$ cells have to be $CD3^+$TCR α/β^+. Based on this, we are entitled to state that $CD34^+$ adult human bone marrow cells when cultured on thymic stroma possess the ability to proliferate and differentiate into mature $CD4^+CD3^+$TCR α/β^+ and $CD8^+CD3^+$TCR α/β^+ T lymphocytes. The functional ability of these $CD4^+$ and $CD8^+$ T lymphocytes will be addressed in ongoing studies.

It is generally accepted that development of T lymphocytes from early thymocytes ($sCD3^-CD4^-CD8^-$) involves a stage of $CD4^+CD8^+$ cells preceding the appearance of $sCD3^+CD4^+$ and $sCD3^+CD8^+$ T lymphocytes. $CD4^+CD8^+$ cells were present at day 14, but they were hardly detectable at day 28. This suggests that maturation to $sCD3^+CD4^+$ and $sCD3^+CD8^+$ lymphocytes took place through an intermediate stage of $CD4^+CD8^+$ cells in this in vitro assay as well.

We found that a large majority of T lymphocytes developing when adult human $CD34^+$ bone marrow cells were cultured on thymic stroma expressed TCR α/β. This is in contrast to previous reports that postnatal $sCD3^-CD4^-CD8^-$ thymic T lymphocyte precursors develop nearly exclusively into TCR γ/δ^+ cells in cultures without feeder cells (30). However, fetal $sCD3^-CD4^-CD8^-$ thymic T lymphocyte precursors develop into TCR α/β^+ cells (31, 32). Human T lymphocytes found in the peripheral blood of SCID-hu (fetal thymus and liver) mice express TCR α/β (33). Finally, hematopoietic stem cells in the G_0 phase from adult mice differentiate into T lymphocytes expressing TCR β chains in an in vitro organ culture assay with fetal thymic tissue (25). Taken together with the present results, this suggests that thymic stroma is necessary for the development of T lymphocytes expressing TCR α/β from postnatal progenitors. This notion is further supported by another report showing that thymic stromal cells are required for the development of both α/β and γ/δ T lymphocytes from postnatal $CD7^+CD45^+CD1^-CD2^-CD3^-CD4^-CD8^-$ early thymocytes (34). In previous studies where T lymphopoiesis from hematopoietic stem cells was achieved, the three-dimensional structure of the thymic microenvironment was maintained (16, 17, 25). Our studies confirm the necessity of thymic stroma, but the maintenance of the three-dimensional structure is not always necessary. In the absence of thymic stroma we were not able to obtain T lymphocyte differentiation when $CD34^+$ cells were cultured in the presence of several combinations of growth factors (rIL-1, rIL-2, rIL-3, rIL-7, GM-CSF, and rmc-*kit* ligand) or supernatant from thymic stromal cells (27, Steen, R., L. Mørkrid, G. E. Tjønnfjord, and T. Egeland, manuscript submitted for publication, and unpublished observations).

Human $CD34^+$ cells are phenotypically heterogeneous with subsets coexpressing lineage-specific markers. CD7 and CD2 are believed to be expressed by prethymic pre-T cells (7, 11, 12, 31, 35, 36). Therefore, the ability of $CD34^+CD2^+$, $CD34^+CD7^+$, $CD34^+CD2^+CD7^+$, $CD34^+CD2^-$, $CD34^+CD7^-$, and $CD34^+CD2^-CD7^-$ bone marrow cells to differentiate into T lymphocytes was studied in the same assay. Progenitors able to support T lymphophoiesis in vitro were found in all of these subsets. Furthermore, limiting dilution analyses did not demonstrate enrichment of T lymphocyte precursors in any of these subsets. The inability to demonstrate an enrichment for T lymphocyte precursors among different subsets of $CD34^+$ cells may reflect thymus-dependent negative selection and apoptosis. This may be the rate-limiting step even in this in vitro assay and may preclude the finding of a possible difference in T lymphocyte precursor frequency of the subsets studied. In vivo, surface molecules like leukocyte endothelial cell adhesion molecule 1 (LECAM-1) known to be expressed by $CD34^+CD2^+CD7^+$ bone marrow cells, may be involved in homing of these cells to thymus (12). The concept of homing is bypassed in this in vitro assay, and this as well as other culture-associated phenomena may account for our inability to demonstrate a difference in precursor frequency between different subsets of $CD34^+$ cells.

$CD34^+CD7^+sCD3^-CD4^-CD8^-$ and $CD34^+CD2^+$ $sCD3^-CD4^-CD8^-$ cells as well as the other subsets studied were multipotent since they were capable of giving rise to myeloid progeny. This corresponds with the demonstration of differentiation into nonlymphoid lineages of human thymic

$CD7^+$ $sCD3^-CD4^-CD8^-$ T lymphocyte precursors by IL-3, Epo, G-CSF, M-CSF, GM-CSF, and factors produced by thymic epithelial (TE) cells (37).

Both purified native and rc-*kit* ligand show synergistic actions with other factors on whole bone marrow and isolated $CD34^+$ human cells such as IL-3, IL-1, IL-6, G-CSF, GM-CSF, and Epo in various assays (38–40, and Steen, R., L. Mørkrid, G. E. Tjønnfjord, and T. Egeland, manuscript submitted for publication). The results presented here show that rmc-*kit* ligand strongly potentiated the cell multiplication in this assay without changing the phenotypic profile of the progeny. This supports the view that the synergistic effect of rmc-*kit* ligand on growth factors also includes growth factors neccessary for T lymphopoiesis.

In the present study we show that purified $CD34^+$ $sCD3^-CD4^-CD8^-$ bone marrow cells from healthy adults contained progenitors with the ability to proliferate and differentiate into $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes expressing TCR α/β in vitro. The presence of thymic stromal cells seemed to be a prerequisite for T lymphopoiesis to be accomplished from bone marrow progenitors. T lymphocyte progenitors were found both among $CD34^+$ cells expressing CD2 or CD7 surface markers and $CD34^+$ cells devoid of these markers. Furthermore, c-*kit* ligand enhanced T lymphopoiesis.

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