Long-Term Erythropoiesis from Constant Numbers of CD34+ Cells in Serum-free Cultures Initiated with Highly Purified Progenitor Cells from Human Bone Marrow

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Summary

To directly study the biological properties of purified hematopoietic colony-forming cell precursors, cells with a $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype were purified from human bone marrow using density separation and fluorescence-activated cell sorting, and were cultured in serum-free culture medium supplemented with various cytokines. In the presence of interleukin 3 (IL-3), IL-6, erythropoietin, and mast cell growth factor (a c-*kit* ligand), cell numbers increased approximately 10^6-fold over a period of 4 wk, and the percentage of cells that expressed transferrin receptors (CD71) increased from <0.1% at day 0 to >99% at day 14. Interestingly, the absolute number of $CD34^+$ $CD71^{lo}$ cells did not change during culture. When $CD34^+$ $CD71^{lo}$ cells were sorted from expanded cultures and recultured, extensive cell production was repeated, again without significant changes in the absolute number of cells with the $CD34^+$ $CD71^{lo}$ phenotype that were used to initiate the (sub)cultures. These results document that primitive hematopoietic cells can generate progeny without an apparent decrease in the size of a precursor cell pool.

The formation of blood cells in the bone marrow is the result of proliferation and differentiation of hematopoietic cells that differ widely in their proliferative and differentiation potential. Lineage-restricted progenitors as well as multi-lineage progenitors are all thought to be derived from a more primitive population of hematopoietic cells, the majority of which are considered to be quiescent during normal steady-state hematopoiesis (1). The study of such cells in humans has been hampered by the lack of quantitative assays for totipotent human hematopoietic cells, their expected low frequency among nucleated bone marrow cells, and difficulties in obtaining sufficient bone marrow cells for their isolation in large numbers. As a result, major questions regarding the mechanisms of stem cell maintenance, activation, and differentiation remain to be answered, despite an increasing number of indirect studies, implying the role of various cytokines and cell-cell interactions in these processes (1–4).

The identification of the product of the Steel gene (Steel factor; mast cell growth factor, MGF;[1] c-*kit* ligand; stem cell factor) as an important regulatory molecule for immature hematopoietic cells (5–7) has increased interest in the possibility of expanding hematopoietic cells in vitro for a variety of clinical applications. Indeed, several recent papers have described studies with purified hematopoietic cells from mice (8, 9) and humans (2, 10, 11) that were cultured in the presence of Steel factor and other growth factors. All these studies have documented large increases in the production of progenitor cells, but little or no increase in the numbers of the most primitive hematopoietic cells. These results suggest that such cells are either unresponsive to Steel factor or display unusual properties that concomitantly prevent expansion and avoid depletion. We describe here that primitive hematopoietic cells from human bone marrow that participate in extensive and sustained production of cells are themselves maintained at constant numbers in serum-free cultures containing Steel factor, IL-6, IL-3, and erythropoietin (Epo).

Materials and Methods

mAbs. IgG1 mAbs specific for CD34 (8G12 [12]), CD45RA (8d2 [13]), CD71 (0KT9 [14]), and glycophorin A (10F7MN [15]) were purified from hybridoma tissue culture supernatant using protein A or protein G affinity chromatography. 8G12 mAbs were labeled with cyanine 5-succinimidylester as described (16). Cy5 was kindly provided by Dr. A. S. Waggoner (Carnegie Mellon University, Pittsburgh, PA). 0KT9 mAbs were labeled with FITC (F-7250;

[1] *Abbreviations used in this paper:* Epo, erythropoietin; LTC-IC, long-term culture initiating cell; MGF, mast cell growth factor.

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Sigma Chemical Co., St. Louis, MO) at a fluorescein/protein ratio of 5.0. 8d2 and 10F7MN mAbs were labeled with RPE by mixing with, respectively, monoclonal IgG1 anti-RPE antibody 1D3, $F(ab')_2$ fragments of rat anti-mouse IgG1, and RPE at a molecular ratio of 1:1:2:4 as described (13). 8G12-RPE was kindly provided by Drs. A. Jackson and L. Terstappen (Becton Dickinson & Co., San Jose, CA).

Bone Marrow Cells. Heparinized bone marrow was obtained from informed and consenting individuals donating marrow for allogeneic transplantation. Low-density cells (<1.077 g/cm^3) were isolated using Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden), resuspended in Iscove's medium with 50% FCS and kept overnight at 4°C. In some experiments, suspensions of bone marrow cells (1–3 × 10^{10} cells total) retrieved from vertical bodies of organ donors were also used (kindly provided by Dr. M. Strong, North West Tissue Center, Seattle, WA). These cells were aliquoted and frozen in Iscove's medium containing 2.5% human serum albumin and 7.5% DMSO either after density separation using Percoll as described (17) or without further manipulation. Vials of frozen marrow cells were rapidly thawed and slowly diluted with Iscove's medium containing 0.1 mg/ml DNA-se (type II-S, D4513; Sigma Chemical Co.) and 2% FCS. Cells were then washed once and resuspended in Hank's hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) for subsequent staining.

Three-Color Cell Analysis and Sorting. Bone marrow cells (10^7/ml) were incubated simultaneously with 8G12-Cy5, 8d2-RPE complexes, and 0KT9-FITC at, respectively, 20, 4 and 1 μg/ml for 30 min at 4°C. Controls consisted of single-stained suspensions and three-color staining with anti-TNP-RPE complexes instead of 8d2-RPE complexes. Cells were washed twice in HFN and resuspended in HFN containing 2 μg/ml propidium iodide (p-5264; Sigma Chemical Co.) before sorting. Throughout the procedure, cells were kept at 4°C. Cells were sorted on a FACStar Plus® (Becton Dickinson & Co.) equipped with a 5-W argon and a 30-mW helium neon laser. Specific fluorescence of FITC, RPE, PI, and Cy5 excited at 488 nm (0.4 W) and 633 nm (30 mW), as well as forward and orthogonal light scatter signals, were used to establish sort windows. Positivity for each probe was defined as fluorescence that exceeded 99% of controls. Cells were collected in serum-free medium. For phenotypic characterization of cells upon culture, suspensions of cells were labeled with 8G12-Cy5, 10F7MN-RPE complexes, and 0KT9-FITC. For other resorting experiments cultured cells were labeled with 8G12-RPE and 0KT9-FITC.

Serum-free Cultures. The serum-free medium used in this study was a modification of serum-free medium described for murine erythroid progenitor cells (18). IMDM powder, (17633; Sigma Chemical Co.) was stored at −20°C in 0.177-g aliquots. Single aliquots were dissolved in 9.8 ml double-distilled water plus 0.2 ml 7% (wt/vol) $NaHCO_3$. A volume of 7.57 ml of this solution was used to prepare 10 ml complete serum-free medium by adding 2.3 ml of an albumin/insulin/transferrin/pen/strep mixture (see below), 50 μl of 10 mM 2-ME, and 80 μl of low density lipoproteins (5 mg/ml, L-2139; Sigma Chemical Co.). Complete serum-free medium was filter sterilized (0.2 μm) before use and prepared fresh from powder for each experiment. The ingredients of the albumin/insulin/transferrin/pen/strep mixture were prepared as follows. A solution of 20% (wt/vol) of BSA (fraction V, A4503; Sigma Chemical Co.) in double-distilled water was deionized using 60 g of AG-501-X8(D) resin (Bio-Rad Laboratories, Richmond, CA), filtered and mixed with an equal volume of double-strength IMDM (plus bicarbonate) to give a 10% (wt/vol) stock solution, which was filter sterilized, and kept at −20°C in aliquots. Insulin (40205; Collaborative Research, Bedford, MA) was stored in aliquots at 1 mg/ml in IMDM at −20°C. Human transferrin (iron-saturated, 82-343; ICN ImmunoBiological, Costa Mesa, CA) was stored in aliquots at 20 mg/ml in IMDM at −20°C. A stock solution of penicillin (10^4 U/ml) and streptomycin (5 mg/ml) was also kept at −20°C in aliquots (pen/strep). To 20 ml of the 10% albumin solution, 1 ml of pen/strep stock solution, 1 ml of insulin stock solution, and 1 ml of transferrin stock solution were added to obtain BSA/insulin/transferrin/pen/strep mixture. Aliquots of 2.3 ml of this mixture were kept at −20°C to prepare serum-free medium as described above. The final concentrations of various ingredients in serum-free medium were 2% BSA, 10 μg/ml insulin, 200 μg/ml transferrin, 40 μg/ml low density lipoproteins, 5 × 10^{-5} M 2-ME, and pen/strep (at, respectively, 10^2 U and 50 μg/ml). It should be noted that this medium is not truly serum-free in that some contamination of the BSA and transferrin preparations with serum proteins is to be expected.

Cultures were initiated in 24-well tissue culture plates (Nunc, Kamstrup, Denmark) in serum-free medium at a concentration of 10^4 cells/ml at 37°C in a water-saturated atmosphere of 5% CO_2 in air. The medium was supplemented with (combinations of) the following recombinant human growth factors: IL-6, IL-3, MGF, and Epo. IL-6 and MGF were kindly provided by Dr. D. E. Williams (Immunex, Seattle, WA). Tissue culture supernatants of transfected Cos cells containing human IL-3 at 20 μg/ml and human Epo at 150 U/ml were provided by colleagues in our laboratory. The final concentrations of the various cytokines used in serum-free cultures were 20 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml MGF, and 3 U/ml Epo. Cultures were diluted when the total number of cells per well reached confluency at ~10^6 cells/ml. Every 5–7 d, cultures were subjected to half medium change with fresh medium and growth factors. After variable time intervals the cells of each culture were harvested by pipetting, spun, and resuspended in a small measured volume of serum-free medium. The numbers of viable and dead cells were counted in a hemocytometer using trypan blue before subsequent studies. For some experiments purified cells were cultured in Iscove's medium alone or in medium containing 10% FCS (A-1111-L; HyClone Labs, Logan, UT).

Progenitor Cell Assays. Clonogenic and long-term culture assays were performed as described previously (19), with the exception that 20 ng/ml GM-CSF/IL-3 fusion protein (20) and 50 ng/ml human MGF were added to methylcellulose cultures for assays of clonogenic cells with purified cells. The total number of clonogenic cells present in pooled adherent and nonadherent cell fractions of 5-wk-old long-term cultures using regular methylcellulose cultures was used to obtain an estimate of long-term culture initiating cell (LTC-IC) frequency (21).

Thymidine Incorporation Assays. Thymidine incorporation assays were performed in 96-well round-bottomed microtiter plates (Nunc). Purified $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ bone marrow cells (1,000/well) were cultured for 8 d in 200 μl serum-free medium supplemented with and without growth factors at the concentrations indicated above. Cells were cultured for the last 6 h in the presence of 10 μM [^{3}H]thymidine (2 Ci/mmol, Net-027A; New England Nuclear, Boston, MA). Cells were then harvested on filter mats and counted using a scintillation counter (LKB Instruments, Turku, Finland).

Results

Resolution of $CD34^+$ Cells into Functionally Distinct Subpopulations by Correlated CD45RA and CD71 (Transferrin Receptor) Expression. The small fraction (1–3% of cells) of

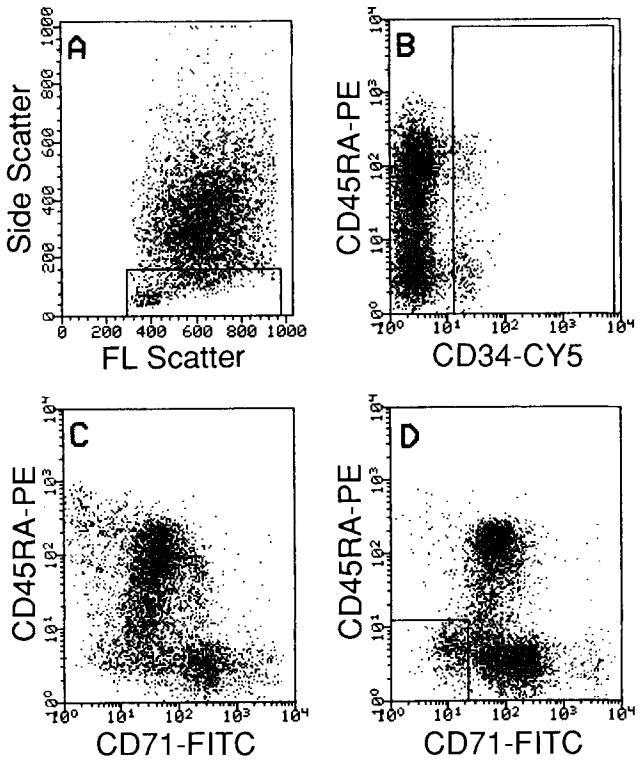

Figure 1. Selection of CD34$^+$ CD45RA^{lo} CD71^{lo} cells from organ donor bone marrow by flow cytometry. (*A*) Selection of the light scatter window used in all subsequent analyses and sorts, (*B*) selection of CD34$^+$ cells, (*C*) correlated expression of CD45RA and CD71 of cells in scatter window, and (*D*) correlated expression of CD45RA and CD71 of CD34$^+$ cells. The boxed area in *D* indicates the criteria used for selection of CD34$^+$ CD45RA^{lo} CD71^{lo} cells among CD34$^+$ cells. All dot plots are derived from low-density cells that are propidium iodide (PI) negative. Fluorescence is plotted on a log scale.

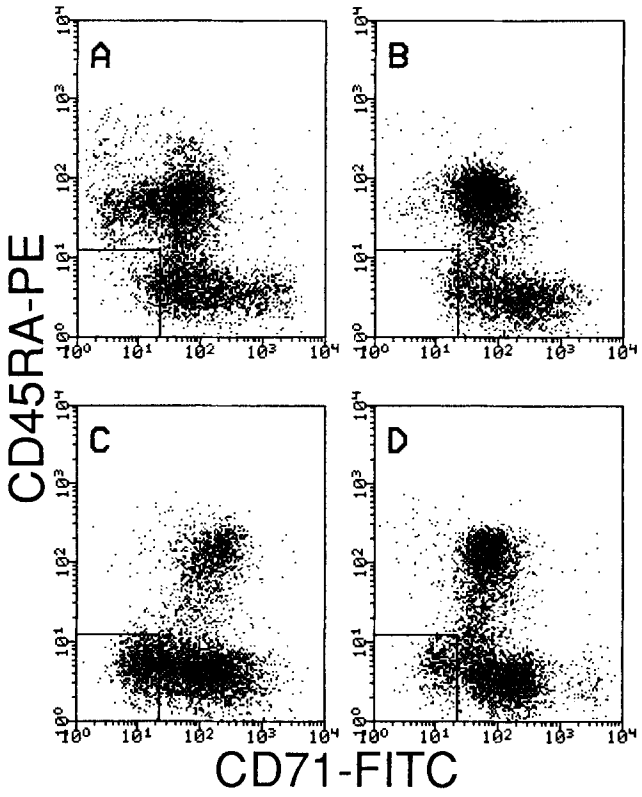

Figure 2. Correlated expression of CD45RA and CD71 on CD34$^+$ bone marrow cells. Marrow aspirates from two different donors (*A* and *B*) and bone marrow cells from two different organ donors (*C* and *D*) were analyzed. Cells were gated with respect to light scatter properties and CD34 expression as shown by the boxed areas in Fig. 1, *A* and *B*. The windows used to sort CD34$^+$ CD45RA^{lo} CD71^{lo} cells are represented by the boxed areas in *A–D*. The thresholds set to discriminate between CD71^{lo} and CD71$^+$ and CD45RA^{lo} and CD45RA$^+$ cells (Table 1) are those used to create boxed areas in *A–D*.

low-density cells in bone marrow that expresses CD34 can be subdivided into distinct subpopulations using antibodies against CD45RA and CD71, as is shown in Figs. 1 and 2. Although the relative size of the CD34$^+$ subpopulations defined by CD45RA and CD71 varied to some extent between donors and between bone marrow aspirates (Fig. 2, *A* and *B*) and bone marrow cells from vertebral bodies of cadaveric organ donors (Fig. 2, *C* and *D*), the overall resolution of CD34$^+$ cells using this three-color staining strategy was very similar and reproducible. Note that the proportion of CD34$^+$ cells with a CD34$^+$ CD45RA^{lo} CD71^{lo} phenotype was higher among gated bone marrow cells from organ donors (18.4% and 8.0%, respectively in Fig. 2, *C* and *D*) as compared with marrow aspirates (respectively, 4.5% and 2.6% in Fig. 2, *A* and *B*). The distribution of CD34$^+$ cells detected by clonogenic and long-term culture assays over the subpopulations defined by correlated expression of CD45RA and CD71 was studied in more detail. The results of these experiments are summarized in Table 1. Despite considerable variation in the results of these biological assays, a number of consistent findings were obtained. Cells capable of initiating long-term cultures (LTC-IC) were highly enriched in CD34$^+$ CD45RA^{lo} CD71^{lo} cell fractions, whereas CFU-GM and BFU-E were enriched in CD34$^+$ CD45RA$^+$ CD71$^+$ and CD34$^+$ CD45RA^{lo} CD71$^+$ cell fractions, respectively. This relation between phenotype and function was observed irrespective of whether marrow aspirates or vertebral body bone marrow cells were studied.

Survival of CD34$^+$ CD45RA^{lo} CD71^{lo} Cells in Serum-free and Serum-containing Tissue Culture Media. The survival of purified CD34$^+$ CD45RA^{lo} CD71^{lo} cells in Iscove's medium containing 10% FCS (22) and serum-free tissue culture medium is shown in Fig. 3. Without addition of exogenous growth factors the number of viable cells decreased much more rapidly in serum-containing medium than in serum-free medium. In view of this finding, further studies on the functional properties of purified CD34$^+$ CD45RA^{lo} CD71^{lo} cells were performed in serum-free culture medium.

Induction of Erythropoiesis from CD34$^+$ CD45RA^{lo} CD71^{lo} Cells. The effect of IL-6, IL-3, MGF, and Epo in serum-free cultures of CD34$^+$ CD45RA^{lo} CD71^{lo} cells from previously frozen bone marrow cells was studied using a [^{3}H]thymi-

Table 1. *Correlated Expression of CD71 and CD45RA Allows Separation of Functional Subpopulations of CD34⁺ Cells*

Exp.	Fraction Sorted	Percent* sorted	BFU-E Frequency‡ (per 10^5)	BFU-E Enrichment	BFU-E Recovery	CFU-GM Frequency‡ (per 10^5)	CFU-GM Enrichment	CFU-GM Recovery	LTC-IC Frequency‡ (per 2×10^6)	LTC-IC Enrichment	LTC-IC Recovery
	Bone marrow aspirates:										
1	Unsorted, stained	–	230	1	100	42	1	100	30	1§	100
	CD34⁺ CD45RA^{lo} CD71^{lo}	0.3	1,130	4.9	1.5	500	12	3.6	93,000	3,100§	930
	CD34⁺ CD45RA^{lo} CD71⁺	0.8	22,630	98	78	2,250	54	43	12,500	417	334
	CD34⁺ CD45RA⁺	1.1	750	3.2	3.5	750	18	20	0	0	0
2	Unsorted, stained	–	270	1	100	380	1	100	300	1	100
	CD34⁺ CD45RA^{lo} CD71^{lo}	0.23	1,500	5.6	1.3	2,750	7.2	1.4	96,500	322	64
	CD34⁺ CD45RA^{lo} CD71⁺	0.54	60,500	224	112	11,130	29	15	21,250	71	36
	CD34⁺ CD34RA⁺	0.8	500	1.9	1.5	31,250	82	66	11,500	38.6	30
3	Sorted, stained	41.0	140	1	100	330	1	100	40	1§	100
	CD34⁺ CD45RA^{lo} CD71^{lo}	0.6	0	0	0	250	0.8	0.5	70,250	1,756§	1,053
	CD34⁺ CD45RA^{lo} CD71⁺	2.2	10,130	72	158	4,000	12	26	19,500	488	1,014
	CD34⁺ CD45RA⁺	3.2	2,250	16	51	16,500	50	160	7,500	188	602
	Organ donor bone marrow:										
4	Sorted, stained	62.2	2,060	1	100	660	1	100	13,300	1	100
	CD34⁺ CD45RA^{lo} CD71^{lo}	3.1	660	0.3	0.9	1,670	2.5	7.8	368,000	28	87
	CD34⁺ CD45RA^{lo} CD71⁺	8.0	22,340	11	88	12,360	19	152	103,400	7.8	62
	CD34⁺ CD45RA⁺	2.5	0	0	0	16,300	25	63	3,600	0.3	0.8
5	Sorted, stained	42.3	1,420	1	100	600	1	100	5,300	1	100
	CD34⁺ CD45RA^{lo} CD71^{lo}	0.3	1,130	0.8	0.2	660	1.1	0.3	200,000	38	11
	CD34⁺ CD45RA^{lo} CD71⁺	2.7	17,800	13	35	9,070	15	41	100,600	19	51
	CD34⁺ CD45RA⁺	2.5	0	0	0	14,500	24	60	0	0	0

* The percentage of low-density, PI-negative cells in the light scatter window (Fig. 1*A*, sorted stained) or the percentage of cells with the indicated phenotype within these gates.
‡ The number of colonies per 10^5 cells (clonogenic assays) or 2×10^6 cells/dish (LTC-IC assays). Results for sorted cell fractions (fewer cells plated) were calculated from actual colony counts for comparisons. The number of colonies present in 5-wk-old long-term cultures per 2×10^6 cells plated (data shown) should be divided by four to derive at a more accurate estimate of the actual LTC-IC frequency (21).
§ The high enrichment/recovery of LTC-IC in these experiments probably reflects removal of inhibitory cells present in unseparated marrow cells.

dine incorporation assay (Table 2). Maximal stimulation was obtained if all four growth factors were present, and the effect of these culture conditions on purified CD34⁺ CD45RA^{lo} CD71^{lo} cells were studied in more detail. The total number of cells in such cultures increased rapidly and exponentially for a period of up to 20 d (Fig. 4). It was calculated that for each CD34⁺ CD45RA^{lo} CD71^{lo} cell, a total of 0.9 and 3.3×10^6 cells was recovered for, respectively, BM1 and BM2 at day 30 and many thousand-fold more at day 50 (Fig. 4). This large increase in cell numbers was accompanied by marked changes in the overall cell surface phenotype of the cultured cells, as is illustrated for four time points in Fig. 5. After 3 d in culture, the majority of the cells (99%) were still CD34⁺, but some cells coexpressed CD71. At day 6 the majority of the cells were CD71⁺ and about half the cells were CD34⁻. Practically all cells (99%) were CD71⁺ and CD34⁻ by day 9, and at day 14 the majority of cells expressed glycophorin, indicative of a relatively late erythroid differentiation stage (Fig. 5). At day 10 most of the cells were erythroblasts and (pro) normoblasts with a prominent Golgi and basophilic to grey cytoplasm upon staining with Giemsa.

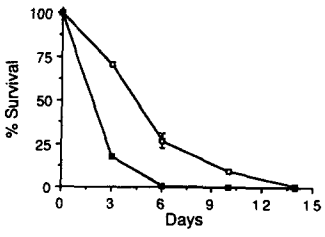

Figure 3. Survival of purified hematopoietic cells in medium with and without FCS. Cultures were initiated with FACS®-purified CD34⁺ CD45RA^{lo} CD71^{lo} bone marrow cells from two different organ donors in serum-free medium (□) and Iscove's medium containing 10% FCS (■). The percentage viable cells (mean ± SD) was determined using trypan blue.

Table 2. *Combinations of Growth Factors Induce Maximal Stimulation of Purified Hematopoietic Cells*

Condition	BM1	BM2	BM3
Serum-free medium only	301 ± 232	100 ± 0	85 ± 12
IL-3	1,274 ± 263	784 ± 128	1,686 ± 174
IL-6	304 ± 232	86 ± 8	106 ± 16
MGF	813 ± 407	169 ± 35	139 ± 30
Epo	91 ± 18	85 ± 7	88 ± 17
IL-6 + MGF	350 ± 31	222 ± 23	296 ± 7
IL-3 + MGF	9,573 ± 4,030	3,118 ± 297	6,194 ± 425
MGF + Epo	410 ± 92	222 ± 45	266 ± 94
IL-3 + IL-6 + MGF	8,209 ± 1,130	5,109 ± 220	6,160 ± 1,824
IL-6 + MGF + Epo	920 ± 228	247 ± 33	946 ± 314
IL-3 + MGF + Epo	38,704 ± 17,235	7,163 ± 1,205	49,162 ± 7,724
IL-3 + IL-6 + MGF + Epo	56,264 ± 12,491	8,654 ± 4,500	70,055 ± 18,703

Thymidine incorporation (mean ± SD) of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ bone marrow cells (1,000 cells/well) cultured for 8 d in serum-free culture medium supplemented with the indicated growth factors.

Many mitotic figures and occasional cells with (pro) myelocyte morphology were seen at this stage. At day 20 most cells had morphological features of (pro) normoblast with distinct vacuoles in their cytoplasm that showed various degrees of hemoglobinization. Fully hemoglobinized enucleated red cells were not observed at any point in time, and it appeared that cells accumulated at a late normoblast-reticuloblast stage.

Maintenance of $CD34^+$ $CD71^{lo}$ Cells in Cultures Supplemented with Recombinant Growth Factors. The phenotype and the number of cells produced in serum-free culture medium supplemented with IL-3, IL-6, MGF, and Epo was analyzed in relation to the number of the purified $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells that were used to initiate the cultures. In eight separate experiments, it was found that the absolute number of $CD34^+$ $CD71^{lo}$ cells present in the cultures between day 9 and 13 remained at input values (1.26 ± 0.4; mean ± SD), whereas the total number of cells in such cultures had increased between 16 and 4,700 times input values. This maintenance of $CD34^+$ $CD71^{lo}$ cells in rapidly proliferating cultures was studied in more detail by resorting of $CD34^+$ $CD71^{lo}$ cells from the cultures (Figs. 4 and 6). $CD34^+$ $CD71^{lo}$ cells present in 10-d cultures in which most of the cells were rapidly proliferating were sorted (Fig. 6 *A*, *box*) and used to initiate secondary cultures. Analysis of secondary cultures at day 19 revealed that cells with the $CD34^+$ $CD71^{lo}$ phenotype were still present at undiminished numbers. Such cells were sorted again (Fig. 6 *B*, *box*) and used to initiate tertiary cultures. This process was repeated two more times with very similar results in that on each occasion $CD34^+$ $CD71^{lo}$ cells, capable of initiating subsequent cultures, were identified at seeded cell numbers (Fig. 4). Concomitant production of cells and maintenance of $CD34^+$ $CD71^{lo}$ cells was observed over a period of 5–7 wk and a total of four rounds of sorting and initiation of new cultures. After this time the absolute number of $CD34^+$ $CD71^{lo}$ cells (of which ~50% were recovered upon each round of staining and sorting) became too low to continue the experiments (<1,000 $CD34^+$ $CD71^{lo}$ cells). In some experiments the number of colony-forming cells and LTC-IC present in the suspension cultures was measured. On each occasion the total number of clonogenic cells and LTC-IC present correlated with the total number of $CD34^+$ and $CD34^+$ $CD71^{lo}$ cells, respectively (data not shown), at ratios similar to those observed at the start of the culture (Table 1).

Discussion

In this report we have used a novel procedure to purify primitive hematopoietic cells from human bone marrow together with a serum-free culture system to study the biological properties of such cells in vitro. Cells with an immature $CD34^+$ $CD71^{lo}$ phenotype were found to repeatedly generate large numbers of erythroid progeny without decreasing in number. These observations are in agreement with several recent studies that have documented extensive Steel factor–dependent production of progenitor cells in the absence of expansion of more primitive hematopoietic cells (2, 8, 10, 23). Our finding that cells with a $CD34^+$ $CD71^{lo}$ phenotype participate in cell production while being maintained in number suggest that the modest effect on very primitive hematopoietic cells observed in these previous studies could be the result of inherent biological properties of such cells rather than their unresponsiveness to the added growth factors. The balance between production and maintenance of cells that was observed in our system could thus be indicative of a biological mechanism that limits expansion and yet avoids depletion of a primitive hematopoietic precursor cell pool. Several models for the observed $CD34^+$ $CD71^{lo}$ cell maintenance at a population, as well as at a single-cell level, are currently being considered. These models range from: (*a*) slow recruit-

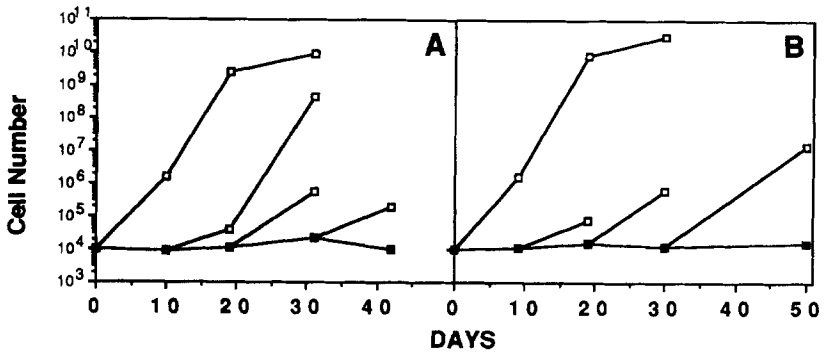

Figure 4. Maintenance of primitive hematopoietic cells that produce extensive progeny. Kinetics of total cells (□) and CD34+ CD71lo cells (■) in serum-free cultures supplemented with IL-3, IL-6, MGF, and Epo. Cultures were initiated with purified CD34+ CD45RAlo CD71lo cells from (previously frozen) bone marrow from two different organ donors (*A* and *B*). CD34+ CD71lo cells were resorted from the cultures at the indicated time intervals. The total number of cells produced was calculated from the number of cells produced in diluted cultures times the dilution factor; the number of CD34+ CD71lo cells present at each time interval was calculated from the percentage of cells with this phenotype times the total number of cells in each culture. The actual number of CD34+ CD71lo cells recovered after sorting was typically 50% of this calculated figure as a result of losses of cells upon staining and sorting.

ment of quiescent cells, and (*b*) balanced self-renewal versus loss of cells, to (*c*) asymmetrical divisions (24), and (*d*) combinations of the above. Although intrinsic factors in control of the overall size of a population of very primitive hematopoietic cells in adult bone marrow are in agreement with the difficulties encountered to expand the absolute number of these cells in vitro, such restrictions do not explain their generation in vivo. One possibility is that the production of these very primitive hematopoietic cells themselves is restricted to early stages of development (e.g., fetal life) and does not typically occur in adult bone marrow.

Our cell purification strategy was based on the use of FACS® to selectively enrich for cells capable of producing colonies after 5 wk in long-term culture (LTC-IC), arguably the best in vitro assay for human hematopoietic stem cells that is currently available (2, 19, 21, 25). Previous studies have shown that LTC-IC can be differentially enriched in CD71lo (26) and CD45RO+ (12) cell fractions of normal bone marrow. These previous observations were confirmed and extended in the studies described here using a three-color immunofluorescence staining procedure. LTC-IC were highly enriched in the CD34+ CD45RAlo CD71lo cell fraction, irrespective of whether bone marrow aspirates or bone marrow cells from vertebral bodies of (cadaver) organ donors were analyzed (Table 1). The finding that LTC-IC are enriched in the CD45RAlo cell fraction (this study) as well as in CD45RO+ cell fractions, as reported previously (12), is in agreement with the proposed mutual exclusive expression of these CD45 isoforms on cells that express CD34 (12). By sorting of cells on the basis of CD45RA, CD71, and CD34, the rare subpopulations of cells that express CD34 could be separated into functionally distinct subpopulations (Table 1). The possibility of

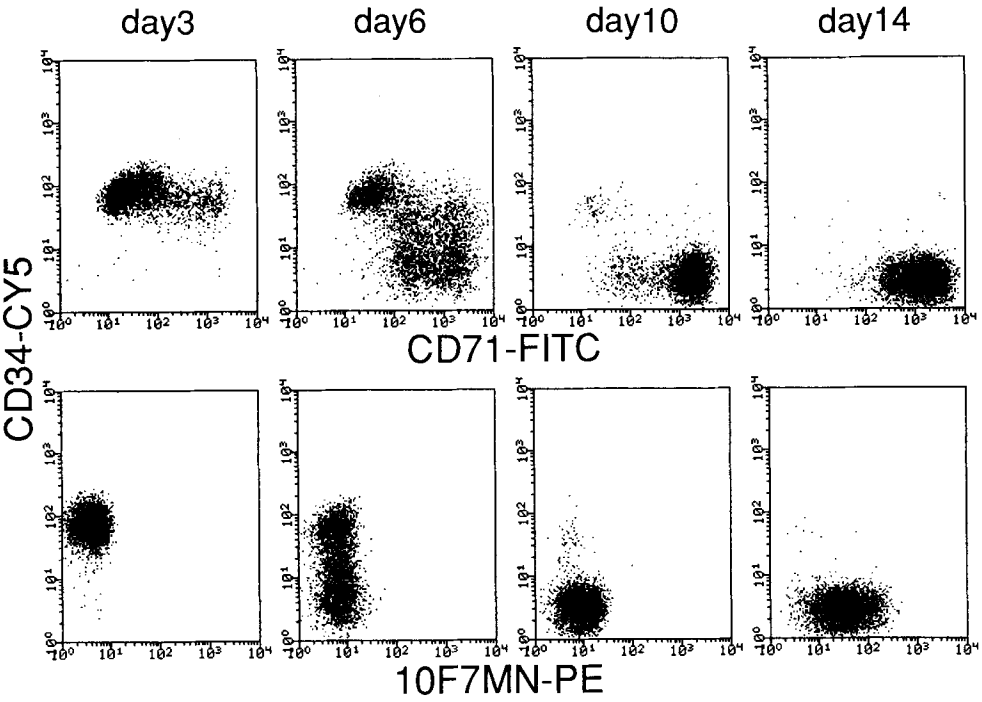

Figure 5. Erythroid differentiation of primitive hematopoietic cells in culture. Phenotypic characterization of cells in cultures initiated with purified CD34+ CD45RAlo CD71lo bone marrow cells at the indicated time intervals. Serum-free culture medium was supplemented with IL-3, IL-6, MGF, and Epo. Note that expression of CD71 precedes loss of CD34 but that loss of CD34 precedes expression of glycophorin A.

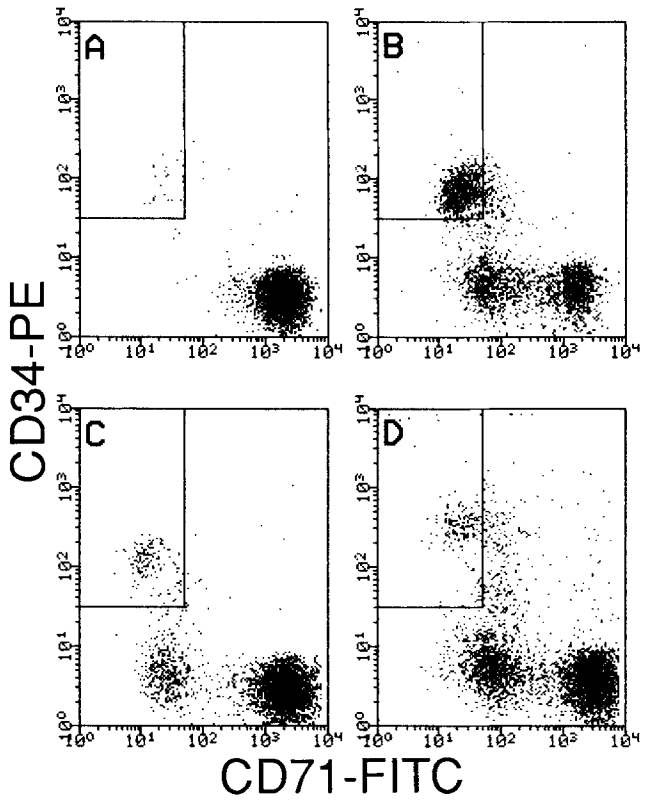

Figure 6. Sorting strategy used for maintenance of serum-free long-term cultures. Cells grown in serum-free culture medium supplemented with IL-3, IL-6, MGF, and Epo were analyzed on day 10 (*A*), day 19 (*B*), day 31 (*C*), and day 42 (*D*). $CD34^+$ $CD71^{lo}$ cells (*boxed area*) were in each case sorted and used to initiate subsequent cultures (e.g., cells sorted from boxed area in *A* on day 10 gave rise to cells with phenotype shown in *B* at day 19, etc.).

separating the majority of myeloid from erythroid progenitors, as well as from LTC-IC, may offer advantages over previously described purification procedures (19, 27–29).

The use of bone marrow cells from organ donors facilitated the studies described in this paper. Not only were these cells enriched for $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells and LTC-IC, as compared with cells from marrow aspirates (Fig. 2 and Table 1), but the availability of relatively large numbers of (frozen) cells allowed reproducible experimentation with immature hematopoietic cells at cell numbers that would have been difficult to obtain from marrow aspirates. Also, some consistent differences between normal marrow aspirates and bone marrow cells from organ donors were observed, in that marrow aspirates contained fewer low-density myeloid cells with an intermediate to high side scatter, and variable numbers of $CD34^+$ $CD45RA^+$ $CD71^{lo}$ cells that coexpressed CD19 and CD10 (30). The latter were rare or absent in cell suspensions from vertebral bodies of organ donors (results not shown). These differences between bone marrow cells from aspirates and vertebral bodies may be indicative of a functional compartmentalization of hematopoietic tissue in vivo, perhaps in combination with some selection of more mature (less adherent) cells in marrow aspirates.

Despite considerable enrichment of LTC-IC in a fraction of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, LTC-IC were not purified to homogeneity (Table 1). Two alternative explanations for this observation were considered. LTC-IC could indeed represent only a minor fraction of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, or, alternatively, inefficiencies in the LTC-IC assay system could underestimate the frequency of functionally similar or identical cells (e.g., similar to purified $Sca\text{-}1^+$, Lin^-, $Thy\text{-}1^{lo}$ cells in the mouse, of which <10% gave rise to colonies after 4 wk in murine long-term cultures [25]). Some interesting results were obtained when the functional in vitro properties of purified $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ were studied in more detail. To our surprise, we found that in the absence of growth factors $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells survived much better in serum-free culture medium than in serum-containing medium (Fig. 3). Furthermore, we observed that in serum-free cultures without stromal cells and with a much larger output of cells as compared with regular long-term cultures, cells with a $CD34^+$ $CD71^{lo}$ phenotype were maintained at constant levels (Fig. 4). These results indicate that the stromal cell requirement for hematopoietic cell maintenance in regular long-term cultures can be substituted as previously reported by cytokines (22), serum-free medium (31), or a combination of cytokines and serum-free medium, as reported here.

Apart from studies on the biology of primitive hematopoietic cells and further studies of erythropoiesis, the techniques and observations reported in this communication could have a number of practical applications in the development of gene transfer protocols and clinical protocols aimed at expansion of hematopoietic cells for diagnostic and therapeutic purposes. In view of such clinical applications, it will be important to obtain more information on the number and type of cells required by transplant recipients for short- and long-term hematopoietic reconstitution in relation to the number and type of cells that can now be produced in vitro.

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