JCI The Journal of Clinical Investigation

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J Clin Invest. 1990;86(3):932-941. https://doi.org/10.1172/JCI114795.

Research Article

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Cytokine-dependent Long-Term Culture of Highly Enriched Precursors of Hematopoietic Progenitor Cells from Human Bone Marrow

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Abstract

Human marrow cells positive for the CD34 antigen but not expressing HLA-DR, CD15, or CD71 antigens were isolated. In a liquid culture system supplemented with 48-hourly additions of recombinant interleukins IL-1α, IL-3, IL-6, or granulocyte/macrophage colony-stimulating factor (GM-CSF), these cells were capable of sustaining in vitro hematopoiesis for up to eight weeks. The establishment of an adherent cell layer was never observed. Cultures containing no exogenous cytokine produced clonogenic cells for only 1 wk. IL-1α and IL-6 were alone able to support hematopoiesis for 2 or 3 wk. Cells maintained with GM-CSF proliferated and contained assayable colony-forming cells for 3 or 4 wk, while maximal cellular expansion and generation of assayable progenitor cells occurred in the presence of IL-3 for 4–5 wk. When IL-3 was combined with IL-1α or IL-6, hematopoiesis was sustained for 8 wks. Basophil numbers were markedly increased in the presence of IL-3. These studies indicate that marrow subpopulations can sustain hematopoiesis in vitro in the presence of repeated additions of cytokines. We conclude that a major function of marrow adherent cells in long-term cultures is that of providing cytokines which promote the proliferation and differentiation of primitive hematopoietic cells. (*J. Clin. Invest.* 1990. 86:932–941.) Key words: cell sorting • differentiation • growth factors • proliferation • stem cells

Introduction

Human bone marrow is composed of heterogeneous populations of hematopoietic precursor and progenitor cells. A variety of in vitro clonal assay systems have been utilized to detect such progenitor cells (1–4). An extensive body of information is currently available which indicates the existence of more primitive hematopoietic cells termed stem cells (5–10). Stem cells have not only the ability to supply mature hematopoietic cells for prolonged periods of time but also are able to generate a continuous supply of additional stem cells, a process referred to as self-renewal (11, 12).

Recently, pure populations of murine hematopoietic stem cells have been isolated which are capable of rescuing lethally irradiated mice and reconstituting all hematopoietic and lymphoid lineages in survivors (5, 6). Detection and eventual isolation of the human equivalent of a pluripotent hematopoietic stem cell have been hindered due in part to the lack of availability in humans of a suitable assay system for this cell. Primitive hematopoietic progenitor cells capable in vitro of producing colonies composed of blast cells (CFU-B1) have, however, been detected in human umbilical cord blood and normal human bone marrow (7–10). These cells possess many of the features of stem cells in that they are capable of both self-renewal and commitment to various hematopoietic lineages. Preliminary phenotypic characterization of the human CFU-B1 has already been accomplished using monoclonal antibody staining and fluorescence-activated cell sorting (10). Since assessment of the self-renewal capacity of the CFU-B1 is dependent on the tedious replating of individual blast cell colonies, routine application of this assay has proven difficult.

An alternative in vitro methodology that has been utilized to study the behavior of primitive hematopoietic elements has been the long-term marrow culture system originally described by Dexter et al. (13). This system requires the preestablishment of a marrow adherent cell layer composed primarily of marrow stromal cells. This in vitro model has been utilized to study stem cell proliferation and differentiation under conditions where close-range interactions with marrow stromal elements are presumably required. Stromal cell-dependent hematopoiesis has been postulated to result from either direct contact between stromal and stem cells or progenitor cells (14), from the trophic effects of the stromal cell-derived extracellular matrix (15), or from stromal cell secretion of hematopoietic growth factors (16–18). Difficulties in defining the relative contributions of these proposed functions have resulted from lack of availability of both pure populations of individual stromal cell types and primitive hematopoietic cellular elements. Recently, a growing number of recombinant cytokines that affect hematopoietic cellular differentiation have become available (19). We have developed a suspension system to study the effects of these cytokines on purified populations of primitive hematopoietic progenitor cells. These long-term marrow culture systems have been established in the absence of a preestablished adherent cell layer. The information presented here provides new insight into factors that control the development of pluripotential hematopoietic cells.

Methods

Cell separation techniques. Bone marrow aspirates were collected from the posterior iliac crests of normal volunteers after informed consent was obtained according to the guidelines of the Human Investigation Committee of the Indiana University School of Medicine. Low-density mononuclear bone marrow (LDBM)[1] cells were obtained by density

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Received for publication 14 February 1990 and in revised form 1 May 1990.

J. Clin. Invest.

0021-9738/90/09/0932/10 $2.00
Volume 86, September 1990, 932–941

1. *Abbreviations used in this paper:* GM-CSF, granulocyte/macrophage colony-stimulating factor; HPP-CFC, high proliferative potential colony-forming cell; LDBM, low-density mononuclear bone marrow (cell).

centrifugation of the heparinized marrow over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) at 500 *g* for 25 min. LDBM cells were suspended in PBS-EDTA (PBS, pH 7.4, containing 5% FBS, 0.01% EDTA wt/vol, and 1.0 g/liter D-glucose) and injected into an elutriator system at 10°C at a rotor speed of 1,950 rpm using a JA-17 rotor and standard separation chamber (Beckman Instruments, Inc., Palo Alto, CA). A fraction of the LDBM eluted at a flow rate of 12–14 ml/min (FR 12–14), enriched for hematopoietic precursors, was collected as previously described (10).

Two- and three-color cell sorting. FR 12–14 cells were incubated with mouse monoclonal anti-HPCA-1 (CD34) of the IgG_1 subclass (Becton Dickinson Immunocytometry Systems, San Jose, CA), washed, and stained with Texas red-conjugated, subclass-specific goat anti-mouse IgG_1 (Southern Biotechnology Associates, Inc., Birmingham, AL). Cells were next incubated with mouse serum to block any unbound active sites on the second-step antibody. Cells were finally stained with phycoerythrin-conjugated mouse anti-HLA-DR either alone or in combination with FITC-conjugated CD33 (My9, Coulter Immunology, Hialeah, FL), CD15 (Leu-M1), or CD71 (transferrin receptor) (Becton Dickinson Immunocytometry Systems). CD15 is present on cells of the granulocytic and monocytic lineages, and an anti-CD15 monoclonal antibody was employed in the hope of eliminating these cellular components from the cell populations (20). CD71 is present on actively proliferating cells and an anti-CD71 antibody was utilized to separate actively proliferating cells from more quiescent marrow elements (21). Controls consisted of the corresponding isotype-matched, nonspecific myeloma proteins used in parallel with the staining monoclonal antibodies. Cells were stained at a concentration of 2×10^7/ml and washed after each step in 1% BSA in PBS. A temperature of 4°C was maintained throughout the procedure.

Immediately after staining, cells were sorted on a Coulter Epics 753 dual-laser flow cytometry system (Coulter Electronics, Inc., Hialeah, FL). Texas red was excited by 590-nm light emitted from a rhodamine 6G dye laser. FITC and phycoerythrin were excited using the 488 nm wavelength from a dedicated 6-W argon laser. Sorting windows were first established for forward angle light scatter (FALS) and Texas red fluorescence. Positivity for each fluorochrome was defined as fluorescence > 99% of that of the controls. Cells were next gated on the presence or absence of detectable HLA-DR-phycoerythrin and CD33-FITC, CD15-FITC, or CD71-FITC.

Hematopoietic growth factors. All cytokines were obtained from the Genzyme Corp., Boston, MA. Recombinant IL-1α and IL-3 each had a specific activity of 10^8 CFU/mg protein, while that of IL-6 was 10^7 and granulocyte/macrophage colony-stimulating factor (GM-CSF) 5×10^7 CFUc/mg protein.

Hematopoietic progenitor cell assays. Cells were suspended at various concentrations in 35-mm plastic tissue culture dishes (Costar Data Packaging, Cambridge, MA) containing 1 ml of 30% FBS, 5×10^{-5} M 2-mercaptoethanol, 1 U human purified erythropoietin (50 U/mg protein, Toyobo Co. Ltd., Osaka, Japan), 50 U GM-CSF, and 1.1% methylcellulose in Iscove's modified Dulbecco's medium. The cultures were incubated at 37°C in a 100% humidified atmosphere of 5% CO_2 in air. After 14 d, erythropoietic bursts (BFU-E), granulocyte-macrophage (CFU-GM), and mixed lineage (CFU-GEMM) colonies were scored in situ on an inverted microscope using standard criteria for their identification (10).

High proliferative potential colony-forming cell (HPP-CFC)–derived colonies were enumerated after 28 d in culture according to the recently published criteria of McNiece and co-workers (22). The human HPP-CFC–derived colony is a late-appearing, very large (0.5 mm or more in diameter) colony composed primarily of granulocytes with a lesser number of monocytes; cell numbers frequently exceed 50,000.

Cells removed from suspension cultures were assayed for CFU-megakaryocyte (CFU-MK) colonies using the serum-depleted method described in detail by Bruno et al. (23). 5×10^3 cells per point were suspended in a 1-ml serum-substituted fibrin clot with 100 U of IL-3 in 35-mm culture dishes and incubated at 37°C in a 100% humidified atmosphere containing 5% CO_2 in air. At 18–24 d, cultures were fixed in situ and stained using rabbit anti-human platelet glycoprotein antisera, and fluorescein-conjugated goat $F(ab')_2$-specific anti-rabbit IgG (Tago, Inc., Burlingame, CA) and megakaryocyte colonies were enumerated on a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York, NY). A positive colony was defined as a cluster of three or more fluorescent cells.

Long-term marrow cultures. Plastic 35-mm tissue culture dishes were seeded with 2×10^6 LDBM cells in 1 ml of Iscove's with 10% FBS and 2×10^{-5} M methylprednisolone. Cultures were incubated at 37°C in 100% humidified 5% CO_2 in air and fed weekly by total replacement of media. Stromal cells were confluent by 4–6 wk. The stromal cultures were then irradiated with 1,500 rad, the media were replaced, and the cultures were inoculated with 5×10^3 sorted bone marrow cells from autologous donors. The media in these cultures were removed at 7–10-d intervals and replaced with fresh media. Suspended, nonadherent cells were then counted and assayed for progenitors.

Long-term suspension cultures. Plastic 35-mm tissue culture dishes containing 1 ml of Iscove's with 10% FBS were inoculated with 5×10^3 cells obtained by sorting and incubated at 37°C in 100% humidified 5% CO_2 in air. At this time, and every 48 h thereafter, cultures received nothing (1% BSA/PBS), 2.5 U/ml IL-1α, 50 U/ml IL-3, 75 U/ml IL-6, 12.5 U/ml GM-CSF, or combinations of the above. At 7-d intervals, cultures were demi-depopulated by removal of one-half the culture volume which was replaced with fresh media. Cells in the harvested media were counted, transferred to slides for staining and morphological examination, and assayed for various progenitor cells.

Cell cycle analysis. DNA content and distribution was determined flow cytometrically using propidium iodide by a modified method of Vindelov et al. (24). Briefly, cells were suspended in equal volumes of staining buffer (0.1 mg/ml propidium + 0.6% NP-40 in PBS) and RNAse (2 mg/ml in PBS, Boehringer Mannheim Biochemicals, Indianapolis, IN) for a total volume of 500–800 μl, agitated, and incubated on ice for 30 min. Analysis was performed on a FACScan using the SFIT software program (Becton Dickinson Immunocytometry Systems) for determination of G, S, and G_2 + M fractions; 5×10^3 cells were analyzed per determination.

Results

$CD34^+DR^+$ and $CD34^+DR^-$ marrow cells were seeded at a concentration of 5×10^3 cells/ml onto preestablished, irradiated, autologous marrow adherent cell layers. The $CD34^+DR^+$ population produced the highest number of nonadherent cells (Fig. 1 *a*) and assayable CFU-GM (Fig. 1 *b*) after 10 days of culture. All subsequent data revealed, however, that the DR-negative cells were more effective in sustaining hematopoietic proliferation as measured by total cell production over the next forty days of observation (Fig. 1 *a*). Cumulative production of assayable progenitor cells was also far greater when $CD34^+DR^-$ cells were used as a recharge inoculum than when $CD34^+DR^+$ cells were utilized (Fig. 1 *b*). Control cultures receiving no recharge with sorted cells released smaller numbers of cells into the supernate which upon examination were macrophages; no assayable progenitor cells of any kind were produced by these cultures. In similar experiments with media replacement occuring every 7 d, $CD34^+DR^-$-derived CFU-GM output exceeded that of $CD34^+DR^+$ cells by day 14 (data not shown). In all studies, small numbers of BFU-E were generated over the first 2–3 wk by both marrow subpopulations. Cell cycle analysis of the marrow subpopulations obtained at various stages of cell separation demonstrated that over 97% of the $CD34^+DR^-$ cells resided in G_0 + G_1 stage while higher percentages of LDBM, FR12–14, and

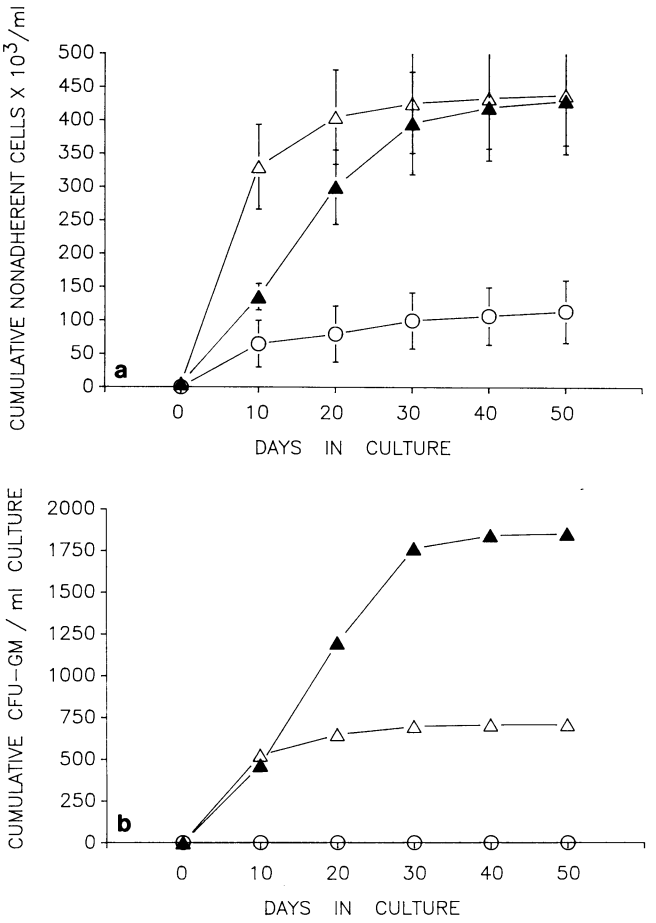

Figure 1. (*a*) Cumulative supernatant cell counts of 4-wk-old, irradiated stromal cultures re-seeded with no cells (○), or autologous $CD34^+DR^+$ (△) or $CD34^+DR^-$ (▲) cells at 5×10^3/ml. Each point represents the mean±SD of duplicate cultures. (*b*) Cumulative production of CFU-GM by $CD34^+DR^+$ or $CD34^+DR^-$ cells seeded onto autologous, irradiated stromal cultures. Each point represents the mean number of CFU-GM assayed±SD of duplicate cultures (all standard deviation values fall within symbol areas).

$CD34^+DR^+$ fractions were in more active phases of the cell cycle (Table I).

Multiparameter sorting was utilized in an effort to further phenotype those $CD34^+DR^-$ cells responsible for long-term in vitro hematopoiesis. Virtually all cells residing in the $CD34^+DR^-$ population failed to express the CD33 antigen (Fig. 2 *a*). Anti-CD15 and anti-CD71 were next examined as sorting probes (Fig. 2, *b* and *c*). Morphological analysis of these sorted populations are shown in Table II. Well over 90% of $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ cells appeared as hematopoietic blasts showing no evidence of characteristics indicating lineage commitment; the subpopulations expressing CD15 or CD71 contained more differentiated cell types. Table III illustrates the progenitor cell content of these various cell populations. It can be seen that the $CD34^+DR^-CD33^+$ and $CD34^+DR^-CD15^+$ populations contained few assayable progenitors while significant numbers were contained in the other cell fractions. The overwhelming majority of BFU-E present in $CD34^+DR^-$ cells resided in the $CD34^+DR^-CD71^+$ population. It was notable that both $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ cells contained HPP-CFC while those $CD34^+DR^-$ fractions expressing either CD15 or CD71 did not.

A liquid culture system supplemented with repeated 48-hourly cytokine additions was utilized to further study these cell populations. Total cell production by both $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ cells is shown in Tables IV and V while assayable CFU-GM in these cultures over time are recorded in Tables VI and VII. In the absence of exogenous cytokines, total cell numbers declined over a 2-wk period and assayable CFU-GM persisted for only 1 or 2 wk. The repeated addition of IL-1α did not significantly enhance total cell production or generation of CFU-GM by either $CD34^+DR^-CD15^-$ or $CD34^+DR^-CD71^-$ cells. IL-6 did not alter total cell numbers or numbers of assayable CFU-GM in cultures initiated with $CD34^+DR^-CD71^-$ cells. By contrast, IL-6 increased total cell numbers over sevenfold by week 3 by $CD34^+DR^-CD15^-$ initiated cultures but did not appreciably extend the interval over which CFU-GM were detected. In both sets of experiments, GM-CSF promoted increased total cell production for 6 wk, by which time cell numbers represented 20–80 times the number present in the initial seeding populations. Assayable CFU-GM persisted for 3–4 wk and cumulatively surpassed those assayable in the initial populations. The single most effective cytokine in terms of promoting cellular expansion, increasing the number of CFU-GM, and lengthening the duration of time over which CFU-GM were assayable was IL-3. Both $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ cells experienced 200-fold increases in cell numbers by day 28, and, after 1 or 2 wk in culture, contained equal or slightly greater numbers of CFU-GM than present in the initial inoculi. Assayable progenitors were produced for 4–5 wk in the system when maintained with IL-3, and viable cell counts remained high at 8 wk. IL-1α or IL-6 prolonged and enhanced these effects when added in combination with IL-3. CFU-GM were assayable after 8 wk in suspension culture after continued treatment with these two cytokine combinations. No adherent cell layer was established in any of the suspension cultures over the 8-wk period of observation.

In a separate experiment, $CD34^+DR^-CD71^-$ cells were grown in this suspension culture system in the presence of a combination of both IL-3 and IL-6 and assayed for CFU-MK from days 7 through 28 of culture. CFU-MK were detected over this 28-d period (Table VIII). Utilizing this IL-3/IL-6 cytokine combination, the ability of $CD34^+DR^-CD15^+$ and

Table I. Cell Cycle Analysis of Human Bone Marrow Fractions

Phase	LDBM	FR12–14‡	$CD34^+DR^+$	$CD34^+DR^-$
		*percentage of cell population**		
$G_0 + G_1$	88.2	92.1	96.0	97.5
S	10.2	7.0	3.8	2.5
$G_2 + M$	1.6	0.9	0.1	0.0

DNA distribution was determined flow cytometrically by propidium iodide staining of cell preparations and analysis on a FACScan using SFIT program.

* 5×10^3 cells per sample were analyzed.

‡ LDBM was fractionated by counterflow centrifugal elutriation; cells eluted at a flow rate of 12–14 ml/min at a rotor speed of 1,950 rpm using an elutriation system with standard separation chamber.

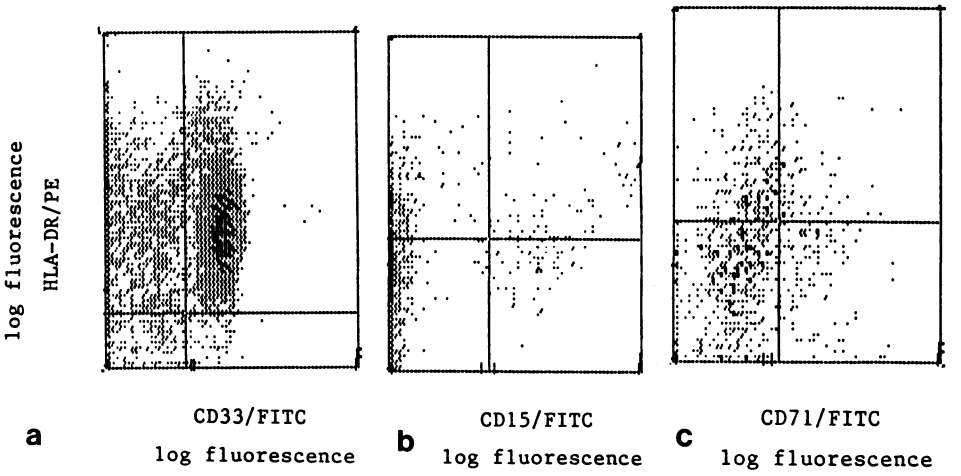

Figure 2. Two-parameter histograms representing density distributions of (*a*) CD33 vs. HLA-DR, (*b*) CD15 vs. HLA-DR, and (*c*) CD11 vs. HLA-DR by human marrow cells. FR 12–14 marrow cells were gated on forward angle light scatter and gates were set for cells positively stained by CD34 antibody second-step conjugated to Texas red. Cells positive for CD34 were analyzed for evidence of staining by PE-conjugated anti-HLA-DR and FITC-conjugated anti-CD33, CD15, or CD71. Vertical bars represent positivity for HLA-DR and horizontal bars positivity for CD33, CD15, or CD71 defined as fluorescence above that of 99% of isotype-matched nonspecific myeloma proteins used in parallel with the staining monoclonal antibodies.

$CD34^+DR^-CD71^+$ cells to sustain long-term hematopoiesis was compared to that of the $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ fractions (Table IX). Both the CD15-positive and CD71-positive cells failed to generate CFU-GM after 2 wk, and the CD71-positive population, which initially included the overwhelming majority of BFU-E, failed to produce assayable BFU-E after only 7 d in culture.

Morphological analysis of the cells in these suspension cultures during the period of observation revealed changes in the cellular composition of the populations following the addition of various cytokines (Tables X and XI). IL-1α– and IL-6–containing cultures behaved very similarly to the control samples. Cultures to which no cytokines were added were composed of 90–100% blasts after 1 wk; the $CD34^+DR^-CD15^-$ cells did not survive 2 wk in the absence of cytokine whereas the $CD34^+DR^-CD71^-$ initiated cultures were composed of 40% blasts and 60% monocytes by week 2. Cultures receiving IL-1α had a similar cellular composition. IL-6 facilitated some differentiation to the granulocytic series by both cell populations; the $CD34^+DR^-CD15^-$ cells produced a significant number of mature granulocytic elements by week 2. GM-CSF, as well as IL-3, reduced the percentage of blasts in these suspension cultures appreciably by day 7. GM-CSF-containing cultures of $CD34^+DR^-CD15^-$ and $CD34^+DR^-CD71^-$ cells consisted primarily of metamyelocytes through 4 wk, with a shift to monocytes occurring by week 6.

IL-3 was unique in that, at 3 wk, suspension cultures initiated by either $CD34^+DR^-CD15^-$ or $CD34^+DR^-CD71^-$ cells were composed of 48% basophils in the presence of this growth factor (Tables X and XI). Addition of IL-1α or IL-6 did not alter this trend, all IL-3-containing cultures being composed of about 50% basophils by 3 wk and retaining significant numbers of basophils throughout the duration of culture.

The cellular composition of hematopoietic colonies assayed from aliquots of the suspension cultures was comparable to those assayed from the original sorted populations with a few notable exceptions. Blast cell colonies, as well as HPP-CFC–derived colonies, were routinely obtained by directly assaying $CD34^+DR^-CD15^-$ or $CD34^+DR^-CD71^-$ cells while these colony types were not observed in subsequent clonal assays of cellular aliquots obtained from the long-term liquid cultures. Distribution of GM colony subtypes, however, remained fairly consistent with roughly 40% being granulocyte-macrophage, 40% monocyte-macrophage, and 20% basophil or eosinophil colonies in either assays initiated with sorted cells of those initiated on days 7 through 42 of liquid culture.

Table II. Cellular Composition of Sorted Cell Populations

Population	Blasts	Myelo	MM	Band	Seg	Baso	Lymph	Norm	Mo
					%				
$CD34^+DR^-$	65.0	0.0	13.0	0.0	21.0	1.0	0.0	0.0	0.0
$CD34^+DR^+$	88.0	1.0	5.0	2.0	0.0	3.0	0.0	1.0	0.0
$CD34^+DR^-CD15^-$	96.0	1.0	1.0	0.0	0.0	0.5	0.5	1.0	0.0
$CD34^+DR^-CD15^+$	36.0	10.0	40.0	10.0	2.0	0.0	0.0	2.0	0.0
$CD34^+DR^-CD33^-$	59.0	2.5	18.0	15.5	2.5	0.0	0.0	0.0	2.5
$CD34^+DR^-CD33^+$	6.5	6.5	52.5	6.5	2.0	0.0	0.0	4.5	21.5
$CD34^+DR^-CD71^-$	91.5	2.5	4.0	0.5	0.0	0.5	0.0	1.0	0.0
$CD34^+DR^-CD71^+$	82.0	1.5	5.0	1.0	1.5	0.0	0.0	7.0	0.0

Differential cell counts were performed on Wright-Giemsa stained cytocentrifuge preparations of cells obtained by monoclonal antibody staining and fluorescence-activated cell sorting. 200 cells per slide were classified. Abbreviations: Band, neutrophil band form; Baso, basophils; Lymph, lymphocytes; MM, metamyelocytes; Mo, monocytes; Myelo, myelocytes; Norm, normoblasts; Seg, segmented neutrophils.

Table III. Hematopoietic Progenitor Cells Present Initially in Sorted Cell Populations

Population	Enumeration at day 14			Enumeration at day 28:
	CFU-GM	BFU-E	CFU-GEMM	HPP-CFC
	*colonies/10^3 cells**			
$CD34^+DR^-$	26.8±17.6‡	34.5±57.0	1.0±1.4	ND§
$CD34^+DR^+$	65.3±13.5	55.8±72.6	2.4±2.8	ND
$CD34^+DR^-15^-$	111.0±18.8	41.5±4.9	3.3±0.5	28.0±2.8
$CD34^+DR^-15^+$	24.0±3.8	0.5±0.6	0.0±0.0	0.0±0.0
$CD34^+DR^-CD33^-$	27.3±25.6	19.5±24.1	1.2±1.9	ND
$CD34^+DR^-CD33^+$	1.2±1.6	0.0±0.0	0.0±0.0	ND
$CD34^+DR^-CD71^-$	137.5±19.6	8.3±4.4	2.0±1.8	27.5±2.1
$CD34^+DR^-CD71^+$	20.8±3.1	151.3±2.8	3.3±2.8	0.0±0.0

* 10^3 cells/ml were cultured in methylcellulose with 50 U/ml GM-CSF and 1 U/ml purified human urinary erythropoietin (Toyobo Co., Ltd., Osaka, Japan). ‡ Each point represents the mean±SD of quadruplicate assays or duplicate assays performed on at least two separate occasions. § Not determined.

These CFU-GM-derived colonies ranged in size from 100 to 2,000 cells with the average colony containing between 200 and 400 cells. After 8 wk of suspension culture, monocyte-macrophage colonies were the predominant colony type observed in the clonal assays.

Discussion

Mammalian hematopoiesis has been studied in vitro through the use of various long-term marrow culture systems (13, 25–27). Dexter and co-workers (13) described a murine system from which CFU-S and CFU-GM could be assayed for several months, with erythroid and megakaryocytic precursors appearing for a more limited time. Maintenance of these cultures was dependent on the formation of an adherent stromal cell layer composed of endothelial cells, adipocytes, reticular cells, and macrophages. These methods were soon adapted for the study of human bone marrow. Human long-term culture systems were reported to generate assayable hematopoietic progenitor cells for 8 or 9 wk (25, 26) and, later, for up to 20 wk (27, 28). Such cultures are again reliant on the preestablishment of a stromal cell layer which is frequently reinoculated with a large, heterogeneous population of marrow cells. Hematopoietic stem cells have been shown to home and adhere to this adherent cell multilayer before generating and releasing more committed progenitor cells (8, 29, 30). Stromal cells are thought to provide not only a physical matrix on which stem cells reside, but also to produce membrane-contact signals

Table IV. Total Cell Production of $CD34^+$, DR^-, $CD15^-$ Cells after Addition of Various Cytokines

Cytokine	Day							
	0	7	14	21	28	35	42	56
	viable cell count × 10^3							
None	5	1	4	0	0	0	0	0
IL-1a*	5	2	2	0	0	0	0	0
IL-3‡	5	53	140	591	1,085	533	678	781
IL-6§	5	3	4	36	26	16	0	0
GM-CSF‖	5	8	14	44	169	213	118	0
IL-1α/IL-3	5	32	167	556	1,360	1,387	758	1,069
IL-6/IL-3	5	47	171	471	854	1,440	1,200	1,216

Total cells = cells/ml culture/$(½)^n$, where n = number of previous demi-depopulations.
* 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein.
‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein.
§ 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein.
‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

Table V. Total Cell Production of $CD34^+$, DR^-, $CD71^-$ Cells after Addition of Various Cytokines

Cytokine	Day							
	0	7	14	21	28	35	42	56
	viable cell count × 10^3							
None	5	1	2	0	0	0	0	0
IL-1α*	5	3	0	0	0	0	0	0
IL-3‡	5	40	226	964	746	1,190	1,120	851
IL-6§	5	1	2	0	0	0	0	0
GM-CSF‖	5	3	34	44	45	445	438	0
IL-1α/IL-3	5	23	202	684	1,112	835	800	1,067

Total cells = cells/ml culture/$(½)^n$, where n = number of previous demi-depopulations.
* 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein.
‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein.
§ 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein.
‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

Table VI. Total CFU-GM Production by $CD34^+$, DR^-, $CD15^-$ Cells after Addition of Various Cytokines

Cytokine	Week 1	2	3	4	5	6	8
	CFU-GM/ml culture						
None	40	0	0	0	0	0	0
IL-1α*	22	14	0	0	0	0	0
IL-3‡	432	696	591	325	0	0	0
IL-6§	42	242	96	0	0	0	0
GM-CSF‖	273	200	219	0	0	0	0
IL-1α/IL-3	254	397	444	408	139	152	64
IL-6/IL-3	98	342	236	768	864	1,080	384

Total CFU-GM = CFU-GM/ml culture/$(½)^n$, where n = number of previous demi-populations.
Cells were seeded at 5×10^3/ml. CFU-GM in initial (day 0) population = $555/5 \times 10^3$ cells. Colonies grown in methylcellulose containing 50 U/ml GM-CSF and enumerated after 14 d.
* 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein.
‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein.
§ 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein.
‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

and/or hematopoietic growth factors necessary for stem cell proliferation and differentiation (14, 17, 31, 32). This heterogeneous mixture of cells comprising the adherent cell layer presents an inherently complex system from which the isolation of discrete variables affecting stem cell growth has proven difficult.

Table VII. Total CFU-GM Production by $CD34^+$, DR^-, $CD71^-$ Cells after Addition of Various Cytokines

Cytokine	Week 1	2	3	4	5	6	8
	CFU-GM/ml culture						
None	15	4	0	0	0	0	0
IL-1α*	20	0	0	0	0	0	0
IL-3‡	664	272	96	448	119	0	0
IL-6§	51	14	0	0	0	0	0
GM-CSF‖	402	360	135	28	0	0	0
IL-1α/IL-3	347	324	342	334	167	240	214

Total CFU-GM = CFU-GM/ml culture/$(½)^n$, where n = number of previous demi-populations.
Cells were seeded at 5×10^3/ml. CFU-GM in initial (day 0) population = $690/5 \times 10^3$ cells. Colonies grown in methylcellulose containing 50 U/ml GM-CSF and enumerated after 14 d.
* 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein.
‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein.
§ 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein.
‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

Table VIII. Assayable CFU-MK in Long-Term Suspension Cultures of $CD34^+DR^-CD71^-$ Cells Receiving a Combination of IL-3 and IL-6

Days in culture*	CFU-MK/ml culture‡
7	42.6±7.6§
14	67.6±56.6
21	17.0±11.8
28	20.2±10.4

50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFUc/mg protein. 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein.
* Cultures were demi-depopulated every 7 d.
‡ CFU-MK were assayed in serum-free fibrin clot culture containing 100 U/ml IL-3 colonies enumerated at days 18–24 of culture.
§ Each point represents the mean±SD of triplicate assays. Values are not corrected for the effects of demi-depopulation.

The use of fluorescence-activated cell sorting has facilitated further characterization of the hematopoietic stem cell in the mouse (5, 6, 33, 34). In humans, monoclonal antibody identification of a number of cell surface antigens has permitted partial phenotypic analysis of the human marrow cell responsible for long-term hematopoiesis (10, 35–37). The CD34 antigen, present on 1–4% of LDBM cells, has been repeatedly demonstrated to be present not only on unipotent and multipotent progenitor cells but their precursors as well (35, 36). Cells responsible for the long-term generation of assayable progenitors in marrow culture have been shown by Keating and co-workers (37) to express undetectable quantities of HLA-DR. The data presented here indicate that the cells responsible for long-term in vitro hematopoiesis express the CD34 antigen but not detectable amounts of HLA-DR. The CFU-B1 has also recently been partially phenotyped and shown to be $CD34^+DR^-$ (10). The presence of both cell types responsible for these in vitro functions in the $CD34^+DR^-$ marrow cell population suggests that they are closely related.

The study of various marrow subpopulations by measure-

Table IX. Total CFU-GM and BFU-E Production by Sorted Cell Populations Stimulated with a Combination of IL-3 and IL-6

Population	Week 1	2	3	4	6	8
	CFU-GM (BFU-E)/ml culture					
$CD34^+DR^-CD15^-$	275 (10)	286 (4)	64	32	75	0
$CD34^+DR^-CD15^+$	7 (1)	26	0	0	0	0
$CD34^+DR^-CD71^-$	220 (5)	330 (4)	132	18	43	0
$CD34^+DR^-CD71^+$	13	16	0	0	0	0

Total CFU = CFU/ml culture/$(½)^n$, where n = number of previous demi-depopulations. 50 U/ml recombinant human IL-3, specific activity 10^8 CFU/mg protein and 75 U/ml recombinant human IL-6, specific activity 10^7 CFU/mg protein were added every 48 h. Cells were seeded at 5×10^3/ml.

Table X. Differential Analysis of CD34$^+$, DR$^-$, CD15$^-$ Cells after Addition of Various Cytokines

Cytokine	Day	Blasts	Pro	Myelo	MM	Band	Seg	Eo	Baso	E	Mo
						%					
Control	7	100									
IL-1α*	7	100									
	14	78									22
IL-6‡	7	100									
	14	27	11		9		13		38		2
	21	9			48	2	7		17		17
	28				30		4				66
GM-CSF§	7	25	24		27	3	21				
	14	9	1		46	3	21		13		7
	21	3	2	1	62	3	5		22		2
	28	6		1	43	7	3		6	2	32
	35			4							96
	42			1							99
IL-3‖	7	21	44		35				1		
	14	7	7		53				33		
	21	8			44				48		
	28	5			35	3	9		35		13
	35	2			16	5	20		25		32
	42				15		2		20		63
IL-1α/IL-3	7	1	5	1	53	12	14		14		
	14	5			34	9			52		
	21	1			53	4	3		31		8
	28	1			42	12	5		32		8
	35				20				27		53
	42				8				8		84
	56								11		89
IL-6/IL-3	7	19	26	2	40	5	4		4		
	14	2	2		46	3	1		46		
	21	5	1		37	1	7		48		1
	28	4	1		37	10	8		35		5
	42	1			8		1		9		81
	56				2				3		95

Differential cell counts were performed on Wright-Giemsa stained cytocentrifuge preparations of cells removed from liquid culture. 200 cells per sample were classified; if < 200 cells appeared on a slide, all were classified. Abbreviations: E, erythrocytes; Eo, eosinophils; Pro, promyelocytes; other abbreviations as in Table II. * 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein. ‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein. § 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein. ‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

ment of their hematopoietic cellular output provides an alternative method of characterizing primitive multipotential hematopoietic progenitor cells. Irradiated stromal cultures recharged with CD34$^+$DR$^+$ cells contained more than twice the number of supernatant cells and a significantly greater number of CFU-GM than parallel cultures recharged with CD34$^+$DR$^-$ cells after 7–10 d. Cells expressing both CD34 and HLA-DR have previously been demonstrated to contain the majority of hematopoietic progenitor cells present in human marrow (38). By 14–20 d, however, the CD34$^+$DR$^-$ cultures had surpassed their CD34$^+$DR$^+$ counterparts in ability to generate hematopoietic cells, suggesting the presence of a more quiescent progenitor with higher proliferative capacity within the CD34$^+$DR$^-$ population.

The almost total absence of CD33-positive cells in the CD34$^+$DR$^-$ subpopulation is consistent with the reports of Andrews et al. (39, 40), who demonstrated the absence of CD33 on the precursors of colony-forming cells. Removal of cells expressing the CD15 antigen from the CD34$^+$DR$^-$ population eliminated virtually all of the contaminating, differentiated granulocytic cells. The presence of the CD71 antigen on actively proliferating cells was exploited in order to select a population of quiescent CD34$^+$DR$^-$ cells. The CD34$^+$DR$^-$CD15$^-$ and CD34$^+$DR$^-$CD71$^-$ populations contained HPP-CFC, a primitive, quiescent cell capable in the mouse of differentiating into several lineages and possessing in vivo repopulating potential (41). Based upon the data presented here, human marrow cells expressing CD34 but not CD33, HLA-DR, CD15, or CD71 antigens share many biological properties that would likely be associated with human hematopoietic stem cells. Such cell populations are quiescent, capable of initiating long term hematopoiesis and generating HPP-CFC–derived colonies in vitro and represent only about 0.01% of nucleated marrow cells.

During the 8 wk of observation in the stroma-free suspen-

Table XI. Differential Analysis of CD34$^+$, DR$^-$, CD71$^-$ Cells after Addition of Various Cytokines

Cytokine	Day	Blasts	Pro	Myelo	MM	Band	Seg	Eo	Baso	E	Mo
						%					
Control	7	90									10
	14	40									60
IL-1α*	7	82									18
IL-6‡	7	43	4								13
	14	33	20								47
GM-CSF§	7	39	33		9	5	6		5		2
	14	18	5		42	3	12		20		
	21	4		1	66	9	7				4
	28	2			61	3	1	8			24
	35	14			18	8	8	9			52
	42										100
IL-3‖	7	52	40		1	2	2		2	1	
	14	29	26		26	2	3		14		
	21	13	4	2	28	2	3		48		
	28	14	3		35	5	1		35		7
	35	9			20	7	6		27		31
	42	2			5		4		16	2	71
IL-1α/IL-3	7	48	42		6	2	1		2		
	14	4	1		53	4	5		33		
	21	3			44	1	1		49		2
	28	21	3		34	4	3	1	27		8
	35	3			23	4	29		20		21
	42	1			7	3	3		16		70
	56						1		8		91

Differential cell counts were performed on Wright-Giemsa stained cytocentrifuge preparations of cells removed from liquid culture. 200 cells per sample were classified; if < 200 cells appeared on a slide, all were classified. Abbreviations as in Tables II and X. * 2.5 U/ml recombinant human IL-1α were added every 48 h; specific activity 10^8 CFU/mg protein. ‡ 50 U/ml recombinant human IL-3 were added every 48 h; specific activity 10^8 CFU/mg protein. § 75 U/ml recombinant human IL-6 were added every 48 h; specific activity 10^7 CFU/mg protein. ‖ 12.5 U/ml recombinant human GM-CSF were added every 48 h; specific activity 5×10^7 CFU/mg protein.

sion system, an adherent cell layer was never observed and sustained proliferation in these cultures was entirely dependent upon the addition of cytokines. One can infer from such data that an important function of the adherent cell layer is the secretion of a number of hematopoietic growth factors. Human stromal cell lines have been shown to produce cytokines including GM-CSF, G-CSF, IL-6, but not IL-3 (16, 42). It is therefore intriguing that IL-3 alone or especially in combination with IL-1 or IL-6 has been shown in this report and that of others (43) to best promote the proliferation of primitive hematopoietic progenitor cells. IL-3 appears to be exclusively produced by T cells and is presently thought not to be a product of marrow stromal cells. The ST-1 stromal fibroblastoid cell line and several murine adipocyte lines that have been utilized by a number of investigators to study cytokine production by marrow stromal elements apparently do not produce IL-3 mRNA (16, 44). Such cell lines do not necessarily reflect the capacity of all stromal cell types to produce IL-3. It remains possible that a rare but important stromal cell subtype may secrete IL-3. An alternative explanation is that another cytokine distinct from IL-3 but possessing similar biological activity may be produced by marrow stromal cells and provide under physiological conditions, similar functions to IL-3 (44).

The maintenance of hematopoiesis in vitro for 8 wk in the absence of an adherent cell layer should not lead to conclusions that stromal cell-derived matrix proteins or cell–cell interactions are not important in sustaining in vivo or in vitro hematopoiesis. Further dissection of this complex system will be required to identify the importance of these stromal components. Gualtieri et al. (45) have shown that after in vitro irradiation, colony-stimulating activity elaborated by such stromal cell layers was markedly increased, while Alberico et al. (18) have provided evidence that GM-CSF is one cytokine responsible for this postirradiation effect. GM-CSF alone was capable in our studies of sustaining hematopoiesis in suspension culture alone for only 3 or 4 wk. Although GM-CSF is likely an important cytokine elaborated by those stromal cell cultures, its limited ability to sustain long-term hematopoiesis alone suggests that additional cytokines, matrix proteins, or cellular interactions are necessary for more prolonged cell production within the marrow microenvironment.

IL-3 is thought to be necessary both for the continued proliferation of hematopoietic multipotential cells (46) and for the continued viability of cells that reside in G_0 and/or G_1 phase of the cell cycle (47). IL-3 alone was able to sustain hematopoiesis in the suspension culture system for 4 or 5 wk, while combinations of IL-1 or IL-6 and IL-3 extended hematopoietic activity to 8 wk. The prolongation of the period of quantifiable hematopoiesis by these combinations of cytokines is consistent with the hypothesis that such cytokine combina-

tions actually are affecting a more primitive precursor cell of hematopoietic progenitor cells than are influenced by IL-3 alone (48, 49). Ikebuchi et al. (48) have shown that IL-6 synergizes with IL-3 probably by shortening the G_0 period of murine stem cells and that the synergistic activity of IL-6 is stronger than that of IL-1α. Leary et al. (50) have confirmed this synergistic action of IL-6 with IL-3 in the human system by documenting the earlier appearance of blast cell colonies in the presence of both growth factors than in the presence of IL-3 alone. They were, however unable to demonstrate a synergistic effect of IL-1 with IL-3 in promoting human blast cell colony formation. In our studies IL-1 and IL-6 were both effective in enhancing the action of IL-3. The action of IL-1 in potentiating the function of IL-3 may be directly on the primitive hematopoietic multipotential cells as suggested by Stanley et al. (49) and Moore and Warren (51), or perhaps may be due to an indirect effect involving marrow accessory cells. This potential indirect action of IL-1 has been suggested to be in part mediated by IL-6 by Leary and co-workers (50). It remains possible that the cytokines or cytokine combinations tested in this report do not provide the optimum environment for the propagation long term in vitro hematopoiesis. Further assessment of additional cytokines alone or combinations will be necessary to determine the most efficient humoral environment.

The presence of significant numbers of basophils in suspension cultures containing IL-3 alone or in combination with IL-1 and IL-6 indicates the capability of IL-3 to promote basophil development. The induction of basophilic differentiation by IL-3 has been previously demonstrated in vivo in primates by Mayer et al. (52) and in vitro in marrow suspension cultures by Valent et al. (53).

In addition to its ability to enhance IL-3–dependent proliferation of stem cells, human IL-6 is known to be capable alone of supporting the development of murine granulocyte and monocyte progenitors (54). This effect of IL-6 was observed to a limited degree in the presently utilized suspension cultures. IL-6 alone was able to support granulocytic and monocytic differentiation for a limited time. IL-1α is known to induce various cell types to elaborate other cytokines, among them IL-6, and to potentiate the direct actions of other cytokines (16). The inability of IL-1 alone to promote the proliferation or differentiation of $CD34^+DR^-CD15^-$ or $CD34^+DR^-CD71^-$ cells is consistent with the findings of others that indicate that this cytokine alone has no colony stimulating ability (23, 55).

In the present studies BFU-E were generated for 2 or 3 wk in the suspension cultures. In most human long-term marrow culture systems, the duration of erythropoiesis is far exceeded by that of granulopoiesis and monocytopoiesis (32, 56). The etiology of the defect in long-term erythropoiesis in the suspension system described here and in other long-term marrow culture systems containing adherent cell layers is only now being unraveled (15, 32).

The suspension culture system utilized in the present studies provides a new investigative tool to explore the biological behavior of primitive multipotential hematopoietic precursor cells. Utilizing highly enriched populations of primitive hematopoietic stem cells, factors influencing hematopoiesis may be isolated and studied. In this way, cytokine and extracellular matrix requirements as well as selective cell–cell interactions necessary for development of the human hematopoietic stem cell might be better examined and defined.

Acknowledgments

We wish to thank Stephanie McGillem for secretarial assistance during the preparation of this manuscript.

This work was supported by grants from the National Cancer Institute.

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