

Dependence of Highly Enriched Human Bone Marrow Progenitors on Hemopoietic Growth Factors and Their Response to Recombinant Erythropoietin

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Abstract

Human bone marrow cells were sequentially fractionated by three negative selection steps to remove adherent cells and Fc receptor-bearing cells, followed by immune adsorption (panning) to deplete maturing cells that react with a panel of monoclonal antibodies. This nonadherent Fc receptor and antibody negative fraction could be further enriched by a positive selection "panning" step, using an antibody to HLA-DR antigen; 12–27% of the cells formed erythroid burst-forming unit (BFU-E), erythroid colony-forming unit, granulocyte-monocyte colony-forming unit, and erythroid and granulocyte and/or monocyte colony-forming unit-derived colonies with recovery of 0.5–1% of the cells and 20–100% of the colony-forming cells.

Sequential fractionation resulted in increasing dependence of a subset of BFU-E-derived colonies on exogenous burst-promoting activity (BPA) for proliferation in culture, but the most enriched progenitor fraction still contained a proportion of accessory cell or BPA-independent BFU-E that responded to either natural or biosynthetic erythropoietin when added to cultures on day 0 in the absence of BPA. If the addition of erythropoietin was delayed until day 3, the data suggest that this population of BFU-E either died or became unresponsive to erythropoietin. Delayed addition of erythropoietin to cultures of enriched progenitors provided a sensitive BPA assay, since BPA-independent but erythropoietin-responsive BFU-E were eliminated. The surviving BFU-E that were dependent for their proliferation on the presence of both BPA and erythropoietin showed a characteristic dose response to increasing BPA concentrations.

Introduction

Hemopoietic progenitors depend on specific glycoprotein growth factors for their survival, proliferation, and terminal differentiation in vitro. Insight into the regulation of hemopoiesis at a cellular level requires purification of both progenitors and hemopoietic growth factors (HGFs).¹ To date, at least three human

HGFs have been purified to homogeneity and their genes cloned (1–5): Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the formation of colonies derived from granulocyte and/or monocyte progenitors (CFU-GM). We have recently demonstrated (6) that purified recombinant GM-CSF also has burst-promoting activity (BPA) for erythroid burst-forming units (BFU-E) and stimulates the formation of colonies derived from multipotent progenitors (CFU-MIX); erythroid-potentiating activity (EPA) (2, 3) increases BFU-E and erythroid colony-forming unit (CFU-E) numbers above background in a low serum assay; and erythropoietin (EP) (4, 5) acts on committed erythroid progenitors and is essential for the terminal maturation of erythroid cells.

Progenitor purification is necessary because accessory "helper" cells present in unfractionated bone marrow produce factors that result in significant background noise in colony-forming assays for growth factor activity. Progenitors comprise <1% (usually <0.5%) of human bone marrow cells. Because of the heterogeneity of bone marrow tissue, attempts to purify them using physical parameters have been only partially successful (7, 8), although murine CFU-E have been greatly enriched (9). Monoclonal antibodies and/or lectins have been used with more success for purifying mouse (10) and human (11–14) progenitors using negative and/or positive selection by complement lysis (13, 14), immune rosetting (14), or fluorescence-activated cell sorting (FACS) (10, 11, 13) techniques. While significant enrichment using these methods has been obtained, disadvantages include nonspecific cell loss with poor progenitor recovery, and long sorting times with the FACS that make difficult the selection of these rare cells in sufficient numbers for further study. Immunoabsorption to plastic dishes (panning) combined with the FACS has been used to partially enrich for murine bone marrow progenitors (15), and recently we have shown that the progenitors present in human fetal liver can be greatly enriched with excellent recovery using immunoabsorption to immunoglobulin-coated plates (16).

In this report, we show that sequential fractionation of human bone marrow cells results in increasing dependence of BFU-E on an exogenous source of BPA for proliferation in culture. Panning, using negative selection with a combination of monoclonal antibodies, followed by positive selection with an antibody

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1. *Abbreviations used in this paper:* Ab⁻ and Ab⁺, cells without and with antigens recognized by a panel of monoclonal antibodies, respectively; Ad⁻ and Ad⁺, plastic nonadherent and adherent cells, respectively; BFU-E, erythroid burst-forming unit; BPA, burst-promoting activity; CFU-E, erythroid colony-forming unit; CFU-GM, granulocyte-monocyte CFU; CFU-MIX, erythroid and granulocyte and/or monocyte CFU; CM, con-

ditioned medium; DR⁺ and DR⁻, cells expressing or not expressing HLA-DR antigens, respectively; EP, erythropoietin; EPA, erythroid-potentiating activity; FACS, fluorescence-activated cell sorting; Fc⁻ and Fc⁺, cells without and with receptors for the Fc portion of immunoglobulins, respectively; FCS, fetal calf serum; GCT, giant cell tumor; GCT-CM, GCT cell line-conditioned medium; GM-CSF, granulocyte-monocyte colony-stimulating factor; HGF, hemopoietic growth factor; IMDM, Iscove's modified Dulbecco's medium; Mo-CM, Mo T lymphoblast cell line-conditioned medium; REC, recombinant EP; U, unfractionated Ficoll-Hypaque bone marrow mononuclear cells.

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to HLA-DR antigens, can greatly enrich for progenitor cells with good recovery. Among these progenitors are two distinct BFU-E populations. One subset is initiated into terminal differentiation by EP alone; the second and somewhat larger subset requires both BPA and EP to differentiate. (Indeed, this class of BFU-E cannot survive 3 d in culture if BPA is withheld.) Manipulation of these subsets by delayed addition of either partially purified human urinary or recombinant erythropoietin (REC) to enriched progenitors thereby provides a method to perform a sensitive BPA assay with no or minimal background colony formation.

Methods

Bone marrow samples. Normal human bone marrow was obtained by aspiration from adult volunteers. Approximately 5–15 ml from 1 to 2 aspirations was collected into sterile syringes containing preservative-free heparin.

Cell separation procedures. The marrow suspensions were separated over Ficoll-Hypaque (1.077 g/cu cm) (Pharmacia Fine Chemicals, Piscataway, NJ) at 400 g for 40 min at 20°C and the interface mononuclear cells collected, washed three times, and resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 20% fetal calf serum (FCS). The cells were incubated in 100-mm tissue culture dishes (Lux, Miles Laboratories, Naperville, IL) overnight at 37°C and the nonadherent cells (Ad^-) removed with two gentle washes and centrifuged.

Fc receptor-positive cell depletion. In some experiments, the Ad^- cells were resuspended in phosphate-buffered saline (PBS) containing 5% FCS and incubated on immunoglobulin-coated 100-mm plastic Petri dishes for 1 h at 4°C. Fc receptor negative (Fcr^-) cells were removed with two gentle washes and the cells centrifuged.

Immunoadsorption to immunoglobulin-coated plates (panning). The bone marrow cells were incubated at 4°C for 30 min with previously determined optimal concentrations of either Leu 1 and 5b (Becton-Dickinson Monoclonal Centre Inc., Mountain View, CA) to label T lymphocytes or a panel of 10–12 monoclonal antibodies directed against myeloid, erythroid, and lymphoid maturation antigens (Table I). The cells were washed twice in IMDM containing 2% FCS and once in PBS/5% FCS, resuspended at $5-6 \times 10^6$ /ml in PBS/5% FCS, and 4–5-ml aliquots were incubated at 4°C for 1 h on 100-mm plastic bacteriological petri dishes (Fisher Scientific Co., Pittsburgh, PA) that had been previously coated with rabbit anti-mouse immunoglobulin (see below). The non-adherent cells (T^- [Leu 1/5b]) or antibody-negative cells (Ab^- [multiple antibodies]) were removed and washed twice by gently swirling, tilting, and decanting, and then a second incubation on another antibody-coated plate was carried out to ensure removal of all antibody labeled cells. In some experiments, the Ab^- cells were incubated with a monoclonal antibody to HLA-DR antigen (Becton-Dickinson & Co., clone L243), washed, and incubated at 4°C for 1 h on a third immunoglobulin-coated plate. Nonadherent HLA-DR-negative cells were removed with two gentle washes. The plates were then washed three times and the adherent HLA-DR-positive cells were removed by vigorous pipetting using 10 ml of IMDM/2% FCS. Most of the adherent cells detached with this procedure, but occasionally it was necessary to remove the remaining cells with a rubber policeman. Cytocentrifuge preparations of the different cell fractions were stained with Wright-Giemsa for differential counting.

Preparation of immunoglobulin-coated plates. Panning was carried out by minor modifications of the method of Wysocki and Sato (21). 100 × 15-mm polystyrene bacteriological petri dishes (Fisher Scientific Co.) (catalogue 8-751-12) were incubated at room temperature in 10 ml affinity-purified rabbit anti-mouse IgG, and A & M heavy and light chain immunoglobulin (Zymed Laboratories Inc., San Francisco, CA) diluted to 10 µg/ml in PBS. After 40 min, the plates were washed three times in PBS and then incubated at 4°C in 5 ml PBS/1% FCS until required.

Culture procedures. The bone marrow cells were cultured in a mixture containing 30% FCS, 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO), 10^{-4} M mercaptoethanol (Sigma Chemical Co.), penicillin/streptomycin, and 0.9% methyl cellulose. Mo T lymphoblast

Table I. Monoclonal Antibodies Used to Fractionate Bone Marrow

Antibody	Specificity	Source and reference
Leu 1	T lymphocyte	Becton-Dickinson & Co.
Leu 5b	T lymphocyte, some NK cells	Becton-Dickinson & Co.
Leu 9	T lymphocytes	Becton-Dickinson & Co.
Leu 12	B lymphocytes	Becton-Dickinson & Co.
Campath 1	T and B lymphocytes	Hale et al. (17)
TG1	Myeloid maturation	Beverly et al. (12)
Leu M1	Monocytes, granulocytes	Becton-Dickinson & Co.
Leu M3	Monocytes	Becton-Dickinson & Co.
Mo 1	Monocytes, myeloid maturation, NK cells	Todd et al. (18)
My 8	Monocytes, myeloid maturation	Griffin et al. (19)
YTH 89.1.8	Glycophorin A	H. Waldmann
LICR.LON.R18	Glycophorin A	Edwards (20)
L243	HLA-DR	Becton-Dickinson & Co.

NK, natural killer.

cell line-conditioned medium (Mo-CM) (22) prepared from the Mo T lymphoblast cell line was kindly provided by Drs. D. Golde and J. Gasson, UCLA School of Medicine, Los Angeles, CA, and was added with GCT-cell line-conditioned medium (GCT-CM) (23) (Gibco, Grand Island, NY) at 10% of the final volume (5% each) as a source of BPA/GM-CSF. In one experiment, highly purified recombinant GM-CSF was used instead. Either human urinary EP (Terry Fox Laboratories, Vancouver, British Columbia, Canada) at 2 U/ml, or human REC obtained from transfected COS or CHO cells (5) was added to the cultures on day 0 or day 3. The day 3 additions were made either directly to the methylcellulose cultures or, in some experiments, to cells that had been suspended in IMDM/2% FCS with Mo/GCT CM in a tissue culture incubator at 37°C/5% CO₂ for 3 d. The total volume of these suspension cultures was 0.25 ml, and on day 3, 1 ml of the methylcellulose culture mixture was added to the suspension cultures and the mixture plated and returned to the incubator. The final concentration of cells was 1,000–50,000 cells/ml depending on the degree of enrichment expected for the different fractions. The cells were plated in 0.5-ml duplicates in flat-bottomed 24-well tissue culture plates (Linbro, Flow Laboratories Inc., McLean, VA) and incubated at 37°C in a high humidity 5% CO₂, 95% air incubator. CFU-E were counted on day 7 and BFU-E, CFU-GM, and CFU-MIX, comprising granulocytes and/or monocytes and erythroid cells, were counted on day 14.

Calculation of progenitor recovery. The number of colonies present in the Ficoll-Hypaque mononuclear cell fraction cultured in the presence of Mo-GCT CM was taken as 100%, and recovery of progenitors in all fractions related to this by multiplying colony number in each fraction by the cumulative proportion of cells recovered after that fractionation step, i.e., recovery = colony number × proportion of cells recovered × 100 ÷ colony number in the unfractionated sample (+ Mo/GCT).

Results

Effect of sequential fractionation of human bone marrow cells on colony growth. Fig. 1 shows the recovery and enrichment

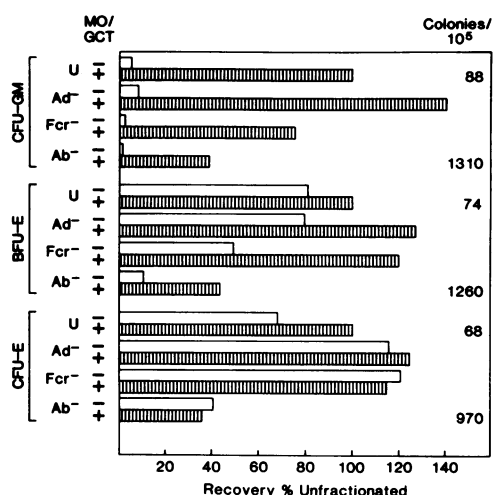


Figure 1. Fractionation of human bone marrow. U were sequentially fractionated by overnight adherence to plastic (Ad⁻); depletion of cells with receptors for the Fc portion of immunoglobulin by adherence (panning) to immunoglobulin coated petri dishes (Fcr⁻); and a second panning step to remove cells reacting with a panel of monoclonal antibodies recognizing maturation antigens (Ab⁻). Each fraction was cultured for progenitor colony growth without (open bars) and with (hatched bars) Mo/GCT CM, and EP was added day 0. The bar diagram indicates colony recovery as percentage of the colonies recovered in U cultured with Mo/GCT (100%). CFU-GM (upper panel) are almost completely dependent on Mo/GCT for proliferation in all fractions. In marked contrast to CFU-E (lower panel), BFU-E show increasing Mo/GCT dependence as the fractionation proceeds. U and Ab⁻ colony number per 10⁵ cells plated is shown on the right of the appropriate bars.

observed in a representative experiment when Ficoll-Hypaque separated mononuclear cells (U) were sequentially depleted of monocytes (Ad⁻), Fc receptor-bearing cells (Fcr⁻), and maturing precursor cells (Ab⁻), and each fraction was cultured in the presence or absence of Mo/GCT CM as a source of HGF. Human urinary EP was added on day 0. Recovery for all three progenitor classes was >70% through the first three steps, but dropped to ~40% after antibody panning. CFU-GM-derived colonies show marked dependence on Mo/GCT for proliferation in culture,

even in the culture of the unfractionated cell population before monocyte depletion. In contrast, CFU-E were not dependent on Mo/GCT for proliferation in this assay. A subset of BFU-E showed increasing dependence on Mo/GCT CM as monocytes, Fc receptor-bearing cells and maturing cells, including lymphocytes, were removed. Note that even the final Ad⁻ Fcr⁻ Ab⁻ fraction contained a proportion of BFU-E that were responsive to EP in the absence of Mo/GCT CM. A 14–17-fold enrichment was obtained, with 3.5% of the final fraction capable of colony formation. Representative cyto-spin differential counts of the cell fractions are shown in Table II. The majority of adherent cells are monocytes; Fc receptor-positive cells are mostly lymphoid cells, but include myeloid precursors and a significant number of monocytes; and antibody-positive cells comprise myeloid precursors, lymphocytes, and variable numbers of erythroid precursors and monocytes. The final Ad⁻ Fcr⁻ Ab⁻ fraction is enriched for myeloblasts and large mononuclear cells with prominent nucleoli, indented or oval nuclei, and basophilic fairly abundant cytoplasm. Variable numbers of promyelocytes/myelocytes contaminate this fraction.

Enrichment of progenitors using combined negative and positive selection. Fig. 2 shows the enrichment and recovery observed when bone marrow cells were depleted of adherent cells and maturing precursors, followed by a second panning step in which adherent HLA-DR-positive cells were detached from the plastic dishes after removal of the HLA-DR-negative nonadherent cells. 60–100% of progenitors were recovered in the HLA-DR-positive fraction. Virtually all CFU-GM and BFU-E were recovered in the positive fraction in contrast to CFU-E, of which a minority (10%) were recovered in the negative fraction. These results are similar to those previously reported using FACS analysis (24). All progenitor classes were greatly enriched, with BFU-E comprising 16%, and CFU-E and CFU-GM 5.7 and 5.5% of the final fraction, respectively (39–65-fold enrichment). The enrichment observed in this and two other experiments is summarized in Table III. A similar pattern of growth dependence on Mo/GCT was observed for the three progenitor classes to that shown in Fig. 1, and even the highly enriched HLA-DR-positive fraction, plated at very low density (1,000 cells/ml) contained BFU-E responsive to EP alone (data not shown).

Effect of delaying the addition of EP on BFU-E. When EP was added to either unfractionated or highly enriched bone mar-

Table II. Cyto-centrifuge Differential Counts (%)

Experiment		M		E		L	Mo	LMC	B
		l	e	i	p				
1	U	46	6	16	3.5	19	2.5	4.5	2.5
	Ab ⁻	5.5	8.5	2	0.5	5.5	1.5	68	8.5
2	U	24	12		14	44	4	0	2
	Ab ⁻	14	16		7	26	0	29	8
	Ad ⁺	2				13	85		
	Fcr ⁺	13	4		1	55	26	1	0
	Ab ⁺	19	6		3	67	2	2	1

Results from two representative experiments in which 200 cells from each fraction were counted. U and Ab⁻ fractions were separated as indicated in Fig. 1. Ad⁺ and Fcr⁺ cells were removed using a rubber policeman and Ab⁺ cells by repeated pipetting. M, myeloid; l, late precursors-myelocytes and metamyelocytes; e, early precursors-promyelocytes; E, erythroid; i, intermediate and late normoblasts; p, pronormoblasts and early normoblasts; L, lymphoid; Mo, monocyte; LMC, large basophilic mononuclear cells; B, blast.

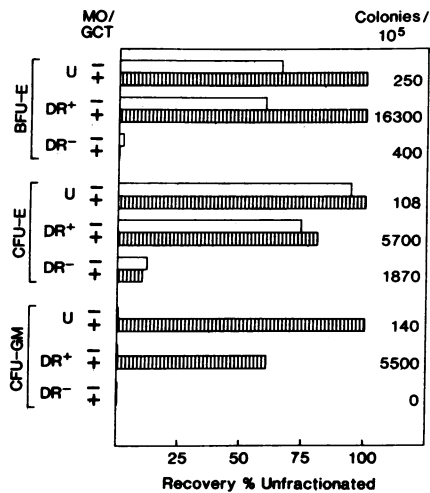


Figure 2. Enrichment of progenitors in human bone marrow. Progenitor recovery and colony growth (see Fig. 1) are shown for HLA-DR positive (DR⁺) and negative (DR⁻) cells obtained after sequential fractionation (see Methods). EP was added on day 0. 60–100% of progenitors were recovered; virtually all the BFU-E and CFU-GM were in the DR-positive fraction, whereas a minority of CFU-E were negative. Marked enrichment was obtained, especially for BFU-E in this experiment, but note that this enriched HLA-DR⁺ BFU-E population contained progenitors responsive to EP alone (open bar).

row BFU-E cultures on day 0 (Fig. 3, upper panel), considerable “background” BFU-E-derived colony formation was observed in both fractions. The background was unusually low for unfractionated cells in this experiment, and the results depicted in Fig. 1 are more typical. In contrast, when exposure to EP was delayed until day 3 and added either directly to the methylcellulose cultures (middle panel) or to preincubated suspension cultures, which were then plated in methylcellulose (lower panel), background colony formation in the absence of Mo/GCT CM was reduced, markedly in the highly enriched fractions. Results from the particular experiments depicted in Figs. 2 and 3 do

not show increasing dependence of enriched progenitors on Mo/GCT, as illustrated in Fig. 1. We therefore analyzed a larger series of experiments to investigate the effect of sequential depletion more thoroughly. The results for a series of 10 experiments comparing day 0 to day 3 EP addition are summarized in Fig. 4, with the ordinate showing the ratio of BFU-E-derived colonies observed without or with Mo/GCT CM. In this series of experiments, adherent cells, Fc receptor-positive cells, and T lymphocytes (Leu 1, 5b positive) were sequentially removed. It is evident from Fig. 4 that unfractionated cells to which EP was added on day 0 usually showed a response to Mo/GCT. Delayed addition of EP enhances this BPA dependence in all fractions, and BFU-E in Fc⁻ and T cell-depleted fractions are almost completely dependent on exogenous BPA for proliferation in culture.

Comparison of the effect of human urinary and recombinant EP on enriched progenitors. It was possible that the BFU-E-derived colonies observed in the highly enriched progenitor fractions without added Mo/GCT CM were responding to BPA contaminating the partially purified human urinary EP used in these experiments. The EP gene has recently been cloned and expressed in COS cells (5). We therefore compared the effect of human urinary EP with REC on BFU-E-derived colony formation in such enriched fractions. Fig. 5 showed that both human urinary and REC added at 2 U/ml on day 0 (upper panel) stimulated similar numbers of BFU-E-derived colonies in the presence or absence of Mo-CM. Since mock COS CM has no BPA (data not shown), these data suggest that a proportion of BFU-E respond to EP alone. Both types of EP induced very low background BFU-E-derived colony formation when the addition of EP was delayed until day two (lower panel).

We therefore wished to determine whether the fraction of BFU-E that were able to respond to day 0 addition of EP alone could survive in its absence if BPA was substituted instead. Hence, we compared (Fig. 6) the number of BFU-E-derived colonies induced by day 0 EP alone (abscissa) to the decline in colony number (Δ) observed in the presence of BPA when EP was added on day 3 compared with day 0 (ordinate). Linear regression analysis of the results of eight experiments (13 ob-

Table III. Enrichment of Progenitors Using Combined Negative and Positive Selection

Exp.	Cell fraction	Derived colonies/10 ⁵				CFC*	Recovery
		BFU-E	CFU-E	CFU-GM	CFU-MIX		
						%	%
1	U	250	108	140	ND†		
	DR ⁺	16,300	5,700	5,500	0	27.5	61–100
	DR ⁻	400	1,870	0	0		
2	U	118	98	100	4		
	DR ⁺	5,000	2,500	4,200	100	11.7	25–43
	DR ⁻	200	1,300	80	0		
3	U	142	72	212	6		
	DR ⁺	5,000	2,800	7,800	200	15.6	18–20
	DR ⁻	0	20	0	0		

Enrichment obtained for three experiments, including exp. 1, the results of which are shown in more detail in Fig. 2, in which bone marrow cells were sequentially fractionated to remove adherent cells and antibody-positive cells and then separated on the basis of HLA-DR expression.

* CFC, total colony-forming cells as a percentage of cells plated.

† ND, not done. Colony density was too high in this experiment to confidently enumerate CFU-MIX.

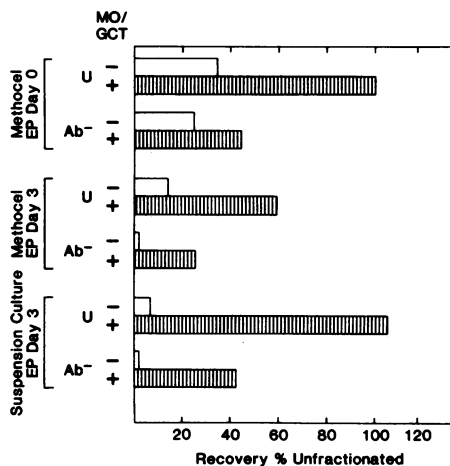


Figure 3. Effect of delayed EP addition on BFU-E derived colonies: recovery of BFU-E before (U) and after (Ab⁻) sequential fractionation. When EP was added on day 0 to methylcellulose cultures (*upper* panel), a proportion ($\frac{1}{3}$ – $\frac{1}{2}$) of BFU-E-derived colonies formed in the absence of Mo/GCT (open bars). However, when EP addition to either methylcellulose cultures (*middle* panel) or suspension cultures (*lower* panel) was delayed until day 3, very few BFU-E were observed in the Ab⁻ fraction in the absence of Mo/GCT. In this experiment, recovery in both day 3 EP additions is related to U + Mo/GCT day 0 EP. 176 BFU-E-derived colonies/10⁵ cells formed in this fraction.

servations) showed a significant correlation ($r = 0.88$, $P < 0.0001$ by a two-sided t test [25]) between the BFU-E-derived colony numbers for the individual experiments, whether the EP used was natural or biosynthetic, or whether EP was added on day 3 directly to methylcellulose cultures or to suspension cultures. These data suggest that the BPA-independent BFU-E population is the same subset of BFU-E that require EP from the initiation of progenitor culture. They will not survive 72 h in BPA alone.

BFU-E dose response to Mo/GCT CM. The dependence of a subset of highly enriched Ab⁻ BFU-E on an exogenous source of BPA when EP addition was delayed suggested that this would provide a useful assay for BPA. Pooling data from five experiments, Fig. 7 shows a normalized dose response of BFU-E to increasing concentrations of Mo-CM. A plateau was observed at 5% CM, and the mean maximal colony number at 10% Mo-

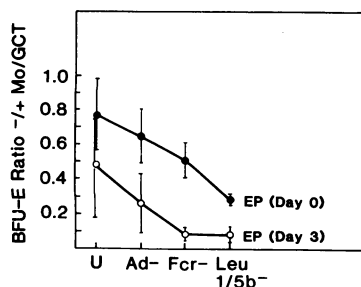


Figure 4. Summary of the effect of delayed EP addition on sequentially fractionated bone marrow. In this series of 10 experiments, the final step consisted of panning with two antibodies to T lymphocytes (Leu 1/5b), and the graph shows the ratio of BFU-E-derived colonies observed without or with

Mo/GCT for each fraction when EP was added on either day 0 (solid circles) or day 3 (open circles). In all fractions, delay of the addition of EP increased dependence on Mo/GCT and the day 3 addition Fcr⁻ and Leu 1/5b⁻ fractions were almost totally dependent on Mo/GCT for proliferation (ratio < 0.1). Colony number per 10⁵ U ranged from 17 to 142 BFU-E/10⁵ and recovery from 40 to 122%.

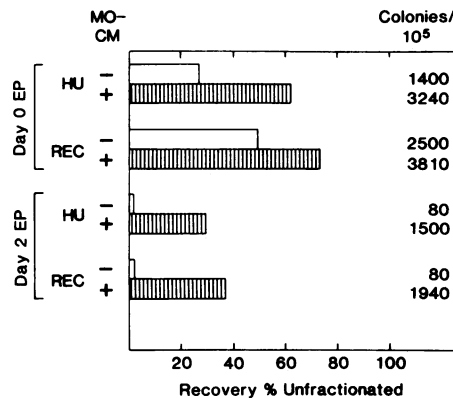


Figure 5. Comparison of the effect of human urinary (Terry Fox Laboratories) and REC on Ab⁻ enriched BFU-E. The histogram shows recovery of enriched Ab⁻ bone marrow BFU-E in relation to U + Mo-CM taken as 100% (not shown). BFU-E-derived colony number in this control fraction was 120/10⁵. Both sources of EP stimulated similar numbers of BFU-E-derived colonies in the presence or absence of Mo-CM, and very few colonies were observed in the absence of Mo-CM when EP addition was delayed until day 2 (open bars). CM from mock transfected COS cells had no EP or BPA activity.

CM was 1,415 BFU-E/10⁵ Ab⁻ BM cells (range 320–1,970). Background colony formation was very low in the absence of Mo-CM (mean 75, range 13–180 BFU-E/10⁵ cells).

Effect of increasing REC concentration on CFU-GM-derived colonies. In view of the hypothesis that EP can act as an instructive molecule and promote stem cell or bipotent progenitor commitment to erythropoiesis at the expense of other haemopoietic lineages, we carried out a dose response on highly enriched progenitors using REC (Fig. 8). We have recently shown that recombinant GM-CSF has BPA (6), and we used highly purified (specific activity $1-4 \times 10^7$ U/mg in an agar bone marrow CFU-GM assay [1]) recombinant GM-CSF as the source of BPA for this experiment to define the culture conditions as carefully as possible. The result showed no inhibitory effect of increasing

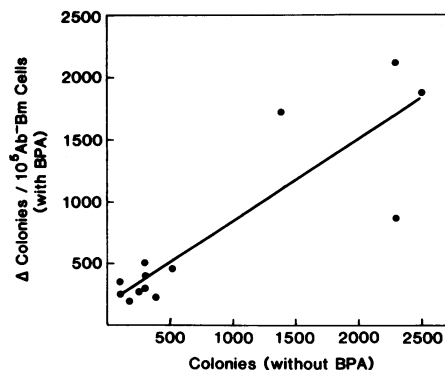


Figure 6. Relationship of EP to BPA requirement of a subset of BFU-E. The ordinate represents the difference (Δ) between the frequencies of BFU-E-derived colonies that appear in the presence of BPA when EP is added either on day 0 or day 3 to duplicate cultures of a BM progenitor concentrate. The abscissa represents the colonies that appear in the same cultures in the absence of BPA when EP is added on day 0. Linear regression analysis reveals a close correlation ($r = 0.88$, $P < 0.0001$ by a two-sided t test).

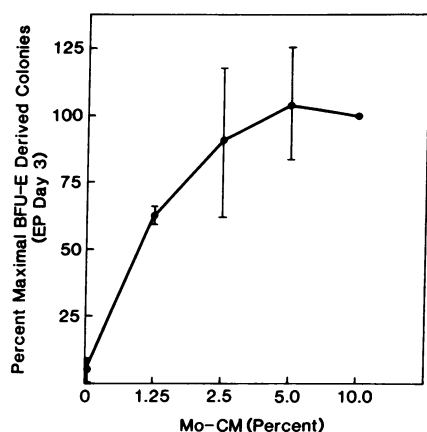


Figure 7. Normalized Mo-CM dose responses of enriched (Ab^-) bone marrow BFU-E after delayed addition of EP. Data were pooled from five experiments and shows mean \pm 1 SD. There are very few BFU-E-derived colonies (mean 75, range 13–180/ 10^5 cells) in the absence of Mo-CM and a plateau was observed at 5% Mo-CM. The maximal BFU-E number at 10% Mo-CM was 1,415 (range 320–1,970)/ 10^5 cells.

doses of erythropoietin on CFU-GM-derived colony numbers. At very high doses, the REC is apparently inhibitory, but this may have been due to the larger volume necessary to incorporate this dose in the cultures, or to other inhibitory components present in the EP preparation.

Discussion

In this report, we demonstrate that normal human bone marrow cells can be sequentially depleted of BPA-producing accessory cells to provide a final nonadherent, Fc receptor and multiple antibody-negative cell fraction that is considerably enriched for progenitors. A second positive selection panning step using an antibody to HLA-DR antigens results in further enrichment, with total colony-forming cells comprising 12–27% of this fraction, and progenitor and cell recovery, 19–100% and 0.5–1%, respectively. To our knowledge, this is the highest concentration of a workable population of normal human bone marrow progenitors achieved to date.

Previous attempts to enrich for progenitors in bone marrow have been beset with difficulties relating mainly to progenitor yield. Thus, although the FACS can be used in conjunction with

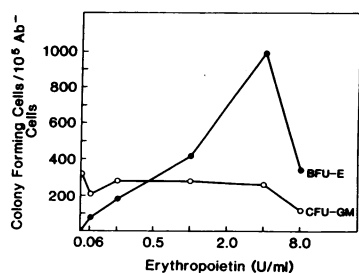


Figure 8. Dose response of enriched bone marrow progenitors to REC. Although a progressive linear BFU-E response was observed from 0.63 to 4.0 U/ml, CFU-GM-derived colony numbers remained constant over this range. Cultures contained 1 ng/ml recombinant GM-CSF (maximal stimulation for this material) and $5 \times 10^3 \text{ Ab}^-$ bone marrow cells/ml. EP was added on day 0.

tion for this material) and $5 \times 10^3 \text{ Ab}^-$ bone marrow cells/ml. EP was added on day 0.

monoclonal antibodies to select positively for progenitors (11, 12), impractically prolonged sorting times are necessary to select rare cells, and yields are often reduced (20–50%). Complement lysis is associated with significant nonspecific cell loss (13), and normal bone marrow progenitors may be sensitive to nonspecific damage by CrCl_3 -coupled erythrocytes that are used for immune rosetting. Immunoabsorption to antibody-coated plates (panning) can be used for negative and positive selection (21), and is a simple, reproducible procedure that can provide highly enriched progenitor fractions, with moderately reduced yields. In this respect, our results are not as good as those we obtained when separating human fetal liver progenitors (yields 60–100%) (16), but fetal liver cells are two- to fivefold enriched for progenitors compared with bone marrow, and do not require such extensive fractionation. The results do, however, compare very favorably with other attempts to enrich human bone marrow progenitors (12, 14, 26).

The sequential fractionation data demonstrate that the majority of bone marrow BFU-E require a source of exogenous BPA for their survival in vitro. In unfractionated marrow, the BPA requirement for these BFU-E is partially met by adherent cells, $\text{Fc}\gamma$ cells, and T lymphocytes. The increasing dependence of this subset of BFU-E in sequentially fractionated bone marrow cells on an exogenous source of BPA supports previous evidence and recent data from this laboratory (14) that bone marrow monocytes (14, 27–29), Fc receptor-bearing cells (14), and T lymphocytes (14, 29–32) produce BPA or collaborate in its production. However, even highly enriched progenitor fractions contain a fraction of BFU-E responsive to EP in the absence of BPA and unaffected by accessory cell depletion. That this BFU-E population is responsive to EP and not a BPA contaminant in human urinary EP is shown by the similar results obtained when REC was used. Furthermore, the number of day 0 EP-responsive BFU-E-derived colonies correlates significantly with the decline in BFU-E observed when the addition of EP is delayed until day 3 in the presence of Mo/GCT CM. This suggests that once BFU-E have become EP sensitive, they require exposure to the hormone for survival and terminal differentiation. They appear to die or become unresponsive in its absence. Thus, although most BFU-E survive in the presence of BPA for 3 d, a smaller proportion are BPA independent and require exposure to EP for terminal differentiation to occur. We presume that the former represent immature, and the latter, mature, BFU-E. The results support previous evidence suggesting that BFU-E are heterogeneous with respect to their BPA and EP responsiveness (33, 34), and are consistent with recent short-term bone marrow liquid culture results suggesting that day 15 BFU-E are sensitive to EP (35).

The EP responsive BPA-independent BFU-E population has made it difficult to assay for BPA in the past, since EP is essential for terminal maturation of all erythroid colonies. When EP is added to cultures on day 0, the result is a high background of BPA-independent BFU-E-derived colonies that are indistinguishable from the BFU-E-derived colonies forming in the presence of BPA. However, if accessory cell-depleted progenitors are used as target cells, the simple maneuver of delaying the addition of EP until day 3 can obviate this problem by eliminating BFU-E responsive to EP alone. A subset of (presumably) less mature BFU-E then show a typical dose response to increasing concentrations of Mo/GCT CM, and provide a sensitive BFU-E population for BPA assay. We have recently used this assay to show

that highly purified recombinant GM-CSF, cloned from the Mo cell line, has significant BPA (6).

The availability of recombinant GM-CSF and EP, and enriched progenitors, has made it possible to reexamine the question of whether EP can act as an instructive molecule and influence progenitor commitment toward erythropoiesis at the expense of granulopoiesis, as suggested by van Zant and Goldwasser (36, 37). Our results do not support this view, since the frequency of CFU-GM-derived colonies was constant despite a wide variation in EP concentration and resultant erythroid colonies.

In conclusion, the ability to obtain highly enriched human bone marrow progenitors using a relatively simple method will be valuable for investigating the regulation of hemopoiesis at a cellular level. This will be particularly relevant as more highly purified and recombinant human growth factors become available. The ability to assay more accurately for BPA, and for BPA and erythropoietin-responsive progenitors, should allow an improved evaluation of these parameters in hypoplastic anemias and myeloproliferative disorders, and thereby offer opportunities to gain further insight into their pathophysiology and effective treatment.

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