Proliferative Responses to Interleukin-3 and Granulocyte Colony-Stimulating Factor Distinguish a Minor Subpopulation of CD34-Positive Marrow Progenitors That Do Not Express CD33 and a Novel Antigen, 7B9

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Human hematopoietic colony-forming cells (CFC) express the CD34 antigen ($CD34^+$) as well as differentiation antigens such as CD33 and HLA-DR. $CD34^+$ cells that do not express these latter differentiation antigens have been shown to contain few CFC in direct culture, but generate increasing numbers of CFC when cultured over a marrow stromal cell layer in the long-term culture system. In this study we determined if $CD34^+$ cells with low or absent expression of CD33 and a novel antigen, 7B9 ($CD34^+CD33^-7B9^-$), could be distinguished from $CD34^+$ cells expressing these antigens ($CD34^+CD33^+7B9^+$) based on their proliferative responses to interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) in a short-term liquid culture system. These two populations were separated by fluorescence-activated cell sorting, cultured with IL-3 (10 ng/mL), G-CSF (100 ng/mL), or IL-3 and G-CSF, and ^{3}H-thymidine uptake was measured. $CD34^+CD33^-7B9^-$ cells proliferated in the presence of IL-3, but not G-CSF. However, a synergistic response to the combination of IL-3 and G-CSF was seen in most experiments. In contrast, $CD34^+CD33^+7B9^+$ cells proliferated in the presence of either IL-3 or G-CSF but did not display an additive or synergistic response to the combination of IL-3 and G-CSF. In colony-forming assays performed before and after liquid culture, the $CD34^+CD33^-7B9^-$ cells in two experiments contained 0.3% and 2.2% of all sorted marrow CFC before liquid culture and generated 40-fold and ninefold increases in the number of granulocyte-macrophage colony-forming units (CFU-GM), respectively, after liquid culture with IL-3 and G-CSF. In contrast, the $CD34^+CD33^+7B9^+$ cells contained 99.7% and 97.8% of all sorted marrow CFC before liquid culture and had no change or a threefold increase in the number of CFU-GM, respectively, after liquid culture with IL-3 and G-CSF. Single-cell liquid cultures containing IL-3 and G-CSF with cells that were either $CD34^+CD33^-7B9^-$ and depleted of mature lymphoid cells ($CD34^+lin^-$) or were $CD34^+lin^+$ showed that a higher proportion of wells containing a $CD34^+lin^-$ cell gave rise to one or more CFC (8.7%) than did wells containing a $CD34^+lin^+$ cell (2.9%), with the responding cells in the former population giving rise to an average of 2.9 ± 0.6 CFC and in the latter population, 2.0 ± 1.0 CFC. Thus, we have identified a presumably immature precursor population that is $CD34^+CD33^-7B9^-$ and is distinguishable from a population containing the vast majority of CFC that is $CD34^+CD33^+7B9^+$ based on its proliferative responses to IL-3 and G-CSF and its ability to generate increased numbers of CFC upon culture in the presence of these factors.

WITHIN THE hematopoietic hierarchy, cells at different stages of development can be distinguished on the basis of their proliferative and differentiative potential. The development of clonal semisolid culture assays for the quantitation and characterization of colony-forming cells (CFC) has provided important insights into this hierarchy.[1,2] Studies from this laboratory and others have shown that, in humans, CFC are present in a population of cells that expresses the CD34 antigen and a number of cell surface differentiation antigens such as CD33 and HLA-DR.[3,4] There are also rare precursors that express the CD34 antigen ($CD34^+$), but not antigens such as CD33 and HLA-DR. These rare precursor populations contain few CFC, but have been shown to generate increasing numbers of CFC, including multiple CFC from single cells, when cultured in the presence of a marrow stromal layer in the long-term culture system.[3-5] Other primitive hematopoietic progenitor cells that give rise to blast cell colonies have also been shown to express CD34 and lack expression of CD33 or HLA-DR.[6-8]

In the present study, we used a short-term liquid culture system in the absence of a marrow stromal layer to ask if marrow cells that express the CD34 antigen, but not antigens usually associated with CFC, were distinguishable from the $CD34^+$ population expressing these antigens and containing the majority of CFC. To separate the majority of CFC from more primitive precursors we selected $CD34^+$ cells based on their expression or lack thereof, of CD33 and a novel antigen recognized by monoclonal antibody (MoAb), 7B9 (referred to as 7B9 antigen). CD33 is known to be expressed by virtually all granulocyte-macrophage colony-forming units (CFU-GM) and a portion of erythroid burst-forming units (BFU-E), but not their precursors.[3,9,10] MoAb 7B9 identifies a novel antigen present on virtually all BFU-E and a portion of CFU-GM, but not on the precursors of CFC detectable in long-term culture (Brashem-Stein et al, manuscript in preparation). We demonstrated that $CD34^+$ marrow cells with low or absent expression of CD33 and/or 7B9 antigen contained $\leq 2\%$ of CFC and could be distinguished from $CD34^+$ cells that expressed both of these antigens and contained the vast majority of CFC. These differences were based on their proliferative responses to interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) and their potential to generate CFC progeny.

MATERIALS AND METHODS

Bone marrow samples. Marrow samples were obtained from normal donors after informed consent under an Institutional

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Review Board-approved protocol at the Fred Hutchinson Cancer Research Center, Seattle, WA. Erythrocytes and mature granulocytes were depleted by Ficoll-Hypaque density gradient centrifugation (d, 1.077) and residual erythrocytes were further depleted by lysis with a 0.85% ammonium chloride solution.

Antibody preparation and purification. Monoclonal IgM antibodies 12-8 (anti-CD34) and isotype control H12C12 (anti-mouse Thy 1.2) and monoclonal IgG antibodies p67-6 (anti-CD33), 7B9, HD37 (anti-CD19), 1F5 (anti-CD20), 35.1 (anti-CD2), 24.1 (anti-CD10) and isotype control 31.A (anti-mouse Thy 1.1) were purified from ascites (except H12C12, 35.1, and 24.1, which were used at a 10^{-3} dilution of ascites) and used at 20 μg/mL for the IgM and 10 μg/mL for the IgG antibodies as previously described.[3]

Staining and sorting. Cells were stained using indirect immunofluorescent antibody staining techniques and separated using fluorescence-activated cell sorting (FACS) as previously described.[3] All staining was done with cells suspended in sterile phosphate-buffered saline (PBS) supplemented with 2% human AB serum (GIBCO Laboratories, Grand Island, NY). Two-color staining was performed by incubating cells with antibodies 12-8, p67-6, and 7B9 (and in some experiments HD37, 1F5, 24.1, and 35.1). Control cells were incubated with: (1) H12C12 and 31.A; (2) H12C12, p67-6, and 7B9 (and HD37, 1F5, 24.1, and 35.1 in some experiments); or (3) 12-8 and 31.A. Control and experimental cells were incubated with the primary antibodies for 20 to 30 minutes at 4°C, washed twice, then incubated with a 1:40 dilution of phycoerythrin-conjugated goat anti-mouse IgM antisera (μ-chain specific) (Calbiochem Corp, La Jolla, CA) and a 1:40 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (γ-chain specific) (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD) for 20 to 30 minutes at 4°C, and washed twice. Cells were analyzed and sorted on a FACS II (Becton Dickinson and Co, Oxnard, CA). Cells stained with antibody 12-8 ($CD34^+$) had higher fluorescence levels than 99.7% ± 0.2% (range 99.3% to 99.9%) of the cells stained with control antibody, H12C12. These $CD34^+$ cells were isolated using two-color FACS based on the amount of their staining with p67-6 and/or 7B9. Only those cells with low right angle light scatter were selected.

Two groups of cells were isolated. One group was p67-6 and 7B9 low or negative with fluorescence less than the upper 10% of cells stained with control antibody (31.A) and referred to hereafter as $CD34^+CD33^-7B9^-$. The second group, which was separated from the first by five channels, was p67-6 ($CD33^+$) and/or 7B9 positive with fluorescence greater than 92% to 97% of $CD34^+$ cells stained with the control antibody (31.A) and referred to hereafter as $CD34^+CD33^+7B9^+$. After sorting, cells were collected in RPMI supplemented with 10% calf bovine serum.

In single-cell cloning experiments, $CD34^+CD33^-7B9^-$ cells were also stained with antibodies against lymphoid-associated antigens including HD37 (CD19), 1F5 (CD20), 35.1 (CD2), and 24.1 (CD10) and sorted into two groups. We collected groups of cells that were $CD34^+CD33^-7B9^-$ and $CD19^-$, $CD20^-$, $CD2^-$, $CD10^-$ ($CD34^+$, lineage negative or $CD34^+lin^-$), or $CD34^+$ and positive for one or more of the lineage antigens ($CD34^+lin^+$). In these experiments, cells were further selected based on their forward light scatter properties with only the blast size cells (those with high forward light scatter) collected. The FACS II was equipped with a modified single-cell deposition unit and single cells from these two groups were sorted directly into individual U-bottom microtiter wells of 96-well plates (Costar, Cambridge, MA). To confirm that single events were sorted, fluorescent beads (Epics Division of Coulter Corp, Hialeah, FL) were selected for their bright fluorescence and sorted at one bead per well. The number of wells with beads was visually assessed on a fluorescent microscope and the cloning efficiency was calculated. Of 192 wells cloned in two experiments, 93% contained single beads and 7% contained no beads. Wells with more than one bead were never observed.

Liquid cultures. Immediately after sorting, aliquots of $CD34^+CD33^-7B9^-$ and $CD34^+CD33^+7B9^+$ cells were placed in triplicate or quadruplicate in individual microtiter wells in 200 μL of Iscove's modified Dulbecco's medium (IMDM; GIBCO) supplemented with 20% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) alone, or with IL-3, G-CSF, or IL-3 and G-CSF. The recombinant human IL-3 and G-CSF, produced in yeast expression systems and purified as previously described,[11] were kindly provided by Dr David Urdal, Immunex Corporation, Seattle, WA. The plates were then incubated in a humidified environment at 37°C with 5% CO_2 in air. The same growth conditions were used for the single cell experiments with $CD34^+lin^-$ and $CD34^+lin^+$ cells.

^{3}H-thymidine (^{3}H-TdR) assay. After varying intervals of time, 20 to 25 μL of IMDM containing 1 μCi of ^{3}H-TdR (74 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added to each microtiter well. After overnight incubation, the plates were frozen, thawed, and the cells harvested onto glass-fiber filters with a cell harvester (Cambridge Technology, Inc, Watertown, MA). Filters were placed into vials (Wheaton Scientific, Millvale, NJ) with Ready Safe liquid scintillation cocktail (Beckman Instruments, Inc, Fullerton, CA), and radioactivity was measured as counts per minute (cpm) in a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL).

Colony-forming assays. The ability of cells to form granulocytic and monocytic (CFU-GM) and erythroid (BFU-E) colonies was determined by culturing cells in IMDM supplemented with 20% FCS, 20% human placental conditioned medium (HPCM), 10 ng/mL IL-3, 2 U/mL human recombinant erythropoietin (Amgen, Thousand Oaks, CA), 10^{-4} mol/L 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA) and 0.3% agar (Seaplaque; FMC Corp, Rockland, ME). In some experiments 0.9% methylcellulose (Terry Fox Laboratories, Vancouver, BC) and 2% bovine serum albumin (Intergen Company, Purchase, NY) were substituted for the agar. All cultures were incubated at 37°C in 5% CO_2 in air in a humidified incubator. After 14 to 16 days of culture, colonies of different types were scored using an inverted microscope.

Statistical methods. For the ^{3}H-TdR and colony-forming assays, the data was transformed by taking the square root of each value of cpm or colony number to normalize the variance and the mean and standard deviation of these transformed values determined. A pooled variance was calculated for each population for each experiment and the significance of differences estimated with a two-sided *t*-test.

RESULTS

Proliferative responses of isolated $CD34^+CD33^-7B9^-$ and $CD34^+CD33^+7B9^+$ cells to IL-3 and G-CSF in liquid culture. Normal marrow cells were selected by two-color fluorescence based on their expression of the CD34 antigen and the presence or absence of CD33 antigen and/or 7B9 (Fig 1). In a series of 13 experiments, $CD34^+$ cells represented 1.2% ± 0.1% (range 0.5% to 1.7%) of all mononuclear cells remaining after Ficoll-Hypaque separation. The $CD34^+CD33^-7B9^-$ population was 21% ± 3% (range 3% to 35%) of all $CD34^+$ cells, or 0.23% ± 0.03% of all cells sorted, and the $CD34^+CD33^+7B9^+$ population was 77% ± 3% (range 61% to 95%) of all $CD34^+$ cells, or 0.85% ± 0.10% of all cells sorted.

Sorted cells were placed in liquid culture in microtiter wells with or without IL-3 (10 ng/mL) or G-CSF (100 ng/mL). After 3 or 4 days, their proliferative activity was

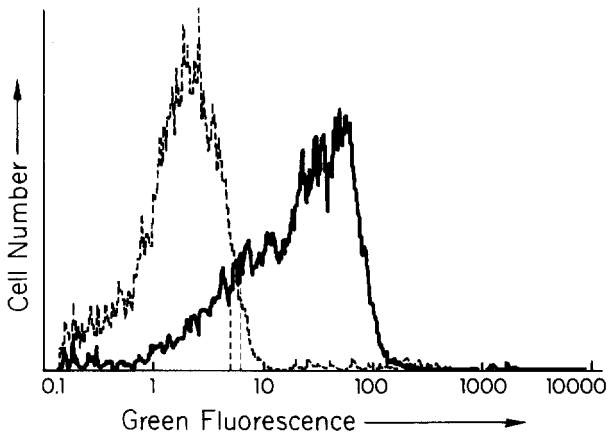

Fig 1. Gated fluorescence histogram of CD34⁺ marrow cells, identified by staining with antibody 12-8, simultaneously stained with the anti-CD33 antibody, p67-6, and antibody 7B9 (—) or with the control antibody 31.A (----). Fluorescence was analyzed on a FACS II equipped with 5 log amplifiers for the photomultipliers. The cell number is displayed using a linear scale. The thin vertical dashed lines indicate the locations of the sorting windows. The CD34⁺CD33⁻7B9⁻ population is to the left of the sorting windows and the CD34⁺CD33⁺7B9⁺ population is to the right.

measured by incorporation of ^{3}H-TdR. As shown in Table 1, the CD34⁺CD33⁻7B9⁻ population cultured with IL-3 displayed a twofold to threefold increase in ^{3}H-TdR incorporation compared with cells cultured with medium or G-CSF. Significant proliferation ($P < .05$) in response to IL-3 was seen in a total of five of six experiments. In four experiments where G-CSF was studied, an increase in ^{3}H-TdR incorporation in the presence of G-CSF was noted on only one occasion.

In contrast, the CD34⁺CD33⁺7B9⁺ population proliferated in response to either G-CSF or IL-3 and displayed a twofold to threefold greater response to G-CSF than to IL-3 (Table 1). This same result was observed in four additional experiments.

Table 1. ^{3}H-TdR Incorporation (cpm) of CD34⁺CD33⁻7B9⁻ and CD34⁺CD33⁺7B9⁺ Cells After Incubation With IL-3 and G-CSF

Growth Factors	Experiment No. 1	Experiment No. 2
CD34⁺CD33⁻7B9⁻ Cells		
None	237	573
G-CSF	225	810
IL-3	704*	1,481*
CD34⁺CD33⁺7B9⁺ Cells		
None	269	1,032
G-CSF	2,228†	22,693†
IL-3	1,163†	8,352†

CD34⁺CD33⁻7B9⁻ and CD34⁺CD33⁺7B9⁺ cells were incubated in triplicate for 3 to 4 days in liquid culture followed by overnight incubation with ^{3}H-TdR. Values are adjusted to represent mean cpm per 2,000 cells plated.

*IL-3 responses significant at $P < .05$ level compared with control and G-CSF.

†IL-3 and G-CSF responses significant at $P < .01$ level compared with control.

Time course of proliferative responses of isolated CD34⁺CD33⁻7B9⁻ and CD34⁺CD33⁺7B9⁺ cells in liquid culture to IL-3, G-CSF, and the combination of IL-3 and G-CSF. Isolated CD34⁺CD33⁻7B9⁻ and CD34⁺CD33⁺7B9⁺ cells were cultured in microtiter wells (2,000 cells/well) with or without IL-3, G-CSF, or the combination of IL-3 and G-CSF. The course of the proliferative activity of the two cell populations at days 3, 6, 9, and 12 was assessed in two experiments (Fig 2). In the first experiment, CD34⁺CD33⁻7B9⁻ cells (Fig 2A) displayed significantly greater proliferation in response to IL-3 than to G-CSF, but in this experiment the increased proliferation observed when the two growth factors were combined was not statistically different from that seen with IL-3 alone. However, in a second experiment, the CD34⁺CD33⁻7B9⁻ cells (Fig 2C) did not respond to IL-3 alone, but displayed a synergistic response to IL-3 plus G-CSF. This synergistic response was seen in two of three additional experiments (data not shown). In contrast, the

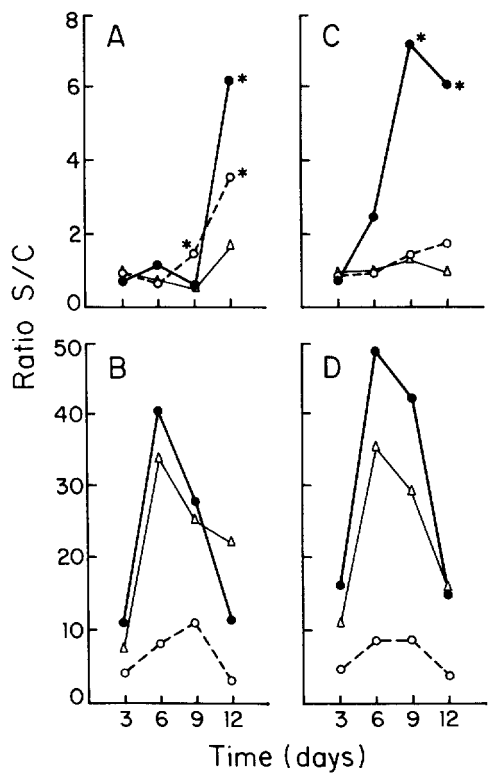

Fig 2. Time course of growth factor responses. Data is presented as the ratio (stimulated/control [S/C]) of the means of ^{3}H-TdR cpm values for cultures stimulated with growth factors (stimulated) compared with medium (control). Cultures were in triplicate or quadruplicate at each of the time points tested. (A) and (C) represent CD34⁺CD33⁻7B9⁻ cells in each of two experiments, respectively, and (B) and (D) represent the CD34⁺CD33⁺7B9⁺ cells in those same experiments. Cells were cultured in the presence of: G-CSF (△); IL-3 (○); and IL-3 and G-CSF (●). Values that were statistically different ($P < .02$) from medium control are marked by an asterisk (*) for the CD34⁺CD33⁻7B9⁻ cells. For the CD34⁺CD33⁺7B9⁺ cells all growth factor responses were significantly different from medium control.

$CD34^+CD33^+7B9^+$ cells (Fig 2B and D) again displayed a greater response to G-CSF than to IL-3 and the combination induced proliferation that, while greater than that seen with either growth factor alone, was neither additive nor synergistic. The tempo of response of the $CD34^+CD33^+7B9^+$ cells was more rapid with the peak proliferative response observed at day 6 compared with that displayed by the $CD34^+CD33^-7B9^-$ cells that had a peak proliferative response at days 9 to 12. The absolute magnitude of response displayed by the $CD34^+CD33^+7B9^+$ cells was also greater. It is possible that further increases in ^{3}H-TdR incorporation after day 6 of the $CD34^+CD33^+7B9^+$ cells were prevented by limiting culture conditions.

Dose titration of growth factors. Dose titration studies of IL-3 with $CD34^+CD33^-7B9^-$ and $CD34^+CD33^+7B9^+$ cells showed that maximal proliferation was observed at concentrations of 10 to 100 ng/mL (Fig 3). When IL-3 was titered in the presence of a constant dose of G-CSF of 100 ng/mL, the $CD34^+CD33^-7B9^-$ population displayed peak ^{3}H-TdR incorporation at IL-3 doses of 50 to 100 ng/mL, which was significantly different from that seen with IL-3 alone, and the response seen was a synergistic one. G-CSF induced little proliferation by itself (Fig 3A). However, when IL-3 was titered in the presence of 100 ng/mL of G-CSF with the $CD34^+CD33^+7B9^+$ population, neither synergistic nor additive responses were found (Fig 3B). G-CSF doses of 10, 50, 100, and 200 ng/mL were also titered in the presence of a constant dose of IL-3 of 10 ng/mL. For each cell population no significant difference in ^{3}H-TdR uptake was noted between the doses of G-CSF tested (data not shown).

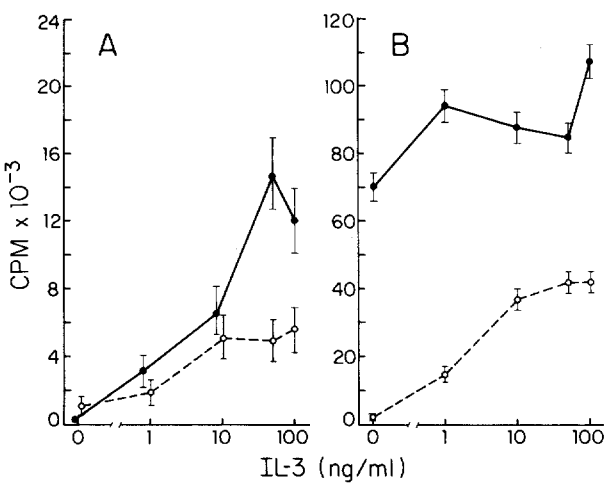

Fig 3. Dose titration of growth factors. The proliferative response of $CD34^+CD33^-7B9^-$ cells (A) and $CD34^+CD33^+7B9^+$ cells (B) is shown. Data is presented as the mean ± 1 SEM of triplicate ^{3}H-TdR cpm values after 9 days of incubation with growth factors in liquid culture. IL-3 was titered with (●) and without (○) G-CSF at a constant concentration of 100 ng/mL. For the $CD34^+CD33^-7B9^-$ cells the IL-3 and IL-3 plus G-CSF curves were significantly different ($P < .01$) from each other at IL-3 dose levels of 50 and 100 ng/mL. For the $CD34^+CD33^+7B9^+$ cells, values for IL-3 and IL-3 plus G-CSF were significantly different from each other ($P < .001$) at all concentrations of IL-3 tested. The values for the IL-3 and G-CSF curve were all significantly different from G-CSF alone ($P < .05$), but not from each other.

Generation of CFC by $CD34^+CD33^-7B9^-$ and $CD34^+CD33^+7B9^+$ cells in liquid culture with IL-3 and G-CSF. Aliquots of sorted cells were cultured directly in a semisolid medium CFC assay or placed in liquid culture with medium only or medium supplemented with IL-3, G-CSF, or IL-3 and G-CSF. After 9 days in liquid culture, cells were plated in semisolid medium to enumerate CFC. In two experiments (Table 2), $CD34^+CD33^-7B9^-$ cells contained 0.3% and 2.2% of all sorted marrow CFC in direct culture. In the first experiment, the $CD34^+CD33^-7B9^-$ cells displayed a threefold to fourfold increase in CFU-GM after liquid culture containing IL-3 and a 40-fold increase of CFU-GM with appearance of BFU-E after liquid culture containing IL-3 and G-CSF. No increase was seen in cultures with G-CSF alone in the numbers of CFC. In the second experiment, the number of CFU-GM in the $CD34^+CD33^-7B9^-$ cells increased ninefold without a change in the number of BFU-E after culture with IL-3 and G-CSF.

In contrast, the $CD34^+CD33^+7B9^+$ cells, which contained 99.7% and 97.8% of marrow CFC in the two experiments before liquid culture, showed either no change or a decrease in colony numbers after culture with IL-3 and/or G-CSF in the first experiment and a modest threefold increase in CFU-GM, but a sixfold decrease in the number of BFU-E in the second experiment.

Generation of CFC by single $CD34^+lin^-$ and $CD34^+lin^+$ cells in liquid culture with IL-3 and G-CSF. We next determined the proportion of $CD34^+CD33^-7B9^-$ cells capable of generating CFC in liquid culture, and whether each CFC was generated from an individual precursor cell or whether multiple CFC were generated from precursors with high proliferative potential. This was accomplished by further enriching for myeloid precursors in the $CD34^+CD33^-7B9^-$ population by also depleting cells expressing the lymphoid antigens CD2, CD10, CD19, and CD20. We also sorted only those cells with light scatter properties of blast cells. These $CD34^+lin^-$ cells were sorted directly into microtiter wells at one cell per well (216 total cells cloned) in liquid culture with IL-3 (10 ng/mL) and G-CSF (20 ng/mL). $CD34^+$ cells expressing one or more of the above antigens ($CD34^+lin^+$) were also cloned at one cell per well with IL-3 and G-CSF (72 cells cloned). The $CD34^+lin^-$ and $CD34^+lin^+$ cells were also directly plated at 250 cells per culture dish to assay for the number of CFC in the starting cells. After 7 days in liquid culture, cells were plated in methylcellulose to enumerate CFC.

In the presence of IL-3 and G-CSF, 8.7% of wells with $CD34^+lin^-$ cells produced one or more CFC, while only 2.9% of wells containing a $CD34^+lin^+$ cell produced CFC (Table 3). When an individual cell gave rise to one or more CFC in the $CD34^+lin^-$ population, an average of 2.9 ± 0.6 CFC was observed and the $CD34^+lin^+$ cells produced an average of 2.0 ± 1.0 CFC per well giving rise to CFC. At the initiation of this experiment the $CD34^+lin^-$ population had 6.2 CFC per 100 starting cells as compared with 25.2 CFC per 100 starting cells after liquid culture with IL-3 and G-CSF. In contrast, the $CD34^+lin^+$ cells, which had 20.8 CFC per 100 starting cells at the initiation of cultures, had

Table 2. Colony Formation Before and After Incubation With IL-3, G-CSF, or IL-3 + G-CSF

Experiment No.	Cell Type	Direct Culture (CFC per 2,000 cells plated)		After 9 Days in Liquid Culture With: (CFC per 2,000 cells plated in liquid culture)							
				Medium		G-CSF		IL-3		IL-3 + G-CSF	
		CFU-GM	BFU-E	CFU-GM	BFU-E	CFU-GM	BFU-E	CFU-GM	BFU-E	CFU-GM	BFU-E
1	$CD34^+CD33^-7B9^-$	3 ± 2	0	0	0	1 ± 1	0	11 ± 4	0	124 ± 92	9 ± 9
	$CD34^+CD33^+7B9^+$	136 ± 14	13 ± 3	69 ± 7	2 ± 0	79 ± 6	3 ± 0	110 ± 8	10 ± 3	124 ± 18	2 ± 0
2	$CD34^+CD33^-7B9^-$	5 ± 1	2 ± 1	0	0	0	0	8 ± 3	1 ± 1	47 ± 1	3 ± 2
	$CD34^+CD33^+7B9^+$	96 ± 5	19 ± 3	93 ± 8	1 ± 1	141 ± 5	6 ± 2	342 ± 13	5 ± 0	214 ± 14	3 ± 1

After sorting, $CD34^+CD33^-7B9^-$ and $CD34^+CD33^+7B9^+$ cells were directly cultured (2,000 cells/plate) in agar with IL-3 (10 ng/mL), 20% HPCM, and erythropoietin (2 U/mL), or were placed in liquid culture (2,000 cells/well) with medium, IL-3 (10 ng/mL), G-CSF (100 ng/mL), or IL-3 and G-CSF (same concentration) for 9 days and then cultured in agar with the same growth factors as above. Colony counts are expressed as the mean ± 1 SEM.

only 5.8 CFC per 100 starting cells after liquid culture with IL-3 and G-CSF. These same results for single $CD34^+lin^-$ and $CD34^+lin^+$ cells were confirmed in a second experiment (data not shown).

DISCUSSION

These studies examined the proliferative and differentiative responses of subsets of $CD34^+$ human marrow cells that express or lack antigens usually associated with committed progenitors. These antigens include CD33 and a novel antigen defined by antibody 7B9. We have previously shown that anti-CD33 antibody and complement will lyse nearly all CFC of both myeloid and erythroid type.[10] However, because CD33 is expressed in low amounts on a portion of BFU-E and may not be detected by immunofluorescence assays, $CD33^+$ cells obtained by FACS contain a variable portion of BFU-E.[3,9] Therefore, we also used the recently developed MoAb, 7B9, which stains most BFU-E in sufficiently high amounts to permit virtually complete selection of these cells by FACS (Brashem-Stein et al, manuscript in preparation). Cell separation studies demonstrated that 98% or more of CFC are present in the $CD34^+CD33^+7B9^+$ marrow population.

Our results indicate that the $CD34^+CD33^-7B9^-$ population contains progenitors distinguishable from the vast majority of progenitors that are present in the $CD34^+CD33^+7B9^+$ population, both on the basis of their pattern of proliferative responses to recombinant IL-3 and G-CSF in liquid culture and their ability to give rise to increased numbers of CFC after liquid culture with these two growth factors.

Table 3. Single-Cell Cloning of $CD34^+lin^-$ and $CD34^+lin^+$ Cells

Cells	CFU-GM (% wells containing CFC)	BFU-E (% wells containing CFC)	Average No. CFC/ Positive Well*	Total No. CFC/100 Starting Cells†
$CD34^+lin^-$	6.8	1.9	2.9 ± 0.6	25.2
$CD34^+lin^+$	2.9	0	2.0 ± 1.0	5.8

Single $CD34^+lin^-$ or $CD34^+lin^+$ cells were sorted directly into microtiter wells containing IMDM, 20% FCS, IL-3 (10 ng/mL), and G-CSF (20 ng/mL). After 7 days the contents of wells were cultured in methylcellulose with IL-3 (10 ng/mL), 20% HPCM, and erythropoietin (2 U/mL). Colony counts were performed at 14 days.

*A positive well was one containing CFC.

†For the $CD34^+lin^-$ and $CD34^+lin^+$ cells there were 6.2 and 20.8 CFC/100 cells, respectively, at the initiation of culture.

The $CD34^+CD33^-7B9^-$ population was found to contain cells that generally do not proliferate in the presence of G-CSF and proliferate variably in the presence of IL-3. However, a synergistic response to these two factors in combination was observed in most experiments. Importantly, culture of these enriched cells in medium containing IL-3 and G-CSF amplified the number of detectable CFC present in this population. In contrast, the $CD34^+CD33^+7B9^+$ fraction of cells that contained virtually all marrow CFC, proliferated in the presence of either IL-3 or G-CSF alone, but did not display an additive or synergistic response to the combination of the two factors. Importantly, this cell population did not show a substantial increase in detectable CFC after culture with IL-3, G-CSF, or the combination of IL-3 and G-CSF. These findings demonstrate the more limited proliferative potential of the vast majority of the $CD34^+CD33^+7B9^+$ cells that responded to the growth factors.

Experiments where single cells were cultured in the presence of IL-3 and G-CSF and then tested for CFC activity, demonstrated that a higher portion of $CD34^+lin^-$ cells as compared with $CD34^+lin^+$ cells formed colonies, and that multiple CFC were generated from individual precursors. As the number of individual $CD34^+lin^-$ cells yielding CFC approximated the numbers of CFC present before liquid culture, it was not possible to determine whether these precursors represented the same CFC detected in the population before liquid culture or were previously nonactive cells. This finding was in contrast to the single-cell cloning of $CD34^+lin^+$ cells where a decrease in the number of CFC was found during the liquid culture period. However, the few single $CD34^+lin^+$ cells that did give rise to CFC gave rise to approximately the same average number of CFC per individual precursor. It is possible that these latter cells are those expressing the lowest amounts of CD33 and 7B9 and still retain their substantial proliferative potential, analogous to those more frequently found in the $CD34^+lin^-$ population.

The enhanced proliferation of isolated $CD34^+CD33^-7B9^-$ cells observed with IL-3 and G-CSF precursors is reminiscent of the observed synergy of these factors in the

development of blast cell colonies.[12] In collaboration with Leary and Ogawa, we have shown that blast CFC express the CD34, but not the CD33 antigen.[7] The $CD34^+CD33^-$ as well as the $CD34^+7B9^-$ populations also contain cells that, upon culture over a previously established irradiated marrow stromal cell layer, give rise to colony-forming activity over time[3,5] (Brashem-Stein et al, manuscript in preparation).

Studies in mice have demonstrated that primitive hematopoietic cells termed high proliferative potential CFC (HPP-CFC) require more than one hematopoietic growth factor to proliferate in contrast to more differentiated progenitors that proliferate in the presence of only one growth factor.[13] In humans, HPP-CFC have also been shown to require combinations of growth factors such as IL-3 and GM-CSF (with or without CSF-1 or IL-1) for colony formation. These human HPP-CFC also express the CD34 antigen.[14,15]

Brandt et al have recently studied $CD34^+$ cells that lack lineage-associated antigens including HLA-DR and CD15 or CD71 and demonstrated the ability of these cells to be maintained in culture and generate CFC for up to 8 weeks in the presence of IL-3 and IL-1, or IL-6 in a liquid culture system.[16] The authors noted that an adherent marrow stromal cell layer was never observed in their liquid culture system, although stromal cells are known to express the CD34 antigen.[17] To what extent such cells played a role in the maintenance of their culture system is unknown. The single-cell studies described here exclude the possibility of a stromal cell interaction while demonstrating the high proliferative potential of $CD34^+lin^-$ cells.

The exact relationship between cells that form blast cell colonies, cells that initiate long-term cultures, HPP-CFC, and the $CD34^+lin^-$ populations evaluated in liquid culture systems without stroma remains to be determined. Nonetheless, each of these assays provides a measure of rare cells with high proliferative potential. These cell populations may represent appropriate targets for gene insertion therapy and may contain transplantable cells with long-term repopulating ability, but further studies will be required to address these issues. The methods presented in this report provide a quantitative means of assessing these populations and determining the effects of specific growth factors and culture conditions on primitive hematopoietic progenitor cells.

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