

PRECURSORS OF COLONY-FORMING CELLS IN HUMANS  
CAN BE DISTINGUISHED FROM COLONY-FORMING CELLS  
BY EXPRESSION OF THE CD33 AND CD34 ANTIGENS  
AND LIGHT SCATTER PROPERTIES

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In the hematopoietic stem cell hierarchy, the earliest cell is pluripotent and capable of extensive self-renewal (1). Later cells have lesser amounts of self-renewal capacity and a greater degree of differentiative commitment (1, 2). In humans, most studies of hematopoietic stem cells have relied on assays of unipotent and multipotent colony-forming cells (CFC),<sup>1</sup> populations that are relatively mature and capable of only limited self-renewal. Recent data suggest that a less mature cell gives rise to CFC in long-term marrow cultures (LTMC), and that cell surface antigens are markers by which these primitive precursors can be distinguished.

Antigens expressed by different progenitor cell populations have been characterized in several laboratories (3-22). Of particular interest are mAbs to the CD33 and the CD34 antigens. Antibodies L4F3(5), L1B2(5), and MY-9(9) identify the CD33 antigen, while antibodies 12-8(6), MY-10(10,11), BI-3C5(12-14), and ICH3(15) recognize the CD34 antigen. The CD33 antigen is found on immature myelocytic cells in marrow, while the CD34 antigen is only expressed by 1-4% of marrow cells. Importantly, both of the antigens are expressed by virtually all CFC (5, 6, 9-11, 13, 14). The CD34 antigen also is expressed by cells that give rise to CFC during LTMC (6), while the CD33 antigen is not (7).

In the studies presented here, we used mAbs to the CD33 and CD34 antigens to separate cells on the basis of expression of CD33, CD34, and light scattering properties. The ability of the cells to form colonies and generate CFC in LTMC was assessed.

### Materials and Methods

*Bone Marrow Samples.* Marrow samples were obtained from normal healthy donors following informed consent under an institutional review board approved protocol. RBC and

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<sup>1</sup> Abbreviations used in this paper:

mature granulocytic cells were depleted by density gradient centrifugation over Lymphocyte Separation Medium (LSM) (sp gr 1.078; Litton Bionetics, Kensington, MD) at 400 *g* for 20 min at room temperature.

**Antibody Preparation and Purification.** The generation and characterization of the 12-8 and L4F3 antibodies used in these experiments have been described in detail (5, 6). Antibody 12-8 is a murine IgM mAb that identifies the CD34 antigen, an *M*, 115,000 glycoprotein. Antibody L4F3 is a murine IgM mAb that recognizes the CD33 antigen, an *M*, 67,000 glycoprotein. IgM mAbs were partially purified from ascites fluids by boric acid precipitation as previously described (7). The p67-5 antibody is an IgG2a mouse mAb generated against a transfected mouse cell line that expresses the CD33 antigen (22) (the FMY9S5C17 and parental L-cell lines were kindly provided by Dr. T. Look, St. Jude's Research Hospital, Memphis, TN). The p67-5 antibody competitively inhibits binding of L4F3 to the CD33 antigen (our unpublished observations). As isotype controls for staining we used the anti-mouse Thy-1.1 mAb 1A14 (23), an IgG2a, and H12C12, a murine monoclonal IgM against the mouse Thy-1.2 antigen.

**Staining and Cell Sorting.** Cells were stained using indirect immunofluorescent antibody staining techniques and separated using FACS as previously described (5, 6). All staining was done with cells suspended in sterile PBS supplemented with 2% human AB serum (Irvine Scientific, Irvine, CA). For single-color fluorescence studies,  $10^7$  cells/ml were incubated with antibody (25  $\mu$ g/ml) for 30 min at 4°C, washed twice, and then incubated in a 1:20 dilution of FITC-conjugated goat anti-mouse IgM ( $\mu$  chain specific) antiserum (Tago Inc., Burlingame, CA) for 30 min at 4°C.

For two-color staining, cells were incubated with both antibody 12-8 (25  $\mu$ g/ml) and p67-5 ascites (1:10<sup>3</sup> dilution). Control cells were incubated with: (a) H12C12 (25  $\mu$ g/ml) and 1A14 ascites (1:10<sup>3</sup>); (b) H12C12 (25  $\mu$ g/ml) and p67-5 (1:10<sup>3</sup>); or (c) 12-8 (25  $\mu$ g/ml) and 1A14 (1:10<sup>3</sup>). Control and experimental cells were incubated with the primary antibodies for 30 min at 4°C, washed twice, then incubated with a 1:80 dilution of biotin-conjugated goat anti-mouse IgM antisera ( $\mu$  chain specific; Tago, Inc.) and a 1:20 dilution of FITC-conjugated goat anti-mouse IgG ( $\gamma$  chain specific, Southern Biotechnology Associates, Inc., Birmingham, AL.) for 30 min at 4°C, and then washed twice. Finally, cells were incubated with a 1:20 dilution of phycoerythrin-conjugated avidin (Becton-Dickinson & Co., Oxnard, CA.) for 30 min at 4°C, and washed once. Cells were analyzed and sorted using either a FACS-440 or FACS-II (Becton Dickinson & Co.). Cells were collected into RPMI-10% FCS.

The flow cytometers were set using fixed chicken red blood cells and Calibrite beads (Becton Dickinson & Co.) as standards for forward and right angle light scatter. Compensators and photomultiplier tube voltage were set using a mixture of equal amounts of unconjugated, FITC-conjugated, and PE-conjugated Calibrite beads. The compensators were adjusted so that PE-labeled beads had no more green fluorescence than unconjugated beads and the FITC-labeled beads had the same red fluorescence as unconjugated beads.

**Cytotoxicity Assays.** Cells were treated with antibody and complement (C') in cytotoxicity assays as previously described (5, 7). After the initial C' incubation, cells were pelleted, the supernatant was removed, and cells were resuspended in C' (1:4 dilution of rabbit serum) and incubated for 60 min at 37°C. In one experiment, a third 60-min incubation with C' was also performed. Residual viable cells were isolated by density gradient centrifugation over LSM.

**Colony-forming Assays.** Progenitor cells capable of forming granulocytic and monocytic (CFU-GM), erythroid (BFU-E), and mixed lineage (CFU-MIX) colonies were identified by culturing cells in Iscove's modified Dulbecco's MEM (IMDM) (Gibco Laboratories, Grand Island, NY) supplemented with 20% FCS (HyClone Laboratories, Logan, UT), 10% human placental conditioned medium, 3 IU/ml human urinary erythropoietin (Terry Fox Cancer Research Center, Vancouver, BC, Canada),  $10^{-4}$  M 2-ME (Bio-Rad Laboratories, Richmond, CA), and 0.3% agar (Seaplaque; FMC Corp., Rockland, ME). All cultures were incubated at 37°C in 5% CO<sub>2</sub> in air, in a humidified incubator. At 14-16 d of culture the colonies of different types were scored using an inverted microscope using the criteria for identification of BFU-E and CFU-MIX as previously described (24).

Human placental conditioned medium was prepared using the methods of Schlunck and Schleyer (25) and screened for the ability to support the growth in vitro of the desired types of CFC.

**Long-Term Marrow Cultures.** Separated as well as unseparated marrow cells were cultured in a long-term culture system previously described (6). Irradiated adherent cell layers from 2-4-wk-old LTMC were used as "feeder layers" for isolated marrow cells (7). Cells were inoculated onto irradiated layers and cultured for 5-7 d at 37°C, after which time cultures were maintained in a 33°C incubator. At weekly intervals the cultures were fed by removing half of the culture supernatant and replacing it with fresh medium. The nonadherent cells removed with the culture supernatant were assayed for the presence of CFC.

## Results

**Positive Selection of CD34<sup>+</sup> Cells After Lysis of CD33<sup>+</sup> Cells.** Marrow cells were treated with the anti-CD33 antibody L4F3 and C'. In three experiments, L4F3 plus C' lysed  $93 \pm 1\%$  (mean  $\pm$  SEM) of CFU-GM,  $82 \pm 4\%$  of BFU-E, and  $95 \pm 2\%$  of CFU-MIX, confirming that most CFC express the CD33 antigen (5, 7). Analysis by flow cytometry after lysis demonstrated that cells with high forward and high right-angle light scatter characteristics were predominantly depleted (Fig. 1, A and B), and that detectable numbers of CD33<sup>+</sup> cells were no longer present (Fig. 1 C). Antibody 12-8 stained 1.8-6.0% of the CD33-depleted cells (Fig. 1 D), representing 0.4-0.5% of marrow cells present before cytolysis.

We then separated the CD33<sup>-</sup> cells into CD34<sup>+</sup> and CD34<sup>-</sup> populations by FACS. In a representative experiment (Fig. 2), the CD33<sup>-</sup>CD34<sup>+</sup> cells produced unipotent and multipotent CFC for more than 5 wk in LTMC (Fig. 2). As expected, this CD34<sup>+</sup> fraction also contained the few CFC that remained after lysis with the anti-CD33 antibody. In contrast, CD33<sup>-</sup>CD34<sup>-</sup> cells in LTMC produced fewer CFU-GM, and virtually no CFU-MIX or BFU-E. These results were confirmed in the two additional experiments (data not shown).

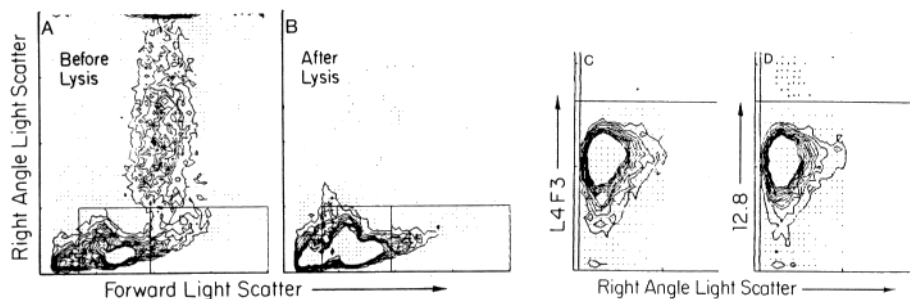


FIGURE 1. (A and B) Bone marrow cells were analyzed by flow microfluorimetry for forward and right angle light scatter characteristics before (A) and after (B) lysis with L4F3 and C' (analysis of 30,208 cells is shown in each panel). Cells with high right angle and forward light scattering characteristics were predominantly depleted. (C and D) Viable cells isolated after lysis with L4F3 + C' were analyzed for reactivity with mAbs L4F3 (C) and 12-8 (D) (analysis of 6,000 cells is shown in each panel). Antibody 12-8 positively stained 1.8% of the remaining cells, while no detectable staining was observed with L4F3 or H12C12, the IgM control antibody (not shown). The dots representing positively stained cell populations in C and D have been enhanced for purposes of reproduction.

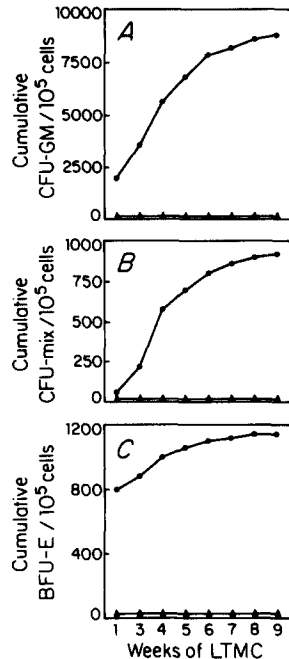


FIGURE 2. Long-term marrow cultures of CD34<sup>+</sup> and CD34<sup>-</sup> cells resistant to lysis with L4F3 and C'. Data points are the cumulative number of colonies produced from weeks 1-9 per 10<sup>5</sup> cells placed in each LTMC at week 0. For each time point, the data are the mean of two to four replicate cultures. Treatment with L4F3 + C' lysed 92 ± 1% of CFU-GM, 100% of CFU-MIX, and 83 ± 17% of BFU-E. The remaining CD33<sup>-</sup> cells were 1.8% CD34<sup>+</sup> (see Fig. 1). The CD34<sup>+</sup> and CD34<sup>-</sup> cells were separated by FACS. At the initiation of the LTMC the CD34<sup>+</sup> population contained 1,430 CFU-GM, 20 CFU-MIX, and 1,280 BFU-E per 10<sup>5</sup> cells, and the CD34<sup>-</sup> population contained 3 CFU-GM, 0 CFU-MIX, and 220 BFU-E per 10<sup>5</sup> cells. (A) CFU-GM; (B) CFU-MIX; (C) BFU-E. (●) CD34<sup>+</sup> cells; (▲) CD34<sup>-</sup> cells.

*Direct Isolation of CD33<sup>-</sup>CD34<sup>+</sup> and CD33<sup>+</sup>CD34<sup>+</sup> Cells Using Two-Color Immunofluorescence and Cell Sorting.* Cells were labeled with the anti-CD34 and anti-CD33 antibodies 12-8 and p67-5, respectively. The latter anti-CD33 antibody was used because it is of the IgG2a isotype and in two-color fluorescent antibody binding studies it could be distinguished from the IgM antibody 12-8 by use of  $\gamma$ - and  $\mu$ -specific second-step reagents (see Materials and Methods). Three groups of CD34<sup>+</sup> cells were isolated using two-color FACS. The first group had virtually no detectable CD33 (fluorescence <99% of cells stained with p67-5 and <90% of control stained cells), and represented 0.3% of all marrow cells analyzed and 12% of all CD34<sup>+</sup> cells (Fig. 3). Most of these CD33<sup>-</sup>CD34<sup>+</sup> cells had the appearance of small to intermediate sized lymphocytes or small blast-like cells as determined by examination of Wright-stained slides prepared by cytocentrifugation. The third group was CD33<sup>+</sup>, with fluorescence >95% of cells stained with the control antibody. These CD33<sup>+</sup>CD34<sup>+</sup>

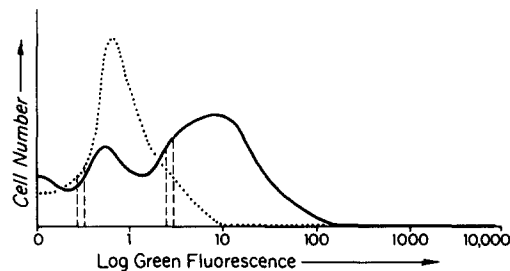


FIGURE 3. Gated fluorescence histogram of CD34<sup>+</sup> marrow cells, identified by staining with antibody 12-8 simultaneously stained with the anti-CD33 antibody p67-5 (solid line) or with the control antibody 1A14 (dotted line). Fluorescence was analyzed on a FACS II equipped with 5 log amplifiers for the photomultipliers. The cell number is displayed using a linear scale. The vertical dashed lines indicate the locations of sorting windows. Moving from left to right in the figure: group I, group II, and group III.

cells accounted for 2.3% of marrow cells and 64% of the CD34<sup>+</sup> cells. The second group contained CD34<sup>+</sup> cells that had<sup>h</sup>CD33 expression intermediate between the first and third groups (CD33). These accounted for 1% of marrow cells analyzed and 27% of CD34<sup>+</sup> cells. A fourth group consisting of CD34<sup>-</sup> cells also was collected, and represented 96% of marrow cells analyzed.

When these groups were tested for colony forming ability, the CD33<sup>-</sup>CD34<sup>+</sup> and CD33<sup>±</sup>CD34<sup>+</sup> populations were found to contain >90% of all CFC in the four separated groups, and therefore in unseparated marrow as well.

Cells from these groups were grown in LTMC and the nonadherent cells tested weekly for colony growth. The CD33<sup>-</sup>CD34<sup>+</sup> group was found to generate more unipotent and multipotent CFC than did the CD33<sup>±</sup>CD34<sup>+</sup> population (Fig. 4), while the CD33<sup>±</sup>CD34<sup>+</sup> cells generated intermediate numbers of CFC. Although they constituted the majority of marrow cells, the CD34<sup>-</sup> cells generated virtually no CFC. These findings were confirmed in two further experiments (data not shown).

Although the precursors of CFC were found in the CD33<sup>-</sup>CD34<sup>+</sup> population, some CFC were still present in this group. To define a population of precursors devoid of CFC activity, CD33<sup>-</sup>CD34<sup>+</sup> cells were further fractionated on the basis of light scatter properties.

*Fractionation of CD33<sup>-</sup>CD34<sup>+</sup> Cells by Light Scatter Properties.* To study the light scatter properties of precursors of CFC in LTMC we initially separated marrow cells into subpopulations based solely on their right angle and forward angle light scattering characteristics on the cell sorter. Four discrete populations of cells were detectable (Fig. 5). Cells in the first population (A) had high right angle light scatter and consisted primarily of morphologically maturing granulocytic and monocytic

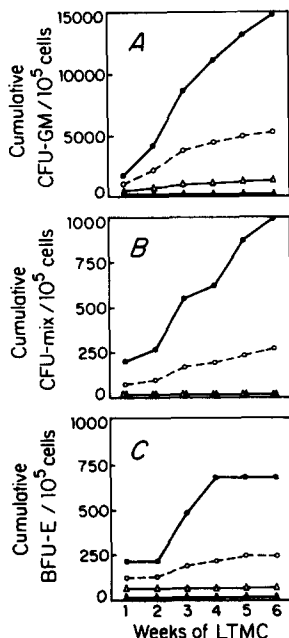


FIGURE 4. Long-term cultures of marrow cells isolated by two-color immunofluorescent antibody staining and FACS. Data points are the cumulative number of colonies harvested from LTMC on weeks 1-6, per  $10^5$  starting cells. For each time point the data are the mean of two to four replicate cultures. At the initiation of cultures the colony numbers per  $10^5$  cells were as follows: CD33<sup>±</sup>CD34<sup>+</sup> (group III, 64% of CD34<sup>+</sup> cells) CFU-GM = 5,754, CFU-MIX = 80, BFU-E = 1,678; CD33<sup>±</sup>CD34<sup>+</sup> (Group II, 27% of CD34<sup>+</sup> cells) CFU-GM = 4,745, CFU-MIX = 495, BFU-E = 5,245; CD33<sup>-</sup>CD34<sup>+</sup> (group I, 12% of CD34<sup>+</sup> cells) CFU-GM = 3,466, CFU-MIX = 33, BFU-E = 4,400; CD34<sup>-</sup> (group IV) CFU-GM = 19, CFU-MIX = 0, BFU-E = 2. group I, CD33<sup>-</sup>CD34<sup>+</sup> (●); group II, CD33<sup>±</sup>CD34<sup>+</sup> (○); group III, CD33<sup>±</sup>CD34<sup>+</sup> (△); group IV, CD34<sup>-</sup> (▲).

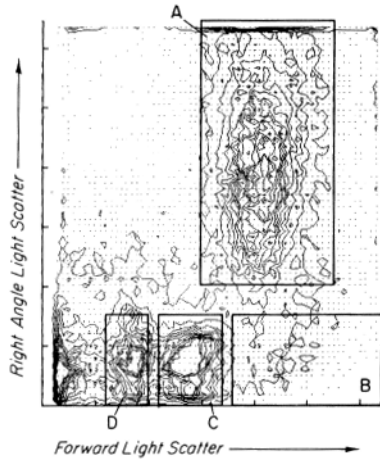


FIGURE 5. Light scatter analysis of unseparated Ficoll-hypaque marrow cells (analysis of 60,416 events) is shown as a two-dimensional representation of the right angle,  $y$ -axis, versus forward,  $x$ -axis, light scatter of unseparated bone marrow cells, using linear amplification of signals is shown. The windows used for sorting cells have been outlined and labeled. (A) Cells with high right angle light scatter (50.9% of all events); (B) cells with low right angle and high forward light scatter (2.7% of all events); (C) cells with low right angle and low forward light scatter (17.5% of all events); and (D) cells with low right angle and very low forward light scatter (11.1% of all events).

cells. The second population (B) had cells with low right angle and high forward light scattering and consisted of immature monocytic cells and myeloblasts morphologically. The third population (C) contained cells with low right angle and low forward light scattering properties. Morphologically these cells were lymphocyte sized and had the appearance of mature lymphocytes and small blast cells. The fourth population (Fig. 5 D) of cells had low right angle and still lower forward angle light scattering and contained primarily nucleated and anucleate RBCs.

Cells from these four separated populations were cultured directly to assay colony formation and cultured over irradiated marrow stromal cells in wells of microtiter plates. In the direct colony assays the majority of colony forming cells were present in the population of cells with low right angle and high forward angle light scattering properties (Table I). We then asked which population contained precursors for CFC in LTMC. Cells from the long-term cultures were harvested after 4 wk of culture and assayed for CFU-GM as a measure of generation of CFC. The cultures of cells with low right angle and low forward light scattering showed a 27-fold increase in detectable CFU-GM. In contrast, the population that contained most of the CFC in direct colony assays showed a nearly 50% decrease in detectable CFU-GM. The cells with high right angle scatter and cells with light scatter properties of nucleated RBC generated almost no CFU-GM in LTMC. This suggested that precursors of CFC in LTMC should have the light scattering properties that would allow them to be further distinguished in the  $CD33^-CD34^+$  population.

The  $CD33^-CD34^+$  marrow cell population is heterogeneous primarily with respect to forward angle light scatter characteristics. We therefore asked if precursors of CFC in LTMC could be further distinguished within the  $CD33^-CD34^+$  population based on light scattering characteristics. The  $CD33^+$  cells were first depleted by lysis with L4F3 and C', and the remaining  $CD34^+$  cells with low right angle light scatter were separated into two populations by FACS: one subset with low forward light scatter, similar to that of lymphocytes, and a second subset with high forward light scatter similar to that of larger blast-like cells. The cells with light scatter prop-

TABLE I  
*Separation of Marrow Cells Based on Right Angle and Forward  
 Light Scatter: Direct Colony Forming Activity and  
 Generation of CFU-GM in LTMC*

Light scatter	Sort window*	CFU-GM/10 <sup>5</sup> cells	
		Direct culture	Week 4 LTMC
High right angle	A	2 ± 2	0
Low right angle High forward	B	1,437 ± 52	807 ± 148
Low right angle Low forward	C	7 ± 3	190 ± 27
Low right angle Very low forward	D	2 ± 2	3 ± 4

Isolated cells were cultured directly in soft agar for the ability to form colonies, and an identical number of each population was cultured in wells of microtiter plates over irradiated marrow stromal cell layers. After 4 wk of culture, all the cells in individual LTMC wells were harvested and cultured in soft agar to assay for the presence of CFU-GM. Data are expressed as the mean ± SEM of CFU-GM per 10<sup>5</sup> cells placed in culture, either directly in soft agar or in the LTMC wells.

\* The sort windows are shown in Fig. 5.

erties similar to those of lymphocytes formed fewer CFU-GM upon direct culture (0 colonies/10<sup>5</sup>) than did cells with high forward light scatter (10 colonies/10<sup>5</sup>). Although both populations of cells generated CFC in LTMC, the cells with low forward light scatter generated fewer CFC in the first two weeks of LTMC than did the cells with high forward light scatter (Fig. 6). In contrast, after 4 wk of LTMC, the cells with low forward light scatter generated greater numbers of CFC than did the cells with high forward light scatter. These results were confirmed in two further studies (data not shown). Thus, within the CD33<sup>-</sup>CD34<sup>+</sup> population it is possible

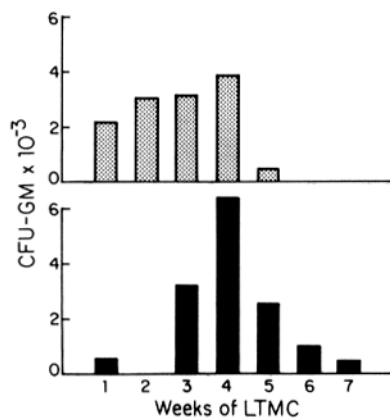


FIGURE 6. CD33<sup>-</sup>CD34<sup>+</sup> cells with low right angle light scatter were separated by FACS into populations with low forward or high forward light scatter. Data points are CFU-GM in the nonadherent cell fraction per 2 × 10<sup>6</sup> LTMC starting cells, and represent the mean of two to four replicate cultures at each time point. (Bottom) LTMC of cells with low forward light scatter. (Top) LTMC of cells with high forward light scatter.

TABLE II  
*CFC from Long-Term Marrow Cultures of CD33<sup>-</sup> CD34<sup>+</sup> Cells:  
 Cytolysis with L4F3 (Anti-CD33 Antibody) And Complement*

Week		CFU-GM	BFU-E	CFU-MIX
5	L4F3 + C'	150 ± 52 (87)*	0 (100)	0 (100)
	H12C12 + C'	1,180 ± 121	210 ± 106	90 ± 17
7	L4F3 + C'	10 ± 7 (98)	0 (100)	0 (100)
	H12C12 + C'	640 ± 0	50 ± 7	100 ± 0

Data are the mean ± SEM for colonies at each time point, and are expressed as total colonies per culture. H12C12 is a control isotype matched mAb with irrelevant specificity, anti-mouse Thy-1.2.

\* Percent lysis compared with control cultures.

to distinguish precursors of CFC from cells with CFC activity on direct culture based on light scatter properties.

*Expression of the CD33 Antigen by CFC from Long-Term Cultures of CD33<sup>-</sup> CD34<sup>+</sup> Marrow Cells.* If the CD33 antigen is acquired at the time of differentiation to a CFC, then CFC generated in LTMC from CD33<sup>-</sup>CD34<sup>+</sup> cells should express the CD33 antigen. We therefore tested the ability of L4F3 and C' to lyse CFC generated in LTMC of CD33<sup>-</sup>CD34<sup>+</sup> cells, the CD34<sup>+</sup> cells isolated after lysis of marrow with L4F3 and C' to deplete cells expressing the CD33 antigen. The percent of CFC from these long-term cultures, that were sensitive to lysis by L4F3 and C' was similar to that of fresh marrow (Table II). These results were confirmed in two additional experiments (data not shown).

### Discussion

The aim of the present studies was to provide direct evidence for the hypothesis that precursors of CFC are distinguishable from CFC on the basis of expression of differentiation antigens. Data indicate that precursors of CFC are separable from CFC on the basis of expression of CD33 and CD34 antigens and physical properties.

Lysis of CD33<sup>+</sup> marrow cells with antibody L4F3 and C' depleted 83–95% of the CFC. Of the remaining cells, those that expressed the CD34 antigen generated CFC in LTMC. That the CD33<sup>-</sup>CD34<sup>+</sup> marrow cells contained the precursors for CFC was demonstrated using two-color FACS to directly isolate CD33<sup>-</sup>CD34<sup>+</sup> and CD33<sup>+</sup>CD34<sup>+</sup> populations. In contrast, both the CD33 and CD34 antigens were expressed on most CFC, and these CD33<sup>+</sup>CD34<sup>+</sup> cells lacked self-renewal capacity in LTMC.

Importantly, as in fresh marrow, most CFC detected in LTMC of CD33<sup>-</sup>CD34<sup>+</sup> cells expressed the CD33 antigen as determined by lysis with antibody L4F3 and C' (5, 7). This demonstrated that maturation of CD33<sup>-</sup> cells to CFC in LTMC resulted in the expression of the CD33 antigen.

Light scatter properties provided additional parameters for discriminating cells. Most CFC had low right angle and high forward light scattering, while precursors for CFC appeared to have low right angle and low forward light scattering properties. We used light scattering characteristics to discriminate CD33<sup>-</sup>CD34<sup>+</sup> cells with different proliferative properties. It was then shown that cells requiring longer to generate CFC in LTMC had the light scatter properties of small lymphocytes, while



cells with high forward light scatter, associated with larger cells, generated CFC after only a short time in LTMC. It is possible that these differences in light scatter, a measure of cell size, reflect cells in different stages of the cell cycle or with differing self renewal potential.

The importance of CD34<sup>+</sup> marrow cells is underscored by our recent observations that the CD34 antigen also is expressed by a minor subset of baboon bone marrow cells. These cells, when isolated by immunoadsorption chromatography and FACS can reconstitute lymphohematopoiesis in lethally irradiated baboons (26, 27), while CD34<sup>-</sup> cells appear incapable of doing so (27). In initial clinical trials in patients with acute nonlymphoblastic leukemia (ANL), autologous marrow cells treated with an anti-CD33 mAb and C' to deplete ANL cells, which lysed nearly all CFC, engrafted patients receiving marrow ablative chemoradiotherapy (our unpublished observations; and Griffin, J. D., and J. Ritz, personal communication). These observations suggest that cells responsible for reconstituting hematopoiesis *in vivo* are CD33<sup>-</sup>CD34<sup>+</sup>.

An important unresolved question is whether CD33<sup>-</sup>CD34<sup>+</sup> progenitors are pluripotent and can generate lymphoid as well as myeloid progeny. In normal bone marrow there are subsets of CD34<sup>+</sup> cells that express T cell-associated (CD2, CD7) and B cell-associated (CD10, CD19, CD20) antigens, suggesting that CD34 is also expressed by immature B and T lymphocytes (10, 12, 15, 28, 29, our unpublished observations). Whether these cells, or CD34<sup>+</sup> cells that do not express these lymphocyte-associated antigens can undergo lymphoid maturation cannot, however, be determined until methods to culture human lymphoid precursor cells are developed, as they have been for murine cells (30-33). The presence of CD34 subpopulations expressing lymphoid associated antigens also suggests that depletion of these cells can allow further enrichment of precursors of CFC using methods as described in the present studies.

### Summary

We determined whether human marrow cells that directly form colonies *in vitro* could be distinguished from cells that generate or become CFC only after LTMC in the presence of irradiated marrow stromal cells. In previous studies, an anti-CD33 antibody, L4F3, and complement (C') were used to lyse nearly all CFC in marrow, and the remaining cells generated CFC in LTMC. In the present studies, marrow cells were treated with L4F3 + C' and the remaining CD33<sup>-</sup> cells were separated into CD34<sup>+</sup> and CD34<sup>-</sup> populations and placed in LTMC. Only the CD34<sup>+</sup> cells were found to generate significant numbers of CFC. To compare the CD33<sup>-</sup>CD34<sup>+</sup> and CD33<sup>+</sup>CD34<sup>+</sup> cells, we isolated each cell population using two-color FACS. Only LTMCs of the CD33<sup>-</sup>CD34<sup>+</sup> cells generated CFC for >5 wk. In contrast, cells that expressed both the CD33 and CD34 antigens, which contained most of the CFC, generated few CFC in LTMC. Fractionation of marrow cells based on right angle and forward light scattering suggested that precursors for CFC have low right angle and low forward light scattering properties. The CD33<sup>-</sup>CD34<sup>+</sup> marrow cells were therefore further fractionated based on light scatter characteristics. Cells with low right angle and low forward light scatter formed few or no colonies on direct culture, yet generated greater numbers of CFC after 4 wk of LTMC than did cells with low right angle and high forward light scatter. Most (87-98%) CFC generated in the

LTMCs that were initiated with CD33<sup>-</sup>CD34<sup>+</sup> cells were found to express the CD33 antigen. Thus, hematopoietic progenitors with differing proliferative and differentiative potentials can be directly separated on the basis of their expression of the CD33 and CD34 cell surface antigens and their light scatter properties.

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### References

1. Till, J. E., and E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14:213.
2. Dick, J. E., M. C. Magli, D. Huszar, R. A. Phillips, and A. Bernstein. 1985. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system in *W/W<sup>v</sup>* mice. *Cell.* 42:71.
3. Reinherz, E. L., B. F. Haynes, L. M. Nadler, and I. D. Bernstein, editors. 1986. Leukocyte Typing II. Vols. I-III. Springer-Verlag, New York.
4. McMichael, A. J., P. A. C. Beverley, S. Cobbold, et al., editors. 1987. Leukocyte Typing III: White Cell Differentiation Antigens. Oxford University Press, Oxford.
5. Andrews, R. G., B. Torok-Storb, and I. D. Bernstein. 1983. Myeloid associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood.* 62:124.
6. Andrews, R. G., J. W. Singer, and I. D. Bernstein. 1986. Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent colony-forming cells and their precursors. *Blood.* 67:842.
7. Andrews, R. G., M. Takahashi, G. M. Segal, J. S. Powell, I. D. Bernstein, and J. W. Singer. 1986. The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. *Blood.* 68:1030.
8. Griffin, J. D., J. Ritz, L. M. Nadler, and S. F. Schlossman. 1981. Expression of myeloid differentiation antigens on normal and malignant cells. *J. Clin. Invest.* 68:932.
9. Griffin, J. D., D. Linch, K. Sabbath, P. Larcom, and S. F. Schlossman. 1984. A monoclonal antibody reactive with normal and leukemic myeloid progenitor cells. *Leuk. Res.* 8:521.
10. Civin, C. I., L. C. Strauss, C. Brovall, M. J. Fackler, S. J. Schwartz, and J. S. Shaper. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J. Immunol.* 133:157.
11. Strauss, L. C., S. D. Rowley, V. F. La Russa, S. J. Sharkis, R. K. Stuart, and C. I. Civin. 1986. Antigenic analysis of hematopoiesis. V. characterization of MY-10 antigen expression by normal lymphohematopoietic progenitor cells. *Exp. Hematol.* 14:878.
12. Tindle, R. W., R. A. B. Nichols, L. Chou, D. Campana, D. Catovsky, and G. D. Birnie. 1985. A novel monoclonal antibody B1-3C5 recognizes myeloblasts and non-B non-T lymphoblasts in acute leukemias, CGL in blast crisis, and reacts with immature cells in normal marrow. *Leuk. Res.* 9:1.
13. Katz, F. E., R. W. Tindle, R. Sutherland, and M. F. Greaves. 1986. Reactivity of a monoclonal antibody defining human haematopoietic multipotential progenitors. In Leukocyte Typing II. E. Reinherz, B. F. Haynes, L. M. Nadler, and I. D. Bernstein, editors. Springer-Verlag, New York. 305-314.
14. Katz, F. E., R. Tindle, R. P. Sutherland, and M. F. Greaves. 1985. Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. *Leuk. Res.* 9:191.
15. Watt, S. M., K. Karhi, K. Gatter, A. J. W. Furley, F. E. Katz, L. E. Healy, L. J. Altass, N. J. Bradley, D. R. Sutherland, R. Levinsky, and M. F. Greaves. 1987. Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human

- hemopoietic progenitor cells. *Leukemia (Baltimore)*. 1:417.
16. Papayannopoulou, Th., M. Brice, T. Yokochi, P. S. Rabinovitch, D. Lindsley, and G. Stamatoyannopoulos. 1984. Anti-HEL cell monoclonal antibodies recognize determinants that are also present on hemopoietic progenitors. *Blood*. 63:326.
  17. Strauss, L. C., L. C. Stuart, and C. I. Civin. 1983. Antigenic analysis of hematopoiesis. I. Expression of the MY-1 granulocyte surface antigen on marrow cells and leukemic cell lines. *Blood*. 61:1222.
  18. Strauss, L. C., C. Brovall, M. J. Fackler, J. F. Schwartz, J. H. Shaper, M. R. Loken, and C. I. Civin. 1986. Antigenic analysis of hematopoiesis. IV. The MY-11 hematopoietic cell surface antigen is expressed by myelomonocytic and lymphoid, but not erythroid, progenitor cells. *Exp. Hematol.* 14:935.
  19. Bodger, M., C. A. Izaguirre, H. A. Blacklock, and A. V. Hoffbrand. 1983. Surface antigenic determinants on human pluripotent and unipotent hematopoietic progenitor cells. *Blood*. 61:1006.
  20. Ferrero, D., H. E. Broxmeyer, G. L. Pagliardi, S. Venuta, B. Lange, S. Passano, and G. Rovera. 1985. Antigenically distinct subpopulations of myeloid progenitor cells (CFU-GM) in human peripheral blood and marrow. *Proc. Natl. Acad. Sci. USA*. 80:4114.
  21. Ferrero, D., M. Gabbianelli, C. Peschle, B. Lange, and G. Rovera. 1985. Surface phenotypes of human hemopoietic progenitor cells defined by monoclonal antibodies. *Blood*. 66:496.
  22. Look, T. A., S. C. Peiper, E. C. Douglas, J. M. Trent, and C. J. Scherr. 1986. Amplification of genes encoding human myeloid membrane antigens after DNA-mediated gene transfer. *Blood*. 67:637.
  23. Denkers, E. Y., C. C. Badger, J. A. Ledbetter, and I. D. Bernstein. 1985. Influence of antibody isotype on passive serotherapy of lymphoma. *J. Immunol.* 135:2183.
  24. Powell, J. S., P. J. Fialkow, and J. W. Adamson. 1984. Human mixed cell colonies: unicellular or multicellular origin-analysis by G-6-PD. *Br. J. Haematol.* 57:89.
  25. Schlunck, T., and M. Schleyer. 1980. The influence of culture conditions on the production of colony stimulating activity by human placenta. *Exp. Hematol.* 8:179.
  26. Berenson, R. J., W. I. Bensinger, R. G. Andrews, D. Kalamasz, R. Hill, and I. D. Bernstein. 1987. Hematopoietic stem cell transplants. *UCLA (Univ. Calif. Los Angel.) Symp. Mol. Cell. Biol. New Ser.* 61:527.
  27. Berenson, R. J., R. G. Andrews, W. I. Bensinger, D. Kalamasz, G. Knitter, C. D. Buckner, and I. D. Bernstein. 1988. CD34+ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* 81:951.
  28. Loken, M. R., O. V. Shah, K. L. Dattilio, and C. I. Civin. 1987. Flow cytometric analysis of human bone marrow. II. normal B lymphocyte development. *Blood*. 70:1316.
  29. Ryan, D. H., C. W. Chapple, S. A. Kossover, A. A. Sandberg, and H. J. Cohen. 1987. Phenotypic similarities and differences between CALLA-positive acute lymphoblastic leukemia cells and normal marrow CALLA-positive B cell precursors. *Blood*. 70:814.
  30. Davis, K.A., and O. N. Witte. 1986. In vitro development of B lymphocytes from long-term cultured precursor cells. *Proc. Natl. Acad. Sci. USA*. 83:441.
  31. Johnson, A., and K. Dorshkind. 1986. Stromal cells in myeloid and lymphoid long-term bone marrow cultures can support multiple hematopoietic lineages and modulate their production of hematopoietic growth factors. *Blood*. 68:1348.
  32. McKearn, J. P., J. McCubry, and B. Fagg. 1987. Enrichment of hematopoietic precursor cells and cloning of multipotent B-lymphocyte precursors. *Proc. Natl. Acad. Sci. USA*. 82:7414.
  33. Spangrude, G. J., S. Heimfeld, and I. L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science (Wash. DC)*. 241:58.