$CD34^+/CD33^-$ Cells Reselected From Macrophage Inflammatory Protein 1α + Interleukin-3–Supplemented "Stroma-Noncontact" Cultures Are Highly Enriched for Long-Term Bone Marrow Culture Initiating Cells

By Catherine M. Verfaillie and Jeffrey S. Miller

Human hematopoietic stem cells are thought to express the CD34 stem cell antigen, low numbers of HLA-DR and Thy1 antigens, but no lineage commitment antigens, CD38, or CD45RA antigens. However, fluorescence-activated cell sorted $CD34^+$ subpopulations contain not more than 1% to 5% primitive progenitors capable of initiating and sustaining growth in long-term bone marrow culture initiating cells (LTBMC-ICs). We have recently shown that culture of fresh human marrow $CD34^+/HLA\text{-}DR^-$ cells separated from a stromal layer by a microporous membrane ("stroma-noncontact" culture) results in the maintenance of 40% of LTBMC-ICs. We hypothesized that reselection of $CD34^+$ subpopulations still present after several weeks in stroma-noncontact cultures may result in the selection of cells more highly enriched for human LTBMC-ICs. Fresh marrow $CD34^+/HLA\text{-}DR^-$ cells were cultured for 2 to 3 weeks in stroma-noncontact cultures. Cultured progeny was then sorted on the basis of CD34, HLA-DR, or CD33 antigen expression, and sorted cells evaluated for the presence of LTBMC-ICs by limiting dilution analysis. We show that (1) LTBMC-ICs are four times more frequent in cultured $CD34^+/HLA\text{-}DR^-$ cells (4.6% ± 1.7%) than in cultured $CD34^+/HLA\text{-}DR^-$ cells (1.3% ± 0.4%). This suggests that HLA-DR antigen expression may depend on the activation status of primitive cells rather than their lineage commitment. We then sorted cultured cells on the basis of the myeloid commitment antigen, CD33. (2) These studies show that cultured $CD34^+/CD33^-$ cells contain 4% to 8% LTBMC-ICs, whereas cultured $CD34^+/CD33^{+bright}$ cells contain only 0.1% ± 0.03% LTBMC-ICs. Because LTBMC-ICs are maintained significantly better in stroma-noncontact cultures supplemented with macrophage inflammatory protein 1α (MIP-1α) and interleukin-3 (IL-3) (Verfaillie et al, *J Exp Med* 179:643, 1994), we evaluated the frequency of LTBMC-ICs in $CD34^+/CD33^-$ cells present in such cultures. (3) $CD34^+/CD33^-$ cells present in MIP-1α + IL-3–supplemented cultures contain up to 30% LTBMC-ICs. The increased frequency of LTBMC-ICs in cultured $CD34^+$ subpopulations may be the result of terminal differentiation of less primitive progenitors, loss of cells that fail to respond to the culture conditions or recruitment of quiescent LTBMC-ICs. The capability to select progenitor populations containing up to 30% LTBMC-ICs should prove useful in studies examining the growth requirements, self-renewal, and multilineage differentiation capacity of human hematopoietic stem cells at the single-cell level.

LONG-TERM BONE MARROW culture initiating cells (LTBMC-ICs) are thought to represent a very primitive hematopoietic progenitor population.[1] The phenotypic characteristics of such cells in normal human marrow are partially defined. Human adult marrow LTBMC-IC express high numbers of the CD34 stem cell antigen,[2-8] lack lineage commitment antigens such as CD7,[3] CD19,[2] CD33,[3,6] CD15,[4,7] and CD71[4,5,7] or the CD38[6] or CD45RA[5] antigens, and fail to express high levels of the HLA-DR,[2,4,7] or Thy1[8] antigens. Culture of such highly selected cell populations in stroma-dependent[2,3,4] or cytokine-dependent long-term cultures devoid of stroma[5,6,7] results in prolonged in vitro hematopoiesis. However, the frequency of cells capable of initiating and sustaining in vitro hematopoiesis in these cell populations is not higher than 1% to 5%.[5,6,9,10] This indicates that such highly selected populations are still heterogeneous and that additional selection criteria will be required to fully characterize the phenotype of human LTBMC-ICs.

A fraction of primitive progenitors can be maintained in cultures using mixtures of cytokines in the absence of stroma.[5,6] Alternatively, primitive progenitors are maintained in stroma-based culture systems in which progenitors are cultured either in direct contact with stroma[9,10,11] or separated from the stroma by a microporous transwell.[10,12,13] Because almost all these culture systems have a finite life span, and a gradual loss of primitive progenitors occurs over time,[5,6,9,10] it is clear that the artificial in vitro microenvironment poses a significantly greater differentiation pressure than that of the in vivo BM microenvironment. Therefore, more mature progenitors are likely to have differentiated in myeloid-committed progenitors within several weeks of in vitro culture, whereas more primitive progenitors may prevail. We hypothesized that reselection by fluorescence-activated cell sorting (FACS) of $CD34^+$ subpopulations still present after several weeks of culture might result in a cell population that is highly enriched for the more primitive LTBMC-ICs. Such highly enriched populations of primitive LTBMC-ICs may then allow study of growth requirements and differentiation capabilities of sorted human hematopoietic stem cells at the single-cell level. Moreover, further phenotypic identification of the primitive LTBMC-ICs present in long-term cultures will provide a useful tool in evaluating culture systems aimed at expansion of primitive human hematopoietic progenitors.

Culture of primitive progenitors in transwell inserts of "stroma-noncontact" cultures either in the absence of cytokines[10] or, even more so, in the presence of macrophage

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Submitted October 25, 1993; accepted April 22, 1994.

Supported in part by National Institutes of Health Grants No. R01-CA-4581401, 1-R24-HD30511-01, and PO1-CA-21737, The Paul Christiansen Foundation, The Minnesota Medical Foundation, The Leukemia Task Force, The Graduate School of the University of Minnesota and the University of Minnesota Hospitals and Clinics. C.V. is a Special Fellow of the Leukemia Society of America and a Special Fellow of the "Fundacion Internacional Jose Carreras Para La Lucha Contra La Leucemiea."

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0006-4971/94/8405-0021$3.00/0

inflammatory protein 1α (MIP-1α) + IL-3[13] maintains primitive LTBMC-ICs better than when cultured in contact with stroma[9,10] or cytokine-supplemented stroma-free cultures.[12,13] Therefore, we elected to examine the LTBMC-IC frequency in several CD34+ subpopulations reselected from transwell inserts of stroma-noncontact cultures. We show that cultured CD34+/HLA-DR+ rather than cultured CD34+/HLA-DR- cells are enriched for LTBMC-IC. CD34+/CD33- cells present in "stroma-noncontact" cultures contain 6% LTBMC-IC, whereas CD34+/CD33- cells recovered from MIP-1α + IL-3 supplemented stroma-noncontact cultures contain up to 30% cells capable of initiating and sustaining LTBMCs.

MATERIALS AND METHODS

Cell Separations

Initial cell selection. BM was aspirated from the posterior iliac crest in heparin from healthy volunteers after informed consent. BM mononuclear cells were separated by sequential Ficoll-Hypaque centrifugation (specific gravity, 1.077) (Sigma Chemical Co, St Louis, MO), centrifugal counterflow elutriation,[14,15] sheep erythrocyte rosette T-cell depletion[16] and immunomagnetic bead depletion of cells expressing CD11b, CD19 (Becton Dickinson, Mountain View, CA), MY8 (Coulter Cytometry, Hialeah FL), and glycophorin-A antigens (AMAC Inc, Westbrook, MA).[4] The resultant lineage-negative cells were labeled with anti-CD34 (Becton Dickinson) and anti-HLA-DR antibodies (Becton Dickinson). Cells were sorted on a FACStar-Plus laser flow cytometry system equipped with a Consort 32 computer (Becton Dickinson). Cells were selected for low-forward and side-scatter properties and expression of CD34 and HLA-DR antigens using mouse IgG1-phycoerythrin (PE) and mouse IgG2a-fluorescein isothiocyanate (FITC) antibodies as control.[4]

Secondary cell selection. Cells recovered from transwell inserts of stroma-noncontact cultures initiated with CD34+/HLA-DR- cells 2 to 3 weeks earlier were labeled with anti-CD34-PE or anti-CD34-FITC antibodies either alone or in conjunction with anti-HLA-DR-FITC antibodies or anti-CD33-PE antibodies (Becton Dickinson). Cells were selected on a FACStar-Plus laser flow cytometry system for low-forward and side-scatter properties and expression of CD34 and HLA-DR or CD33 antigens using mouse IgG1-PE, IgG1-FITC or mouse IgG2a-FITC antibodies as control (see Figs 1, 3, 4, 5). When freshly selected CD34+/HLA-DR- cells were cultured simultaneously in cytokine-free and MIP-1α + IL-3 supplemented cultures, sorting windows to reselect CD34+/CD33- and CD34+/CD33$^{bright+}$ cells were identical for the two culture conditions. Reselected cell populations will be termed cultured CD34+, cultured CD34+/HLA-DR+ or CD34+/HLA-DR-, and cultured CD34+/CD33+ or CD34+/CD33- cells.

Short-Term Methylcellulose Progenitor Culture

Cells were cultured at 0.5 to 2 × 10^3 cells/mL in methylcellulose containing Iscove's modified Dulbecco's medium (IMDM) (GIBCO Laboratories, Grand Island, NY), supplemented with 30% fetal calf serum (FCS) (Hyclone, Logan, UT), 3 IU erythropoietin (Epoietin, Amgen, Thousand Oaks, CA) and 5% to 10% 5637 bladder carcinoma-conditioned media as described.[16] The cultures were assessed at day 14 to 18 of culture for the presence of colony-forming cells (CFCs).

Long-Term Culture Systems

All culture media consists of IMDM with 12.5% FCS, 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), 2 mmol/L L-glutamine (GIBCO), penicillin 1,000 U/mL, streptomycin 100 U/mL (GIBCO) and 10^{-6} mol/L hydrocortisone.[4]

Initial stroma-noncontact cultures. Freshly sorted CD34+/HLA-DR- cells were plated in collagen-coated transwell inserts (0.4-μm pore size) (Costar, Cambridge MA) placed above irradiated marrow stromal layers (15 to 30 × 10^3/5 mL).[10] Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. Cultures were fed by removing half the media from the bottom well and replacing it with fresh complete media with or without cytokines as indicated.

Enumeration of the absolute number of LTBMC-ICs by limiting dilution assays (LDAs). M2-10B4 feeders can successfully replace human adult marrow stromal layers for stroma-contact cultures.[9] Therefore, this cell line was used to examine the absolute number of LTBMC-ICs present in selected and reselected CD34+ subpopulations. At day 0, fresh CD34+/HLA-DR- cells (22 replicates per concentration; 400, 150, 50, and 15 CD34+/HLA-DR- cells/well) were plated onto 3 × 10^4 irradiated M210-B4 cells subcultured in 96-well plates. Similarly, cultured CD34+ subpopulations (22 replicates per concentration; 100, 25, 6.25, and 1.56 cells/well) were plated onto irradiated M210-B4 cells in 96-well plates. Cultures were maintained in a humidified atmosphere, at 37°C and 5% CO_2 and fed weekly with 75 μL fresh media. After 5 to 6 weeks all media was removed and the stromal layers overlayed with methylcellulose containing media supplemented with erythropoietin (3 IU/mL) and supernatant of the bladder carcinoma cell line 5637 (5% to 10%).[10,12] Wells were scored for the presence or absence of secondary CFC at day 14. The absolute number of LTBMC-ICs present in the different cell populations was calculated as the reciprocal of the concentration of test cells that gives 37% negative cultures using Poisson statistics[17] and the weighted mean method.[18]

Stromal Layers

Marrow stromal layers. Marrow stromal layers were generated as previously described.[4] In short, marrow mononuclear cells from normal individuals were suspended in complete LTBMC media and incubated at 33°C in a humidified atmosphere with 5% CO_2. Cultures were demipopulated weekly and media was replaced with fresh complete media. Once confluent (3 to 5 weeks after initiation of the culture) stromal layers were irradiated with 1,250 Rad using a Mark-1 Cesium irradiator (Shepard and Associates, Glendale, CA). Stromal layers were then subcultured in 6-well plates before use.

M210-B4. M210-B4, a murine marrow stroma cell line, was maintained in RPMI-1640 (GIBCO) supplemented with 10% FCS. M210-B4 was subcultured in the wells of a 96-well plate. When confluent, plates were irradiated with 6,000 Rad using a Mark-1 Cesium irradiator, and all media replaced by complete LTBMC media. Irradiated plates were used 2 to 7 days later. Several studies have shown that maintenance of LTBMC-ICs and their differentiation into CFC is similar when CD34+/HLA-DR- cells are cultured either on marrow stromal layers or on murine M210-B4 feeders.[9,19]

Cytokines

Recombinant human (rh) IL-3 (Genetics Institute, Cambridge, MA) was added to the initial stroma-noncontact cultures as indicated to obtain a final concentration of 5 ng/mL. RhMIP-1α (R&D systems, Minneapolis, MN) was added to the initial stroma-noncontact cultures as indicated to obtain a final concentration of 100 ng/mL.

Statistics

Results of experimental points obtained from multiple experiments were reported as the mean ± 1 SEM. Significance levels were determined by two-sided Students *t*-test analysis.

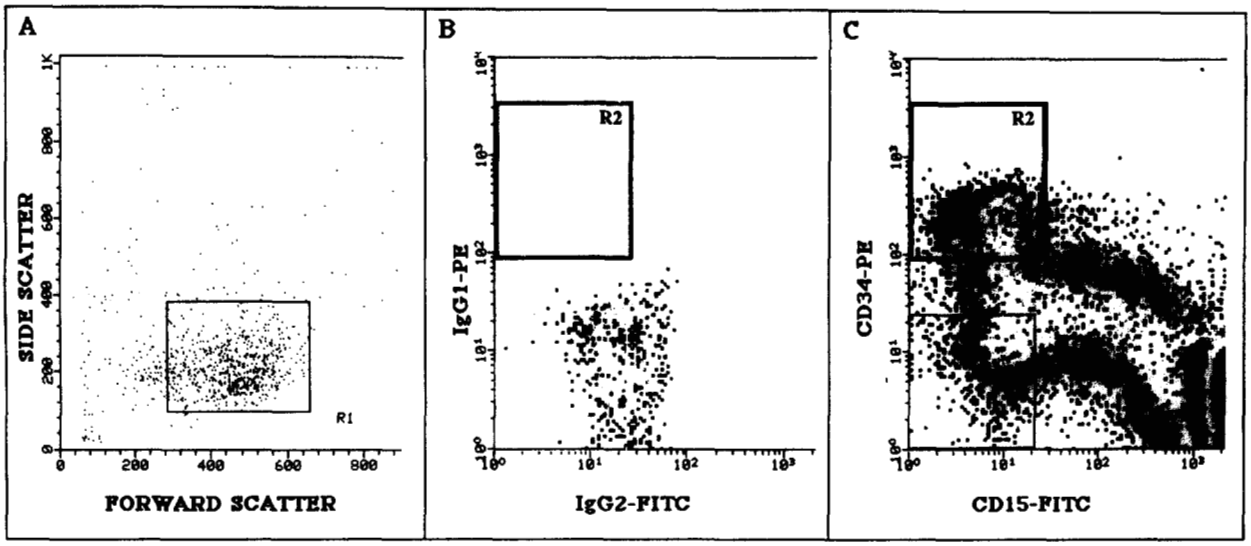

Fig 1. Representative FACS analysis of cells recovered from transwell inserts of cytokine-free stroma-noncontact cultures initiated with freshly sorted CD34$^+$/HLA-DR$^-$ cells 2 weeks earlier. Cells expressing high numbers of the CD34-PE antigen (C, R2) were reselected based on IgG1-PE control staining profile (B).

RESULTS

In an initial set of experiments, cells harvested after 2 to 3 weeks from cytokine-free stroma-noncontact cultures were reselected by FACS for cells expressing high levels of the CD34 antigen. 3.3% ± 0.5% of cultured cells were CD34$^+$ (n = 7) (Figs 1 and 2). Because the total cell number had increased 33 ± 8-fold, the total number of cultured CD34$^+$ cells recovered was 84% ± 18% of fresh CD34$^+$ cells used to initiate the cultures. Cultured CD34$^+$ cells were then replated in either methylcellulose culture to determine the frequency of CFC (n = 7) or in limiting dilution onto secondary stromal feeders for 5 weeks to determine the absolute number of LTBMC-ICs (n = 7). We showed that cultured CD34$^+$ cells contained 11.1% ± 0.6% CFC, not significantly different from that seen in fresh CD34$^+$ cells in normal marrow (Fig 2, Table 1). However, 3.9% ± 0.6% of cultured CD34$^+$

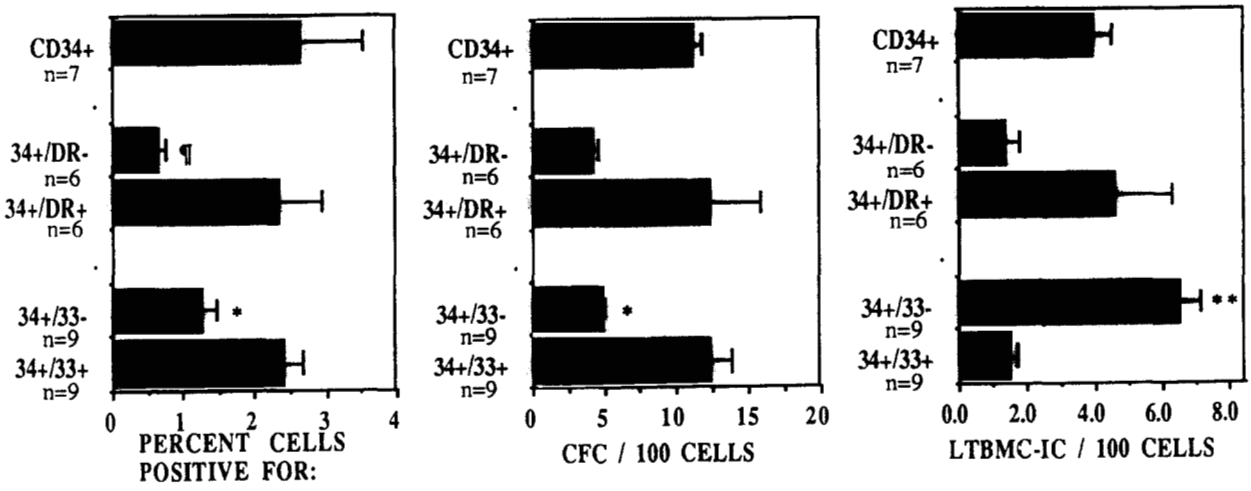

Fig 2. Freshly sorted CD34$^+$/HLA-DR$^-$ cells were plated for 2 weeks in cytokine-free stroma-noncontact cultures. Cells were collected and CD34$^+$ cells, CD34$^+$/HLA-DR$^-$ or CD34$^+$/HLA-DR$^+$ or CD34$^+$/CD33$^-$ or CD34$^+$/CD33$^+$ cells were reselected using FACS as shown in Fig 1, 3, and 4. The percent CD34$^+$ cells or CD34$^+$ subpopulations present in the CD34$^+$/HLA-DR$^-$ progeny was determined as the percent of cells present in R2 and R3. Cultured CD34$^+$ cells or CD34$^+$ subpopulations were then plated in methylcellulose progenitor assay to determine the number of committed progenitors present. Alternatively, cultured CD34$^+$ cells or CD34$^+$ subpopulations were plated in limiting dilution onto irradiated M210-B4 cells. After 5 to 6 weeks, M2-10B4 layers were overlayed with methylcellulose containing media supplemented with erythropoietin and supernatant of the cell line 5637 for an additional 2 weeks before scoring the wells for the presence or absence of CFC. The frequency of LTBMC-ICs in the reselected population was then calculated as described in Materials and Methods. Statistical analysis: Comparison between CD34$^+$/HLA-DR$^-$ and CD34$^+$/HLA-DR$^+$ populations or CD34$^+$/CD33$^-$ and CD34$^+$/CD33$^+$ populations: **, $P \leq .001$; *, $P \leq .01$; ¶, $P \leq .05$.

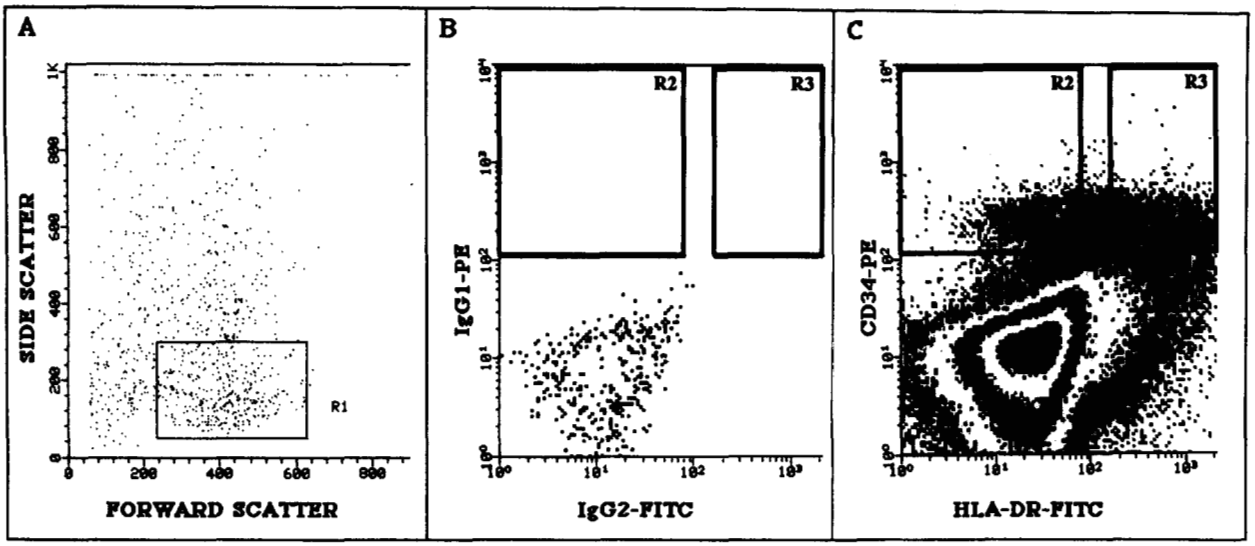

Fig 3. Representative FACS analysis of cells recovered from transwell inserts of cytokine-free stroma-noncontact cultures initiated with freshly sorted CD34+/HLA-DR- cells 2 weeks earlier. Cells expressing high numbers of the CD34-PE antigen and expressing no or low numbers of the HLA-DR-FITC antigen (CD34+/HLA-DR-) or high numbers of the HLA-DR-FITC antigen (CD34+/HLA-DR+) (C, R2 and R3, respectively) were reselected based on the IgG1-PE and IgG2a-FITC control-staining profile (B).

cells were capable of initiating and sustaining long-term cultures, which is significantly higher than what is observed for fresh CD34+ or even fresh CD34+/HLA-DR- cells (LTBMC-IC frequency = 0.86% ± 0.08%, n = 12; $P \leq .01$) (Fig 2, Table 1).

To further characterize the phenotype of LTBMC-ICs present in vitro, cultured CD34+ cells were reselected according to their expression of the HLA-DR (Fig 3) or CD33 (Fig 4) antigen. Cultured CD34+ cells (72.6% ± 6%) expressed the HLA-DR antigen (Fig 2). Committed progenitors were highly concentrated in the cultured CD34+/HLA-DR+ cell population and, similar to fresh CD34+/HLA-DR- cells, committed progenitors were relatively depleted from the cultured CD34+/HLA-DR- cell population (Fig 2). Somewhat surprisingly, primitive LTBMC-ICs were four times more frequent in cultured CD34+/HLA-DR+ cells than in cultured CD34+/HLA-DR- cells (Fig 2). Therefore, the phenotype of LTBMC-ICs present in stroma-noncontact cultures differs

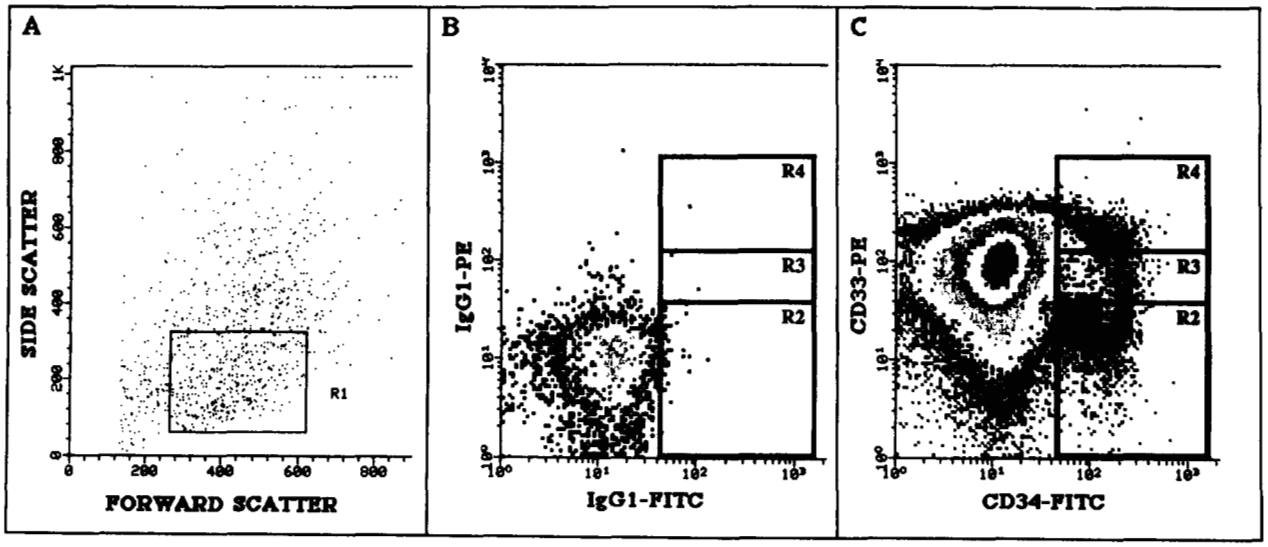

Fig 4. Representative FACS analysis of cells recovered from transwell inserts of cytokine-free stroma-noncontact cultures initiated with freshly sorted CD34+/HLA-DR- cells 2 weeks earlier. Cells expressing high numbers of the CD34-FITC antigen and expressing no CD33-PE antigen (CD34+/CD33-) or low or high numbers of the CD33-PE antigen (CD34+/CD33+) (C, R2 and R3 + R4, respectively) were reselected based on the IgG1-FITC and IgG1-PE control staining profile (B). In two additional experiments, CD34+/CD33$^{bright+}$ cells (C, R4) were selected separately.

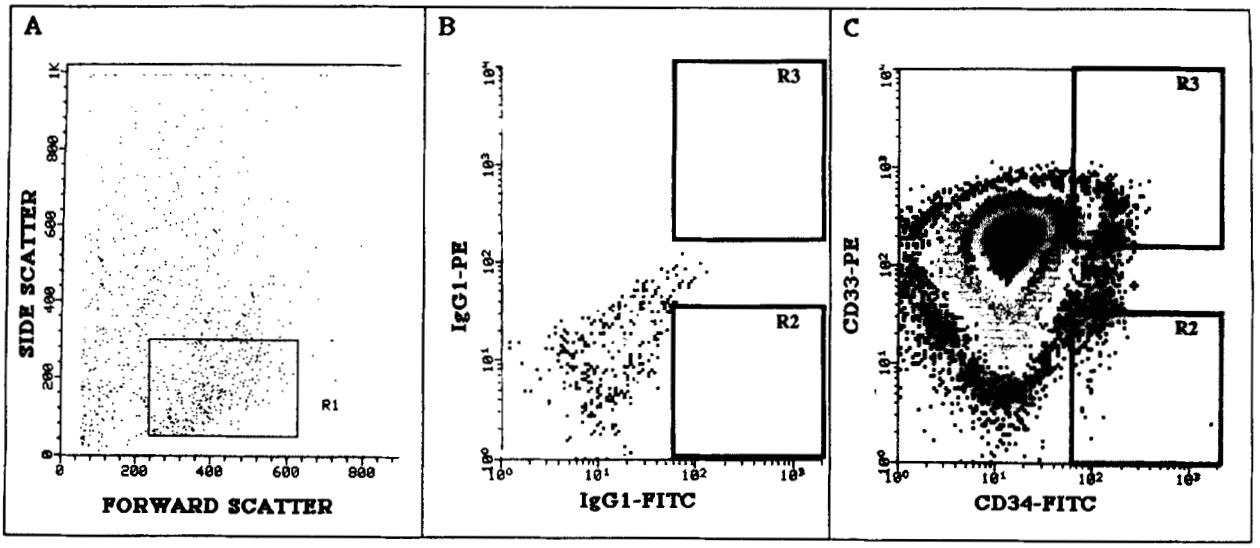

Fig 5. Representative FACS analysis of cells recovered from transwell inserts of MIP-1α + IL-3 supplemented stroma-noncontact cultures initiated with freshly sorted CD34⁺/HLA-DR⁻ cells 2 weeks earlier. Cells expressing high numbers of the CD34-FITC antigen, but no CD33 antigens (CD34⁺/CD33⁻ cells) or no high numbers of the CD33 antigen (CD34⁺/CD33$^{bright+}$) (C, R2 and R3, respectively) were reselected based on the IgG1-FITC and IgG1-PE control staining profile (B).

from that in fresh adult marrow, where LTBMC-ICs are almost exclusively present in cells that express no or low levels of HLA-DR antigens (Table 1).[2,4,7]

Because HLA-DR expression may reflect the activation status of progenitors rather than commitment to the myeloid lineage, we then reselected CD34⁺ cells from 2-week-old stroma-noncontact cultures according to their expression of the myeloid commitment antigen, CD33.[20] 66.5% ± 3% of cultured CD34⁺ cells expressed the CD33 antigen (Figs 2 and 4). Cultured CD34⁺/CD33⁺ cells were highly enriched for CFC, whereas cultured CD34⁺/CD33⁻ cells were relatively depleted of CFC (Fig 2). LTBMC-ICs were highly enriched in the cultured CD34⁺/CD33⁻ population (6.5% ± 0.7%) and comprised only 1.5% ± 0.2% of cultured CD34⁺/CD33⁺ cells (Fig 2). The observation that some cells coexpressing CD34 and CD33 antigens could initiate LTBMC prompted us to evaluate this population further. In two experiments, we selected CD34⁺/CD33$^{bright+}$ cells only (Fig 4) and examined the number of LTBMC-ICs by limiting dilution on stromal layers. These studies showed that cultured CD34⁺/CD33$^{bright+}$ cells are almost devoid of LTBMC-IC (0.17% ± 0.03%). This indicates that the presence of LTBMC-IC in cells expressing both low and high levels of the CD33 antigen could be the result of contamination with CD34⁺/CD33⁻ cells or that low levels of CD33 antigens may be found on some LTBMC-IC, as has been shown by other investigators (Landsdorp et al and Terstappen et al, personal communication, October 1993 and March 1994).

Table 1. Frequency of CFC and LTBMC-ICs in Sorted Cell Populations

Cell Population	CFC Frequency	LTBMC-IC Frequency
Fresh BM		
CD34⁺	5.4 ± 0.15 (3)	0.19 ± 0.06 (3)
CD34⁺/HLA-DR⁻	2.2 ± 0.3 (21)	0.86 ± 0.08 (12)
CD34⁺/HLA-DR⁺	12.6 ± 0.66 (25)	0.01 ± 0.003 (14)
Cytokine-free culture		
CD34⁺	11.1 ± 0.6 (3)	3.9 ± 0.6 (7)
CD34⁺/HLA-DR⁻	4.1 ± 0.5 (2)	1.3 ± 0.4 (6)
CD34⁺/HLA-DR⁺	12.2 ± 3.5 (2)	4.6 ± 1.7 (6)
CD34⁺/CD33⁻	4.9 ± 0.2 (5)	6.5 ± 0.7 (9)
CD34⁺/CD33⁺	12.3 ± 1.6 (5)	1.5 ± 0.2 (9)
CD34⁺/CD33$^{bright+}$	ND	0.17 ± 0.03 (2)
MIP-1α + IL-3		
CD34⁺/CD33⁻	ND	20.6 ± 3.7 (5)
CD34⁺/CD33$^{bright+}$	ND	0.18 ± 0.04 (5)

CD34⁺, CD34⁺/HLA-DR⁻ or CD34⁺/HLA-DR⁺ cells or CD34⁺, CD34⁺/HLA-DR⁻, CD34⁻/HLA-DR⁻, CD34⁺/CD33⁻ or CD34⁺/CD33⁺ cells reselected from stroma-noncontact cultures were plated in either methylcellulose cultures for 14 to 18 days to enumerate the frequency of CFC or in long-term culture on M2-10B4 feeders in limiting dilutions to enumerate the absolute frequency of LTBMC-ICs. Numbers in parentheses represent the number of replicate experiments.

Abbreviation: ND, not determined.

We have recently shown that culture of fresh CD34⁺/HLA-DR⁻ cells in stroma-noncontact cultures supplemented with MIP-1α + IL-3 results in the complete maintenance of LTBMC-ICs for at least 8 weeks.[13] Therefore, we examined the LTBMC-ICs cloning efficiency of CD34⁺/CD33⁺ or CD34⁺/CD33⁻ cells present in 2-week-old MIP-1α + IL-3 supplemented stroma-noncontact cultures (Fig 5). Because of the higher cell expansion in MIP-1α + IL-3 supplemented cultures (67-fold ± 10-fold expansion) the percent of CD34⁺ cells was significantly lower than that in cytokine free cultures (1.5% ± 0.4% *v* 3.3% ± 0.5%, $P = .017$). However, because of the higher expansion induced by MIP-1α + IL-3, the total number of cultured CD34⁺ cells present in MIP-1α + IL-3 supplemented cultures (93.4% ± 19% of the

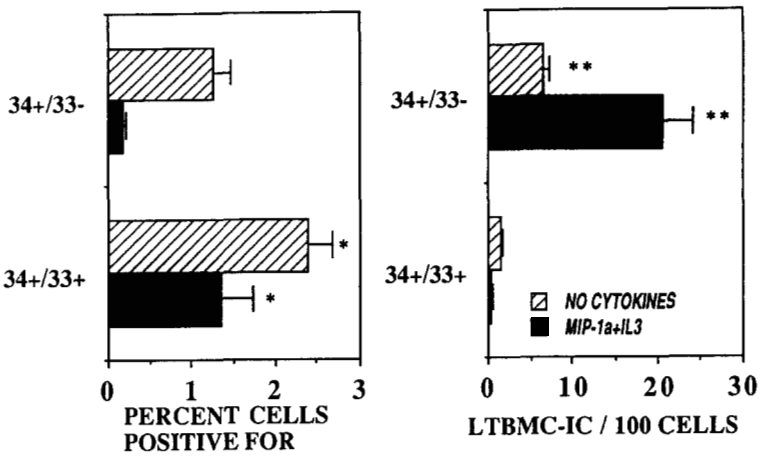

Fig 6. Freshly sorted CD34+/HLA-DR− cells were plated for 2 weeks in MIP-1α + IL-3 supplemented stroma-noncontact cultures. CD34+/CD33− or CD34+/CD33bright+ cells were reselected using FACS as shown in Fig 5. The percent CD34+ cells present in the CD34+/HLA-DR− progeny was determined as the percent of cells present in R2 and R3 (Fig 5). Cultured CD34+/CD33− or CD34+/CD33bright were plated in limiting dilution onto irradiated M210-B4 cells. After 5 to 6 weeks, the layers were overlayed with methylcellulose containing media supplemented with erythropoietin and supernatant of the cell line 5637 for an additional 2 weeks before scoring the wells for the presence or absence of CFC. The frequency of LTBMC-IC in the reselected population was then calculated as described in Materials and Methods. Statistical analysis: Comparison between CD34+/CD33− and CD34+/CD33+ populations: **, $P \leq .001$; *, $P \leq .01$.

initial CD34+ cells) was similar to that seen in cytokine-free cultures (84% ± 18%). The relative frequency of CD34+/CD33− cells present in the CD34+ fraction (11.8% ± 2.1%) was also significantly lower than that seen in cytokine-free cultures (33.5% ± 3%) ($P = .001$) (Fig 6). Evaluation of the cultured CD34+/CD33bright+ population in LDA showed that this population was practically devoid of LTBMC-ICs (0.18% ± 0.04%; Fig 6). In contrast, up to 30% of cultured CD34+/CD33− cells were LTBMC-ICs (LTBMC-IC frequency for the five different experiments = 7.5%, 21%, 25%, 26%, and 30%).

DISCUSSION

In an attempt to further purify human hematopoietic stem cells, we reselected CD34+ subpopulations from in vitro stroma-noncontact cultures initiated 2 to 3 weeks earlier with fresh CD34+/HLA-DR− cells. CD34+/HLA-DR− cells sorted from fresh, normal, adult marrow contain ≈1% LTBMC-ICs.[9,10,12,13] This indicates that CD34+/HLA-DR− cells constitute a heterogeneous population in which 99% of cells either fail to proliferate in the commonly used culture systems or do not have the capability of initiating and sustaining long-term hematopoiesis. The heterogeneity in this population may at least in part be caused by progenitors of intermediate maturity not capable of generating colonies in methylcellulose or agar assays or of initiating long-term hematopoiesis. We hypothesized that the differentiation pressure of in vitro long-term cultures might induce terminal differentiation of less primitive progenitors and hence their depletion. In contrast, more primitive progenitors might be spared. Therefore, reselection of CD34+ cells and CD34+ subpopulations from in vitro cultures may result in the recovery of a population more highly enriched for primitive progenitors. CD34+ cells present in 2- to 3-week-old stroma-noncontact cultures were still heterogeneous. Cultured CD34+ cells contained greater than 10% CFC. However, such cultured CD34+ cells contained also threefold to fourfold more LTBMC-ICs compared with fresh marrow-derived CD34+/HLA-DR− cells. Cultured CD34+/CD33− cells were relatively devoid of CFC, but enriched further in LTBMC-ICs. Compared with fresh marrow CD34+/HLA-DR− cells, LTBMC-ICs were 6 times more frequent in cultured CD34+/CD33− cells present in cytokine-free stroma-noncontact cultures and up to 30 times in CD34+/CD33− cells present in MIP-1α + IL-3–supplemented cultures. This indicates that the here-used culture conditions are favorable for maintenance of primitive LTBMC-ICs present in the starting CD34+/HLA-DR−, but not for a large fraction of fresh CD34+/HLA-DR− cells that fail to initiate LTBMC. LTBMC-ICs were significantly more frequent in CD34+/CD33− cells present in MIP-1α + IL-3–containing cultures than cytokine-free cultures. This suggests that the enrichment may at least in part be the result of terminal differentiation of progenitors of intermediate maturity within the initially sorted population resulting from the differentiation inducing culture conditions. Alternatively, the combination of MIP-1α + IL-3 and stroma-derived factors may recruit significantly more quiescent LTBMC-ICs than stroma-derived soluble factors alone.

Interestingly, reselection of cultured CD34+ cells based on their HLA-DR antigen expression showed that 80% of LTBMC-ICs reside in the cultured CD34+/HLA-DR+ fraction, whereas cultured CD34+/HLA-DR− cells were relatively depleted of LTBMC-ICs. These results differ from fresh, uncultured adult human marrow where LTBMC-IC are almost exclusively present in the CD34+/HLA-DR− fraction and absent from the CD34+/HLA-DR+ fraction.[2,4,7] Presence of high numbers of HLA-DR antigens on LTBMC-IC present in stroma-noncontact cultures could be a reflection of a more differentiated stage of these progenitors compared with LTBMC-ICs in the CD34+/HLA-DR− fraction of fresh marrow. However, we have shown that although CD34+/HLA-DR+ cells selected from fresh, adult marrow give rise to CFC during the initial 3 weeks of culture in stroma-noncontact culture, no CFC are recovered after 5 weeks of culture (unpublished observations). This indicates that the freshly sorted CD34+/HLA-DR+ cell population does not contain LTBMC-ICs. Therefore, the observation that cultured CD34+/HLA-DR+ cells give rise to sustained in vitro generation of clonogenic progenitors for at least 5 to 6 weeks indicates that these progenitors are functionally similar to LTBMC-ICs present in fresh marrow-derived CD34+/HLA-

DR^- cells. The phenotype of LTBMC-ICs in stroma-noncontact cultures is reminiscent of that of LTBMC-ICs found in human cord blood[21] or in human fetal liver.[22] The $CD34^+$/HLA-DR^+ phenotype of LTBMC-ICs present in stroma-noncontact cultures also resembles that of malignant LTBMC-IC found in the marrow of patients with chronic myelogenous leukemia.[14,23] It has been shown that malignant CML LTBMC-ICs proliferate significantly more than their quiescent counterparts present in normal marrow.[24] Similarly, LTBMC-ICs present in human cord blood or fetal liver $CD34^+$/HLA-DR^+ cells may be more proliferative than their counterparts in normal adult marrow.[21] Because HLA-DR antigen expression is cell cycle-related and increases when cells evolve from G_0 to G_1,[25,26] the increased expression of HLA-DR antigens may be caused by differences in proliferative status. We have shown that LTBMC-ICs cultured in stroma-noncontact cultures proliferate significantly more than when cultured in direct contact with stromal layers.[27] It is possible that the concentration of negative growth regulators, such as transforming growth factor β, which retain primitive hematopoietic progenitors in a nonproliferative, resting state,[28] are lower in stromal supernatants than in the extracellular matrix surrounding the stromal cells. Lack of growth inhibitory substances in stromal supernatants may then explain the increased proliferation of hematopoietic progenitors cultured in transwell inserts above the stroma and hence their increased HLA-DR antigen expression. Therefore, it will be interesting to evaluate the HLA-DR antigen status of primitive LTBMC-ICs cultured in contact with stromal layers.

In conclusion, we show that LTBMC-ICs are significantly more frequent in cultured $CD34^+$/HLA-DR^+, but not cultured $CD34^+$/HLA-DR^- cells, indicating that absence or presence of HLA-DR antigens on primitive hematopoietic progenitors may reflect the proliferative status rather than lineage commitment of these progenitors. We show further that the phenotype of cultured LTBMC-IC is $CD34^+/CD33^-$ and that up to 30% of $CD34^+/CD33^-$ cells present in MIP-1α and IL-3–supplemented stroma-noncontact cultures are capable of initiating and sustaining in vitro hematopoiesis for 5 to 6 weeks. These highly purified populations of LTBMC-ICs may now allow us to evaluate at the single-cell level multilineage differentiation and self-renewal capabilities of primitive human hematopoietic progenitors. The phenotypic characterization of human LTBMC-ICs present in cultures will also prove useful in studies evaluating culture systems designed to expand human hematopoietic stem cells.

ACKNOWLEDGMENT

We thank Dr C. Eaves for providing the murine marrow stromal cell line M210-B4; Dr G. Wong for providing rhIL-3; Brad Anderson, Jori Burhoughs, Peter Catanzarro, Wen-na Li, and Ranjita Sengupta for their excellent technical help; and Dr Philip McGlave for his critical review of the manuscript.

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