

Characterization and Partial Purification of Human Marrow Cells Capable of Initiating Long-Term Hematopoiesis In Vitro

By Heather J. Sutherland, Connie J. Eaves, Allen C. Eaves, Wieslawa Dragowska, and Peter M. Lansdorf

To develop a purification strategy for isolating the most primitive hematopoietic stem cells present in normal human marrow we have combined cell separation techniques with an assay for cells that initiate sustained hematopoiesis in vitro in the presence of irradiated human marrow adherent cells. These "feeders" were established by subculturing 2- to 6-week-old primary long-term marrow culture adherent layers at a density of 3×10^4 irradiated cells per square centimeter. Test "long-term culture (LTC)-initiating cells" were plated on top of the feeders and the cocultures then maintained as standard long-term marrow cultures with half-media changes and removal of half of the nonadherent cells each week. The total number of myeloid, erythroid, and multilineage clonogenic progenitors present after 5 weeks was used to provide a quantitative assessment of the number of LTC-initiating cells originally added. Using this assay, the density, light scatter, and two cell surface antigen properties of LTC-initiating cells have been defined and compared with cells capable of directly forming colonies in methylcellulose. While the majority of the clonogenic cells were found in the high forward light scatter (FLS) "blast" window, LTC-initiating cells had significantly lower FLS properties and in this respect were more similar to lymphocytes.

LTC-initiating cells also expressed less HLA-DR antigen than clonogenic cells. The majority of LTC-initiating cells were found in the top 2% of the CD34 (My10) fluorescence profile, whereas clonogenic cells were found throughout the top 5% of the CD34 fluorescence profile. By combining low FLS, low orthogonal light scatter (OLS), low HLA-DR expression, and high CD34 expression, a population could be obtained that was enriched for LTC-initiating cells ~800-fold over unseparated marrow. This population contains only 0.06% of the marrow cells and 2% of the total clonogenic cells, but retains 50% to 60% of the LTC-initiating cells present in the original marrow. The ability to purify these two populations independently shows that the LTC and clonogenic assays identify distinct, although not necessarily nonoverlapping cell types in human marrow. Since clonogenic cells are derived from LTC-initiating cells, the LTC assay clearly detects a more primitive population. The availability of a simple approach that allows the purification of such cells by three orders of magnitude in high yield should be useful for the investigation of early events in hematopoiesis as well as for the definitive isolation of human hematopoietic stem cells with long-term in vivo repopulating potential.

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PURIFICATION of the most primitive cells in human marrow capable of sustained repopulation of hematopoietic tissues in vivo has been hampered by the inability to assay these cells directly. Thus, strategies for human hematopoietic stem cell purification have had to rely on available in vitro assays, whose validity has been based on cross-comparisons with similar assays for murine hematopoietic cells. In contrast pluripotential repopulating cells in murine marrow can be measured directly in quantitative transplantation assays and procedures that yield murine marrow cell suspensions that are highly enriched in their content of repopulating cells have been described by several groups.¹⁻⁴ Analysis of the in vitro clonogenic potential of these highly purified populations has suggested that most cells capable of long-term repopulation in vivo may not be detected as clonogenic cells in standard semisolid culture systems.^{1,5} Nevertheless, most efforts to date to obtain enriched populations of primitive human hematopoietic progenitors have focused on the use of in vitro clonogenic assays to define selection criteria for repopulating cells on the assumption that they are either identical or would be copurified^{6,7} and populations of cells have now been isolated from normal human marrow that show a cloning efficiency in vitro of up to 47%.⁸

Recently, studies of marrow cells exposed in vitro to 4-hydroperoxycyclophosphamide (4-HC) have provided evidence that in humans as well as in rodents, most if not all clonogenic cells differ from cells capable of regenerating hematopoiesis in vivo by the much lower sensitivity of the latter type of cells to this drug.^{9,10} Human marrow cells capable of initiating prolonged hematopoiesis in the long-term marrow culture system have also been found to have a similar insensitivity to 4-HC.^{11,14} (For simplicity, we will

refer to this population of hematopoietic cells as "long-term culture (LTC)-initiating cells.") Experiments with mouse marrow have shown that both LTC-initiating cells and in vivo repopulating cells are rhodamine-123 dull, whereas all day 8 colony-forming units in the spleen (CFU-S) and the majority of day 12 CFU-S are rhodamine bright.¹⁵ In human marrow clonogenic cells and LTC-initiating cells have been found to differ in their expression of CD33 (My9).¹⁶ Recent data from our laboratory suggest that some human marrow cells derived from ten-day-old LTCs are capable of re-engraftment upon autologous transplantation.¹⁷ Initiation of sustained hematopoiesis in the LTC human marrow system would appear to offer a procedure for detecting cells that give

From the Terry Fox Laboratory, B.C. Cancer Research Centre, Vancouver, and The Departments of Pathology, Medical Genetics, and Medicine, University of British Columbia, Vancouver.

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Address reprint requests to Heather J. Sutherland, 5110 Terry Fox Laboratory, B.C. Cancer Research Centre, 601 West 10th Avenue, Vancouver, B.C. V5Z 1L3.

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rise clonogenic progenitor cells but which may, themselves, be distinct from most clonogenic cells and therefore developmentally more closely related to repopulating cells.

The present study is part of a larger effort we have begun in this center to develop procedures for obtaining homogeneous populations of human cells capable of long-term repopulation in transplant recipients. In this report we describe the results of experiments in which four features of LTC-initiating cells were characterized and used as a basis for their enrichment. To develop a quantitative measure of the number of LTC-initiating cells in a given cell suspension, we assessed the total (nonadherent plus adherent) number of clonogenic cells present in LTC "assays" 5 weeks after their initiation. The selection of this time point was based on previous studies with human marrow indicating that most of the clonogenic cells present initially would have disappeared (by differentiation and/or death) within 5 weeks,^{16,18} and that a period of this duration might be necessary to detect significant numbers of newly produced clonogenic cells.¹⁷ To eliminate effects due to variable degrees of purification of supportive cells, all LTCs were initiated by plating the test cells on pre-established, irradiated normal marrow adherent layers.¹⁶

MATERIALS AND METHODS

Cells. Human marrow was obtained from informed and consenting individuals donating marrow for autologous or allogeneic marrow transplants and was collected in TC 199 with 60 U/mL of preservative free heparin. Although marrow was normal in all patients by FACS analysis, production of clonogenic cells in LTC by some autologous marrow harvests was too low to allow meaningful analysis of the data and these experiments were then discarded. Both clonogenic progenitor content and LTC-initiating cell content were lower in the marrow from autologous donors as compared with normal allogeneic donors; however, the difference in the LTC-initiating cell content in the autologous and allogeneic Percoll cells was the only one to reach significance (Table 1).

Density separation. Discontinuous Percoll density gradient centrifugation was performed using a modification of the methods of Frickhofen¹⁹ and Roos and de Boer.²⁰ An isotonic Percoll stock solution was prepared by mixing Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) with a 10× concentrated phosphate buffered saline to obtain a Percoll stock solution with a measured osmolality of 290 ± 5 mosm and a pH of 7.4.

Marrow cells were washed twice in Iscove's medium with 2% fetal calf serum (FCS), suspended in Iscove's 2% FCS at 10⁷ cells/mL, a microhematocrit determined and the cells then mixed with an appropriate volume of Percoll to suspend the cells in a solution with a

density 1.066 g/mL or 1.068 g/mL, as follows:

Volume of Percoll Stock Solution to be Added (mL)	Density Desired (1.066 or 1.068 g/mL)	Density of Iscove's 2% FCS
	Density of Percoll Stock Solution	Desired Density
	$\frac{\text{Volume of Cells \& Wash (mL)}}{\times 1} = \frac{\text{Hematocrit}}{100}$	

The suspension was overlaid with 5 mL of Iscove's 2% FCS and centrifuged at 600 × g for 15 minutes. The cells floating on top of the Percoll solution were removed and washed twice in 2% FCS. Percoll cells were stored at 4°C overnight in Iscove's 30% FCS before staining and sorting. Most experiments were performed with cells with a density <1.066 g/mL. However for the last five experiments with double-stained cells, low density cells with a density <1.068 g/mL were used. Nucleated cell counts were performed on a Coulter Counter Model ZM (Coulter Electronics Limited, Luton, England).

Staining and flow cytometry. Low density cells were washed twice in Hank's buffered saline with 2% FCS and 0.1% sodium azide (HFN) and suspended in HFN at 5 × 10⁶ cells/mL with 2 µg/mL propidium iodide (PI) and 100 µg/mL DNase I (Type II-S; Sigma Chemical Co, St. Louis) and sorted on the basis of their light scatter properties. For labeling with (fluorescent) antibodies low density cells were washed and suspended in HFN at 2 × 10⁷ cells/mL. Cells stained with anti-CD34 (HPCA-1; Becton Dickinson, Sunnyvale, CA) were incubated with 2 µg of antibody/10⁷ cells for 30 minutes on ice, washed with HFN, and then stained with fluorescein isothiocyanate (FITC)-conjugated F(ab)₂ fragments of sheep anti-mouse IgG (SAM-FITC, Cappel 1711-3244; Organon Teknica Corp, West Chester, PA) at 2 µg of antibody/10⁷ cells for 30 minutes on ice. Cells were then washed again and suspended in HFN with PI and DNase I. Cells stained with anti-HLA-DR conjugated directly to phycoerythrin (PE) (anti-HLA-DR-PE, Becton Dickinson) were incubated with 1 µg antibody/10⁷ cells for 30 minutes on ice, then washed and suspended in HFN with DNase I. Double-stained cells were first stained with anti-CD34 and SAM-FITC as above, then washed, incubated with anti-HLA-DR-PE for 30 minutes at 4°C, and finally washed and suspended in HFN with DNase I.

Cells were analyzed and sorted on a Becton Dickinson FACS 400 using a single argon laser at 488 nm. FITC fluorescence was measured using a 530/30 band pass filter, PE fluorescence using a 630 long pass filter, and PE fluorescence using a 575/26 band pass filter. The FACS was calibrated before each run using 10 µm fluorescent microspheres (Coulter Corp, Hialeah, FL). Compensation for double-stained specimens was set up in both FITC and PE fluorescence with single-stained samples using Stanford counters (Stanford University, Pasadena, CA). Cells were sorted at 10⁵

Table 1. Frequency of Clonogenic Cells and LTC-Initiating Cells in Human Marrow Before and After Density Centrifugation on 1.066 to 1.068 g/mL Percoll

Donor	Marrow Buffy Coat		Marrow Cells ± 1.066 to 1.068 g/mL	
	Clonogenic Cells	LTC-Initiating Cells	Clonogenic Cells	LTC-Initiating Cells
Allogeneic	95 ± 14 (5)	12 ± 3 (5)	640 ± 87 (11)	72 ± 13 (11)
Autologous	63 ± 23 (3)	5 ± 1 (2)	439 ± 56 (9)	20 ± 5 (9)

All values shown are the mean ± SEM (number of experiments) per initial 10⁵ nucleated cells plated in methylcellulose on day 0 (clonogenic cells) or day 0 (LTC), in which case the clonogenic cell content was measured 5 weeks later (LTC-initiating cells). Differences between allogeneic and autologous marrow values are not significant except in the case of the reduced LTC-initiating cell concentration found in low density suspensions of autologous marrow harvests ($P = .03$).

full drop envelope mode at 2,000 to 3,000 cells per second. Data was collected in list mode, 10,000 cells per sample, on a VAX 730 computer. Contour plots and histograms were generated using EDESK software (Stanford University). Cells were maintained at 4°C before, during, and after the sort and collected in Iscove's 50% FCS medium.

Clonogenic assays. Primitive erythropoietic (BFU-E), granulopoietic (CFU-GM), and multilineage (CFU-GEMM) progenitors were assayed in methylcellulose cultures containing 3 U/ml human urinary erythropoietin (>1,000 U/mg) and 10% agar-stimulated human leukocyte conditioned media as described.²¹ Progenitor numbers were derived from colony counts after incubation of the culture for 18 to 21 days at 37°C. Marrow buffy coat cells were plated at 2×10^5 cells/mL. Marrow cells recovered after Percoll density centrifugation were plated at 5×10^4 cells/mL. Sorted fractions were plated at lower cell concentrations depending on the enrichment of progenitors anticipated from preliminary experiments. All assays were set up in duplicate or quadruplicate 1-mL volumes. Progenitor enrichment relative to the unsorted low density cells was calculated by dividing the progenitor frequency/ 10^5 cells in the sorted fraction by the progenitor frequency/ 10^5 cells in the unsorted, Percoll separated, suspension. Progenitor recovery in a given sorted fraction was calculated by multiplying the calculated enrichment by the percentage of cells in the fraction sorted. In the experiments where cells were sorted according to their light scatter properties or expression of HLA-DR, the sum of the individual progenitor recoveries for all fractions was, on average, $115 \pm 20\%$ and $112 \pm 22\%$, respectively. Accordingly, in these experiments the recovery in individual fractions was normalized so that the sum of all fractions in an individual experiment was 100%.

Long-term marrow cultures. These were established and maintained in general according to procedures previously described.²¹ Briefly, cells were suspended in 2.5 mL of LTC media (alpha medium supplemented with 40 mg/L inositol, 10 mg/L folic acid, 400 mg/L glutamine, 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L hydrocortisone sodium succinate), which was then placed in 35-mm Corning tissue culture dishes (Corning Glassworks, Corning, NY) on pre-established, irradiated, normal marrow feeders. To prepare feeders, allogeneic marrow was used to initiate primary LTCs in 60-mm tissue culture dishes 2 to 6 weeks before the experiment and these were then maintained as for regular LTCs. One to seven days before setting up cocultures, nonadherent cells were removed and the adherent cells trypsinized,²¹ irradiated with 15 gray of 250 KVP x-rays, and plated in 35-mm Corning tissue culture dishes at 3×10^4 cells per dish (ie, 1×10^4 cells/cm²).²²

Unprocessed marrow or marrow buffy coat cells were plated at 8×10^6 cells/LTC. Percoll separated cells at 10^7 cells/LTC, and sorted fractions at proportionately lower cell concentrations depending on the degree of enrichment anticipated from preliminary experiments. Cultures were incubated for the first three to four days at 37°C and thereafter at 33°C, in 5% CO₂. At weekly intervals, half of the nonadherent cells were removed and at the same time half of the medium was replaced. After 5 weeks all nonadherent cells were removed and the cells in the adherent layer suspended by trypsinization. Nonadherent and adherent cells were then washed and aliquots assayed for clonogenic cells. The total number of progenitors present in the 5-week-old LTCs was calculated, and enrichment and recovery values determined as for clonogenic cells, using the number of clonogenic cells at 5 weeks to provide an indirect measure of the number of LTC-initiating cells present at day 0. In experiments where cells were sorted according to their light scatter properties or expression of HLA-DR, the sum of the recoveries from all fractions was $168 \pm 30\%$ and $128 \pm 22\%$, respectively. While these recoveries are slightly greater than 100%, this represents the range of error inherent in these measurements. As for the clonogenic cells, the

LTC-initiating cell recovery in an individual fraction was then normalized so that the sum of all fractions in each experiment was 100% to allow more accurate comparisons between fractions. Differences between the mean recovery of LTC-initiating cells in a given fraction and the mean recovery of clonogenic cells in that fraction were compared by a two-tailed Student's *t* test.

RESULTS

Density separation. Recovery of nucleated marrow cells >1.066 g/mL and <1.068 g/mL was $11 \pm 3\%$ and $14 \pm 6\%$, respectively. Recovery of clonogenic progenitors was $73 \pm 25\%$ and $94 \pm 29\%$, respectively, and recovery of LTC-initiating cells was $49 \pm 32\%$ and $97 \pm 8\%$. Because of the higher recovery of LTC-initiating cells using 1.068 g/mL, a switch was made to the use of this density in later experiments. The average enrichment of clonogenic cells was 6.5 ± 2 and 9 ± 4 fold, and of LTC-initiating cells was 3 ± 1 and 6 ± 3 fold after density separation at 1.066 and 1.068 g/mL.

Light scatter properties of clonogenic and LTC-initiating cells. Low density marrow cells from five individuals (two allogeneic donors and three autologous donors) were sorted into four fractions according to their forward light scatter (FLS) and orthogonal (90°) light scatter (OLS) properties (Fig 1A). Morphologically, fraction I contains primarily small lymphocytes and some normoblasts, fraction II contains larger lymphocytes, fraction III contains monocytes, blasts, and some promyelocytes and myelocytes, and fraction IV contains primarily maturing granulocytic cells. In each experiment aliquots of cells from the four sorted fractions as well as from the starting low density cell suspension were plated in the clonogenic assay and were also used to initiate LTCs for determination of LTC-initiating cell content. The progenitor recovery in each fraction was determined and then expressed as a percentage of total progenitor recovery for that experiment. The mean recovery of clonogenic cells for each fraction is compared with the corresponding LTC-initiating cell recovery values in Fig 1B. By comparing fractions I and III it can be seen that LTC-initiating cells are significantly different from clonogenic cells ($P < .05$) with respect to their light scatter characteristics. Selection of cells with low FLS (fraction I) selectively enriched for LTC-initiating cells, whereas sorting in the blast window²³ (fraction III) selectively enriched for clonogenic cells. This result was not altered when data from autologous or allogeneic marrow harvests were analyzed separately.

HLA-DR expression on clonogenic and LTC-initiating cells. Low density marrow cells from six individuals (two autologous donors and four allogeneic donors) were stained with anti-HLA-DR-PE and sorted into four fractions according to their phycoerythrin fluorescence intensity (Fig 2A). Light scatter gates were set to include only cells with low OLS and FLS gates were left open (ie, to include all cells in fractions I, II, and III in Fig 1A). The first two HLA-DR-PE sort fractions (I and II) overlap with the unlabeled unstained control and correspond to 95% and 5% of cells in the control profile, respectively. Fractions III and IV are HLA-DR positive. Almost all (~90%) of the clonogenic cells were found in the latter two fractions. In contrast, approxi-

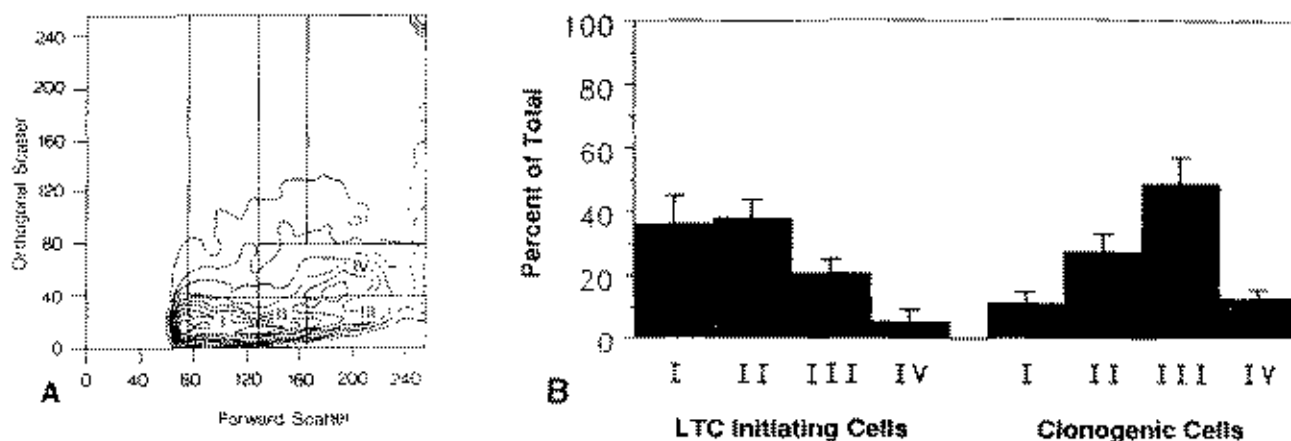


Fig 1. Light scatter properties of clonogenic and LTC-initiating cells. (A) Bivariate probability contour histogram of low density marrow cells, plotting FLS (channel number) vs OLS (channel number) is presented in a representative experiment. Ten percent of cells fall in the space between each adjacent pair of contour lines. (B) The mean progenitor recovery \pm SEM is expressed as a percent of the total recovery in each of five experiments. The content of LTC-initiating cells and directly clonogenic cells was compared for each of the four fractions sorted as outlined in A. Differences between the recoveries of clonogenic and LTC-initiating cells in fractions I and III are significant ($P < .05$).

mately half (55%) of the LTC-initiating cells were found in fractions I and II, and these cells were virtually absent from fraction IV (Fig 2B). The differences between clonogenic cell recovery and LTC-initiating cell recovery are significant at the $P < .05$ level in fractions II and IV.

Progenitor enrichment by sorting for high CD34 expression. Low density marrow cells were also sorted for high CD34 expression after staining with anti-CD34 and SAM-FITC. In a first series of experiments, the cells were concomitantly sorted with open FLS gates and low OLS gates (fractions I, II, and III in Fig 1A), and the top 5% of viable (PI negative) FITC fluorescent cells collected. Table 2 shows the results obtained for clonogenic cell and LTC-initiating cell numbers per 10^5 cells assayed, and the corresponding

enrichment obtained by comparison to unsorted, low density cells. In experiments 3 and 5, it was possible to compare internally the top 5% of CD34-positive cells with the top 2% of CD34-positive cells. The frequency of clonogenic cells and their enrichment was not different in the two CD34-positive fractions; however, their recovery decreased approximately twofold when only the top 2% of CD34-positive cells were sorted. In contrast, LTC-initiating cells doubled in both frequency and enrichment when the top 2% was compared with the top 5% of CD34-positive cells and the same high recovery was obtained in both fractions.

Using Percoll density separation the overall average enrichment of clonogenic cells was 8 ± 3 -fold and of LTC-initiating cells was 4.5 ± 2 -fold over original marrow buff

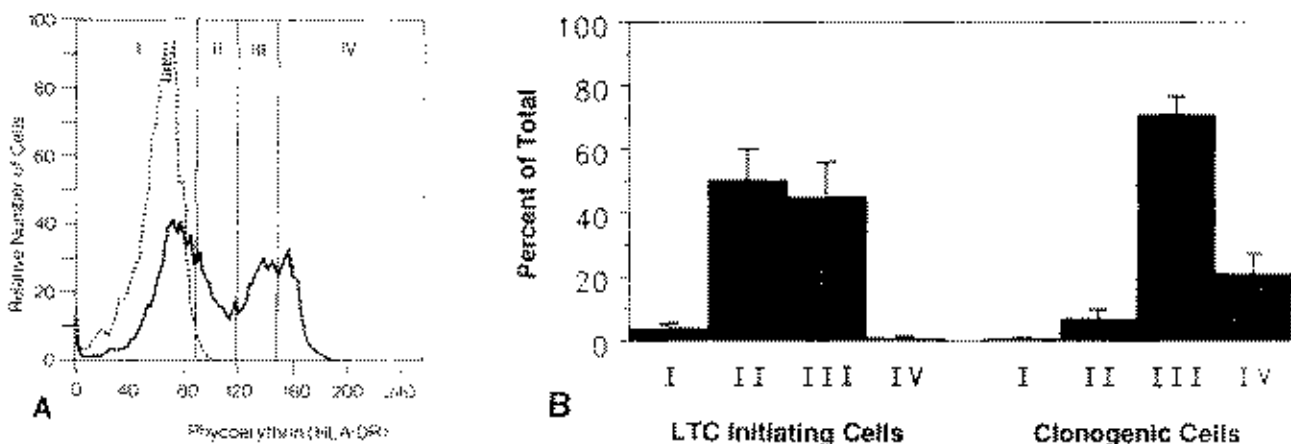


Fig 2. Expression of HLA-DR on clonogenic and LTC-initiating cells. (A) The fluorescence profile (log scale) of low density, unsorted marrow cells (dotted line) is compared with the same cell suspensions after labeling with anti-HLA-DR conjugated to PE (solid line) in a representative experiment. Relative number of cells is presented vs the phycoerythrin fluorescence channel number. The fluorescence intensity remained relatively constant between experiments and the gating of fractions I to IV was maintained constant at the fluorescence channels shown. (B) Mean progenitor recovery \pm SEM is expressed as a percent of the total number recovered in each of five experiments. The content of LTC-initiating cells and directly clonogenic cells was compared for each of the four fractions sorted as outlined in A. Differences between the recoveries of LTC-initiating cells and clonogenic cells in fractions II and IV are significant ($P < .05$).

Table 2. Degree of Enrichment of Clonogenic and LTC-Initiating Cells After Sorting of Low Density Human Marrow for High My 10 Expression

Experiment No.	Top % Sorted	Clonogenic Cells			LTC-Initiating Cells*		
		No. Per 10 ⁵ Cells	Enrichment v Percoll	Recovery (%)	No. Per 10 ⁵ Cells	Enrichment v Percoll	Recovery (%)
1	5	11,000	25X	44	2,800	55X	68
2	5	14,600	68X	47	600	58X	65
3	5	11,100	19X	57	500	19X	67
4	5	15,100	31X	70	1,100	107X	98
5	5	18,850	23X	58	2,145	32X	80
Mean		14,100	33X	57	1,440	58X	78
3	2	16,100	28X	38	1,350	50X	69
5	2	15,500	19X	21	4,060	61X	67
Mean		15,850	24X	30	2,700	56X	68

*Values refer to the number of clonogenic cells in LTC at 5 weeks per 10⁵ nucleated cells initially plated in LTC.

coat cells. Multiplying these numbers by the average enrichment obtained for clonogenic and LTC-initiating cells after sorting for CD34 (top 5%) gives an enrichment of 260 ± 120 fold for both clonogenic and LTC-initiating cells as compared with the original marrow buffy coat suspensions.

Partial purification of clonogenic and LTC-initiating cells. Using the FLS, OLS, HLA-DR, and CD34 results obtained above, appropriate gates were then chosen for use in four parameter sorts to selectively isolate either clonogenic or LTC-initiating cells. To enrich for clonogenic cells, cells were sorted for high HLA-DR expression, with or without the addition of high FLS (Table 3). A maximum enrichment of 74-fold over the Percoll cells was obtained, or ~600-fold enrichment over the concentration of clonogenic cells in the original marrow buffy coat. Such suspensions had an average cloning efficiency of $30 \pm 2\%$ and were relatively depleted of LTC-initiating cells.

Table 4 shows the results obtained using gates to enrich selectively for LTC-initiating cells. In this case cells were sorted for low HLA-DR expression and high CD34 expression (top 2%) with low or very low FLS. The concentration of LTC-initiating cells was enriched 170- to 190-fold over that measured in Percoll cells, and ~800-fold over marrow buffy coat. These fractions were found to contain only 0.4% to 0.8% of the low density nucleated cells and only 2% of the clonogenic cells. Recovery of LTC-initiating cells was 50% to 59%. One hundred of these highly purified cells produced, on

average, eight clonogenic progenitors in 5-week-old LTC, even though only four of the initial 100 cells were initially capable of colony formation in methylcellulose. As shown in Table 5 (the relative proportions of different types of clonogenic cells detected either initially or after 5 weeks in LTC was not significantly different between unsorted low density cells and the fractions most enriched for primitive cells (second and third rows, Table 4).

DISCUSSION

In this report we have described a purification procedure for the cells in human marrow that are capable of initiating long-term hematopoiesis *in vitro*. These cells could be enriched ~800-fold by a combination of density centrifugation and a single step 4 parameter FACS sort. To quantify LTC-initiating cells, we measured the total number (adherent and nonadherent) of clonogenic progenitors present after a culture period of 5 weeks. The validity of this approach is based in part on the assumption that there is a linear relationship between the number of clonogenic progenitors present after 5 weeks and the number of cells in the original suspension tested that are capable of generating clonogenic progeny. Experimental data validating this assumption has been obtained²² and will be presented in detail elsewhere. For simplicity we have referred to the number of clonogenic progenitors present in 5-week-old LTC assays as the "CFU-E"

Table 3. Selective Enrichment of Clonogenic Cells in Normal Human Marrow by Multi-Parameter Sorting

Cells Tested	Total Nucleated Cells	Clonogenic Cells			LTC-Initiating Cells*		
		Per 10 ⁵ Cells	Enrichment	Recovery (%)	Per 10 ⁵ Cells	Enrichment	Recovery (%)
My10 + ve (top 5%), HLA-DR high (fraction III)	1.3 ± 0.2	17,000 ± 3,000	32 ± 5	44 ± 12	940 ± 30	21 ± 7	32 ± 11
My10 + ve (top 5%), HLA-DR high (fraction III) FLS-high (fraction III)	0.8 ± 0.6	30,000 ± 2,000	74 ± 16	78 ± 58	1,700 ± 100	30 ± 3	26 ± 11

Values shown are the mean ± SEM for seven (first row) and two (second row) experiments by comparison with low density Percoll cells

*Values refer to number of clonogenic cells in LTC at 5 weeks per 10⁵ nucleated cells initially plated in LTC.

Table 4. Selective Enrichment of LTC-Initiating Cells in Normal Human Marrow by Multi-Parameter Sorting

Cells Tested	Total Nucleated Cells	Clonogenic Cells			LTC-Initiating Cells*		
	Recovery (%)	Per 10 ⁶ Cells	Enrichment	Recovery (%)	Per 10 ⁶ Cells	Enrichment	Recovery (%)
My10 + ve (top 5%) HLA-DR low (fraction III)	0.8 ± 0.2	10,000 ± 3,000	20 ± 3	17 ± 6	5,300 ± 1,800	110 ± 30	70 ± 30
My10 + ve (top 2%) HLA-DR low (fraction III) FLS-low (fraction I & II)	0.4 ± 0.1	4,500 ± 1,100	9 ± 3	2 ± 1	7,100 ± 2,000	190 ± 80	59 ± 20
My10 + ve (top 2%) HLA-DR low (fraction III) FLS-very low (fraction I)	0.4 ± 0.2	3,500 ± 1,200	7 ± 2	2 ± 1	8,900 ± 3,700	170 ± 60	50 ± 23

Values shown are the mean ± SEM for seven (first row), six (second row), and four (third row) experiments by comparison with low density Percoll cells.

*Values refer to number of clonogenic cells in LTC at 5 weeks per 10⁶ nucleated cells initially plated in LTC.

initiating cell content", recognizing that the actual number of progenitor cells detected will likely be somewhat greater than the number of original LTC-initiating cells, as the latter would each give rise to more than one clonogenic cell detectable after 5 weeks in LTC.

The separate characterization of LTC-initiating cells and directly clonogenic cells with respect to a number of physical and antigenic properties revealed consistent differences that could be exploited to allow their differential enrichment. Previous reports have shown that the modal density of clonogenic progenitors occurs between 1.063 and 1.064 g/mL.¹⁷ As confirmed here, essentially all such cells were found to be lighter than 1.066 g/mL. Less is known about the density profile of LTC-initiating cells, but our results indicate that substantial numbers of these cells may be slightly more dense than clonogenic progenitors. With respect to light scatter properties, previous studies have also shown that the majority of clonogenic progenitors exhibit a high FLS and a low to intermediate OLS^{24,25} and are thus concentrated in an area referred to as the blast window.²⁷ Again, our data confirm these findings; however, we have now shown that LTC-initiating cells have a significantly lower FLS than the majority of clonogenic cells, and are therefore found in the light scatter fractions that contain small-to-medium sized lymphocytes.

Expression of the HLA-DR antigen on primitive hematopoietic cells has been a point of some controversy in the

literature.²⁶⁻³⁰ Most clonogenic cells express detectable levels of HLA-DR antigens,^{26,28} although the "blast colony-forming cell" has been found to lack surface HLA-DR antigens.²⁶ LTC-initiating cells have been reported to both express²⁸ and lack^{27,29} HLA-DR antigens. In the present experiments, the HLA-DR fluorescence profile was subdivided into four fractions, the first two corresponding to no or very low HLA-DR antigen expression and the latter two to increasingly higher HLA-DR expression. Separate analysis of each of these fractions confirmed that the majority of clonogenic cells express readily detectable levels of HLA-DR, whereas approximately 50% of LTC-initiating cells showed no or very low HLA-DR expression.

Sorting of human marrow cells with high CD34 antigen expression allowed both clonogenic and LTC-initiating cells to be significantly enriched in agreement with previous observations by others.^{13,21} Recent studies have suggested that cells responsible for hematopoietic reconstitution after lethal irradiation are also retained in the CD34 positive fraction although quantitative measurements and enrichment calculations were not performed.³¹ In the present study, we have obtained evidence that LTC-initiating cells express higher levels of CD34 than do the majority of clonogenic cells. This is consistent with the previously reported progressive decline in CD34 antigen expression with increasing hematopoietic cell differentiation at later stages of myelopoiesis.³²

Previous attempts to obtain enriched populations of primitive human hematopoietic cells have focused on the isolation of cells capable of directly forming colonies of different myeloid and erythroid cells in semisolid media containing soluble growth factors. We have shown that the majority of these cells differ in their light scatter properties, and CD34 and HLA-DR expression from the cells that give rise to clonogenic progenitors in the LTC system. Analogous studies with murine marrow would predict that it might be possible to further separate human LTC-initiating cells from residual clonogenic cells.¹ Similarly it may be possible

Table 5. Relative Proportions of Different Types of Clonogenic Cells Detected Before and After 5 Weeks in LTC (% of Total)

	Clonogenic Cells			LTC-Initiating Cells*		
	SPU-E	CPU-GM	CPU-GRM	SPU-E	CPU-GM	CPU-GRM
Unsorted low density cells	36 ± 3	87 ± 4	12 ± 0.7	9 ± 2	91 ± 2	0.9 ± 0.3
LTC-initiating cell-enriched (rows 2 and 3 Table 4)	24 ± 5	78 ± 5	0.4 ± 0.3	5 ± 1	80 ± 4	1.4 ± 0.6

Values represent mean ± SEM for ten experiments.

*Values refer to clonogenic cells derived from LTC at 5 weeks.

obtain suspensions that are more enriched in human clonogenic cells than those described here (30%, Table 3) which are completely devoid of LTC-initiating cells. Comparative studies of these populations should be useful for the identification of growth factors that stimulate the most primitive hematopoietic cells in normal human marrow and to charac-

terize molecular events that distinguish their response patterns.

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