Phenotypic Analysis of Murine Long-Term Hemopoietic Reconstituting Cells Quantitated Competitively In Vivo and Comparison with More Advanced Colony-forming Progeny

By Maryanne Trevisan and Norman N. Iscove

From The Ontario Cancer Institute, and the Department of Medical Biophysics, University of Toronto, Toronto, Canada M4X 1K9

Summary

Early hemopoietic precursors have been extensively studied using short-term assays based on colony formation or in vivo reconstitution that do not run beyond a few weeks. However, little information is available on the phenotype of the stem cells that are detectable in 6–12 mo transplantation assays, and their relationship to cells detected in short-term assays is not known. In this study, we investigated the phenotype and separability by cell sorting of a spectrum of hemopoietic precursor cells in normal adult mouse marrow, including cells quantitated in a 1 yr competitive transplantation assay in vivo as well as in short-term colony assays in vitro and in vivo. Two principal findings emerged. The first was that cells detected in a variety of short-term assays—CFU-S12 (spleen colony-forming cells), CFCmulti (multilineage colony-forming cells), pre-CFCmulti (precursors of CFCmulti), CFC-E/Mg (erythroid/megakaryocyte CFC) and CFC-G/M (granulocyte/macrophage CFC)—were phenotypically similar and could not be separated from one another using a panel of markers useful in segregating them from more differentiated cells, including buoyant density, sedimentation velocity, adhesiveness to plastic, light scatter, high rhodamine-123 retention, and expression of surface wheat-germ agglutinin (WGA)-binding carbohydrate, H-2K, CD45, AA4.1, heat stable antigen (HSA), CD71, and Ly6A/Sca-1 antigens. Long-term reconstituting (LTR) cells quantitated in vivo differed little from the other precursors in expression of many of the above markers. However, they differed somewhat in lower sedimentation velocity and lower expression of WGA-binding surface carbohydrate, and most strikingly in their conditional adhesiveness to plastic, very low retention of Rh123 and high level expression of Ly6A/Sca-1, to a degree that would permit the quantitative separation of the two precursor classes from each other. The results provide a comprehensive characterization of LTR cells measured to 12 mo in vivo and a direct and quantitative analysis of their separation from cells detected in colony assays.

The permanence of hemopoiesis derives from the ability of rare precursor “stem” cells to generate continuously not only differentiating cells of finite proliferative capacity, but also progeny that retain the stem properties of the original parent. In the mouse, individual genetically marked stem cells can reconstitute and maintain recipient hemopoietic systems for at least 15 mo (1–3). Moreover, clones that are maintained into the long-term (beyond 4 mo) practically invariably contain cells in all the lymphoid and myeloid lineages (2, 4–6). However, while clonal longevity appears strictly coupled to multipotentiality, multipotentiality is not necessarily accompanied by longevity; like their more restricted progeny, most multipotential precursors generate clones that do not persist more than a few weeks in irradiated recipient mice (1, 2, 4, 7, 8). Thus, of the two stem cell hallmarks, longevity and multipotentiality, only longevity defines uniquely those stem cells able to sustain the system.

Two alternative models have been proposed to account for differing stem cell longevity. Decision models generate differing clonal lifespans on the basis of probabilistic (9) or environmentally conditioned decisions between self-renewal and differentiation (death) in initially identical stem cells. In contrast, maturational models postulate initially distinct cells that differ intrinsically in their ability to self-renew (10, 11). The models differ starkly in their major predictions: physical separation of short- from long-lived precursors would be feasible in maturational but not in decision-only models. Despite the clearly differing predictions, decisive separation experiments have yet to be reported. One key, unfulfilled requirement is for detection of long-term reconstituting...
(LTR) cells in assays running beyond a few months. A second is for direct comparison between LTR cells and cells detected in shorter time frames within the same separation experiments. A third is for quantitative measures for precursors that allow meaningful comparison of their distributions over the fractions yielded by various separative procedures.

Quantitative assays have been extensively described for precursors detectable in short time frames, and are mainly based on ability to generate discrete colonies within 1–2 wk either in spleens of irradiated mice or in culture. Quantitative measures of LTR cells have also been described. One approach is based on long-term cure (to 1 yr) of the anemia of unirradiated W (c-kit) mutant mice in limiting dilution assays (12), making use of the competitive advantage of normal precursors over the c-kit–defective host cells. An alternative approach is the competition assay developed by Harrison (13) which determines LTR activity from the proportion of myeloid or lymphoid populations arising from test cells cotransplanted into lethally irradiated mice together with a reference number of normal host-type marrow cells. The test cells differ from the host cells by a quantifiable electrophoretic mobility marker such as hemoglobin or glucose phosphate isomerase-1 (GPI-1) (14). Both limiting dilution analysis in the W model (12, 15) and fluctuation analysis in the competition model (7, 8, 14) yield LTR cell frequency estimates between 1 and 4 per 100,000 adult marrow cells. In contrast, cells detected in short-term colony assays number 100 or more per 100,000 marrow cells. Although the latter have been extensively characterized in terms of physical properties and expression of surface markers (16–21), relatively little information is available on the properties of cells detected directly by long-term in vivo reconstitution.

In this study, we investigated the phenotype and separability of a spectrum of hemopoietic precursor cells in normal adult marrow measured in the long-term in vivo as well as in short-term assays in vitro and in vivo. We chose seven operationally distinct assays to represent stages along a path from earliest stem cell to definitive commitment into distinct myeloid lineages. LTR cells were detected by their capacity to supply erythrocytes for 1 yr after transfer into irradiated recipients in competition with a standard marrow inoculum (13). Short-term reconstituting (STR) cells were quantitated in similar assays 4 wk after transfer (7). Spleen colony-forming cells (CFU-S12) were enumerated in day 12 spleens (22). Multilineage colony-forming cells (CFCmulti) and more restricted erythroid/megakaryocyte (CFC-E/Meg) or granulocyte/macrophage (CFC-G/M) precursors were enumerated in semisolid cultures (23, 24), and precursors of CFCmulti (pre-CFCmulti) were detected by their ability to generate new daughter CFCmulti in short-term liquid culture (23, 24). Our working assumption was that these assays detected distinct stages in a developmental pathway leading to definitive lineage commitment, and that they were related temporally in the sequence LTR-STR-pre-CFCmulti–CFU-S12/CFCmulti–committed CFC. Our goal was to use cell separation to determine which if any of these operationally defined precursors could be separated from one another and therefore be established as intrinsically distinct.

We report here the results of a comprehensive phenotypic analysis of normal adult marrow LTR cells measured in 12-mo assays in vivo, and direct quantitative comparison of their distributions in fractionation experiments with precursors detected in shorter-term assays. The results revealed remarkable phenotypic similarity among cells detected in any of the short-term assays, and a distinctly differing phenotype of LTR cells sufficient for their quantitative separation away from more advanced progeny.

### Materials and Methods

**Mice.** C57BL/6j (Gpi-1s/b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Congenic C57BL/6j-Gpi-1s/b and C57BL/6j-W journalists were obtained originally from The Jackson Laboratory and bred at the Oregon Cancer Institute animal facility. Marrow was harvested from femora and tibiae of untreated 12–16-wk-old mice.

**Factors.** Purified human rIL-1β was obtained from A. R. Shaw and J.-J. Mermod (Glaxo Research Institute, Geneva, Switzerland) (25). Mouse rIL-3 was supplied as the conditioned medium of X63 Ag8-653 myeloma cells expressing IL-3 from a transduced vector (26). Its activity was measured in methyl cellulose cultures of mouse marrow, where 1 U/ml stimulated half maximum numbers of total colonies at 7 d. Purified human recombinant erythropoietin and rHu-11 were gifts from Kirin Brewery (Tokyo, Japan) and Genetics Institute (Boston, MA), respectively. Murine c-kit ligand (KL) was supplied as the conditioned medium (KLCM) of Chinese hamster ovary (CHO) cells transfected with a vector expressing KL (Deborah Donaldson, Genetics Institute) and used at concentrations determined to be optimal. Conditioned medium from the human bladder carcinoma cell line 5637 (CM5637, [27]) was similarly used at concentrations titrated to maximum activity.

**Antibodies.** Monoclonal antibodies 14.8 (anti-B220) (28), M1/70.15.11.5. HL (anti-Mac-1) (29), M1/9.3.4. HL-2 (anti-CD45) (29), J11.d.2 (anti-heat stable antigen (HSA)) (30) and R17 217.1.3 (anti-transferin receptor/CD71) (31), AA.4.1 (32), and E13 161.7 (anti-Ly6A.2) (33) were precipitated from serum-free hybridoma culture supernatants with 45% saturated ammonium sulphate. Purified monoclonal antibodies AF6-88.5 (anti-H-2K*, [34]) and RB6-8CS (anti-Gr-1, [35]) were obtained in purified form from Pharmingen (San Diego, CA). Monoclonal antibody 53-6.7 (anti-CD8, [36]) was obtained in purified form from Becton Dickinson & Co. (Mountain View, CA). A cocktail of lineage marker antibodies (“LIN”) contained 14.8, M1/70, RB6-8CS, and 53-6.7.

**CFC.** Marrow cells were cultured in IMDM containing methyl cellulose, 4% FCS, 0.5% serum fraction V, transferrin, insulin, lipids, α-thioglycerol, 15 U/ml IL-3, 1 ng/ml IL-1β, 1 IU/ml erythropoietin, 3% KLCM, 10% CM5637, and 30 ng/ml IL-11 (23). Seeding density was 2 × 10^6/ml for unfractionated marrow and 5–100 × 10^6/ml for enriched populations. Colonies were
blood was sampled from the tail vessels of recipient mice and packed cells were injected for each experimental point. At the indicated times, chloride, 2.22 mg/ml fructose-6-phosphate, 60 μg/ml phenazine methosulphate, 220 μg/ml α-nicotinamide adenine dinucleotide were added for enhancement of recipient survival (37) and did not themselves generate macroscopic spleen colonies. Mice were killed at 12 d and their spleens fixed in Bouin's solution. In a typical separation experiment, five mice were injected with cells from each experimental group. Since each separation was run generally three or more times, experimental results shown in the figures were derived from at least 15 mice/group. In control experiments, GPI-1 was typed in individually dissected spleen colonies. After lysis of red cells in 170 mM ammonium chloride and subsequent washing, nucleated cells were suspended in 20 mM Tris/TAPSO, pH 7.4, (Sigma Chemical Co., St. Louis, MO) containing 2 mM EDTA, 0.1% BSA, and 5 mM dithiothreitol (DTT) and twice frozen and thawed. The lysates were electrophoresed and the proportion of GPI-1A to total GPI-1 determined as above. Of 48 macroscopic spleen colonies, 46 were predominantly (generally >70%) GPI-1A/A while 9/10 samples of inter-colony tissue were generally <20% GPI-1A/A and peripheral erythrocytes had no detectable GPI-1A/A. We thus estimate that in our conditions 96% of macroscopic colonies originated from injected GPI-1-α cells and not from W-α/α or host cells. 

LTR Cells. C57BL/6-J-GPIt-α marrow cells were tested for their LTR cell content by injecting lethally γ-irradiated (900 rad, 137Cs) C57BL/6-J (GPI-t-α) mice. 105-C57Bl/Hμ-α (GPI-t-β) marrow cells were added for estimation of recipient survival (37) and did not themselves generate macroscopic spleen colonies. Mice were killed at 12 d and their spleens fixed in Bouin's solution. In a typical separation experiment, five mice were injected with cells from each experimental group. Since each separation was run generally three or more times, experimental results shown in the figures were derived from at least 15 mice/group. In control experiments, GPI-1 was typed in individually dissected spleen colonies. After lysis of red cells in 170 mM ammonium chloride and subsequent washing, nucleated cells were suspended in 20 mM Tris/TAPSO, pH 7.4, (Sigma Chemical Co., St. Louis, MO) containing 2 mM EDTA, 0.1% BSA, and 5 mM dithiothreitol (DTT) and twice frozen and thawed. The lysates were electrophoresed and the proportion of GPI-1A to total GPI-1 determined as above. Of 48 macroscopic spleen colonies, 46 were predominantly (generally >70%) GPI-1A/A while 9/10 samples of inter-colony tissue were generally <20% GPI-1A/A and peripheral erythrocytes had no detectable GPI-1A/A. We thus estimate that in our conditions 96% of macroscopic colonies originated from injected GPI-1-α cells and not from W-α/α or host cells.

LTR Cells. C57BL/6-J-GPIt-α marrow cells were tested for their LTR cell content by injecting lethally γ-irradiated (900 rad, 137Cs) congenic GPI-t-α recipients along with a competing reference dose of 2 x 105 normal marrow GPI-t-α cells. Three mice were injected for each experimental point. At the indicated times, blood was sampled from the tail vessels of recipient mice and packed red cells were lysed by 30-fold dilution in 20 mM Tris/TAPSO, pH 7.4, containing 1 mM EDTA and 5 mM DTT. Volumes of 2 μl were electrophoresed on cellulose/Mylar membranes (Super Sepharose; Gelman Sciences, Inc., Ann Arbor, MI) in 20 mM Tris/TAPSO buffer, pH 7.4, with 0.25 mM DTT. The membranes were developed at room temperature under a thin overlay of 0.37% agarose containing 18 mM Tris-HCl, pH 8, 30 mM magnesium chloride, 2.22 mg/ml fructose-6-phosphate, 60 μg/ml phenazine methosulphate, 220 μg/ml β-nicotinamide adenine dinucleotide phosphate, 220 μg/ml nitroblue tetrazolium, and 1.11 U/ml glucose-6-phosphate dehydrogenase (38). The relative amounts of GPI-1 A/A and B/B were determined by scanning digital densitometry (ImageQuant system; Molecular Dynamics, Sunnyvale, CA). Defining 1 LTR unit as the reconstituting capacity present in 2 x 106 normal unseparated competing reference cells, the number of GPI-t-α erythroid reconstituting units in the tested sample was given by the ratio of the amount of GPI-1A/A to the amount of GPI-1B/B. 

For determination of the proportion of GPI-t-α reconstitution in nonerythroid lineages, cell suspensions were prepared from recipient thymus, spleen, and bone marrow. Red cells in the thymus and spleen cell suspensions were eliminated by centrifugation over Lymphocyte-M (Cedarlane, Hornsby, Ont., Canada). Splenic B cells were further enriched by complement-mediated lysis of Thy1.2+ T cells. Bone marrow monocytes and neutrophils were isolated by immunoadherence to M1/70- and Gr-1–coated plates, respectively (28). For all populations, analysis by flow cytometry confirmed 85–100% purity. Lyases prepared in 5 μl of Tris/TAPSO/EDTA/0.1% BSA/DTT from 105 cells were electrophoresed and GPI-1 isoenzyme ratios were determined as above.

Separation of Marrow Cells on Density Gradients. Cells were separated at room temperature on Percoll (Pharmacia LKB, Piscataway, NJ) step gradients consisting of various proportions of isotonic Percoll stock (ISP) mixed with a balanced salt solution (IBSS: 153 mM NaCl, 4.5 mM KCl, 1 mM Na2PO4, 1 mM CaCl2, 0.9 mM MgCl2, 6 mM glucose). Both ISP and the diluent were adjusted to 300 mOsM (measured by freezing point depression) and pH 6.4 (for reduced cell clumping and higher loading capacity). ISP and further dilutions were allowed to reach density equilibrium at room temperature for several days before use. 2 x 107 marrow cells were suspended in 2 ml 65% ISP with subsequent overlays of 2–3 ml each of 50, 45, and 45% ISP and finally IBSS alone. The cells were floated upward for 20 min during centrifugation at 2,000 g. Fraction I was collected from the interface above the 45% phase, fraction II from the interface between the 45 and 55% phases and fraction III from the pool of interfaces above the 60 and 65% phases, corresponding to densities of <1.068, 1.068–1.078, and 1.078–1.090 (39). 

Separation of Marrow Cells by Countercurrent Centrifugal Elutriation (CCE). CCE was performed using a centrifuge (I-6 ME; Beckman Instruments, Palo Alto, CA) with a JE-5.0 rotor and a standard chamber. A masterflex peristaltic pump (Cole-Parmer, Chicago, IL) was used to adjust and record flow rates. Elutriation was performed at a constant rotor speed of 3,000 rpm (1,260 g) at 22–25°C. Marrow cells (1–3 x 107) in 20 ml of elutration buffer (IBSS/0.1% BSA, pH 6.4) were loaded into the chamber at a flow rate of 15 ml/min. Cells were eluted by increasing the flow rate to 25, 29, and 33 ml/min and collecting 200 ml at each flow rate. Cells remaining in the chamber at the end of the run were pooled and designated the rotor-off fraction.

Adherence. Bone marrow cells (5 x 107/ml) were incubated overnight in plastic 90-mm tissue culture plates (Nunc, Roskilde, Denmark) in 10 ml IMDM containing 5% NBS, 15 U/ml IL-3, and 15% CM5637 at 37°C in 5% CO2. Nonadherent cells were harvested and pooled together with two washes of the plates. Adherent cells were recovered by mild trypsinization (0.25% wt/vol porcine trypsin, without EDTA; GIBCO BRL, Gaithersburg, MD) at room temperature for <5 min. PBS containing 20% NBS was added and cells were removed with the help of a rubber scraper.

Immunofluorescence of Bone Marrow Cells. Cells at a density of 107/ml were incubated for 10 min on ice with 150 μg/ml purified nonspecific mouse IgG (Cedarlane) in IBSS/5% NBS/3% KLCM to saturate Fe receptors. Specific antibody was then added and incubation continued for another 20 min. Antibody concentrations were in excess of those already determined to be required for maximum staining. Cells were washed and reincubated with FITC-conjugated mouse anti-rat IgG (Jackson Immunochemicals, West Grove, PA) or FITC-conjugated goat anti–rat IgM (Southern Biotechnology Associates, Birmingham, AL) in IBSS/5% NBS/3% KLCM for 20 min on ice. Cells were washed twice and analyzed.

Rhodamine-123 Staining. Rhodamine-123 (Eastman-Kodak Co., Rochester, NY) preparation and cell staining were carried out as described by Bertoncello et al. (40). Briefly, cells at a density of 107/ml were incubated with 0.1 μg/ml rhodamine-123 in IBSS/5% NBS/3% KLCM at 37°C for 20 min, washed twice, resuspended in IBSS/5% NBS/3% KLCM to 107/ml and rein-

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cubated for 15 min at 37°C to remove unbound fluorochrome. After washing, cells were kept in the dark on ice until analysis.

Wheat Germ Agglutinin (WGA) Binding. WGA binding was carried out as described by Visser et al. (16). Briefly, cells at a density of 10⁶/ml in IBSS/5% NBS/3% KLCM were incubated for 20 min with 0.2 µg/ml FITC-conjugated WGA (Polysciences, War-lington, PA) on ice, then washed twice and analyzed.

Cell Sorting. A cell sorter (Epics V; Coulter Corp., Hialeah, FL) was used with a 400 mW argon laser emitting at 488 nm. Rhodamine-123 and FITC fluorescence was detected through a 525- nm bandpass filter. Cells were maintained below 10°C to prevent capping and protected from light to limit fluorescence extinction. Sorted populations were collected into 5 ml IBSS/20% NBS/3% FCS. Sorted populations were reanalyzed to determine purity.

Sorted populations were reanalyzed to determine purity. KLCM in 15-ml conical polypropylene tubes precoated with 1 ml KLCM in 15-ml conical polypropylene tubes precoated with 1 ml of 10⁷/ml in IBSS/5% NBS/3% KLCM were incubated for 20 min with 0.2 µg/ml FITC-conjugated WGA (Polysciences, War-lington, PA) on ice, then washed twice and analyzed.

Results
Assay Characteristics

Linearity. The general approach taken in this study was to fractionate marrow on the basis of individual parameters and determine the distribution profiles of each of the precursor classes over the fractions obtained. Straightforward comparison of the profiles would depend on linearity of the corresponding assays. While linearity of the various clonogenic assays has been repeatedly documented in the past, it was important to test for it in the LTR and pre-CFCmulti measurements as performed in our experiments.

Pre-CFCmulti activity was quantitated simply on the basis of the number of new CFCmulti generated in 4-d liquid cultures of test cell populations. In our experiments, the absolute number of CFCmulti recovered from liquid culture was typically 8–10-fold increased over the number originally seeded into liquid cultures in these conditions. Fig. 1 A documents the linear relationship between the number of marrow cells seeded into liquid culture and the number of CFCmulti harvested.

LTR activity in test cell populations from Gpi-1a/a donors was determined by coinjection of the cells along with a fixed reference of 2 × 10⁶ Gpi-1b/k marrow cells into lethally irradiated Gpi-1b/k hosts, and determining the proportion of Gpi-1A/A in blood erythrocytes sampled at various times thereafter. Theoretically, the ratio of the amount of Gpi-1A/A to the amount of Gpi-1B/B should bear a linear relationship to the number of injected Gpi-1a/a cells. As shown in Fig. 1 B, the expectation was confirmed experimentally.

Multipotentiality of LTR. Control experiments were performed 1 yr after engraftment in which GPI-1 was analyzed in monocyte, neutrophil, splenic B and thymic T cell populations as well as in blood erythrocytes in individual recipients. In agreement with the observations of Harrison et al. (14), we found close concordance between the proportion of Gpi-1A/A in erythrocytes and the proportion of Gpi-1A/A in each of the other myeloid and lymphoid populations examined (data not shown).

Phenotypic Features That Did Not Distinguish between Precursor Classes

Our initial approach was to divide marrow into three fractions on the basis of individual parameters and measure each fraction for total amounts of activity in each of the precursor assays. When preliminary experiments localized activities predominantly into one fraction, subsequent analyses focused on simpler two-fraction comparisons of activities in the precursor-containing and precursor-depleted fractions.

Cells were separated on the FACS® (Becton Dickinson & Co.) on the basis of forward light scatter and expression of a variety of surface antigens known to be markers of hemopoietic precursors measured in short-term assays (6, 16, 18, 21, 41–43). Distributions of some of these properties among unseparated and medium density marrow cells are shown in Fig. 2, along with the windows chosen for analysis of precursor content.

Before sorting on the FACS®, marrow was initially separated according to buoyant density. The results are shown in the first column (labeled “density”) of Fig. 3, and exemplify the means of presentation of recovery profiles used throughout Figs. 3, 5, and 7. The first box shows that about 70% of recovered nucleated cells localized in the highest density fraction, 23% in the medium density fraction, and 7% in the lowest density fraction. The second box shows the recovery of CFC-G/M, indicating that 97% of those recovered were found in the medium density fraction. The lowermost box shows the results of quantitative assay of LTR activity. The medium density fraction contained about 85% of the total recovered units of reconstituting activity (defined in Materials and Methods), while the low and high density fractions contained the remaining 15%. Most of the precursors detected in the various assays segregated together into the medium

Figure 1. Linearity of assays for pre-CFCmulti and LTR cells. (A) Pre-CFCmulti. Normal unseparated marrow cells were seeded in varying numbers into liquid cultures with IL-1 and IL-3. After 4 d, nonadherent cells were harvested and plated into duplicate methyl cellulose cultures. Multilineage colonies were scored after 10–12 d. The mean CFCmulti ± 1 SEM recovered/1 ml liquid culture at each density is indicated. The least squares line fitted to the logarithms of the arithmetic means vs. the logarithms of the seeding densities had a slope of 1.1 indicating a simple linear relationship. (B) LTR. The indicated numbers of unseparated Gpi-1a/a marrow cells were injected with 2 × 10⁶ normal unseparated Gpi-1b/k bone marrow cells into lethally irradiated Gpi-1b/k mice. The ratio of Gpi-1A/A to Gpi-1B/B was determined in red cell lysates at 52 wk. Means ± 1 SEM are shown. The result was typical of three independent experiments. The mean slope of the line fitted as above to the logarithms of the data was 1.14 ± 0.20 over the three experiments.
density range (1.068-1.078) regardless of their biological potential and away from the majority of marrow cells which were more dense. The general property of precursors provided a useful means of initial enrichment. Although in vivo reconstituting cells were somewhat lower in mean density than pre-CFCh multi and CFCh multi, little usable separation was achieved among various precursor classes.

All of the markers were useful in segregating hemopoietic precursors from the bulk of medium density cells (Fig. 3). LTR differed from other precursor classes to a subtle degree in expression of somewhat higher levels of CD45 and HSA, and somewhat lower levels of forward angle light scatter (FALS), H-2K, AA4.1, and CD71 than the more advanced classes, especially GM-CFC, but not to an extent that could provide useful separation. Although expression of AA4.1 is known to mark hemopoietic precursors from fetal liver (6) or from marrow recovering from depopulation after treatment with 5-fluorouracil (44), we found expression in normal marrow precursor populations to be low to undetectable. Total precursor recoveries from the various separations generally exceeded 50% of input. The exception was the antibody to H-2K, whose application to marrow led to loss of 70% of LTR and 90% of STR activity detected in vivo without corresponding adverse effect on precursors detected in culture. The consensus phenotype of all the various functionally defined precursors in normal adult marrow was H-2K high; FALS and CD45 intermediate; and HSA, CD71, AA4.1, and lineage marker low.

Phenotypic Features That Differed between LTR Cells and Other Precursor Classes

Adherence. The ability of the various classes of precursor cells to adhere to plastic tissue culture plates was directly compared. Unseparated marrow cells were incubated for 16 h with 5% serum, IL-3, and CM5637. Nonadherent cells, and adherent cells recovered by trypsinization were collected and assayed separately. The proportion of all recovered precursors present in the adherent fraction is indicated in Fig. 4. Only small proportions of total marrow cells, clonogenic cells and pre-CFCh multi adhered. In contrast, 30% of STR and 60% of total LTR activity was recovered in the adherent fraction.

When a highly enriched LTR fraction (medium density/FALS low-med/LIN-Ly6A hi) was similarly incubated on plastic, only 18% of recovered LTR adhered. The result suggested that adherence of LTR might involve the participation of accessory cells. This possibility was tested by addition of irradiated unseparated marrow cells to the enriched LTR fraction (Fig. 4). In the presence of the irradiated cells, the proportion of adherent LTR increased to 77% without change in the total amount of recovered LTR activity. The result indicates that accessory cells mediate the adherence property, functioning as intermediate adherence substrates, or as sources of modulating cytokines, or as suppliers of soluble adhesion molecules. The result also identifies adherence as a significant discriminator between LTR cells and more advanced progeny.

Sedimentation Velocity. Counterflow centrifugal elutriation was used to separate whole marrow cells on the basis of sedimentation velocity, a parameter determined by both buoyant density and the square of cell diameter. Five fractions were analyzed containing cells of progressively greater sedimentation velocity (Fig. 5). Most of the recovered in vitro clonogenic cells were found in the more rapidly sedimenting "RO" fraction. In contrast, most of the recovered 52-wk LTR activity reproducibly located in the more slowly sedimenting 29 and 33 ml/min fractions. STR and CFU-S12 activity sedimented in a more rapid range than LTR but somewhat
more slowly than in vitro clonogenic cells. The results were compatible with the subtly lower density and FALS profiles of LTR cells noted above, and likely reflected gain in discriminating power resulting from the compounding of the two parameters and the higher sensitivity to size. Although the modal sedimentation velocity of LTR was slower than that of more advanced progeny, it was not sufficiently so to provide more than a modest enrichment of LTR relative to later cells.

**WGA Binding.** Medium density marrow cells were incubated with fluorescent WGA and separated into fractions of low, medium, and high binding levels as indicated in Fig. 6. The various precursor classes distributed as shown in Fig. 7. Most of the precursors detected in shorter term clonogenic assays, including CFU-S12, labeled strongly. In contrast, staining of STR and particularly LTR cells was distinctly weaker, in agreement with previously reported observations (45).

**Ly6A Expression.** Medium density marrow cells were similarly separated on the basis of Ly6A expression (Fig. 6). As shown in Fig. 7, clonogenic precursors distributed about evenly into fractions of low, intermediate, and high expression levels.

In contrast, STR and particularly LTR activity were detected almost exclusively in the brightly labeled population.

**Rhodamine-123 Staining.** Medium density cells were separated into fractions of weak, intermediate, or strong staining intensity as indicated in Fig. 6. 80-90% of clonogenic precursor activity, including CFU-S12, was found in the brightly labeling fraction (Fig. 7). In contrast, 50% of STR activity, and 85% of LTR activity, segregated with the most weakly labeled cells.

**Discussion**

This study was designed to determine and compare the physical characteristics of LTR cells from normal marrow with cells detectable in a variety of shorter-term colony-forming assays. Two principal findings emerged, neither of which has been explicitly detailed before.

The first observation was that the various cells detected in short-term colony assays had a remarkable degree of phenotypic similarity. Among the markers explored in this study, no parameter or combination of parameters was identified.
that could have achieved quantitative separation of one clonogenic class from another. These markers were chosen because of their capacity to distinguish hemopoietic precursors from the bulk of maturing marrow cells. For the purpose of separating various clonogenic cells, lineage-specific maturational markers that appear early in commitment may turn out to be more effective than the nonspecific markers examined here.

The second, contrasting finding was that cells detected by long-term in vivo reconstitution differed distinctly in phenotype from the cells detected in the short-term assays. Practically all marrow LTR activity was associated with cells having a class I phenotype: conditional adherence to plastic, slow/intermediate sedimentation velocity, high expression of surface Ly6A, intermediate amount of surface WGA-binding carbohydrate, and weak staining with rhodamine-123. In contrast, the majority of precursor activity detected in the shorter-term assays was attributable to class II cells that were not plastic adherent and had higher sedimentation velocity, low/intermediate Ly6A, higher WGA-binding carbohydrate, and strong rhodamine-123 staining. Both classes were similar in all other markers examined: intermediate buoyant density, low/medium forward light scatter, low or undetected levels of AA4.1 and CD71, low levels of lineage marker and HSA expression, intermediate CD45 and high levels of H-2K.

These observations are generally congruent with earlier findings correlating expression of Ly6A/Sca-1 (18, 46-48) in some but not all mouse strains (49), and weak staining with rhodamine-123 (19, 40, 47, 50-52) with capacity for self-renewal, and reports of plastic adhesiveness of in vivo reconstituting cells (53-55). The CCE results are also in general agreement with the qualitative findings of Jones et al. (56) and the quantitative analysis of Orlic et al. (57). However, for most of the markers characterized, our findings extend...
the 48-h interval, LTR are normally at rest and are slow to enter into active cycle after marrow cell depletion (61). In our study, this difference in cycle regulation between LTR and later cells was not reflected in detectable differences in transferrin receptor (CD71) expression in precursors from untreated marrow. However, the major phenotypic differences that we did identify strongly reinforce the evidence from the 5-fluorouracil studies that significantly differing cells are detected in long- and short-term assays.

Since LTR cells differ intrinsically and structurally from class II precursors, their greater longevity can not be accounted for simply on the basis of stochastic outcome of initial divisions or of differing environmental stimuli. As is evident in Fig. 7, class II precursors (Ly6A<sub>low</sub>/intermediate, rhodamine-123<sub>intermediate/high</sub>) appear intrinsically incapable of long-term reconstitution, at least when measured in competition against a normal marrow standard. On the other hand, the estimates of absolute numbers of LTR and clonogenic cells in the Ly6A<sub>high</sub> and rhodamine-123<sub>low</sub> fractions (legend to Fig. 7) were close enough to be compatible with the notion that class I precursors may be capable of both colony formation in short-term assays as well as reconstitution in the long term. If LTR cells were able to initiate colony formation and self-renew in culture, then colonies grown from class I cells should be capable of reconstituting murine hosts in the long term. This prediction has been confirmed in experiments currently in progress. It may similarly be possible for LTR cells to form 12-d spleen colonies. CFU-S12 number about 100 in 100,000 marrow cells, taking into account their spleen seeding efficiency (62, 63). LTR cells number between 1 and 4 in 100,000 marrow cells (12, 14) and are class I in phenotype. If they were detected in clonogenic assays such as CFU-S12, between 1 and 4 of every 100 spleen colonies obtained from unseparated marrow would derive from class I LTR cells. This estimate is compatible with the frequency of reconstitution of murine recipients from single spleen colonies measured by Kitamura et al. (64). It is also compatible with results from experiments in progress in which multiparameter sorting of class I cells segregates practically all LTR activity from more than 98% of recovered CFU-S12, into a fraction in which absolute numbers of LTR cells and CFU-S12 are about equal. Based on these considerations and the results from experiments in progress, we favor the view that a very high proportion of the cells detectable in short term colony assays including CFU-S12 are of class II phenotype and are intrinsically incapable of long-term reconstitution in vivo. Long-term reconstitution is almost exclusively associated with cells of class I phenotype. These cells may also be capable of generating colonies in short-term assays, but would account numerically for only a very small proportion of the total colony-forming cells in unseparated marrow samples.

The nature and phenotype of multipotential precursors having only transient reconstituting capacity (7) remains to be determined. We quantitated STR activity based on erythrocyte production for 4 wk after transfer into irradiated hosts, but did not address the differentiative repertoire of the responsible precursors. Like LTR as well as about a third of colony-forming cells, STR precursors uniformly expressed a high

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**Figure 7.** Distribution of hemopoietic precursor cells in sorted cell fractions: parameters that distinguish precursor classes. Medium density cells were sorted on the FACS<sup>®</sup> on the basis of each of the single parameters shown. The fractions correspond to the sorting windows indicated in Fig. 6. The mean proportions of total precursor activity recovered in each fraction are shown + 1 SEM from at least two independent analyses of each parameter. The LTR results are those obtained after 1 yr of reconstitution. Average absolute numbers of recovered LTR cells were 531 and 706 in the Ly6A<sub>high</sub> and Rh123<sub>low</sub> fractions, respectively, estimated on the assumption that 2 x 10<sup>6</sup> normal competitor marrow cells contained 20 LTR cells (14). The Ly6A<sub>high</sub> fraction also contained 4864 CFU-S12 (assuming a seeding factor of 0.16) and 3313 CFCmulti. The Rh123<sub>low</sub> fraction also contained 4556 CFU-S12 and 614 CFCmulti.

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In the steady state in normal adult mouse marrow, half or more of LTR resist the cytotoxic action of injected 5-fluorouracil for the 48-h duration of its effectiveness (58, 59). In contrast, CFU-S12 (60) and pre-CFCmulti (59) are extensively depleted in the same time frame. The observation indicates that while nearly all colony-forming cells cycle within...
level of surface Ly6A. In WGA binding and rhodamine-123 retention on the other hand, half of STR resembled LTR while half were more similar to colony-forming cells. The observations might reflect the existence of a transitional STR precursor population of intermediate phenotype. However, the findings are more simply explained on the basis of equal contributions to short-term erythropoiesis from two distinct populations, one an Ly6A+ subcomponent of class II, the other identical with class I LTR cells. The observations also add further support to the notion that class I LTR cells may be as capable as class II precursors in short-term reconstitution as well. These properties, however, would be effectively dominated in unseparated marrow by the large numeric excess of class II precursors having all of these capabilities. Although the various colony assays are considered to detect precursors possessing a variety of different biological potentials, corresponding phenotypic differences did not emerge in this study. Nevertheless, analysis of the differentiative potential of individual sibling progeny sampled from growing clones (Suda et al. [65], and our experiments in progress) provides compelling evidence for the existence of differing states of commitment prior to culture. For investigators interested in the mechanisms of lineage commitment, the absence of strong phenotypic differences between various kinds of clonogenic cells highlighted in the present study makes the search for mechanisms of commitment more difficult and suggests that paradigms other than purification will need to be explored. Study of gene expression in individual cells would provide a different means to achieve the desired homogeneity, and the availability of a method for global amplification of mRNA from individual precursor cells now offers an exciting and powerful alternative (66).

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Address correspondence to Dr. Norman Iscove, The Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Canada M4X 1K9.

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