Cycle Initiation and Colony Formation in Culture by Murine Marrow Cells With Long-Term Reconstituting Potential In Vivo

By Maryanne Trevisan, Xiao-Qiang Yan, and Norman N. Iscove

This investigation was directed at separating long-term reconstituting (LTR) stem cells in normal murine marrow from hematopoietic precursors detectable in short-term assays in vitro and in vivo, and then at determining whether purified LTR cells could themselves form colonies in culture. To do so, it was first necessary to identify culture conditions that would induce their growth while preserving their long-term reconstituting capacity. Marrow was cultured with various cytokines in liquid suspension for 4 days, after which the surviving LTR activity was quantitated in a competitive in vivo assay. Activity was preserved near input levels with combined murine c-kit ligand (KL), interleukin-1 (IL-1), IL-6, and IL-11. When the cultures also included tritiated or unlabeled thymidine, LTR potential was eliminated, indicating that essentially all LTR cells were induced into cell cycle with these cytokines. To purify them, marrow was sorted on the basis of Ly6A expression and Rhodamine123 retention. The Ly6A<sup>+</sup>Rh123<sup>-</sup> fraction contained 85% of total recovered LTR activity but only 1% of the recovered cells measured by multilineage colony formation in spleens or in vitro. This fraction was cultured in methyl cellulose with KL, IL-1, IL-6, and IL-11 for 4 to 6 days, after which colonies were isolated and injected into mice. High levels of permanent reconstitution were achievable in sublethally irradiated W<sup>55</sup>/ W<sup>55</sup> mice after the injection of a single reconstituting unit, and limiting dilution analysis estimated the frequency of multilineage LTR at 1 in 11,200 unpurified adult marrow cells. In either lethally irradiated normal or sublethally irradiated W<sup>55</sup>/ W<sup>55</sup> mice, 1-year lymphomyeloid reconstitutions were obtained from 1 in 65 to 84 colonies of 2 to 16 dispersed cells, but not from larger colonies or those with clumped cells. The results establish that resting marrow LTR cells can be separated from almost all of the more advanced clonogenic cells that are still pluripotential, can be induced to cycle in culture by defined cytokines with preservation of their reconstituting potential, and can be manipulated and assayed efficiently at single-cell and colony levels.

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ment within nascent clones, solutions to these problems would substantially facilitate retroviral gene transfer and analysis of differential gene expression in single LTR cells and their immediate progeny. In this report, we describe experiments directed at achieving these goals.

**MATERIALS AND METHODS**

**Mice.** C57BL/6J (Gpilb') mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Congenic C57BL/6J-Gpilb' and C57BL/6J-W4'/W4' (Gpilb') mice were obtained originally from The Jackson Laboratory and bred at the Ontario Cancer Institute animal facility. Marrow was harvested from the femora and tibiae of untreated 12- to 16-week-old mice.

**Cytokines.** Purified recombinant human interleukin-1β (rhIL-1β) was obtained from A.R. Shaw and J.-J. Mermod (Glaxo Research Institute, Geneva, Switzerland). Murine rIL-3 was supplied as the conditioned medium of X63 Ag8-653 myeloma cells expressing IL-3 from a transduced vector. Its activity was measured in methyl cellulose cultures of mouse marrow, in which 1 U/mL stimulated half-maximum numbers of total colonies at 7 days. Purified human recombinant erythropoietin was a gift from Kirin Brewery (Tokyo, Japan). rhIL-6 and rhIL-11 were gifts from Genetics Institute (Boston, MA). Murine c-kit ligand (KL) was supplied as the conditioned medium (KLCM) of Chinese hamster ovary (CHO) cells transfected with a vector-expressing KL (D. Donaldson, Genetics Institute) or as recombinant murine mast cell growth factor (rmMGF; supplied by Immunex, Seattle, WA). Conditioned medium from the human bladder carcinoma cell line 5637 (CM5637) was used, where indicated, as a source of IL-6.

**Antibodies.** Monoclonal antibodies 14-8 (anti-B220),12 M1/70,15.11.15.1H (anti–Mac-1),23 and E13 161-7 (anti-Ly6A.2)24 were precipitated from serum-free hybridoma culture supernatants with 45% saturated ammonium sulphate. Purified monoclonal antibody RB6-8C5 (anti–Gr-1) was obtained in purified form from Pharmingen (San Diego, CA). Purified monoclonal anti-Thyl.2 antibody was purchased from Cedarlane (Hornby, Ontario, Canada).

** Colony-forming cells (CFC).** Marrow cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) containing methyl cellulose, 4% fetal bovine serum (FBS), 0.5% bovine serum fraction V, transferrin, 10 μg/mL insulin, lipids, α-thioglycerol, 15 U/mL IL-3, 1 ng/mL IL-1, 0.5 U/mL erythropoietin, 3% KLCM, 10% CM5637, and 30 ng/mL IL-11. The seeding density was 2 × 10^5 mL for unfractionated marrow and 5 to 10 × 10^5/mL for enriched populations. Colonies were scored after 9 to 12 days. Erythroid colonies at least 1 mm in diameter containing at least two other lineages were attributed to multilineage CFC (CFCellMulti). Colonies containing at least 1,000 erythroid cells alone or with megakaryocytes were attributed to erythrocyte/megakaryocyte CFC (CFCE/Meg), and those with at least 500 granulocytes and/or macrophages were attributed to granulocyte/macrophage CFC (CFGM).

**Pre-CFCMulti.** Marrow cells were incubated for 4 days in 1 mL volumes of IMDM, 5% newborn bovine serum (NBS), 0.1% bovine serum fraction V, 1 ng/mL IL-1, and 15 U/mL IL-3. The seeding density was 7.5 × 10^5/mL for unfractionated marrow and 5 to 500 × 10^5/mL for enriched populations. Nonadherent cells were harvested after 4 days and appropriately diluted into secondary methyl cellulose cultures for the enumeration of CFCellMulti, as described above.

**Day-12 spleen colony-forming units (CFU-S12).** Congenic C57BL/6J-Gpilb' marrow cells were injected into γ-irradiated (900 rad, 137Cs) C57BL/6J-Gpilb' mice (5 per experimental point). C57BL/6J-W4'/W4' (Gpilb') marrow cells (1 × 10^7) were added for the enhancement of recipient survival and did not themselves generate macroscopic spleen colonies. Mice were killed at 12 days, and their spleens were fixed in Bouin’s solution.

**Reconstituting activity.** Congenic C57BL/6J-Gpilb' marrow cells were injected into γ-irradiated (900 rad, 137Cs) Gpilb' recipients (3 per experimental point) along with a competing reference dose of 2 × 10^6 normal marrow Gpilb' cells. Short-term reconstitution (STR) was measured after 4 weeks. Long-term reconstitution was measured at 32 to 52 weeks. At the indicated times, blood was sampled from the tail vessels of recipient mice and packed red blood cells were lysed and electrophoresed on cellulose/Mylar membranes. After staining for GPII activity,12,25 relative amounts of GPIIIAA and BB were determined by scanning digital densitometry, all as detailed earlier.15 Defining 1 STR or LTR Unit as the reconstituting capacity present in 2 × 10^6 normal unseparated competing reference cells, the number of Gpilb' erythroid reconstituting units in the tested sample was given by the ratio of the amount of GPIIIAA to the amount of GPIIIB, a quantity already shown to vary linearly with the number of injected Gpilb' cells.15 For the determination of the proportion of Gpilb' reconstitution in nonerythroid lineages, cell suspensions were prepared from recipient thymus, spleen, and bone marrow. Red blood cells in the thymus and spleen cell suspensions were eliminated by centrifugation over Lymphocyte-M (Cedarlane). Splenic B cells were further enriched by complement-mediated lysis of Thy-1.2+ T cells. Bone marrow neutrophils were isolated by florescence-activated cell sorting (FACS) of Gr-1+ cells after ammonium chloride lysis of red blood cells. Bone marrow macrophages were prepared29 by culturing unseparated bone marrow in IMDM, 5% FBS, 15 U/mL IL-3, and 15% L929 fibroblast conditioned medium for 3 days, after which nonadherent cells were harvested and recultured for an additional 2 days. Nonadherent cells were then discarded, fresh medium was added, and the cultures were continued for another 3 days. Adherent cells were harvested with 0.6 mmol/L EDTA in phosphate-buffered saline and a scraper. For all populations, analysis by flow cytometry confirmed 85% to 100% purity. Lysates prepared in 5 μL of Tris/TAPS/EDTA/0.1% bovine serum albumin/dithiothreitol from 1 × 10^6 cells were electrophoresed and GPIi iso-enzyme ratios were determined as described above.

**LTR cell growth in suspension culture.** Unseparated Gpilb' bone marrow cells were cultured in 90-mm tissue culture (Nunc, Glostrup, Denmark) plates in a 10 mL total volume of IMDM/5% FBS at a density of 10^5 cells/mL with recombinant cytokines as indicated (50 to 100 ng/mL MGF, 1 ng/mL IL-1, 200 half-maximum hybridoma growth units/mL IL-6, 7.5 ng/mL IL-11). After 4 days, adherent cells were harvested by brief treatment with 0.25% trypsin (without EDTA) and scraping,3 pooled with the nonadherent cells, and assayed competitively for LTR activity. Where indicated, cultures also contained 5 μCi/mL (methyl-3H)TdR (80 Ci/mmol/L, Amersham, Arlington Height, IL).

**Separation of marrow cells.** Medium buoyant density (1.068 to 1.074) cells were isolated on iso-osmotic Percoll (Pharmacia, Uppsala, Sweden) step gradients as detailed in Trevisan and Iscove,15 stained with rhodamine123 (Rh123),12,26 and incubated with nonspecific mouse IgG and bovine serum to saturate Fc receptors before reaction with monoclonal anti-Ly6A.2 antibody. Washed cells were then labeled with biotin-conjugated mouse antirat IgG, stained with streptavidin-phycocerythrin, and finally washed, all as detailed in Trevisan and Iscove.15

**Sorting.** Cells were separated on Coulter Epics V (Coulter, Hialeah, FL) or Becton Dickinson FACStar Plus (Becton Dickinson, Mountain View, CA) sorters using argon lasers emitting at 488 nm at 400 or 40 mW. Rh123 and phycoerythrin signals were detected through 525- and 570-nm bandpass filters, respectively. Cells were maintained on ice to prevent capping and protected from light. Sorted populations were collected into 20% NBS/3% KLCM in polyurethane tubes precoated with FBS. Purity was confirmed by reanalysis.
Our first objective was to identify conditions of culture that could initiate cycling of resting LTR cells from normal marrow. We found that cycling could be initiated by the cytokine combinations shown schematically in Fig IA, unseparated marrow cells, distinguishable by the GPIIAAA' marker, were cultured at a concentration of 0.5 X 10^6 cells per milliliter of IMDM containing methyl cellulose, 4% FBS, 0.05% serum fraction V, transferrin, 10 μg/mL insulin, lipids, α-thioglycerol, 1 ng/mL IL-1, 3% KLCM, 10% CMS5637, and 30 ng/mL IL-11. At the indicated times, colony starts were individually harvested with a finely drawn pipette, pooled, and added to 1 X 10^4 C57BL/W64/W64 (Gpil/−/−) bone marrow cells (as a carrier and for enhancement of recipient survival) before injection into mice.

RESULTS

Cycling and maintenance of LTR cells in liquid culture.

Our first objective was to identify conditions of culture that could initiate cycling of resting LTR cells from normal marrow while maintaining the capacity of their progeny for long-term reconstitution of lethally irradiated mice. As shown schematically in Fig IA, unseparated marrow cells, distinguishable by the Gpil/−/− marker, were cultured at a concentration of 1 X 10^7/mL for 4 days in the presence of various cytokine combinations. Cells from cultures initiated in total with 2 X 10^6 cells were then pooled and injected into each lethally irradiated syngeneic Gpil/−/− recipient, together with 2 X 10^6 fresh competitor marrow cells of recipient genotype. Peripheral blood was sampled at intervals to 32 weeks for electrophoretic quantitation of erythrocyte GPII isoforms. The results are shown in Fig 1B, expressed as the ratio of Gpil/AA to Gpil/BB, an index that varies linearly with the number of injected Gpil/−/−-reconstituting cells.15

Figure 1B shows the amounts of reconstituting activity recovered from cultures relative to that initially placed in culture, indicated by the shaded grey zones. Measurements at 4 weeks in vivo represent the recovery of STR activity, and measurements after 24 weeks represent LTR activity. In cultures established without KL KL (left panel), recovery of STR activity was strongly dependent on combined IL-1 and IL-3, conditions that also increased CFUmulti 10- to 20-fold above input numbers (Iscove and Yan2 and data not shown). In contrast, at 32 weeks, LTR activity decreased in the cultures to only one tenth of input and was not influenced by the presence of IL-1 and IL-3. As shown in the middle panel, the addition of KL to the cultures improved the recovery of both STR and LTR activities, and the inclusion of IL-1 further enhanced the LTR recovery to about 50% of input. The further addition of IL-3 markedly increased total cellular proliferation, with an attendant increase in cell concentrations, but had only a detrimental effect on the recovery of LTR activity from the cultures. The inclusion of IL-11 (right panel) together with KL provided further enhancement of STR recovery. Of greatest interest, the combination of KL, IL-11, IL-1, and IL-6 yielded LTR activity that was close to input levels.

Because these cultures were initiated with normal adult marrow in which LTR cells are known to cycle only rarely, it was of considerable interest to determine whether the maintenance of their numbers in culture was associated with the induction of cell cycling. To test for cycling, a small amount of high specific activity 3HTdR was included in 4-day cultures containing KL, IL-6, and IL-11 with or without IL-1. As shown in Fig 1B, no reconstituting activity was recoverable from cultures containing 3HTdR (5 μCi/mL, 6 X 10^-11 mol/L) regardless of whether they contained IL-1. Additional controls for the specificity of the 3HTdR effect on cycling cells confirmed the expected partial protective effects of excess unlabeled thymidine on harvested clonogenic precursors measured in vitro. Furthermore, cycle blockade with 100 μg/mL (4 X 10^-14 mol/L) of unlabeled thymidine similarly eliminated 24-week reconstituting cells from the cultures, an effect that was antagonized by unlabeled cytidine26,31 (data not shown).

Together, these results show that initially quiescent LTR cells are induced quantitatively into cell cycle in cultures containing KL, IL-11, IL-1, and IL-6 and that these conditions are also associated with recoveries of LTR activity near input levels.

Efficient detection of single LTR cells in vivo.

Analysis of self-renewal and commitment of LTR within individual clones would be greatly facilitated if single LTR cells could be detected at high efficiency and with relative ease. W-series (c-kit−/−) mice are attractive recipients for such purposes because they can be reconstituted with limiting...
numbers of wild-type stem cells without need for prior marrow ablative conditioning.27 W4i/W4i homozygotes27 offer the additional advantages of ease of breeding and a uniform C57BL/6 genetic background. We therefore investigated the repopulation sensitivity of W4i/W4i mice by performing limiting dilution measurements on normal congenic B6 marrow cells bearing a distinguishable GpiL isoenzyme marker that is universally expressed and readily detected in small numbers of cells.28 Reconstitution of W4i/W4i recipients was monitored by measurement of GpiL isoforms in recipient blood erythrocytes 32 to 52 weeks after the injection of marrow. Mice that had detectable donor erythrocytes were considered to be positive for reconstitution.

Figure 2A shows the extent of erythrocyte reconstitution achieved after injection of varying numbers of normal GpiL+ marrow cells into unconditioned W4i/W4i (GpiL100) recipients. In the limiting range of 3,000 to 30,000 injected cells, erythrocyte reconstitutions ranged from 0% to 70%. From this data set, the frequency of reconstituting cells was estimated at 1 in 23,100 nucleated marrow cells (Fig 2A, inset). However, the median value in successfully reconstituted mice was only about 10%. Statistically, about 20% of non-zero values were predicted to fall below the experimental threshold of 1% to 2% for reliable detection, leading probably to an underestimate of the true frequency of reconstituting cells.

A second series of reconstitutions was performed using W4i'/W4i recipients that had been lightly γ-irradiated (300 rad) before marrow cell injection. All mice survived this irradiation dose independently of whether they reconstituted. As shown in Fig 2B, the median level of donor erythrocyte reconstitution in successfully reconstituted recipients that received limiting amounts of marrow was near 65%, and no values were encountered close to the detection threshold. Statistically, only 3% of non-zero values would be predicted to fall below the detection threshold in this data set. The estimated frequency of reconstituting cells in this series was 1/11,200 marrow cells (Fig 2B, inset), which is significantly different from the estimate derived from unirradiated recipients.

The same reconstituted hosts were similarly analyzed at 4 weeks to extract frequency estimates for cells with STR capacity. In lightly irradiated W4i'/W4i recipients, 1 in 2,618 marrow cells (95% confidence limits, 1,072 to 6,392; χ2 = 1.31 for fit to single-hit Poisson distribution; P > .25) yielded detectable erythroid reconstitution. Although persistence of host erythrocytes present at the time of irradiation makes underestimation of STR frequencies likely, our measurement of STR cell frequency (at least fivefold higher than that of LTR cells) confirms the earlier conclusions of Harrison and Zhong26 based on measurements of variance.

Mice reconstituted in the long term at limiting numbers of unseparated marrow cells were further tested for donor contribution to granulocytic and lymphoid populations. Figure 3 (top row) shows representative kinetics of erythroid reconstitution and proportions of donor cells at 52 weeks in purified granulocytes, macrophages, and T and B lymphocytes in recipients of unseparated marrow inocula containing an average of 1 reconstituting cell. In 4 of 4 recipients in which erythroid reconstitution was not detectable at 52 weeks, donor contribution was also undetected in the other myeloid or lymphoid lineages.

These experiments establish the lightly irradiated W4i'/W4i mouse as a simple, sensitive, and efficient host for detection of single injected stem cells.

Separation of LTR cells from other clonogenic precursors. Colonies originating in culture from cells with long-term in vivo reconstituting potential could be identified by testing the colonies for reconstituting ability in vivo. Identification of individual colonies by such screening could only be feasible if LTR cells represent a significant proportion of the originally seeded population. Physical separation of LTR cells from more advanced clonogenic populations is therefore a practical prerequisite to detailed clonal analysis of LTR cell self-renewal and commitment in vitro.

In a previous report, we identified strong expression of
Ly6A (Sca-1) and weak Rh123 staining as the best single parameters distinguishing LTR cells from other primitive precursors such as in vivo STR cells, CFU-S12, and in vitro CFCmulti. In this study, we subjected the medium buoyant density cells from normal marrow to multiparameter sorting on the basis of light scatter (Fig 4A) as well as Ly6A expression and Rh123 staining (Fig 4B) to obtain a selected FLSmedLy6AhiRh123b population and a remainder fraction composed of the recovered cells excluded from this window. The two populations were tested for their content of various precursors measured in short-term colony assays as well as for cells detectable by short (4 weeks) and long-term erythroid reconstitution in the competitive assay in vivo.

Of all colony-forming cells recovered from the sorter, greater than 98%, including CFU-S12 and multilineage in vitro CFC, were localized in the remainder fraction (Fig 5). In contrast, 85% of total recovered LTR activity detected at 1 year was localized in the FLSmedLy6AhiRh123b fraction.
These results reflect a nearly complete separation of cells having long-term reconstituting potential from cells detected in conventional colony assays. Most cells with erythroid reconstituting activity detectable at 4 weeks in the competitive assay were found in the remainder fraction, although a smaller amount of STR activity was also found in the $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction. Figure 3 (middle row) shows representative multilineage reconstitutions obtained in lethally irradiated mice that received limiting numbers of $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ LTR cells.

Because clonogenic cells are detectable in marrow at frequencies 10- to 100-fold higher than that of LTR cells, the presence in the $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction of even 1% to 2% of total conventional CFC could still represent a significant component of the fraction that could exceed LTR cells in absolute numbers. The absolute numbers of LTR and clonogenic cells in the $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction are also indicated in Fig 5. LTR numbers were calculated on the basis of direct measurement in the competition assay and on our frequency estimate of 1 LTR cell in 11,200 unseparated normal marrow cells. LTR cells were estimated to exceed the measured numbers of CFU-S12 and CFUmulti in the $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction, but were still outnumbered twofold by CFC-G/M. However, the numbers of LTR and STR cells in this fraction were similar, suggesting that much of the 4-week reconstituting activity in this fraction may have derived from the LTR population itself.

These separation results led us to expect that, although LTR cells might still be a minority of the total growth-competent cells in the $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction, they might now represent as many as 1 in 5 of such cells, a frequency that should make practical the detection of clones in vitro with long-term in vivo reconstituting potential.

**Long-term multilineage reconstitutions of mice by cells from nascent colonies generated in culture.** $\text{FLS}^\text{med}$, $\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ cells were seeded sparsely into methyl cellulose cultures containing KL, IL-11, IL-6, and IL-1. Nascent colonies observed at various times from 2 to 9 days were harvested individually and pooled for injection into lethally irradiated normal or lightly irradiated $\text{W}^{+/-}\text{IL}^{-/-}$ hosts. Typically, about 50 well-separated colony starts were identified in a single 35-mm petri plate, and the total sampled volume represented less than 1% of the culture volume.

As shown in Table 1 (data set I), colonies pooled without selection criteria from 2- to 6-day cultures yielded long-term reconstitutions, whereas colonies harvested at 7 to 9 days did not. Maximum likelihood Poisson statistics estimated a reconstituting frequency of 1 in 65 colonies harvested between 2 and 6 days. In some experiments, the cells remaining in a plate after all identified colony starts had been removed were harvested and also injected into irradiated mice. Of 14 2- to 6-day cultures tested in this way, 14 yielded long-term reconstitutions (not shown in Table 1). The result indicates that not all LTR cells were associated with identifiable nascent colonies. However, because the assay can respond strongly to a single LTR cell, the result is compatible with a residue as small as a single remaining LTR cell per plate.

![Fig 5. Distribution of cells and precursors between the LTR-enriched $\text{FLS}^\text{med}\text{Ly6A}^\text{b}\text{Rh123}^\text{b}$ fraction and the remaining cells recovered from the FACs.](image)
Table 1. Reconstituting Potential of Colonies Grown in Culture

<table>
<thead>
<tr>
<th>Colony Class</th>
<th>Clones Injected per Mouse</th>
<th>Proportion of Mice Reconstituted</th>
<th>Frequency of Reconstituting Clones (99% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All colonies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-6 d</td>
<td>45-130</td>
<td>9/14</td>
<td>65 (32-132)</td>
</tr>
<tr>
<td>7-9 d</td>
<td>10-250</td>
<td>0/9</td>
<td>&gt;988 (&gt;127)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Disperse, 2-4 d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-16 cells</td>
<td>7-94</td>
<td>20/46</td>
<td>84 (49-143)</td>
</tr>
<tr>
<td></td>
<td>10 exactly</td>
<td>1/20</td>
<td></td>
</tr>
<tr>
<td>8-32 cells</td>
<td>9-41</td>
<td>1/11</td>
<td>202 (28-1,443)</td>
</tr>
<tr>
<td>Clumped 2-4 d</td>
<td>11-53</td>
<td>1/22</td>
<td>575 (78-4,230)</td>
</tr>
</tbody>
</table>

I. Unirradiated \(W^{+/+}\) mice were injected with all identified colonies from semisolid cultures of low density, FALS<sup>-</sup>Rh<sup>-</sup>123<sup>-</sup>Ly<sub>6</sub><sup>a</sup> mice seeded at \(5 \times 10^3\) /mL. Donor GP1 was measured in blood erythrocytes after 40 to 52 weeks, and animals were scored as positive if donor GP1 was detected. The average proportion of donor erythrocytes in positive recipients was 36.5% (range, 2% to 80%). II. Lethally irradiated or sublethally irradiated \(W^{+/+}\) mice were injected with pooled colonies based on size and morphology. The average proportion of donor erythrocytes in positive recipients was 40% (range, 0.3% to 100%). Frequencies of reconstituting clones were estimated by the method of maximum likelihood applied to the Poisson model. For data entry into the iterative algorithm, each mouse represented 1 entry. For example, a mouse that received 45 colonies and had detectable donor erythrocytes was considered as a cohort of 1, with 0 negatives and an input of 45. The first line of the table describes 14 mice individually entered, of which 9 had donor erythrocytes.

In data set II, 2- to 4-day colonies were pooled according to size and whether cells adhered to one another. Most of the observed reconstitutions were obtained from small, disperse colony starts containing 2 to 16 cells (Fig 6, panels 5 through 8). In this series, long-term reconstitutions were obtained from about 1 in 84 such starts. In contrast, starts of larger average size (8 to 32 cells) were estimated to reconstitute at a lower frequency, although the difference did not reach statistical significance. Reconstitution from clones containing clumped cells (Fig 6, panels 1 through 4) was achieved in only 1 instance of 22 pools tested, representing a total of nearly 600 individual colonies. The estimated frequency was 1 in 575 colonies, a result that differed at a significance level of 5%. The correlation between start morphology and frequency of successful reconstitution further suggested that the reconstitutions were more likely to have been derived from colony cells than from bystander cells unintentionally included in the colony pools.

Representative kinetics of erythroid reconstitutions obtained from positive colony pools and proportions of donor cells reached in the long-term in granulocytic, macrophage, and B- and T-lymphoid populations are shown in Fig 3 (bottom row). Because the reconstitutions were obtained from pool sizes containing on average less than 1 reconstituting clone, most of the reconstitutions were likely to have been clonally derived in individual recipients.

These results suggested that LTR cells from normal marrow are able to initiate colony formation in cultures containing KL, IL-11, IL-6, and IL-1 and that such colonies can contain cells that themselves can provide recipient mice with multilineage reconstitution into the long term.

**DISCUSSION**

These experiments were undertaken to determine whether LTR cells, which are at rest in normal marrow, could be induced to proliferate in short-term culture and, if so, whether their progeny would retain the capacity for long-term multilineage reconstitution in recipient mice. Using a quantitative 32-week competitive erythroid reconstitution assay, we established that LTR cells from normal marrow were quantitatively recruited into cell cycle by 4 days in liquid cultures containing combined KL, IL-6, IL-11, and IL-1 and that the amount of LTR activity finally recovered was close to the amount initially placed in the cultures. These results have important practical implications, most notably for the design of retroviral infection protocols.

Only a small proportion of unseparated marrow cells responded to this cytokine mixture. Most of the cultured cells died, and the small recovered subpopulation of healthy, dividing cells was much smaller than the original number.
plated. In these conditions, inclusion of IL-1β consistently improved recovery of LTR activity with little effect on overall cell numbers. When IL-3 was included, cell growth was explosive and cell numbers soon exceeded those plated. However, as noted by others, the effect of IL-3 on recovery of LTR cells was adverse. A deleterious effect of IL-1 on LTR cell recovery has also been reported in 7-day cultures containing a final cell density greater than those reached in our 4-day cultures. These observations suggest that the adverse effects on LTR recovery may depend on high cell concentrations and could therefore reflect accumulation or induction of inhibitory mediators.

These results further suggested that LTR cells might be capable of generating colonies in semisolid medium, even though potential multilineage reconstitution of mice by cells from colonies grown from normal marrow had not previously been described. Because marrow contains many cells with colony-forming ability that are developmentally advanced and lack reconstituting capacity, we sought to enhance the likelihood of identifying colony formation from rare LTR cells by physically separating them from more advanced CFC before culture. Isolation of marrow cells strongly expressed Ly6A/E and weakly staining with Rh123 yielded a fraction that contained 85% of the recovered LTR activity measured in the competitive assay in vivo, whereas 99% of the recovered precursor cells measured in short-term colony assays, including CFU-S12 and in vitro CFCmulti, were detected in the fraction excluded from the sort window. This result strongly reinforces earlier evidence that the long-term reconstituting cells in resting marrow are phenotypically distinct from other pluripotential precursor cells measured in short-term assays in vitro or in vivo.

Because the LTR cell-enriched fraction also contained small numbers of cells detectable in short-term colony assays, there is the possibility that LTR cells themselves may have the capacity to generate colonies in short time frames. It was therefore of considerable interest to estimate and compare absolute numbers of the various operationally measured cells. Overall, the measured number of colony-forming cells, mainly CFC-G/M, outnumbered LTR cells by about twofold, suggesting that at least some of the residual CFC-G/M were distinct from LTR cells. However, it was of particular interest that the estimated absolute number of LTR cells exceeded the number of residual CFCmulti and CFU-S12 (t-corrected), an observation that suggests that at least some LTR cells would not be detected in such short-term assays.

To prepare for analysis of possible colony formation in culture by LTR cells, we wanted to ensure that we would be able to detect single LTR cells at high efficiency. W-series, c-kit-defective mice are uniquely suitable recipients for this purpose, because they can be substantially repopulated by injected wild-type cells without any requirement for prior myeloablation. Small numbers of injected stem cells can therefore be detected without concern for host survival. The use of an electrophoretic variant of GP11 as a marker of donor cells provides additional advantages. GP11 is expressed in all cells, including erythrocytes, and its enzymatic measurement provides unequivocal, sensitive, and quantitative detection of donor cells in any desired lineage. In this study, we show that this system is further enhanced by sublethal irradiation of the W4*/W4* recipients, to the point that virtually all recipients of single LTR cells at limiting dilution reconstitute donor erythropoiesis at high proportions that are comfortably above the threshold of detection.

Our limiting dilution experiments in sublethally irradiated W4*/W4* recipients yielded a frequency estimate of 1 LTR cell in 11,200 normal marrow cells measured 1 year after marrow injection, in agreement with the measurements of Szilvassy et al. The frequency estimated in unirradiated W4*/W4* hosts was twofold lower. This difference may have arisen from the lower detection sensitivity in the unirradiated model, and statistical analysis of the data indeed suggested that 20% of non-zero reconstitution values would have fallen below our threshold of detection of the donor component. The estimate obtained from sublethally irradiated hosts was also higher than the 1 in 20,000 to 30,000 estimate obtained by assessing cure of anemia in unirradiated W/W hosts. It appears likely that donor GP11, which can be detected at as little as 1% of total erythrocytes, would be more easily detectable at lower reconstitution levels than cure of anemia or reduction of macrocytosis, and the difference in sensitivity could account for the difference in estimates of stem cell frequency.

The proportion of donor cells achieved in hosts reconstituted at limiting dilution, ie, in recipients of single reconstituting entities, varied extensively from recipient to recipient. The basis of the variation can only be speculated on at present, although it is a predicted outcome of probabilistic stem cell self-renewal. Regardless of its source, the observation has significant implications. For example, Harrison et al estimated the frequency of LTR cells in marrow to be 1 in 100,000, with the assumption that the variation observed among recipients of competitive marrow transplants was due principally to binomial variance in the numbers of stem cells injected. However, if the observed variation arises not only from binomial variance in stem cell numbers, but also from stem cell-to-stem cell variation in eventual contribution, then observed variation would exceed the binomial component and hence yield an underestimate of the true LTR cell frequency.

In recipients of limiting numbers of reconstituting cells, all hosts that were positive in the long term for donor erythrocytes were also positive for donor lymphocytes, whereas identical recipients that did not have detectable donor erythrocytes also lacked detectable donor lymphocytes. However, the extent of B- and T-lymphoid repopulation was frequently less than the amount of erythroid, monocytic, and granulocytic reconstitution. Although we did not specifically investigate the issue, it could in part reflect a stronger competitive capacity of host or co-injected W4*/W4* cells in repopulating lymphoid relative to myeloid lineages. The requirements for successful rearrangement of the antigen receptor genes could also, in a stochastic fashion, restrict the amount of lymphopoiesis relative to myelopoiesis at the level of single clones.

When cells from the LTR-enriched fraction were plated in methyl cellulose containing KL, IL-1, IL-6, and IL-11, nascent colonies formed that proved capable of sustained...
multilineage reconstitution in murine hosts. Although small disperse clones of less than 16 cells had the highest likelihood of successful reconstitution, the frequency of reconstituting clones even among those meeting these morphologic criteria was only about 1 in 60 to 80. Furthermore, the total recovery of LTR cells from methyl cellulose cultures, measured by competitive reconstitution, comprised only a small fraction of the LTR activity initially cultured. A variety of possible reasons for the poor recovery can be considered, including details that varied from those providing high recoveries from liquid cultures such as the use of conditioned media rather than purified cytokines and the likelihood that some of the clones originated from cells with LTR capacity that failed to self-renew. Nevertheless, the observed reconstitutions establish that at least some LTR cells are able to initiate colony formation in culture and, therefore, that at least some of the short-term colony-forming capacity found in the LTR-enriched fraction is likely to derive from cells with reconstituting capacity.

There is strong current interest in the possibility of expanding stem cell numbers in culture, particularly because of the implications expansion could have for clinical transplantation. The biologic capacity of LTR cells to multiply in numbers in vivo can be deduced from the quantitative experiments reported by Harrison and Astle. Although their interpretations focussed on the failure of transplanted mice to reconstitute to a normal marrow complement of LTR capacity, a severalfold increase in LTR activity over the injected amount with time is implicit in their data; our own work in progress as well as that of others confirms this conclusion. It is likely only a matter of time before stem cell increase will be achieved in culture, and there is no shortage of interesting cytokines to explore, including VEGF, the FGF family, LIF, and the ligands for c-mpl, flk-2, and TIE. Capture and general amplification of cDNA specifically attributable to LTR cells, following the paradigm already described for more advanced cells in the hematopoietic precursor hierarchy, now appears feasible in principle on the basis of our findings and should need only modest further improvement in clonogenic efficiency of LTR cells in culture.

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