Phenotypic and Functional Characterization of Competitive Long-Term Repopulating Hematopoietic Stem Cells Enriched From 5-Fluorouracil-Treated Murine Marrow

By Stephen J. Szilvassy and Suzanne Cory

Lymphomyeloid stem cells from the bone marrow of C57BL/6 mice treated with 5-fluorouracil (5-FU) were characterized with respect to 12 parameters using fluorescence-activated cell sorting and a competitive long-term repopulation assay. Stem cells were larger than lymphocytes and exhibited side light-scatter characteristic of blast cells. Most expressed low levels of Thy-1.2, high levels of Sca-1 (Ly6-A/E), H-2K, and AA4.1 antigens and stained brightly with rhodamine-123. Significantly, most long-term repopulating cells also expressed CD4, some at high density. In a significant proportion displayed low to medium levels of the "lineage-specific" markers CD45R (B220), Gr-1, and TER-119. A simple and rapid multiparameter sorting procedure enriched the stem cells 100-fold and substantially removed most other clonogenic cell types, including day 12 spleen colony-forming cells. Cells able to generate cobblestone colonies on stromal cells in vitro were co-enriched. Lethally irradiated mice transplanted with limiting numbers of the sorted stem cells did not survive unless cotransplanted with "compromised" marrow cells prepared by prior serial transplantation and shown to be depleted of long-term repopulating activity. A significant number of recipients transplanted with 25 to 100 sorted cells contained donor-derived B and T lymphocytes and granulocytes in their peripheral blood for at least 6 months. Limiting dilution analysis in vivo indicated that the frequency of competitive long-term repopulating units (CRU) in the sorted population was at least 1 in 80 cells. The calculated frequency of CRU was largely independent of the time of recipient analysis between 10 and 52 weeks, indicating that highly enriched stem cells can be recruited relatively early in certain transplant settings. This simple enrichment and assay strategy for repopulating hematopoietic stem cells should facilitate further analysis of their regulation in vivo.

SUSTAINED production of mature blood cells throughout adult life requires the continued activity of hematopoietic stem cells, the most primitive of which are defined operationally by their potential for self-renewal and their capacity to regenerate and maintain both lymphoid and myeloid lineages on transplantation into lethally irradiated or genetically deficient mice. Although the paucity of stem cells has made their characterization difficult, major advances have recently been made toward their purification. Most methods use immunofluorescence and flow cytometry, and exploit the differential expression of specific cell-surface markers to separate stem cells from their more differentiated progeny. Antigens that have been used to remove mature cells include the B-lymphocyte marker CD45R (B220), the T-lymphocyte markers CD4 and CD8, and the myeloid markers Gr-1 and Mac-1. Bone marrow (BM) stem cells have been positively selected for expression of high levels of Sca-1 (now called Ly6-A/E) and low levels of Thy-1, while fetal liver stem cells have been selected by staining with the antibody AA4.1. Differential accumulation of the supravital fluorescent dye rhodamine-123 (Rh-123) in mitochondrial membranes has also proved to be a powerful discriminator. Primitive stem cells are stained less brightly than more mature progenitor cells because they express higher levels of the multidrug resistance efflux pump, P-glycoprotein, and therefore expel Rh-123 more rapidly.

Reconstitution of hematopoiesis in irradiated recipients occurs in two phases. Because the relatively mature hematopoietic cells recruited early after transplantation can only transiently repopulate the mature blood cell compartments, long-term maintenance of hematopoiesis ultimately depends on the activation of more primitive stem cells. Highly purified stem cells may be ineffective for radioprotection because they may require several weeks to generate sufficient numbers of mature effector cells. To avoid this potential problem, a competitive repopulation assay has recently been introduced for the identification and quantitation of long-term repopulating stem cells. In this assay, histocompatible but genetically distinguishable "test" stem cells are injected into lethally irradiated mice together with a large excess of marrow cells whose competitive long-term repopulating ability has been markedly reduced by two previous cycles of serial transplantation and regeneration. The latter "compromised" population contains a normal frequency of relatively mature clonogenic cells, and is capable of reconstitution when transplanted alone. However, such cells can be readily outcompeted for long-term reconstitution of hematopoiesis when transplanted together with a population of normal or 5-fluorouracil (5-FU)-treated stem cells. Thus, compromised cells provide short-term support for recovering transplant recipients, but in the later stages of engraftment exert less competitive pressure upon test stem cells than normal marrow. The assay also provides a selective pressure at the level of stem cell recruitment that serves to identify a more primitive class of stem cell with a greater capacity for competitive long-term repopulation. The stem cell defined by this assay is termed a competitive repopulating unit (CRU).

The competitive repopulation assay has been used to quantitate stem cells in marrow from mice treated 4 days previously with 5-FU. 5-FU treatment is selectively cy-
totoxic for cycling hematopoietic cells, thus enriching the starting population for stem cells.6-9 5-FU marrow cells have been further enriched for CRU by sorting for blast cells expressing low levels of Thy-1.2 antigen and very high levels of the class I major histocompatibility antigen H-2K.1

In the present study, we have further characterized 5-FU CRU for expression of additional cell-surface antigens. Several of these markers have been used to enrich for stem cells from normal adult BM or fetal liver, either by positive (Sca-1, AA4.1) or negative (B220, CD4, Gr-1) selection, but their expression pattern on competitively repopulating 5-FU marrow stem cells has not previously been investigated. Two other potentially discriminatory markers analyzed were Qa-1m,2 which is reportedly expressed at high levels on stem cells able to generate spleen colonies 14 days after transplantation (CFU-S),3 and TER-119, a recently described erythroid cell marker.25 The ability of 5-FU CRU to retain the dye Rh-123 was also investigated.

Based on the phenotype of 5-FU CRU established by these experiments, we have developed a novel procedure for their rapid enrichment by six-parameter fluorescence-activated cell sorting (FACS). The frequency of CRU in the starting and enriched population was determined by limiting dilution analysis in vivo using the competitive long-term repopulation assay, and compared with the frequency of more mature classes of hematopoietic cells. The implications of the results for stem cell recruitment in vivo are discussed.

**MATERIALS AND METHODS**

**Animals.** Six- to 12-week-old male C57BL/6-Alpha-17 or C57BL/6 mice were used as marrow donors. Both strains express the Thy-1.2 and Ly-5.2 alleles, and are of the H-2H haplotype. Age-matched female C57BL/6-Ly-5.1-Pep26 mice (Thy-1.2, Ly-5.1, H-2H) were used as recipients. All mice were bred and maintained on sterilized food and acidified water at The Walter and Eliza Hall Institute of Medical Research animal facility.

**Cell labeling and sorting.** Femoral BM cells were obtained from groups of 10 to 15 Ly-5.2 mice that had been injected intravenously (IV) 4 days previously with a sterile solution of 5-FU (David Bull Laboratories, Melbourne, Australia) in phosphate-buffered saline (PBS) at a dose of 150 mg/kg body weight. Cell suspensions were prepared in balanced salt solution (SFN) containing 2% fetal calf serum (FCS) and 0.02% sodium azide by repeated flushing of the femoral marrow (FBS). After a further 35 minutes on ice, the cells were pelleted, and then, to reduce subsequent Fc-receptor-mediated binding, a wash was performed using SFN with 10 μg/mL propidium iodide (PI) before flow cytometry. Staining of cells with Rh-123 was performed as described.26 Unstained cells or cells incubated only with streptavidin conjugates were used as background controls. Cell sorting was performed using an unmodified dual-laser FACSStar-Plus instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA). PI-negative cells were sorted into siliconized glass tubes containing 50% FCS in PBS.

**In vitro colony-forming cell (CFC) assays.** 5-FU BM cells were plated in 35-mm Petri dishes in 1.0 mL of 0.8% methylcellulose in Iscove’s medium containing 20% FCS, 2 U/mL erythropoietin, and predetermined maximal stimulatory concentrations of pokeweed mitogen-stimulated mouse spleen cell-conditioned medium (kindly provided by Dr G. Johnson, The Walter and Eliza Hall Institute of Medical Research).32 Colonies of greater than 50 cells were counted in situ after 14 days incubation at 37°C in a humidified atmosphere of 10% CO2 in air.

**CFU-S and pre-CFU-S assays.** Mice were exposed to 10.0 Gy of total body gamma-irradiation administered in two equal doses 3 hours apart from a 60Co source at a dose rate of 5.5 cGy/min, injected IV with 105 to 1.5 × 105 sorted or unsorted day 4 5-FU BM cells, depending on the response anticipated, and killed 9 or 12 days later for macroscopic spleen colony counts.33 Spleen colony numbers were corrected for the two-fold reduction in colony formation caused by antibody labeling of cells,17 but not for seeding efficiency (which was not measured). Endogenous day 12 spleen colony formation was less than 0.1 per spleen. For pre-CFU-S assays, femoral marrow cells obtained from primary recipients 12 days after transplantation were injected into secondary mice (0.5 to 1.0 femur/recipient) irradiated as above. The number of secondary spleen colonies counted 12 days later was normalized to the fraction of primary marrow injected, assuming each femur represents 6% of marrow,40 and again correcting for the two-fold reduction in pre-CFU-S seeding caused by antibody labeling. The frequency of pre-CFU-S within the original 5-FU marrow population is expressed as the number per primary marrow per cell injected into the primary mice.

**CRU assays.** Compromised marrow cells (see text) were produced by transplanting Ly-5.1 marrow into irradiated (10.0 Gy) syngeneic recipients (106 cells/mouse). After 5 weeks, 105 marrow cells from the primary reconstituted recipients were transplanted into each of a second group of irradiated Ly-5.1 mice to be used as a source of compromised cells 5 to 12 weeks later. Competitive repopulation assays were then performed as previously described, with some modifications. Ly-5.1 recipients irradiated as above were cotransplanted with limiting numbers of sorted or unsorted Ly-5.2 5-FU BM cells together with 2 × 105 compromised Ly-5.1 marrow cells. Reconstitution by donor cells was assessed for each recipient at ~10-week intervals by analysis of peripheral blood (PB) obtained from the retro-orbital sinus. After depletion of erythrocytes, 60 μL samples were stained with a FITC-conjugated anti-Ly-5.2 MoAb (clone AL4A224) and an R-PE-conjugated MoAb specific for either B220, Thy-1.2 or Gr-1 antigens. Two-color flow cytometry performed using a FACScan instrument (see Fig 3 for example) enabled the detection of as few as 1% donor cells. The frequency of CRU in the test cell suspension was determined by limiting dilution analysis. Groups of 4 to 8 lethally irradiated Ly-5.1 mice were injected with 2 × 105 compromised Ly-5.1 marrow cells together with 300, 900, 2,700, or 8,100 unfractionated, or 25, 50, 100, or 300 sorted Ly-5.2 test cells per mouse. Five to 52 weeks later, the proportion of nonrepopulated (>99% Ly-5.1) recipients in each group was determined by FACS analysis as described above and plotted against the number of Ly-5.2 test cells injected (see Fig 4 for example). A line of best fit was generated using the maximum-likelihood method without forcing the data through the origin, and the frequency of CRU was then determined using standard statistical methods by interpolation of the number of test cells required to obtain a 37% negative response.41
Cobblestone area-forming cell (CAFC) assays. Using an automated cell deposition unit (ACDU) (Becton Dickinson Immuno-cytometry Systems), limiting numbers of BM cells were sorted directly into individual wells of 96-well flat-bottom tissue-culture plates seeded 1 day previously with 5,000 unirradiated MC3T3-G2/Pa6 stromal cells (Pa6) per well in 200 µL Dulbecco’s modified Eagle’s medium containing 10% FCS and 0.5% supernatant from the hybridoma cell line X63/040 expressing 6 × 10^5 U/mL interleukin (IL)-3. Cultures were fed twice weekly by replacement of half the medium and the development of cobblestone colonies was monitored by phase-contrast microscopy every 3 to 4 days. The frequency of input CAFC was determined by limiting dilution analysis of the proportion of negative wells at each test cell concentration after 4 weeks of culture.

RESULTS

Phenotypic characterization of competitive long-term repopulating stem cells. To characterize the phenotype of competitive long-term repopulating stem cells, day 4 5-FU marrow cells were sorted on the basis of either forward (FSC) or side (SSC) light-scattering properties, Rh-123 retention or the level of expression of nine hematopoietic cell surface markers (Fig 1). For each parameter, four fractions were collected and 10^4 cells from each fraction were injected into lethally irradiated mice, together with sufficient compromised marrow cells (2 × 10^3 per recipient) to ensure survival. Reconstitution was very effective and 98% of the transplanted animals survived 5 weeks. To facilitate identification of the progeny of the sorted stem cells, we adopted the strategy of Spangrude et al.2 and used C57BL/6 mouse strains congenic for the Ly-5 locus, which encodes an antigen detectable by flow cytometry on the surface of all hematopoietic cells except mature erythroid cells.44 The fractionated cells were derived from Ly-5.2 mice, while both the compromised cells and recipient mice were Ly-5.1 in origin.

The degree of donor engraftment of 271 transplanted animals was assessed at 5 weeks by flow cytometric analysis of PB, or of marrow and thymus cells. The proportion of mice that contained ≥15% donor-derived (Ly-5.2+) leukocytes at that time is shown below each fraction in Fig 1. One hundred fifty-eight of these mice were subsequently monitored by blood cell analysis for 12 months, and all but 12 survived the entire period. Those fractions of 5-FU marrow cells in which ≥15% donor engraftment was sustained for at least 6 months in at least half of the analysed recipients are highlighted by shading in Fig 1. The competitive long-term repopulating stem cells (CRU) defined by these criteria exhibited high FSC and medium to high SSC, suggesting they were blast cells. They displayed very high levels of Sca-1 and medium to high levels of CD45R (B220) or Gr-1. Most expressed low levels of Thy-1.2, although some mice transplanted with Thy-1.2-negative cells also displayed a high degree of donor engraftment. Most CRU expressed low levels of Qa-m2 antigen. Greater than 99% of the 5-FU marrow cells stained brightly with Rh-123 and CRU were detectable in all fractions analyzed, albeit apparently at lower frequency in fraction 3. No CRU activity was detected in fractions expressing high levels of the “lineage-specific” markers CD45R (B220) or Gr-1. However, significant levels were apparent in fractions displaying very high levels of CD4 or TER-119. Furthermore, a significant level of CRU activity was reproducibly detected in fractions expressing low to intermediate levels of CD4, TER-119, B220 or Gr-1. These results indicate that not all long-term repopulating stem cells are “lineage-negative” (Lin-).

For each parameter analyzed, the average level of donor engraftment in mice repopulated with cells from the fractions shaded in Fig 1 usually either increased after 5 weeks, or remained stable for up to 1 year after transplant (Table 1). Lineage analysis (see Fig 3 for an example) established that the donor-derived (Ly-5.2+) leukocytes in such mice always included both B and T lymphocytes as well as granulocytes. The level of donor B cells in individual mice remained relatively constant after 5 weeks, and varied from 40% to 74% of all donor cells. The proportion of T cells usually stabilized after 10 weeks at 14% to 32% of all donor cells. The myeloid component of the donor compartment typically diminished with time, decreasing from 7% to 44% at 5 weeks to 6% to 14% by 26 weeks, but disappeared in only two mice.

Enrichment of competitive long-term repopulating stem cells by multiparameter sorting. Based on these results, we decided that the most effective purification strategy would be to retain the criteria used previously for CRU enrichment13 (high FSC, intermediate SSC, high H-2Kb, low Thy-1.2), and to incorporate selection for cells expressing high levels of Sca-1. The availability of a facility for six-parameter sorting also enabled exclusion of damaged cells stained with propidium iodide. One of the most attractive features of the procedure is its speed. Because of the 5-FU treatment in vivo, no preliminary negative separation step is required to remove mature cells, so the presort preparation time is less than 2 hours. Approximately 10^4 cells can then be routinely isolated from 20 to 24 femurs with a single FACS sort.

Typical contour plots of the distribution of 5-FU marrow cells relative to the sort windows are shown in Fig 2. The light-scatter window (panel A) selected 35% of the cells, and their expression profile for Thy-1.2 and H-2Kb antigens (panel B) was similar to that obtained previously for the equivalent fraction of cells from (C57BL/6 × C3H/HeJ)F1 mice.13 The advantage of imposing the criterion of high Sca-1 expression for further separation of both the Thy-1.2 low and the H-2Kb high subpopulations is readily apparent from panels C and D, respectively. Gating on these parameters localized the CRU to a relatively small fraction representing 0.2% to 0.3% of the total population of viable 5-FU BM cells.

To assess the competitive long-term repopulating potential of the sorted Ly-5.2 marrow cells, they were injected at limiting numbers (25 to 300 cells per mouse) into cohorts of lethally irradiated Ly-5.1 mice together with excess (2 × 10^3) compromised Ly-5.1 cells. A total of 84 mice were transplanted in three experiments; 70 survived at least 5 weeks and 45 survived for 12 months. PB was analyzed at 5 weeks, and at ~10-week intervals thereafter. Donor-derived cells were detected in 31 mice on at least one of these occasions. This frequency of positive mice is consistent with the injection of limiting numbers of stem cells and ensures that the determination of CRU frequency (see below) is based on the linear portion of the limit-dilution curve. Table 2 summarizes the level of donor engraftment in the 24 mice in which it was sustained beyond 5 weeks. At doses of 100 cells or fewer per mouse, donor cells usually represented less than 20% of PB leukocytes. With 300 cells per mouse, the level of donor en-
Fig 1. Phenotype of competitive long-term repopulating stem cells (CRU) in 5-FU BM. BM cells from Ly-5.2 mice injected 4 days previously with 5-FU were separated into four fractions according to the indicated parameters and 10^6 cells from each fraction were injected together with 2 x 10^5 Ly-5.1 compromised marrow cells into lethally irradiated Ly-5.1 mice. Each parameter was fractionated at least twice. The proportion of Ly-5.2 donor cells in the PB, or marrow and thymus, was determined in all survivors at 5 weeks, and the proportion of mice exhibiting > 5% donor engraftment at that time is indicated below each fraction. One cohort was monitored for 12 months by periodic blood-cell analysis. Those fractions that sustained a level of >15% donor engraftment at that time are indicated by shading and bolding. The 13 positive mice identified in four independent SSC fractionation experiments exhibited only 10% to 15% donor cells, but 9 of these were transplanted with cells from the fractions shaded. Dotted lines represent background profiles of unstained or second-step reagent stained cells.

Quantitation of CRU. The frequency of CRU in 5-FU marrow before and after sorting was determined by plotting the proportion of donor-negative survivors versus the number of test cells injected, and analyzing the resulting graph using limiting-dilution statistics. Figures 4A and 4B show this analysis for mice transplanted with (unstained) unsorted and (stained) sorted cells, respectively, and analyzed 10 and 52 weeks after transplantation. The CRU frequencies calculated from such data are shown for each time of recipient analysis in Table 3. Because the frequency did not significantly vary with time, these data suggest that 10 weeks is sufficient for assessing competitive repopulating stem cell activity in 5-FU BM.

The fractionation procedure achieved a 100-fold enrichment of CRU from day 4 5-FU marrow with a recovery of 31% ± 4% (Table 3). The average frequency of CRU in the sorted population was 1.7% ± 0.9% (ie, 1 in 59 cells), com-
Table 1. Long-Term Engraftment of Ly-5.1 Mice Competitively Repopulated With Single Parameter Sorted Ly-5.2 5-FU BM Cells

<table>
<thead>
<tr>
<th>Parameter Fractionated</th>
<th>No. of Mice</th>
<th>%Ly-5.2* (Donor)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5w</td>
</tr>
<tr>
<td>FSC</td>
<td>6</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>H-2Kb</td>
<td>2</td>
<td>50 ± 31</td>
</tr>
<tr>
<td>Thy-1.2</td>
<td>5</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>Sca-1</td>
<td>2</td>
<td>76 ± 0</td>
</tr>
<tr>
<td>AA4.1</td>
<td>7</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>Rh-123</td>
<td>7</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>B220</td>
<td>8</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>CD4</td>
<td>6</td>
<td>46 ± 10</td>
</tr>
<tr>
<td>Gr-1</td>
<td>7</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>TFR-119</td>
<td>2</td>
<td>29 ± 20</td>
</tr>
<tr>
<td>Qa-m2</td>
<td>7</td>
<td>50 ± 8</td>
</tr>
</tbody>
</table>

The degree of donor engraftment in PB is shown as the mean percent of Ly-5.2+ cells ± SEM (or ± range for groups of 2 mice) in all mice that exhibited ≥15% donor cells for at least 6 months after injection of cells from the fractions shaded in Fig 1. The number of such mice is indicated in column 2.

* One of the 2 positive mice died between 10 and 26 weeks. The value for the remaining survivor is shown.

pared with an average frequency of 0.017% ± 0.009% (ie, 1 in 6,000 cells) in the starting 5-FU marrow-cell population. This frequency of sorted CRU has been corrected by a factor of 3 to allow for the reduction in stem cell frequency caused by antibody labeling. The correction factor was determined by comparing the frequency of CRU in the unsorted 5-FU marrow cell population before and after staining. CRU represented 0.006% ± 0.003% (ie, 1 in 16,000 cells) of stained but unsorted 5-FU marrow (determined by evaluation of 81 mice from three independent experiments 17 weeks after transplantation). This three-fold reduction in detectable CRU is thought to reflect opsonization of antibody-coated hematopoietic cells by reticuloendothelial cells, as documented originally for day 12 CFU-S.39

Quantitation of other clonogenic cell types in the sorted population. To determine the extent to which more mature clonogenic cell types were present in the sorted population, we performed assays for in vitro colony-forming cells (CFC) and for spleen colony-forming cells (CFU-S) (Table 4). In vitro colony assays were performed using spleen cell-conditioned medium as the stimulus for growth of erythroid burst-forming units (BFU-E), granulocyte-macrophage progenitors (CFU-GM), high proliferative potential colony-forming cells (HPP-CFC) and multipotential progenitors (CFU-GEMM). CFC were present only at very low frequencies (0.02% of total cells) in the initial 5-FU marrow suspension and the sorted population contained essentially none; only 2 colonies, both of mixed composition, were obtained from more than 3 × 10⁴ cells plated over five experiments. Day 9 CFU-S were undetectable in both the starting and the sorted population, and although the more primitive day 12 CFU-S were moderately enriched in the latter (0.2% v 0.01%), their overall recovery was only 7%.

A still more primitive stem cell, the pre-CFU-S, was assayed by determining the number of day 12 CFU-S in the marrow of primary mice 12 days after injection of test cells.45 The frequency of pre-CFU-S in the unsorted marrow suspension was determined to be approximately 0.3% (Table 4). As for all spleen colony assays, this value is corrected for the twofold reduction in colony formation caused by antibody staining,13,39 but not for seeding efficiency (which was not

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Fig 2. Multiparameter sorting of day 4 5-FU BM cells. Panels B, C, and D show the patterns of Thy-1.2, H-2Kb, and Sca-1 antigen expression for blast cells in the light-scatter window shown in panel A. The gates used for cell sorting, and the proportion of cells contained in each window, are indicated by the boxes.
In vitro cobblestone colony-forming activity in the repopulating stem cell population. The potential to form "cobblestone" colonies on pre-established marrow stromal layers in liquid long-term culture has been suggested as an assay for primitive hematopoietic cells. We tested the 5-FU BM cells for their potential to form cobblestone colonies on monolayers of PA6 cells, a cloned preadipocyte cell line that supports the growth and differentiation of day 12 CFU-S, and maintains both pre-[/B]--B-cell precursors and erythropoietic stem cells. Both the unfractionated and sorted population contained cells able to generate colonies of flattened, optically-dense cells associated with the adherent cell layer. These cobblestone colonies arose at different times, increased in size over several days, and generated semi-adherent, round, refractile cells that appeared to be myeloid blasts by May Gr"{u}wald-Giemsa staining (data not shown). Mature granulocytes and monocytes/macrophages typically became apparent in the wells approximately 4 days after the initial detection of cobblestone cells.

The frequency of CAFC was estimated by limiting dilution analysis to be 0.1% (ie, 1 in 1,019 cells) for the starting population of 5-FU BM, and 7% (ie, 1 in 14 cells) for the fraction enriched for long-term repopulating activity (Table 4). Thus, the enrichment after sorting was about 70-fold, and the overall recovery about 22%. It has been suggested, but not proven, that the subset of CAFC detected 3 to 4 weeks after initiating in vitro culture may be closely related to repopulating stem cells. In the fraction enriched for repopulating cells, 20% to 50% of the cobblestone colonies developed late (Table 5).

Secondary transfer of donor-derived stem cells. The most primitive hematopoietic stem cells have a capacity for self-renewal that is operationally defined by their potential to maintain lymphomyelopoiesis upon secondary transplantation. One year after primary transplantation, BM cells from 15 mice exhibiting a range (3% to 85%) of donor cells in their PB were transplanted into pairs of lethally irradiated Ly-5.1 mice (0.8 femur/mouse). In general, the level of donor-derived leukocytes in the marrow of the primary mice was somewhat lower than in their blood (Table 6). When the PB of secondary mice was analyzed 5 or 10 weeks later, donor-derived leukocytes were detected in all but one of the 10 pairs that had received marrow containing $\geq$4% Ly-5.2$^+$ cells. Except for one pair, donor cells remained detectable for at least 17 weeks. As in the primary mice, the donor compartment was predominantly lymphoid (22% to 86% B cells, 12% to 68% T cells at 10 weeks). Granulocyte levels varied from 0% to 46% (mean 11% at 10 weeks) of donor cells, the level in individual secondary mice at 5 weeks mirroring that in the primary animal at death, with some reduction over time (not shown).
supportive cotransplant of compromised marrow cells. In an attempt to minimize deaths caused by infection, one cohort of mice was given antibiotic (neomycin B) in their drinking water. Five weeks after transplantation, most mice maintained without antibiotic had died, including those receiving 300 sorted cells (Table 7). The mortality of mice given antibiotic was significantly lower, but was still about 60%. These results contrast markedly to those described above, where 70 of 84 mice receiving a cotransplant of compromised cells survived 5 weeks.

Most surviving mice exhibited donor cells in their PB 5 weeks after transplantation (Table 7) and the proportion that remained positive at 26 weeks was higher than had been seen in mice receiving a transfusion of compromised cells. However, somewhat surprisingly, the degree of donor engraftment in individual mice was usually no greater than that seen with a competitive transplant. These results indicate that, despite the 10 Gy of total body irradiation, recovering host cells can play a significant role in maintaining both short- and long-term hematopoiesis. As with the standard protocol, the donor compartment contained more lymphoid than myeloid cells: 2% to 76% B cells, 12% to 90% T cells, 0% to 76% granulocytes at 10 weeks; 16% to 76% B cells, 12% to 69% T cells, 0% to 20% granulocytes at 26 weeks.

Frequency of CRU in compromised marrow. We determined the frequency of CRU in the compromised marrow population by competitive repopulation assays in which 6 × 10⁴, 2 × 10³, 6 × 10⁵, or 2 × 10⁶ compromised Ly-5.2 cells were cotransplanted with the standard supporting population of 2 x 10⁵ Ly-5.1 compromised marrow cells. Limiting dilution analysis of mice assessed 5 weeks after transplantation indicated that CRU represented 1 in 250,000 compromised marrow cells (data not shown). In contrast to CRU assays of sorted and unfractionated 5-FU marrow, this frequency was reduced by 17 weeks to 1 in 1,235,000 cells, with a level of donor engraftment of only 4% ± 1%. Thus, while serial transplantation does not significantly affect the number of short-term repopulating hematopoietic cells present, both the frequency and competitive repopulating ability of more primitive stem cells are reduced. Cotransplantation of compromised marrow, which contains a much lower ratio of long- to short-term repopulating cells, thus enables most mice in-
Table 3. Frequency of CRU in Day 4 5-FU BM

<table>
<thead>
<tr>
<th>Week of Analysis</th>
<th>Unfractionated BM</th>
<th>Fractionated BM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CRU Frequency (x10^-4)</td>
<td>Total No. Survivors</td>
</tr>
<tr>
<td>5</td>
<td>1.9 ± 0.9</td>
<td>74</td>
</tr>
<tr>
<td>10</td>
<td>1.8 ± 0.9</td>
<td>73</td>
</tr>
<tr>
<td>17</td>
<td>1.5 ± 0.8</td>
<td>70</td>
</tr>
<tr>
<td>26</td>
<td>1.6 ± 0.8</td>
<td>61</td>
</tr>
<tr>
<td>43</td>
<td>1.8 ± 0.9</td>
<td>51</td>
</tr>
<tr>
<td>52</td>
<td>1.3 ± 0.7</td>
<td>45</td>
</tr>
<tr>
<td>Average</td>
<td>1.7 ± 0.9</td>
<td>Average: 170 ± 90</td>
</tr>
</tbody>
</table>

The CRU frequency expressed as mean ± SEM was determined by limiting dilution analysis (see Materials and Methods) of pooled data from three independent experiments (20 to 30 mice per experiment). For each time point, the total number of mice on which the calculation is based is indicated.

* The response of the sorted population shown in Fig 4B is corrected here for the threefold reduction in CRU frequency caused by antibody labeling of cells (see text).

jected with very few purified stem cells to survive 5 weeks while reducing competition against such cells in the later stages of engraftment.

DISCUSSION

Phenotype and frequency of competitive repopulating stem cells. Analysis of the phenotype of stem cells with competitive long-term repopulating potential (CRU) in 5-FU-treated C57BL/6 BM showed that most exhibited high FSC and intermediate SSC, stained brightly with Rh-123, and expressed high levels of H-2Kb and Sca-1, medium to high levels of AA4.1, and a low level of Qa-m2 antigen. Most 5-FU CRU expressed a low level of Thy-1.2, although a significant proportion were Thy-1.2 negative, as previously reported for C57BL/6 BM.26 It is unclear to what extent the results reflect intrinsic heterogeneity in the stem cell compartment as opposed to stochastic processes that regulate stem cell recruitment in vivo. Our

Table 4. Frequency of Short-Term Clonogenic Cell Types in Day 4 5-FU BM

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Unfractionated BM</th>
<th>Fractionated BM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (x10^-5)</td>
<td>No. Expts</td>
</tr>
<tr>
<td>CFC*</td>
<td>2.0 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>d9 CFU-S*</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>d12 CFU-S*</td>
<td>1.1 ± 0.1</td>
<td>5</td>
</tr>
<tr>
<td>Pre-CFU-S</td>
<td>28 ± 10</td>
<td>3</td>
</tr>
</tbody>
</table>

Frequency is expressed as the mean ± SEM for unfractionated, unstained 5-FU BM and for the fraction (0.3% of starting BM) sorted as indicated in Fig 2.

* Unfractionated and fractionated cells were plated at 3 x 10^4 and 10^5 cells per dish, respectively. Assay conditions are described in Materials and Methods.

† Unfractionated and fractionated cells were injected at 5 x 10^4 and 10^5 cells per mouse, respectively. The frequency is corrected for the twofold reduction in spleen colony formation caused by antibody labeling of cells but is not corrected for seeding efficiency.

§ Unfractionated and fractionated cells were plated at 3 x 10^4 and 1 to 2 x 10^5 cells per mouse, respectively. Data are expressed as pre-CFU-S per primary marrow per injected cell (see Materials and Methods).

* CFU-5 by limiting dilution on PA6 stroma. Unfractionated cells were plated at 300, 1,000, and 3,000 per well; fractionated cells were plated at 3, 10, and 30 per well.
5-FU BM cells were seeded at limiting dilution onto monolayers of unirradiated PA6 cells in 96-well tissue culture plates. The number of new wells containing cobblestone colonies each week is indicated only for those cultures seeded with 300 stained, unfractionated cells/well or with 3 sorted cells/well. At these plating densities, 19% to 32% of wells sorted population was singularly deficient in CFC capable of proliferation in vitro. However, in all enrichment procedures described to date, 1-14%57 Al-enriched in our sorted population, their low overall recovery had been genuinely depleted by sorting. Therefore, it is likely that such cells had been genuinely depleted by sorting.

The phenotypic similarity of day 12 CFU-S to long-term repopulating stem cells has resulted in their copurification in all enrichment procedures described to date.11-14,57 Although day 12 CFU-S and pre-CFU-S were about 25-fold enriched in our sorted population, their low overall recovery nevertheless implies that these cell types are largely separable from CRU, and supports the findings of Jones et al.23 Both the enrichment and recovery of CAFC after sorting approached that of CRU (Table 4). A correlation between the frequency and phenotype of cells able to generate late-appearing cobblestone colonies and that of stem cells with short-term marrow repopulating ability has previously been noted.46,49 In our experiments, such CAFC represented 30% of all CAFC activity in the sorted population (Table 5). However, because we used PA6 cells rather than normal marrow stromal cells, and included a high level of IL-3 in the cultures, it is difficult to compare our results with those obtained previously.49,58

Kinetics of recruitment of stem cells in vivo. A significant finding of this study was that the frequency of CRU was largely independent of the time of recipient analysis (Fig 4 and Table 3). That is, despite variations in the degree of donor engraftment in individual mice, the proportion of animals with detectable levels of donor leukocytes remained the same for each test cell dose whether 5 or 52 weeks had elapsed since transplantation. For at least those mice transplanted at limiting dilution (<100 sorted cells per mouse), it is likely that the donor-derived cells were of clonal origin. The data imply that the CRU from 5-FU marrow were activated relatively early after transplantation.

A different pattern of stem cell recruitment has been observed upon transplantation of retrovirally marked stem cells.5,7,19 In those studies, there was a transient period of clonal instability, presumed to represent the proliferation of relatively mature hematopoietic progenitors, followed eventually by the emergence of a few stable totipotent clones ~20 weeks later. The inference from these data was that detection of the most primitive stem cells requires at least 6 months. However, because large numbers of (unfractionated or AA4.1+) cells were transplanted, the high frequency of relatively mature radioprotective progenitor and/or stem cells present may have delayed recruitment of the most primitive stem cells. In contrast, the cell population we have isolated contains very few cells more mature than pre-CFU-S and, relatively mature radioprotective progenitor and/or stem cells. In contrast, the cell population we have isolated contains very few cells more mature than pre-CFU-S and, as such cells, is not radioprotective (Table 7). Because the frequency of CRU in the supporting transplant is only 1 per 250,000 cells,
most short-term repopulating cells become limiting 5 to 10 weeks after transplantation and stem cells in the “test” population are recruited early. Thus, our data suggest that delayed activation of long-term repopulating cells is not an obligatory consequence of their primitive nature, but merely a reflection of the time at which their recruitment is required to sustain the output of mature cells in a particular transplant setting. In many mice, donor-derived lymphoid cells remained prominent for longer than donor-derived granulocytes, particularly when fewer than 100 cells were injected (Table 2). This pattern of engraftment has also been observed in mice transplanted with limiting numbers of normal marrow stem cells purified by alternate procedures. Although the rapid decrease in Ly-5.2+ granulocyte production between 5 and 10 weeks probably reflects the initial generation of exhaustible progenitors by donor stem cells, the reasons for the later decline are unclear. While it may signify the presence of lymphoid-restricted stem cells within the sorted populations, we obtained similar results when limiting numbers (<8,100 cells per mouse) of unseparated day 4 5-FU marrow cells were competively transplanted (Table 2). Alternatively, because granulocytes have a life span of less than 24 hours, their continued production may require the activity of a larger cohort of stem cells than that required for lymphopoiesis. At limiting numbers, the probability that some donor (Ly-5.2) clones may ultimately be succeeded by those of host or compromised (Ly-5.1) origin will thus be higher. Unlike multipotential stem cells purified from normal marrow, post-5-FU stem cells are in cycle. Therefore, CRU from 5-FU-treated marrow represent superior targets for retrovirus infection. Retroviral tagging has already been used to show that the CRU assay can detect lymphomyeloid repopulating stem cells. Our current efforts are directed toward using this technology to explore the effect of overexpression of key regulatory genes within this primitive hematopoietic cell compartment.

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Phenotypic and functional characterization of competitive long-term repopulating hematopoietic stem cells enriched from 5-fluorouracil-treated murine marrow

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