Isolation in a Single Step of a Highly Enriched Murine Hematopoietic Stem Cell Population With Competitive Long-Term Repopulating Ability

By Stephen J. Szilvassy, Peter M. Lansdorp, R. Keith Humphries, Allen C. Eaves, and Connie J. Eaves

A simple procedure is described for the quantitation and enrichment of murine hematopoietic cells with the capacity for long-term repopulation of lymphoid and myeloid tissues in lethally irradiated mice. To ensure detection of the most primitive marrow cells with this potential, we used a competitive assay in which female recipients were injected with male “test” cells and 1 to 2 x 10^6 “compromised” female marrow cells with normal short-term repopulating ability, but whose long-term repopulating ability had been reduced by serial transplantation. Primitive hematopoietic cells were purified by flow cytometry and sorting based on their forward and orthogonal light-scattering properties, and Thy-1 and H-2K antigen expression. Enrichment profiles for normal marrow, and marrow of mice injected with 5-fluorouracil (5-FU) four days previously, were established for each of these parameters using an in vitro assay for high proliferative potential.

The functional properties of the most primitive hematopoietic cells, defined by their capacity for long-term blood cell production in vivo, are poorly understood. Transplantation experiments have clearly shown that such cells persist in the marrow throughout adult life and constitute a population with a very slow rate of turnover. Direct analysis of the clonal progeny of individual stem cells, as well as indirect analyses of the progeny of stem cells carrying unique chromosomal or retroviral markers, have revealed some evidence of heterogeneity in the differentiative as well as the proliferative potential of normal hematopoietic cells with in vivo repopulating ability. However, the extent to which such heterogeneity may be attributed to intrinsic cellular differences as opposed to stochastic or biologic processes that regulate stem cell recruitment in vivo is not known.

An obvious approach to the resolution of these questions is to obtain pure populations of hematopoietic stem cells whose functional attributes can then be evaluated with a variety of methodologies. Three key considerations to such an approach are the choice of assay used to define the stem cell population to be purified, the cellular characteristics used to select this population, and the method used for cell separation. In the present study, we introduced the use of a stringent, competitive long-term repopulation assay to ensure that the isolation procedure was selective for the most primitive type of hematopoietic stem cell. This involved transplanting irradiated female recipient mice with limiting numbers of separated (or unseparated) populations of marrow cells from male donors together with a much larger number of “compromised” marrow cells from female mice. The compromised cells contained an almost normal frequency of CFU-S and in vitro clonogenic cells, but their competitive long-term repopulating ability had been markedly reduced by two previous cycles of successive marrow transplantation and regeneration. The presence of male cells in the lymphoid and myeloid tissues of the reconstituted female recipients was then assessed 1 to 3 months later by Southern or spot blot analysis of extracted DNA using a Y-chromosome–specific probe. This approach is based on the hypothesis that a population of very primitive hematopoietic stem cells exists that may not begin to proliferate and differentiate for several weeks after transplantation and would therefore not be detected in a transplantation assay unless sufficient short-term repopulating cells were also transplanted. The injection of ≥10^5 compromised female marrow cells thus serves the dual purpose of ensuring survival of recipients injected with limiting numbers of purified stem cells, and providing a selective pressure to identify a class of stem cells with a greater capacity for long-term repopulation.

The characteristics used for stem cell purification were forward and orthogonal light-scattering properties (FLS, OLS), and Thy-1 and H-2K antigen expression. Recent studies have suggested that low levels of Thy-1 are expressed on primitive hematopoietic cells and that this decreases with differentiation. Comparisons of the level of class I major histocompatibility complex (MHC H-2K) antigen on different hematopoietic cell types have also suggested a progressive decrease of this antigen with differentiation.
To develop a simple purification procedure that could be used with a single laser FACS, we devised a cell labeling technique in which Thy-1-positive cells are indirectly stained with one fluorochrome (FITC) by a biotin–avidin complex, and H-2K²–positive cells are indirectly stained with a second fluorochrome (R-phycocerythrin, R-PE) by a bispecific tetramolecular antibody complex. The tetramolecular complex consists of two different mouse IgG, monoclonal antibodies (MoAbs; in this case, one being directed against the H-2K² antigen, the other against R-PE) linked together by two different component antibodies (to favor formation of bispecific molecular antibody complexes for flow cytometry were described previously.

Sorting experiments were undertaken with suspensions of normal marrow as well as marrow from mice that had been treated four days previously with 5-fluorouracil (5-FU). The latter were included because 5-FU selectively decreases the number of mature hematopoietic cells present, and we therefore anticipated that marrow from 5-FU–treated mice might serve as an already enriched starting population.

**MATERIALS AND METHODS**

**Animals.** Six- to 12-week old (C57BL/6J × C3H/HeJ)-F₁ (B6C3F₁) male and female mice were used in all experiments. B6C3F₁ mice are homozygous for the Thy-1.2 allele and are of the H-2K²/H-2K¹ haplotype.

**Preparation of marrow cell suspensions.** Bone marrow (BM) cells were obtained from the femurs of either normal male mice or male mice injected intravenously (IV) four days previously with a sterile solution of 5-FU in phosphate-buffered saline (PBS) at a dose of 106 cells/mL. Cells were suspended in Hank’s balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) and 0.02% sodium azide (HFN-buffer) with a 21-gauge needle and then were filtered through a double layer of 20 μm nylon mesh. Cells were washed in HFN, resuspended in NH₄Cl-Tris (pH 7.2) for five minutes at room temperature to lyse erythrocytes, washed once again in HFN, and then diluted to a concentration of >5 x 10⁶ cells/mL HFN for immunostaining and subsequent plating or injection.

**Cell labeling.** Biotin-conjugated, purified rat anti-Thy-1.2 MoAb (clone 30-H12, Becton Dickinson, Mountain View, CA) was used at a dilution of 1:10 in HFN for staining in green. Mouse anti–H-2K² MoAb (IgG₁) (hybridoma TIB 139, American Type Culture Collection) purified from ascites fluid generated in pristane–primed BALB/c mice was labeled with R-PE by generating tetramolecular antibody complexes (tetramers) with anti–R-PE MoAbs by simple mixing of the component antibodies as previously described. In this case, anti–H-2K² IgG₁ (~500 μg/mL) was mixed with anti–R-PE IgG₁ (~500 μg/mL) and rat anti-mouse IgG₂ (~500 μg/mL) to give a final molar ratio of 1:10:11, respectively, of the different component antibodies (to favor formation of bispecific tetramers containing anti–H-2K²). The solution of tetramers was then used as the primary reagent for H-2K²-specific staining in red.

Control cells stained with tetramers containing only R-PE antibodies (anti–R-PE × anti–R-PE), prepared by omitting the anti–H-2K² reagent, were used to measure the level of nonspecific red fluorescence.

For staining, 50-μL aliquots of washed cells (at 5 x 10⁶ cells/mL) were mixed with an equal volume of either biotinylated anti-Thy-1.2 MoAb, anti–H-2K² × anti–R-PE tetramers, or both in the case of double-stained samples, incubated for 30 to 45 minutes on ice, washed twice in HFN, resuspended in a solution of avidin–FITC (5 μg/mL) and/or R-PE (2 μg/mL) in HFN, and then incubated for 30 to 45 minutes more on ice, washed three times in HFN, resuspended to >5 x 10⁶ cells/mL in HFN, and passed through a nylon mesh (20 μm) to remove clumps and debris before FACS analysis and sorting. Controls were treated identically substituting HFN or monospecific anti–R-PE tetramers for specific antibodies as required. Cell viability was determined by staining with 0.1% nigrosin and was consistently >90%.

**FACS analysis and sorting.** Red and green fluorescence and FLS (0.5 to 13°) and OLS (65°–115°) were analyzed with a FACS 440 (Becton Dickinson, Sunnyvale, CA) with the laser at 488 nm (300 mW). FITC fluorescence was separated from R-PE fluorescence with 525/10-nm and 755/26-nm band pass filters in combination with a dichroic mirror (560 nm). For double-stained cells, the signal from the green fluorescence into the detector used to measure red fluorescence was electronically compensated to background levels with cells stained with biotinylated anti–Thy-1.2 and avidin–FITC only. A logarithmic amplifier was used for all fluorescence signals. The horizontal position of the profiles obtained in separate experiments was standardized as follows: For FLS, the peak of the second major population was set at channel 120; for OLS, the major lymphocyte peak was set at channel 30; and for Thy-1.2 fluorescence, the major peak of Thy-1.2–negative cells was set at channel 90. No adjustments were made in the position of the H-2K² fluorescence profile. For analysis, 2 x 10⁶ cells were evaluated per sample. In sorting experiments, cells were sorted at a rate of not more than 2 x 10⁶ cells/sec, and inlet and collection tubes were cooled on ice. Sorted cells were collected in 50% FCS in HBSS. All sorts were performed with the FACS in FDE mode, and the abort rate for coincident cells was reproducibly <10%.

**Competitive repopulation assay.** All recipients were female mice administered 800 to 850 cGy total body irradiation (TBI; 124 cGy/min) with a Phillips 250 kVp X-ray machine. Three to 15 hours after irradiation, recipients were injected IV with male test cells and 1 to 2 x 10⁶ compromised marrow cells from female mice that contained normal numbers of CFU-S and in vitro clonogenic cells (Table I) but had been compromised in their competitive long-term repopulating ability by having been previously subjected to two cycles of marrow regeneration. This was achieved by transplanting 10⁶ female B6C3F₁ marrow cells/mouse into a group of irradiated (800 to 850 cGy) syngeneic female recipients, and then 5 to 8 weeks later transplanting 10⁶ marrow cells from these primary recipients into each of a second group of irradiated syngeneic female recipients. The secondary recipient mice were then used as donors of compromised marrow cells 1 to 3 months later. Recipients of mixed transplants of male test marrow cells and compromised female marrow cells were killed 1 to 3 months after injection as indicated. Isolated marrow, spleen, and thymus cells were pelleted in PBS and stored at −20°C before DNA extraction. Compromised marrow cells (10⁶) alone were sufficient to allow injected recipients to survive

![](https://www.bloodjournal.org/)

**Table 1. Frequencies of Clonogenic Progenitors in Unstained Suspensions of Normal and Compromised Marrow Cells**

<table>
<thead>
<tr>
<th>Progenitor Assayed</th>
<th>Normal Donors</th>
<th>Compromised Donors</th>
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<tr>
<td>Day 12 CFU-S†</td>
<td>20 ± 3 (10)</td>
<td>14 ± 2 (3)</td>
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<tr>
<td>Day 9 CFU-S</td>
<td>26 ± 2 (4)</td>
<td>17 ± 2 (3)</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>13 ± 6 (18)</td>
<td>5 ± 1 (18)</td>
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<tr>
<td>CFU-GM</td>
<td>340 ± 20 (18)</td>
<td>290 ± 40 (18)</td>
</tr>
<tr>
<td>BFU-E</td>
<td>23 ± 5 (18)</td>
<td>23 ± 5 (18)</td>
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*Mean ± SEM of values measured in (n) different experiments.†Not corrected for seeding efficiency.
long-term, but doses up to 10^6 were insufficient to outcompete the marrow, spleen, and thymus repopulating capacity of a graft of 10^4 unseparated day 4 5-FU marrow cells (data not shown).

**DNA analyses.** DNA was purified by proteinase K digestion and phenol-chloroform extraction followed by dialysis against 1 × Tris-EDTA buffer (3 mmol/L Tris, 0.2 mmol/L EDTA, pH 7.5; TE). Samples of DNA (10 μg) were digested with PvuII (2 U/μg) for four to 12 hours at 37°C in the buffer recommended by the manufacturer [Bethesda Research Laboratories (BRL), Gaithersburg, MD]. For Southern blot analyses, samples of male and female DNA from normal B6C3F1 mice were used as positive and negative controls. Ethanol precipitated DNA was dissolved in 300 μL spotting buffer [60 mmol/L Tris, 0.2 N NaOH, 6 × saline sodium citrate (SSC)], heat denatured, and neutralized with 80 μL 1 mol/L Tris, pH 7.5, before being spotted directly onto nitrocellulose membranes. Wells were then rinsed with an equivalent volume of 5 × SSC. 5-μg samples of undigested DNA were ethanol precipitated and dissolved in 300 μL spotting buffer [60 mmol/L Tris, 0.2 N NaOH, 6 × saline sodium citrate (SSC)], heat denatured, and neutralized with 80 μL 1 mol/L Tris, pH 7.5, before being spotted directly onto nitrocellulose membranes. Wells were then rinsed with an equivalent volume of 5 × SSC. 5-μg mixtures of male and female DNA in serially (threefold) decreasing ratios from 100% male DNA to 1% and 0% male DNA were prepared and included in each spot blot as a titration standard. Under the conditions used, ≥10% male DNA in such mixtures could be consistently detected. All blots were air-dried and baked under vacuum at 80°C for two hours. Membranes were prehybridized for two hours at 68°C in 50 mL of a buffer containing 3 × SSC, 4 × Denhardt’s solution, and 0.5 mg/mL denatured salmon sperm DNA. Hybridization conditions were the same except for the inclusion of 0.1% sodium dodecyl sulfate (SDS), 3 mmol/L Tris, and the reduction to 0.1 mg/mL denatured salmon sperm DNA. The pY2 plasmid contains a 720-base pair (bp) Mbol fragment of the Y-chromosome from male BALB/c mice cloned into the BamH1 site of pBR322.18 pY2 probe was 32P-labeled to high specific activity by nick-translation with a kit purchased from BRL. After hybridization for 18 to 20 hours at 68°C, filters were washed at a final stringency of 0.1% SDS, 0.1 × SSC, and 0.1% sodium pyrophosphate at 65°C. Autoradiography was performed at −70°C with Kodak XAR-5 film for 24 to 72 hours.

**Methylcellulose assays.** All sorted and unsorted marrow cells were plated in 35-mm Petri dishes in 1.1-mL culture mixtures consisting of 0.8% methylcellulose in α-methyl medium containing 30% FCS, 1% bovine serum albumin (BSA), 10^−4 mol/L β-mercaptoethanol, 3 U/mL partially purified human urinary erythropoietin (Epo) and 2% pokeweed mitogen (PWM)-stimulated mouse spleen cell-conditioned medium (CM).19 For day 4 5-FU marrow cells, 10% agar-stimulated human leukocyte conditioned medium (LCM)22 was also added. Unsorted marrow cells from both normal and day 4 5-FU mice were plated at a concentration of 3 × 10^6 cells per dish. Sorted cells were plated at lower concentrations down to 500 cells per dish depending on the degree of enrichment anticipated from preliminary experiments. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and colonies were counted in situ after 12 to 14 days (normal marrow) or 21 days (day 4 5-FU cells). Macroscopic multilineage colonies (termed CFU-HMGM) were scored as previously described.23 All other colonies (>20 cells) present were categorized as follows: Those containing more than four clusters of small erythroblasts but not of macroscopic size are called BFU-E derived (even though many of these colonies contained cells of other lineages). Those that did not contain detectable erythroblasts and were composed primarily of granulocytes and macrophages are called CFU-GM derived.

**CFU-S assays.** Female B6C3F1 mice were administered 800 to 850 cGy TBI as for competitive repopulation experiments. This dose was sufficient to completely eliminate endogenous spleen colony formation (to <0.1/spleen). Irradiated animals were injected IV with 0.5 to 50 × 10^5 sorted or unsorted marrow cells per mouse, depending on the number of spleen colonies anticipated, and were killed 9 or 12 days later as indicated for macroscopic spleen colony counts.24 Preliminary experiments in which mice were injected with double-stained but unseparated marrow cells showed that the number of spleen colonies produced both 9 and 12 days later was reduced relative to controls by ~50%. Data for day 12 spleen colony formation are shown in Table 2. To determine whether staining also reduced the capacity of the injected cells to regenerate hematopoiesis in the marrow, we compared the femoral content of in vitro clonogenic progenitors in mice injected with stained or unstained cells. Twelve days after transplantation of stained cells, the number of clonogenic cells per femur was also decreased to approximately half of the control value (data not shown). Baum et al.25 reported a comparable reduction in detectable CFU-S after standard indirect antibody staining of marrow cells and showed that the reduction could be partially abrogated by pretreating mice with antimacrophage agents such as λ-carrageenan.26 Although we were able to confirm this with our double-stained cells (Table 2), we chose not to adopt this “correctional” procedure since the effect was only partial and the long-term consequences of such treatments on host stem cell survival and/or reactivation are unknown. The fraction of unstained cells able to colonize the spleen of irradiated but otherwise untreated recipients was assumed to be 10% and for stained cells was therefore assumed to be 5%.

**RESULTS**

**Light scatter properties, Thy-1, and H-2K antigen expression of in vitro clonogenic cells.** Representative FLS profiles for normal and day 4 5-FU marrow (total nucleated cells) are shown in Fig 1A and B, respectively. The corresponding distributions of CFU-GEMM, CFU-GM, and BFU-E are shown in Fig 1C and D. CFU-GEMM in normal marrow were maximally enriched in the fraction isolated between channels 121 and 150 (eg, 2.7-fold in the experiment shown in Fig 1C). Corresponding enrichments of

<table>
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<th>Table 2. Reduction in CFU-S Detectable After Double Staining of Marrow Cells</th>
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<tr>
<td>Treatment of Cells Injected*</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Double stained</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Double stained</td>
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Abbreviation: ND, not done.

*All mice were injected IV with 10^8 unstained or double stained normal syngeneic marrow cells.

†λ-Carrageenan 0.5 mg per mouse was injected intraperitoneally immediately after irradiation and ~20 hours before transplantation of cells.

‡Values are mean ± SEM of counts from (n) spleens.
CFU-GM and BFU-E in the same fraction were 1.6-fold and 3.0-fold. As expected, day 4 5-FU marrow showed a depletion of larger cells (Fig 1B), an increased frequency of CFU-GEMM, and a decreased frequency of CFU-GM and BFU-E (Fig 1E). All three of these progenitor types showed a higher FLS than their counterparts in normal marrow. As a result, optimal enrichment of CFU-GEMM from day 4 5-FU marrow was obtained in the fraction gated between channels 151 and 255 (4.1-fold in the experiment shown). Enrichment of day 4 5-FU CFU-GM in this fraction was similar (3.9-fold).

The OLS properties of CFU-GEMM, CFU-GM, and BFU-E in normal and day 4 5-FU marrow were similarly analyzed (Fig 2). Maximum enrichment of CFU-GEMM and CFU-GM (4.2-fold and 2.4-fold, respectively, in the experiment shown) from normal marrow was obtained in the region between channels 46 and 90. Maximum enrichment of BFU-E was observed in a window of cells with slightly lower OLS (4.1-fold in the region between channels 31 and 60). Day 4 5-FU marrow was also, as expected, depleted of cells with higher OLS properties (Fig 2B). In vitro clonogenic cells in day 4 5-FU marrow showed a higher OLS than those in normal marrow and were maximally enriched in the fraction gated between channels 61 and 120. In the experiment shown, CFU-GEMM were enriched 2.0-fold in this region.

Staining of normal marrow cells with biotinylated anti-Thy-1.2 followed by avidin-FITC revealed a small population including both weakly and strongly fluorescent cells (~8% of the total marrow population, Fig 3A). The proportion of such cells in day 4 5-FU marrow was higher (~24%, Fig 3B), facilitating better resolution of the subpopulation expressing low levels of Thy-1.2. May-Grünewald-Giemsa staining of cytospin preparations of sorted cells showed that most cells expressing low levels of Thy-1.2 antigen had a blast cell morphology, whereas most in the peak of strongly Thy-1.2-positive cells were lymphocytes. In vitro clonogenic cells in both normal and day 4 5-FU marrow were maximally enriched in the weakly Thy-1.2-positive fraction distributed between channels 121 and 150 (Fig 3C and D). Sorting of cells in this region gave an enrichment of CFU-GEMM from normal and day 4 5-FU marrow of 6.9-fold and 2.3-fold, respectively, in the experiment shown. CFU-GM and BFU-E were also maximally enriched in this fraction, although their distributions extended further into the negative region. This is consistent with the reported decreased expression of Thy-1 on the more mature classes of murine hematopoietic progenitors.

H-2K<sup>+</sup> expression was measured by incubating cells with preformed bispecific tetramolecular antibody complexes containing an anti-H-2K<sup>+</sup> antibody coupled to an anti-R-PE antibody followed by staining with R-PE as described in the Materials and Methods section. The resultant red fluorescence profile was compared with that obtained by staining...
Fig 2. Representative OLS profiles of normal and day 4 5-FU marrow cells. (A and B) Total nucleated cells. (C and D) Frequencies of in vitro clonogenic cells in sequential fractions (5 through 30, 31 through 45, 46 through 60, 61 through 90, 91 through 120, 121 through 150, 151 through 255).

Fig 3. Representative Thy-1.2 fluorescence profiles of normal and day 4 5-FU marrow cells. (A) Total nucleated cells; control fluorescence profiles of normal (---) and day 4 5-FU marrow cells (---) stained with HFN + avidin-FITC. Profiles of cells stained with biotinylated anti-Thy-1.2 MoAb and avidin-FITC (----). (C and D) Frequencies of in vitro clonogenic cells in sequential fractions (20 through 90, 91 through 120, 121 through 150, 151 through 286).
cells with control tetrarmers monospecific for R-PE. These control profiles indicated a low but significant degree of nonspecific staining, probably due primarily to Fc-receptor-mediated binding. Nevertheless, in comparison, \( \text{anti}-\text{H-2K}^b \times \text{anti}-\text{R-PE} \) tetramers gave a much stronger staining of all cells, as shown by the marked shift of the entire profile (Fig 4A). The staining characteristics of normal and day 4 5-FU marrow cells with respect to H2Kb antigen expression were indistinguishable.

Cells were sorted from increasingly selective fractions representing increasingly higher levels of H-2Kb antigen expression. Results from a representative experiment are shown in Fig 4B and C. Although only 15% and 3% of all CFU-GEMM in normal and day 4 5-FU marrow, respectively, were found in the fraction containing the 2% of cells expressing the highest levels of H-2Kb, maximum enrichment of CFU-GEMM (7.7-fold and 1.7-fold in the experiment shown in Fig 4) was obtained in this fraction. In contrast, CFU-GM and BFU-E numbers were not significantly enriched in this fraction, although some selection in favor of CFU-GM of higher proliferative potential (ie, yielding colonies containing > 5,000 cells) was noted.

Characterization of cells with competitive long-term repopulating ability. Each of the four types of analyses performed using assays for clonogenic cells to identify primitive hematopoietic cells was repeated using the competitive repopulation assay as follows: \( 10^4 \) male cells from each sorted fraction were injected with \( 2 \times 10^5 \) compromised female marrow cells (obtained as described in the Materials and Methods section) into each of two irradiated female recipients. Five weeks later, the repopulated marrows, spleens, and thymuses of the recipients were individually analyzed for the presence of male cells. The results obtained with the various fractions of the FLS profile of day 4 5-FU marrow showed that cells with competitive long-term repopulating ability were present in all fractions above channel 90, whereas fractions of smaller cells (channels 31 through 90) were relatively depleted of this activity. The FLS fractionation results for normal marrow were inconclusive. Similar analysis of the OLS profile of day 4 5-FU marrow showed that competitive long-term myeloid and lymphoid repopulating cells were maximally enriched in the fraction gated between channels 61 and 150. For normal marrow, these cells, like in vitro clonogenic cells, appeared to have lower OLS properties and were concentrated in the window between channels 31 and 90. Competitive repopulating hematopoietic cells in both normal and day 4 5-FU marrow were concentrated in the fraction corresponding to low Thy-1.2 expression (channels 121 through 150, although some of these cells do appear to be Thy-1.2 negative, channels 91 through 120), and in the fraction corresponding to the 2% to 5% of cells expressing the highest levels of H-2Kb. An example of the data obtained in these experiments is shown for the OLS analysis (Fig 5).

Fig 4. Representative H-2Kb fluorescence profiles of normal and day 4 5-FU marrow cells. (A) Total nucleated cells in day 4 5-FU marrow: unstained cells (---) (ie, incubated with HFN + R-PE or HFN only); nonspecifically stained cells (· · · · ) (ie, after incubation with monospecific anti-R-PE × anti-R-PE control tetrarmers followed by R-PE); specifically stained cells (——) (ie, after incubation with bispecific anti-H-2Kb × anti-R-PE tetrarmers followed by R-PE). Identical profiles were obtained with normal marrow cells and are not shown separately. (B and C) Frequencies of in vitro clonogenic cells in the 2% to 20% of cells showing the highest level of H-2Kb specific fluorescence.
as follows: intermediate to high FLS (channels 91 through 255), intermediate OLS (channels 31 through 90 for normal marrow and channels 61 through 150 for day 4 5-FU marrow), low Thy-1.2 (channels 121 through 150), and high H-2Kb (top 2% to 5%). Approximately 0.1% of the starting marrow cells (both normal and day 4 5-FU) were found in the window defined by these gates, and from $1.5 \times 10^7$ cells, 4 to $6 \times 10^5$ cells could be routinely isolated in a 1.5- to 2-hour sort. A typical two-dimensional (Thy-1.2 v H-2Kb) contour plot of the distribution of day 4 5-FU marrow cells relative to the sort window is shown in Fig 6. As shown in Table 3, all clonogenic cells were enriched in the sorted population. For day 4 5-FU marrow, enrichment and recovery values for CFU-GEMM and CFU-S (both day 9 and day 12) were similar. For normal marrow, both types of CFU-S showed somewhat lower enrichment and recovery values, suggesting that the gates used in this case were not optimal for selection of these cells. The most likely explanation for this is that the FLS window used was defined exclusively on the basis of preliminary CFU-GEMM enrichment data and excluded cells in the low FLS region in which other investigators have shown some of the CFU-S to be present. 

Because the gates used gave better enrichment of more primitive cells (ie, day 12 CFU-S) from day 4 5-FU marrow, we focused on this population to define more quantitatively the extent to which competitive long-term repopulating cells had been purified. In an initial series of six experiments, 500 sorted male day 4 5-FU marrow cells were injected together with $10^6$ compromised female marrow cells into irradiated female recipients. Subsequent analysis showed that male cells constituted a readily detectable (typically >80%) proportion of the cells in the repopulated marrow, spleen, and thymus in all of >20 such mice killed 30 to 90 days after transplantation, although the proportion of cells in the repopulated myeloid

### Table 3. In Vitro and In Vivo Assayable Clonogenic Cell Content of Marrow Cells Isolated by Four-Parameter Sorting

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>CFU-GEMM</th>
<th>Day 9 CFU-S*</th>
<th>Day 12 CFU-S*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal bone marrow</td>
<td>0.4 ± 0.1 (5)</td>
<td>7 ± 1 (5)</td>
<td>1.3 ± 0.1 (3)</td>
<td>14 ± 6 (5)</td>
<td>9 ± 1 (3)</td>
</tr>
<tr>
<td>Frequency (% purity)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrichment</td>
<td>18 ± 4</td>
<td>19 ± 3</td>
<td>100 ± 28</td>
<td>53 ± 23</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>27 ± 7</td>
<td>14 ± 6</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Day 4 5-FU bone marrow</td>
<td>0.01 ± 0.003 (3)</td>
<td>3 ± 1 (4)</td>
<td>4 ± 1 (3)</td>
<td>10 ± 3 (5)</td>
<td>23 ± 3 (5)</td>
</tr>
<tr>
<td>Frequency (% purity)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Enrichment</td>
<td>2 ± 1</td>
<td>32 ± 14</td>
<td>89 ± 44</td>
<td>93 ± 38</td>
<td>75 ± 27</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>0.4 ± 0.2</td>
<td>9 ± 4</td>
<td>24 ± 8</td>
<td>25 ± 10</td>
<td>20 ± 7</td>
</tr>
</tbody>
</table>

Gate selection was as described in the text. Values are mean ± SEM of values from (n) different experiments.

*Assuming a seeding efficiency to the spleen, f, of 5% for antibody-labeled cells (based on experiments described in the Materials and Methods section).
Fig 7. Demonstration by Southern analysis of male DNA in bone marrow (b), spleen (s), and thymus (t) of female mice transplanted with 500 double stained male day 4 5-FU marrow cells isolated by four-parameter sorting (as described in the text) together with 10⁶ compromised female marrow cells. Recipients were killed 30 (A, B, D, E) or 90 (C, F) days after transplantation.

and lymphoid tissues that originated from the 500 sorted male cells did show some variation both within and between mice in different experiments. Results for six mice from two experiments are shown in Fig 7.

To obtain a more precise measure of the frequency of repopulating cells in the sorted day 4 5-FU marrow cell population, groups of irradiated mice were injected with 2 x 10⁵ compromised female marrow cells and decreasing numbers of sorted male cells, and then killed 35 days later for analysis. The results, shown in Fig 8, indicate that 100 sorted male day 4 5-FU marrow cells were sufficient to generate ≥ 50% of the cells present in the repopulated marrow of 50% of the recipients, whereas 1,450 unstained, unseparated day 4 5-FU cells were required to achieve the same result. From these data, the frequency of competitive long-term repopulating stem cells in the sorted day 4 5-FU marrow population was calculated to be 1 in 170 cells (95% confidence limits: 1 in 90 to 1 in 300). By comparison, the frequency of competitively

Fig 8. Frequency of recipients (eight animals per group) showing ≥50% repopulation of the marrow with male cells 35 days after transplantation of varying numbers of male day 4 5-FU marrow cells before (O) or after (•) staining and four-parameter sorting, together with 2 x 10⁵ compromised female marrow cells. The proportion of male DNA was determined by Southern analysis of 10 μg PvuII digested marrow cell DNA from each recipient using pY2 as described. "Projected" frequency for the same sorted cells assuming a 50% loss in vivo due to antibody staining (— — —) (Table 2).
repopulating cells in the unstained, unseparated cell suspension was 1 in 2,300 cells (95% confidence limits: 1 in 1,100 to 1 in 4,600). Assuming a 50% loss of stem cells in vivo due to the staining procedure alone (Table 2), the enrichment obtained is ~28-fold. The recovery of competitive repopulating cells based on these data was calculated to be 7.4%.

DISCUSSION

In this study, we first analyzed in vitro clonogenic cells present in normal marrow and marrow from 5-FU-treated mice with respect to four unique parameters that can be discriminated using a single-laser FACS. Cells capable of generating macroscopically visible multilineage colonies (called CFU-GEMM) were maximally enriched (100-fold) in a fraction of normal marrow cells that showed intermediate to high FLS and low to intermediate OLS ("the blast window") and that bound low levels of anti-Thy-1 antibodies and very high levels of anti-H-2K antibodies. Previous studies of these progenitors of large mixed colonies present in normal marrow and marrow from 5-FU-treated mice have shown that some have a significant capacity for self-renewal and for generating day 9 CFU-S,19,21,26 suggesting overlap with cells detected as day 9 or day 12 CFU-S. This finding is further supported by the present finding that both types of CFU-S were copurified in fractions maximally enriched in CFU-GEMM. However, in the case of normal marrow, both recovery and enrichment of day 9 and day 12 CFU-S in the sorted population was somewhat lower than for CFU-GEMM, suggesting that the cells defined by these operational criteria may include mutually exclusive subpopulations. Other investigators have suggested that day 12 CFU-S in normal marrow are heterogeneous with respect to their uptake of rhodamine and that this allows distinction of a subset of cells able to promote the 30-day survival of lethally irradiated mice.31 Some CFU-S also exhibit a lower FLS27,28 than that which we selected. These and our data provide additional support for the concept of heterogeneity in the CFU-S population.

The concentration of primitive cells in the marrow of mice pretreated with 5-FU was two- to threefold higher than that in normal marrow. Because of this and the increased power of CFU-GEMM light-scattering properties to predict the distribution of more primitive hematopoietic cells in 5-FU-treated marrow, the day 12 CFU-S content of sorted day 4 5-FU marrow was significantly higher than that of sorted normal marrow (Table 3). As reported by other researchers,21 labeling of cells with antibody resulted in an apparent twofold reduction in stem cell numbers detected by in vivo assays (Table 2). Assuming that this resulted in a seeding efficiency of 5% for CFU-S, one in four cells in the sorted day 4 5-FU marrow population is intrinsically capable of forming a macroscopic spleen colony visible 12 days after injection.

Long-term repopulation of mice could be reproducibly achieved with as few as 500 stained, sorted day 4 5-FU marrow cells despite the requirement to compete with a compromised but protective graft of female marrow cells, and this could be demonstrated in a proportion of mice receiving as few as 50 of these cells (Fig 8). Limiting-dilution analysis indicated that the frequency of long-term repopulating cells detectable in this stringent competitive assay was approximately one in 170 of the stained, sorted day 4 5-FU marrow cell population. However, this assumes no cell loss during the transplantation process and a 100% efficiency of activation of biologically comparable stem cells in the presence of the cotransplanted compromised marrow cells. Assessment of either of these assumptions is difficult, but both may lead to a considerable underestimation of the actual content of stem cells with competitive long-term repopulating potential. With respect to the former, we already showed a 50% loss due to staining alone (Table 2), indicating a real purity of at least one in 85. Little is known about the mechanisms that determine which and how many stem cells are recruited in a transplant recipient, but this may be influenced by the total number of cells injected, resulting in a relatively greater competitive pressure in recipients injected with decreasing numbers of test cells and a fixed number of compromised cells.

Nevertheless, the large discrepancy between the recovery and purity of the day 12 CFU-S and cells detected in the competitive repopulation assay suggests that these are not identical populations even in day 4 5-FU marrow. Since the compromised marrow used as a source of competing cells contained near normal numbers of day 12 CFU-S (Table 1), in some situations the capacity for forming day 12 spleen colonies clearly can be biologically separated from the properties that confer a high level of competitive repopulating potential. We recently showed, using appropriate genetic markers, that the compromised cells contribute detectably, although at low levels, to the regenerated hematopoietic populations (unpublished observations). Additional experiments to compare the frequencies of purified repopulating cells assayed under different conditions and at later times should help to clarify further the extent of heterogeneity that may exist among cells with stem cell potential and to devise additional strategies to permit their differential isolation and detection.

Our results show that a population of very primitive hematopoietic cells can be obtained by a simple, single-step isolation procedure, although this population is probably not yet as homogeneous as that which can be obtained by more complicated procedures that may require a dual-laser FACS.28,31 Although we, like Spangrude et al,28 demonstrated the presence of both lymphoid and myeloid repopulating cells in the cell suspensions isolated, direct evidence that these are dual functions of a single stem cell type has not yet been provided. Preliminary experiments in this laboratory using retrovirus-mediated gene transfer to uniquely mark sorted day 4 5-FU marrow cells sorted as we described indicate that the competitive repopulation assay may detect such a cell. Extension of this approach should therefore be an important avenue to further analysis of hematopoietic cells with stem cell properties.

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Isolation in a single step of a highly enriched murine hematopoietic stem cell population with competitive long-term repopulating ability

SJ Szilvassy, PM Lansdorp, RK Humphries, AC Eaves and CJ Eaves