Isolation and Functional Properties of Murine Hematopoietic Stem Cells that are Replicating In Vivo

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Summary

Hematopoietic stem cells (HSC) are multipotent cells that reside in the bone marrow and replenish all adult hematopoietic lineages throughout the lifetime of the animal. While experimenting with staining of murine bone marrow cells with the vital dye, Hoechst 33342, we discovered that display of Hoechst fluorescence simultaneously at two emission wavelengths revealed a small and distinct subset of whole bone marrow cells that had phenotypic markers of multipotential HSC. These cells were shown in competitive repopulation experiments to contain the vast majority of HSC activity from murine bone marrow and to be enriched at least 1,000-fold for in vivo reconstitution activity. Further, these Hoechst-stained side population (SP) cells were shown to protect recipients from lethal irradiation at low cell doses, and to contribute to both lymphoid and myeloid lineages. The formation of the Hoechst SP profile was blocked when staining was performed in the presence of verapamil, indicating that the distinctly low staining pattern of the SP cells is due to a multidrug resistance protein (mdr) or mdr-like mediated efflux of the dye from HSC. The ability to block the Hoechst efflux activity also allowed us to use Hoechst to determine the DNA content of the SP cells. Between 1 and 3% of the HSC were shown to be in S-G_2M. This also enabled the purification of the G_0-G_1 and S-G_2M subsets of fresh purified HSC. Transplantation of these subsets of HSC revealed that S-G_2M HSC had a reconstitution capacity equivalent to quiescent stem cells. These findings have implications for models of hematopoietic cell development and for the development of genetic therapies for diseases involving hematopoietic cells.

Hematopoietic stem cells (HSC)[1] are multipotent cells that reside in the bone marrow and give rise to all adult hematopoietic lineages of mice. HSC are thought to be inherently quiescent, dividing little or not at all until required to differentiate, yet capable of self-renewal. Probably the best evidence in support of the self-renewal of stem cells comes from studies that have examined the recovery of mouse bone marrow after repeated ablation with cytotoxic agents (1) as well as from retroviral marking studies (2). Substantial evidence also exists that suggests that the capacity for self renewal of stem cells may be limited. Serial transplantation of either whole bone marrow or highly purified HSC results in a loss of multilineage reconstituting capacity (3–8). This loss of HSC function could be due to effects of the transplantation process itself, or to a lack of regeneration of HSC after proliferation. The inability to establish in vitro culture conditions that result in the significant expansion of stem cells able to engraft lethally irradiated recipients may also indicate that the self-renewal capacity of stem cells is limited.

Resolution of the issue of the capacity of HSC for self-renewal may be facilitated through an examination of the functional properties of HSC that have undergone a cycle of cell division. We sought to address this issue through the physical isolation of live HSC that were undergoing cell division, and by comparison of the engraftment of the cycling cells to that of quiescent HSC by long-term bone marrow transplantation in mice. Toward this end, we attempted to use the fluorescent vital dye Hoechst 33342, which binds DNA in live cells, and is therefore an indicator of cell cycle as it relates to DNA content (9), to isolate replicating and quiescent cell populations. In preliminary studies, however, we found that both unfractionated and enriched bone marrow populations yielded an uninterpretable DNA staining profile. In the course of those studies, we observed a complex fluorescence pattern of Hoechst dye in

[1] *Abbreviations used in this paper:* CFU-s(12), day 12 spleen colony-forming activity; HSC, hematopoietic stem cell(s); mdr, multidrug resistance protein; PI, propidium iodide; Rh123, Rhodamine-123; SP, side population.

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whole bone marrow when Hoechst fluorescence was displayed simultaneously at two emission wavelengths. This led us to identify a small subset of Hoechst-staining cells that were well separated from the rest of the bone marrow, contained a majority of cells that had cell surface markers of HSC, and were enriched for HSC activity at least 1,000-fold. We show that this distinct staining pattern by the HSC is due to a high level of dye efflux activity that may be mediated by *p*-glycoprotein or a related molecule. We have used this enrichment strategy and associated observations to further develop a method to purify subsets of HSC that are in G_0-G_1 or in S-G_2M and to show that these populations appear to have equivalent abilities to reconstitute the hematopoietic system of lethally irradiated recipients.

Materials and Methods

Mouse Strains. Mice were obtained at 5–8 wk of age and were used between 6 and 12 wk of age. C57Bl/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and C57Bl/6-Ly-5.1 mice were obtained from the National Cancer Institute (Bethesda, MD). All mice were maintained on acidified water.

Preparation of Bone Marrow Cells for FACS®. Murine bone marrow was extracted from the femurs and tibias of C57Bl/6 mice, a single cell suspension was made by passage of the bone marrow through an 18-gauge needle, and the cells were pelleted by centrifugation. The bone marrow cells were resuspended at 10^6 cells per ml in prewarmed DMEM containing 2% FCS, 1 mM Hepes, penicillin, streptomycin, and 5 μg per ml Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) and were incubated for 90 min at 37°C. When verapamil was used, bone marrow was stained as described in the presence of 50 μM verapamil (Sigma Chemical Co.). The resolution of the various Hoechst populations is highly sensitive to the staining time and the Hoechst dye concentration (10). After Hoechst staining, cells were pelleted and maintained at 4°C before antibody staining or FACS® analysis (Becton Dickinson & Co., Mountain View, CA). For antibody staining, Hoechst-stained bone marrow was suspended in HBSS containing 2% FCS, 1 mM Hepes, penicillin, and streptomycin (HBSS+) at 10^8 cells per ml. The antibodies were added at 1/50 to 1/100 dilutions. The lineage cocktail used was comprised of the following: CD4 (GK1.5, Becton Dickinson & Co.); CD8 (53-6.7, Becton Dickinson & Co.); CD5 (53-7.3; PharMingen, San Diego, CA); B220 (RA3-6B2; Caltag Laboratories, South San Francisco, CA); Mac-1 (M1/70.15, Caltag Laboratories); and Gr-1 (RB6-8C5, PharMingen). The mixture was incubated on ice for 10 min, the bone marrow was then washed once in excess HBSS+ and the cells were pelleted through a serum cushion. All washes were performed in this manner. The cells were resuspended in media containing goat anti–rat antibody conjugated to PE (mouse serum adsorbed; Caltag Laboratories), and incubated for 10 min on ice. After washing, the cells were resuspended in 1/3 volume rat serum (Cappel Laboratories, Cochranville, PA) and 2/3 HBSS+. After 10 min on ice, biotinylated Sca-1 antibody (E13 161-7) was added for 10 min on ice. After washing, the cells were stained with avidin-FITC (Becton Dickinson & Co.) for 10 min on ice. After the final wash, the bone marrow cells were filtered through a 70-μM nylon filter (Falcon Plastics, Cockeysville, MD) and resuspended in HBSS+ containing 2 μg/ml propidium iodide (PI). For sorting experiments, the bone marrow was sometimes magnetically preenriched for Sca-1^+ cells using the MACS® (Miltenyi Biotec, Sunnyvale, CA) and streptavidin microbeads. This resulted in a 5–10-fold enrichment for Sca-1^+ cells.

Flow Cytometry. Analysis and sorting were performed on a dual-laser FACStar Plus® flow cytometer (Becton Dickinson & Co.). The Hoechst dye was excited at 350 nm and its fluorescence was measured at two wavelengths using a 450 BP 20 (450/20 nm band pass filter) and a 675 EFLP (675 nm long pass edge filter) optical filter (Omega Optical Inc., Brattleboro, VT). A 610 DMSP (610 nm short pass dichroic mirror) was used to separate the emission wavelengths. PI fluorescence was also measured through the 675 EFLP (having been excited at 350 nm). Hoechst "blue" represents the 450 BP filter, the standard analysis wavelength for Hoechst 33342 DNA content analysis. Cells positive for PI were seen on the far right of the Hoechst "red" (675 EFLP) axis shown in Fig. 1 *A*, and excluded. The addition of PI did not affect the Hoechst staining profile, but allowed exclusion of dead cells. Both Hoechst blue and red fluorescence are shown on a linear scale. The gating on forward and side scatter was not stringent; only erythrocytes and debris were excluded. Reanalysis of sorted populations showed purity >98%. Cells were sorted into glass tubes containing 100% FCS, and an aliquot was removed at the end of the sort and reanalyzed to establish high purity. The side population (SP) sorting gates were established as follows. A live gate was defined on the flow cytometer using Hoechst red and blue axes to exclude dead cells, red cells (no Hoechst stain), and debris. After collecting 10^5 events within this live gate, the SP population was able to be clearly defined. A new gate was established on this population, as shown boxed in Fig. 1 *A*. After the SP region was defined, this region was used as a live gate to display the Sca-1^+ and lineage marker fluorescence of this subset of cells. The Sca-1^+ $lin^{neg/low}$ population stands out clearly with this gate. A sorting gate was then defined as cells that fell into both of the regions boxed in Fig. 1, *A* and *B*.

Transplantation. Typically, bone marrow was extracted from femurs and tibias of several male 6–10-wk-old C57Bl/6-Ly-5.1 mice, stained and purified as described above. Sorted cells were washed, counted, and mixed (in competitive repopulation experiments) with unfractionated bone marrow obtained from two male 6–10-wk-old C57Bl/6-Ly-5.2 mice, and the mixtures were introduced into female C57Bl/6-Ly-5.2 recipient mice (all from the Jackson Laboratory). The recipients were 6–12-wk-old and were maintained on acidified water. The recipients were irradiated with 1,100 rad given in two doses (620 and 480 rad) at least 2 h apart. Bone marrow cells were given intravenously by retro-orbital injection under methoxyflurane anesthetic (Pittman Moore, Mundelein, IL). All animal care was in accordance with institutional guidelines.

Analysis. Peripheral blood was taken from transplant recipients by retro-orbital puncture under methoxyflurane anesthesia 4 mo or more after transplant. Red blood cells were removed and the nucleated peripheral blood cells were stained with biotinylated anti-Ly-5.1 antibody that was detected with streptavidin-PE (Molecular Probes, Inc., Eugene, OR). For lineage analysis, the blood samples were subdivided and costained with directly conjugated lineage-specific antibodies against Thy-1, B220, Gr-1, and/or Mac-1 (PharMingen). These blood samples were analyzed on the FACStar Plus® or a FACScan® flow cytometer (Becton Dickinson & Co.). The anti-Ly-5.2 and anti-Ly-5.1 hybridomas used were 104.2.1 and A20.1.7, respectively (gifts from D. Pardoll, Johns Hopkins University, Baltimore, MD).

Separation of G_0-G_1 from S-G_2M Stem Cells. SP cells were purified from 10 mice as described above. An aliquot of these cells

was removed and transplanted as the "1× sorted" group in Table 4, column 1. The rest of the sorted cells were incubated for 60 min in DMEM as described above, containing 10 μg/ml Hoechst 33342 and 50 μM verapamil. These were resuspended in HBSS including 2 μg/ml of PI, and sorted on the flow cytometer as described above. Several thousand cells were first allowed to pass through the laser without subfractionation. These were transplanted as the "2× sorted" group. The remaining cells were sorted into 2n (G_0-G_1) or >2n (S-G_2M) groups and transplanted accordingly. For PI analysis, stem cells purified as described above were pelleted by centrifugation and resuspended in 0.1% Na citrate, 50 μg/ml PI. After incubation on ice for 10 min, the cells were analyzed by standard flow cytometry procedures using 488-nm excitation.

Results

A Distinct Population of Hoechst 33342–stained Cells Is Sca-1$^+$ Lin$^{neg/low}$. Hoechst 33342 is a fluorescent dye readily taken up by live cells where it binds to DNA. The quantity of Hoechst 33342 fluorescence in a cell is therefore an indicator of cell cycle as it relates to DNA content. However, when it was used to examine DNA content in whole murine bone marrow by standard methods (11), a complex fluorescence pattern was observed. When dye fluorescence was observed simultaneously at two emission wavelengths (red and blue), several distinct populations could be resolved (Fig. 1 *A*). These populations appeared to represent multiple cell cycle profiles that are shifted relative to one another with regard to red and blue Hoechst fluorescence.

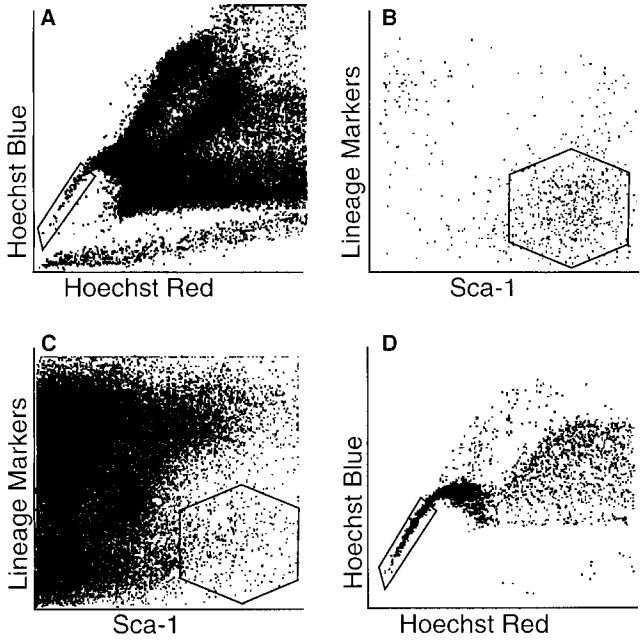

Figure 1. Characterization of Hoechst 33342 fluorescence on whole murine bone marrow. (*A*) Hoechst fluorescence on whole normal bone marrow. The boxed region is the SP region and represents 0.1% of the total bone marrow. (*B*) Sca-1$^+$ and lineage marker profile of cells that fall exclusively into the region indicated in *A*. The percentage of cells that fall in the indicated window is 75% in this example, and ranges from 75 to 90% depending on precisely where the gate in *A* is drawn. (*C*) Sca-1 and lineage marker fluorescence on whole murine bone marrow. The indicated region is identical to that in *B* and contains 1% of the nucleated cells in the bone marrow. (*D*) Hoechst fluorescence of the cells from whole bone marrow that fall into the region indicated in *C*. The SP region drawn is identical to that in *A*, and 10% of the cells fall into this region.

In the course of our analysis of hematopoietic cells in this staining profile, we observed that one population of cells, boxed in Fig. 1 *A* (SP), was predominantly Sca-1$^+$ lin$^{neg/low}$ Fig. 1 *B* displays the Sca-1$^+$ and lineage marker expression of the cells from whole bone marrow that fall into the region boxed in Fig. 1 *A*. The region boxed in Fig. 1 *B* contains cells that express Ly-6A.2 (Sca-1$^+$), and a low level of six lineage antigens (detected by a cocktail of antibodies against B220, Gr-1, Mac-1, CD4, CD5, and CD8) and represents 75% of the cells displayed. These cell surface characteristics have previously been used to identify murine HSC (12–14). The SP population boxed in Fig. 1 *A* represents 0.1% of total bone marrow. Fig. 1 *C* shows that 1% of whole unfractionated bone marrow are Sca-1$^+$ lin$^{neg/low}$ cells when using the region defined in Fig. 1 *B*. Fig. 1 *D* shows that SP cells comprise a subset (~10%) of this 1% fraction of Sca-1$^+$ lin$^{neg/low}$ cells from whole bone marrow.

Competitive Repopulation with SP Cells. To examine the functional properties of the SP cells, long-term bone marrow transplantation experiments were performed. We used a competitive repopulation assay in which defined numbers of purified cells were transplanted into lethally irradiated recipients along with unfractionated but distinguishable whole bone marrow cells. In this semiquantitative method (15, 16), the relative stem cell activity of sorted vs. unfractionated cells was assessed by determining the percentage of peripheral blood cells derived from each of the two input populations in the bone marrow transplant recipients. These recipients were analyzed 4 mo after transplant when the majority of peripheral blood cells are derived from long-term reconstituting stem cells introduced in the transplant instead of committed progenitors (2). In addition, differentiated cells and progenitors in the unfractionated competitor bone marrow rescue recipients from the otherwise lethal irradiation. This allows the long-term multilineage repopulating functions of the input HSC to be examined separately from short-term activities. For this competitive repopulation assay, we used congenic C57Bl/6 mouse strains that differ at the Ly-5 locus (17). This antigen is found on the surface of all nucleated peripheral blood cells and the two allelic variants are readily distinguished by specific monoclonal antibodies. Thus peripheral blood can be collected from transplant recipients at multiple time points and assayed by flow cytometry for the proportion of the two Ly-5 alleles present in each blood lineage.

Data from two competitive repopulation experiments with fractionated cells are shown in Table 1. In the first example, the following three populations of cells were tested for stem cell activity: (*a*) SP cells; (*b*) cells that fell distinctly outside of the SP region (*NOT SP*); and (*c*) cells that are part of the SP region, but would be contaminated by non-SP cells (*Upper SP*). In this experiment, in order to provide large amounts of starting material, bone marrow cells were

Table 1. *Competitive Repopulation with Hoechst-purified SP Cells*

Fraction	No. sorted Ly-5.1 × 10^{-3}	No. compet. Ly-5.2 × 10^{-3}	*n*	Mean percent Ly-5.1 PBL	SD	Enrichment
				%		
Experiment 1						
NOT SP	20	500	5	8	3	2
Upper SP	8.2	500	5	31	11	28
SP	2	1,000	6	56	5	640
SP	1	1,000	7	41	5	700
SP	0.5	1,000	7	30	8	960
Experiment 2						
SP	1.0	200	5	79	11	720
SP	0.3	200	4	54	14	780
SP	0.1	200	4	43	3	1,500
SP	1.0	400	4	52	2	430
SP	0.3	400	3	26	9	470
SP	0.1	400	5	29	15	1,600

The first column displays the fraction of cells purified by flow cytometry (C57Bl/6-Ly-5.1 derived). The second column displays the number of purified cells transplanted. The third column displays the number of competitor cells introduced (C57Bl/6-Ly-5.2 derived). *n* represents the number of transplant recipients at the time of analysis (usually the same as the number initially transplanted) in each group. The mean is the mean number of nucleated peripheral blood cells derived from the sorted population (Ly-5.1$^+$) present in recipients 4 mo after transplant. The enrichment is calculated as the mean percent Ly-5.1$^+$ cells in the peripheral blood per purified cell introduced, divided by the mean percent contribution of Ly-5.2$^+$ cells per unfractionated Ly-5.2 cell introduced. All numbers are rounded to two significant digits.

preenriched for Sca-1$^+$ cells, which have been shown to mark all stem cells in these mice and provide a 7–10-fold enrichment for HSC activity (13, 14, 18, and data not shown). Preenrichment with Sca-1$^+$ was not necessary, however, for obtaining the enrichments observed. As shown in Table 1, the SP fraction was highly enriched for stem cell activity. In contrast, there is little detectable activity in the NOT SP fraction and only a small amount of activity in the upper SP fraction. Numerous other competitive repopulation experiments with or without Sca-1 enrichment revealed almost no additional stem cell activity outside of the SP region (data not shown). In the second experiment shown in Table 1, three doses of SP cells were tested against two doses of competitor cells. The SP cells gave rise to average enrichments of HSC activity of ~1,000-fold in these and other experiments. Since the SP cells represent 0.1% of the total bone marrow, these data are also consistent with the above finding that most if not all reconstituting cells in normal mouse bone marrow reside in the SP fraction.

Radioprotection and Spleen CFU Content of SP Cells. Having established by competitive repopulation that all stem cell activity fell into the SP region, we investigated the ability of these cells to protect recipients from lethal irradiation (radioprotection). Varying doses of purified SP cells were transplanted into lethally irradiated recipients without competitor bone marrow. 4 mo after transplant, peripheral blood was collected and analyzed for donor (Ly-5.1) contribution. As shown by data from three separate experiments in Table 2, on average, ~150 purified cells radioprotected 50% of recipients. The mean contribution of the SP cells to hematopoiesis in the peripheral blood of survivors was ≥81% except in one animal transplanted with 50 cells, where the PBL contribution was 12%. Radioprotection by whole unfractionated bone marrow is also shown. Approximately 5 × 10^4 whole bone marrow cells will reproducibly protect ~50% of recipients from lethal irradiation. This constitutes an enrichment of radioprotection activity of ~300-fold.

Day 12 spleen colony-forming activity, or CFU-s(12), of the SP cells was also measured. In three separate experiments, we found an average of about 1 CFU-s(12) per 35 SP cells injected (5.5 ± 1.7 per 200 cells injected, $n = 18$; 6.1 ± 1.6 per 200 cells, $n = 9$; and 7.1 ± 1.8 per 250 cells, $n = 11$). This compares with 1.3 CFU-s (12) per 10^4 whole bone marrow cells (6.6 ± 1.3 per 5 × 10^4 cells injected, $n = 8$). These data indicate that CFU-s activity is enriched ~200-fold in the SP population.

Multilineage Contribution by SP Cells. The SP cells were also shown to contribute to both lymphoid and myeloid lineages in the transplant recipients. Table 3 shows a lineage analysis 4 mo after transplant from the third experiment shown in Table 2. For each group, the mean percentage of peripheral blood cells with the given marker is indicated. It is evident that in all of the surviving recipients, there was a very consistent number of Thy-1, B220, and Gr1 positive cells, indicating no major hematopoietic anomalies in the transplant recipients. The percentage of each marker subset derived from the purified cells is also indicated. Except in

Table 2. *Radioprotective Ability of Purified SP Cells*

	No. sorted Ly-5.1	*n*	Percent survival	Mean percent Ly-5.1 PBL	SD
Purified SP Cells			%		
Experiment 1					
	25	10	0	0	–
	50	10	10	12	–
	100	20	25	81	11
	200	10	80	87	10
	500	10	70	89	6
Experiment 2					
	25	20	4	1	–
	50	19	0	0	–
	100	29	24	83	18
	200	20	60	89	6
Experiment 3					
	0	15	0	0	–
	100	20	40	85	16
	250	20	45	95	4
	500	20	70	97	1
	1,000	19	68	97	1
Whole bone marrow ($\times 10^{-3}$)	50	8	50	76	31
	100	9	88	92	4
	500	9	100	94	1
	1,000	10	100	94	1

Results from three independent experiments are shown. The indicated numbers of purified HSC (male C57Bl/6-Ly-5.1 derived, Hoechst SP, Sca-1$^+$, lin$^{neg/low}$) cells were introduced into female C57Bl/6-Ly-5.2 recipients as described in the legend to Table 1. The number of animals initially transplanted in each group is shown in the second column (*n*). The percentage of animals surviving at least 4 mo after transplantation is shown in the third column. The mean percent SP cell–derived Ly-5.1 nucleated peripheral blood cells in the transplant recipients 4 mo after transplant is shown in column four. All numbers are rounded to two significant digits.

some of the animals surviving with only 100 purified cells, where some host-derived contribution was evident, all the lineages are overwhelmingly derived from the purified cells.

Verapamil Blocks Formation of the SP region. HSC activity has been seen in Hoechst "low" staining regions before (see Discussion), but since we had found the stem cell activity in such a specific staining region, we wondered if it would be possible to account for the staining pattern. It is known

Table 3. *Lineage Analysis of SP Cell Contribution*

			Percent PBL with given marker			Percent of each marker that is 5.1$^+$		
No. SP	*n*	% 5.1 + PBL	Thy-1	B220	Gr1	Thy1	B220	Gr1
100	7	84.7 ± 16.1	33.0 ± 8.1	37.7 ± 9.9	13.1 ± 6.4	86.3 ± 7.5	94.2 ± 13.9	62.3 ± 25.8
250	9	94.7 ± 3.5	27.3 ± 8.2	37.1 ± 6.7	14.5 ± 3.5	85.9 ± 4.0	99.0 ± 1.9	78.8 ± 6.3
500	14	96.5 ± 1.0	29.2 ± 7.0	37.1 ± 7.8	11.9 ± 3.7	89.7 ± 2.0	99.7 ± 0.3	79.3 ± 4.5
1,000	13	97.0 ± 0.7	23.4 ± 4.6	40.7 ± 7.3	11.5 ± 4.3	88.9 ± 1.9	99.8 ± 0.2	81.0 ± 4.3

The analysis presented is from the third radioprotection experiment shown in Table 2. The number of transplanted SP cells is shown in column 1, with the number of recipients analyzed in column 2 (*n*). The mean percent SP-derived PBL is shown in the third column. The next three columns show the means of the proportion of cells in the peripheral blood displaying the indicated cell surface markers. These contain both donor and host-derived cells. The final 3 columns display the proportion of cells that display the indicated marker and also the Ly-5.1 marker, indicating that they are SP cell derived.

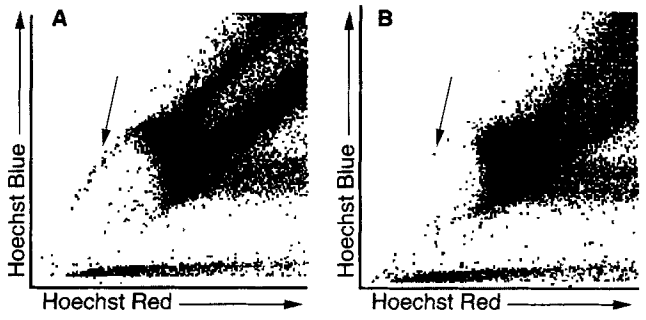

Figure 2. Inhibition of mdr activity with verapamil. (*Arrows*) SP region that contains the stem cells. (*A*) Whole bone marrow stained with Hoechst 33342. (*B*) Whole bone marrow stained with Hoechst 33342 in the presence of verapamil.

that several vital dyes are actively pumped out of cells by the multidrug resistance protein (mdr, or *p*-glycoprotein), so we investigated the possibility that the low Hoechst fluorescence was due to higher mdr activity in murine HSC. As shown in Fig. 2, the stem cell (SP) population was specifically eliminated when bone marrow cells were stained with Hoechst 33342 in the presence of the drug verapamil, an inhibitor of mdr. Therefore, it is likely that the cells are uniquely low in Hoechst fluorescence because of mdr or a mdr-like–mediated efflux of the dye.

G_0-G_1 and S-G_2M Cells Contribute to Long-Term Reconstitution. In whole bone marrow or in the Sca-1^+ $lin^{neg/low}$ cell populations, the efflux of Hoechst dye prohibits its use as an indicator of cell cycle. However, we reasoned that the ability to specifically block the efflux of the dye in the presence of verapamil should allow full uptake of the dye and appropriate staining of DNA. This should allow cell cycle analysis on live stem cells from either purified or heterogeneous populations of hematopoietic cells. We tested this possibility on purified SP cells. After obtaining SP cells as described above, we restained the sorted cells with Hoechst 33342 in the presence of verapamil. Hoechst fluorescence in SP cells now exhibited a typical cell cycle profile, with the number of cells in S-G_2M between 1 and 3% of the purified cells (Fig. 3 *A*). This figure correlates well with the number shown to be in S-G_2M by PI staining of the purified cells (Fig. 3 *B*). This number is much lower than the

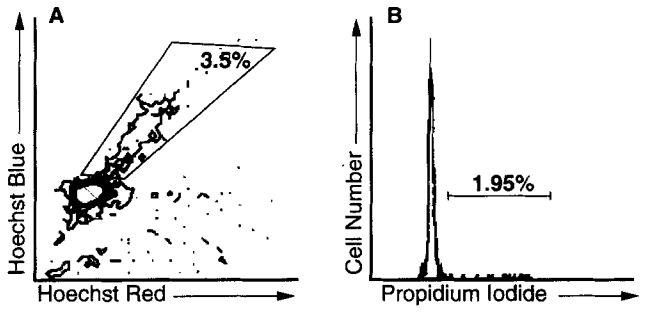

Figure 3. Analysis of the cell cycle status of purified HSC. (*A*) Purified HSC restained with Hoechst 33342 in the presence of verapamil. (*B*) Purified HSC stained with PI.

fraction of cells in S-G_2M in whole bone marrow (20%, data not shown), and in other populations enriched for stem cells (19). Our data, therefore, support the widely held view that HSC from normal bone marrow are largely quiescent (20).

The ability to isolate viable, replicating stem cells made it possible to determine whether the cells were capable of reconstituting lethally irradiated recipients and, if so, to compare their engraftment properties with cells in G_0-G_1. For these studies, the two subsets of the SP population were purified by flow cytometry, and tested in the competitive repopulation assay. Table 4 shows data from one such experiment. The 1× sorted population represents transplantation of an aliquot of cells that have been purified once, and serves as a control for comparison with enrichment activity obtained in other experiments. The 2× sorted population represents SP cells that have been restained with Hoechst in the presence of verapamil, and run through the flow cytometer without subfractionation. A large discrepancy between the activity of the 1× and 2× sorted samples would have indicated possible damage to the stem cells incurred in the second staining or sorting procedure. In fact, the activity of the 1× and 2× sorted populations was very similar. We found that the contribution of the G_0-G_1 HSC to the peripheral blood was almost identical to that of the S-G_2M HSC, and both populations were virtually identical in activity to HSC that have not been subfractionated (1× and 2× sorted HSC). The percentage of peripheral blood cells derived from the sorted cells was almost identical at 2 and 11 mo, indicating the long-term engraftment capacity of both populations of cells.

Discussion

The above studies describe the development of a method for the enrichment of murine HSC that involves a novel use of the vital dye Hoechst 33342 and the use of the method to isolate both quiescent and replicating populations of stem cells. The Hoechst-purified SP cell population described above comprises 0.1% of the bone marrow and is highly homogeneous with respect to cell surface markers found on murine HSC (12–14). Furthermore, the cells are enriched ~1,000 fold for HSC activity, as determined in competitive repopulation experiments, and provide high long-term multilineage contribution in radioprotection experiments.

Numerous strategies for the enrichment or purification of HSC have been described previously (11, 12, 21–24). However, the Hoechst SP region is the first use, to our knowledge, of a single dye or manipulation to define a population with HSC activity that is so strikingly small and homogeneous. Further, the SP region is highly reproducible and easily identifiable, and therefore should be able to be used advantageously by many experimenters. In addition, the SP region can be used to allow independent verification of cell surface characteristics, as shown in Fig. 1 *B*.

Although we have demonstrated a high level of stem cell activity in the competitive repopulation assay, we do not

Table 4. *Competitive Repopulation with G_0-G_1 and S-G_2M SP Cells*

	Group	No. sorted Ly-5.1	No. compet. Ly-5.2 cells × 10^{-3}		Mean percent Ly-5.1 PBL	SD	Enrichment
					%		
At 2 mo	1× sorted	400	400	9	44	10	800
	2× sorted	400	400	7	56	6	1,300
	2× sorted	200	200	6	52	16	1,100
	G_0-G_1	400	400	6	47	9	870
	G_0-G_1	200	200	7	62	17	1,600
	S-G_2M	200	200	5	64	2	1,800
At 11 mo	1× sorted	400	400	7	51	14	1,000
	2× sorted	400	400	6	60	8	1,500
	2× sorted	200	200	6	56	26	1,300
	G_0-G_1	400	400	6	54	7	1,200
	G_0-G_1	200	200	7	60	11	1,500
	S-G_2M	200	200	5	63	20	1,700

One experiment is shown with the analysis performed at both 2 and 11 mo after transplant. The number of purified or subfractionated SP cells (Ly-5.1$^+$) transplanted per mouse is shown in the second column. The number of unfractionated Ly-5.2 competitor bone marrow cells cotransplanted is indicated in the third column, and the number of mice at the time of analysis (*n*) in the fourth column. The mean shown is the mean percentage of Ly-5.1$^+$ nucleated peripheral blood cells at the time after transplant indicated. The enrichment is calculated as in Table 1. All numbers are rounded to two significant digits.

observe substantial radioprotection with very small numbers of cells as has been described with other populations of purified cells (12, 14, 25, 26). This could be interpreted to reflect a lower level of purity of our stem cell population than others, differences in animal husbandry practice, or a lack of inherent radioprotective activity in SP cells, a function that may be very different from long-term reconstitution. Although the SP cells do not exhibit radioprotective activity at small cell doses, they do show very high levels of contribution in almost all of the animals that are rescued. In contrast, Spangrude and Scolly (26) report ~30% radioprotection with 20–33 purified Sca-1$^+$ lin$^{neg/low}$ cells, but the donor contribution in the rescued animals remains very low (3%) until larger numbers of purified cells are introduced (1,000 cells were reported to give an average of 98% contribution). Since SP cells appear to be a subset of Sca-1$^+$ lin$^{neg/low}$ cells, these results indicate that the Hoechst purification strategy may allow at least partial separation of radioprotection activity from long-term reconstitution activity.

In support of this notion, we have compared SP cell radioprotection activity with that of whole bone marrow. On average, we see about 150 SP cells conferring radioprotection to 50% of lethally irradiated transplant recipients. This compares with 5×10^4 whole bone marrow cells conferring 50% radioprotection in the same conditions (Table 2). This is an enrichment of 50% radioprotection activity of ~300-fold. Furthermore, we observe the CFU-s (12) activity to be ~1 per 35 SP cells injected. Compared with the CFU-s (12) content of whole unfractionated bone marrow (~1.3 in 10^4 injected cells), this represents an enrichment for CFU-s (12) of only ~200-fold. Since we observe an enrichment of long-term stem cell activity of ~1,000-fold, this indicates that although there is some radioprotection activity, and some CFU-s (12) in the SP region, the majority of cells with these functions do not appear to be present in this population, even though the majority of long-term reconstitution activity is present.

Previous observations of HSC activity being associated with a low level of Hoechst dye have been made using solely the Hoechst blue wavelength in combination with other sorting parameters (11, 27–29). However, the dual wavelength analysis described here allows an exquisite separation of multiple populations, and clear identification of the discrete SP region that is enriched for stem cell activity at least 10-fold higher than what had previously been achieved using Hoechst 33342. It is possible that using the Hoechst red wavelength alone, the SP region could be identified.

The observation that cells stained with Hoechst 33342 can show distinct patterns of fluorescence when examined at different wavelengths had been made previously, using cell lines and thymus tissue (10, 30). In those studies, as in our own, the fluorescence patterns were shown to be dependent on both the Hoechst concentration and staining times. These findings may be interpreted to reflect, at least in part, the existence of different binding states of the Hoechst dye that occur as a consequence of different chromatin conformations, and that result in different fluorescence spectra from the bound dye. This would potentially give rise to a unique Hoechst "fingerprint" for different cell types that

would reflect the whole set of binding states in a given cell type. In addition to the two to three major populations seen in Fig. 1 *A*, we have observed other minor populations with unique states of red and blue Hoechst fluorescence when the major populations have been removed (data not shown). Whereas this phenomena may account for the distribution of the major bone marrow populations accross the two emission wavelengths as seen in Fig. 1 *A*, it does not explain the uniquely low Hoechst fluorescence exhibited by the SP cells (see below).

The fact that the SP region was so well separated from the rest of the bone marrow cells suggested either that the Hoechst dye was inefficiently taken up by stem cells, or that it was being actively pumped out of the cells. The verapamil-blocking experiment shown in Fig. 2 suggests that it is the efficiency of efflux of the dye that accounts for the uniquely low fluorescence of the SP population. Although verapamil has very broad activities, it is known to inhibit *p*-glycoprotein. Therefore, this very high dye efflux activity which can be blocked with verapamil may be due to a higher level of *p*-glycoprotein on the surface of HSC, a higher level of activity of the *p*-glycoprotein present, or a mdr-like activity not yet identified. Antibody studies on the expression of the product of the *MDR1* gene in human hematopoietic cells suggest that *p*-glycoprotein is in fact expressed quite widely, and that ≤65% of bone marrow cells may express mdr (31, 32). If this is also true in the mouse, then mdr expression alone may not account for the unique Hoechst-staining properties of SP cells. In light of this, a purification strategy based on functional properties, such as the one we have developed, will likely be more powerful than a scheme based on the level of mdr cell surface expression (e.g., antibody based).

Rhodamine-123 (Rh123) is another fluorescent vital dye that has been used to enrich for HSC in combination with other antibody-based approaches (24, 33–35). Like the low Hoechst 33342 staining of HSC, cells that stain with low levels of Rh123 contain the most primitive hematopoietic cells. Although this may be an indicator of low mitochondrial activity (and hence quiescence), Rh123 is also known to be a substrate for mdr (31). It is possible that the utility of both Rh123 and Hoechst 33342 is based on their mdr-mediated efflux from stem cells. Although powerfully used in combination with other approaches, Rh123 alone is not a useful tool for HSC purification, as the stained stem cell population is not distinct from the rest of the bone marrow as is the case with the Hoechst SP. It is possible that Hoechst 33342 is an exceptionally good or specific substrate for mdr, allowing superior resolution of cells with extremely high mdr activity over those with simply high mdr activity. If this is indeed the case, other fluorescent non-DNA–binding substrates for mdr may be able to be identified that allow an equally powerful purification. The $Rh123^{low}$ subset shown to contain the most primitive stem cells has been defined as the 10% of Sca-1^+ $lin^{neg/low}$ Thy-1.1^{low} cells that contain the lowest amount of Rh123 after staining (33, 34). Hoechst SP cells also comprise a 10% subset of Sca-1^+ $lin^{neg/low}$ cells. The 12-d spleen colony formation of the SP population is ~1 CFU-s(12) per 35 purified cells injected. This frequency correlates extremely well with the CFU-s(12) forming frequency of the $Rh123^{low}$ subpopulation of Sca-1^+ $lin^{neg/low}$ Thy-1.1^{low} cells (7, 33). We suspect that these populations will prove to be very similar.

Although self-renewal is a property traditionally associated with HSC (20), the ability of stem cells to proliferate without concomitant differentiation has not been formally demonstrated. The ability to block Hoechst efflux in SP cells with verapamil allowed us to examine DNA content with Hoechst 33342 and thereby physicially isolate live stem cells that had undergone or were undergoing DNA replication and mitosis. As shown in Fig. 3, purified SP cells restained with Hoechst in the presence of verapamil exhibit a typical cell cycle profile, with ~3% of the cells in S-G_2M. This is very similar to the percentage obtained with PI, and considerably lower than the number in S-G_2M in whole bone marrow (~20%). This supports the notion that HSC are largely quiescent. Our long-term reconstitution studies involving S-G_2M and G_0-G_1 fractions of stem cells clearly show that both fractions are equally competent for reconstitution. These results strongly suggest that entry of stem cells into cycle does not necessarily lead to an absolute loss of self-renewal or reconstitutive activity. Whether or not the S-G_2M fraction of cells isolated by our procedure represents cells that are continuously in cycle or simply cells that are captured in a rare state of replication remains to be determined. It will be interesting to determine whether quiescent and dividing stem cells from normal mice will possess distinct functional properties as revealed by specific assays such as secondary transplantation or growth factor responsiveness.

It is noteworthy that Fleming et al. (25) also compared the reconstitution potential of G_0-G_1 and S-G_2M fractions of Sca-1^+ $lin^{neg/low}$ Thy1.1^{low} stem cells from normal mice, using Hoechst 33342 staining in the absence of verapamil. In contrast to our results, they observed 18% of the cells to be in S-G_2M, and reported a lower reconstitution capacity of this subset of cells relative to G_0-G_1 cells. However, as shown above, Hoechst fluorescence in HSC is dramatically influenced by mdr-like efflux activity, unless the efflux activity is blocked with inhibitors such as verapamil. Consequently, the Hoechst content of the HSC analyzed by Fleming et al. (25) would be lower than expected and it is likely that a significant proportion of cycling HSC would be found in the G_0-G_1 peak of their staining profile (and perhaps below it). Therefore, the reconstitution potential of the cycling fraction was probably underestimated.

In summary, we have developed a novel approach to the enrichment of murine HSC. The stem cell activity has been defined solely on the basis of its ability to contribute to all peripheral blood lineages in long-term bone marrow reconstitution of lethally irradiated recipients. The dye-based fractionation allows independent determination of the cell surface characteristics of HSC, a feature of the purification method that may be particularly useful for characterizing the surface phenotype of stem cells from other species (see below). We have also shown that this purification strategy

may rely on a high level of mdr-like activity present in stem cells. This finding allowed us to establish a strategy for separating cycling from quiescent stem cells, to show that cycling cells capable of the long-term engraftment of lethally irradiated recipients exist (and can be physically isolated), and to show that their reconstituting capacities are equivalent to those of quiescent stem cells.

The stem cell purification strategy described above may be able to be extended to other species. In particular, we have stained human adult bone marrow, human umbilical cord blood, and porcine bone marrow with Hoechst 33342 and observe a staining pattern remarkably similar to that which we observe with murine cells (data not shown). We are currently characterizing stained SP cells from human bone marrow with respect to cell surface markers found on human progenitor cells (36–39) and in in vitro culture assays. It will be of considerable interest to determine whether the same markers defined through studies of the progenitor activities of human cells are present on cells purified by our method, and whether the replicating cells isolated by our methods may show responses to hematopoietic growth factors distinct from those observed with quiescent stem cell populations.

The ability to obtain populations of cycling stem cells may also ultimately have important implications for HSC gene therapy. Previous attempts to infect unmanipulated murine and human HSC with recombinant retroviruses have met with mixed success, presumably at least in part because the inability of retroviral vectors to integrate in quiescent cells. As shown here, only 1–3% of HSC are in cycle. The use of the rare population of stem cells that are naturally replicating in vivo as targets for gene transfer may provide an important alternative to transduction strategies based on attempts to influence the cell cycle status of stem cells. Studies to test this possibility are in progress.

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