

Functional Heterogeneity Is Associated with the Cell Cycle Status of Murine Hematopoietic Stem Cells

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Abstract. Hematopoietic stem cells (HSCs) are characterized by their ability to differentiate into all hematopoietic cell lineages while retaining their capacity for self renewal. One of the predictions of this model is the existence of a heterogeneous pool of HSCs, some members of which are destined to become lineage restricted progenitor cells while others function to renew the stem cell pool. To test whether HSCs are heterogeneous with respect to cell cycle status, we determined the fraction of phenotypically defined murine HSCs (Thy1.1^{lo} Lin^{-lo} Sca-1⁺) that contain >2*n* amount of DNA as measured by propidium iodide staining, Hoechst dye uptake and [³H]thymidine labeling; that fraction is 18–22%. In contrast, in the developing fetal liver, 40% of HSCs are in the

S/G2/M phases of the cell cycle. Those HSCs which exhibit a low level of staining with rhodamine 123 are almost exclusively in G0/G1 (97%) whereas only 70% of HSCs which stain brightly for rhodamine 123 are in G0/G1. The injection of 100 G0/G1 HSCs rescued 90% of lethally irradiated mice in contrast to 100 S/G2/M HSCs, which protected only 25% of lethally irradiated recipients. Enhanced long-term donor-derived multilineage reconstitution of the peripheral blood was observed in recipients of 100 G0/G1 HSCs compared to recipients of 100 S/G2/M cells. These data indicate that a significant proportion of HSCs are actively proliferating during steady state hematopoiesis and that this subpopulation of cells exhibits reduced stem cell activity.

HEMATOPOIETIC stem cells (HSCs)¹ are a population of cells, each of which can give rise to all blood cell lineages; contained within this population are cells that are radioprotective and are capable of self-renewal (Spangrude et al., 1991). This model predicts the existence of a heterogeneous pool of HSCs, some members of which are destined to become lineage restricted progenitor cells while others function to renew the stem cell pool. To test for this functional heterogeneity we first examined the cell cycle status of phenotypically defined murine hematopoietic stem cells. A rare (~0.05%) population of mouse bone marrow cells characterized phenotypically by the surface marker profile Thy1.1^{lo} Lin^{-lo} Sca-1⁺ has been isolated previously and shown to contain all the above properties (Spangrude et al., 1988). These cells are 1,000–2,000-fold enriched for day 12 CFU-S (Till and McCulloch, 1961; Magli et al., 1982), radioprotection, and long-term multilineage reconstitution of lethally irradiated congenic hosts (Spangrude et al., 1988; Uchida and Weissman, 1992). Single cells give rise to all

lineages tested including T-cells, B-cells, and myelomonocytic cells and can self-renew (Smith et al., 1991; Uchida, N., and I. L. Weissman, manuscript in preparation).

We had reported previously that only 2–3% of bone marrow cells of this phenotype contained >2*n* amounts of DNA as shown by propidium iodide staining (Spangrude et al., 1988). This finding was consistent with several studies including the relative resistance of inferred HSCs to cell cycle specific chemotherapeutic agents such as 5-FU (Bruce and Meeker, 1967); the demonstration that in whole bone marrow transplants, repopulation can be oligoclonal (Keller et al., 1985; Dick et al., 1985; Jordan and Lemishka, 1990); the general proposition that there is a limit of ~40 doublings for nontransformed cells (Hayflick, 1965) as well as a corresponding limit to the number of serial bone marrow transplants that are radioprotective (Ross et al., 1982). In conflict with this conclusion is the demonstration that by several measures of hematopoiesis, inferred HSCs contain 20–30% of cells that are susceptible to the effects of tritiated thymidine suicide or hydroxyurea (Necase et al., 1989). Furthermore, significant heterogeneity in the mouse HSC pool in terms of self renewal potential has been found in experiments on single cell Thy1.1^{lo} Lin^{-lo} Sca-1⁺ seedings (Smith et al., 1991). When these cells were stained with the vital dye

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1. *Abbreviation used in this paper:* HSCs, hematopoietic stem cells.

rhodamine-123 (Rh-123) (Bertoncello et al., 1985), Rh-123^{lo} cells appear to self renew to a much greater degree than the Rh-123^{hi} cells (Spangrude and Johnson, 1990).

We therefore examined the functional significance of the cell cycle status of C57BL/Ka Thy1.1, Thy1.1^{lo} Lin^{-lo} Sca-1⁺ hematopoietic cells. We show here that the frequency of bone marrow derived S/G2/M HSCs is 18–22%, while at least 40% of fetal liver HSCs are actively proliferating. The S/G2/M fraction of HSCs demonstrate a reduced capacity for both radioprotection and long-term, donor-derived multilineage reconstitution of the peripheral blood. These findings are consistent with the proposition that many of these S/G2/M cells are now destined to complete a program of terminal differentiation and are no longer capable of renewing the stem cell pool.

Materials and Methods

Mice

The C57BL/Ka-Thy1.1 (Thy1.1, Ly5.2) strain was used as the source of whole bone marrow, fetal liver and bone marrow derived stem cells in these studies. The C57BL/6J-Ly5.1-Pep^{3b} strain was used as congenic recipients. Animals were bred and maintained in the mouse facility at Stanford University (Stanford, CA).

Cell Preparations

Bone marrow stem cell preparations were obtained by flushing the tibias and femurs of 4–6 wk old animals followed by a series of purification steps outlined previously (Spangrude et al., 1988). Briefly, cells that express low levels of the Thy1.1 antigen, high levels of Sca-1 (Ly6A/E) antigen, and negative or low levels of the following lineage markers: CD3, CD4, CD8, Mac-1, GR-1, B220, and TER-119, were isolated using fluorescence-activated cell sorting (FACS). Labeled cells were analyzed and sorted using a modified dual laser FACS[®] II (Becton Dickinson, San Jose, CA). Day 14 fetal liver cells were harvested and Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells enriched as described previously (Ikuta and Weissman, 1992). In some experiments, bone marrow derived stem cells were stained with rhodamine 123 as described previously (Bertoncello et al., 1985; Spangrude and Johnson, 1990) before cell cycle analysis.

Cell Cycle Analysis

Cell suspensions were lysed in a solution containing 1% Triton X-100, RNase 100 µg/ml, and propidium iodide (PI) 10 µg/ml. Nuclei were then analyzed for DNA content by FACS[®] (Taylor, 1980). Alternatively, cells were incubated with Hoechst 33342 dye (Sigma Chemical Co., St. Louis, MO) at a concentration of 5 µg/ml then analyzed for DNA content by FACS (Hamori et al., 1980).

Thymidine Labeling

Bone marrow derived Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells were incubated with 10 µCi of [³H]thymidine (Specific activity 20 Ci/mmol, New England Nuclear, Boston, MA) for 30 min at 37°C, rinsed twice in HBSS, and then cytospin preparations were made. Slides were coated with photographic emulsion (NTB2, Eastman Kodak Co.) and then stored at 4°C for 8–14 d developed, then counter stained with hematoxylin. Percent labeling was determined after counting 400 cells. Those cells with more than 10 grains above background were considered positive (average background was two grains/cell).

Radioprotection Assay

Recipient mice were lethally irradiated using a Phillips 250 kV x-ray machine at 1 Gy/min in two fractions at least 3 h apart to a total of 8.5 Gy. Suspensions of sorted cells from adult bone marrow were injected in a volume of 200 µl into the retro-orbital sinus under methoxyflurane anesthesia. Animals were maintained on aqueous antibiotics (polymyxin B sulphate 10⁶ U/liter and neomycin sulphate 1.1 g/liter) in their drinking water. Recipient mice were monitored daily for survival for 30 d.

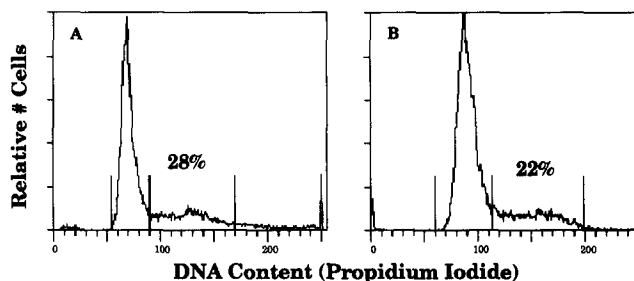


Figure 1. DNA content of bone marrow derived stem cells. Cells were lysed and the nuclei stained with propidium iodide and the DNA content determined by FACS. (A) Total bone marrow cells. (B) Purified Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells (HSCs). Percentage of PI labeled nuclei in S/G2/M is indicated.

Long-Term Reconstitution Assay

The donor and recipient mice used were congenic at the Ly-5 locus (CD45). Peripheral blood was obtained from the retroorbital sinus under methoxyflurane anesthesia and stained with a panel of antibodies. Using FACS analysis the percentage of B cells (B220⁺), T cells (CD3⁺) and myeloid cells (Gr-1⁺/Mac-1⁺), that were donor derived (Ly5.2⁺) was determined (Spangrude et al., 1988).

Competitive Repopulation Assay

Lethally irradiated Ly5.1 recipient mice were injected with 2 × 10⁵ syngeneic bone marrow cells and either 10 G0/G1 or 100 S/G2/M Thy1.1^{lo} Lin^{-lo} Sca-1⁺ stem cells (Ly 5.2). The degree of donor-derived long-term reconstitution was determined as described above.

Results

Cell Cycle Status of Bone Marrow-derived Hematopoietic Stem Cells

During the course of experiments designed to evaluate the

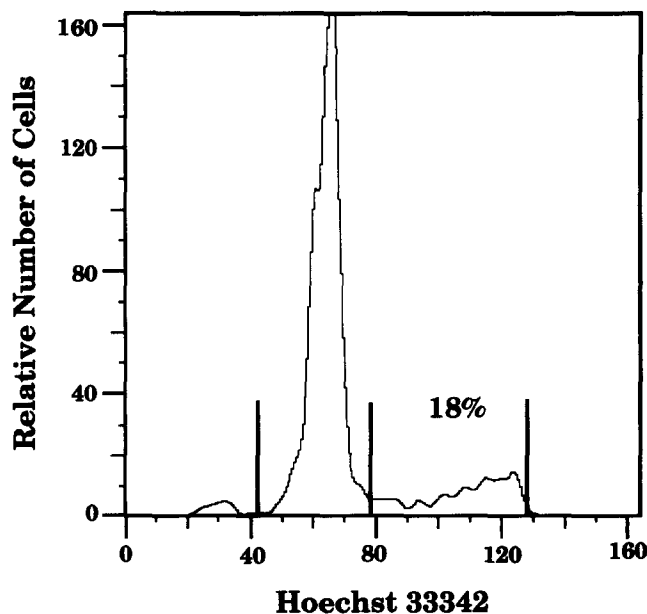


Figure 2. Hoechst 33342 staining of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells. Sorted viable HSCs were incubated with Hoechst dye and uptake was analyzed by FACS[®]. The percentage of cells in S/G2/M is indicated.

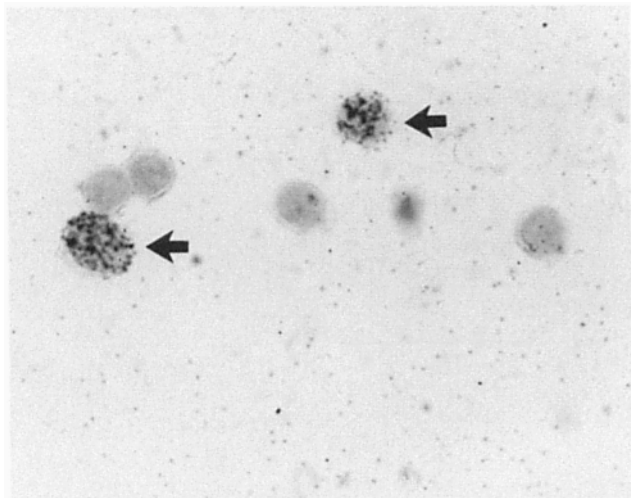


Figure 3. DNA synthesis in Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells. Autoradiograph of tritiated thymidine labeled HSCs from adult bone marrow. Of a total of 400 cells examined, 12–15% were labeled (arrows). Exposure time was 8 d. (× 800)

cell cycle status of Thy^{lo} Lin^{-lo} Sca-1⁺ cells, the observation was made by Mr. Tim Knaak, Stanford FACS[®] operator, that a large number of events recorded by the cell sorter during the analysis of propidium iodide stained nuclei were neither excluded by forward scatter gates nor readily apparent in the major peak of 2*n* nuclei. Gating around the S/G2/M population and expressing this value as a percentage of total events lead to a significant underestimation of the proportion of cells in S/G2/M (Spangrude et al., 1988). These extra signals are presumably due to the presence of magnetic beads in the cell lysate. Sorting these cells without magnetic beads as shown in Fig. 1, demonstrates that 28% of unfractionated bone marrow cells and 22% of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells are in the S/G2/M phase of the cell cycle based on their content of propidium iodide stained DNA. The DNA content of viable HSCs was determined using the vital dye Hoechst 33342, and as shown in Fig. 2, 18% of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells contain >2*n* amounts of DNA. The similarity in the PI and Hoechst 33342 staining effectively rules out any underestimation of cycling cells that may result from the active transport of Hoechst dye from cells by the MDR gene product (Chaudhary and Roninson, 1991). This high percentage of cycling cells is confirmed by tritiated thymidine labeling of purified Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells followed by autoradiography. An analysis of 400 cells per experiment indicates that 12–15% of these cells are actively synthesizing DNA (Fig. 3).

Cell Cycle Status of Fetal Hematopoietic Stem Cells

Analysis of the DNA content of day 14 fetal liver cells by propidium iodide staining indicates that 44% of these cells are in the S/G2/M phase of the cell cycle (Fig. 4 *b*). Depletion of those cells which express the erythroid marker TER-119 results in a cell population enriched for HSCs and other progenitors which are predominantly (59%) in S/G2/M (Fig. 4 *c*). Subsequent purification of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells from this enriched population yields a subset of highly purified HSCs, 40% of which are in S/G2/M (Fig. 4 *d*). The percentage of day 14 fetal liver derived Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells in S/G2/M is therefore nearly twice that found in normal adult bone marrow derived HSCs (40% vs 22%). This finding is consistent with the rate of increase in fetal liver derived HSCs observed during this period of fetal development (Ikuta and Weissman, 1992).

Cell Cycle Status of Rhodamine 123 Stained Hematopoietic Stem Cells

Rhodamine-123 uptake has been used to fractionate hematopoietic progenitors into Rh-123^{lo} vs Rh-123^{hi} subpopulations (Bertoncello et al., 1985; Spangrude and Johnson, 1990; Visser et al., 1981). The Rh-123^{lo} subset appears to be enriched for more primitive HSCs in both the mouse (Spangrude and Johnson, 1990; Baines and Visser, 1983) and human (Baum et al., 1992). We therefore wished to analyze cell cycle status of Rh-123^{lo} and Rh-123^{hi} Thy1.1^{lo} Lin^{-lo} Sca-1⁺ adult bone marrow cells. The further fractionation of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells on the basis of Rh-123 staining followed by analysis of DNA content demonstrates that only 2.9% of Rh-123^{lo} cells are in S/G2/M (Fig. 5). In contrast, 30% of Rh-123^{hi} cells contain >2*n* amounts of DNA, and therefore at least that percentage are actively cycling.

Stem Cell Activity of G0/G1 and S/G2/M Thy1.1^{lo} Lin^{-lo} Sca-1⁺ Hematopoietic Stem Cell Subsets

To test directly the functional capacity of proliferating Thy1.1^{lo} Lin^{-lo} Sca-1⁺, these cells were fractionated based on Hoechst 33342 staining, injected into lethally irradiated recipient mice and then survival was monitored daily for 30 d. Fig. 6 demonstrates that 100 G0/G1 cells provided radio-protection to 90% of lethally irradiated recipients while 100 S/G2/M cells protected only 22% of recipients (animals that survived for 30 d continued to survive for at least 6 mo). At 6 mo post transplantation, recipient mice were examined for the presence of donor-derived multilineage reconstitution of the peripheral blood (Table I). In mice reconstituted with

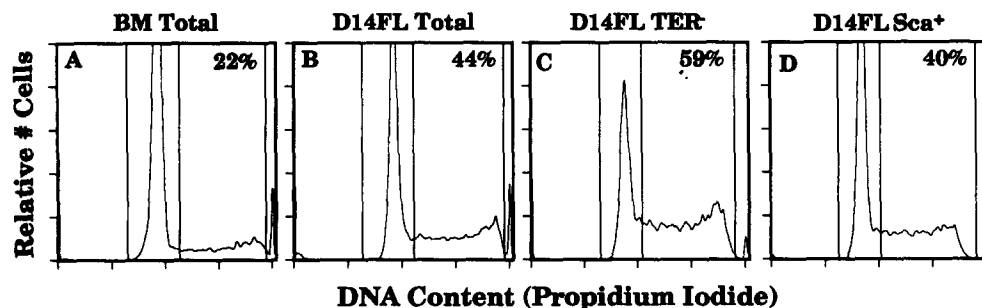


Figure 4. DNA content of fetal liver HSCs. (A) Total adult bone marrow. (B) Total day 14 fetal liver cells (D14FL). (C) D14FL cells after depletion of erythroid cells. (D) D14FL Thy^{lo} Lin^{-lo} Sca-1⁺ cells. The percentage of PI labeled nuclei in S/G2/M is indicated.

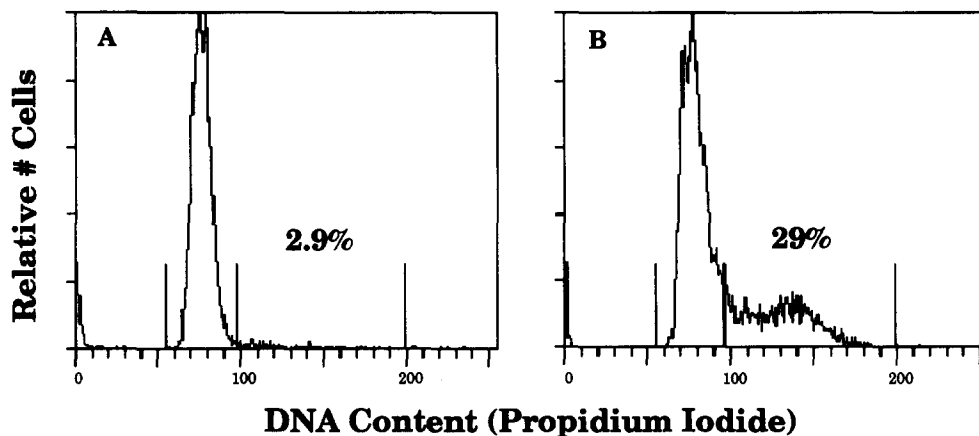


Figure 5. DNA content of rhodamine 123 stained HSCs. Thy1.1^{lo} Lin^{-lo} Sca-1⁺ were further sorted based on rhodamine 123 staining, lysed and the nuclei were stained with propidium iodide. (A) Rh-123^{lo} HSCs. (B) Rh-123^{hi} HSCs. Percentage of PI labeled nuclei in S/G2/M is indicated.

100 G0/G1 cells, 34% of B cells, 16% of myeloid cells and 19% of T cells were donor-derived. In contrast, in recipients of 100 S/G2/M cells, only 7.8% of B cells and <2.0% of both myeloid cells and T cells were donor-derived. When 300 G0/G1 cells were injected, survival was 100% ($n = 10$) and the majority of cells in all three lineages were donor-derived. After the injection of 300 S/G2/M cells, similar levels of donor-derived reconstitution were observed and survival was increased to 50% ($n = 8$) (Table I).

To demonstrate that the decreased radioprotective capacity of the S/G2/M cells was itself not responsible for the decrease in donor-derived long-term reconstitution capacity of S/G2/M cells, we employed a competitive repopulation assay (Harrison, 1980). Lethally irradiated recipient mice were injected with 2×10^5 syngeneic bone marrow cells and either 100 G0/G1 or 100 S/G2/M cells. This dose of syngeneic bone marrow provides radioprotection to the recipient mice while permitting the G0/G1 and the S/G2/M cell subsets to undergo lineage specific differentiation. Recipient mice were analyzed 9 mo postinjection. When 100 G0/G1

cells were injected along with syngeneic bone marrow (Table II), 24% of B cells, 19% of T cells, and 23% of myeloid cells were donor derived. Recipients of 100 S/G2/M cells and syngeneic bone marrow demonstrated less than 2% donor-derived peripheral blood cells. Together these results demonstrate that stem cells in the S/G2/M phases of the cell cycle have a diminished capacity for both radioprotection and long-term multilineage reconstitution of the peripheral blood.

Discussion

Using three independent techniques we have provided evidence that during steady state hematopoiesis in adult bone marrow, and in the developing fetal liver, a significant fraction of Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells are actively proliferating.

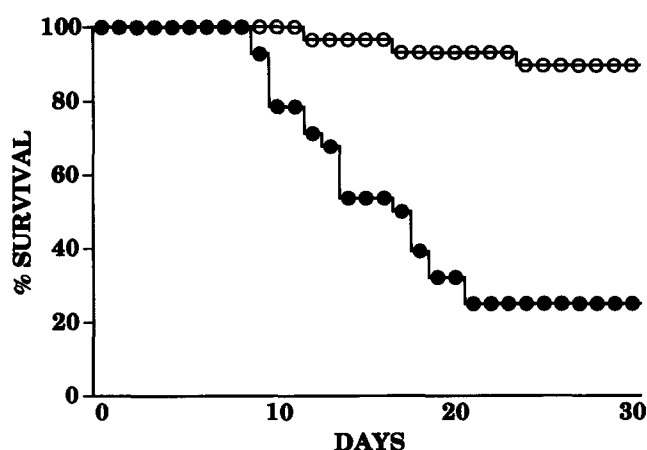


Figure 6. Radioprotective capacity of G0/G1 and S/G2/M HSCs. Thy^{lo} Lin^{-lo} Sca-1⁺ cells were incubated with Hoechst 33342 dye and further sorted based on their DNA content. Either G0/G1 or S/G2/M HSCs fractions were then injected into lethally irradiated recipient mice and survival was monitored daily for 30 d. (100 G0/G1 HSCs \circ ; 100 S/G2/M HSCs \bullet).

Table I. Donor-derived Multilineage Reconstitution

	B220	MAC-1/GR-1	CD3
	% Donor		
100 G0/G1	34.1 \pm 11.7	15.6 \pm 12.6	18.9 \pm 9.0
100 S/G2/M	7.8 \pm 4.7	1.1 \pm 0.4	1.6 \pm 0.5
300 G0/G1	70.3 \pm 18.5	58.0 \pm 21.8	53.4 \pm 15.4
300 S/G2/M	73.7 \pm 23.3	36.0 \pm 24.2	43.9 \pm 18.2

Lethally irradiated congenic recipient mice were injected with either 100 or 300 G0/G1 or S/G2/M HSCs. The percentage of donor-derived (Ly5.2⁺) B cells (B220⁺), myeloid cells (Mac-1/GR-1⁺), and T cells (CD3⁺) in the peripheral blood was determined after 24 wk. The mean \pm SEM is shown ($n = 4-10$ mice per group).

Table II. Competitive Repopulation with G0/G1 and S/G2/M HSCs

	B220	MAC-1/GR-1	CD3
	% Donor		
100 G0/G1	23.5 \pm 20.4	23.1 \pm 20.6	19.1 \pm 17.2
100 S/G2/M	1.7 \pm 0.5	1.9 \pm 0.5	1.6 \pm 0.4

Lethally irradiated recipient mice (Ly5.1) were injected with 2×10^5 syngeneic BM cells and coinjected with either 100 congenic G0/G1 HSCs or 100 congenic S/G2/M HSCs. The percentage of HSC-derived (Ly5.2⁺) B cells (B220⁺), myeloid cells (Mac-1/GR-1⁺), and T cells (CD3⁺) in the peripheral blood was determined after 9 mo. The mean \pm SEM is shown ($n = 4$ mice per group).

These findings are in agreement with previous studies which indicate that 20–30% of stem cell activity is susceptible to tritiated thymidine suicide or hydroxyurea treatment (Necase et al., 1989; Quesenberry and Stanley, 1980; Schofield, 1979). These data are also similar to the cell cycle studies of the hematopoietic progenitors CFUs and HPP-CFC, which reveal that 12–24% of these cells are in S/G2/M phases of the cell cycle (Visser et al., 1981; Baines and Visser, 1983).

As judged by the functional differences between G0/G1 and S/G2/M HSCs, there is considerable heterogeneity within this phenotypically defined stem cell compartment. Previous studies have shown that it is possible to divide this highly enriched population into two groups based on staining with the vital fluorescent dye Rh-123 (Spangrude and Johnson, 1990). Rh-123 initially was thought to stain mitochondria preferentially and therefore be a marker for metabolically active cells (Bertoncello et al., 1985); however, it has been shown that low levels of Rh-123 staining of human hematopoietic progenitor cells may be the result of the expression of the P-glycoprotein (Chaudhary and Roninson, 1991) which confers the multidrug resistance phenotype (MDR). Those Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells which take up low levels of Rh-123 are enriched for the properties of radioprotection and long-term reconstitution of the peripheral blood of lethally irradiated mice after transfer to secondary recipients (Spangrude and Johnson, 1990). In contrast, those cells which have a high level of Rh-123 staining do not exhibit this activity. While the mechanism of low levels of Rh-123 staining in these cells is not known, from this experiment it is apparent that Rh-123 staining permits the separation of Rh-123^{lo}, mainly noncycling Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cells from the total population. It is important to note that in the experiments cited above, the secondary transfer experiments were performed only 14 d after the first transfer (Spangrude and Johnson, 1990), a situation which likely places a marked proliferative stress on these cells. Recently, Li and co-workers have fractionated Lin⁻ Sca-1⁺ cells on the basis of Rh-123 staining and have reported significantly greater long-term, donor-derived, multilineage reconstitution in the Rh-123^{lo} (67–73%) vs Rh-123^{hi} (1–2%) subsets (Li and Johnson, 1992). Taken together, these studies suggest that the majority of stem cell activity in these enriched populations is confined to the Rh-123^{lo} subset.

When we directly tested the G0/G1 and S/G2/M Thy1.1^{lo} Lin^{-lo} Sca-1⁺ cell subsets, we found them to be heterogeneous in terms of both radioprotective capacity and long term multilineage reconstitution potential. The decreased long-term reconstitution capacity of the S/G2/M subset was also observed when these cells were coinjected with a radioprotective dose of syngeneic bone marrow. Whether the decreased radioprotection and long-term reconstitution capacity associated with the S/G2/M subpopulation is the result of a loss of their differentiation potential/self-renewal capacity, or due to a defect in the homing of these cells to a favorable microenvironment remains to be determined. These data are consistent with the hypothesis that S/G2/M HSCs are themselves heterogeneous, the majority of these cells differentiating into the committed progenitor cell pool while a smaller subset is responsible for self renewal. Alternatively, S/G2/M HSCs may be functionally homogeneous but

have a reduced capacity to provide long-term multilineage reconstitution on a per-cell basis.

As hematopoietic growth factors are more frequently utilized to enhance the recovery of peripheral blood counts following high dose chemotherapy, and as retroviral vector systems are being evaluated for gene therapy, it becomes increasingly important to understand the developmental potential of actively proliferating versus resting hematopoietic stem cells. Furthermore, the experiments that lead to the conclusion that the hematopoietic system may operate by uni/oligoclonal expansion and clonal succession would predict that the proliferating fraction of HSCs at any one time interval should be uniclonal, a hypothesis that could now be tested outside the context of retroviral marking of bone marrow transplants.

We thank Drs. David Vaux, William Tse, and Xincheng Liao for helpful discussions and for reviewing the manuscript. We are also grateful to Mr. T. Knaak for operating the cell sorter, Mr. L. Hidalgo and staff for maintaining the animal care facility and Ms. L. Jerabek for managing the laboratory.

William H. Fleming M.D., PhD is a Fellow of the Medical Research Council of Canada. This work was supported by National Institutes of Health grant CA-42551 (to I. L. Weissman).

Received for publication 15 April 1993 and in revised form 25 May 1993.

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