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Resting and activated subsets of mouse multipotent hematopoietic stem cells

(splenic colony-forming units/T-lymphocyte development/rhodamine 123)

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ABSTRACT **The fluorescent vital dye rhodamine 123 (Rh-123), which preferentially accumulates in mitochondrial membranes, can be used as a probe to indicate mitochondrial and hence cellular activity. In this study, mouse bone marrow hematopoietic stem cells were subdivided into Rh-123^{lo}, Rh-123^{med}, and Rh-123^{hi} populations. The Rh-123^{lo} (resting) population was significantly enriched in cells with a higher proliferative potential compared to the Rh-123^{hi} (activated) population. The resting population exhibited a 20-fold greater ability to differentiate into splenic colony-forming units (CFU-S) relative to the activated population, whereas the activated population contained about 4-fold more day 13 CFU-S on primary transfer relative to the resting population. The two populations produced morphologically distinct splenic colonies; however, the frequency and morphology of *in vitro* colonies were very similar. Only the resting population provided sufficient stem cells to transfer long-term hematopoietic repopulation to secondary recipient animals after lethal irradiation. On a single cell level, the resting and activated populations exhibited an equivalent ability to differentiate into lymphoid and myeloid progeny. These observations provide further insight into the heterogeneous nature of CFU-S and directly demonstrate that multipotent hematopoietic stem cells are heterogeneous with regard to their clonogenic capacities.**

The hematopoietic process provides a unique opportunity to study the execution of an ongoing developmental program in normal adult mammals. One approach to this problem has been to identify and isolate the various cells that contribute to the hematopoietic system (1–3) and to reconstruct the hematopoietic process from the isolated cells, either *in vitro* using cell culture with defined growth factors (1, 4, 5) or within irradiated animals (3, 6, 7). Although this approach has illuminated various aspects of the terminal stages of hematopoiesis (8), the details of the earliest stages remain obscure owing to the small number of cells within the most primitive hematopoietic compartments and the lack of a defined culture system to support them.

An additional complication to the study of primitive hematopoietic stem cells has been the demonstration that splenic colony-forming unit (CFU-S) activity, once thought to be a specific characteristic of these cells (9), does not adequately represent the most primitive of stem cells (10–12). Thus, workers have turned to indirect assays that measure the repopulation of irradiated hematopoietic tissues with CFU-S or with *in vitro* colony-forming cells (CFCs) (7, 13). Unfortunately, these assays do not allow a direct clonal readout of the initiating primitive hematopoietic stem cell. The most primitive of hematopoietic stem cells may be unable to directly form spleen colonies within a 13-day period (7). This suggests that the hematopoietic stem cell compartment may be more complex and heterogeneous than previously supposed (14, 15).

In a previous study, the isolation of a highly enriched population of multipotent hematopoietic stem cells from normal mouse bone marrow was reported (3). These cells were characterized by low-level expression of the Thy-1 antigen (Thy-1^{lo}), failure to express lineage-specific antigens characteristic of differentiated blood cells (Lin^{neg}), and expression of the Ly6A/E antigen [previously termed Sca-1 (16)]; in the present communication these cells will be termed Ly6A/E$^+$. We now demonstrate a functional heterogeneity within the population of Ly6A/E$^+$ cells. This heterogeneity can be revealed by rhodamine 123 (Rh-123), a fluorescent vital dye that preferentially accumulates in mitochondrial membranes proportionately to the activation state of the individual cells (17). Isolation of the "resting" (Rh-123^{lo}) and "activated" (Rh-123^{hi}) subpopulations of Ly6A/E$^+$ bone marrow cells permits the direct demonstration that mouse multipotent hematopoietic stem cells can differ in their ability to produce more multipotent hematopoietic stem cells.

MATERIALS AND METHODS

Animals. The C57BL/Ka-Thy-1.1 (Thy-1.1, Ly-5.2) and C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2, Ly-5.1) mouse strains used in this study were cesarean derived and foster reared under specific pathogen-free conditions at the Walter and Eliza Hall Institute animal facility. All mice were regularly maintained on acidified water.

Enrichment of Bone Marrow Subpopulations. Ly6A/E$^+$ bone marrow cells were enriched from 4- to 12-week-old C57BL/Ka-Thy-1.1 animals as described (3). Fluorescence-activated cell sorting (FACS) was performed using an unmodified dual-laser FACStar-Plus instrument (Becton Dickinson).

After isolation of the Ly6A/E$^+$ cells, the cell suspension was concentrated by centrifugation and resuspended in 1.0 ml of balanced salt solution with 5% fetal calf serum (BSS/FCS) for Rh-123 staining, which was performed exactly as described by Bertoncello *et al.* (13). The cells were then resuspended in 1 ml of BSS/FCS for a second round of FACS selection, based on Rh-123 staining intensity. A sample of normal bone marrow cells was also stained and was used to compare the staining intensity of the Ly6A/E$^+$ cells from experiment to experiment.

Animal Irradiation and Hematopoietic Cell Transplantation. Recipient animals, 6–12 weeks of age, were exposed to 11.0 Gy of γ radiation from a ^{137}Cs source (Atomic Energy, Ottawa) at a dose rate of 31 cGy/min. The 11.0-Gy dose was

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Abbreviations: CFU-S, splenic colony-forming unit(s); CFC, *in vitro* colony-forming cell; Rh-123, rhodamine 123; FACS, fluorescence-activated cell sorting.

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delivered in two equal exposures, given 3 hr apart. Animals were maintained on aqueous antibiotics (polymyxin B sulfate, 10^6 units/liter; and neomycin sulfate, 1.1 g/liter, both from Sigma) for 2 weeks after irradiation. Secondary transfers of bone marrow and spleen cell suspensions were performed 13 days after primary transplantation. The number of day 13 spleen colonies in each secondary animal was normalized to a per donor spleen or per donor femur basis and to the number of cells (per 10^3) injected into the primary animals.

Hematopoietic reconstitution was indicated by the percentage of donor-derived cells in peripheral blood of animals 8 weeks after irradiation and reconstitution. Donor-derived cells were identified by FACS analysis of recipient animals, which differed from the donors at the Ly-5 (CD45) allelic locus.

CFC Assays. Bone marrow cells were seeded at 5×10^4 and spleen cells were seeded at 1×10^5 cells per culture in triplicate 35-mm plastic Petri dishes. Cells were cultured for 8 days in semi-solid agar medium containing predetermined maximal stimulatory concentrations of pokeweed mitogen-stimulated spleen cell-conditioned medium (18) plus 1 unit of erythropoietin per ml.

Intrathymic Injections. Recipient animals (C57BL/6-Ly-5.1-Pep^{3b}) were exposed to 7.5 Gy of γ radiation in a single dose 6–8 hr prior to intrathymic transfer. Anesthetized animals were secured by elastic bands while the sternum was cut, and cell suspensions were introduced into one lobe of the thymus in a 5-μl volume of BSS/FCS. Fourteen to 18 days after injection, the animals were sacrificed and a single-cell suspension from each lobe was screened by immunofluorescence for the presence of donor-derived cells based on the expression of the Ly-5 marker. Donor-derived cells were phenotyped by two-color analysis using monoclonal antibodies specific for T-, B-, and myeloid cell lineages in combination with the Ly-5 allelic marker.

RESULTS

Heterogeneity in Phenotype and Function of Ly6A/E$^+$ Hematopoietic Stem Cells. The cationic fluorescent dye Rh-123 is a cell-permeant molecule that preferentially binds to mitochondrial membranes in living cells (19). The intensity of staining is related not only to the number of mitochondria per cell but also to the state of cellular activation (17). When Ly6A/E$^+$ hematopoietic stem cells were stained with Rh-123, the results shown in Fig. 1 were obtained. About 50% of Ly6A/E$^+$ cells stained significantly brighter with Rh-123 than did the majority of normal mouse bone marrow cells; these cells can be identified in Fig. 1*B* as the Rh-123^{hi} population. The remaining 50% of Ly6A/E$^+$ cells roughly coincided with normal bone marrow cells in their staining intensity and were arbitrarily subdivided into equal subpopulations containing the dullest 25% (Rh-123^{lo}) and the intermediate 25% (Rh-123^{med}). In some experiments, these two fractions were considered together (Rh-123^{lo+med}).

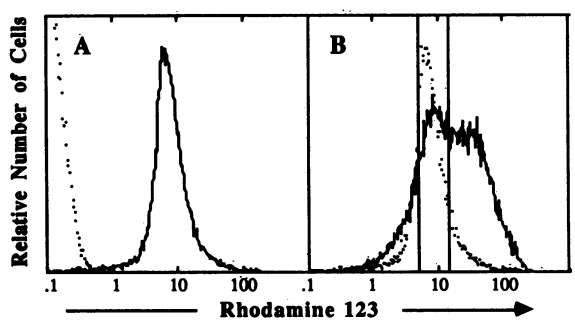

FIG. 1. Separation of Ly6A/E$^+$ bone marrow cells by Rh-123 staining. (*A*) Normal bone marrow cells unstained (dotted line) and stained (solid line). (*B*) Stained normal bone marrow cells (dotted line); stained Ly6A/E$^+$ cells (solid line). The vertical lines indicate the approximate gate settings for collection of the Rh-123^{lo}, Rh-123^{med}, Rh-123^{hi} subpopulations of cells.

Table 1. Spleen colony-forming potential of Ly6A/E$^+$ bone marrow fractions

Population	CFU-S activity per 1000 injected cells		Day 13/day 8
	Day 8	Day 13	
Rh-123^{lo}	ND	22.8 ± 7.6	—
Rh-123^{med}	ND	58.3 ± 11.2	—
Rh-123^{lo+med}	0.75 ± 1.1	43.1 ± 10.0	57.5
Rh-123^{hi}	5.1 ± 2.5	104.6 ± 36.1	20.5

The numbers indicate means ± SD, with 6–16 animals and at least two independent experiments represented by each value. ND, not done.

To assess colony-forming potential, the subpopulations of cells were tested for CFU-S activity. As shown in Table 1, most of the day 8 CFU-S activity mediated by the Ly6A/E$^+$ cells was found to be due to the Rh-123^{hi} subpopulation, which contained about 5-fold more day 8 CFU-S on a per cell basis than did the combined Rh-123^{lo+med} fractions. The Rh-123^{hi} subpopulation was also relatively enriched in day 13 CFU-S activity compared to the other fractions, containing about 1.8- and 4.6-fold more day 13 CFU-S per cell than the Rh-123^{med} and Rh-123^{lo} subpopulations, respectively. Considered together, the Rh-123^{lo} and Rh-123^{med} subpopulations contained almost 60-fold more day 13 CFU-S than day 8 CFU-S, indicating a relatively more primitive differentiation state compared to the Rh-123^{hi} subpopulation.

The cellular composition of day 8 and day 13 spleen colonies developing from the various populations of cells was determined. At both time points, colonies developing from Ly6A/E$^+$ cells included erythroid, neutrophil, megakaryocyte, mixed, and undifferentiated types (Table 2). Ly6A/E$^+$Rh-123^{lo+med} cells generated day 8 spleen colonies that were predominantly undifferentiated and megakaryocytic, whereas at day 8 the colonies generated by Ly6A/E$^+$Rh-123^{hi} cells were predominantly neutrophilic and mixed cell types. At day 13, the majority of colonies developing from Ly6A/E$^+$Rh-123^{lo+med} cells were mixed and megakaryocytic, whereas Ly6A/E$^+$Rh-123^{hi} bone marrow cells generated mostly erythroid and mixed colonies.

The sorted populations of cells were also cultured *in vitro* to determine the content of committed progenitor cells. Fractionation of the Ly6A/E$^+$ population with Rh-123 did not result in any major differences in frequency or distribution of colony types, as a cloning efficiency of about 30% was observed, and 80–90% of the colonies consisted of neutrophils and/or macrophages in all cases (data not shown). Very few erythroid colonies were observed, most likely because of

Table 2. Differential morphology of spleen colonies formed by Thy-1^{lo}Lin^{neg}Ly6A/E$^+$ subpopulations

Colony morphology	% of total colonies			
	Day 8		Day 13	
	Rh-123^{lo+med}	Rh-123^{hi}	Rh-123^{lo+med}	Rh-123^{hi}
Erythroid	3	3	8	53
Neutrophil	13	47	14	6
Megakaryocyte	37	13	30	4
Mixed	0	37	48	30
Undifferentiated	47	0	0	7
No. of colonies	30	38	27	30

Colony morphology was determined by microscopic examination of serial hematoxylin/eosin-stained sections of spleens.

an inefficiency of the CFC assay for supporting erythroid development from very primitive hematopoietic cells since parallel cultures of unseparated bone marrow cells contained many erythroid colonies.

Repopulation of Hematopoietic Tissues by Isolated Cell Populations. Thirteen days after intravenous infusion of sorted populations into lethally irradiated mice, the animals were sacrificed and suspensions of bone marrow and spleen cells were assessed for the number of cells per organ and for the content of CFCs and day 13 CFU-S. The three Rh-123 subpopulations were equivalent within a factor of 2 in their ability to mediate cellular repopulation of irradiated hematopoietic organs (Fig. 2*A*) and were about 200- to 300-fold more effective than unseparated bone marrow in this assay. The results of CFC assays on the same cell suspensions generally paralleled the cellularity of the organs, as shown in Fig. 2*B*. However, the CFC content of femoral marrow from animals reconstituted with Ly6A/E$^+$Rh-123^{hi} cells was 10-fold lower than that of marrow from animals reconstituted with Ly6A/E$^+$Rh-123^{med} or Ly6A/E$^+$Rh-123^{lo} cells, a much greater difference than could be accounted for by the differences in cellularity.

An analysis of day 13 CFU-S content of splenic and femoral compartments 13 days after the primary reconstitution revealed a more dramatic gradient of activity resolved by Rh-123 staining intensity. As shown in Table 3, Ly6A/E$^+$Rh-123^{lo} cells generated 42-fold more CFU-S in marrow and about 13-fold more CFU-S in spleen relative to Ly6A/E$^+$Rh-123^{hi} cells. By extrapolation to the total marrow mass of the recipient animals, 1000 Ly6A/E$^+$Rh-123^{lo} cells generated >5000 CFU-S in the hematopoietic tissues of the recipient animals compared to 230 CFU-S generated by 1000 Ly6A/E$^+$Rh-123^{hi} cells. Since the CFU-S assay is about 10–20% efficient due to the efficiency of cell seeding to the spleen, it is likely that in each case the actual number of stem cells generated by the 1000 injected cells is at least 5- to 10-fold greater than the data in Table 3 indicate.

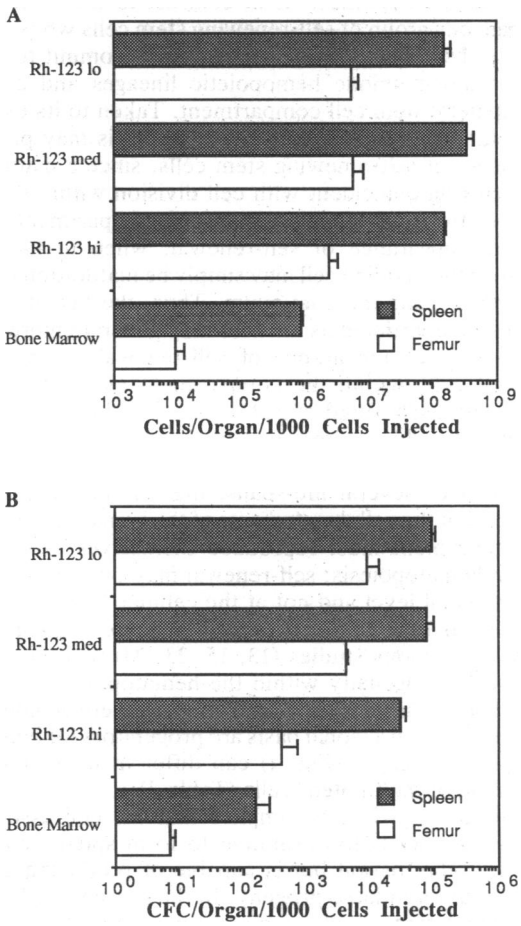

FIG. 2. Repopulation of hematopoietic tissues by bone marrow subpopulations. Irradiated recipient mice received 2000 cells of each population, or 10^6 normal bone marrow cells. Thirteen days later, the mice were sacrificed and suspensions of cells prepared from bone marrow and spleen were assayed for cellularity (*A*) and CFC content (*B*). The mean values (±SD) from three independent experiments are presented after normalization to 10^3 injected cells.

Table 3. Pre-CFU-S and day 13 CFU-S activity of Ly6A/E$^+$ bone marrow subpopulations

Population	CFU-S per 1000 cells	CFU-S generated in 13 days			Recovered/ input
		Spleen	Marrow	Total	
Rh-123^{lo}	23	1900	3350	5250	228
Rh-123^{med}	58	820	1360	2180	38
Rh-123^{hi}	105	150	80	230	2

Irradiated primary animals received 2000 cells of the indicated population intravenously. Thirteen days later, spleen and bone marrow cells were harvested and assayed for the presence of day 13 CFU-S. The mean values of three experiments are shown, indicating the number of CFU-S per 1000 injected cells. The bone marrow values are calculated on the basis of one femur representing 6% of the total marrow mass of a mouse (20). The primary day 13 CFU-S numbers are from Table 1.

The Ly-5 allelic determinant of the CD45 molecule, which is expressed by all bone marrow-derived cells with the exception of erythrocytes and erythroblasts (21), was utilized to indicate hematopoietic reconstitution by the subpopulations of cells. The cells were FACS-isolated from C57BL/Ka-Thy-1.1 mice (Ly-5.2) and intravenously transferred into lethally irradiated C57BL/6-Ly-5.1-Pep^{3b} mice (Ly-5.1). Thirteen days later, the recipient mice were sacrificed and bone marrow cells isolated from them were intravenously transferred into a second group of lethally irradiated C57BL/6-Ly-5.1-Pep^{3b} mice. Surviving animals were bled 8 weeks after reconstitution, and peripheral blood samples were stained with monoclonal antibodies specific for the donor and host alleles of CD45. A summary of the results is shown in Table 4. Secondary recipients of bone marrow from Ly6A/E$^+$Rh-123^{lo}-reconstituted animals contained mostly donor-derived cells in their peripheral blood, whereas reconstitution with marrow derived from recipients of Ly6A/E$^+$Rh-123^{hi} cells resulted in virtually no peripheral blood progeny 8 weeks later. These results indicate that the resting population of Ly6A/E$^+$Rh-123^{lo} cells is much more capable of repopulating hematopoietic tissues with primitive stem cells relative to the activated population of Ly6A/E$^+$Rh-123^{hi} cells.

Table 4. Long-term repopulation of animals by progeny of various bone marrow populations

Primary recipient animals		Secondary recipient animals	
Population transferred	No. of cells	Dead/total	% donor-derived PBL (*n*)
Ly-6A/E$^+$Rh-123^{lo}	2000	0/7	91.8 ± 1.9 (5)
Ly-6A/E$^+$Rh-123^{med}	1000	0/7	21.1 ± 16.4 (5)
Ly-6A/E$^+$Rh-123^{hi}	2000	4/9	0.06 ± 0.04 (4)
None	—	10/10	—

The indicated populations of cells were intravenously transferred into lethally irradiated mice as indicated in the text. Thirteen days later, 0.1 or 0.2 femoral equivalent from these animals was transferred into secondary groups of lethally irradiated mice. The % donor-derived peripheral blood leukocytes (PBL) of secondary recipients 8 weeks after transplant is presented as the mean ± SD based on cell surface staining for the donor allele of the Ly-5 molecule. *n*, Number of animals screened in each group.

Rh-123^{lo} and Rh-123^{hi} Subpopulations of Ly6A/E$^+$ Cells Contain Multipotent Hematopoietic Stem Cells. To test whether the subsets of Ly6A/E$^+$ cells were equally efficient at initiating T-cell development, intrathymic transfers were performed at limiting dilution. The results, shown in Fig. 3, indicate that two populations of donor-derived cells could be identified in the irradiated thymus after injection of five Ly6A/E$^+$Rh-123^{lo+med} cells. The donor-derived cells were phenotyped as T lymphocytes expressing the Thy-1 allele of the donor strain (Fig. 3*A*) and as myeloid cells expressing either of two antigens specific for the myeloid lineages (Mac-1 and Gr-1, Fig. 3*B*). FACS isolation of the donor-derived cells enclosed in the box in Fig. 3*B* confirmed that they were normal-appearing neutrophils and macrophages at various stages of development (data not shown). B lymphocytes were not detected in thymuses that had been injected with limiting numbers of Ly6A/E$^+$Rh-123^{lo+med} cells (Fig. 3*C*); however large numbers of presumptive B lymphocytes were observed in thymuses that had received 5000 Ly6A/E$^+$ cells (Fig. 3*D*).

The intrathymic injection assay was utilized to compare the frequency of cells within the Rh-123 subsets of the Ly6A/E$^+$ population that could respond to the thymic microenvironment. The ability of the irradiated thymus to support T-lymphoid and myeloid development also allowed an evaluation of the multipotent nature of the injected cells. The results of the experiment are shown in Fig. 4. When five cells of either the Ly6A/E$^+$Rh-123^{lo+med} or the Ly6A/E$^+$Rh-123^{hi} subset were intrathymically injected into groups of irradiated mice, an equal frequency of thymic lobes in the two experimental groups contained progeny of the injected cells. These results agree well with limiting-dilution analysis of the total Ly6A/E$^+$ population (3) and indicate that the two Rh-123 subpopulations are equally efficient in this assay. Furthermore, an analysis of the cell surface phenotype of the donor-derived cells in each clone indicated that the majority of clones contained lymphoid and myeloid cells (Fig. 4). Since, according to the Poisson distribution, the probability of any thymic colony being derived from two or more injected cells (about 40%) is much smaller than the frequency of colonies containing lymphoid and myeloid components (about 80%), these results argue that many of the cells contained within the two populations are multipotent.

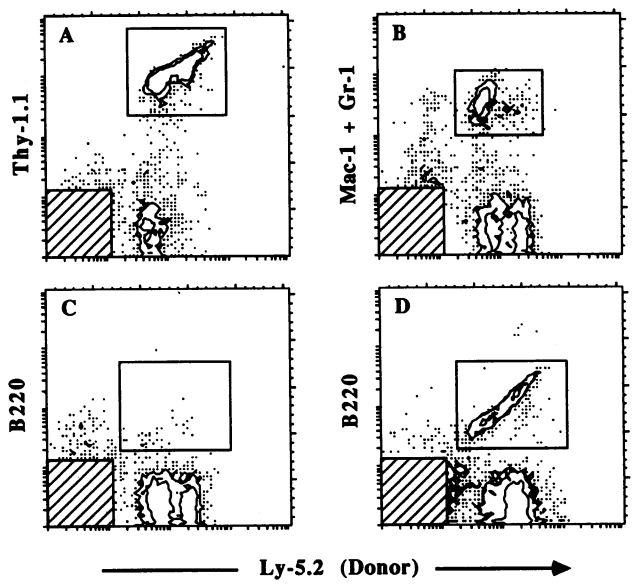

FIG. 3. Development of multiple hematopoietic lineages after intrathymic injection of bone marrow subpopulations. (*A–C*) Analyses of cells from one single thymic lobe that was injected with five cells. (*D*) One lobe that received 5000 cells.

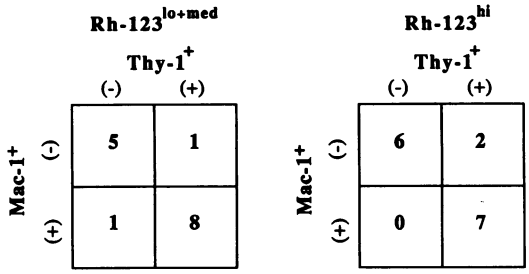

FIG. 4. Resting and activated fractions of Ly6A/E$^+$ cells contain multipotent cells. Five cells of either the Rh-123^{lo+med} or the Rh-123^{hi} subpopulation of Ly6A/E$^+$ cells were intrathymically injected into groups of irradiated (7.5 Gy) mice. Fourteen to 18 days later, thymuses were removed and screened for donor-derived cells by two-color immunofluorescence, as shown in Fig. 3. The numbers represent the number of thymic lobes that contained donor-derived cells expressing the indicated antigen. The average clone size of Thy-1$^+$ cells was 3.3×10^5, whereas that of Mac-1$^+$ cells was 1.6×10^5. All thymic lobes contained from 1 to 7×10^7 host-derived thymocytes.

DISCUSSION

The results presented here lend support to an evolving concept, originally termed the generation-age hypothesis, that visualizes the source of adult mammalian hematopoiesis as a continuum of maturing multipotent stem cells (22). The generation-age hypothesis is in contrast to the notion of a homogeneous group of self-renewing stem cells whose members lose their multipotent nature as they commit to differentiation along unique hemopoietic lineages and exit the hematopoietic stem cell compartment. Taken to its extreme interpretation, the generation-age hypothesis may preclude the concept of self-renewing stem cells, since a maturation step occurring coincident with cell division within the continuum of the hematopoietic stem cell compartment would have the appearance of self-renewal, when in fact both progeny of the dividing cell may simply be maturationally one step further along the continuum. Thus, the hematopoietic stem cell compartment as a whole may generate more members, giving the appearance of self-renewal, even though individual cells within the compartment may differentiate slightly with each round of cell division. If the continuum is long enough, and its most primitive member is present in sufficient supply to meet the hemopoietic demands of the organism over several life-spans, there is no necessity to postulate that the cellular elements of the hematopoietic stem cell compartment must reproduce themselves in order to maintain hematopoiesis; self-renewal may only occur at the compartmental level and not at the cellular level.

The results of the present experiments are in good agreement with previous studies (13, 15, 23, 24) that have demonstrated heterogeneity within the hematopoietic stem cell compartment. Further, the results reported here demonstrate that cells which on a clonal basis are progenitors for lymphoid and myeloid lineages (Fig. 4) can differ in their ability to generate more multipotent cells (Table 3).

The term "pre-CFU-S" implies that the cell associated with this activity is too primitive to form splenic colonies within a 13-day period but is capable of producing day 13 CFU-S as differentiated progeny. In the present studies, the resting population of cells seems to have pre-CFU-S and day 13 CFU-S activities, although the day 13 CFU-S activity is relatively depleted compared to the activated population (Table 1). It is possible that the resting cells as isolated in these studies have a higher affinity for splenic seeding due to the presence of antibodies and immunomagnetic particles on their surface membranes, since no attempt was made to remove these foreign substances prior to intravenous infu-

sion. The spleen, in its role as a blood filter, may sequester the cells due to the surface-bound molecules and thus allow splenic colonization by a larger number of cells than would normally seed there. Ploemacher and Brons (7, 23) have reported isolating a population of cells that contains pre-CFU-S activity in the absence of CFU-S. In those studies, no cell surface labeling methods were utilized to identify the population of cells which were isolated.

Although some evidence has been presented to argue that primitive hematopoietic stem cells primarily seed to irradiated marrow cavities and that only after a period of residence in the marrow do their progeny seed to the spleen (10, 12), Lord and colleagues (25) have recently shown that bone marrow cells that form late (day 12) spleen colonies probably do not have a requirement to seed into marrow spaces initially. Since Lord and colleagues obtained similar results using normal bone marrow cells or cells derived from animals treated with 5-fluorouracil or cyclophosphamide, populations thought to be representative of more primitive hematopoietic stem cells (10), marrow homing may not be a prerequisite for engraftment and survival of these cells.

An understanding of the process of hematopoiesis, including precursor–product relationships, maintenance and regulation of the differentiation of hematopoietic stem cells by growth factors and the stromal cells that produce them (26–29), and the factors dictating the commitments to specific blood lineages, is critical to an adequate control over therapeutic applications of bone marrow transplantation. In addition, contemplation of gene therapy for many blood disorders must include precautions regarding the target cell for gene transfer; not all multipotent hematopoietic stem cells are sufficiently primitive to mediate the sustained production of the products of a transferred gene over many generations (Table 4). It is now critical to devise better methods to identify, isolate, and manipulate the primitive members of the spectrum of multipotent hematopoietic stem cells.

This paper is dedicated to the memory of Cheryl A. Whitlock, who lost her battle with acute myelogenous leukemia on January 21, 1990. Cheryl will be long remembered as an enthusiastic colleague and as a good friend. Her important contributions to the field of experimental hematology include development of a culture system to study B-cell differentiation, generation of unique bone marrow-derived stromal cell lines, and a significant influence in the early studies that led to the work reported here. In the midst of her illness, Cheryl remained enthusiastic and interested enough in scientific conversations to suggest the terminology of "resting" and "activated" stem cells. Cheryl will not be forgotten by those of us who were touched by her life.

Special thanks are given to Roland Scollay and Ken Shortman for providing laboratory space and encouragement during the course of this work, to Frank Battye for operation of the flow cytometer, and to Liz Viney for assistance with the cell cultures. Conversations with Ivan Bertoncello, George Hodgson, and Ray Bradley of the Peter MacCallum Cancer Institute in Melbourne were instrumental to the initiation of this work. G.J.S. is a Special Fellow of the Leukemia Society of America.

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