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Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells

(precursor cells/continuously active stem cells/binomial formula)

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ABSTRACT We estimated numbers of transplantable primitive stem cells (PSCs) and found evidence that the same PSC continuously produced circulating erythrocytes and lymphocytes. These estimations used the binomial formula on data from recipients of identical portions of marrow mixtures containing two distinguishable cell types. Analysis of variance was used to compare repeated tests within each recipient. Values of π_s or π_c, probabilities that two independently sampled cells were descended from the same PSC, were also estimated, as this does not require the unverified condition that all PSCs contribute equally to the differentiated cell population. Several months after transplantation, erythrocytes were descended from only a single PSC per 1–2 × 10^5 marrow cells injected, several times rarer than previously reported. Percentages of erythrocyte and lymphocyte types in each recipient were closely correlated, with *r* values ranging from 0.86 to 0.94, in groups receiving 2–8 × 10^5 marrow cells; apparently the same precursors repopulated both myeloid and lymphoid lines in each recipient, as expected of true PSCs. Our data did not fit the clonal succession model, which predicts sequential activation of new PSCs and deactivation of old. Between 76 and 154 days, differentiated erythrocyte precursors were probably exhausted, with no evidence for new precursor activation or for further change between 154 and 250 days. The percentage of newly produced erythrocytes (reticulocytes) of each donor type varied little when individual recipients were followed between 165 and 295 days after transplantation, and variances within recipients were similar at marrow doses from 8 to 200 × 10^5 cells, further contradicting models of sequential activation and deactivation of PSC clones. Thus, transplanted PSCs were continually active during much of the recipient's lifespan.

Circulating erythrocytes and most lymphocytes are short-lived and must be continually replenished. In producing them, the immune and hemopoietic systems provide a model of mammalian cellular differentiation that continues throughout life. All blood cells are derived from the primitive hemopoietic stem cell (PSC), which has maximal ability to repopulate the immune and hemopoietic systems and to differentiate into both myeloid and lymphoid lineages, as diagramed in Fig. 1. The PSC is an unusual somatic cell type, as it shows no loss of repopulating ability with age (1–3), and stem cells obtained from old mice repopulate the hemopoietic systems of irradiated hosts as well as those obtained from fetal donors (4). Thus the PSC may be a model for primitive cells usually found in embryonal development.

PSCs are difficult to study, as they are extremely rare and cannot be identified or isolated by currently available technology. They are easily confused with more differentiated precursors, such as P (precursor) myeloid [the spleen colo-

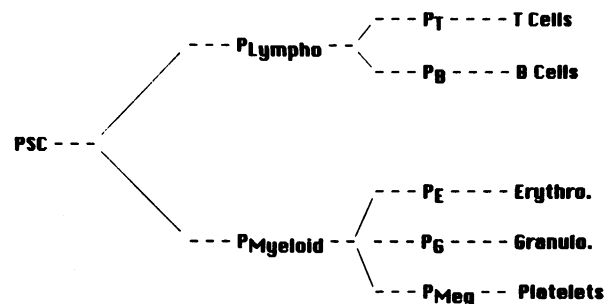

FIG. 1. The differentiation pathways followed by PSCs and more differentiated precursors (P). The PSC is defined by its functions of repopulation and differentiation. Meg, megakaryocyte.

ny-forming unit (CFU-S) of Till and McCulloch (5)] or other precursor (P) cell types outlined in Fig. 1. PSC numbers have been estimated at 1 per 10^4 transplanted bone marrow cells by limiting dilution analysis (6), and their activities have been defined in long-term repopulating assays *in vivo* (1–4, 7). That stem cells are capable of repopulating both myeloid and lymphoid pathways has been demonstrated in three different systems: in recipients of cells with radiation-induced chromosome markers (8, 9); in fetal mice injected with mixtures of cells from strains distinguished by hemoglobin, isozyme, and immunoglobulin variants (10–12); and in progeny of cells identified by unique retroviral vector integration sites (13–18).

Mintz *et al.* (12) found that some recipients showed dominance followed by decline of cells of particular genotypes, suggesting that PSCs are sequentially activated, then exhausted, to be replaced by newly activated PSCs in a clonal succession model as proposed by Kay (19) and Hellman *et al.* (20). Micklem *et al.* (7) reported data supporting this model in recipients of small numbers of marrow cells, as did Lemischka *et al.* (18) in recipients of retrovirus-mediated gene transfer-marked cells.

Numbers of PSCs that form clones contributing to a population made up of two cell types may be estimated by using the binomial formula, in which the number of PSCs (*n*) depends inversely on the variance of the observations (7, 21–23). One of these studies (23) found no evidence for clonal succession in normal (untransplanted) individuals. Instead, PSCs appeared to continuously produce erythrocytes throughout the lifespans of the individuals studied.

Abbreviations: PSC, primitive stem cell; PSC clone, a clone derived from a successfully transplanted PSC; CFU-S, spleen colony-forming unit, a myeloid precursor; B6, C57BL/6J; B6-*Hbb^d^ Gpi-1^a^*, double congenic from B6.C-*H-1^b^*/By and B6.CAST-*Gpi-1^a^*/Ei; S^2, variance; SD, standard deviation; π_s, the one-aggregate probability estimated from the variances that two erythrocytes or two lymphocytes drawn independently at random from a recipient were produced by the same PSC; π_c, the two-aggregate probability estimated from the covariances that a randomly drawn erythrocyte and a randomly drawn lymphocyte, or a randomly drawn erythrocyte at time 1 and another at time 2, were produced by the same PSC.

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In the present study, we transplanted mixtures of bone marrow cells from two types of congenic mouse donors. Use of congenic mice avoids rejection or resistance that may occur with mixtures of cells from different stains or from parent and F_1 hybrid. A wide range of cell doses were used, with 17–21 recipients at each dose. The number of PSCs was estimated by using the variances in percentages of cells descended from each donor type among the recipients of identical marrow doses. Excellent correlations between percentages of each donor type in both myeloid (erythroid) and lymphoid repopulation suggested that true PSCs were being measured.

We tested the clonal succession model in two ways. First, we determined variances in the percentage of each donor hemoglobin type within each recipient between successive samplings at 2- to 3-month intervals. Second, we measured variances in the percentage of newly synthesized reticulocytes tested in the same individual at five successive 13- to 16-day intervals plus one 70-day interval. High variances would be expected between cohorts of reticulocytes produced by different PSCs if only a few PSCs were active at a time.

MATERIALS AND METHODS

Marrow Donors. Since PSC development may be affected by adverse interactions of cells from different mouse strains with disparate alleles affecting histocompatibility or hybrid-allogeneic resistance, congenic C57BL/6J (B6) and B6-*Hbb*^{d} *Gpi-1*^{a} donors and B6 recipients were used. Those in experiment 1 were 10- to 12-week-old males, and those in experiment 2 were 11- to 16-week-old females. All mice were produced and maintained at The Jackson Laboratory, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. B6-*Hbb*^{d} *Gpi-1*^{a} donors were prepared from B6-*Hbb*^{d}/*Hbb*^{d} (B6.C-*H-1*^{b}/By, N15F18) and B6-*Gpi-1*^{a}/*Gpi-1*^{a} (B6.CAST-*Gpi-1*^{a}/Ei, N10F5) congenic lines. The congenic lines were crossed, their F_1 hybrids were interbred, and the B6-*Hbb*^{d} *Gpi-1*^{a} congenic mice were selected.

Marrow Transplantation. B6 recipients were lethally irradiated with 1100 rads (1 rad = 0.01 Gy) in a ^{137}Cs γ irradiator (Shepherd Mark I; J. L. Shepherd & Associates, Glendale, CA) at a dose rate of 220 rads/min 15–18 hr before the marrow was transplanted. Marrow cell suspensions were prepared by using femurs and tibias from each donor, as previously described (3, 4). Cells were injected through 26-gauge needles into the lateral tail veins of warmed mice.

Hemoglobin Electrophoresis. Details of the hemoglobin electrophoretic techniques were similar to those in previous reports (24, 25). B6 mice have *Hbb*^{s} (s, single) hemoglobin, which forms a band migrating farthest from negative to positive on the standard cellulose acetate gels used, while *Hbb*^{d} (d, diffuse) hemoglobin has the *Hbb-b1*^{d} and *Hbb-b2*^{d} bands, which are intermediate and slowest migrating, respectively.

Electrophoresis of Glucosephosphate Isomerase. B6 mice have the *Gpi-1*^{b} allele at the glucosephosphate isomerase (*Gpi-1*) locus; this type forms a band migrating more rapidly from positive to negative than does the *Gpi-1*^{a} allele. Details of the enzyme electrophoretic techniques were similar to those previously reported (26).

Circulating Lymphocyte Separation. Circulating lymphocytes were separated by density as follows: The centrifuged erythrocyte pellet from 0.2 ml of blood was resuspended in 2.0 ml of the saline/citrate mixture, layered over 1.75 ml of Ficoll (LSM solution, Litton Bionetics no. 8410-01) in 10 × 75 mm tubes, and centrifuged for 1 hr at 1600 rpm (IEC Centra-7 swinging-bucket centrifuge with a 6-inch radius). The supernatant was discarded without disturbing the white band of lymphocytes atop the Ficoll. Erythrocytes from the same blood sample were in a pellet at the bottom of the tube and were collected by removing the Ficoll; hemoglobins were analyzed as detailed previously. Erythrocytes and platelets contaminating the lymphocytes were removed by osmotic shock with distilled water, and lymphocytes were collected by centrifugation; on microscopic examination of 2000 cells, all appeared to be lymphocytes by morphological criteria.

Hemoglobin Synthesis by Reticulocytes. Circulating reticulocytes were radiolabeled to quantitate their hemoglobin synthesis by using techniques described previously (23). Peripheral blood was collected from the retroorbital sinuses and incubated with ^{35}S-labeled methionine for 3 hr. Hemoglobins were separated by standard procedures, hemoglobin-containing bands were cut from the cleared gel, and the ^{35}S activity was determined. Data were expressed as the percentage B6 type (cpm in the single band)/(total cpm in the diffuse and single bands).

Calculations. The numbers of precursors that produced erythroid and lymphoid clones (n) were estimated by using the binomial formula, $n = P(100 - P)/S^2$, where P is the mean percentage of B6-type erythrocytes or lymphocytes and S^2 is the variance. The binomial formula can be derived by applying Pearson's (27) method of moments. Its use requires that each measurement of the percentage B6 type be independent, a criterion clearly met for variation between recipients, since each was given a separate portion of the same cell dose. Use of this calculation also assumes that the genotypes measured in circulating erythrocytes or lymphocytes reflect the proportions of active PSCs of those genotypes. These assumptions are reasonable because the strains used have uniform genetic backgrounds, minimizing possible genotypic selection of proliferating cells.

Estimates using the binomial formula make the unverified assumption that all PSCs contribute equally to the differentiated cells that are sampled. A model not requiring equal contributions from each PSC was developed by Stone (28). π_s is defined as the probability that two cells drawn independently at random from a recipient were produced by the same PSC. $\pi_s = S^2/[P(100 - P)]$; thus $1/\pi_s$ is n, the number of PSCs calculated by the binomial formula in the special case when all precursors contribute equally to the differentiated cell population.

Pearson's product-moment correlation coefficient (r) was used to measure the relationships between percentages of B6-type erythrocytes and lymphocytes in the same blood sample, or erythrocytes in successive samples, from each recipient. Covariances were calculated as r(SD erythrocytes)(SD lymphocytes), or r(SD time 1)(SD time 2). π_c = covariance/$[P(100 - P)]$, where π_c is the probability that a randomly drawn erythrocyte in a recipient was produced by the same PSC as a randomly drawn lymphocyte, or that an erythrocyte drawn at time 1 was produced by the same PSC that produced one drawn at time 2. For estimates of numbers of equally sized clones from $1/\pi_c$ to be meaningful, the two SDs should be similar and r should be high.

RESULTS

In experiment 1, erythrocytes and lymphocytes were tested 104 days after marrow transplantation (Table 1). In recipients of 2, 4, and 8 × 10^5 cells, π_s values were 1/1.5, 1/2.6 and 1/7.3, giving concentrations of erythrocytes per 10^5 injected marrow cells of 0.7 to 0.9. Concentrations of lymphocyte precursors were similar, 1.1 to 1.5, for the same recipients, and percentages of B6 erythrocyte and lymphocyte types were closely correlated. Calculated π_c values for common erythroid-lymphoid precursors were 1/2.2, 1/3.5, and 1/9.7, giving PSC concentrations of 0.9 to 1.2 per 10^5

Table 1. Numbers of PSCs producing circulating erythrocytes and lymphocytes (experiment 1)

No. cells injected $\times 10^{-5}$	Erythrocytes				Lymphocytes				Erythrocytes and lymphocytes*			
	% B6 type				% B6 type							
	Mean	SD	$1/\pi_s$	PSCs per 10^5 injected	Mean	SD	$1/\pi_s$	PSCs per 10^5 injected	r	Co-variance	$1/\pi_c$	PSCs per 10^5 injected
2	46	40.4	1.5	0.8	53	33.1	2.2	1.1	0.86	1150	2.2	1.1
4	51	31.2	2.6	0.7	58	24.0	4.3	1.1	0.94	704	3.5	0.9
8	58	18.4	7.3	0.9	66	14.0	12	1.5	0.94	242	9.7	1.2
20	53	7.8	45	2.2	63	6.5	67	3.4	0.71	36	68	—
80	53	5.4	104	1.3	62	4.9	146	1.8	0.47	12.4	197	—

Lethally irradiated B6 mice were given doses of 1×10^5, 2×10^5, 4×10^5, 10×10^5, or 40×10^5 cells from each donor marrow type, B6 and B6-*Hbb*^{d} *Gpi-1*^{a} from the same pools. Thus marrow doses totaled 2, 4, 8, 20, or 80×10^5 cells and all recipients of a given dose were injected i.v. with identical portions from the same mixture. Numbers of recipients in groups receiving each dose ranged from 18 to 21. The same blood samples were tested for both erythrocyte and lymphocyte values at 104 days. Controls for erythrocytes were independently sampled WBB6F1 hybrids, with one parent having single and the other having diffuse type hemoglobin giving an SD of 2.4, for 22 samples with a mean of 54%. Controls for lymphocytes were repeated, independent samplings from a 50:50 mixture of B6 and B6-*Hbb*^{d} *Gpi-1*^{a} blood giving an SD of 2.7 for 20 samplings with a mean of 48%. SD is the standard deviation about the mean. $1/\pi_s$ was estimated as $P(100 - P)/[(\mathrm{SD})^2 - (\mathrm{SD\ control})^2]$, where P is mean % B6-type hemoglobin, SD is standard deviation about the mean, and SD control is the standard deviation of the control group. π_s is the probability that two erythrocytes or two lymphocytes drawn independently at random from a recipient were produced by the same PSC. $1/\pi_s$ is n (the number of PSCs) calculated by the binomial formula, which is valid if the PSCs contribute equally to the differentiated cell population. This value is used to derive the number of PSCs per 10^5 injected marrow cells.

*The r values are Pearson's product moment correlation coefficients relating percentages of B6-type erythrocytes and lymphocytes in the same recipients. $1/\pi_c$ is estimated as $P(100 - P)$/covariance, where P is the mean % B6 type in erythrocytes and lymphocytes; π_c is the probability that a randomly drawn erythrocyte was produced by the same PSC as produced a randomly drawn lymphocyte. The number of common erythroid–lymphoid precursors per 10^5 transplanted marrow cells is given only when the two SDs are similar and $r > 0.75$.

(Table 1). Apparently most erythrocytes and lymphocytes were produced by the same precursors; thus these precursors appear to be true PSCs. Correlations for recipients of higher marrow cell numbers were lower, probably because variances were smaller so that random noise was more important.

Data on hemoglobin types in a second experiment were analyzed by covariance to relate erythrocyte populations sampled at days 76 and 154 or at days 154 and 250 (Table 2). Covariances and other values were not determined when correlations between percent B6-type hemoglobin at the two different times were not significant. Correlations were much better at 154 and 250 days than at 76 and 154 days. Values of π_c were 1/6.5 and 1/12.6, giving 0.8 and 0.6 precursors per 10^5 cells at 154 and 250 days, for recipients of 8 and 20×10^5 cells. Correlations were less good in groups with high cell doses, especially 200×10^5 marrow cells, probably because variabilities were small and mostly resulted from random noise; thus precursor numbers in these groups were not estimated. The poor correlations between 76 and 154 days may have resulted from exhaustion by 154 days of differentiated erythrocyte precursors from the original marrow transplant. These may have remained active at 76 days.

To directly test the clonal succession model, we measured variances within 20 recipients by repeatedly sampling 5 recipients of each cell dose in experiment 2 (Table 3). Percentages of freshly synthesized B6-type hemoglobin in newly released erythrocytes (reticulocytes) were measured so that each sample would test an independent cohort of erythrocytes. Blood samples were taken at five 14-day intervals and one 70-day interval from 165–295 days after the transplantation. Variabilities during that period are shown by the SDs and variances within each individual in Table 3. Variances (S^2) within each recipient were separated from variances between individuals and between the six different measurements. Variances within individuals were small, ranging from 13.6 to 18.3, and did not change with cell dose. If sequential activation of different PSCs were taking place, variances within recipients would have been much higher in recipients of low marrow cell numbers.

DISCUSSION

PSC Numbers. Estimates of PSC numbers made by using the binomial formula are based on a model in which each PSC contributes equally to the differentiated cells sampled. This assumption is not required to calculate π_s or π_c (28). Probabilities that an erythroid cell and a lymphoid cell drawn at random from the same recipient were produced by the same PSC were inversely proportional to the numbers of

Table 2. Covariance analysis of B6 hemoglobin type (experiment 2)

No. cells injected $\times 10^{-5}$	Days 76 and 154							Days 154 and 250						
	% B6-type Hb							% B6-type Hb						
	Mean	SD_1	SD_2	r	Co-variance	$1/\pi_c$	PSCs per 10^5 injected	Mean	SD_2	SD_3	r	Co-variance	$1/\pi_c$	PSCs per 10^5 injected
8	45	11	21	0.59*	130	19	—	49	21	21	0.89	382	6.5	0.8
20	45	7.2	15	0.29†	—	—	—	45	15	14	0.92	196	12.6	0.6
80	44	3.5	4.5	−0.19†	—	—	—	46	4.5	5.9	0.60*	15.8	157	—
200	46	3.5	3.2	0.22†	—	—	—	47	3.2	3.4	0.40*	4.3	578	—

Experiment 2 was similar to experiment 1 (Table 1) except that recipient groups of 18, 17, 20, and 21 were given doses of 4×10^5, 10×10^5, 40×10^5, and 100×10^5 cells, respectively, of each marrow type from the same pool. Hemoglobin was identified after 76, 154, and 250 days. SD_1, SD_2, and SD_3 are the standard deviations about the mean at 76, 154, and 250 days, respectively. The r values are correlation coefficients between percentages of B6-type hemoglobin in the same recipients after times 1 and 2 or times 2 and 3. Covariance is calculated as correlation coefficient (r) between erythroid % B6 types × both SDs. $1/\pi_c$ is calculated and used as described for Table 1 and π_c is the probability that a randomly drawn erythrocyte at one time was produced by the same PSC as produced an independent randomly drawn erythrocyte at the other time (28).

*When correlation coefficients were not high (0.75 or more), and the two SDs were not similar, concentrations of PSCs were not estimated.

†These correlation coefficients are not significant, with $P > 0.10$ that they were found by chance. Therefore covariances, $1/\pi_c$, and numbers of PSCs are not calculated. Significances of r were calculated by using table A11 of ref. 29.

Table 3. Variabilities of newly synthesized hemoglobins made by reticulocytes of individual recipients

No. cells injected $\times 10^{-5}$	SD of % B6-type Hb					Mean SD	Mean % B6 Hb	S^2 within recipient*
	Animal no. 1	Animal no. 2	Animal no. 3	Animal no. 4	Animal no. 5			
8	3.0	5.4	3.4	4.6	3.3	3.9	54	15.2
20	3.7	3.6	4.1	7.4	0.8	3.9	57	18.3
80	5.9	3.6	4.8	2.9	2.8	4.0	50	16.0
200	4.4	3.7	3.6	2.4	7.5	4.3	47	13.6
0 (control F_1)	1.9	1.5	2.5	—	—	2.0	48	—

Five recipients from experiment 2 that had the % B6 type closest to 50% at 154 days were selected from each dose group. Starting at 165 days, for the next 60 days, blood samples were taken at five 13- to 16-day intervals and percentages of newly synthesized B6-type hemoglobin were determined after incubation with [^{35}S]methionine. After 70 days more, a sixth point was taken, with only minor changes. Thus each SD (standard deviation) above represents six determinations in each recipient, and the mean values of SD, % B6-type Hb, and S^2 within each group represent 30 determinations.

*Variances (S^2) within each recipient in each group were calculated by an analysis of variance for repeated measures, correcting for the variance between the five recipients in each group and for the group trend between each of the six time periods when tests were done.

marrow cells injected, for recipients of 2, 4, and 8 $\times$ 10^5 marrow cells (Table 1). The same was true for the probabilities that an erythrocyte drawn at 154 days was produced by the same PSC that produced one drawn at 250 days for recipients of 8 and 20 $\times$ 10^5 marrow cells (Table 2). If each PSC contributes equally to the population, $1/\pi_c$ will be the PSC concentration, ranging from 0.6 to 1.2 per 10^5 in the foregoing examples. If PSCs contribute unequally, the numbers of PSCs estimated will tend to reflect the major contributors and underestimate numbers of precursors that produce fewer differentiated cells (28).

The number of transplantable PSCs estimated by this study, 1 per 1–2 $\times$ 10^5 marrow cells, is 1/10th to 1/20th the estimate by Boggs *et al.* (6). Perhaps the rigorous selection of forcing a single precursor cell to cure the W/W^v anemia allowed early differentiated cell types, such as myeloid precursors, to function as erythroid stem cells in the limiting dilution experiments of Boggs *et al.* These cells would not have repopulated the lymphoid system and would have been more numerous than true PSCs. They would not have been able to repopulate the erythroid system in competition with a true PSC in the mixtures of cells that we used. A recent report by Micklem *et al.* (30) suggested that 2.5 precursors per 10^5 marrow cells injected produced clones, a concentration much closer to the concentrations found by us. The calculations of Micklem *et al.* used the binomial formula on variances between individual recipient organs. Visser *et al.* (31) showed that 30-day radioprotective ability copurified with 65% pure 12-day CFU-S with an ED_{50} of 170 sorted cells; however, there was no measure of long-term repopulation. Thus, true PSC functions were not tested, and the purified CFU-S may have provided 30 days of radioprotection although they are differentiated myeloid precursors.

Precursors defined in our study met both criteria for true PSCs (Fig. 1). They repopulated and produced differentiated cells for at least 250 days (Tables 2 and 3), and the same precursors produced both myeloid and lymphoid descendants, as shown by the close correlations between percentages of donor erythrocytes and lymphocytes in the same recipients (Table 1). In this study, as in all transplantation studies, some precursor cells may not have survived after being transplanted. This possibility is suggested by the deleterious effects of a single transplantation on long-term repopulating ability (3).

Continuous Activity of the PSC. The extremely high correlations in recipients of 8 and 20 $\times$ 10^5 cells between 154 and 250 days (Table 2) suggest that the transplanted PSC clones continuously produce descendants. This is also suggested by the small variances in mice repeatedly sampled between 165 and 295 days (Table 3). If sequential activation of PSCs were taking place, correlations would have been poorer in Table 2, and variances within recipients would have been much higher in Table 3, especially in recipients of low marrow cell numbers.

The conclusion that active PSCs continuously produce progeny contradicts findings by Mintz *et al.* (12) and Lemischka *et al.* (18) that stem cell clones are sequentially activated and inactivated. In the former study, mixtures of cells from two different mouse strains were engrafted in fetal hosts of a third strain, and the genetic differences between cell types may have caused the rise and fall of particular cell types. In the study by Lemischka *et al.* (18), donor cells were cocultivated for 48 hr with virus-producing cells, then nonadherent cells were harvested. This treatment may have removed the true PSCs, as nonadherent cells have reduced repopulating ability in marrow cell cultures (32), leaving differentiated precursors that became sequentially exhausted. Such precursors may be able to resume function if levels of hemopoietic stimuli increase, as would happen if they were the only precursors remaining. A second possibility is that viral integration occasionally causes cells to proliferate abnormally, producing high percentages of differentiated cell types, but only for a limited time. Even if this were a rare event, the procedures used by Lemischka *et al.* would tend to detect such cells.

When variance among recipients of identical mixtures is used to study precursor cells, results are based on entire populations. Conclusions are not overly influenced by exceptional cases as may occur when only a few marked cells are followed. Our results are consistent with the conventional picture that hemopoietic stem cells are organized in a complex hierarchy, with the most primitive cells having the most repopulating and differentiating ability (Fig. 1; ref. 15). Transplants of 2–20 $\times$ 10^5 marrow cells appeared to exhaust the differentiated precursors after 104–154 days, leaving PSCs at a concentration of 1 per 1–2 $\times$ 10^5 marrow cells that functioned continuously to produce circulating erythrocytes and lymphocytes over much of the recipient's lifespan. The techniques in this report should be useful to define PSC numbers and proliferative behavior in many types of immunohemopoietic cell populations.

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