Hematopoietic Stem Cells with High Proliferative Potential

ASSAY OF THEIR CONCENTRATION IN MARROW BY THE FREQUENCY AND DURATION OF CURE OF W/W^v MICE

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ABSTRACT This study was designed to approach two primary questions concerning hematopoietic stem cells (HSC) in mice: what is the concentration of HSC with extensive proliferative potential in marrow, and how long can an HSC continue to function in an intact animal? The assay system was the W/W^v mouse, a mouse with an inherited HSC defect, reflected in a reduction in all myeloid tissue and most particularly in a macrocytic anemia.

A single chromosomally marked HSC will reconstitute the defective hematopoietic system of the W/W^v. The concentration of HSC in normal littermate (+/+) marrow was assayed by limiting dilution calculation using cure of W/W^v as an end point (correction of anemia and erythrocytes' macrocytosis) and found to be $\sim 10/10^5$. This is significantly less than spleen colony forming cell (CFU-S) concentration: $\sim 220/10^5$ in +/+ and ranging from 50 to $270/10^5$ in various other studies. Blood values were studied at selected intervals for as long as 26 mo. Of 24 initially cured mice, which were observed for at least 2 yr, 75% remained cured. However, of all cured mice, 17 lost the cure, returning to a macrocytic anemic state. Cured mice had normal numbers of nucleated and granulocytic cells per humerus and a normal concentration of CFU-S. However, cure of secondary W/W^v recipients by this marrow was inefficient compared with the original +/+ marrow. These studies suggest the CFU-S assay overestimates extensively proliferating HSC or perhaps does not assay such a cell. A single such HSC can not only cure a W/W^v, but can sustain the cure for 2 yr or more, despite a relative deficit of cells capable of curing other W/W^v. However, the duration of sustained cure may be finite.

INTRODUCTION

Hematopoietic stem cells (HSC)[1] are capable of self-replication and of differentiation into mature blood cells. The exact structure of the HSC system has not been delineated, but in both mouse and man there appears to be a concatenated series of HSC with an increasingly limited degree of pluripotentiality as cells move through HSC compartments. Chromosomal and morphologic studies in mice (1) and clinical, chromosomal, and isoenzyme studies in human diseases (2) indicate the presence of an HSC totipotent (THSC) for all common classes of blood cells, the lymphocytic as well as the myeloid series. In mice, there is also evidence for a cell pluripotent for myeloid (PMSC) but not for lymphoid tissue (1); a presumed descendant of the THSC. The only reported method that may assay THSC or PMSC quantitatively in vivo is the spleen colony system in irradiated mice (CFU-S, colony-forming units in spleen) (3).

The W/W^v mouse has a recessively inherited syndrome characterized by a hypoplastic marrow accompanied by macrocytic anemia, the result of a poorly defined defect in its THSC and/or PMSC (4, 5). If an adequate amount of marrow from a normal histocom-

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[1] *Abbreviations used in this paper*: CFU-S, colony-forming units in spleen, in irradiated mice; Hct, hematocrit; HSC, hematopoietic stem cells; PMSC, pluripotent myeloid stem cells; RBC, erythrocytes; THSC, totipotent HSC.

patible mouse is injected into W/W^v, the normal stem cells grow preferentially, repopulate the entire hematopoietic system and cure the mouse with respect to its hematopoietic defects (5, 6). Breivik (7) used the technic of limiting dilution to determine the concentration of progenitor cells in marrow that produce granulocytes and macrophages in diffusion chambers. Multiple small volumes of a homogenous marrow suspension were placed in chambers and progenitor cell concentration was calculated from the proportion of resultant empty chambers. We have used this type of assay in W/W^v mice to estimate the concentration of THSC and PMSC in normal littermate (+/+) marrow by determining the proportion of W/W^v that were or were not cured by 10^2–10^7 +/+ cells. The major assumption underlying this study was that a single THSC or PMSC could cure the myeloid system defect of W/W^v mice, an assumption subsequently shown to be probably true by Abramson et al. (1) by injecting chromosomally marked cell clones into W/W^v mice.

The second purpose of this study was to further investigate the proliferative potential of THSC, PMSC, and their progeny. The work of Hayflick (8) and others (reviewed in references 9 and 10) has suggested that nontransformed, nonmalignant mammalian cells may have a limited and finite proliferative potential when in culture. There is somewhat conflicting data on the proliferative potential of HSC in vivo (Discussion). To further study this question W/W^v mice cured by small doses of marrow cells were followed for maintenance of cure for as long as 2 yr and, in selected mice, total nucleated and peroxidase-positive cells per humerus were determined and marrow was tested for CFU-S content and ability to cure other W/W^v mice.

METHODS

Mice were W/W^v or their normal (+/+) or heterozygous (W/+ or W^v/+) littermates, purchased from Jackson Laboratories, Bar Harbor, ME, or bred in our laboratories from parents purchased from Jackson Laboratories (C57BL/6J-W^v × WB)F$_1$. W^x/W^v mice (C3H/HeJ-W^x × C57BL/6J-W^v)F$_1$ have a defect seemingly identical to W/W^v and in one experiment W^x/W^v or their normal or heterozygote littermates were used. In addition (C57BL/6J × WB)F$_1$ and (C3H/HeJ × WB)F$_1$ +/+ mice were used as recipients in certain CFU-S studies. Mice of either sex and of various ages were used, but for groups being compared in each experiment, sex and age of recipients and/or donors were matched. They were housed 1–10/cage and food and HCl water were available ad lib.

For sequential examination of the blood of individual mice, the retroorbital sinus was punctured with a heparinized microhematocrit tube and an attempt was made, usually successful, to restrict blood loss to <0.15 ml with each puncture. Erythrocytes (RBC) count and relative RBC size (cell size) were determined electronically on an aliquot of blood (Coulter counter, model ZBI, Coulter Electronics Inc., Hialeah, FL) and a hematocrit (Hct) determined on the remainder in a microhematocrit centrifuge calibrated to yield values similar to those obtained by the Wintrobe method (11). On each occasion, values in W/W^v were compared with values in age-matched +/+ controls, both of which had been bled the same number of times. Since control values varied modestly from time to time, all values were normalized to a mean control value of 100%; mean absolute control values for RBC (10±1 million/mm^3) and Hct (49±1%). Cell size was determined from the peak (mode) of a plot using a model ZB Coulter counter. Position of the peak is determined in arbitrary units and peak value for treated mice was determined in relation to peak value for +/+ controls studied on the same day. Experiments in which W/W^v and +/+ blood was mixed in variable proportions in vitro indicated that the presence of 20% W/W^v cells was easily detected by visual inspection of these size curves, in agreement with recent reports (12, 13).

Cure was defined as a loss of anemia and macrocytosis (Hct and/or RBC count and cell size within 95% confidence limits of +/+ values) on at least two consecutive studies done >4 wk apart. When such values were normal on only one occasion or when there was loss of macrocytosis with persistent anemia, the mouse was said to have a questionable cure (?cure). On rare occasions, anemia was corrected in the presence of persistent macrocytosis and this was considered to be an uninterpretable observation. Loss of cure was defined as a return to anemic, macrocytic values on two consecutive determinations in a previously cured mouse, and ?loss as a return to anemia and macrocytosis on only one determination.

To eliminate the possibility of iron deficiency being responsible for development of recurrent anemia without macrocytosis, mice at risk were given iron dextran, 0.5 mg/mouse, i.m., but this had no effect on anemia in any mouse.

Marrow cells for injection were flushed from a femur into Hanks' balanced salt solution and passed six times through a 21-gauge needle to produce a unicellular suspension. Concentration of nucleated cells was determined electronically and appropriate dilutions were made with Hanks' solution to produce the desired number of cells in 0.25 or 0.5 ml for i.v. injection. Total nucleated cells per humerus were determined by a similar, but quantitatively more precise flushing technic (14), and the percentage of peroxidase positive cells was determined by filtering an aliquot of the suspension, staining the cells on the millipore filter and counting 500 consecutive cells.

The ability of marrow to produce hematopoietic spleen colonies in irradiated +/+ recipients was tested by injecting 10^5 to 10^6 nucleated marrow cells, prepared in the above manner, into recipients within 1–2 h after 875 to 950 rads whole body irradiation. Recipients were irradiated in a cesium small animal irradiator (Mark IV, J. L. Sheperd & Associates, Glendale, CA) in a lucite chamber with a source to mouse distance of 19 cm and with a dose rate of 126 rads/min, measured by Fricke ferrous sulfate dosimetry. Recipients were killed 8 d later, having been injected with 1.0 μCi of ^{59}Fe 16–18 h before. Their spleens were removed, weighed, placed in Bouin's fixative, and the radioactivity per spleen was determined in a well-type scintillation counter and expressed as a percentage of the total injected dose of ^{59}Fe; macroscopic spleen colonies were counted. Selected spleens were sectioned, stained with hematoxylin and eosin and longitudinal midsections examined for the type of cells contained within colonies.

Breivik (7) has reviewed the basis of limiting dilution analysis in detail and briefly, it is carried out as follows. Random samples of a homogeneous cell population are assayed for

the presence or absence of stem cells and such cells should be distributed between samples in a Poisson fashion. The finite probability that a sample will not contain a stem cell is defined by $P_{(0)} = e^{-\phi x}$, where ϕ is the fraction of stem cells in the sample of x cells. P is the proportion of samples devoid of stem cells and estimates $P_{(0)}$. The concentration of stem cells in the sample can then be expressed as $\phi = -(\ln P)/X$.

In the CFU-S method, cells from a single donor may be given to a large number of recipients and the mean and variance of the resultant number of colonies primarily reflects the reproducibility of the assay. Since we were interested in variability of the concentration of CFU-S in donor marrow, comparisons between groups of more than three donors were made using Student's *t* test and were based on the means of the colonies seen in recipients from each donor, rather than the individual colony number seen in recipients.

RESULTS

150 W/W^v or W^x/W^v mice were injected intravenously with 10^2–10^7 nucleated marrow cells from +/+ controls (frequently from littermates). RBC size, Hct, and RBC count were determined in each mouse on at least three occasions, 1–5 mo later. Examples of these data from groups of mice are shown in Fig. 1. Cure was observed in ~5% of those given 10^2 or 10^3 cells, in 50% given 10^4 cells, and in virtually all given 5×10^4–10^7 cells (Table I). Limiting dilution calculation of the stem cell concentration in marrow suspensions ranged from 0 to 36 with a median of $9/10^5$ and a mean of $11/10^5$ (Table I).

If an unequivocal cure was achieved, (normal cell size and no anemia on two consecutive studies [Methods]), it usually was evident by 3 mo after cell injection. Macrocytosis tended to disappear before anemia was fully corrected. 47 of the eventually cured mice were studied at 3 mo and 44 were cured by that time (94%). In contrast, 78% of those eventually cured were cured by 2 mo (43/55 studied) and only 25% were cured by 1 mo (7/28 studied). There was a suggestion that mice given a relatively large dose of cells were cured faster than those given a smaller dose. Of mice eventually cured, studied at 2 mo, 88% of those given 10^5–10^7 cells were cured (22/25), 79% of those given 5×10^4 (15/19), and 55% of those given 10^2–10^4 (6/11).

Cured mice were sequentially studied for a minimum of 12 mo from cell injection and some surviving mice were studied for as long as 26 mo. Of 24 initially cured mice, observed for at least 2 yr, 75% remained cured (Table II). Of all cured mice, 17 were observed to return to a macrocytic, anemic state; i.e., lost their cure (Methods) (Table II). The probability of losing the cure was not significantly higher in mice cured by a relatively large as compared with a small cell dose, nor was loss of cure obviously more frequent as duration of cure increased (Table II).

Cured mice were killed for further studies at selected intervals from 12 to 26 mo after cell injection or were killed for further study on an ad lib. basis if they began to appear ill. Total nucleated cells per humerus and peroxidase-positive cells (granulocytes) per humerus are reduced in W/W^v as compared with +/+ (15), and these values were found to be normal in the cured W/W^v. In 11 cured W/W^v, total cells per humerus were 11.1±0.5 million compared with 9.1±0.5 in 4 age-matched +/+ and 8.8±0.2 in 10 previously studied +/+. Three age-matched W/W^v that had not been cured had 5.9±0.9 million cells/humerus, similar to the values previously reported of 6.2±0.3 (15). Peroxidase-positive cells per humerus in cured W/W^v were 4.3±0.3 million compared with 4.0±0.4 in age-matched +/+, and 1.9±0.3 in age-matched, uncured W/W^v. Four previously cured W/W^v were studied when anemia had recurred, but macrocytosis had not (?cured, see Methods). These mice probably were still "cured" since total nucleated cells were 12.8±0.2 million/humerus and peroxidase-positive cells were 4.5±0.8 million/humerus. As W/W^v age, many develop a chronic, inflammatory skin rash associated with spotty hair loss and at times with frankly infected (or at least necrotic) skin lesions. Mice that were ?cured or those that were cured and then became ?cured without again becoming macrocytic (Fig. 1) often suffered from such inflammatory skin afflictions. However, results from mice that appeared to have frank infections were discarded. In a healthy-appearing, noninfected W/W^v, which had clearly lost its cure, total nucleated cells were 6.2 and peroxidase-positive 1.6 million/humerus.

Marrow cells from 12 cured W/W^v were used in attempts to cure other W/W^v (Table III) and found to be less effective than +/+ marrow cells. No difference in the effect of cells from mice cured by different cell doses or for different periods of time could be discerned, so results from all mice were combined. The 10^5 cell dose, which would be routinely curative if from the original +/+ donors (Table I), cured no secondary W/W^v recipients, and $1–2 \times 10^6$ cells was less effective than expected (Table III). However, when larger doses ($5–15 \times 10^6$ cells from cured W/W^v were given, virtually all secondary W/W^v recipients were cured. Large doses ($5–8 \times 10^6$) of cells from four previously cured W/W^v that had again become anemic, but not macrocytic (?cured), cured secondary recipients more effectively than cells from those in whom the cure was lost or probably lost (Table III, $P < 0.05$). As expected, no cures were seen with marrow from W/W^v that were injected with +/+ cells but had not ever been cured (Table III).

Marrow cells from most of the above mice also were studied for their ability to produce CFU-S in irradiated +/+, W/+ or $W^v/+$ recipients (Table IV). The num-

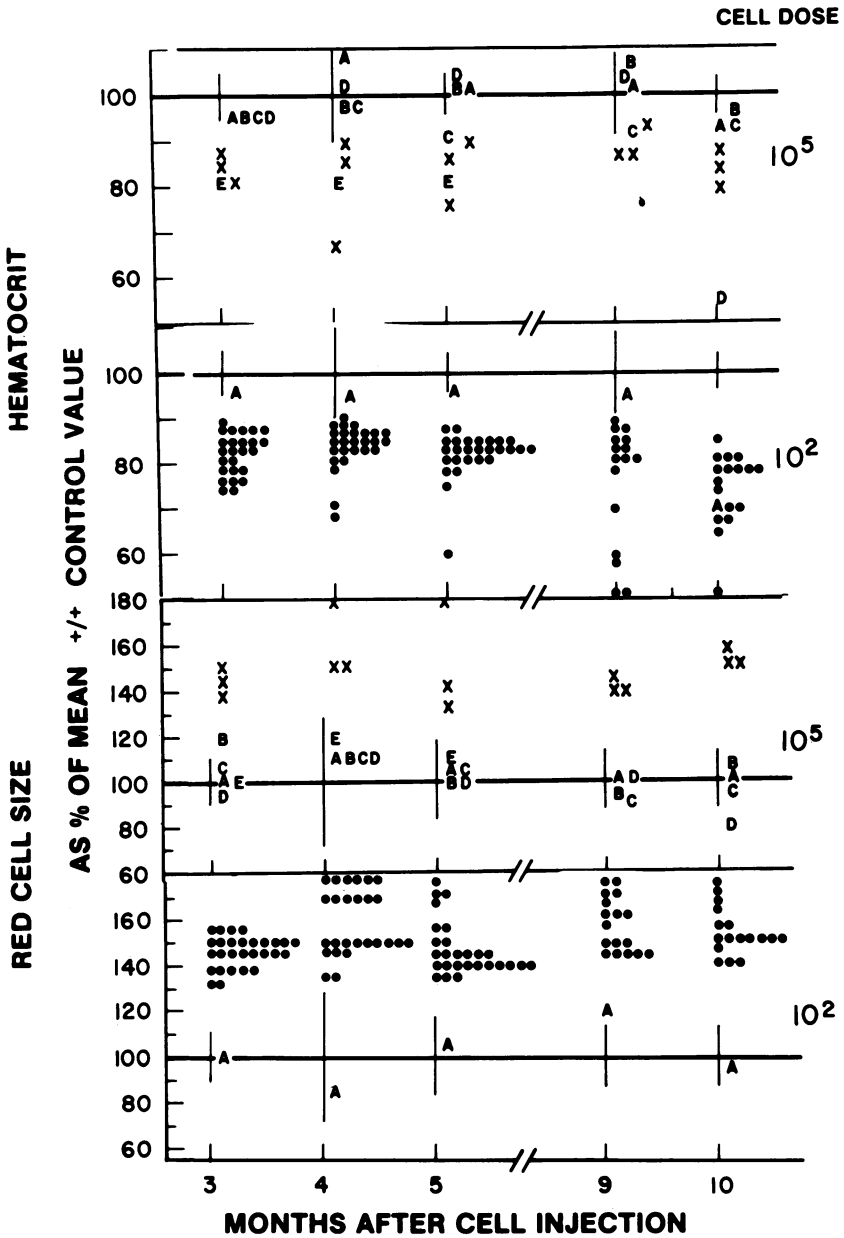


FIGURE 1 Hct and RBC size in W/W^v mice 3–10 mo following injection of 10^2 or 10^5 +/+ cells. The letters represent values from individual mice cured or ?cured; solid dots from mice injected but not cured; and X from age-matched W/W^v that were not injected. The 100% line represents the mean value of 3–9 age-matched +/+ mice and the vertical bars the 95% confidence limits for the group on that day of study.

ber of CFU-S per 10^5 injected cells was not significantly different in W/W^v that were cured or that had been cured and were still questionably cured, than in +/+ donors (14–20 CFU/10^5 cells). Spleen weight and uptake of ^{59}Fe per spleen reflected colony number (Table IV). In four mice that had lost the cure CFU-S were still present, but in slightly reduced concentration (8–9 CFU/10^5) as compared with cured W/W^v or +/+ ($P < 0.05$) (Table IV). As expected, no macroscopic colonies were produced by marrow from W/W^v that had been given +/+ marrow without being cured, and the spleen weight and ^{59}Fe uptake of recipients of these cells did not differ from that of irradiated mice given no cells (Table IV).

TABLE I

Cure Rate of Anemia and Macrocytosis in W/W^v Given Varying Doses of +/+ Marrow Cells

Cell dose	No. cured/injected* Experiment 1	Experiment 2	Experiment 3§	Calculated‡ stem cells/10^5 cells Experiment 1	Experiment 2	Experiment 3
10^2	1/28			36		
10^3	1/11		0/15	10		0
10^4	7/11	11/20	4/10	10	8	5
5×10^4		19/19				
10^5	5/5	5/5				
10^6		7/7	7/7			
10^7		7/8				

* Excludes a few mice that died after cell injection and before first study.

‡ Limited dilution technique, see Methods (7).

§ W^x/W^v recipients; W/W^v in experiment 1 and 2.

The histologic appearance of spleen colonies produced by cells from cured W/W^v was examined. Of 109 colonies in spleens of recipients of cells from seven cured W/W^v, 67% were composed predominantly of erythroid cells, 10% of granulocytic cells, 1% were megakaryocytic, and 22% were predominantly immature, morphologically unidentifiable cells. This proportion of colony type is approximately that expected in 8-d spleen colonies derived from normal marrow cells (16).

TABLE II

Persistence or Loss of Correction of Anemia and Macrocytosis in W/W^v Mice Initially Cured by Injection of +/+ Marrow Cells

Cell dose	Observation period (months) 6–11	12–18	19–24	Total‡
	Number known to have lost cure during period/total number observed during period*			
10^2–10^4	6/20 (30%)	1/17 (6%)	2/4 (50%)	9/41 (22%)
5×10^4	1/19 (6%)	0/15 (0%)	2/15 (13%)	3/49 (6%)
10^5–10^7	2/18 (11%)	1/8 (13%)	2/5 (40%)	5/31 (16%)
Total‡ mice observed during period	9/57 (16%)	2/40 (5%)	6/24 (25%)	17/121 (14%)

* Total number observed equals those known to be cured at beginning of period and still cured at end of period, plus those known to have lost cure during the period.

‡ The total number of mice in these columns exceeds the actual number of W/W^v cured in this report, since a mouse may appear in more than one period of study.

TABLE III

The Ability of Marrow from W/W^v, Cured Previously by Injection of +/+ Cells, to Cure Other W/W^v

Status of W/W^v donor at time of study	Cell dose	No. of donors	Recipient Cured	?Cured	Not cured	Cured %
Cured	10^5	(1)	0	0	5	0
	1–2 $\times$ 10^6	(7)	9	2	13	38
	5–15 $\times$ 10^6	(4)	14	1	0	93
?Cured	5–8 $\times$ 10^6	(4)	17	0	1	94
?Lost cure	6–7 $\times$ 10^6	(2)	2	4	4	20
Lost cure*	10^6	(1)	0	0	3	0
	15 $\times$ 10^6	(1)	0	0	5	0
Never cured	10 $\times$ 10^7	(2)	0	0	10	0

* Three other mice with lost cures were studied; >5 $\times$ 10^6 cells from them cured no W/W^v, but they were all infected (skin and ears).

The concentration of cells forming spleen colonies is an underestimate of overall CFU cells in the suspensions, because the majority of such stem cells that seed and grow do so in the bone marrow rather than in the spleen. An estimate of the fraction of CFU cells measured by spleen colonies (f_2) was obtained by the technic described by Siminovitch et al. (17) as modified by Fred and Smith (18). In brief, CFU-S are measured in one aliquot of a suspension of cells and a larger aliquot is given to a second set of irradiated recipients. Spleens are removed a few hours later from the second set and CFU-S per spleen were measured (Fig. 2). The assumption is made that on the first transfer a fraction (f_1) of the actual number of CFU (N) makes colonies (i.e., f_1N = colonies). On the next transfer $f_2(f_1N)$ colonies are formed. Thus:

$$\frac{\text{2nd colony count}}{\text{1st colony count}} = \frac{f_2(f_1N)}{f_1N} = f_2.$$

The f_2 was measured using both +/+ and W/W^v as recipients of +/+ cells with resultant values of 14 and 12%, respectively (Fig. 2).

DISCUSSION

Certain of these observations concerning HSC provide new information and others confirm, extend, and com-

TABLE IV

Splenic Hematopoiesis in Irradiated Recipients of Marrow

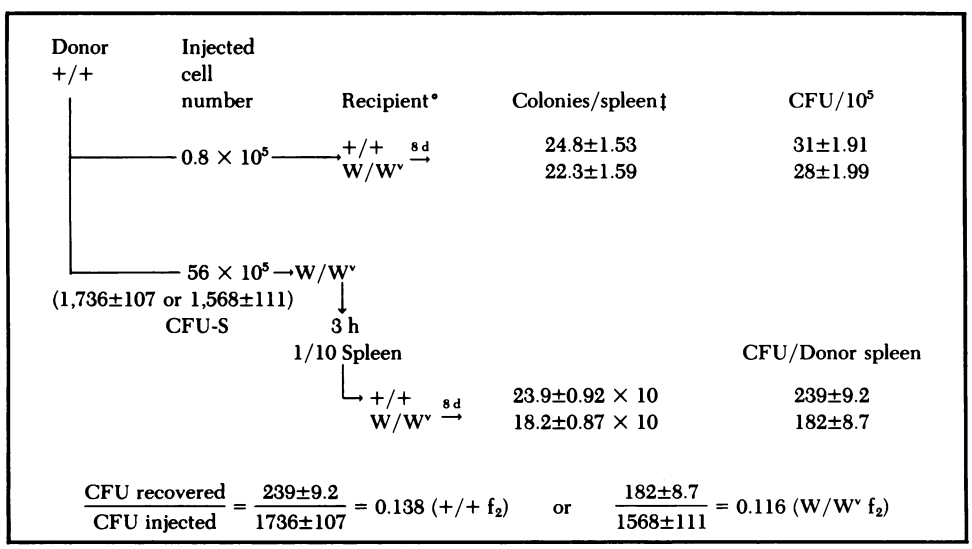
Donors		Cell dose	No. of recipients	Recipients (+/+, W/+ or W^v/+) Spleen Colonies*	Spleen Weight*	Spleen Iron uptake*
	n			*n*	*mg*	*% injected dose*
None		—		0	32±0.9	0.4±0.09
+/+	(6)	10^5	18‡	14±2.1	37±2.2	1.6±0.41
Cured W/W^v	(5)	10^5	7‡	16±2.0	38±2.0	2.1±0.82
		10^6	9‡	C§	80±5.1	8.8±2.11
	(5)	2×10^5	22‡	34±3.2	57±4.7	5.6±1.81
?Cured W/W^v	(4)	2×10^5	20	40±2.0	63±8.1	4.8±0.92
?Lost cure W/W^v	(2)	2×10^5	10	27±3.6	42±3.0	3.0±0.58
Lost cure W/W^v	(1)	10^5	4	8±1.7	30±2.2	1.0±0.72
	(3)	2×10^5	11	18±4.1	39±3.0	2.5±0.39
Never cured W/W^v	(2)	2×10^5	10	0	30±0.2	0.3±0.07

* Data represent geometric mean±SE.

‡ Mean based on sum of mean figure for groups of recipients, i.e., an estimate of mean number produced by each donor (Methods).

§ C, confluent colonies too numerous to count.

pliment observations of others. These can be summarized as follows: (*a*) the concentration of HSC, as measured by cure of W/W^v mice, is <10% of that estimated from the fraction of HSC that form CFU-S; (*b*) a prolonged cure in hematopoietic defects of the W/W^v mice can be induced by adult, +/+ marrow cells; (*c*) certain mice eventually lose their induced cure; (*d*) marrow from cured W/W^v will, in turn, cure



* All +/+ recipients received 875 rad before cell injection.

‡ All control groups given no cells had <0.4 colonies/spleen.

FIGURE 2 Method and result of measurement of the fraction of +/+ CFU lodging and growing in spleens of W/W^v recipients.

other W/W^v, but the concentration of curative HSC is much smaller than in +/+ marrow; (*e*) however, in cured W/W^v, the concentration of HSC is normal as measured by the CFU-S technic; and (*f*) CFU-S were still detectable in W/W^v that had lost their cure. The possible significance of each of these observations will be discussed.

From a variety of experimental (1, 19–21) and clinical (2) observations, it can be deduced that the HSC compartment in mouse and man consists of concatenated HSC compartments of decreasing degrees of pluripotentiality, and each compartment presumably has a relationship to the next as parent to progeny. In this scheme, the totipotent HSC (THSC) gives rise to a pluripotent myeloid HSC (PMSC) as well as to committed lymphoid HSC. The PMSC in turn gives rise to a series of HSC of more limited proliferative potential; the so-called committed progenitor cells that produce colonies of neutrophils/monocytes, eosinophils, erythrocytes, and megakaryocytes in semisolid media (22). The cell producing spleen colonies in irradiated mice (CFU-S) is often considered to be synonymous with the PMSC, but in fact can be a THSC (1, 19, 20), a PMSC (1), and perhaps a committed progenitor (21). Either a THSC or a PMSC can cure the myeloid defect of the W/W^v (1). In other words, the current operational definition of a HSC is not synonymous with its place within the overall structure of the HSC compartment, but is dependent upon the method by which it is measured.

The concentration of HSC in marrow capable of effecting a cure of anemia and macrocytosis in W/W^v mice was significantly less than HSC concentration as calculated by measuring CFU-S and transplantation factor (f_2). Calculations of HSC number by seven different laboratories using the spleen colony technic are shown in Table V. There are many reasons why concentration of cells producing CFU-S might exceed the concentration of HSC with very extensive proliferative potential. The limited dilution assay may give an underestimate or the CFU-S assay may give an overestimate or the two assays may measure different populations. The assumption that animals can be cured by one HSC might be false. Studies by Abramson et al. (1) using chromosomal markers, strongly suggest that cure of the myeloid defect by one THSC or PMSC is possible. Although cells with chromosomal markers were derived from irradiated animals and their normality may be questioned, it seems unlikely that irradiation would increase pluripotentiality. A second factor affecting this calculation is the relatively small number of mice studied, which adversely affects the precision of the calculation.

A number of factors might lead to overestimation of concentration of HSC with extensive proliferative potential as estimated by spleen colony-forming assays. An extensive review of relevant CFU-S studies is beyond the scope of this paper, but, in brief, a number of observations indicate that more than one class of cells is measured by the CFU-S technic and these CFU-S subdivisions vary in their proliferative potential. For example, the number of cells in a spleen colony that will in turn produce colonies in secondary recipients is distributed randomly (3); treatment of donors or donor marrow with drugs, such as 5-fluorouracil (28), with an antibody killing most, but not all, CFU-S (29), and various physical means of fractionating marrow (30, 31), will all selectively result in isolating a fraction of CFU-S with greater or lesser proliferative potential than found in normal marrow. From such data, hy-

TABLE V

Calculated Concentration of CFU-S in Marrow from Six Different Laboratories Compared with Concentration of Stem Cells with Extensive Proliferative Potential as Determined in the Present Study

Reference	Mouse strain	CFU-S/10^5 nucleated marrow cells	Transplantation fraction (f)*	Calculated no. of stem cells per 10^5
Siminovitch et al. (17)	?	17–19	0.17	100–110
Fred and Smith (18)	BALB/c × DBA	56	0.20	280
Schooley (23)	C3H	22	0.24	90
Matioli et al. (24)	C3H	6	0.11	50
Shadduck et al. (25)	CF_1	13–35	0.13–0.20	65–270
Chertkan et al. (26)	C57BL × CBA	11–24	0.15–0.17	60–160
Our Laboratories				
Previous studies (18, 27)	$B_6D_2F_1$	~30	~0.15	~200
Present study	+/+ → +/+	31	0.14	220
	+/+ → W/W^v	28	0.12	230
+/+ cells curing W/W^v, measured by limiting dilution				~10

* Secondary transfer within 1–5 h of original cell injection.

potheses have been formulated to suggest that self-replication vs. differentiation by CFU-S is a random event (3), is related to the previous proliferative history of the CFU-S (10, 32, 33), is related to HSC compartment size (34, 35) or to overall size of the myeloid compartment (36) or is primarily subject to the level of stimuli for differentiation in the environment (37, 38). Irrespective of the reason, it would appear that certain cells assayed by the CFU-S technique undergo only limited subsequent proliferation.

Whereas the concentration of cells in marrow that produce CFU-S can be measured with reasonable accuracy, colonies also are formed in the marrow and deep in the spleen, so that CFU-S reflect only a fraction of colony-forming cells in the suspension. To estimate total effective HSC concentration in the suspension, the fraction of cells that seeds the spleen (f_2) must be determined. This fraction has been calculated by secondary and even tertiary transplantation experiments (25), but the calculation is somewhat controversial (18, 26, 39, 40). In general, when experimental conditions other than those used in our study and in the studies summarized in Table V are used, lower f_2 values are found (40, 41). Thus, if anything, the figures derived for HSC by spleen colony techniques in Table V technically underestimate HSC concentration.

With either the W/W^v assay or the CFU-S assay, a significant number of potential HSC may fail to lodge and grow after intravenous injection. Those that do lodge and grow in a favorable environment could be termed the effective "seeding fraction", a fraction quite different from that measured by the f_2 value studies of CFU-S. There are no known means of measuring such a seeding fraction in either technique.

There are still other assays that measure classes of HSC that may or may not relate to THSC and PMSC. Certain cells that seed and grow in "Dexter-type" flask cultures of hematopoietic tissue are HSC with rather extensive proliferative potential (42), but as yet, no reports of attempts to measure the concentration of such cells in marrow have appeared. Cells that produce hematopoietic cells in diffusion chambers placed in the peritoneal cavity also are cells with fairly extensive proliferative potential (7). These "DC stem cells" generally are thought to be somewhat differentiated offspring of the PMSC, but their concentration in marrow has been estimated as less than concentration of CFU-S (7). From the in vitro assays for HSC number in semisolid media, only those that contain mixtures of myeloid cells (CFU-MIX) are considered candidate PMSC. The concentration of cells forming all types of hematopoietic colonies in adult CBA mouse marrow was $150/10^5$, but $<10/10^5$ marrow cells formed mixed colonies (43).

Perhaps the simplest interpretation of the discrepancy between concentration of CFU-S and of cells capable of curing W/W^v in marrow is that only a small fraction of CFU-S will exhibit great enough proliferative potential to cure, and maintain cure, in a W/W^v. Obviously, an alternative suggestion is that the cell curing the W/W^v is not measured as part of the CFU-S population.

We think that the markedly reduced concentration of HSC in marrow of cured W/W^v, which will cure other W/W^v, as compared with the concentration in $+/+$ marrow in the presence of a normal concentration of CFU-S is compatible with the conclusion that the curative HSC represents a minor population of CFU-S. A similar conclusion could be drawn from the studies of Sharkis et al. (44, 45) in which antitheta serum abolished the ability of marrow to cure W/W^v but did not reduce CFU-S concentration significantly.

A major question relevant to our data is the following: are HSC and stem cells for other cellular systems, in which constant mature cell replacement is required throughout life, capable of inexhaustible proliferation, or is there a programmed, finite number of divisions within a system? This is a question of crucial importance with respect to the ability to survive repeated insults to the system and the ability of transplanted marrow to produce life-long cures as well as determining whether limitation of proliferation in a system(s) might define a maximal theoretic life-span for the animal.

Prolonged cure of W/W^v by injection of normal marrow has been reported from many laboratories (5), and has been most extensively studied by Harrison (6, 46). However, previous studies have used a much larger cell dose than used herein.

Hayflick (8) and others (9, 47, 48) have carried out extensive studies on the ability of fibroblasts from different species, including man and mouse, to regenerate their number on serial transfer in in vitro liquid culture systems, and proliferation ceased following <100 transfers. However, the serial transfer studies of fibroblasts have been criticized conceptually. When a flask is used for secondary transfer, only a small portion of the cells contained therein are transferred and studied further; so it has been suggested that by simple chance dilution, cells with extensive proliferative ability will eventually be discarded from the system (38, 48–50).

Previously published data relative to the question of cellular proliferative potential derived from studies of HSC in intact animals are also subject to difficulties in interpretation. Certain studies suggest that HSC routinely can be demonstrated to have limited proliferative potential; chief among these are studies of the

ability of marrow, serially transferred in lethally irradiated mice, to produce spleen colonies (9, 46, 51). In all instances, colony-forming ability of the original marrow has declined after the second transfer and has eventually disappeared. Furthermore, the rate at which marrow cells forming CFU-S regenerate is less rapid with sequential marrow transfer (52). The technical and interpretative problems concerning the CFU-S assay, which were discussed above, complicate interpretation of these studies. Furthermore, the milieu of the lethally irradiated mouse into which these HSC are introduced may promote their extinction. There is a major stimulus for the HSC to differentiate and produce mature cells in the irradiated mouse. Unless an adequate number of HSC differentiate and produce mature blood cells in a relatively brief time, the mouse dies as a consequence of pancytopenia. Differentiation of an HSC is equivalent to its death. We (34) and others (35) have presented evidence that a protective mechanism is operative in a markedly reduced HSC compartment in which self-replication is favored markedly over differentiation until significant regrowth of the HSC compartment has been achieved. However, this protection may not be absolute, and serial transfer of marrow in hosts given potentially lethal whole body irradiation may promote HSC extinction by the presence of strong stimuli for differentiation. Harrison et al. (46) compared the ability of chromosomally marked marrow from young and old mice to reconstitute irradiated mice and in turn compared the repopulating ability of each following serial transplantation. They found that marrow repopulating ability declined to a greater degree following a single transplantation than it did during the entire life-span of the animal. Therefore, they suggested that the apparent loss of proliferative capacity, as defined by serial transplantation, was a characteristic of the system used for study rather than physiologic loss of proliferative ability of the cells as they aged. The problems of dilution with serial in vitro transfer of cells mentioned above are also applicable to serial in vivo transfer experiments.

Other in vivo studies fail to yield any evidence for a limitation of HSC proliferation. Although there appears to be a subtle decline in immune function in aged mice, their myeloid hematopoietic system and their concentration of cells forming CFU-S are not appreciably different from those of young mice (9, 53, 54).

Still other in vivo studies indicate that extensive replication of the HSC and its progeny occurs but that there may be an extensive but finite limit to this proliferation. In previous studies from our laboratory (55), we gave whole body irradiation in a dose that reduced the HSC compartment to very low levels, but a level that still allowed the mouse to survive by repopulating its own hematopoietic system without addition of exogenous cells. A number of mice survived six such treatments at 6-wk intervals and their hematopoietic system was only modestly depleted following recovery from the sixth treatment. The modest reduction in hematopoiesis could have reflected natural exhaustion of proliferative ability, with the proviso concerning stimulus for differentiative stem cell extinction discussed above and/or damage to stem cells by irradiation that limited their proliferative ability (56).

The present studies indicate that certain HSC and their progeny are capable of very extensive proliferation, but also suggest that there may be a finite limit to this ability. It seems likely that some mice were cured by a single HSC. Even if this assumption is incorrect, cure by a few, rather than a single HSC, still indicates that very extensive proliferation is required to cure and maintain cure in a W/W^v. A crude calculation of the number of divisions in HSC and differentiated cell compartments required to maintain erythropoiesis for a year in cured W/W^v is presented in the Appendix.

Two lines of evidence suggested that proliferation of transplanted +/+ HSC and their progeny may have been finite, although admittedly very extensive. Certain mice failed to maintain a cure throughout the period of study and the marrow of cured mice did not cure as many secondary W/W^v recipients as would be anticipated with normal +/+ marrow. Loss of cure was also reported by Harrison (6), but loss of cure was evidenced by recurrence of anemia, and our data suggest that this does not necessarily reflect loss of cure unless also accompanied by a return of macrocytosis.

These data indicate that there was a loss, or at least a decline, in the function of transplantable +/+ HSC with extensive proliferative potential. However, this cannot be assumed unequivocally to represent a programmed limitation in proliferative potential of the stem cell. If such a limitation was imposed upon each cell, loss of cure would be anticipated more frequently in mice cured by a small as compared to a large cell dose, and loss of cure would be expected to become increasingly frequent as duration of observation of cured mice increased. Neither of these events was observed, but considering the relatively small number of mice involved in such studies, we would in no sense consider them to be definitive observations. Once a W/W^v is cured, stimuli for HSC differentiation would presumably return to normal so the possibility of HSC extinction as postulated above for serial transfer of cells in lethally irradiated recipients should not be applicable. A very subtle graft-versus-host reaction by

the coisogenic +/+ marrow might account for loss of cure in the W/W^v. Although the hematopoietic microenvironment appears normal in relatively young W/W^v (4), it is conceivable that selective inhibition of transplanted +/+ HSC is imposed by the overall environment of the aging W/W^v. The possible role of the lymphoid system in regulating production of myeloid cells is very complex (56). Although the thymus is not required (57), an antitheta antibody-sensitive lymphoid cell may be necessary for the cure of W/W^v in addition to HSC (44, 45, 58). Loss of cure could represent exhaustion of that cell rather than HSC exhaustion.

In any event, it is evident that further study is required before firm conclusions regarding the mortal or immortal life-span of the HSC can be reached.

APPENDIX

A crude estimate of the total number of divisions required in HSC compartments and of sequential doubling divisions in differentiation compartments by their offspring to cure and maintain cure in a W/W^v for a year can be derived. However, there is no way to determine how many cell divisions take place in each concatenated HSC compartment. For example, since there are at least three concatenated stem cell compartments (THSC, PMSC, committed progenitor), three consecutive stem cell divisions within each compartment would result in feedout of one cell as would a single stem cell division in the most differentiated compartment, while the other two remained at rest.

The proliferative ability of a HSC and its progeny to reconstitute and maintain the erythroid system of a mouse was calculated as follows. RBC concentration is $\sim 9 \times 10^9$/ml, blood volume is ~7% of body weight, and RBC survival is ~45 d (59). Thus, a 20-g mouse will have $20 \times 0.07 \times 9 \times 10^9$ or $\sim 13 \times 10^9$ mature RBC. 34 doubling divisions will yield 9×10^9 cells. Doubling divisions reconstituting HSC compartments, which undoubtedly occurred since the CFU-S forming compartment was reconstituted, would not contribute directly to mature cells, so 34 doubling divisions to replace the RBC compartment is an underestimate. $^1/_{45}$th of RBC must be replaced per day or $\sim 0.3 \times 10^9$ cells for a 20-g mouse. 29 doubling divisions will supply $\sim 0.3 \times 10^9$ cells/d. To maintain the system for 12 mo would require at least 8,000 doubling divisions. Doubling divisions required to maintain HSC would be in addition to this figure. Divisions required to maintain the granulocytic and other hematopoietic systems would not add to the total required doubling steps unless more such steps are imposed between the functioning HSC and the postmitotic cell in these compartments as compared with RBC. It should be emphasized that these calculations do not refer to the total number of cell divisions in the system, but rather to sequential population doubling divisions; i.e., a cell undergoing division may progress in number from 1:2:4:8, three doubling divisions, although the total cell divisions are seven.

These calculations are not presented as an attempt to calculate the actual number of divisions that a stem cell and its concatenated system of cohort progeny are capable, but are presented to indicate that this in vivo proliferative potential of a single HSC vastly exceeds that estimated for fibroblasts in vitro. Further, no estimate of the self-replication ability of specific HSC compartments can be derived from these data. One can hypothesize that the potential number of self-replicative divisions might be finite in each successive HSC compartment, but such speculation would not influence the extensive overall proliferative potential of a HSC and its progeny implied from the above data.

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