

LOSS OF STEM CELL REPOPULATING ABILITY  
UPON TRANSPLANTATION  
Effects of Donor Age, Cell Number,  
and Transplantation Procedure\*

BY DAVID E. HARRISON AND CLINTON M. ASTLE

*From The Jackson Laboratory, Bar Harbor, Maine 14609*

The earliest precursor cell or stem cell that populates the immunohemopoietic system in mammals must have a high proliferative potential. Stem cells can be transplanted by intravenous injections of single cell suspensions and can repopulate stem cell-depleted recipients (1, 2). Therefore, stem cells have been used to test the hypothesis that mammalian aging is genetically programmed to cause loss of cell proliferation in all somatic cells of adults (3, 4). Yet hemopoietic stem cells from old donors transplanted into young recipients proliferate as well as cells from young donors, implying that aging is not an intrinsically timed phenomena (2, 5-11).

However, after four to six successive serial transplantations, stem cells lose their ability to repopulate and proliferate normally, even if given long periods of time to recover after each transplantation (1, 2, 8-15). This loss may result from acceleration of the normal aging process, or perhaps from unique stresses caused by transplantation. It is important to distinguish between these possibilities: proof that transplantation causes unique stresses would add credibility to the theory that stem cell lines do not age. An understanding of how transplantation affects stem cells is also important in understanding their functioning, and is of clinical significance as the use of marrow transplantation in medicine increases (16, 17).

Using the technique of competitive repopulation, we have shown that there is a substantial loss of proliferative capacity after a single marrow transplantation (10). This loss is detected even when large numbers of marrow cells are used, and the interval between transplantations is long (10, 18). Other techniques, such as measuring macroscopic spleen colony-forming cell (CFU-S)<sup>1</sup> numbers, detect little or no change after a single transplantation under these conditions (1, 2, 5-10, 12).

Micklem and his colleagues developed the earliest competitive repopulation techniques (1). They transplanted mixtures of genetically distinguishable (19) stem cells and compared their abilities to repopulate lethally irradiated recipients and maintain continuous function for long periods of time. They showed that fetal liver cells were better than adult marrow cells (20), cells from the marrow were better than circulating cells (21) and young and old marrow cells functioned identically (9). Recently, this

\* Supported by research grant AM-25687 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, and research grants AG-00594 and AG-01755 from the National Institute on Aging. The Jackson Laboratory is fully accredited by the American Association for Accreditation Animal Care.

<sup>1</sup> Abbreviations used in this paper: CFU-S, macroscopic spleen colony-forming cell; LPS, *Escherichia coli* lipopolysaccharide; PHA, phytohemagglutinin; T6, T(14;15)6 chromosome translocation.

group has confirmed our results (10) showing that a single transplantation causes a major loss of stem cell-repopulating ability (22). Micklem's studies (9, 20–22) differed from those reported here in two ways: they compared stem cells from only two genetically distinguishable donors at a time; and specific cell populations were not studied.

In our studies we needed to simultaneously compare many different donors of the same genotype to determine the effects of age and transplantation while varying transplantation procedures. Therefore, in our competitive repopulation assays we mix stem cells from each donor with a specific dose of genetically distinguishable stem cells from a single pool (10, 18). Cells from this pool are called competitors, and several lethally irradiated recipients are given portions of each donor-competitor cell mixture. Cells from many different donors are mixed with cells from the same competitor pool. Thus the relative repopulating abilities of these donors are simultaneously determined by percentage of donor-type cells produced in each of their recipients.

We also needed to compare repopulating abilities of specific erythroid and lymphoid cell types. When donor and competitor cells are distinguished by chromosome markers (19), this is done by stimulating proliferation in the cell type desired with a specific stimulus (10, 18). When hemoglobin markers are used, the repopulating ability of erythropoietic precursor cells is specifically measured (23).

To explore the deleterious effect of transplantation, we studied the competitive repopulating ability of cells that had been serially transplanted using  $10^5$  to  $10^8$  marrow cells. The vascular systems of mice were surgically joined so that stem cells would circulate by natural processes from the donor to the lethally irradiated carrier. Finally, we sublethally irradiated donors and allowed their stem cell numbers to recover without transplantation.

### Materials and Methods

*Mice.* The following strains of mice were bred and maintained at The Jackson Laboratory, Bar Harbor, ME: CBA/HT6 (CBAT6), CBA/CaJ (CBA), and (CBAT6  $\times$  CBA) $F_1$  mice carrying 2, 0, and 1 T(14;15)6 (hereafter called T6) chromosome translocations, respectively, but otherwise genetically similar; C57Bl/6J (B6) and their  $F_1$  hybrids with WB/ReJ (WBB6F $_1$ );  $F_1$  hybrids between B6 and CBAT6 (B6CBAT6F $_1$ ) or CBA (B6CBAF $_1$ ); and the genetically anemic mutants with defective erythropoietic stem cell lines, WBB6F $_1$ - $W/W^v$ . Mice of both sexes were used and gave similar results. Male donors were only used with male recipients.

*Irradiation.* Mice were irradiated using either a General Electric Maxitron 250 x-ray machine (23) or a Shepherd Mark I Gamma Irradiator with a  $^{137}\text{Cs}$  source. Dose rates were 70 or 150 rad/min, respectively. All but the  $W/W^v$  carriers, and all recipients used in competitive repopulation assays, were lethally irradiated 15–18 h before marrow transplantations with 750–850 rad using the x-ray machine, or with 950–1,100 rad using the Gamma Irradiator. To perform sublethal irradiation, 550 rad (gamma) was given to CBAT6 donors or 600 R (x ray) was given to B6CBAT6F $_1$  donors.

*Marrow Transplantation.* Single cell suspensions of marrow cells were prepared by rinsing both femurs and tibias of each donor with Ks-74 medium (24), a balanced salt solution buffered with Hepes (pH 7.4). Cells were counted (8) and stored on ice until use. Recipients were injected intravenously with the appropriate numbers of cells. The recipients used for serial transplantations are referred to as carriers.

*Competitive Repopulation Assay.* Marrow cells from each donor were mixed with equal numbers of marrow cells from a pool of genetically distinguishable competitors. Mixtures were injected intravenously into appropriate recipients. The following assays were performed to determine the proportions of donor and competitor cells in the recipients. Chromosome markers were

identified in proliferating cells using techniques similar to those of Ford (19). To identify specific cell types, erythroid cells in the marrow were stimulated to proliferate by bleeding in vivo, whereas T and B cells from the spleen were stimulated to proliferate in vitro by phytohemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS), respectively (10, 18).  $10^6$  spleen cells were cultured in RPMI 1640 medium plus 15% of heat-inactivated fetal calf serum (Grand Island Biochemicals, Grand Island, NY), with PHA (HA-17; Burroughs Corp., Detroit, MI) at  $1.25 \mu\text{g}/1 \text{ ml}$ , or with LPS (L2880; Sigma Chemical Co., St. Louis, MO) at  $10 \mu\text{g}/1 \text{ ml}$ . Controls were incubated in mitogen-free medium.

Erythropoietic stem cell proliferation was analyzed using donors and competitors with electrophoretically separable hemoglobins (23). B6 mice have  $\alpha_2\beta_2^s$  hemoglobin, and CBA, CBAT6, and WB mice have  $\alpha_2\beta_2^{\text{d}^{\text{maj}}}$  and  $\alpha_2\beta_2^{\text{d}^{\text{min}}}$  hemoglobins. When mixtures of B6 and WBB6F<sub>1</sub> cells are used in WBB6F<sub>1</sub> recipients, or CBAT6 and B6CBAF<sub>1</sub> cells in B6CBAT6F<sub>1</sub> recipients, there is little or no hybrid resistance against the parental cells (25), and the amount of each hemoglobin type produced is proportional to the number of donor marrow cells of each type in the mixture (23).

*Varying Cell Numbers.* To determine whether the transplantation effect was less when larger marrow cell numbers were transplanted, lethally irradiated carriers were given doses of young marrow ranging from  $10^5$  to  $10^8$ . These cells were allowed to repopulate the carriers for periods of time ranging from 13 d to 4 mo. Thereafter, cells from each carrier were tested in the competitive repopulation assay. The cells were mixed with competitor cells from a single pool, and the mixtures were transplanted into lethally irradiated recipients. After periods of time ranging from 70 to 211 d, the proportions of donor and competitor cells were determined using chromosome or hemoglobin markers as described above.

*Parabiosis.* To determine whether the transplantation effect is reduced when cells are transplanted by natural processes, lethally irradiated carriers were surgically joined to donors through their vascular systems (25). To test how rapidly blood circulated between the partners,  $^{59}\text{Fe}$ -labeled erythrocytes were injected in the tail vein of one partner and blood samples were removed from each partner's orbital sinus. Previous results showed that mixing was typically about one-half complete after 20 min in mice that had been in parabiosis successfully for 2–8 wk. Therefore, circulating stem cells should have been readily transplanted into the carriers by natural processes. These experiments were done in parallel with the transplants in which cell numbers were varied. Donors and carriers remained joined for the same periods of time that transplanted cells repopulated carriers.

*Sublethal Irradiation.* To study the effects on stem cells of proliferation without transplantation, donors were sublethally irradiated with 550–600 rad as previously described. The donors were allowed to recover, and the competitive repopulating ability of their cells was compared with the cells of carriers transplanted at the same time.

*Unirradiated Carrier.* To determine whether the transplantation effect was caused by irradiation, unirradiated WBB6F<sub>1</sub>-*W/W<sup>v</sup>* carriers were used. The erythropoietic systems of *W/W<sup>v</sup>* mice are completely repopulated by transplanted histocompatible normal marrow cells (6).

## Results

*Effects of Serial Transplantation and Age.* We confirmed and extended previous findings that stem cells from old and young donors competed equally well against genetically distinguishable competitor cells from the same pool (Fig. 1). However, after a single serial transplantation, the ability of cells from carriers to compete with untransplanted competitor cells dropped substantially, regardless of the age of the original donor (Fig. 1). In the competitive repopulation assay for cells responding to PHA stimulation, marrow from old donors was >70% as effective as marrow from young donors. After a single serial transplantation in carriers, marrow originally from young donors was only 10%, and that from old donors ~20%, as effective as untransplanted cells. Thus a single transplantation caused three to seven times as great a deleterious effect as a lifetime of normal function (Fig. 1).

In experiments testing whether the degree of repopulation by cells from old donors

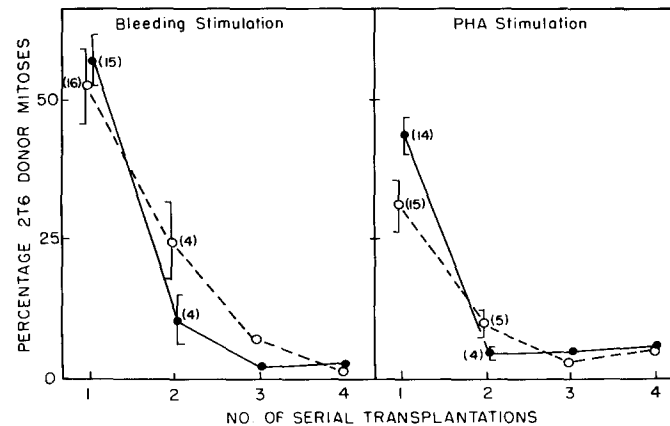


FIG. 1. Effects of age and serial transplantation on competitive repopulating ability. Values in parentheses are the number of donors, with 2.3 recipients per donor. Old donors (○) were 24–31-mo old, and young donors (●) were 3–6-mo old, CBAT6 mice; this genotype has 2T6 chromosomes. For transplantations 2, 3, and 4, their marrow was serially transplanted using  $4 \times 10^6$ – $10 \times 10^6$  marrow cells per lethally irradiated CBA carrier. These were held 4–12 mo before they were used as donors. For competitive repopulation assays, their marrow cells were mixed with young (CBAT6  $\times$  CBA) $F_1$  competitor marrow ( $3 \times 10^6$  cells each) having one T6 chromosome, and intravenously injected in lethally irradiated CBA recipients. Recipients were splenectomized to provide spleen cells for PHA stimulation *in vitro*, and bled 1–4 wk later to stimulate marrow cells *in vivo*. A mean of 48 (range, 20–65) mitoses were scored for each determination after 2–15 mo. Usually, cells from two old and two young donors were studied in each assay; in three experiments, donors at transplantation 2 were included, and in one experiment, donors at transplantations 3 and 4. Means for all donors are plotted; bars give  $\pm$  SE.

declined after long periods of time, we found that the age of the donor cells did not affect the ability to compete. Results of several different experiments are shown in Fig. 2. Stem cells from both old and young donors in each experiment had generally consistent levels of repopulation over the following time periods (in days): 11–135, 60–375, 90–310, and 300–500. Furthermore, old and young donors gave similar results in Tables I–III.

*Effects of Transplanting Various Cell Numbers.* In these experiments, carriers were given  $10^5$ – $10^8$  bone marrow cells. After recovery the repopulating cells from each carrier were made to compete against untransplanted marrow cells. Regardless of whether T cells, B cells, or erythropoietic cells were studied, previously transplanted cells were present at consistently lower percentages than the competing cells. This occurred whether carriers were given  $10^5$ ,  $10^6$ , or  $10^7$  donor marrow cells (Tables I and II). Only carriers given  $10^8$  cells performed substantially better, showing an overall average of 28% donor mitoses, midway between the 39–40% of untransplanted donors and the 15–18% of carriers given  $10^5$  to  $10^7$  cells (Table I).

To claim that the tested mitotic cells are responding to a specific stimulus it is necessary to show that the stimulus greatly increases the number of cells in mitoses. The numbers of mitoses per 1,000 spleen cells were measured and the result is expressed as a percent. Manifold increases were seen in cells exposed to PHA and LPS, but not after bleeding. PHA and LPS generally caused mitoses to increase 5–20-fold (to 2–5%). By comparison, mitoses from marrow were usually only two- to threefold increased in bled recipients as compared with untreated recipients. Therefore, many cells scored as erythropoietic were multiplying without the stimulus of

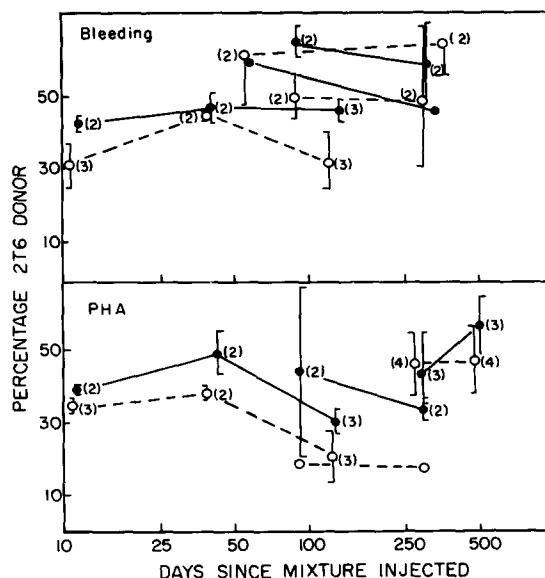


FIG. 2. Effects of time in the recipient on the percentage of donor cells in the competitive repopulation assay. Results using old and young CBAT6 donors are shown in several experiments followed for different periods of time. Results from recipients given the same donor-competitor mixtures are joined by lines. Donor ages (○, old; ●, young) and treatments are the same as in Fig. 1, transplantation 1.

TABLE I  
Effect of Transplantation Procedure on Competitive Repopulating Ability

Donor age*	Transplantation‡		Percent donor mitoses stimulated by§			Average percentage
	Number	Method	PHA	LPS	Bleeding	
O	1	—	34 ± 5 (5)	38 ± 3 (5)	48 ± 6 (4)	40
Y	1	—	35 ± 8 (3)	38 ± 5 (3)	43 ± 9 (3)	39
O	2	10 <sup>7</sup> IV	12 ± 4 (2)	19 ± 4 (3)	14 ± 2 (3)	15
Y	2	10 <sup>6</sup> IV	17 ± 4 (3)	19 ± 6 (4)	10 ± 4 (3)	15
Y	2	10 <sup>6</sup> IV	9 ± 3 (3)	16 ± 4 (4)	29 ± 6 (4)	18
Y	2	10 <sup>7</sup> IV	19 ± 10 (3)	25 ± 6 (3)	9 ± 5 (3)	18
Y	2	10 <sup>8</sup> IV	23 ± 1 (3)	31 ± 3 (3)	29 ± 7 (3)	28
Y	2	Parabiosis	16 ± 3 (7)	17 ± 4 (7)	7 ± 2 (7)	13
Y	1	600 rad	12 ± 2 (3)	8 ± 2 (4)	6 ± 2 (4)	9

\* Old (O) and young (Y) B6CBAT6F<sub>1</sub> donors were 26–28- and 4–6-mo old, respectively.

‡ Lethally irradiated B6CBAF<sub>1</sub> carriers (lines 3–7) were given the donor marrow cell numbers listed intravenously (IV) and were tested in the competitive repopulation assay at transplantation 2. Carriers in line 8 received cells by parabiosis; B6CABT6F<sub>1</sub> donors in line 9 were irradiated with 600 rad (x ray) 4 mo before the assay.

§ In the competitive repopulation assay competitors (pooled marrow from 5-mo olds) and recipients were B6CBAF<sub>1</sub> mice, and mixtures of 3 × 10<sup>6</sup> donor and competitor marrow cells were given to each recipient 75–150 d before its cells were stimulated for the assay. Data are given as mean ± SE. Number of recipients is in parentheses; an average of 48 mitoses were scored per recipient.

|| Mean value from the three different types of stimulation.

TABLE II  
*Effect of Transplantation Procedure on Competitive Repopulating Ability of Erythropoietic Stem Cell Lines*

Donor age*	Transplantation†		Percent CBAT6 hemoglobin in recipients after‡	
	Number	Method	13 d	90 d
Y	1	—	54 ± 13 (4)	42 ± 4 (4)
O	2	10 <sup>7</sup> i.v.	6 ± 1 (4)	6 ± 1 (4)
Y	2	10 <sup>5</sup> i.v.	5 ± 1 (4)	-1 ± 1 (4)
Y	2	10 <sup>6</sup> i.v.	4 ± 1 (4)	1 ± 1 (4)
Y	2	10 <sup>7</sup> i.v.	4 ± 1 (4)	1 ± 1 (4)
Y	2	10 <sup>8</sup> i.v.	8 ± 1 (4)	14 ± 3 (4)
Y	2	Parabiosis	2 ± 1 (4)	9 ± 2 (4)
Y	1	550 rad	2 ± 1 (4)	2 ± 1 (4)
Y	2	10 <sup>5</sup> i.v. vs. transplant 2	—	1 ± 1 (4)
Y	2	10 <sup>6</sup> i.v. vs. transplant 2	9 ± 3 (4)	—
Y	2	10 <sup>7</sup> i.v. vs. transplant 2	25 ± 5 (4)	21 ± 4 (4)

\* Old (O) and young (Y) CBAT6 donors were 24- and 8-mo old, respectively.

† Lethally irradiated B6CBAT6F<sub>1</sub> carriers (lines 2-6) were given donor marrow cell numbers listed intravenously (i.v.), and after 13 and 90 d were tested in the competitive repopulation assay at transplantation 2. Lethally irradiated CBA carriers in line 7 received cells by parabiosis from the CBAT6 donors for 13 and 90 d; CBAT6 donors in line 8 had been irradiated with 550 rad (gamma) 13 and 90 d before the assay. The last three lines show the same carriers as lines 3-5, but are assayed against competitor cells that had also been previously transplanted (vs. transplant 2) using 1 × 10<sup>7</sup> B6CBAF<sub>1</sub> marrow cells per carrier.

‡ In the competitive repopulation assay, competitors (pooled marrow from 3.5- and 6.5-mo-old donors) were B6CBAF<sub>1</sub> mice, recipients were B6CBAT6F<sub>1</sub> mice, and mixtures of 10<sup>6</sup> donor and competitor marrow cells were given to each recipient 70-80 d before its hemoglobin was assayed. Data are given as mean ± SE. Number of recipients is in parentheses.

bleeding.

It is likely that repopulating abilities of erythropoietic stem cells are measured most specifically by using genetically distinguishable hemoglobins, as in Tables II and III. Marrow from carriers given 10<sup>5</sup> to 10<sup>7</sup> donor cells showed no donor (CBAT6) hemoglobin when mixed with pooled, untransplanted B6CBAF<sub>1</sub> marrow (Table II). Repopulating abilities of marrow from these carriers were reduced at least 10-fold in 13-d, and 20-fold in 90-d, carriers. The hemoglobin assay usually detected a minimum of 2-3% donor hemoglobin (23), but by chance the assay on the 13-d carriers could not distinguish ~5% donor hemoglobin from 0%. As found previously, carriers given 10<sup>8</sup> cells did better: their marrow produced 14% donor hemoglobin after 90 days (Table II). This is only one-third of the erythropoietic repopulating ability shown by untransplanted cells, which produced 42% donor hemoglobin after 90 d (Table II).

Erythropoietic function in marrow from carriers of 10<sup>6</sup> and 10<sup>7</sup> CBAT6 cells was measured by competition against B6CBAF<sub>1</sub> cells that had also been previously transplanted (Table II, bottom 3 lines). Under these conditions, marrow from a carrier given 10<sup>7</sup> cells produced about three times as much hemoglobin as that from a carrier given 10<sup>6</sup> CBAT6 marrow cells for 13 d. Marrow from a carrier given 10<sup>5</sup> cells still failed to produce detectable amounts of hemoglobin.

*Effects of Transplantation by Parabiosis.* When the circulatory systems of donor and lethally irradiated recipient are joined, stem cells circulate and seed the recipient. Transplantation is effected without artificial handling procedures. Irradiated carriers

that had been repopulated by stem cells from parabiotic donors were analyzed by competitive repopulation. Marrow cells from such carriers competed poorly. Parabiotic carriers gave an overall average for PHA, LPS, and bleeding stimulation of 13% donor-type mitoses, compared with 15–18% for carriers given  $10^5$ – $10^7$  cells, and 28% for those given  $10^8$  cells (Table I). After 90 d of parabiosis, but not after 13 d of parabiosis, parabiotic carriers produced detectable levels (9%) of hemoglobin when competing against B6CBAF<sub>1</sub> marrow. This was fivefold less than untransplanted donors (Table II).

*Effects of Sublethal Irradiation.* After a single dose of sublethal irradiation, mouse stem cell numbers (measured as endogenous macroscopic spleen colonies) recover and even temporarily exceed original values (27). Therefore, we used sublethal irradiation to test the effects of stem cell proliferation. Marrow from sublethally irradiated donors performed very poorly in competitive repopulation assays. Donors that had been sublethally irradiated 4 mo before their marrow was tested produced an average of only 9% of their types in assays using chromosome markers (Table 1, bottom line). No donor hemoglobin was produced in competitive repopulation assays 13 and 90 d after sublethal irradiation (line 8, Table II).

*Use of Unirradiated Carriers.* The very strong deleterious effect of sublethal irradiation (Tables I and II) suggested that a significant portion of the deleterious effect of transplantation may have resulted from using lethally irradiated recipients. Therefore, we transplanted marrow cells intravenously from young and old WBB6F<sub>1</sub>+/+ donors into unirradiated WBB6F<sub>1</sub>-*W/W<sup>v</sup>* carriers. Marrow cells were also transplanted by parabiosis into *W/W<sup>v</sup>* carriers. In all cases lethally irradiated carriers were used as controls. All *W/W<sup>v</sup>* carriers were cured of their anemias, showing that their erythrocytic systems were successfully repopulated. Their cells were tested by competitive repopulation 111 d after the original transplantation. The stem cells from *W/W<sup>v</sup>* or lethally irradiated carriers failed to produce detectable amounts of hemoglobin in every case (Table III, transplantation 2). With minimum detection limits of 2–3%, this was at least a 20-fold reduction in repopulating ability compared with untransplanted WBB6F<sub>1</sub>+/+ stem cells. Such stem cells produced 51–57% of the circulating hemoglobin when mixed with the same pool of B6 competitor cells (Table III, transplantation 1). As found in the other experiments, there was no deleterious effect of donor age, and transplantation by parabiosis did not have a beneficial effect.

### Discussion

Our major finding is that transplantation is highly deleterious to stem cell-repopulating abilities, especially to erythropoietic stem cells. This effect occurs over a wide range of transplanted cell doses and over a variety of transplantation conditions. When chromosome markers were used to identify donor cells, a single transplantation reduced repopulating abilities by at least three- to sevenfold (Fig. 1). This reduction was similar whether the carrier of the transplanted stem cells received marrow cell doses of  $10^5$ ,  $10^6$ , or  $10^7$ ; a lesser reduction was seen using  $10^8$  cells (Table I). Erythropoietic stem cell functions were identified using hemoglobin markers. Repopulating abilities of these cells were reduced at least 10–20-fold by a single transplantation in carriers that had received  $10^5$  to  $10^7$  marrow cells 13, 90, and 111 d before (Tables II and III). They were reduced threefold in carriers that had received  $10^8$  marrow cells 90 d before (Table II).

TABLE III  
*Effect of Carrier Irradiation and Transplantation Procedure on Competitive Repopulating Ability of Erythropoietic Stem Cell Lines*

Donor age*	Transplantation‡		Carrier treatment	Percent WBB6F <sub>1</sub> hemoglobin§
	Number	Method		
O	1	—	—	57 ± 13 (12)
Y	1	—	—	51 ± 6 (20)
O	2	10 <sup>7</sup> i.v.	<i>W/W<sup>v</sup></i>	-1 ± 1 (12)
O	2	10 <sup>7</sup> i.v.	Irradiated	-1 ± 1 (6)
Y	2	10 <sup>7</sup> i.v.	<i>W/W<sup>v</sup></i>	-2 ± 1 (8)
Y	2	10 <sup>7</sup> i.v.	Irradiated	0 ± 2 (8)
O	2	10 <sup>6</sup> i.v.	Irradiated	-3 ± 0 (8)
Y	2	10 <sup>6</sup> i.v.	Irradiated	-1 ± 1 (8)
Y	2	Parabiosis	<i>W/W<sup>v</sup></i>	-2 ± 1 (12)
Y	2	Parabiosis	Irradiated	-2 ± 1 (8)

\* Old (O) and young (Y) WBB6F<sub>1</sub>+/+ donors were 25-28- and 2-8-mo old, respectively.

‡ Genetically anemic *W/W<sup>v</sup>* or lethally irradiated normal WBB6F<sub>1</sub> carriers (lines 3-8) were given donor marrow cell numbers listed intravenously (i.v.), and after 111 d were tested in the competitive repopulation assay; this was transplantation number 2.

§ In the competitive repopulation assay, competitors (pooled marrow from 4-6-mo old donors) were B6 mice, recipients were WBB6F<sub>1</sub> mice, and mixtures of  $3 \times 10^6$  donor cells with  $7.5 \times 10^6$  competitor marrow cells were given to each recipient 113 to 211 d before its hemoglobins were assayed. Data are given as mean ± SE. Number of recipients is in parentheses. Negative values result from imprecisions in the hemoglobin assay; B6 donors give  $0 \pm 3\%$  WBB6F<sub>1</sub> hemoglobin (23).

Transplantation by parabiosis caused similar deleterious effects as transplanting  $10^5$ - $10^7$  marrow cells (Tables I-III). Apparently, relocation of stem cells, even by natural processes, greatly reduces their repopulating ability. Sublethal irradiation of the donor induces stem cell proliferation, but does not require their relocation. However, it affected repopulating abilities even more severely than transplantation.

There are several possible explanations for these findings: (a) The earliest stem cells with maximum functional capacity may be so rare that none are usually found in  $10^7$  marrow cells, and  $10^8$  cells must be transplanted to achieve a high probability of including at least one. (b) The deleterious effect may be caused by excessive differentiation of stem cells to produce progeny rather than division to produce stem cells shortly after transplantation. This can be mitigated only by using marrow cell doses approaching the total numbers of cells in the marrow. The similarly high losses of repopulating ability in carriers of  $10^5$ - $10^7$  marrow cells (Tables I and II) suggest that the effect is not simply caused by the amount of proliferation. (c) Lethal irradiation may damage the carrier environment permanently, preventing complete stem cell regeneration. (d) The transplantation process may physically damage stem cells.

Each of these explanations has certain weaknesses. The first suggests that  $10^7$  marrow cells usually contain none of the earliest stem cells, yet this dose repopulates and restores normal functions in genetically anemic and lethally irradiated recipients (2, 8). Furthermore,  $10^7$  marrow cells from these recipients function normally when



retransplanted in successive recipients, and this can be repeated for three to six serial transplantations (2, 8). Therefore, doses of  $10^7$  marrow cells have a very substantial degree of repopulating ability, although their recipients are defective in competitive repopulation assays.

If the second explanation is true, damage to stem cells would result from excessive stimuli to differentiate. However, the deleterious effect of transplantation in unirradiated  $W/W^v$  recipients (Table III), may be inconsistent with this idea. Levels of stimuli for early precursor cells to differentiate may be greatly reduced in  $W/W^v$  compared with lethally irradiated recipients, as the former have normal lymphoid cell functions. They live nearly normal life spans, and show normal levels of immune competence throughout (28). A dose of  $10^6$   $W/W^v$  marrow cells saves and repopulates most lethally irradiated recipients (29), and  $W/W^v$  mice have 80% of the hematocrits and hemoglobin concentrations shown by normal littermates (30). Only if this degree of anemia stimulates the excessive differentiation previously discussed would the second explanation be tenable.

The deleterious effects of transplantation in unirradiated  $W/W^v$  recipients disproves the third explanation: that the damage results solely from irradiating the recipients.

The fourth possibility seems unlikely because of the effects of two procedures: transplanting by parabiosis and sublethally irradiating the donor. In the former case all handling is avoided and cells are transplanted by natural processes; in the latter case no transplantation is performed, and stem cells regenerate without any dislocation. However, both procedures result in severe deleterious effects on stem cell function (Tables I-III). If transplantation physically damages cells, these procedures must cause equivalent amounts of damage. For example, parabiosis may not dislodge the earliest stem cells, or they may not be transplantable in the circulation. This is suggested by previous findings (21). Sublethal irradiation may permanently injure the earliest stem cells.

Recently, Micklem et al. (22) reported that long-term stem cell-repopulating abilities were not affected by as many as 25 hydroxyurea treatments given over a year. Each treatment killed 70% of the CFU-S, as well as higher percentages of more-differentiated cells. Deleterious effects may be absent because hydroxyurea treatment avoids the disruption of microenvironments caused by transplantation (22, 31). Yet damage may be caused without transplantation. We find that a single dose of sublethal irradiation severely reduces stem cell-repopulating abilities (Tables I, II). Other workers report that treatments with several other drugs (11, 32, 33), chronic damage from  $^{55}\text{Fe}$  (34), and repeated treatments with phenylhydrazine (35) all cause long-term reductions in CFU-S self-renewal. Possibly, the treatments with sublethal irradiation, the other drugs tested,  $^{55}\text{Fe}$ , and phenylhydrazine all damage the earliest stem cells; hydroxyurea and 5-fluorouracil apparently do not induce this effect (33, 36, 37). Of course, some of the other treatments may damage the stem cell microenvironment (22, 31) as well as the stem cell.

Comparing hemoglobin and chromosome markers for donor cells shows an interesting result. After a single transplantation of  $\leq 10^7$  donor marrow cells undetectable amounts of donor hemoglobin were produced in competitive repopulation recipients (Tables II and III), whereas 10-20% of proliferating cells stimulated by bleeding in similar experiments had the donor chromosome marker (Fig. 1, Table I). Thus, using hemoglobin to identify donor cells showed much more severely deleterious effects of

a single transplantation on erythropoietic stem cell lines than were detected using chromosome markers. Because chromosomes can only be visualized in mitotic cells, chromosome markers can be scored only in proliferating cells. A significant number of marrow cells proliferate in the absence of bleeding, and some of these are probably nonerythroid. Thus there may be a set of proliferating cells not as severely affected by transplantation as are erythropoietic stem cells. It is also possible that erythrocytes produced by transplanted stem cells have a shorter life span in the circulation. However, our preliminary results oppose this, because percentages of the two hemoglobin types are similar in newly synthesized, radiolabeled hemoglobin and in the hemoglobin of the total erythrocyte population.

Our comparisons of marrow cells from old and young mice confirm previous reports that they function equally well when transplanted into young recipients (2, 5-11). Reports to the contrary (38-40) may have resulted from the illness of the old donors. Because no damage results from a lifetime of normal function, marrow stem cells may not age. They are capable of functioning normally for at least five serial transplantations, saving lethally irradiated recipients, or curing genetically anemic recipients (1, 2, 8, 9, 12). The deleterious effects of transplantation may result from accelerating the mechanisms that cause normal aging, such as exhaustion of limited proliferative potential (3, 4). However, a single transplantation causes more damage than 3-7 life spans of normal function on PHA-responsive precursor cell lines and 10-20 life spans on erythropoietic precursor cell lines (Fig. 1; Tables I and III). Therefore these precursors appear to have the potential to function for at least 15, and possibly >50 life spans, because five transplantations are possible. In this case, stem cell lines would not age significantly over a normal lifespan. On the other hand, the deleterious effects of transplantation may result from causes independent of the aging process. Even in this case, the equal functional abilities of stem cells from old and young donors would suggest that they do not age.

### Summary

Long-term functional capacities of marrow cell lines were defined by competitive repopulation, a technique capable of detecting a small decline in repopulating abilities. There was little or no difference between cells from old and young donors, but a single serial transplantation caused a large decline in repopulating ability. Varying the numbers of marrow cells transplanted into the initial carrier from  $10^5$  to  $10^7$  did not alter the ability of the carrier's marrow cells to repopulate in competition with previously untransplanted cells. This ability was improved only in carriers that had received  $10^8$  marrow cells, although deleterious effects of transplantation were still present. These effects were not solely caused by cell damage from the transplantation procedure, because transplantation by parabiosis, or recovery from sublethal irradiation without transplantation, reduced repopulating abilities as much as transplanting  $10^5$  to  $10^7$  marrow cells. The transplantation effect also was not caused solely by irradiation, because the same effect appeared in unirradiated  $W/W^v$  carriers.

The transplantation effect was more pronounced when donors were identified by hemoglobin type than by chromosome markers, implying that nonerythroid cell lines may be less affected by transplantation than erythroid precursor cells. When the effects of a lifetime of normal function and a single transplantation were compared, the latter caused 3-7 times more decline in repopulating abilities of phytohemagglu-

tinin-responsive cell precursors, and at least 10–20 times more decline in erythroid cell precursors. Stem cell lines can be serially transplanted at least five times before losing their ability to repopulate and save lethally irradiated recipients or to cure genetically anemic mice. Therefore, if transplantation causes an acceleration of the normal aging process, these figures suggest that stem cells should be able to function normally through at least 15–50 life spans.

We thank Joan DeLaitre and Janan Eppig for dependable technical assistance, and Dr. Jane Barker for constructive criticism.

*Received for publication 12 August 1982.*

### References

1. Micklem, M. S., and J. F. Loutit. 1966. *Tissue Grafting and Radiation*. Academic Press, Inc., New York.
2. Harrison, D. E. 1979. Proliferative capacity of erythropoietic stem cell lines and aging, an overview. *Mech. Age Develop.* **9**:409.
3. Hayflick, L. 1965. The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37**:614.
4. Hayflick, L. 1968. Human cells and aging. *Sci. Am.* **218**:32.
5. Harrison, D. E. 1972. Normal function of transplanted mouse erythrocyte precursors for 21 months beyond normal life spans. *Nature (Lond.)*. **237**:220.
6. Harrison, D. E. 1973. Normal production of erythrocytes by mouse marrow continuous for 73 months. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3184.
7. Harrison, D. E., C. M. Astle, and J. W. Doubleday. 1977. Stem cell lines from old immunodeficient donors give normal responses in young recipients. *J. Immunol.* **118**:1223.
8. Harrison, D. E. 1979. Mouse erythropoietic stem cell lines function normally 100 months: loss related to number of transplantations. *Mech. Ageing Develop.* **9**:427.
9. Ogden, D. A., and H. S. Micklem. 1976. The fate of serially transplanted bone marrow cell populations from young and old donors. *Transplantation (Baltimore)*. **22**:287.
10. Harrison, D. E., C. M. Astle, and J. A. Delaitre. 1978. Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than aging. *J. Exp. Med.* **147**:1526.
11. Hellman, S., L. E. Botnick, E. C. Hannon, and R. M. Vigneulle. 1978. Proliferative capacity of murine hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:490.
12. Barnes, D. W. H., C. E. Ford, and J. F. Loutit. 1959. Greffes en serie de moelle osseuse chez des souris irradiées. *Sang.* **30**:762.
13. Popp, R. A. 1961. Competence of retransplanted homologous marrow cells in relation to time after original transplantation into irradiated mice. *Int. J. Radiat. Oncol. Biol. Phys.* **4**:155.
14. Siminovitch, L., J. E. Till, and E. A. McCulloch. 1964. Decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice. *J. Cell. Comp. Physiol.* **64**:23.
15. Cudkowicz, G., A. C. Upton, G. M. Shearer, and W. L. Hughes. 1964. Lymphocyte content and proliferative capacity of serially transplanted mouse bone marrow. *Nature (Lond.)*. **201**:165.
16. Doney, K. C., C. D. Buckner, E. D. Thomas, J. Sanders, R. A. Clift, J. A. Hansen, G. E. Sale, J. Singer, and R. Storb. 1981. Allogeneic bone marrow transplantation for chronic granulocytic leukemia. *Exp. Hematol.* **9**:966.
17. Johnson, F. L., E. D. Thomas, B. S. Clark, R. L. Chard, J. R. Hartmann, and R. Storb.

1981. A comparison of marrow transplantation with chemotherapy for children with acute lymphoblastic leukemia in second or subsequent remission. *N. Engl. J. Med.* **305**:846.
18. Harrison, D. E. 1981. Measuring the functional abilities of stem cell lines. In *Immunology Techniques Applied to Aging Research*. W. A. Adler and A. A. Nordin, editors. CRC Press, Inc., Boca Raton, FL. 37-50.
19. Ford, C. E. 1966. The use of chromosome markers. In *Tissue Grafting & Radiation*. H. S. Micklem and J. F. Loutit, editors. Academic Press, Inc., New York. 197-206.
20. Micklem, H. S., C. E. Ford, E. P. Evans, D. A. Ogden, and D. S. Papworth. 1972. Competitive *in vivo* proliferation of foetal and adult haematopoietic cells in lethally irradiated mice. *J. Cell. Physiol.* **79**:293.
21. Micklem, H. S., N. Anderson, and E. Ross. 1975. Limited potential of circulating haemopoietic stem cells. *Nature (Lond.)*. **256**:41.
22. Ross, E. A. M., N. Anderson, and H. S. Micklem. 1982. Serial depletion and regeneration of the murine hematopoietic system. Implications for hematopoietic organization and the study of cellular aging. *J. Exp. Med.* **155**:432.
23. Harrison, D. E. 1980. Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood*. **55**:77.
24. Kaliss, N. 1971. Jerne plaque assay for antibody-forming spleen cells: some technical modifications. *Transplantation (Baltimore)*. **12**:146.
25. Harrison, D. E. 1981. F<sub>1</sub> hybrid resistance to parental marrow grafts: long-term, radiosensitive and age-sensitivity effects over the entire hemopoietic system. *Immunogenetics*. **13**:177.
26. Nisbet, N. W. 1973. Parabiosis in immunology. *Transplant. Rev.* **15**:123.
27. Boggs, D. R., J. C. Marsh, P. A. Chervenick, G. E. Cartwright, and M. M. Wintrobe. 1967. Factors influencing hematopoietic spleen colony formation in irradiated mice. III. The effect of repetitive irradiation on proliferative ability of colony-forming cells. *J. Exp. Med.* **126**:871.
28. Harrison, D. E. 1978. Genetically defined animals valuable in testing aging of erythroid and lymphoid stem cells and microenvironments. In *Genetic Effects on Aging*. D. Bergsma and D. E. Harrison, editors., Birth Defects: Original Article Series, Vol. XIV. Alan R. Liss, Inc., New York. 187-196.
29. Harrison, D. E. 1972. Lifesparing ability (in lethally irradiated mice) of *W/W<sup>v</sup>* mouse marrow with no macroscopic colonies. *Radiat. Res.* **52**:553.
30. Harrison, D. E., and E. S. Russell. 1972. The response of *W/W<sup>v</sup>* and *Sl/Sl<sup>d</sup>* mice to erythropoietic stimuli. *Brit. J. Haematol.* **22**:155.
31. Schofield, R. 1978. The relationship between the spleen colony-forming cell and the haematopoietic stem cell. *Blood Cells*. **4**:7.
32. Popp, D. M., and R. A. Popp. 1979. Hemopoietic stem cell heterogeneity: use of cell cycle-specific drugs to look for age-associated alterations. *Mech. Age Develop.* **9**:441.
33. Botnick, L. E., E. C. Hannon, and S. Hellman. 1979. Nature of the hemopoietic stem cell compartment and its proliferative potential. *Blood Cells*. **5**:195.
34. Reincke, U., D. Brookhoff, H. Burlington, and E. P. Cronkite. 1979. Forced differentiation of CFU-S by iron-55 erythrocytocide. *Blood Cells*. **5**:351.
35. MacMillan, J. R., and N. S. Wolf. Depletion of reserve in the hemopoietic system: decline in CFU-S renewal capacity following prolonged cell cycling. *Stem Cells*. In press.
36. Hodgson, G. S., and R. R. Bradley. 1979. Properties of haemopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell. *Nature (Lond.)*. **281**:381.
37. Hodgson, G. S., T. R. Bradley, R. F. Martin, M. Sumner, and P. Fry. 1975. Recovery of proliferating haemopoietic progenitor cells after killing by hydroxyurea. *Cell Tissue Kinet.* **8**:51.
38. Price, G. B., and T. Makinodan. 1972. Immunologic deficiencies in senescence. II. Characterization of extrinsic deficiencies. *J. Immunol.* **108**:413.

39. Farrar, J. J., B. E. Loughman, and A. Nordin. 1974. Lymphopoietic potential of bone marrow cells from aged mice. *J. Immunol.* **112**:1244.
40. Albright, J. W., and T. Makinodan. 1976. Decline in the growth potential of spleen-colonizing bone marrow stem cells of long-lived aging mice. *J. Exp. Med.* **144**:1204.