LONG-TERM ERYTHROPOIETIC REPOPULATING ABILITY OF OLD, YOUNG, AND FETAL STEM CELLS*

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Stem cells, the earliest precursor cells that populate the lymphoid and hemopoietic systems in mammals, have a very high proliferative capacity. They can be transplanted by intravenous injections of single-cell suspensions. Therefore, they are useful in determining whether somatic cells are timed to age as a result of intrinsic limits on proliferative capacity. Such a prediction has been made after studies with fibroblasts in vitro (1, 2). In many experiments, hemopoietic and lymphoid stem cells from old and young donors have repopulated recipients equally well, implying that changes with age are not intrinsic (3–8). In other cases, especially with lymphoid stem cells, results have suggested intrinsic deficiencies (9–15). Gozes, et al. (16) recently reported that cellular immune responses by old stem cells declined after 8 mo, although the hemopoietic cells functioned normally. Since lymphoid and hemopoietic cells are progeny of the same precursor (17, 18), these findings suggest that stem cell regulation may alter with age. Recent results by Tyan (19) showing that both the helper and suppressor functions of Thy-1-sensitive regulatory cells change with age reinforce this suggestion. In other experiments, marrow cells from old animals failed to maintain normal hemopoiesis and lymphopoiesis (15), but marrow responses to stress were normal (20).

If there is no difference in proliferative capacity between stem cells from old and young animals, then it is possible that such cells do not age. The experiments reported in this paper were designed to test stem cell function by mixing stem cells from old, young, or fetal donors with a standard dose of young competitor cells, and injecting the mixtures into stem cell-depleted recipients. Under these conditions, the two cell types in the mixture, each producing distinguishable hemoglobins, compete to repopulate the host (8, 21, 22). After the recipient is fully repopulated, percentages of donor cells are estimated by measuring the percentage of donor type hemoglobin. This is proportional to the percentage of erythropoietic stem cells of the donor type (22). To test stem cell proliferating abilities more rigorously, we stimulated an additional round of rapid repopulation by sublethal whole body irradiation. This treatment causes a surprisingly severe deleterious effect on stem cell repopulating abilities (8). The relative repopulating abilities of old, young, and fetal stem cells were again determined by the percentage of donor type hemoglobin in the recipients.

Other workers have reported that stem cells from fetal liver have higher proliferative capacities (23) and better competitive repopulating abilities (24) than stem cells from marrow. While this seems plausible, it is not what would be expected if stem cells had

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infinite proliferative capacities. In neither of the studies (23, 24) were the differentiated cells produced by the fetal donor's stem cells identified. The present study shows the contribution to the erythrocyte pool of stem cells from equal doses of old marrow, young marrow, and fetal liver. When B6 donors were used, old stem cells repopulated erythroid compartments more effectively than young or fetal stem cells. This remained true after 500 rad irradiation, and in nonirradiated *W*-anemic recipients. When B6CBAF$_1$ donors were used, stem cells from old marrow were more effective than those from young. However young stem cells were more effective when CBA marrow was tested. These data suggest that aging is not intrinsic in stem cells, and genetic factors appear to determine how their regulation is affected by aging.

Materials and Methods

Mice. C57BL/6J (B6), CBA/CaJ (CBA), CBA/HT6J (CBAT6), the F$_1$ hybrid of WB/ReJ and B6 (WBB6F$_1$), B6CBAF$_1$, and B6CBAT6F$_1$ hybrid mice were bred and maintained at The Jackson Laboratory, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. WB-*W*/+ × B6-*W^v*/+ parents were used to produce WBB6F$_1$-*W*/*W^v* mice, genetically anemic mutants with defective erythropoietic stem cell lines (25). Some mice of genotypes WBB6F$_1$-*W*/+ or *W^v*/+ were thymectomized at 4–6 wk of age by sucking the thymus into a glass pipette and rapidly closing the incision. The thymectomized mice were immunologically crippled by lethal irradiation, and were used as recipients.

Irradiation. All recipients, except the *W*/*W^v* mice, were lethally irradiated with 1,100–1,200 rad in a Shepherd Mark I Gamma Irradiator with a Cs-137 source at dose rates of 150 rad/min 15–18 h before marrow was transplanted. For sublethal irradiation (500–600 rad), donors were treated 90–120 d before they were used. Recipients of mixtures were checked 77–106 d after the sublethal irradiation.

Transplantation and Competitive Repopulation Assay. Marrow cell suspensions were prepared by rinsing both femurs and tibias of each donor with a balanced salt solution buffered by Hepes to a pH of 7.4 (8). Livers from 15–16-d fetal mice were minced in the same type of media. Clumps of cells were dissociated by hydrostatic pressure as they were gently expelled through a 1-ml disposable syringe pressed against the bottom of a 4-ml sterile plastic culture tube. Suspensions were filtered through 100-mesh nylon cloth, and cells were counted using a Coulter model ZBI electronic cell counter (Coulter Electronics Inc., Hialeah, FL). Appropriate numbers of cells from each donor were mixed with equal numbers of marrow cells from a pool of genetically distinguishable competitors. Mixtures were injected intravenously into the appropriate recipients.

Erythropoietic stem cell proliferation was analyzed using donors and competitors with electrophoretically separable hemoglobins (22). B6 mice have $\alpha_2\beta_2^s$ (single) hemoglobin, whereas WB and CBA mice have $\alpha_2\beta_2^{dmaj}$ and $\alpha_2\beta_2^{dmin}$ (diffuse) hemoglobins. There is little or no hybrid resistance from WBB6F$_1$ animals against B6 cells, or from B6CBAF$_1$ animals against CBA cells (26). Chromosome markers were identified in proliferating cells using standard techniques (6). To identify specific cell types, T and B cells from spleens were stimulated to proliferate in vitro by phytohemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS), respectively, while marrow cells were stimulated in vivo by bleeding (6, 8, 21).

Results

In the first experiment, host hemoglobins were quantitated ~90 d after donor and competitor stem cells were injected into lethally irradiated WBB6F$_1$ hosts. B6 stem cell donors were either old marrow, young marrow, or fetal liver; each was mixed with young WBB6F$_1$ marrow competitor stem cells. The B6 cells synthesize only single hemoglobin, whereas the WBB6F$_1$ cells produce equal quantities of single and diffuse hemoglobin. Thus the percentage of hemoglobin produced by B6 cells may be readily calculated from the measured percentages of single and diffuse hemoglobin as

previously described (22); data are given as the percentage of B6 donor hemoglobin. Descendants of stem cells from old B6 donors produced 25% more hemoglobin than those from young donors and 31% more than those from 15–16-d fetal livers (Table I).

The recipients were sublethally irradiated with 500 rad after 120 d, and the hemoglobins were checked 77 d thereafter. Recovery from the sublethal irradiation requires a second round of rapid stem cell proliferation; this might show defects in the proliferative capacity of old stem cells. However, these cells retained their advantage. Descendants of old stem cells produced 15% more B6 hemoglobin than those of young stem cells, and 43% more B6 hemoglobin than those of fetal cells (Table I). After sublethal irradiation, the percentages of B6 hemoglobin fell, regardless of whether the donor stem cells were from fetal liver, young marrow, or old marrow (Table I). Perhaps this occurred because B6 stem cells from all three sources were slightly more radiosensitive than the $WBB6F_1$ competitor stem cells.

A single dose of sublethal irradiation severely damages competitive repopulating ability (8), as is illustrated in Fig. 1. After recovering for 90 d from 550 rad, stem cells from the sublethally irradiated donor produced undetectable amounts (2 ± 1%) of the hemoglobin when mixed with unirradiated competitor cells, whereas stem cells from an untreated donor produced 42 ± 4% of the hemoglobin (Fig. 1). When chromosome markers were used to distinguish donor and competitor, sublethal irradiation 120 d before the assay reduced the ability of stem cells to produce T, B, and erythroid cell precursors three- to sevenfold; these responded to PHA, LPS, and severe bleeding, respectively (Fig. 1). These experiments suggest that recovery from sublethal irradiation is a stress that should uncover subtle defects in stem cells from aging donors. As shown in Table I, no such defect was found.

A second experiment was performed to test the effect of using immune competent recipients. $WBB6F_1$-W/W^v recipients can be repopulated without irradiation, maintaining fully functional lymphoid systems during repopulation (25). B6 stem cells from old marrow, young marrow, or fetal liver donors were each mixed with identical doses of young marrow from $WBB6F_1$ competitors as in the first experiment. Mixtures were injected into $WBB6F_1$-W/W^v recipients, and into $WBB6F_1$-$W/+$ or $W^v/+$

TABLE I

Effect of Stem Cell Age on Erythropoietic Repopulating Ability in Serially Irradiated Recipients

Recipient Hb measured*		Percentage Hb‡ of B6 donor type:		
Days after cell injection	Recipient treatment	Fetal liver	Young marrow	Old marrow
80–100	Lethal irradiation day − 1	53 ± 3 (7)	59 ± 8 (8)	84 ± 4 (8)
187–207	Lethal irradiation day −1 plus 500 rad day 110–130	21 ± 3 (7)	49 ± 10 (7)	64 ± 8 (8)

* Recipient hemoglobins were initially checked 80–100 d after male $WBB6F_1$ recipients were lethally irradiated and grafted with a mixture of 2.5×10^6 cells from the appropriate B6 donor plus 1.0×10^6 marrow cells pooled from 6-mo-old $WBB6F_1$ +/+ competitors. Recipients were given an additional 500 rad gamma (Cs-137) irradiation 30 d later (at 110–130 d), and hemoglobins were checked a second time in 77 d (187–207 d after mixtures were transplanted).

‡ Hemoglobin (Hb) percentages are given as mean ± SE (number of donors with a mean of 3.4 recipients per donor). Liver was taken from 15–16-d fetuses; young marrow donors averaged 4.1 mo and old marrow donors 26.4 mo of age. All donors were B6 mice.

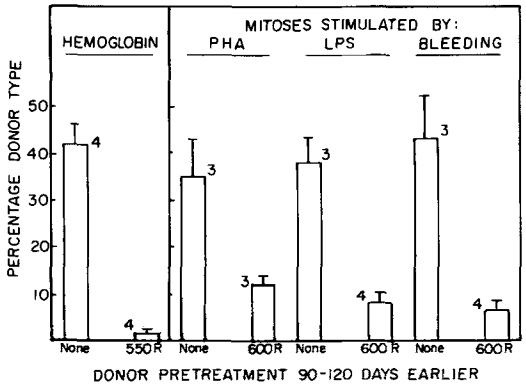

FIG. 1. The effect of sublethal irradiation on stem cell competitive repopulating ability. Donors were tested in the competitive repopulation assay either without pretreatment or after receiving 550–600 rad gamma (Cs-137) 90–120 d previously. Hemoglobin markers were used to distinguish erythrocytes produced by stem cells from 8-mo-old CBAT6 donors and those from 5-mo-old B6CBAF$_1$ competitors in the left hand panel; mixtures of 1×10^6 donor and competitor marrow cells were given to each lethally irradiated B6CBAT6F$_1$ recipient 90 d before hemoglobins were assayed. Chromosome markers were used to distinguish mitoses stimulated by PHA or LPS with spleen cells in vitro or by bleeding with marrow cell in vivo in the next three panels. Donors were 6-mo-old B6CBAT6F$_1$ mice, whereas competitors were 5-mo-old B6CBAF$_1$ mice. Each lethally irradiated B6CBAF$_1$ recipient was given 3×10^6 donor and competitor marrow cells 75–150 d before its cells were stimulated for the assay, and an average of 48 mitoses were scored per recipient. Data bars show mean ± SE; numbers of recipients are given by the top of each bar. In each panel, percentages of donor type were significantly lower after sublethal irradiation ($P < 0.05$, Student-Newman-Keuls multiple range test).

TABLE II

Effect of Recipient Immune Responses on Repopulating Ability

Recipient Hb measured*		Percentage Hb‡ of B6 donor type:		
Days after cell injection	Recipient treatment	Fetal liver	Young marrow	Old marrow
82–98	Thymectomized, lethal irradiated day −1	55 ± 6 (2)	29 ± 5 (5)	75 ± 8 (6)
82–98	Intact W/W^v	43 ± 2 (2)	31 ± 5 (5)	52 ± 3 (6)

* Recipient hemoglobins were initially checked 82–98 d after male WBB6F$_1$ recipients were given a mixture of 2.5×10^6 cells from the appropriate B6 donor plus 1.0×10^6 marrow cells pooled from 4–5-mo-old WBB6F$_1$ +/+ competitors. Thymus glands had been removed from the thymectomized recipients at 5–6 wk of age, so that T cells could not regenerate after lethal irradiation. Intact male WBB6F$_1$-W/W^v recipients were given identical cell mixtures, but were not irradiated.

‡ Hemoglobin (Hb) percentages are given as mean ± SE (number of donors with a mean of 4.5 recipients per donor). Liver was taken from 15–16-d fetuses; young marrow donors averaged 4.3 mo and old marrow donors 25.7 mo of age. All donors were B6 mice.

recipients immunologically crippled by thymectomy followed by lethal irradiation. The results are presented in Table II. Again, stem cells from old B6 mice repopulated most effectively, with their descendants producing 46% more B6 hemoglobin than young stem cells and 20% more than fetal liver in immunologically crippled animals. Old cells produced 21% more B6 hemoglobin than young stem cells and 9% more than fetal liver in immunologically intact W/W^v recipients.

Some of the recipients described in Table II were sublethally irradiated 90 d after

TABLE III

Serial Irradiation of T Cell-deficient and Intact Recipients

Recipient Hb measured*		Percentage Hb‡ of B6 donor type:	
Days after cell injection	Recipient treatment	Young marrow	Old marrow
82	Thymectomized, lethal irradiated day −1	32 ± 5 (3)	87 ± 2 (3)
82	Intact W/W^v	36 ± 4 (3)	55 ± 5 (3)
196	Thymectomized, lethal irradiated day −1 plus 500 rad day 90	37 ± 6 (3)	81 ± 7 (3)
196	Intact W/W^v plus 500 rad day 90	38 ± 6 (3)	60 ± 4 (3)

* As in Table II, except that 8 d after the first hemoglobin samples were taken, all recipients were given 500 rad of gamma (Cs-137) irradiation. The second hemoglobin samples were taken 106 d afterwards.

‡ As in Table II, except an average of 4.6 recipients per donor, and old and young donors were 25.2 and 3.8 mo old, respectively. Some of these data are included in Table II.

injection with donor cells. Old B6 stem cells continued to outperform young ones, with their descendants producing 55 and 44% more B6 hemoglobin than cells from young donors in immunologically crippled recipients before and 106 d after sublethal irradiation, respectively (Table III). They produced 19 and 22% more hemoglobin than young stem cells in immunologically intact W/W^v recipients after the same time periods.

Mice of other genotypes were also tested to determine the effects of donor age. Marrow cells from old and young B6CBAF$_1$ hybrids were mixed with portions from a pool of CBA competitor marrow cells, and mixtures were given to the following types of lethally irradiated B6CBAF$_1$ recipients: old given an infant thymus, young given an infant thymus, and young. Infant thymuses were given to improve immune responses in the old recipients (27).[1] In these experiments, the CBA cells synthesize only diffuse hemoglobin, whereas the B6CBAF$_1$ cells produce equal quantities of single and diffuse hemoglobin. Thus the percentage of hemoglobin produced by B6CBAF$_1$ or CBA cells may be readily calculated from the measured percentages of single and diffuse hemoglobins, as previously described (22). Data are given as the percentage of donor hemoglobin. The amounts of single and diffuse hemoglobin were measured after ~87 d, and rechecked after ~195 d in these recipients; sublethal irradiation was not used. Neither recipient age nor the presence of an infant thymus affected the relative repopulating abilities of stem cells from old and young donors (Table IV). With B6CBAF$_1$ donors, descendants of old stem cells produced ~20% more hemoglobin than descendants of young stem cells. However, with CBA donors, old stem cells produced ~20% less hemoglobin than young ones (Table IV).

[1] Astle, C. M., and D. E. Harrison. Beneficial effects of young but not old stem cells on immune responses of aged mice: studies at the cellular level. Manuscript submitted for publication.

TABLE IV

Effect of Stem Cell Age on Erythropoietic Repopulating Ability In Old and Young Recipients

Donor	Competed against	Time in recipient‡	Percent donor* hemoglobin in:		
			Old + IT recipients§	Young + IT recipients§	Young recipients§
Old $B6CBAF_1$	Young CBA	1	77 ± 7 (3)	75 ± 8 (4)	83 ± 4 (4)
		2	77 ± 4 (3)	72 ± 12 (3)	88 ± 7 (3)
Young $B6CBAF_1$	Young CBA	1	56 ± 3 (3)	50 ± 6 (4)	50 ± 11 (4)
		2	68 ± 4 (3)	70 ± 6 (3)	63 ± 11 (3)
Old CBA	Young $B6CBAF_1$	1	26 ± 5 (4)	17 ± 2 (3)	16 ± 3 (4)
		2	7 ± 2 (3)	8 ± 2 (3)	9 ± 2 (4)
Young CBA	Young $B6CBAF_1$	1	40 ± 4 (4)	44 ± 6 (4)	44 ± 3 (4)
		2	22 ± 8 (3)	22 ± 3 (4)	25 ± 3 (4)

* Old donors were 811–938 d old and young donors were 91–147 d old, while old recipients were 610–680 d old and young recipients 80–100 d old when cells were injected. Equal numbers (1×10^6) of marrow cells from old and young $B6CBAF_1$ donors were mixed with 1×10^6 marrow cells from pooled young CBA competitors (top four lines); the same cell numbers were used with old and young CBA donors and pooled $B6CBAF_1$ competitors (bottom four lines).

‡ Time since grafting is given as 1 (the first hemoglobin analysis after 87 ± 17 d) and 2 (the second analysis after 195 ± 30 d).

§ All recipients were lethally irradiated. Those designated IT were given infant (0–3-d-old) thymus transplants under the kidney capsule (27)[1] 1–5 d after lethal irradiation and marrow transplantation. Results are given as mean ± SE (number of donors) for the percentages of donor hemoglobin. Usually two recipients were averaged for each donor value.

Discussion

This study demonstrates that long-term erythropoietic stem cell functions do not necessarily decline with age. In fact, descendants of stem cells from old B6 mice consistently produced erythrocytes more effectively than those from stem cells of young B6 or fetal B6 mice, when competing against young $WBB6F_1$ stem cells (Tables I–III). The descendants of stem cells from old $B6CBAF_1$ donors also produced erythrocytes more effectively than those from young $B6CBAF_1$ donors in competition with young CBA stem cells. On the other hand, stem cells from old CBA mice were less effective than those from young CBA mice (Table IV). These results suggest that erythropoietic stem cells maintain their proliferative abilities unchanged throughout the lifespan, and that the differences shown in this report result from alterations with age in regulatory functions.

Competitive repopulating abilities are affected by both long- and short-term differences in the ability of stem cells to proliferate and differentiate in lethally irradiated recipients. Maximal functional abilities are tested that allow subtle differences between stem cell types to be demonstrated. For example, using competitive repopulation techniques, 5- to 20-fold differences are demonstrated between repopulating abilities of untransplanted stem cells and those transplanted a single time (6, 8, 21, 28).

The studies described in Tables I and III test proliferation and differentiation even more rigorously, since the cells must recover from sublethal irradiation after repopulating the recipient; this recovery requires another burst of proliferation and differ-

entiation. Fig. 1 shows that sublethal irradiation greatly reduces stem cell repopulating abilities (8). Descendants of old stem cells should recover less well if they have any subtle functional defects. Therefore, the fact that old B6 stem cells maintain their advantage over young or fetal stem cells after sublethal irradiation suggests that the proliferative capacity of B6 stem cells is unimpaired with age.

The advantage of old erythropoietic stem cells in two of three genotypes tested is unexpected. Possibly regulatory changes with age in old B6 donors allow their stem cells to proliferate especially rapidly after transplantation. If the old stem cells have an initial advantage, their competitor cells may never be able catch up. The advantage of old B6 cells is reduced in immunologically intact W/W^v recipients compared with immunologically crippled thymectomized and lethally irradiated recipients (Tables II and III). Old marrow may contain higher concentrations of graft-vs.-host (GVH)-reactive cells that react against the $WBB6F_1$ competitors, reducing their repopulating ability. GVH reactions do not harm *W*-anemic recipients (29), probably because their intact lymphoid cells protect them. This effect would also protect $WBB6F_1$ competitor cells against GVH reactions. It is also possible that the advantage of old stem cells is reduced in immunologically intact W/W^v recipients because abnormalities that develop in erythropoid committed stem cells with age are eliminated by the immune system. Nevertheless, even in these recipients, erythropoietic precursor cells from old donors produce more hemoglobin than those from young or fetal liver donors when competing against the same competitors (Table II), and this advantage is maintained after recovery from sublethal irradiation (Table III). Results with our system would be misleading if erythrocytes produced by descendants of old stem cells had extended lifespans in the circulation; this, however, seems extremely unlikely.

Young adult marrow repopulated as well as fetal liver when seven to eight donors were compared (Table I), though not in a second experiment, using only two fetal donors (Table II). It repopulated better after sublethal irradiation (Table I). These results seem to conflict with previous reports (24) that stem cells from fetal liver repopulate 5–10 times better than those from adult marrow when equal numbers of cells are mixed, and recipients are studied after >50 d. Possibly, the specific mouse genotype tested is important; we used B6 mice, whereas Micklem and his colleagues (24) compared fetal and adult cells from CBA mice. Another possible explanation is that they measured all proliferating cells, while we measured erythropoietic stem cell function specifically.

The most likely explanation for our results is that erythropoietic stem cells do not change their repopulating capacities during the lifespan of the mouse. The differences observed between young, old, and fetal cells probably result from regulatory changes with age. These regulatory changes give stem cells from old B6 or $B6CBAF_1$ mice a temporary advantage on transplantation, whereas changes with age in CBA mice have the opposite effect. Because the stem cells all have the same intrinsic repopulating abilities, the competitor cells are never able to overcome this early advantage, although it probably lasts for only a short time. This explanation implies that stem cells are not affected by aging.

Summary

It is possible that erythropoietic stem cells do not age. This would mean that stem cells from old donors can function as well as those from young or fetal donors. The

competitive repopulation assay has been used to test long-term stem cell function by directly comparing how well competing stem cells repopulate a recipient and produce differentiated cell types. C57BL/6J (B6) mice were used as donors, while recipients and competitors were $WBB6F_1$ hybrids with genetically distinguishable hemoglobin. Lethally irradiated young $WBB6F_1$ recipients were given a mixture of 2.5×10^6 cells from B6 old marrow, young marrow, or fetal liver donors; each recipient also received a standard dose of 1×10^6 marrow cells from a pool of young $WBB6F_1$ competitors. Surprisingly, the old marrow cells competed the best in repopulating the recipients. This pattern was maintained even after recovery from sublethal irradiation, a treatment that severely stresses stem cells. This stress was demonstrated when sublethal irradiation caused a 20-fold decline in repopulating ability measured using hemoglobin markers, and a 3- to 7-fold decline using chromosome markers. Stem cells from old marrow competed better than young or fetal cells in similar experiments using immunologically crippled recipients or using unirradiated W/W^v recipients that are immunologically intact. In both types of recipients, the advantage of old marrow cells again persisted after recovery from sublethal irradiation. Other genotypes were tested, and marrow cells from old $B6CBAF_1$ donors competed better than those from young donors of that genotype. However, marrow cells from young CBA donors completed better than those from old CBA donors. These results support the hypothesis that stem cells do not age, and suggest that regulatory changes with age promote rapid stem cell repopulation in B6 and $B6CBAF_1$ mice, but inhibit it in CBA mice.

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