

DECLINE IN THE GROWTH POTENTIAL OF SPLEEN-COLONIZING BONE MARROW STEM CELLS OF LONG-LIVED AGING MICE

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The decline in humoral and cell-mediated immune responses of aging mice has been attributed to changes both intrinsic and extrinsic to cells of the immune system (1-3). The pluripotent stem cells have been investigated very extensively (4, 5) because it is obvious that any deficit in them will result in pronounced deficiencies in mature, functional progeny (6-8). Furthermore, they can be analyzed independently (9-11). These age-related studies of the stem cells revealed the following: (a) The relative number of stem cells in the bone marrow, which constitute 90% of the total stem cell population (12), declines moderately with age in long-lived mice (12, 13) but their total number remains constant or even increases slightly (12, 14). (b) When subjected to *in vivo* serial cell transfer, stem cells gradually lose their ability to self-replicate with increasing passage, independent of the time interval between passages (4, 15, 16). (c) When given sufficient time to repopulate *in vivo*, young and old stem cells are equally capable of repopulating stem cell-deficient mice (16-19). However, preliminary studies suggest that young stem cells may be more proficient than old stem cells in their ability to self-replicate and generate bone marrow-derived (B) cells shortly after transfer (19-21).

The present studies were undertaken to investigate the extent to which various kinetic parameters of stem cells change with advancing age. Further, we studied not only the reversibility of such changes by culturing old stem cells in young recipients for extended times, but also determined whether young stem cells cultured in old recipients would precociously acquire defects.

Materials and Methods

Animals. BC3F1, B6D2F1, and C3H mice with a mean lifespan of 27-30 mo (22-23) were purchased from Cumberland View Farms, Clinton, Tenn., at 6 wk of age and reared in a conventional animal facility. Both male and female animals were used without regard to sex. Young mice were 4-6 mo of age and old mice were 22-26 mo.

Radiation. Mice were given 850 R total body irradiation with a Ridge Instrument Company 300 KVP Biological Specimen X-Ray Irradiator (Ridge Instrument Company, Atlanta, Ga.). The machine was operated at 250 kV, 10 mA, and 70 R/min with a filtration of 1.1 mm Cu at a skin-target distance of 29 inches.

Cell Suspensions. Mice were killed by cervical dislocation, and a gross pathological examina-

tion was routinely performed on all old mice. Cells were taken only from animals judged to be free of tumors and other diseases. The bone marrow was flushed with RPMI 1640 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) using a 3 ml syringe with a 23 gauge needle, and the viable cells determined in a hemocytometer by the dye exclusion test using 0.01% Trypan blue.

Spleen Colony Assays. Spleen colony-forming units (CFU-S)¹ were determined by the method of Till and McCulloch (7). Young or old irradiated recipient mice each received intravenously 5.0×10^4 donor marrow cells in 0.5 ml medium, and the number of colonies was determined 10 days later when the colonies attained their maximum size. Colony size was estimated by counting the number of cells in individual spleen colonies. Generally, two to four of the largest noncoalesced individual colonies were removed surgically from each spleen with a pair of 27-gauge needles and teased in 1 ml of medium. Nucleated cells were counted in a hemocytometer. A minimum of four recipients was used per experimental group.

¹²⁵I-Labeled Iododeoxyuridine (¹²⁵IUdR) Uptake. The growth rate of marrow cells in the spleens of recipient mice was evaluated by measuring incorporation of ¹²⁵IUdR (24, 25) (sp act, 1.13 mCi/mg; New England Nuclear, Boston, Mass.). Recipients were injected with 10^6 young donor marrow cells or 2×10^6 old donor cells intravenously because Chen showed that the concentration of stem cells decreased by twofold with age in these long-lived BC3F1 mice (12). At daily intervals thereafter a group of recipients was given 1 ml of 10^{-7} mol of fluorodeoxyuridine (FUdR), followed 1 h later by an intraperitoneal injection of 1 μ Ci ¹²⁵IUdR. FUdR injection reduced the competition by endogenous thymidilate for incorporation of ¹²⁵IUdR into DNA (24). 17 h after the ¹²⁵IUdR injection, the spleens of recipients were collected and the incorporated radioactivity (counts per minute per spleen) was determined (Nuclear-Chicago Automatic Gamma Counter; Nuclear-Chicago Corp., Des Plaines, Ill.).

Preliminary experiments showed that the log phase of growth extended for a period of 1 wk after bone marrow cell transfer. Varying numbers of bone marrow cells from young or old donors were then transferred into young, irradiated recipients. Growth of each marrow inoculum was assessed by injecting an excess of ¹²⁵IUdR 7 days later and by determining the amount of radioactivity incorporated into host spleen cell DNA. The purpose was to determine the optimum dose of young and old bone marrow cells for subsequent kinetic studies. These preliminary experiments revealed that a linear relationship exists between bone marrow inoculum size and ¹²⁵IUdR incorporation in the range of 0.5 – 2.0×10^6 cells for both young and old marrow, and the slopes of the regression lines were comparable.

Secondary Marrow Cell Transfer. Young and old irradiated intermediate recipient mice were given 2.5×10^6 young or old marrow cells intravenously, killed 6 or 16 wk later, and the femoral bone marrow cells collected and assessed for CFU-S.

Chromosome Marker. CBA/CaJ (CBA) and histocompatible CBA/H-T6T6J (CBA-T6T6) mice were obtained originally from The Jackson Laboratory, Bar Harbor, Maine, and produced in our conventional animal facility; both have mean lifespans of 27–30 mo. Young mice were 7-mo old and old mice were 24-mo old. Irradiated CBA recipients were given 2.5×10^6 CBA-T6T6 donor cells intravenously. 3 mo later, each recipient received 0.25 ml of 0.05% colchicine (lot 530-1680; Sigma Chemical Co., St. Louis, Mo.) intraperitoneally. Bone marrow cells from each group were pooled 90 min later. The cell suspensions were washed and incubated with 0.56% KCl for 30 min at room temperature, then centrifuged at 500 rpm for 10 min. The pellets were dispersed and fixed in absolute methanol and acetic acid solution (3:1). The smears were stained with 2% Giemsa stain (Harleco, Gibbstown, N. J.) and mitotic cells were counted.

Results

The growth rates of young and old bone marrow cells in the spleens of young and old recipient mice were assessed, based on daily measurement of splenic ¹²⁵IUdR incorporation, for 6 and 7 days, respectively, after injection of 10^6 marrow cells. The results (Fig. 1) showed that in young recipients there was a

¹ Abbreviations used in this paper: CFU-S, spleen colony-forming units; FUdR, fluorodeoxyuridine; ¹²⁵IUdR, ¹²⁵I-labeled iododeoxyuridine.

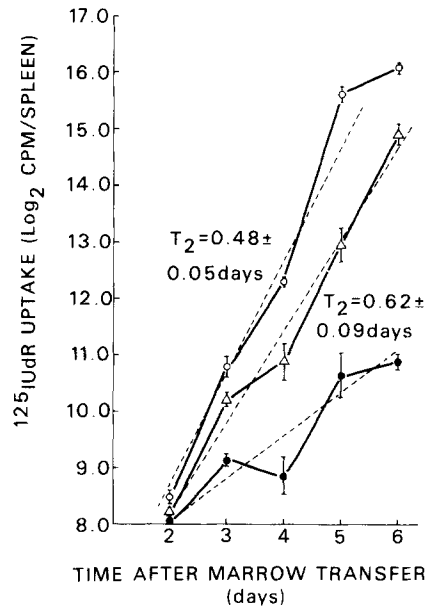


FIG. 1. Incorporation of ^{125}I UdR into spleens of young, irradiated recipients at various times after transfer of 10^6 donor marrow cells. Dashed lines are fitted to points on linear portions of curves by method of least squares ($Y = a + a_1X$). (○), young marrow cells ($a_1 = 2.02 \pm 0.11$); (△), old marrow cells ($a_1 = 1.61 \pm 0.28$); (●), control, no cells ($a_1 = 0.76 \pm 0.16$). Difference in slopes between young and old was significant at the 1.0% level. Vertical bars represent one standard error. Sample size, ≥ 4 animals per group.

slight difference in the growth rate between young and old marrow cells with doubling times of 0.48 ± 0.05 and 0.62 ± 0.09 days, respectively ($P < 0.05$). Results similar to those shown in Fig. 1 were obtained after transfer of four times as many young and old marrow cells into young recipients (data not shown). The difference between young and old marrow cells was also displayed when cultured in old recipients (Fig. 2). There was a noticeable delay in the proliferation of old bone marrow cells and the number of proliferating cells in old marrow was less than that of young marrow. However, the growth rates did not differ significantly. In general, the results presented in Figs. 1 and 2 indicate that old marrow cells seem to display intrinsic defects in their ability to proliferate in the spleen of both young and old recipients.

In subsequent studies, we employed a second method for assessing the proliferative ability of young and old marrow cells. The frequency of CFU-S in the marrow of young and old BC3F1 donors was assessed by the spleen colony assay method, with colony counts made on day 10 after marrow transfer. In the same experiments, the largest individual colonies were dissected out of recipient's spleens on the 10th day after marrow transfer. The cells in individual colonies were dispersed by teasing, and the number of cells was counted. Data concerning frequency of CFU-S and mean cell number of discrete colonies are given in Fig. 3. It is obvious that the frequency of CFU-S in the old marrow was about two-thirds that of young marrow based upon assay in young recipients. Furthermore, the largest colonies formed by old marrow CFU-S contained only half as

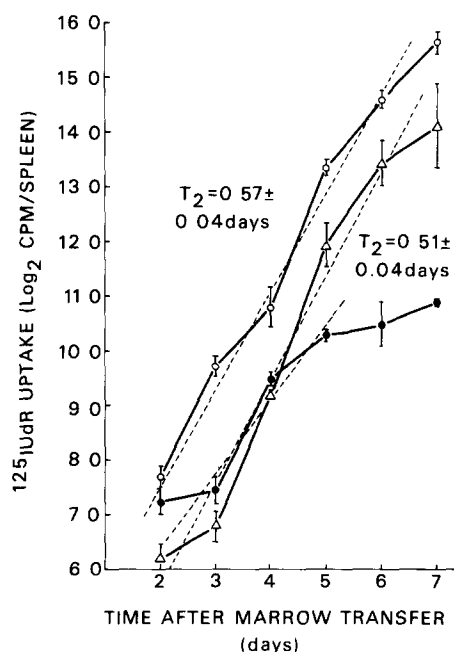


FIG. 2. Incorporation of $^{125}\text{IUdR}$ into spleens of old, irradiated recipients at various times after transfer of 10^6 donor marrow cells. Dashed lines are fitted to points on linear portions of curves by method of least squares using estimating equation $Y = a + a_1X$. (\circ), young marrow cells ($a_1 = 1.74 \pm 0.12$); (Δ), old marrow cells ($a_1 = 1.96 \pm 0.13$); (\bullet), control, no cells ($a_1 = 1.41 \pm 0.17$). Difference in slopes between young and old significant at $<1\%$ level. Vertical bars represent one standard error. Sample size, ≥ 3 animals per group.

many total cells as did the largest colonies formed by young marrow CFU-S. A pronounced decrease in the CFU-S efficiency of young marrow cells was observed when injected into old recipients, i.e., the spleens of old recipients were decidedly less suitable than spleens of young recipients for growth of marrow cells. Thus, for a given inoculum of old marrow cells, only half as many colonies developed in old recipients as in young and the mean number of cells in the largest colonies in old recipients was only one-third as great as in young recipients.

From the standpoint of clonal growth, the deficiency of old CFU-S relative to young CFU-S represents about one less mitotic division. To have a better insight into this problem, the data summarized in Fig. 3 were analyzed in terms of frequency distribution. The results (Fig. 4) showed that the number of mitotic divisions was reduced anywhere from one to four among various colonies derived from old donor CFU-S, i.e., the pattern of distribution was polydispersed in contrast to that of young CFU-S. The average large colony formed by young donor cells in young recipients contained $\sim 2 \times 10^6$ total cells, i.e., 21 divisions were required to produce the total number of cells starting with a single stem cell. By the same reasoning, the colonies produced by old CFU-S underwent 17 to 20 divisions, with a maximum deficit of 4 out of 21 divisions. Such reasoning leads to the conclusion that on the average, aged CFU-S retain at least $\sim 80\%$ of the potential for proliferation that is typical of young CFU-S ($17/21 \times 100$).

GROWTH POTENTIAL OF STEM CELLS

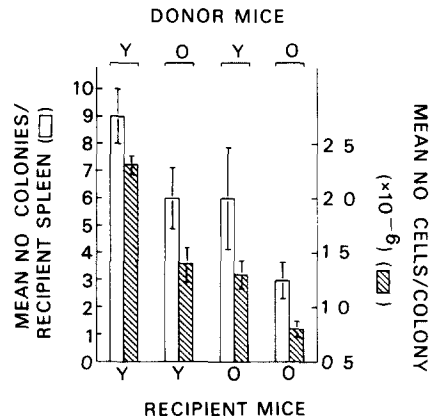


FIG. 3. Formation of colonies in the spleens of irradiated young or old recipient mice after transfer of 5×10^4 young or old donor marrow cells. Number of colonies (□) estimated on day 10 after marrow transfer; number of cells per largest colony (▨) counted on day 10 after marrow transfer. Sample size, ≥ 10 individual colonies per group. Vertical bars represent one standard error.

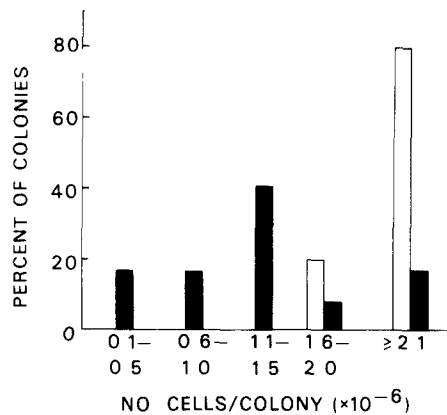


FIG. 4. Variability in colony size in young assay recipients as influenced by the age of marrow cells. (□), young marrow cells; (■) old marrow cells. Number of cells per colony determined by counting a minimum of 10 largest individual colonies per group and the number of colonies was determined from a minimum of 4 animals per group.

In order to be certain that our technique of dissecting out spleen colonies resulted in scoring of virtually all proliferating cells in spleen colonies, we relied on experiments with cells carrying T6 marker chromosomes. Young or old irradiated CBA mice were injected with marrow from young CBA-T6T6 donors. 10 days later, the recipients were sacrificed, splenic colonies were dissected out, and slides were prepared of both the colonies and the tissue immediately surrounding the cavity left after dissecting out the colonies. 5,000 cells from each of the series of slides were scored for mitotic figures and for the presence of the two T6 chromosomes in mitotic figures. It was found that $\sim 2\%$ of the cells present in colonies of young recipients were in mitosis and $>95\%$ of the mitotic figures displayed the T6 chromosomes; among the noncolony cells $\sim 0.02\%$ were in mitosis, and, owing to the very low frequency, an accurate estimate of the occurrence of the T6 chromosomes was impossible. The colonies taken from old

recipients displayed 0.9% mitotic cells of which virtually all had the T6 marker chromosomes; in contrast, among 5,000 noncolony cells scored, only two were found in mitosis, and neither had the T6 marker chromosomes. These studies show that essentially all the proliferating cells are part of the colonies and are of donor origin.

The reduction in growth potential of marrow cells that is associated with aging in BC3F1 mice was estimated by determining the total number of CFU-S cells in young and old hosts per equivalent numbers of young and old marrow cells injected (i.e., number of CFU-S/spleen X cells/CFU-S). The results are summarized in Table I. It can be seen that when the growth of young marrow in young recipients is assigned a value of 100%, the growth potential of equivalent cells cultured in old recipients is able to express only 38% of its growth potential. The growth potential of old marrow cells in old recipients is only 12% of that of an equivalent number of young marrow cells in young recipients. When the growth of old marrow in young recipients is assigned a value of 100%, an equivalent number of old marrow cells expresses only 29% of its growth potential when grown in old hosts. As shown in Table I, two other genetically different types of mice were also studied, one hybrid line and one parental strain. The trend was the same for all regardless of the genetic composition.

The data summarized in Table I clearly suggest that both intrinsic changes and extrinsic influences affect the aging marrow cells. In order to gain further information about these changes and their stability or reversibility, we cultured old and young donor marrow cells *in vivo* in old and young intermediate, syngeneic hosts. Hosts were lethally irradiated and infused with 2.5×10^6 donor marrow cells. They were subsequently sacrificed at either 6 or 16 wk post-transfer, and femoral marrow cells were collected and assayed for spleen colony formation in irradiated recipients. The data from assays involving 10 combinations performed after 6 wk of culture in intermediate hosts are displayed in Table II. The salient findings are as follows: (a) Aging marrow cells are influenced by both intrinsic and extrinsic changes (cf. combinations 1 and 6, and combinations 2 and 7). (b) When young marrow cells are cultured in old hosts, the recovered marrow generally performs as though it had been derived directly from an old donor (cf. combinations 1, 2, and 4; and combinations 6, 7, and 8). (c) On the other hand, when old marrow cells are cultured in young hosts, the recovered marrow performs as though it had been derived directly from an old donor (cf. combinations 1, 2, and 5; and combinations 6, 7, and 9). Results comparable to those present in Table II were also obtained by culturing marrow cells in intermediate hosts for 16 wk. Especially noteworthy was that even after 16 wk of culture in young hosts, old marrow cells still perform as though they had been obtained directly from an old donor. This would suggest that such age-related changes are stable. Thus, this series of studies demonstrates that while the young environment is unable to reverse or improve changes intrinsic to the old marrow cells, the old environment can prematurely induce aging of young marrow cells.

Discussion

Stem cells are not affected by age in their capacity to self-replicate *in situ* and in their capacity to differentiate into immune cells and blood cells (16-18).

TABLE I
*Relative Growth Potential of Young and Old Marrow Stem Cells
 Expressed in Young and Old Recipients' Spleens*

Strain of mouse	Age of donor cells	Relative growth potential in:	
		Young	Old
		%	%
BC3F ₁	Young	100	38
	Old	41	12
B6D2F ₁	Young	100	34
	Old	32	16
C3H	Young	100	22
	Old	22	10

TABLE II
*Growth of Young and Old CFU-S 6 wk after Injection into Young and Old BC3F₁
 Recipients*

Combina- tion no.	Age of donor	Age of inter- mediate host	Age of as- say host	A. No. of CFU- S per spleen (\pm standard error)	B. Cells per largest CFU-S ($\times 10^{-6}$) (\pm standard error)	A \times B
1	Y*	—	Y	9 (± 1.03)	2.3 (± 0.10)	20.7
2	O†	—	Y	6 (± 1.01)	1.4 (± 0.21)	8.4
3	Y	Y	Y	7 (± 1.05)	2.0 (± 0.17)	14.0
4	Y	O	Y	8 (± 0.43)	0.9 (± 0.14)	7.2
5	O	Y	Y	6 (± 0.37)	1.5 (± 0.19)	9.0
6	Y	—	O	6 (± 1.84)	1.3 (± 0.15)	7.8
7	O	—	O	3 (± 0.58)	0.8 (± 0.09)	2.4
8	Y	O	O	2 (± 0.24)	0.4 (± 0.05)	0.8
9	O	Y	O	3 (± 0.29)	0.7 (± 0.08)	2.1
10	O	O	O	3 (± 0.48)	0.51 (± 0.06)	1.5

* Y, young.

† O, old.

However, a recent preliminary finding by Micklem et al. (19), suggests that aging may have reduced the cell transfer self-replicative ability of stem cells, although the deleterious effect was not as easily demonstrable as that between fetal and adult stem cells (20). Similarly, Farrar et al. (21), showed that the relative number of B cells generated by old stem cells 4 wk after bone marrow cells are transferred to young recipients was lower than that of young stem cells. This would suggest that aging may be affecting the rate at which stem cells can generate B cells.

The results presented here confirm and extend the latter observations (19, 21), as they indicate that stem cells do undergo changes with age. The changes that are detectable kinetically and appear stable are reflected in: (a) the reduction in the rate at which stem cells proliferate clonally in young recipients (Fig. 1), (b) a lag before clonal growth commences in old recipients (Fig. 2), and (c) a reduction in the frequency of CFU-S and in the size of the largest colonies at the end of the log phase of growth in young recipients (Figs. 3 and 4, and Table II).

Alterations in the growth parameters suggest that old stem cells are not as efficient as young stem cells in their ability to expand clonally in recipients made conducive for clonal growth by preirradiation treatment. The defect could be in their metabolic properties or in their ability to respond to homeostatic factors, or both. That a defect could exist in their ability to respond to homeostatic factors is supported by the observation that the largest colonies of old stem cells at the end of the growth phase are only half the size of those of young stem cells. If old stem cells were as responsive to homeostatic factors as young stem cells, their colonies should have grown to the size of those of young stem cells, especially in X-irradiated young recipients, an environment most conducive for maximum growth (7). Further, the polydispersed pattern of the colony size of old stem cells, in contrast to the monodispersed pattern of young stem cells, is also consistent with the view that stem cells undergo changes with age that affect their responsiveness to homeostatic factors.

Although we do not know the nature of the age-related changes in stem cells, we have attempted to resolve whether or not they are reversible and can be accelerated. This was done by permitting old stem cells to self-replicate in young recipients for a period up to 16 wk and young stem cells in old recipients for a period as short as 6 wk, respectively. The results revealed that the young host was unable to transform old stem cells into young stem cells in terms of their growth potential. This suggests that these age-related changes are stable reflective of a differentiation event. The results also revealed that young stem cells can be aged precociously with respect to their growth potential by allowing them to self-replicate in old hosts for 6 wk. This indicates that the transformation of young stem cells into old stem cells is not caused by an intrinsic, genetically preprogrammed "aging clock" (26), but by the physiological milieu of the old host.

Based on these findings, we envision that as individuals age humoral (noncellular) changes occur involving hormones and other regulatory and differentiation-inducing factors. They, in turn, induce stem cells to undergo subtle, stable metabolic and structural alterations, thus reducing their ability to colonize efficiently and diminish their clonal growth potential. Obviously, the characterization of these humoral factors and alterations of stem cells is essential to further our knowledge of cellular and molecular processes of aging and of homeostasis. To this end, current studies are focused on both of these problems.

Summary

The growth capacity of femoral bone marrow stem cells from young and old long-lived mice was assessed in the spleen of X-irradiated young and old syngeneic recipients by determining: (a) the number of stem cells colonizing the spleen, (b) the rate of incorporation of ^{125}I -labeled iododeoxyuridine by proliferating colony cells, and (c) the number of cells present in the largest colonies at the end of the growth phase. We found that the growth capacity of stem cells declined with age. We further found that the spleen-seeking and spleen colony growth capacities of old stem cells remained characteristically old even after they were allowed to self-replicate in the bone marrow of young recipients for an extended period of time. On the other hand, the spleen colony growth capacity of

young stem cells could be reduced by allowing them to self-replicate in old recipients. These results suggest that the growth capacity of old stem cells is an intrinsic characteristic which cannot be readily altered, but that of young stem cells can be aged in an accelerated manner by allowing them to self-replicate in old recipients.

An additional reduction was noted in the frequency of both young and old stem cells colonizing the spleen of old recipients and in the cell density of the largest colonies produced. These results indicate that factors extrinsic to the stem cells are also responsible for the decline with age in their spleen colony growth capacity. Thus, the growth capacity of old stem cells in old recipients could be as low as 10% that of young stem cells in young recipients.

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