Brief Definitive Report

LOSS OF PROLIFERATIVE CAPACITY IN IMMUNOHEMOPOIETIC STEM CELLS CAUSED BY SERIAL TRANSPLANTATION RATHER THAN AGING*

By DAVID E. HARRISON, CLINTON M. ASTLE, AND JOAN A. DELAITTRE

(From The Jackson Laboratory, Bar Harbor, Maine 04609)

Normally functioning marrow stem cells have a high proliferative capacity, but can be serially transplanted only three to five times before that capacity is severely diminished (1–4). This loss may demonstrate the natural limit of stem cell proliferative capacity, but it may also result from damage caused by the unnatural procedure of transplantation. The proliferative capacity of nontransplanted stem cells is tested by repeatedly irradiating the animal containing them, and thus forcing the cells to regenerate repeatedly. Under these conditions, marrow stem cells start with a high capacity for regeneration which eventually diminishes with repeated irradiation, and the treated animals die (5). This may be caused by stem cell exhaustion or by other cumulative effects of the irradiation treatments.

The ambiguities caused by damage from the process of transplantation or from treatment to destroy stem cells are removed by comparing the proliferative capacities of stem cells from old and young individuals. This experiment also answers a question that is relevant to the health care of elderly individuals: is a significant amount of stem cell proliferative capacity used up during a lifetime of normal functioning?

Unfortunately, results in this area are conflicting. Some investigators find defects in stem cells from old individuals (6–8), whereas others report no difference in functional abilities of old and young stem cells (9–11). Recently, Albright and Makinodan (8) suggested that old stem cells cannot multiply as rapidly as can young stem cells immediately after transplantation, although they are able to multiply and differentiate into normally functional immunohemopoietic cells if given adequate time. This suggestion was supported by findings that the macroscopic spleen colonies produced by old stem cells contained fewer cells. It conflicted with the recent report by Ogden and Micklem (11) that young marrow cells identified by chromosome markers and mixed with equal numbers of old marrow cells showed no competitive advantage in irradiated recipients. Only two separate old vs. young pairs were used; the young having the advantage in one case, and the old in the other.

The system of mixing old and young marrow cells and determining which better repopulates irradiated recipients compares stem cell proliferative rates immediately after transplantation. If young stem cells are capable of multiplying more rapidly than old cells immediately after transplantation, as suggested by Albright and Makinodan (8), the young cells would have a competitive advantage in repopulating irradiated recipients. We therefore compared the repopulating ability in irradiated recipients of many different young-old pairs,

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and we also measured the rates of spleen colony growth, using stem cells from young and old donors. In both cases we also studied previously transplanted old and young marrow stem cell lines to directly compare the effects of aging and transplantation.

Materials and Methods

Mice. CBA/H-T6J mice carrying two translocated T6 chromosomes (2T6), (CBA/H-T6J × CBA/CaJ)F_1 mice carrying one translocated T6 chromosome (1T6), compatible CBA/CaJ (0T6) mice, (C57BL/6J × CBA/H-T6J)F_1 = B6CBAT6F_1 mice, and (C57BL/6J × CBA/CaJ)F_1 W^x/W^v = B6CBAF_1-W^x/W^v mice were bred and maintained at The Jackson Laboratory, which is fully accredited by The American Association for Accreditation of Laboratory Animal Care. Old mice ranged from 25 to 33 mo of age while young ones were 3–8 mo old. Care was taken in autopsy procedures to use only healthy old mice as previously described (10). The evening before the cells were injected, irradiated recipients received 750–850 R of total body X-irradiation as described previously (10).

Rates of Colony Growth. Macroscopic spleen colonies (12) were counted on unfixed spleens of lethally irradiated histocompatible mice 9 days after irradiation and intravenous infection of 1.0 × 10^5 marrow cells. Then spleen cells were suspended in 2.0 ml of Ks 74, a buffered salt solution (13), after dispersion with a glass tissue homogenizer. The homogenate was filtered through 100 mesh nylon cloth, removing small chunks of gelatinous stroma; aliquots were diluted and counted on a Coulter model ZBI electronic cell counter (Coulter Electronics Inc., Hialeah, Fla.).

Comparative Repopulating Ability. Marrow cells were removed by rinsing both femurs and tibias of each donor with chilled CMRL-1066 media (Grand Island Biological Co., Grand Island, N. Y.) or Ks 74 (13) driven into one end of the bone through a 23-gauge disposable needle. Chunks were broken up by dispersing them through a disposable plastic 1-ml syringe with no needle attached and with its outlet pressed gently against the bottom of a 4-ml sterile plastic tube. Marrow cells were stored on ice, counted as described above for spleen cells, and injected within 1–3 h after removal. Marrow cells from each 2T6 donor were mixed with equal numbers of cells from the same young 1T6 donor in each experiment, and they were injected intravenously into lethally irradiated 0T6 recipients. Amounts were adjusted to contain 3 × 10^6 marrow cells of each chromosomal type. This is a new procedure making it possible to compare the repopulating abilities of marrow cells from several 2T6 donors at once. For example, in four experiments, the 2T6 donors included not only a young and an old individual, but also two irradiated 0T6 recipients previously populated with a young and with an old 2T6 stem cell line, respectively. Cells from all four 2T6 donors were mixed with cells from the same young 1T6 donor. Thus, the percentage of 2T6 cells in each case gave the relative proliferative ability of marrow stem cells from each 2T6 donor immediately after transplantation by measuring how well they competed with the 1T6 cells.

Recipients of the mixtures of 2T6 and 1T6 marrow cells were used 3–16 mo after irradiation and marrow transplantation. Their spleens were removed under anesthesia, and the numbers of T6 chromosomes in mitotic spleen cells were determined after stimulation in vitro with phytohemagglutinin (PHA) by methods previously described in detail (10). Erythropoietic mitoses were stimulated by removing 25–35% of the recipient's blood; marrow cells were removed for study 3 days later. Cells in mitosis were fixed in metaphase for determination of numbers of T6 chromosomes using Ford's methods (14). Only those cells with all 40 chromosomes clearly distinguishable were scored.

Results

There were no differences between young and old donors in colony-forming unit (CFU) numbers (A), numbers of cells per spleen (B), growth potentials (A × B), or cells per CFU (B/A) in mice of the two genotypes tested (Table I) at the initial transplantation. With CBA mice at the third transplantation, the growth potential of colonies from old donors was 2.4 times that of the young; with B6CBAF_1 mice at the second and third transplantations, the growth potential of colonies from young donors was 1.5–1.6 times that of the old (Table I).

TABLE I

Macroscopic Colony Growth in Irradiated Recipient Spleens

Donor		Recipient splenic values (mean ± SE)			
Age	Transplant	(A) Number of colonies	(B) Cell number	A × B	B/A
CBA mice*					
Old	1	6.6 ± 0.4	13.2 ± 7.1	96 ± 58	1.8 ± 0.9
Young	1	7.0 ± 0.7	13.1 ± 3.8	99 ± 36	1.8 ± 0.4
Old	2	6.9 ± 0.3	5.4 ± 0.2	37 ± 3	0.8 ± 0.1
Young	2	6.2 ± 0.7	6.1 ± 2.1	41 ± 18	1.0 ± 0.2
Old	3	6.2 ± 1.0	6.5 ± 1.7	43 ± 15	1.0 ± 0.1
Young	3	4.0 ± 1.5	3.5 ± 1.6	18 ± 9	0.8 ± 0.1
$B6CBAF_1$ mice‡					
Old	1	8.1 ± 0.9	25.0 ± 5.9	224 ± 60	2.9 ± 0.5
Young	1	7.6 ± 0.5	27.8 ± 4.8	220 ± 54	3.6 ± 0.4
Old	2	6.4 ± 0.6	14.2 ± 1.6	88 ± 5	2.4 ± 0.5
Young	2	7.4 ± 0.6	19.0 ± 1.3	142 ± 17	2.6 ± 0.3
Old	3	4.7	10.0	47	2.1
Young	3	5.0	14.0	70	2.8

Each mean ± SE is for 3 or 4 donors with CBA mice or 5 donors with $B6CBAF_1$ mice in three experiments. Values for individual donors were the means of 5 or 6 irradiated recipients of 1.0×10^6 marrow cells each (i.v.) per donor. (A) stands for number of colonies, and (B) for cell number ($\times 10^6$) per spleen given after subtracting values for identical irradiated recipients injected with suspending media containing no cells for CBA recipients, or containing 1.0×10^6 W^z/W^v cells for $B6CBAF_1$ recipients.

(A) and (B) are multiplied (A × B) to give the growth potential, and divided (B/A) to give the number of cells per colony.

* Marrow stem cell lines from CBA/HT6 donors were carried in lethally irradiated CBA/CaJ recipients for transplants 2 and 3, using 4–10 × 10^6 marrow cells per recipient and 4–12 mo between serial transplantations. Age in months given as mean ± SE (n): for old donors, 25.7 ± 0.5 (7); for young donors, 5.0 ± 0.6 (8).

‡ Marrow stem cell lines from $B6CBAT6F_1$ donors were carried in unirradiated $B6CBAF_1$ – W^z/W^v recipients for transplant 2 using 10×10^6 marrow cells per recipient with 11–13 mo between serial transplantations. The same number of cells with 4 mo was used for transplant 3. Age in months is given as mean ± SE (n): for old donors, 29.0 ± 0.8 (8); for young donors, 7.4 ± 0.3 (8).

Much larger differences were evident with increasing numbers of serial transplantations than with increasing age. The number of cells per spleen, growth potentials, and number of cells per colony dropped to 40–70% when stem cell lines serially transplanted once (transplant 2, Table I) were compared with those never transplanted before (transplant 1, Table I). Colony numbers stayed constant between transplant 1 and 2, but generally declined in transplant 3, causing declines in the number of cells per spleen and in growth potentials; the number of cells per colony remained constant between transplants 2 and 3 (Table I).

The percentage of 2T6 cells which populated irradiated recipients in competition with 1T6 cells diminished to a much greater extent in donors whose cells were previously transplanted once (transplant 2) than in old donors (Table II). When mitoses were stimulated by bleeding, there was no difference between cells from old and young 2T6 donors (transplant 1, Table II), but there was a two- to fourfold decline in the ability to compete with cells from the 1T6 donor in stem cell lines that had been transplanted once previously (Transpl. 2, Table II). When mitoses were stimulated by PHA in vitro, stem cell lines from old 2T6 donors showed a slight defect, producing about 70% of the 2T6 cells of young 2T6 donors. However, stem cell lines of both age groups serially transplanted once competed 4- to 10-fold less well than those not previously transplanted (Table II).

TABLE II

Percentage of 2T6 Cells in Lethally Irradiated Recipients of 2T6 and 1T6 Marrow

2T6 Donors		2T6 Mitoses after	
Age	Transplant	Bleeding	PHA
Old	1	55 ± 5 (23)	33 ± 4 (16)*
Young	1	58 ± 4 (21)	47 ± 4 (18)
Old	2	27 ± 8 (6)‡	9 ± 3 (5)‡
Young	2	13 ± 4 (5)‡	4 ± 1 (4)‡

Results are given as mean ± SE (number of recipients scored). A mean of 45 (range 20–64) mitoses was scored for each recipient. Old or young 2T6 (CBA/HT6J) marrow was mixed with equal amounts (3×10^6 cells each) of young 1T6 (CBA/HT6J × CBA/CaJ) marrow and transplanted into lethally irradiated young 0T6 (CBA/CaJ) recipients in transplant 1. In transplant 2, marrow from the 2T6 donors had been previously injected i.v. ($4–10 \times 10^6$ cells) in 0T6 recipients; these recipients were held for 4–8 mo before they were used as donors. Age in months is given as mean ± SE (n): for old 2T6 donors, 28.3 ± 0.6 (16); for young 2T6 donors, 5.4 ± 0.3 (15); for young 1T6 competitors, 5.4 ± 0.3 (7).

* Significantly lower $0.01 < P < 0.05$ by Student-Neuman-Keuls multiple range test.

‡ Significantly lower $P < 0.01$ by Student-Neuman-Keuls multiple range test.

Discussion

We found no loss in growth potential of colony-forming cells from old mice (Table I), directly contradicting Albright and Makinodan (8) who found a 2.5- to 5-fold loss using mice of three different strains. There are three possible reasons for this contradiction. First, we calculated growth potential by a different method, multiplying total colony numbers per spleen by the total number of cells in the spleen which resulted from the injected marrow cells (above background). We used this procedure because essentially all proliferating cells in such a spleen are part of the colony and are of donor origin (8). Albright and Makinodan (8) calculated growth potential by multiplying the total colony number per spleen by the number of cells in the largest colony. This could give falsely high growth potentials for young marrow cells if they produced colonies with greater variability in size than were produced by old marrow cells. Second, the old donors we used may have been more free of factors suppressing colony growth because of differences either in general animal health or in autopsy procedures. Third, we used different strains of mice which may differ in the effects of age on stem cells. Our results are consistent with the finding of Lajtha and Schofield (15) that the growth rates of colonies from young or old donors do not differ significantly during the first 11 days after grafting, even though we measured total numbers of cells and they measured total numbers of colony-forming cells in the recipient spleens.

Our findings that old and young hemopoietic stem cells have equally potent proliferative capacities immediately after transplantation confirm and extend findings reported by Ogden and Micklem (11). These investigators studied recipients from only 2 pairs of old and young donors (11); in contrast, we compared 15 pairs of old vs. young donors from the CBA/H-T6 and the B6CBAT6F_1 genotypes in Table I, and 15 pairs from the CBA/H-T6 strain in Table II. We maximized the number of old donors and young controls because old individuals are highly variable (10), and we wanted to avoid being misled by a rare old individual that gave extreme results.

The evidence that stem cells from old mice were slightly defective in producing PHA-responsive cells (Table II) confirms results previously reported (10). This defect apparently resulted from stem cell residence in the old animal rather than being intrinsic to the stem cells, since it was not present in old stem cells in transplant 2 (Table II).

Although old and young stem cell lines gave similar results, the techniques we used were sensitive enough to detect large defects in stem cell lines after a single serial transplantation, even though we transplanted high numbers of marrow cells ($4–10 \times 10^6$) and waited a lengthy interval (4–12 mo) before the second transplantation. Numbers of spleen cells but not numbers of colonies declined 1.5- to 2.5-fold after one serial transplantation (Table I). The ability to populate irradiated recipients declined even more strikingly, 2- to 4-fold in cells stimulated by bleeding, and 4- to 10-fold in cells stimulated by PHA (Table II). When such high marrow cell numbers and long transplantation intervals are used, other techniques do not detect striking changes until after 2–4 serial transplantations (2–4, 9, 11).

These results suggest that even one transplant is a much more severe stress for marrow stem cells than is normal functioning during a lifetime. The stress may result from damage by the transplantation process, dilution of a nonproliferating reserve of the earliest stem cells, exhaustion of proliferative capacity, or some combination of these. Little or none of the proliferative capacity in immunohemopoietic stem cell lines of mouse marrow appears to be used up by normal functioning throughout the adult lifespan.

Summary

Marrow stem cell lines from old donors and those from young controls gave equally rapid rates of colony growth on spleens of irradiated mice. Old and young stem cell lines competed equally well with chromosomally marked marrow stem cells from a young donor in producing cell types that are stimulated by bleeding; old cells competed 70% as well as young in producing cell types stimulated by phytohemagglutinin (PHA) in vitro. After a single serial transplantation, the rates of colony growth declined 1.5- to 2.5-fold, and the ability to compete declined 2- to 4-fold for bleeding-stimulated and 4- to 10-fold for PHA-stimulated cells. Thus, immediate stem cell proliferative capacities decline much more after one serial transplantation than after a lifetime of normal function.

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References

1. 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.
2. 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.
3. Micklem, H. S., and J. F. Loutit. 1966. Tissue grafting and radiation. Academic Press Inc., New York. 171.

4. Harrison, D. E. 1975. Normal function of transplanted marrow cell lines from aged mice. *J. Gerontol.* 30:279.
5. 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 upon proliferative ability of colony-forming cells. *J. Exp. Med.* 126:871.
6. Price, G. B., and T. Makinodan. 1972. Immunologic deficiencies in senescence. II. Characterization of extrinsic deficiencies. *J. Immunol.* 108:413.
7. Farrar, J. J., B. E. Loughman, and A. Nordin. 1974. Lymphopoietic potential of bone marrow cells from aged mice. *J. Immunol.* 112:1244.
8. 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.
9. Harrison, D. E. 1973. Normal production of erythrocytes by mouse marrow continuous for 73 months. *Proc. Natl. Acad. Sci. U. S. A.* 70:3184.
10. 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.
11. 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.
12. Till, J. E., and E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14:213.
13. Kaliss, N. 1971. Jerne plaque assay for antibody-forming spleen cells: some technical modifications. *Transplantation (Baltimore).* 12:146.
14. Ford, C. E. 1966. The use of chromosome markers. *In* Tissue Grafting and Radiation. H. S. Micklem and J. F. Loutit, editors. Academic Press Inc., New York. 197.
15. Lajtha, L. G., and R. Schofield. 1971. Regulation of stem cell renewal and differentiation: possible significance in aging. *In* Advances in Gerontological Research. B. L. Strehler, editor. Academic Press Inc., New York. 3:141.