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Normal Production of Erythrocytes by Mouse Marrow Continuous for 73 Months

(aging/stem cell lifespan/hematology)

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ABSTRACT **Marrow cell transplants from old and young control donors were carried in genetically anemic W/W^v recipients whose anemias were cured by successful transplants. After a maximum of 36 months and four serial transplants, marrow cell lines from both old and younger control donors continued to produce erythrocytes normally. The oldest marrow cell lines had produced erythrocytes normally for 73 months.**

Normal erythrocyte production was demonstrated by: (1) cure of the anemia in W/W^v recipients, (2) normal rather than delayed recovery rate of cured recipients after severe bleeding, and (3) normal rather than ineffective response of cured recipients to erythropoietin. Hemoglobin patterns, tested in cured W/W^v recipients after the first transplantation, showed that at least 90% of the circulating erythrocytes were of the donor type even in donor lines that had produced erythrocytes continuously for 45 months and were recovering from severe bleeding. Concentrations of cells capable of forming macroscopic spleen colonies were more than two orders of magnitude higher in W/W^v mice cured by old or younger marrow than in uncured W/W^v mice. Nevertheless, colony-forming unit concentrations declined slowly with successive transplants, and the decline seemed more pronounced at the fourth transplant in old than in younger cell lines.

The hypothesis is suggested that senescence is caused by declines in function of only a few vital cell types. The system for comparing old and younger marrow cell lines offers a model for experiments to test this hypothesis and to identify the cell types whose decline causes aging.

When an animal becomes senescent, most of its cells decline in ability to function normally. Much of current research in aging is based on the hypothesis that this deterioration is intrinsically timed within each cell type (1–5). This hypothesis is supported by the limited proliferative capacity *in vitro* of human diploid fibroblasts (1, 6, 7) and the limited proliferative capacities *in vivo* of transplanted mouse mammary gland (8) and marrow (9, 10). These experiments do not completely verify the hypothesis because even the limited proliferative capacities of these cell types may have been sufficient for them to function normally for much longer than the lifespans of their donors. In addition, the cell types studied were required to proliferate abnormally rapidly, and their proliferative capacities may have been exhausted abnormally soon. Such exhaustion may explain why doubling the time interval between successive transplants of mouse marrow increased its apparent proliferative capacity by at least 50% (11).

Abbreviation: CFU, Colony-forming unit—A macroscopic colony on the spleen of an irradiated mouse; it may contain several cell types and over 10^6 cells and is thought to grow from a single stem cell.

The defects in many cell types that deteriorate in senescent animals may be caused by their residence in the inadequate environment of the senescent animal, rather than by aging intrinsically timed within each specific cell type. This alternative hypothesis can be tested by determining whether a variety of cell types can continuously perform their normal function for longer than the lifespan of the donor species. In one such experiment, skin grafts were maintained on successive young hosts for as long as 80 months (12). Unfortunately graft and host cells were not distinguishable so that essential cells may have migrated into the old graft from the young host. In a second experiment, mouse-marrow cell lines were maintained in successive irradiated hosts for a maximum of 60 months, and donor cells were identified by the T6 chromosome (13). Increasing losses of recipients of older cell lines began before the cells had been carried for 40 months and were considered evidence for a decline with age in the ability of the old cell lines to function normally (13). Unfortunately the original cells were obtained from infant spleens so younger controls were not possible. Thus, technical difficulties rather than intrinsic cellular aging may have caused the increasing losses of recipients of older cell lines.

As these examples illustrate, measures of cell lifespan adequate to test the alternative hypothesis should satisfy three criteria: (1) *Function.* Senescence should be determined by loss of the ability to function normally. (2) *Identification.* Tested cells should be unambiguously identified. (3) *Control.* Old cell lines or transplanted tissues should decline while younger controls continue to function normally.

I am reporting a system (14) that is designed to satisfy these criteria. In it, marrow cell lines from old and young normal mouse donors are implanted into genetically anemic recipient mice. Such implants cure the anemic recipients by populating their erythrocyte producing tissues, and become the progenitors of all their circulating erythrocytes (15–18). The three criteria are satisfied as follows: (1) *Function.* Normal function of the marrow cell line is demonstrated by cure of the recipient's anemia and by normal responses to bleeding or erythropoietin. (2) *Identification.* Normal erythrocyte production in cured W/W^v mice demonstrates that the transplanted normal cells are functioning, because the cells native to W/W^v mice have a genetic defect that causes the W/W^v anemia and prevents normal responses to bleeding or erythropoietin. In addition, some normal cell lines were used that produce hemoglobin distinguishable from the type produced by the recipient. (3) *Control.* Marrow transplants from both old and young control donors cure initial genetically anemic recipients; thus limitations on marrow cell lifespans will be demonstrated if old

cell lines decline while younger control lines continue to function normally.

MATERIALS AND METHODS

Experimental Animals. Genetically anemic recipient mice, carrying two dominant mutant alleles at the *W* (dominant spotting) locus, were produced from WB/Re–$W/+$ × C57BL/6J–$W^v/+$ parents, and are herein called WBB6F$_1$–W/W^v recipients (18). They were 3–6 months old when first given normal marrow.

Normal donors were of the (WC/Re × C57BL/6J)F$_1$ (WCB6F$_1$) or C57BL/6J (B6) genotypes. The old donors were 29–39 months old, and the younger donors used as controls were 1–13 months old, and at least 20 months younger than the old donors used in the same experiment. The mean lifespan of a population of 40 WCB6F$_1$ mice, set aside for longevity determination when 3–5 months old, was 29.5 months and one individual lived for 39 months. Lifespans of B6 mouse populations were slightly shorter.

Procedures. Hematocrits, hemoglobin concentrations, erythrocyte numbers, mean cell volumes, and colony-forming unit (CFU) numbers were determined by standard techniques (19–21). Doses of 1 to 10 × 10^6 marrow cells were injected intravenously (20) to cure W/W^v recipients (16). CFU numbers were measured on samples from the same batches of marrow cells. Erythropoietin, the hormone that stimulates erythrocyte production, was partially purified from several rabbits made anemic by phenylhydrazine. The responses to erythropoietin or to bleeding were determined as previously described (18). Hemoglobin patterns were determined by starch-gel electrophoresis with constant amounts of hemoglobin (20).

The standards for classifying a W/W^v recipient as cured have been previously reported (14). They require that the erythrocyte mean cell volume in the recipient be reduced from that characteristic of the W/W^v macrocytic anemia to a mean cell volume at least as low as that resulting from a mixture of equal portions of W/W^v and normal erythrocytes. No untreated W/W^v mice met these standards, although mean cell volumes of more than 350 have been tested. More than 50 W/W^v recipients have been cured by old and young donors with distinguishable starch-gel patterns of hemoglobin. Blood from every W/W^v recipient classified as cured has contained more than 80% of the donor type of hemoglobin.

RESULTS

Cures of Anemic Recipients. Initial transplants of marrow cells from both old and young donors cured approximately the same proportions of anemic recipients. In both cases, most recipients remained cured for at least nine months (Transplant *I*, Table 1). After 9–12 months, marrow cells from W/W^v mice that were still cured were transplanted into a second successive set of W/W^v anemic recipients (Transplant *II*, Table 1). Again no differences were observed between the effectiveness of the old and the younger control marrow cell lines. This procedure was repeated twice more for serial Transplants *III* and *IV* (Table 1). The oldest marrow cell line had produced erythrocytes normally for 73 months as of April, 1973.

After each transplant, some cured mice died or later became anemic, possibly as a nonspecific result of illness; these mice were classified as not cured. In some cases no anemic recipients were cured by cells from a particular donor. This happened with cells from similar proportions of old and younger donors, and such recipients were not included in Table 1.

CFU Numbers. No significant differences were observed between marrow CFU concentrations of initial old and young donors of the WCB6F$_1$ and B6 strains (Transplants *I* and I^e, Table 1). In both old and younger control cell lines, CFU numbers declined slightly with each successive transplant when marrow from cured W/W^v recipients was serially transplanted into successive anemic W/W^v mice (Transplants *II*–*IV*, Table 1) with one exception. The younger control cell lines in Transplant *IV* increased in CFU concentrations and also cured unexpectedly high percentages of W/W^v recipients (footnote[f], Table 1). CFU concentrations in all cured W/W^v recipients were at least two orders of magnitude greater than those in uncured W/W^v mice (20).

TABLE 1. *Effect of marrow cell age on ability to cure W/W^v anemic mice*

Transplant	Original donor[a]	Cell age months[b]	No. CFU (SE)[c]	% cured (*n*) after months[d] 1–3	6–9
I	Old	29–39	10.6 (0.7)	94 (33)	63 (27)
	Young	1–13	10.7 (0.9)	81 (36)	69 (29)
I^e	Old	30–34	10.4 (0.6)	87 (45)	86 (37)
	Young	4–12	11.6 (0.9)	100 (48)	93 (44)
II	Old	37–50	6.3 (1.2)	96 (47)	75 (45)
	Young	14–27	8.7 (0.9)	91 (42)	73 (41)
II^e	Old	43–47	7.7 (0.7)	97 (29)	—
	Young	18–23	9.1 (0.9)	100 (29)	—
III	Old	43–64	5.6 (0.6)	72 (102)	54 (76)
	Young	21–41	7.4 (0.7)	80 (86)	59 (44)
IV	Old	63–70	3.7 (0.3)	64 (36)	53 (30)[g]
	Young	38–43	7.2 (0.9)[f]	87 (23)[f]	80 (10)[g]
Untreated W/W^v		3–6	<0.01	—	—

[a] *Old* and *Young* refer to the ages of the original donors when their cells were first taken (Transplant *I*).

[b] *Cell age* refers to the ages in months of the normal marrow cell lines. These were born with the original donor and have functioned continuously since that time both in the original donor and in successive recipients. Age ranges are given for 1–3 months after the cell transplant.

[c] *No. CFU* is number of macroscopic colonies on spleens of lethally irradiated recipients per 10^5 marrow cells. SE is standard error.

[d] To be scored as cured after 1–3 months, the recipient met the standards for at least months 2 and 3, and to be scored as cured after 6–9 months, the recipient met the standards for at least 3 of those months, including 8 or 9. Results are given as: percentage cured (number checked).

[e] C57BL/6J donors; others were WCB6F$_1$ donors.

[f] After the fourth transplant, the younger control marrow cell lines did not decline in either ability to cure W/W^v recipients or in CFU numbers, while these factors continued to decline in the marrow cell lines from old donors. This may have occurred because only two carriers donated half of the younger marrow. Their marrow contained twice as many CFUs as that of the best of the 11 carriers of old cells. When more younger cell carriers have been transplanted for the fourth time, they may approach the pattern followed for the first three transplants.

[g] Results at 6.5 months only.

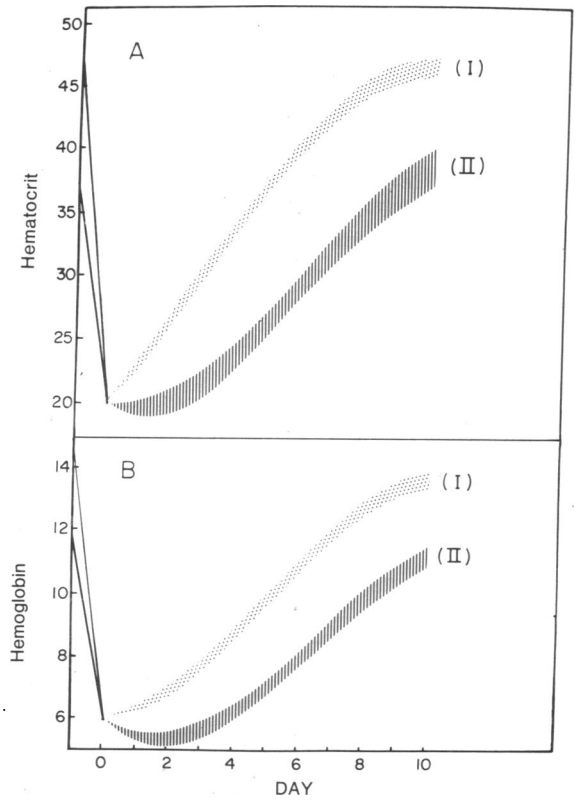

FIG. 1. Mice were bled three times during day 0 to the levels for day 0. On days 3, 4, 5, 6, 7, 10, and 12, hematocrit (*A*, percentage blood volume that is packed erythrocytes) and hemoglobin (*B*, grams hemoglobin per 100 ml of blood, cyanmethemoglobin method, ref. 19) were measured. The recovery curves for each of seven groups of mice were obtained by regression analysis fitting a third degree polynominal through the origin (0, 20 for *A;* 0, 6 for *B*). *Plot I* shows the 95% confidence limits for groups 1–6 combined. Plots for each of groups 1–6 did not differ significantly [variance ratio, F_A (15,165) = 1.06; F_B (15,165) = 1.41]. The groups were: (*1*) ten +/+ mice 6–12 months old, (*2*) four W/W^v mice cured by 63-month-old marrow, (*3*) three W/W^v mice cured by 33-month-old marrow, (*4*) three W/W^v mice cured by 71-month-old marrow, (*5*) three W/W^v mice cured by 45-month-old marrow, and (*6*) four W/W^v mice cured by 73-month-old marrow. Groups *2* and *3* had been serially transplanted three times in 26 months; group *5*, two times in 18 months; and groups *4* and *6*, four times in 36 months. *Plot II* is the 95% confidence limits for group *7*, six uncured W/W^v mice 4–11 months old. Its difference from plot *I* is significant beyond the 0.001 level [F_A (18,203) = 6.27; F_B (18,203) = 7.33].

Recovery from Severe Bleeding. W/W^v recipients cured by marrow cell lines that had produced erythrocytes normally for as long as 73 months responded to severe bleeding as well as did normal mice and much more efficiently than uncured W/W^v mice (Fig. 1). Old and younger control cell lines responded equally well. Even during maximum erythrocyte production, while recovering from this bleeding, only the donor cell line was active. More than 90% donor-type hemoglobin was found in the blood 6 and 12 days after bleeding recipients cured by a 45-month-old donor line whose hemoglobin differed from that of the recipient. Both circulating erythrocyte volumes (hematocrit) (Fig. 1*A*) and hemoglobin concentrations (Fig. 1*B*) recovered at normal rates in the cured recipients.

Response to Erythropoietin. W/W^v recipients cured by old and younger control marrow cell lines responded normally to a standard dose of erythropoietin (Table 2). The cell lines in experiment *II* (Table 2) had produced erythrocytes continuously for totals of 70 and 40 months; those in experiment *I* (Table 2) had done so for 63 and 33 months. All cured W/W^v recipients gave responses to the standard dose of erythropoietin that were two orders of magnitude greater than the responses of uncured W/W^v mice.

Starch-Gel Patterns of Hemoglobin. In those WBB6F$_1$–W/W^v recipients cured by B6 donors, the extent of population of the recipients by donor cells could be estimated from starch-gel patterns of the circulating hemoglobin. Prior to treatment, W/W^v recipients had a 2-banded hemoglobin pattern and B6 donor erythrocytes had a 1-banded hemoglobin pattern. Only the one donor band was detectable in blood from cured W/W^v recipients after erythrocyte precursors produced by the donor cell line had taken over erythrocyte production; as little as 10% recipient hemoglobin produced a detectable second band. Only donor hemoglobin was detected in the starch gel patterns of cured recipients with marrow cells from either old or younger B6 donors when they were tested 13 months after the first B6 marrow transplants (Fig. 2). At this time the old and the younger B6 marrow cell lines had functioned normally for 44 and 18 months, respectively.

DISCUSSION

Normal Erythrocyte Production. Mouse-marrow cell lines have produced erythrocytes normally for at least 73 months. This is 2.5 times the mean lifespan of the cell donors, and 1.6 times the longest recorded mouse lifespan of 45 months (22). Normal erythrocyte production is demonstrated when transplanted marrow cell lines populate and cure genetically anemic recipients (Table 1) and when these cured recipients

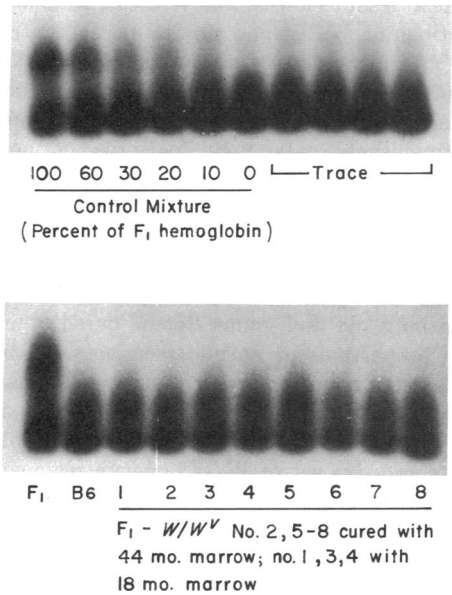

FIG. 2. Starch-gel electrophoretic patterns of donor (B6) and host (WBB6F$_1$) hemoglobin. (*Top*) control mixtures of donor and host hemoglobin. (*Bottom*) replacement of WBB6F$_1$–W/W^v (double-banded) by B6 (single-banded) patterns in anemic recipients cured by marrow from old and young B6 donors.

respond normally to exogenous erythropoietin (Table 2) or to severe bleeding (Fig. 1). Normal responses to erythropoietin demonstrate that marrow cell lines continue to produce normal numbers of cells responsive to erythropoietin for at least 70 months. The normal recovery rate for hematocrits (packed erythrocyte volumes) after severe bleeding (Fig. 1*A*) demonstrates that the maximum rate of production of erythrocyte mass has not declined in marrow cell lines that have functioned normally and continuously for at least 73 months. Normal hemoglobin synthesis after severe bleeding (Fig. 1*B*) demonstrates that the maximum rate of hemoglobin synthesis also has not declined.

Decline in CFU Numbers. Although erythrocyte production in cured recipients remains normal, there is a trend for the CFU number and the cure rate to decline slightly with each successive transplant (Table 1). This indicates that the marrow cell lines may not be maintained for an unlimited time because the CFU is the earliest defined marrow precursor cell (9–11, 21). For the first three transplants and 30 months the declines were similar in marrow cell lines from old donors and younger controls and were therefore not caused by senescence (Transplants *I–III*, Table 1). During this time cure rates and CFU numbers varied between different marrow cell lines. Performance by the lines from both old and younger control donors that declined least were similar, and these lines were selected whenever possible for transplantation into successive recipients. In Transplant *IV* (Table 1, footnote[f]), however, two younger cell lines (38–43 months) remained high in CFU number and cure rate while the old lines (63–70 months) continued to decline. If these results are repeated, and the younger lines also decline after 60–70 months, then an age-related deterioration in marrow cells will be indicated, although it occurs much too late to affect mouse lifespans.

Cause of Senescence—a Hypothesis. Normal erythrocyte production by mouse-marrow cell lines for 2.5 times the donor lifespan demonstrates that an intrinsically timed decline in the functions of this cell type has no part in causing senescence. Finch recently reported that a group of 25- to 28-month-old B6 mice selected for apparent health had significantly lower mean hematocrits than 8- to 10-month-old mice (23). This decline must have been caused by defects of the internal environment within the aged mice, since we have shown that marrow cells from aged donors produced erythrocytes normally in young W/W^v recipients. Perhaps this is true of many cell types. Senescence in an animal may be caused by initial intrinsically timed declines in the functions of only a few vital cell types, resulting in a deleterious internal environment that causes other cells to deteriorate. Researchers who have developed techniques for transplanting various tissues may be able to identify those cell types with initial intrinsically timed declines by transplanting them into young recipients, satisfying the criteria of function, identification, and control previously discussed. If these transplanted cells decline at nearly the same age as they would have in the original donor, then their decline may be an initiating cause of senescence. Such declines should occur after a period of normal functioning in the young host, so that one may be sure that residual effects of previous residence in an aged environment do not cause the declines. When such declines do occur in old cells, identically treated younger cells should continue to function normally until they too reach the age at which the older cells declined.

TABLE 2. *Response to 1.0 unit of erythropoietin*[a]

Experiment[b]	Genotype	*n*	Treatment	Percentage of ^{59}Fe incorporated[c]
I-A	W/W^v	5	Untreated	0.1
I-B	+/+	4	Untreated	22.4 (3.3)
I-C	W/W^v	4	Cured, 63 mo. cells	19.8 (2.3)
I-D	W/W^v	3	Cured, 33 mo. cells	19.1 (0.4)
II-A	+/+	4	Untreated	43.2 (2.6)
II-B	W/W^v	3	Cured, 70 mo. cells	41.3 (8.7)
II-C	W/W^v	3	Cured, 70 mo. cells	25.2 (14.9)
II-D	W/W^v	3	Cured, 40 mo. cells	20.1 (4.5)

[a] Erythropoietin had been calibrated against Standard B several years previously. Each mouse received 1.4 mg of this erythropoietin sample, so the comparison was valid even if the potency in units was not exact. Each mouse had been made polycythemic by transfusion four days before erythropoietin injections.

[b] In experiment *I-C* and *I-D*, the marrow cells had been serially transplanted through three successive W/W^v recipients during 26 months. The recipients were 12 months old and the ages of the original donors at the first transplant were 37 and 7 months. The mice in *I-A* and *I-B* were 6–7 months old and all mice in experiment *I* were females.

In experiment *II-B*, *II-C*, and *II-D*, the marrow cells had been serially transplanted through four successive W/W^v recipients during 33 months. The original donors were the same as in experiment *I*. All mice in experiment *II* were 8 months old. The mice in *II-C* and *II-D* were females, and those in *II-A* and *II-B* were males.

[c] Results given as mean (SE).

Consequences of the Hypothesis. Until it is known that a particular cell type declines whether in old or in young environments, researchers comparing old and young cells should compare them in the same environment. Otherwise any defect noted in old cells may be merely the result of their residence in the deleterious internal environment of the senescent animal and is not evidence pertinent to the mechanism by which senescence develops. For example, the increased numbers of chromosome defects (2) and the accumulation of inactive enzyme molecules (5) in liver cells of old mice may be caused by residence of the livers in the old internal environment rather than by mutations or protein synthesizing errors that develop intrinsically with age in liver cells.

Attention should be concentrated on discovering and studying those cell types that initially decline. To extend the amount of time before health and vigor begin to deteriorate with age, the lifespans of these shorter lived cell types must be extended. The system for studying marrow cell lines described in this paper illustrates how a cell type can be tested to determine whether or not it is an initiating cause of sensecence. Normal mouse marrow cells are not, because they produce erythrocytes normally for more than twice the lifespans of their donors. Also, human fibroblasts are probably not among the cell types whose declines cause senescence because those from old donors proliferate almost as well as those from young donors (6, 7).

If aging is the result of declines in a few vital cell types, senescence would be postponed in an old animal by successful transplantations of those cell types from young histocompatible donors. This would be a most elegant proof of the foregoing hypothesis and would positively identify the cell types whose intrinsically timed declines cause aging.

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