SERIAL DEPLETION AND REGENERATION OF THE MURINE HEMATOPOIETIC SYSTEM

Implications for Hematopoietic Organization and the Study of Cellular Aging*

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The causes of aging of multicellular organisms remain obscure. One of the most influential ideas in recent years has been based on the observation by Hayflick (1), subsequently confirmed by other workers, that diploid fibroblasts do not survive indefinitely under conditions of serial in vitro subculture. These experiments have been interpreted as showing that proliferating cell populations undergo "aging," indicated by a failure to continue dividing once a certain number of population doublings has been achieved. It has been suggested that either there is an inherent, genetically programmed limit to the number of divisions through which a cell and its descendents can pass or that the demise of the cell line is due to an accumulation of copying errors in the DNA (2, 3).

Results from serial in vivo transplantation of normal tissues, including skin (4), bone marrow (5–12), and mammary tissue (13) have also suggested that the potential lifespan of these tissues, although considerably greater than that of an individual animal, is nevertheless finite.

It has been recognized, however, that the eventual death of cells and tissues might be an artifact of the serial transplantation procedure, both in vivo (11, 12, 14) and in vitro (15). If, for example, survival of the transplanted population depended on a relatively small number of "stem" cells capable of extensive self-renewal and proliferation, these might be gradually diluted out during transfer, leaving only cells committed (as a result of differentiation or other factors) to cease dividing. It has been suggested that this may happen even with superficially homogeneous populations of diploid fibroblasts growing in vitro (15). One problem, both here and in vivo, lies in recognizing the putative stem cells. The hematopoietic system is relatively convenient in this respect because the spleen colony assay (16) can provide a numerical estimate of stem cells in any hematopoietic tissue. This has made it possible to provide some control over the number of stem cells being transferred at each stage (11). Unfortunately, however, the stem cells that are measured as spleen colony-forming units (CFU)[1] are a heterogeneous population differing in their degree of "stemness,"

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[1] *Abbreviations used in this paper:* Ara-C, cytosine arabinoside; CFU, spleen colony-forming unit; HBSS, Hanks' balanced salt solution; HU, hydroxyurea; 5 × HU, five injections of HU administered over a 30-h period; i.p., intraperitoneal; i.v., intravenous; IUdR, ^{125}I-labeled 5-iodo-2-deoxyuridine.

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that is, their ability to self-renew as opposed to differentiating (8, 14, 17–20). A further drawback is that serial transfer of hematopoietic cells entails disrupting the microenvironment of the cells, with the attendant possibility of destroying or inducing differentiation of the most powerfully self-renewing stem cells. The apparent limitation of lifespan in serially transferred hematopoietic cell populations may, therefore, be an experimental artifact, which is a view supported by the results of Harrison (21).

In the present experiments, we explore the effects of bone marrow transplantation, per se, on the capacity of stem cells to self-renew, and investigate an alternative system—serial hematopoietic depletion and regeneration *in situ*—for studying cellular aging. The results confirm that transplantation of bone marrow stem cells does adversely affect their potential for self-renewal and suggest that there is no intrinsic limitation in the ability of bone marrow cell populations to regenerate repeatedly.

Materials and Methods

Mice. The chromosomally distinct congenic strains CBA/H (normal chromosome complement), CBA/H-T6 (2 marker chromosomes), and CBA/H × CBA/H-T6 (1 marker chromosome) were bred in our laboratory. For brevity, the marker-carrying mice are usually referred to as T6T6 and T6, respectively. Females were used at 3–4 mo of age. The presence of the marker chromosomes has been shown not to influence the overall proliferative potential of hematopoietic cells (22).

Irradiation. A lethal dose (9.0 Gy) of gamma radiation was delivered from a 137-Cs source at a dose-rate of 0.39 Gy/min.

Cell Suspensions. Bone marrow cell suspensions were prepared by flushing out the marrow cavity with Hanks' balanced salt solution (HBSS) and dissociating by aspiration through a 25 gauge needle. Spleens were dissociated in HBSS and filtered through a stainless steel wire mesh to remove clumps.

Serial Transfer of Bone Marrow. Lethally irradiated CBA mice were injected intravenously (i.v.) with 5×10^6 viable nucleated T6T6 bone marrow cells. 8 wk later, two or more recipients were killed, and 5×10^6 cells from their bone marrow were injected to further lethally irradiated recipients. These were likewise killed as donors for the next transfer generation, and this procedure was repeated for up to six transfers.

Treatment with Cycle-active Drugs. Hydroxyurea (HU) and cytosine arabinoside (Ara-C) were obtained from Sigma Chemical Co., St. Louis, Mo. Serial treatment with HU was performed according to one of two regimes. *(a)* Two intraperitoneal (i.p.) injections of HU (1 mg/g body weight) were given 7 h apart (23). This regime was repeated at 21-d intervals. *(b)* Five intraperitoneal injections of HU (1 mg/g body weight) were given over a period of 30 h (0, 6, 22, 25, 30 h) (24). At intervals after the final injection, recipients were killed and their bone marrow harvested. The cycling status of surviving CFU was estimated by giving a further single injection of HU 2 h before killing to eliminate CFU in S-phase. Control mice received an appropriate series of HBSS injections; these are designated as "normal" in the figure legends.

Assays of CFU. Hematopoietic stem cells were enumerated by means of the spleen colony assay of Till and McCulloch (16). Enough viable nucleated bone marrow cells to yield 8–20 colonies were injected intravenously into groups of 5–20 lethally irradiated syngeneic recipients. No dose-related variation in CFU self-renewal was observed over this range. 8 d later the mice were killed, their spleens fixed in Bouin's fluid, and the macroscopic surface colonies counted. Results were expressed as the CFU ratio (the mean number of colonies found per 10^5 cells injected). Control groups received HBSS only; these showed a colony frequency of <0.2 per spleen. Self-renewal of CFU within the irradiated spleen was measured 8 d after the initial injection of cells. Groups of 10–20 mice were killed. The spleens from one-half were fixed for enumeration of colonies, and one or more pooled cell suspensions were made from the remainder. The cells from a fraction of spleen corresponding to three colonies (three colony equivalents) were injected into groups of 5–10 lethally irradiated secondary recipients that were killed 8 d later. The colonies on their spleens were counted and the results expressed as a self-renewal index (the number of colonies formed per colony-equivalent injected). In some

experiments, CFU self-renewal was also measured in colonies after 10 d in the primary hosts. The same procedure was followed except that only 0.75 colony-equivalent was injected into each secondary recipient.

Competitive Repopulation Assay. The ability of two populations of stem cells to regenerate hematopoiesis in irradiated mice was compared directly using chromosome markers. Two bone marrow cell suspensions were mixed and injected intravenously into lethally irradiated recipients, donors and host being distinguished by the possession of none, one, or two T6 chromosomes. The CFU content of the donor suspensions was determined by the spleen colony assay. Individual recipient mice were killed at intervals for cytological analysis of mitotic populations in the bone marrow (pooled femora, tibiae, ilia, and humeri) and spleen. Chromosome preparations were made as described by Ford (25), and 50–150 mitoses were scored per tissue.

Estimation of Cell Proliferation. Cell proliferation was measured by the incorporation of ^{125}I-labeled 5-iodo-2′-deoxyuridine (IUdR: Radiochemical Centre, Amersham, England). Mice received 1 μCi IUdR i.p. 24 h before killing. Bones were excised and washed in 70% ethanol to remove any non-DNA-bound radioactivity. Samples were counted in an automatic gamma spectrometer (Nuclear Enterprises, Edinburgh, U. K.; model 8311), and radioactivity was expressed as a percentage of the activity injected.

Results

Self-Renewal of Bone Marrow CFU Declines Progressively with Serial Transfer. The self-renewal of CFU from serially transferred bone marrow 8 wk after the final transfer was measured by retransplantation of cells from colony-containing spleens into secondary lethally irradiated recipients (Table I). Once-transferred marrow CFU self-renewed only one-half as well as those from normal marrow, whereas CFU from thrice and four times-transferred marrow self-renewed less than one-tenth as well. Serial transfer also reduced the number of CFU present in bone marrow 8 wk after transfer, but less markedly. The doubling time for CFU calculated from day 8 and day 10 data was in the range of 19–25 h for both normal and transferred bone marrow. Thus, despite the poor overall self-renewal of CFU from serially transferred marrow, a proportion of the CFU were self-renewing at a normal rate. The decline in CFU numbers and self-renewal was associated with the process of serial transfer and not simply with the chronological aging of the marrow population (8 wk per transfer) because these parameters are little affected by age; bone marrow from older CBA mice tends, if anything, to show slightly increased CFU numbers and self-renewal (9; H. S. Micklem and N. Anderson, unpublished observations).

TABLE I

Effect of Serial Bone Marrow Transfer on Numbers and Self-Renewal of Bone Marrow CFU

Source of bone marrow*	Number of CFU per femur ($\times 10^{-3}$)	CFU self-renewal index‡	
		day 8	*day 10*
Normal	3.3	3.5	18.6
Transfer 1	3.5	1.6	8.8
Transfer 2	1.2	0.2	1.1
Transfer 4	0.6	0.2	1.3

* Bone marrow cells were transferred intravenously one, three, or four times at 8-wk intervals through lethally irradiated recipients. Recipients were killed 8 wk after the final transfer as donors for the CFU assay.

‡ Calculated from the number of CFU generated per primary spleen colony in 8 and 10 days, respectively.

The Repopulating Ability of Bone Marrow Is Reduced after a Single Transfer. The ability of stem cells from normal and serially transplanted bone marrow to repopulate the marrow cavities of lethally irradiated mice was compared directly using the T6 chromosome marker system. Lethally irradiated mice were injected with two chromosomally distinct syngeneic bone marrow cell suspensions, one normal and one after transfer through one or more previous irradiated recipients. The number of CFU injected was weighted 6–7 times in favor of the transferred marrow. Mitotic cells in the irradiated recipients were analyzed at intervals. The results are shown in Fig. 1. In two experiments, which were identical except that the chromosome markers were reversed, the proportion of mitoses derived from once-transferred marrow fell to ~10% of that predicted on the basis of the number of CFU injected (Fig. 1 a and b). Three-times transferred marrow gave essentially the same result (Fig. 1 c).

Self-Renewal of CFU Does Not Decline after Serial Treatment with HU. The above data showed that serial transfer of bone marrow resulted in a marked decline in hematopoietic stem cell function. However, because this technique involves disruption of the hematopoietic microenvironment, the decline might be due to the disruption itself and not to any "aging" effect produced by the repeated demands for regeneration. The effect of *in situ* serial depletion and regeneration of the hematopoietic system on stem cell function was therefore studied using the cycle-active drug HU. As a preliminary, the effects of one and ten pairs of HU injections on femoral cellularity and CFU numbers were followed as a function of time. CFU numbers were reduced by 75 and 88%, respectively at 24 h and thereafter recovered to near-normal in 5–7 d.

The self-renewal of marrow CFU from mice given up to 25 pairs of HU injections was tested 21 d after the final pair (Table II). No progressive decline in self-renewal could be detected in the CFU of HU-injected mice. The self-renewal index showed a similar rise between days 8 and 10 in HU-injected (up to at least six pairs of injections) and control mice, implying similar CFU doubling times.

A degree of bone marrow CFU depletion (96–99% within 2 h) comparable to that

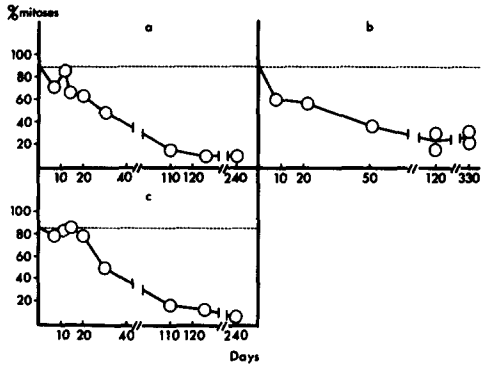

FIG. 1. Competitive repopulation of the bone marrow of lethally irradiated CBA mice by normal and previously transferred bone marrow cells. Ordinate, percentage of total donor mitoses that were derived from the previously transferred marrow donor, the few host mitoses observed being ignored. Broken lines represent the proportions of mitoses predicted on the basis of numbers of CFU injected. (a) 10^6 (230 CFU) normal T6 + 10^7 (1,650 CFU) once-transferred T6T6 cells; (b) 10^6 (250 CFU) normal T6T6 + 10^7 (2250 CFU) once-transferred T6 cells; (c) 10^6 (200 CFU) normal T6 + 10^7 (1,160 CFU) thrice-transferred T6T6 cells. Each point represents a single animal killed between 7 and 330 d after irradiation.

TABLE II

Effect of Serial Exposure to HU on Numbers and Self-Renewal of Bone Marrow CFU

Source of bone marrow*	Number of CFU per femur ($\times 10^{-3}$)	CFU self-renewal index‡	
		day 8	*day 10*
1 pair HU	4.7	2.4	18
4 pairs HU	4.7	1.7	15
4 pairs HBSS	2.9	3.0	20
6 pairs HU	1.8	1.3	10
12 pairs HU	2.5	1.9	—§
12 pairs HBSS	1.8	2.3	—
20 pairs HU	3.5	1.8	—
20 pairs HBSS	2.9	1.8	—
25 pairs HU	4.7	3.4	—
25 pairs HBSS	4.5	3.4	—
Normal 16-wk-old	3.2	3.4	—
5 × HU	1.4	3.5	—
5 × HBSS	2.2	3.5	—

* Pairs of intraperitoneal injections of HU or HBSS 7 h apart were given at 21-d intervals. Alternatively, a single course of five injections was given over a 30-h period (5 × HU). All donors were killed 21 d after the final injection.

‡ Calculated from the number of CFU generated per primary spleen colony in 8 and 10 d, respectively.

§ Indicates that no day 10 assay was done.

obtained after lethal irradiation was induced by five injections of HU administered over a 30-h period (5 × HU). The CFU obtained from the bone marrow 21 d later showed approximately normal 8-d self renewal (Table II).

The Repopulating Capacity of Bone Marrow Is Not Reduced after Serial Depletion by HU. The ability of stem cells taken from recipients of 1, 4, 6, and 18 pairs of HU injections 21 d after the final injection to repopulate the bone marrow of lethally irradiated mice was directly compared with that of normal stem cells using chromosome markers. The results are shown in Fig. 2. The proportion of mitoses derived from HU-injected mice remained around the level predicted from the number of CFU injected, showing that repeated hematopoietic regeneration after injection of HU had no adverse effect on the repopulating capacity of stem cells. Marrow from mice treated 21 d previously with 5 × HU in fact slowly outgrew normal marrow to give twice the expected proportion of mitoses by 100 d (Fig. 3 c).

HU Selectively Removes Stem Cells with Relatively Low Self-Renewal Capacity. An immediate result of a single pair of HU injections was that the self-renewal index of the few CFU remaining 42 h later was raised (Table III); this indicates that the drug selectively destroyed CFU with relatively low self-renewal capacities. 5 × HU treatment also resulted in greater self-renewal of CFU obtained 2 h after the final injection, and the same occurred after a second course of 5 × HU given 21 d after the first (Table III). In confirmation of the CFU data, bone marrow stem cells obtained from mice 42 h after a single pair of HU injections outgrew normal bone marrow in a competitive repopulation assay (Fig. 4).

In a similar assay, marrow cells from mice exposed to 5 × HU rapidly outgrew normal marrow. Fig. 3 a and b shows the results of two experiments in which the

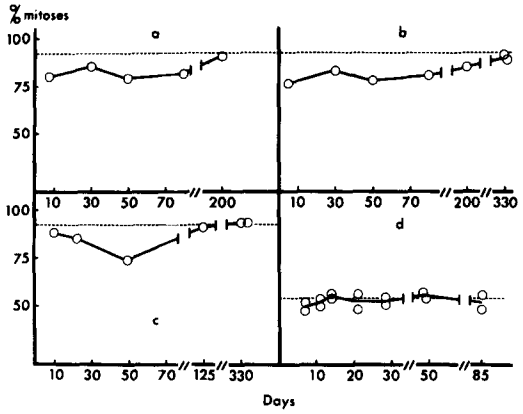

FIG. 2. Competitive repopulation of the bone marrow of lethally irradiated CBA mice by marrow cells from normal (T6T6) and HU-treated (T6) donors. (a) 10^6 (260 CFU) normal + 10^7 (3,200 CFU) HU-treated cells (one pair HU injections); (b) 10^6 (260 CFU) normal + 10^7 (3,000 CFU) HU-treated cells (four pairs HU injections); (c) 10^6 (380 CFU) normal + 10^7 (4,800 CFU) HU-treated cells (six pairs HU injections); (d) 10^6 (650 CFU) normal + 10^7 (540 CFU) HU-treated cells (18 pairs HU injections). Donors were killed 21 d after final HU injection. For further explanations, see legend to Fig. 1.

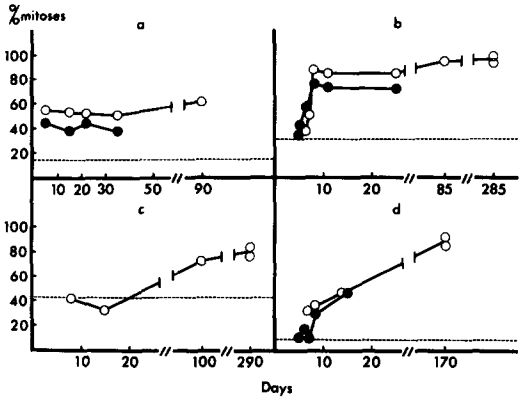

FIG. 3. Competitive repopulation of the bone marrow (○) and spleen (●) of lethally irradiated CBA (a–c) and T6 (d) mice by marrow cells from normal and HU-treated donors. (a) 2×10^6 (450 CFU) normal T6 + 2×10^6 (72 CFU) HU-treated T6T6 cells (one series of 5 × HU; donors were killed 2 h after final injection). (b) 10^6 (220 CFU) normal T6T6 + 5×10^6 (100 CFU) HU-treated T6 cells (one series of 5 × HU: donors were killed 2 h after final injection). (c) 4×10^6 (780 CFU) normal T6 + 2×10^6 (530 CFU) HU-treated T6T6 cells (one series 5 × HU; donors were killed 21 d after final injection). (d) 2×10^6 (540 CFU) normal T6T6 + 2×10^6 (44 CFU) HU-treated CBA cells (three series of 5 × HU; donors were killed 2 h after final injection). For further explanations, see legend to Fig. 1.

number of stem cells (based on CFU numbers) was weighted in favor of the normal marrow by factors of 6 and 2, respectively. The proportion of mitoses derived from the 5 × HU-treated marrow reached three to four times the predicted level within 8 d and remained at this level for up to 100 d. Similar results were obtained in a competition between normal marrow cells and cells taken 2 h after the last of three successive exposures to 5 × HU, suggesting that the extensive hematopoietic depletion and regeneration induced by the two previous exposures to 5 × HU had not significantly depleted the bone marrow of potent stem cells (Fig. 3 d). Confirmation

TABLE III

Short-Term Effects of HU on Numbers and Self-Renewal of Bone Marrow CFU

Source of bone marrow*	Number of CFU per femur ($\times 10^{-3}$)	CFU self-renewal index‡	
		day 8	*day 10*
1 pair HU	0.3	4.8	—§
1 pair HBSS	2.5	2.1	—
1 series 5 × HU	0.03	8.4	36
1 series 5 × HBSS	2.2	2.2	8.7
2 series 5 × HU	0.04	8.4	—
2 series 5 × HBSS	1.8	3.1	—

* A single pair of injections or one or two series of five injections were given intraperitoneally. 8 wk separated the two series of five injections. 5 × HU/HBSS donors were killed 2 h after the final injection; other donors were killed 42 h after the second injection.

‡ Calculated from the number of CFU generated per primary spleen colony in 8 and 10 d, respectively

§ Indicates that no day 10 assay was done.

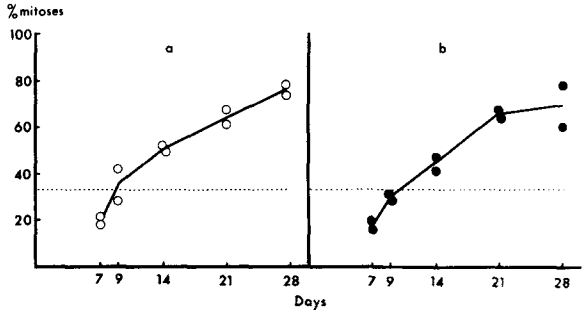

FIG. 4. Competitive repopulation of lethally irradiated CBA mice by marrow cells from normal T6T6 (6×10^6 cells, 1,570 CFU) and HU-treated T6 (1.5×10^6 cells; 790 CFU) donors. Donors received one pair of HU injections 7 h apart and were killed 42 h after the second. For further explanations, see legend to Fig. 1. (a) Bone marrow, (b) spleen of recipients.

of the superior proliferative capacity of bone marrow cells that survived a single course of 5 × HU is shown in Fig. 5. Three groups of CBA mice were lethally irradiated. One group received bone marrow cells from normal syngeneic donors; a second group received bone marrow from donors 2 h after 5 × HU treatment, and the third received HBSS. The donor cell suspensions were also assayed for their content of CFU. Although less than one-half as many CFU were injected from HU-treated donors as from normal donors (40 against 108), cell proliferation in the regenerating bone marrow, assayed by uptake of IUdR, was 1.5–2 times greater in the recipients of 5 × HU-surviving cells.

The Enhanced CFU Self-Renewal Seen Shortly after 5 × HU Is Not Due Merely to Elimination of Cycling Cells. If cycling and noncycling (long G1 or G0) CFU self-renew differently, this alone might account for the above-average self-renewal shown by CFU 2 h after 5 × HU because few of these CFU would be expected to be in cycle. However, 24 h after 5 × HU, when many CFU were evidently cycling rapidly, as indicated by their susceptibility to a further injection of HU, they showed 5–6 times

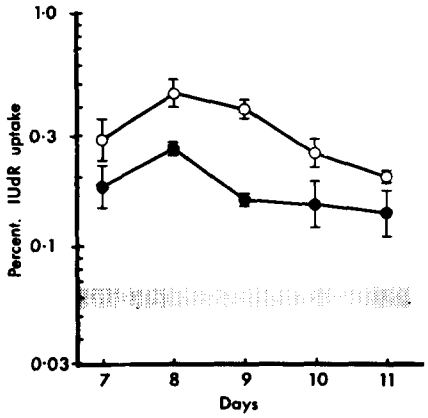

FIG. 5. IUdR incorporation into the bone marrow of lethally irradiated CBA mice injected with 10^6 cells (108 CFU) from normal syngeneic donors (●) or 10^7 cells (40 CFU) from 5 × HU-treated donors (○). Donors were killed 2 h after the final injection. Mean ± SE for groups of three mice. Shaded area represents 95% confidence limits for the mean of 15 irradiated HBSS-injected controls.

TABLE IV

Cycling Status of CFU from HU-treated Donors and Its Relationship to CFU Self-Renewal

Source of bone marrow*	Number of CFU per femur ($\times 10^{-3}$)	CFU self-renewal index‡	
		day 8	*day 10*
24 h after 5 × HU	0.10	8.9	40
24 h after 5 × HU (noncycling cells)§	0.05	5.6	31
24 h after 5 × HBSS	1.9	1.6	6.6
10 d after 5 × HU	1.5	3.4	—‖
10 d after 5 × HU (noncycling cells)§	1.5	3.3	—
10 d after 5 × HBSS	1.4	1.6	—

* Five intraperitoneal injections of HU or HBSS were given over a 30-h period, and donors were killed at the indicated time after the final injection.

‡ A single additional injection of HU was given 2 h before killing.

§ Calculated from the number of CFU generated per primary spleen colony in 8 and 10 d, respectively.

‖ Indicates that no day 10 assay was done.

greater self-renewal than normal (Table IV). The CFU that remained immediately after the additional exposure to HU (presumably including those not in rapid cycle) self-renewed no better than the unseparated population. This was probably not due to any effect of HU on noncycling cells because exposure of a predominantly noncycling population (10 d after 5 × HU) did not influence self-renewal (Table IV). Thus, CFU that were in S-phase during the 2 h before their transfer self-renewed at least as efficiently as those that were not. The superior self-renewal of CFU obtained 2 h after one or two courses of 5 × HU could not, therefore, be attributed to their slow-cycling or G0 state.

Discussion

Adverse Effect of Transplantation on Self-Renewal of Stem Cells. Stem cells are defined by their ability not only to give rise to differentiated progeny, but also to self-renew (26). The decision whether a given stem cell will self-renew (birth) or produce differentiating daughters (death) has been widely regarded as a stochastic process (17), and calculations have been made of the probability of self-renewal under certain experimental conditions (27, 28). However, it is recognized that hematopoietic stem cells, as demonstrated by spleen colony (18, 19, 29) or competitive repopulation (12, 29, 30) techniques, are heterogeneous, some showing a higher probability of self-renewal than others under identical conditions. In the present experiments, we were able to measure self-renewal but not the ability to produce normally differentiated descendants. We therefore consider stem cell function entirely in relation to self-renewal.

The first experiments described showed that there was a marked deficiency of stem cell function as measured by two assays after a single intravenous transfer of bone marrow, confirming the conclusion of Harrison (21). Some further functional decline with additional serial transfers was revealed by spleen colony assays. Previous work showed a progressive decrease in the number of clones contributing to repopulation in recipients of twice-, thrice-, or four times-transferred bone marrow, despite the injection of similar numbers of CFU (12). In these and other (5–8, 10) experiments, no regeneration was detectable after five or six transfers. By 5 wk after a single transfer, the CFU population no longer contained a high frequency of dividing cells and had returned to the G0 or long G1 condition characteristic of normal hematopoiesis, as indicated by resistance to a single dose of 10 mg Ara-C or 25 mg HU (data not shown). This return to G0 was as expected from earlier work (31) and showed that the defective function of stem cells in regenerated bone marrow could not be accounted for simply by their being in cycle at the time of transfer. Data in Table IV also show that cycling at the time of transfer does not militate *per se* against self-renewal. In summary, these data indicate that the combination of lethal irradiation of recipients and reconstitution with intravenously injected single-cell suspensions of bone marrow has lasting effects on hematopoietic stem cell populations. This justifies the view that the serial transfer system is too artificial to allow valid estimates of the longevity of hematopoietic cell populations.

In Situ Regeneration of Bone Marrow after Serial Exposure to HU. HU, an inhibitor of ribonucleotide reductase, selectively kills cells in S-phase of the cell cycle while sparing other cells (32). We used protocols of HU treatment that kill varying numbers of hematopoietic stem cells as well as more differentiated elements (23, 24) to deplete the marrow repeatedly and hence provoke repeated regeneration *in situ* without the need for the disruption of marrow architecture that is inherent in irradiation/reconstitution experiments. Two doses of HU depleted the CFU in the bone marrow by ~70%, and this effect could be produced repeatedly at 3-wk intervals. Because the stem cell function of mice that had received up to 25 pairs of HU injections was unimpaired compared with normal bone marrow, it is clear that this method of achieving repeated extensive hematopoietic regeneration has very different results from those after serial transplantation of bone marrow cells through irradiated recipients. Viewing the arithmetic of stem cell regeneration in the most simplistic terms, it would take ~1.5–2.0 doublings of the stem cell population to achieve

regeneration after each pair of HU injections, or 40–50 in all. On the same basis, regeneration of normal stem cell numbers ($\sim 3 \times 10^5$) from 5×10^3 injected into an irradiated recipient would take 6 doublings, or 30 doublings over 5 transfers. Thus, in terms of the number of population doublings required, three to four pairs of HU injections are equivalent to one transfer. Although these calculations can only be taken at face value, if it is assumed (improbably, as discussed below) that all stem cells have an equal likelihood of contributing to regeneration, they strongly suggest that the decline in the function of stem cells after single or multiple transfers is not attributable simply to an increased mitotic history.

Regeneration after five doses of HU involves an expansion of the stem cell pool comparable to that required after irradiation and reconstitution with bone marrow cells. Here again there is no evidence that this regime adversely affected stem cell function. The small minority of stem cells that survived this regime had greater than average powers of self-renewal as judged both by CFU assays (also reported by others; 24) and by competitive proliferation in the bone marrow. Regeneration of hematopoiesis did not exhaust this population, which was again demonstrable after one or two further exposures to 5 × HU. These data imply that some or all of the most self-replicative stem cells are those that are least responsive to mitogenic signals (33). As the bone marrow regenerated, the average self-renewal of the stem cells returned towards normal, possibly due to dilution by less self-replicative descendants.

Although our data argue against a direct effect of the cycling status of CFU on their capacity for self-renewal, it is probable that rapid cell cycle is related to poor self-renewal in the reverse way: strongly self-renewing stem cells appear to enter cycle rarely, whereas those that self-renew less cycle relatively often (29, 34).

The obstinate refusal of some highly self-replicative stem cells to enter cycle during the 30 h of 5 × HU treatment supports the idea of a stem cell hierarchy (11, 20) in which the tendency both to divide and to differentiate increases with the length of the mitotic history. It also provides a basis for clonal succession (35) in which rare divisions of the most clonogenic stem cells provide progeny that divide more readily and differentiate to satisfy day-to-day hematopoietic requirements. However, clonal succession itself still has the status of an attractive hypothesis rather than an established fact.

In Situ Regeneration vs. Serial Transplantation: Implications for the Study of Cellular Aging. Some interpretations of serial transfer experiments in terms of cellular aging have suggested that hematopoietic stem cells have a finite capacity for self-renewal, a maximum number of mitoses that can be undergone before differentiation and death become inevitable (12, 20). This interpretation has depended on three assumptions. (*a*) Individual members of the stem cell pool acquire approximately equal mitotic burdens; (*b*) all stem cells are equally transplantable, i.e., there is no subclass that is particularly difficult to obtain in single-cell suspension or particularly incompetent at homing to its appropriate microenvironment; (*c*) lethally irradiated recipients provide an environment in which stem cells can proliferate and self-renew normally. Although there is no conclusive evidence against the first assumption, it is difficult to reconcile with the fact that the more self-replicative stem cells are relatively cycle-refractory and is inconsistent with the clonal succession model that this observation implies. Because CFU are evidently heterogeneous in their ability to self-renew and it has not been proven that all stem cells are detectable as CFU, assumptions (*b*)

and (*c*) cannot be verified simply by showing that CFU recover quantitatively in irradiated recipients after injection of bone marrow. On the contrary, the deficient self-renewal of stem cells after even one transfer shows that one or both of assumptions (*b*) and (*c*) are incorrect. It can be concluded that the serial transfer of hematopoietic cells provides an inadequate model for the study of cellular aging.

Assumption (*b*) is irrelevant to serial regeneration induced by HU, and (*c*) is more likely to be satisfied because HU is not, like radiation (36, 37), known to disrupt bone marrow architecture or damage nondividing cells. After lengthy serial HU treatment, we found no evidence of a decline in stem cell function. Moreover, bone marrow from mice that had received 23 pairs of HU injections provided normal stromal supporting elements in long-term in vitro culture (T. M. Dexter and E. Ross, unpublished data). However, the probability still exists that regeneration occurs by the sequential activation of highly clonogenic stem cells, contradicting assumption (*a*). The consequences of such a sequential activation for the stem cell pool as a whole cannot be assessed at present. It could involve irreversible loss from a fixed, nonrenewable store. Alternatively, only one daughter cell might continue to divide and differentiate, whereas the other relapsed into a G0 state. This kind of asymmetric division, possibly determined by microanatomical position (38, 39), could provide a hematopoietic system that was virtually inexhaustible. In either case, the processes of aging in such clones seem to be inseparable from those of differentiation.

Summary

The mouse hematopoietic system was subjected to repeated depletion and regeneration either by serial transfer of bone marrow cells through lethally irradiated recipients or by repeated treatment with the cycle-active drug hydroxyurea (HU).

The capacity of surviving stem cells to proliferate and self-renew was assayed at intervals by two methods: (*a*) the spleen colony method; and (*b*) competitive repopulation of irradiated recipients using chromosome markers, with normal bone marrow cells as an internal control.

The progressive decline in stem cell function that occurred during serial transfer of bone marrow and that had already begun after a single transfer was not seen during HU treatment; up to 25 pairs of HU injections given over more than 1 yr had no discernible effect on the number of stem cells present 3 wk after the final injection or on their capacity to self-renew.

Within 2 d after exposure to HU, the average self-renewal capacity of surviving stem cells was enhanced. This implies that the drug selectively eliminates poorly self-renewing stem cells and hence that these enter cycle more readily than stem cells with a high self-replicative potential. However, the fact of being in cycle at the time of injection did not of itself affect self-renewal.

The results show that serial transfer of bone marrow is not a valid method for studying clonal aging phenomena because it does not fulfill the assumptions on which such studies are based. No evidence was obtained for any intrinsic limitation in the capacity of bone marrow populations for repeated regeneration after HU-induced depletion. However, this does not necessarily imply that individual hematopoietic clones are capable of indefinite expansion because hematopoiesis may (as suggested

by the relative resistance of highly self-replicative stem cells to mitogenic signals) proceed on the basis of clonal succession.

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