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Proliferative capacity of murine hematopoietic stem cells

(serial transplantation/alkylating agents)

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ABSTRACT **The present study demonstrates a decrease in self-renewal capacity with serial transfer of murine hematopoietic stem cells. Production of differentiated cell progeny is maintained longer than stem cell self-renewal. In normal animals the capacity for self-renewal is not decreased with increasing donor age. The stem cell compartment in normal animals, both young and old, appears to be proliferatively quiescent. After apparent recovery from the alkylating agent busulfan, the probability of stem cell self-renewal is decreased, there is a permanent defect in the capacity of the bone marrow for serial transplantation, and the stem cells are proliferatively active. These findings support a model of the hematopoietic stem cell compartment as a continuum of cells with decreasing capacities for self-renewal, increasing likelihood for differentiation, and increasing proliferative activity. Cells progress in the continuum in one direction and such progression is not reversible.**

When hematopoietic cells are removed from the bone marrow and injected into x-irradiated syngeneic recipient mice, they will repopulate the bone marrow and restore normal hematopoietic activity. If these recipient animals are then used as a source of donor marrow for secondary recipients, the marrow restoration is less satisfactory; with further serial transplantation, this capacity is lost (1–5). If one waits a sufficient time between serial transfers, the marrow regains its ability to restore irradiated recipients (6, 7). Micklem *et al.* (8) has shown that, with serial transplantation, one must be careful in assuming it is the descendents of the original donor cells that one is transferring because, in his experiments using chromosomal markers to discern donor cells from residual recipient cells, the proportion of original donor cells in the transplanted marrow decreased to zero after the fourth transfer. These results cast doubt on those studies in which long intervals were allowed between serial transfer because, in this case, there would be sufficient time for residual recipient cell proliferation. By using genetically impaired animals, Harrison (9–11) has demonstrated that, although marrow transfer capacity exists over four or five transfers, extending many times longer than the normal life expectancy of animals, it becomes progressively less satisfactory and eventually fails.

There have been a number of examples of deficient serial transfer in other tissues, including mammary epithelium (12–14), antibody-producing progenitor cells (15), and skin (16, 17). Such observations have been used to support the observation by Hayflick (18) that normal diploid cells have only a limited proliferative capacity *in vitro*. In order to reconcile Hayflick's observation with the rapidly proliferating normal cell renewal system *in vivo*, Kay (19) described a clonal succession theory that would allow normal hematopoietic proliferation for the lifetime of man without requiring more than 50 or so divisions. This has been considered in detail by Reincke *et al.* (20) with a review of the available studies in the literature. Alternative explanations of serial transplantation failure include: the slow proliferation of stem cells, damage to stem cells by the transfer process, and differentiation pressures exceeding those for self-renewal.

The experiments reported here attempted to study serial transplantation of hematopoietic cells from normal mice and those treated with the alkylating agent busulfan. The results suggest that the stem cell compartment is a continuum of cells with decreasing capacities for self-renewal, increasing likelihood for differentiation, and increased proliferative activity.

MATERIALS AND METHODS

Animals used in all experiments were male C3H/HEJ 12 to 14 weeks old. All animal irradiation was performed with a ^{137}Cs irradiator simultaneously irradiating through opposing portals (less than 4% inhomogeneity). Eight to 10 animals were placed in cages and supplied with food and water ad libitum. The radiation given was 1250 rads (12.5 J/kg) in divided doses of 700 and 550 rads, separated by 3 hr. Bone marrow cell suspensions were prepared by flushing the medullary cavity of the tibia and femur with cold sterile Tyrode's solution. Cell suspensions were kept on ice until used and were injected intravenously into the lateral tail vein, not later than 3 hr after removal. Sufficient volume of solution was used so that the injected bolus varied between 0.1 and 0.5 ml. Cell counts were made by using a hemocytometer and counting at least 200 cells.

Serial transplantation was done at 14-day intervals. At each transfer, similarly prepared recipient animals were injected with appropriate numbers of cells and 8 days later were sacrificed for spleen colony determination by the method of Till and McCulloch (21). Thus, at all times, the total cellularity as well as the number of colony forming units (CFU) of any preparation, was known. This technique is described graphically in Fig. 1.

Animals treated with busulfan were randomly selected from littermates who served as the normal controls in these experiments. drug-treated animals received six weekly intraperitoneal injections of 0.18 mg of busulfan [a dose measured to cause a reduction in CFU content of marrow to 0.37 (Do) of control when measured 24 hours after injection]. Five weeks after this treatment, the first serial transfer experiment was performed. By that time both the peripheral blood counts and marrow cellularity had returned to normal.

In order to determine the proportion of CFU in cycle, cytosine arabinoside was administered intravenously to animals (5 mg per mouse). Three hours later, the animals were killed and the femoral CFU was determined. From the ratio of CFU in animals given cytosine arabinoside to that in untreated animals, the proportion of cells not in DNA synthesis could be determined.

In all experiments using the CFU technique, at least 10 re-

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Abbreviation: CFU, colony forming units.

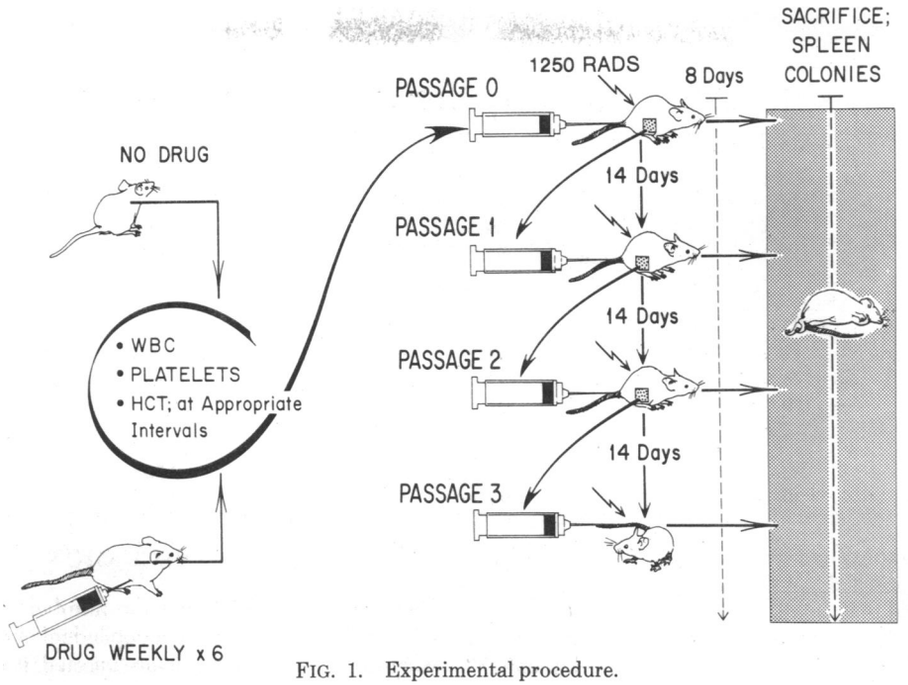


FIG. 1. Experimental procedure.

cipients per point were injected, and no experiment was considered acceptable unless there were at least 5 surviving animals per point.

Determination of R_s and R_c. R_s is the ratio of the CFU at the end of the transfer to the CFU initially seeding the hind limb. R_c is the ratio of bone marrow cells in the limb at the end of transfer to the CFU initially seeding the hind limb.

$$R_s = \frac{S_n}{KS_i}$$

in which S_i = the number of CFU injected, S_n = the number of CFU per hind limb marrow at the time of the next transfer (14 days), and K = correction factor assumed for CFU seeding the hind limb (see below.)

$$R_c = \frac{C_n}{KS_i}$$

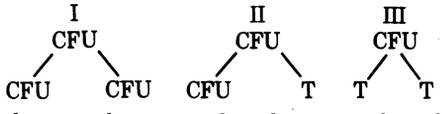
in which C_n = the number of cells in the limb bone marrow at the time of the next transfer (14 days).

Determination of Probability P. This consideration was first described by Till *et al.* (22) and elaborated on by Vogel *et al.* (23). The division of stem cells can be described by using a model with three types of cells: CFU, transition cells, and mature cells. CFU can divide to produce more stem cells or transition cells. If there is a probability P that any daughter cell is a CFU, then after n generations there are S_n CFU progeny from S_i, the initial number of CFU injected:

$$S_n = (2P)^n KS_i \qquad P = 0.5\left(\frac{S_n}{KS_i}\right)^{1/n} \qquad P = 0.5\,(R_s)^{1/n}$$

If $P < 0.5$, the number of CFU cells will diminish with time. If $P > 0.5$, the number of CFU cells will increase.

Note that it does not matter in which way the CFU cells divide. There are three possibilities:



This model cannot determine the relative number of divisions of each type. For example, $P = 0.5$ can mean that there are only divisions of type II or that these are an equal number of type I and type III divisions.

Calculations of R_s, R_c, and P assume that 10% of the CFU that seed hematopoietic sites will seed the hind limb ($K = 0.1$). This latter cannot be determined by using the "f factor" or seeding efficiency (24), which considers the number of CFU that seed the site compared to those injected. What is needed for this calculation is the number that seed the hind limb compared to the number that seed all other hematopoietic sites, and this is not easily obtained. We have assumed this ratio to be 10% because that is the proportion of hematopoietic cells in the hind limb in the normal animal. In any case, the accuracy of this estimate is not important because it is a constant in all the data given and thus will not affect any comparative statements.

RESULTS

In order to determine the appropriate number of cells to be injected, experiments varying the cell inocula were done. R_s, R_c, and P were determined. It is important to note that the calculation of P requires a constant number of divisions in each serial transfer (in this calculation, 28 divisions were assumed). Because Table 1 demonstrates that, at the end of the transfer, the ratio of cells in the hind limb to the CFU initially seeding the hind limb (R_c) decreased with increasing cell inoculum for any serial transfer, this assumption may be incorrect with large cell inocula; therefore, when more than 1×10^6 cells are injected, the calculation of P is of limited significance.

As the size of the cell inoculum decreased, R_s and R_c increased. When the inoculum was less than 1×10^6 cells, there was no further increase in either ratio, and the number of surviving recipient animals decreased. It therefore appears that the cell inoculum best suited for study is 1×10^6 cells. A lesser number of cells injected does not sustain sufficient serial transfer, and a greater number does not result in a maximal proliferative stress.

With serial transfer, R_s and R_c decreased. When this point is amplified serial transfers, with 1×10^6 cells, were studied as a function of the age of the donor, there was a marked decrease in R_s with serial transfer but not in R_c (Table 2). P decreased with each transfer because it is a function of R_s. As animals age,

Table 1. Serial transplantation of different cell inocula of bone marrow derived from young mice

Cells transferred, no. × 10^{-6}	R_s			$R_c \times 10^{-4}$			P		
	1	2	3	1	2	3	1	2	3
				Exp. I					
1-1-1	62	6.7	—	65	20	—	0.579	0.535	—
2-2-2	35	5.5	1.2	39	17	10	0.568	0.531	0.503
4-4-4	17	3	1.8	27	21	20	0.553	0.520	0.510
				Exp. II					
0.25-0.25-0.25	23	—	—	46	—	—	—	—	—
0.5-0.5-0.5	11	—	—	41	—	—	—	—	—
1-1-1	23	—	—	41	—	—	—	—	—
2-2-2	10			31	13	—	—	—	—
				Exp. III					
1-1-1	30	17	—	27	28	—	0.565	0.553	—
1-2-4	30	9	0.2	27	21	7	0.565	0.541	—

there appears to be no consistent change in initial R_s, R_c, or P. Further, the number of serial transfer completed does not decrease. It must be noted that, in all serial transfer experiments, selectivity is occurring because one is scoring marrow derived from survivors and the number of such surviving recipients decreases with subsequent transfer. Therefore, calculation of P for the third transfer may be of limited value because, should it decrease to significantly less than 0.5, it will not be compatible with the animal survival; selection of survivors skews the results to higher estimates of P. Under no circumstances were more than three transfers possible and, under many circumstances, a third transfer was not possible.

In contrast, when marrow was removed from animals that apparently had recovered from the busulfan treatment, the number of transfers was significantly decreased, as we have reported (25). Even with the first transfer, R_s and R_c were significantly reduced (Table 3). With the second transfer, R_s was further reduced, although R_c appeared to be increased. These results may be a matter of the data being skewed by the small number of animals surviving a second transfer.

Table 4 indicates the proportions of CFU not in DNA synthesis, assuming that all those that are killed by the cytosine arabinoside were in DNA synthesis (S). The proportion of cells in S was small in either young or old normals, and considerably more CFU were in cycle in marrow derived from animals apparently recovering from busulfan than in age-matched controls.

DISCUSSION

These results confirm that the number of serial transfers is limited. This appears to be independent of cell inoculum. Even when large numbers of cells are injected, it is difficult to sustain more than three transfers. With successive transfers, R_s appears to decrease whereas R_c is preserved, indicating that the probability P of uncommitted versus differentiated progeny decreases with successive serial transfer.

Calculations of R_c assume that all cells in the bone marrow are derived from CFU injected 2 weeks earlier. This may not be precisely correct because there may be committed progenitor cells in the injected marrow whose progeny are still present in the marrow 2 weeks later. This is likely to be only a small minority of the marrow cells because the time is long and such progeny should have matured and entered the peripheral blood. Furthermore, the number of divisions between such committed progenitors and nondividing progeny would be fewer than between CFU and similar progeny; thus, they would be diluted by the greater exponential growth of clones derived from CFU. In any case, even if the assumption of all marrow cells being derived from injected CFU is not exactly correct, it will affect only the quantitative determinations of R_c and not the qualitative and comparative aspects of the study.

The exact number of divisions in the 2-week period is not critical. Twenty-eight were chosen because the cell-cycle time for proliferative cells in the marrow is thought to be approxi-

Table 2. Serial transplantation of bone marrow (1 × 10^6 cells) derived from normal donors of differing ages

Age, weeks	R_s			$R_c \times 10^{-4}$			P		
	1	2	3	1	2	3	1	2	3
28	28.0	11.0	—	28.0	33.0	39.0	0.563	0.545	—
33	38.0	15.0	—	27.0	28.0	—	0.569	0.551	—
38	47.0	2.4	6.6	20.0	15.0	38.0	0.574	0.516	0.535
43	25.0	7.7	10.3	19.0	18.0	16.0	0.561	0.538	0.543
48	18.9	3.6	0.84	19.0	14.0	37.0	0.555	0.523	0.497
53	45.0	5.4	3.0	63.0	12.0	17.0	0.573	0.531	0.520
67	47.0	12.0	—	16.0	17.0	4.4	0.574	0.546	—
73	83.0	5.0	—	45.0	10.0	—	0.584	0.530	—
92	46.0	10.0	3.5	16.0	29.0	23.0	0.573	0.543	0.523
103	70.0	3.8	—	53.0	17.0	—	0.582	0.524	—
114	91.6	4.8	—	77.1	23.1	—	0.588	0.529	—
129	23.9	3.2	1.1	41.3	46.5	102.1	0.560	0.521	0.520
$\bar{X}$	46.9	7.0	4.2	35.4	21.9	34.6	0.571	0.533	0.520
SEM	6.8	1.2	1.48	5.9	3.0	10.6	0.003	0.003	0.007

Table 3. Serial transplantation of bone marrow derived from busulfan-treated donors

Age, weeks	R_s			$R_c \times 10^{-4}$			P		
	1	2	3	1	2	3	1	2	3
28	0.5	—	—	6.3	—	—	0.488	—	—
33	0.56	—	—	4.5	—	—	0.490	—	—
38	1.83	—	—	10.4	—	—	0.511	—	—
43	1.9	2.6	—	6.2	29.5	—	0.512	0.517	—
48	31.2	9.2	—	46.2	37.0	—	0.565	0.541	—
53	1.3	—	—	17.8	—	—	0.505	—	—
67	10.3	1.9	—	10.4	15.8	—	0.543	0.512	—
73	12.9	6.2	—	48.1	29.6	—	0.548	0.534	—
83	1.3	—	—	6.2	—	—	0.505	—	—
92	4.7	—	—	10.8	—	—	0.528	—	—
103	4.5	2.8	—	12.5	28.5	—	0.528	0.519	—
114	29.4	—	—	40.0	—	—	0.564	—	—
129	1.3	—	—	41.2	—	—	0.505	—	—
$\bar{X}$	7.8	4.5	—	20.0	28.1	—	0.522	0.525	—
SEM	2.9	1.4	—	4.7	3.4	—	0.007	0.005	—

The drug was administered weekly at ages 13–18 weeks.

mately 8–12 hr and there is a known delay in proliferation of 1–3 days after transfer. Thus, for simplicity, a mean cell cycle time of 12 hr was assumed. Although Vogel *et al.* (23) have suggested that P does not change in spleen colonies between day 6 and day 14, in the present experiment the determination of P was as an average and did not necessarily require P to be constant. Evaluation of R_s, R_c, and P when only a few animals survive is difficult. These animals no longer are representative of the entire group because their very survival indicates that they contain cells most capable of producing progeny. This limits the significance of measurements in the last transfer. Despite this, with successive transfers, R_s decreases in both normal and busulfan-treated marrow.

When marrow from busulfan-treated donors is given, the number of cells injected (1×10^6) is held constant and the number of CFU in this marrow is normal or slightly reduced (25, 26). Such an inoculum should result in a maximum value of R_s and, thus, of P as is shown for normals in Table 1. The marked reduction observed in initial R_s and R_c in animals long after busulfan administration can only be explained by there being a nonrecuperable residual decrease in the proliferative capacity of the CFU.

As animals become older, there appears to be no decrease in serial transplantation, R_s, R_c, or P. In contrast, after stem cell depletion by busulfan, there is a marked defect in serial transfer, R_s, R_c, and P. As animals age, the functional capacity of stem cells and marrow proliferation does not decrease. This suggests that the stem cell compartment, under normal circumstances, is called on very little and rather acts as a reserve compartment whose proliferative capacity is only required after cell depletion. Such a notion is supported by there being no change in CFU proliferative activity with age, as demonstrated by there being few, if any, stem cells appearing in DNA synthesis, even in aged animals. In contrast, when significant stem cell depletion and repopulation have occurred after busulfan administration, there is a decrease in serial transfer and in R_s and, concomitantly, there is an increase in the proportion of stem cells in cycle. We have reported (26) that the stem cell proliferative failure after administration of alkylating agents is not limited to the hematopoietic system but can be demonstrated in the immune system, melanocyte, and lens. This suggests that what is applicable in the hematopoietic system may obtain in other cell renewal tissues.

Fig. 2 presents a model of the hematopoietic stem cell compartment as a continuum of cells with changing capacity for self-renewal, differentiation, and proliferation. This model was first presented in order to reconcile various stem cell assays (27). The model suggests that cells measured by the CFU are heterogeneous. The cells at the left are characterized by a great

Table 4. Survival ratio of CFU with and without cytosine arabinoside (Ara C)

Age, weeks	Previous busulfan	CFU with Ara C/ CFU without Ara C*
	Exp. I	
28	−	0.87 ± 0.16
64	−	1.11 ± 0.15
64	+	0.64 ± 0.08
	Exp. II	
116	−	0.78 ± 0.15
116	+	0.48 ± 0.15

Measurements made 3 hr after administration of cytosine arabinoside.
* Mean ± SEM.

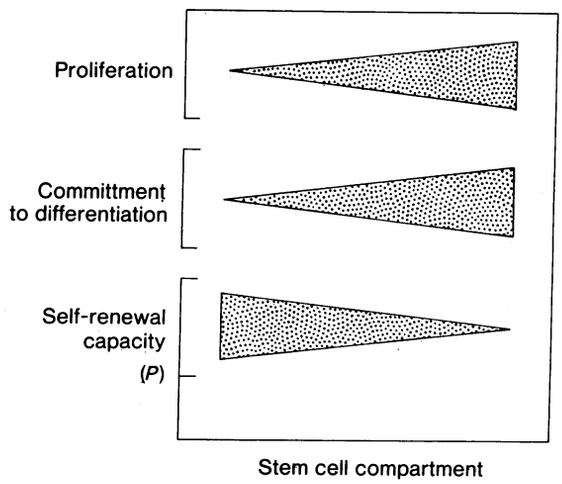


FIG. 2. Model of stem cell compartment as a continuum of cells. Cells progress from left to right after proliferative demands on the compartment. This progression is not reversible.

capacity for self-renewal—that is, a high *P*. Proliferative activity is low, with few cells in cycle, and therefore few if any would be killed by cytosine arabinoside The cells at the right would be characterized by a decrease in *P* with a larger proportion of the progeny being committed to a specific differentiation pathway and a much higher proportion of cells in DNA synthesis and thus killed by cytosine arabinoside.

The results described in these experiments can be fitted to this model by suggesting that, under normal circumstances, the stem cell compartment in the young adult is largely composed of the more primitive cells at the left, which are seldom required to divide during the normal animal life span (28). They function largely during early development; in the adult they serve primarily as a reserve compartment with only a small contribution required during the nonperturbed steady state. When the stem cell compartment has been depleted by noxious agents (in this experiment, busulfan), the stem cells become proliferatively active and attempt to restore the compartment to its original size. The model requires that, once cells move to the right, they cannot move back. Therefore, after recovery from alkylating agents, the distribution of cells within the stem cell compartment has changed, with more cells being proliferatively active and the average *P* decreasing. Daughter CFU are not the same as the parent, at least not as concerns the subsequent probability of differentiated versus undifferentiated progeny. As cell pedigree lengthens, *P* decreases. The suggestion of limited proliferative capacity being important in aging does not appear to be the case in regard to the hematopoietic system because old and young marrow are functionally similar. However, such self-renewal limitations might be quite important in response to cell depletion. This may be important in determining the late effects of either x-irradiation or chemotherapeutic agents (29).

This model is consistent with some known characteristics of stem cells, such as heterogeneity within the CFU compartment. The number of CFU per colony is significantly lower in those colonies derived from alkylating agent-treated animals compared to controls (30), indicating that *P* has decreased under these circumstances. Stem cells surviving vinblastine treatment, and therefore presumably not in cycle, have a greater capacity for self-renewal (31). Micklem (32) has shown that circulating CFU have a markedly reduced ability to give rise to stem cell progeny, compared to those from marrow sources. Spleen-derived CFU have similar limitations (33). Serial transfer experiments (34) using fetal and embryonic tissues have shown an increased number of transfers and, we would suppose, an increase in *P*. This would indicate that the stem cell compartment is required to proliferate during embryonic and fetal life but very little during the normal life span of the animal, unless the stem cell compartment has been sufficiently reduced. Whether this is also the case after loss of mature cells, such as that caused by bleeding or continued infection, is at present unknown.

Thus, the proliferative capacity of stem cells appears to be limited. Although not important in aging, this restriction can be demonstrated with repeated proliferative requirements of serial transplantation and is markedly impaired after apparent recovery from busulfan treatment.

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