STUDIES OF HEMATOPOIETIC STEM CELLS SPARED BY 5-FLUOROURACIL

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Accumulating evidence suggests that the hematopoietic stem cell population is heterogeneous in terms of the probability that at its next division a cell will give rise to either other stem cells or cells with restricted developmental potential (1–5). It has been proposed (6, 7) that the factor governing the choice is the length of the mitotic history of that stem cell. The fewer past divisions, the more likely it is to self-replicate; conversely, a long mitotic history would be associated with a higher probability of forming cells committed to one or another of the developmental pathways culminating in functional blood cells. Stem cells more likely to self-replicate are generally thought of as being more primitive.

Stem cells surviving a single injection of 5-fluorouracil (FU)[1] or multiple injections of hydroxyurea (HU), according to this criterion and others, are on average more primitive than the normal, untreated stem cell population (4–8). Evidence supporting this conclusion comes from several studies including the following: (*a*) Transplanted marrow from HU- or FU-treated mice gave rise to individual spleen colonies containing more spleen colony-forming cells (CFU-S) and more granulocyte/macrophage CFU (CFU-GM) (primitive granulocyte-macrophage precursors) than colonies resulting from normal marrow transplants (6, 7); (*b*) 5-fluorouracil-treated marrow had a greater capacity than did untreated marrow to restore the CFU-GM population in spleens and marrows of irradiated hosts and to restore megakaryocytopoiesis in these organs (4, 9); (*c*) in competitive repopulation studies normal marrow and HU-treated marrow cells were mixed and transplanted into irradiated mice (8). One of the two marrow donors bore a chromosome marker allowing the derivation of mitotic figures in bone marrow or spleen cells in the host to be determined. The HU-treated cells outgrew normal cells even when they were a numerical minority in the transplant.

Hodgson and Bradley (4) found that CFU-S from FU-treated mice followed a temporally different growth pattern in the spleens of irradiated hosts than CFU-S from normal donors. Although the cellular composition of spleen colonies formed by normal marrow cells has been shown to change with time (10–12), the number of colonies remained nearly constant between 8 and 14 d (4, 5). In

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[1] *Abbreviations used in this paper:* AI, atavism index; BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte/macrophage colony-forming unit; CFU-GEMM, granulocyte, erythroid, macrophage, and megakaryocyte CFU; CFU-S, spleen CFU; FU, 5-fluorouracil; 1FU, 7FU, marrow from mice 1 or 7 d after injection of FU; HU, hydroxyurea; MEM, minimum essential medium.

contrast, spleen colonies derived from FU-treated mice steadily increased in number during this period. The usual 8- or 9-d growth period before spleen colonies are counted would, therefore, seriously underestimate the number of CFU-S in FU-treated marrow, but not those in normal marrow. One explanation for the delay in spleen colony growth caused by FU-treated cells is that primitive stem cells surviving FU are unable to directly initiate colony growth. Rather, the population undergoes developmental maturation, giving rise to a cohort of cells capable of spleen colonization and growth. It is this series of requisite developmental steps that could account for the delay in colony growth. We present evidence that this explanation is correct and, moreover, that the locus of at least part of developmental maturation of stem cells from FU-treated mice occurs in the bone marrow of the hosts.

Materials and Methods

Mice. B6D2F1 (C57BL/6 ♀ × DBA/2 ♂)F_1 mice 2–4 mo old and weighing 20–24 g were used throughout these studies. They were bred and housed in our specific pathogen-free animal facility.

Spleen Colony Assay. A single-cell suspension was injected in 0.5 ml of 90% alpha-minimum essential medium (MEM) buffered with morpholinopropane sulfonic acid (MOPS), 10% fetal calf serum. Cells were injected intravenously into a lateral tail vein of lethally irradiated (9.5 Gy) hosts. Spleens were harvested 8–14 d after irradiation and cell injection. Cells were injected within 90 min of irradiation of the host. Macroscopic colonies were counted on the surfaces of Bouins'-fixed spleens.

Irradiation. A lethal dose (9.5 Gy) of gamma radiation was delivered from a ^{137}Cs source at a dose rate of 0.92 Gy/min.

Treatment with Drugs. HU and FU were obtained from the National Cancer Institute, Bethesda, MD. HU and FU were injected intravenously at the specified doses. Control mice in each experiment were injected with the drug vehicle.

Burst Assay. Erythroid burst-forming units (BFU-E) were assayed as previously described (13) in alpha medium containing (in final concentrations) 0.8% methylcellulose (Methocel A4M Premium; Dow Chemical Co., Midland, MI), 30% fetal calf serum (Irvine Serum Co., Irvine, CA), 1% deionized bovine serum albumin (A-4503; Sigma Chemical Co., St. Louis, MO), 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co.), 2.5 U/ml of erythropoietin (Toyobo, New York), and 25 μl/ml of medium conditioned for 5 d by pokeweed mitogen-stimulated spleen cells (14). Benzidine-positive bursts (15) were counted on day 8 and in another set of 35-mm dishes (5221-R; Lux Scientific, Inc., Thousand Oaks, CA) on day 14.

^{89}Sr-treated Hosts. Strontium-89 chloride in 1.5 M HCl (Oak Ridge National Laboratory, Oak Ridge, TN) was neutralized and injected intravenously at a concentration of 2.0 μCi/gm of body weight. 7 d later they were lethally (9.5 Gy) irradiated.

Results

Effects of FU on CFU-S In Vivo: Time Study of Spleen Colony Development. The data in Fig. 1 show that whereas the number of spleen colonies caused by normal marrow did not change significantly between days 8 and 14, spleen colonies increased 67-fold when marrow cells from mice given 3 mg of FU 1 d previously (1FU marrow) were transplanted. Thus when FU toxicity to CFU-S was assessed using an 8-d assay, the surviving fraction was ~0.007; in a 14-d assay the surviving fraction of CFU-S in the same FU-treated marrow was 0.5.

Recovery of CFU-S After FU. From pilot studies we knew that femoral marrow cell counts recovered to nearly normal levels ~10 d after an injection of 3 mg of

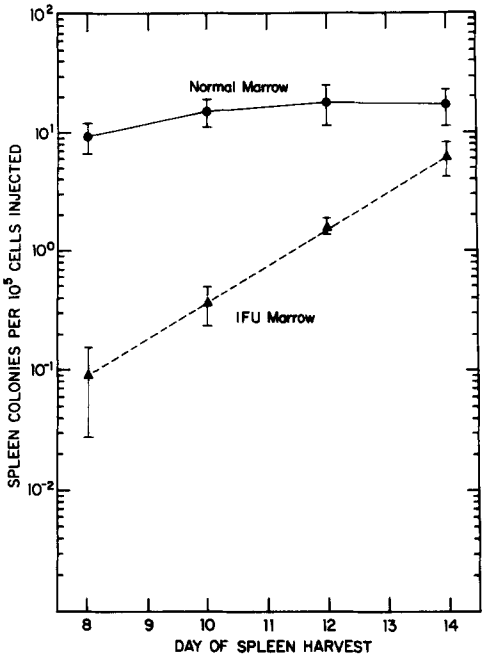

FIGURE 1. Temporal pattern of spleen colony growth caused by normal and FU-treated marrow cells. The following cell numbers were injected into lethally irradiated (9.5 Gy) hosts: 3×10^4 normal marrow cells; 9.4×10^5 1FU marrow cells for days 10, 12, and 14 of spleen harvest; 2.8×10^6 for the day 8 point. 1FU marrow was pooled from femurs of five mice injected 1 d previously with 3 mg of FU. Each represents the mean ± 1 standard deviation of 10 spleens.

FU; therefore, we set up an experiment to temporally study CFU-S numbers and the ratio of day 14/day 8 spleen colony counts, what I will call the atavism index (AI), after FU treatment. The results in Table I show that as the number of CFU-S in the marrow recovered after FU, the AI of these CFU-S decreased until, by day 7, the AI was near the value of 1.3 found in this experiment for untreated normal marrow. The most dramatic change in the AI occurred between days 2 and 3 when it declined from ~21 to 9; from day 3 onward the change was relatively gradual. The small increase in AI between days 9 and 11 is probably not significant; in other experiments we have found that after 12 d the AI is always between 1 and 2.

Frequency of CFU-S and BFU-E in Marrow after FU. A further finding of the temporal study, not readily apparent in Table I, is that the frequency of CFU-S in marrow increased to supranormal levels during recovery from FU. This is graphically illustrated in Fig. 2, where normal, untreated marrow is contrasted with marrow 7 d after FU treatment (7FU marrow). In this experiment we found a 4–10-fold increase in the frequency of CFU-S in 7FU marrow when compared with normal marrow, depending on whether 8- or 14-d spleen colonies were compared (Fig. 2*A*). It should be noted that 7FU marrow, like the normal marrow, had an AI close to unity. We also compared the frequencies of the primitive erythroid precursor, BFU-E, in these marrows. These results in Fig.

TABLE I

CFU-S Numbers and Atavism Index After FU In Vivo

Days after FU	Cells per femur ($\times 10^{-6}$)	Day 8		Day 14		Atavism index
		Fraction of femur injected	Colonies	Fraction of femur injected	Colonies	
0	17.2	0.0025	15.3 ± 3.1	0.0025	20.1 ± 4.2	1.3
1	5.7	0.15	2.1 ± 0.7	0.01	3.3 ± 0.8	24
2	5.9	0.12	6.3 ± 1.2	0.01	11.2 ± 2.0	21
3	4.6	0.10	21.3 ± 3.3	0.01	19.0 ± 5.9	8.9
4	5.0	0.02	3.5 ± 1.7	0.01	9.2 ± 2.5	5.3
5	6.3	0.01	7.6 ± 2.9	0.005	20.4 ± 3.4	5.4
7	7.6	0.002	15.9 ± 4.7	0.002	28.8 ± 4.4	1.8
9	12.4	0.0025	21.6 ± 4.9	0.0025	33.7 ± 3.8	1.6
11	16.0	0.0025	13.4 ± 2.5	0.0025	31.5 ± 4.6	2.4

At each of the time points, groups of three to six mice injected with FU (3 mg, i.v.) were sacrificed and femoral marrow was pooled. The designated femoral fractions were injected into groups of lethally irradiated (9.5 Gy) mice. Spleen colonies were counted on days 8 and 14. Colony counts are presented as the means ± 1 standard deviation of 10–12 spleens per point.

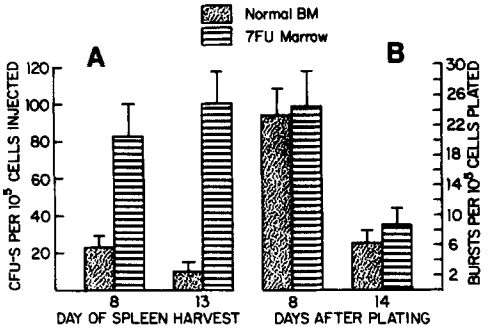

FIGURE 2. Growth of CFU-S and BFU-E from marrow 7 d after FU injection. For CFU-S determinations, 1×10^4 7FU marrow cells were injected; 3×10^4 normal cells were used. Burst counts were made in dishes plated with 1×10^5 cells. Spleen colony counts are the mean ± 1 standard deviation of 10 spleens; four replicate burst cultures were used.

2*B* show that unlike CFU-S, the frequency of BFU-E was not increased above normal values in 7FU marrow. This was true for bursts counted on day 8 and those larger, more heterogeneous and less frequent bursts found after 14 d of culture (14).

Effects of FU on CFU-S In Vitro: Concentration Study. Table II shows that FU in vitro caused the same qualitative and quantitative changes in marrow CFU-S that we found in vivo: in 2 h, FU killed CFU-S in a dose-dependent manner and the AI of the survivors increased dramatically. In this experiment, exposure of CFU-S to a 50 μg/ml concentration of FU, resulted in a surviving population with an AI of 112. These results argue that the effects of FU on CFU-S are direct and rapid; metabolism of the drug in vivo is not required nor are the effects of FU mediated by other tissues. In studies not reported here we have shown that after as little as 15 min, 70% of cells forming 8-d colonies were killed and surviving CFU-S were characterized by an AI of ~3.

TABLE II
Effect of FU Concentration on CFU-S In Vitro

FU concentration	Cells injected ($\times 10^{-4}$)	Day 8		Day 14		Atavism index
		Colonies per 10^5 cells injected	Surviving fraction	Colonies per 10^5 cells injected	Surviving fraction	
μg/ml						
0	3	46.7 ± 6.7	1.0	50.0 ± 6.7	1.0	1.1
5	4	17.5 ± 5.0	3.8×10^{-1}	60.4 ± 9.9	1.2	3.5
10	10	2.3 ± 1.1	4.9×10^{-2}	14.3 ± 2.6	2.9×10^{-1}	6.2
25	20	0.50 ± 0.2	1.1×10^{-2}	4.5 ± 1.0	9.0×10^{-2}	9.0
50	40	0.025 ± 0.0075	5.4×10^{-4}	2.8 ± 1.1	5.6×10^{-2}	112

Marrow cells (5×10^6/ml) incubated 2 h in polystyrene tubes with the designated concentration of FU in 90% α-MEM, 10% fetal calf serum at 37°C. The cells were washed twice in fresh medium before injection into lethally irradiated hosts. Spleen colony counts are presented as means ± 1 standard deviation of 10 spleens per group.

CFU-S Surviving FU Seed in the Marrow Before Colonizing the Spleen. Additional spleen colonies appearing between days 8 and 14 could arise from cells originally seeding in the spleen but not immediately commencing growth, or they could form from cells seeding in the marrow and subsequently seeding the spleen. To distinguish between these possibilities, we assayed marrow from FU-treated mice in two sets of hosts: normal and ^{89}Sr-treated. Strontium-89 is a bone-seeking beta-emitting isotope that causes ablation of marrow hematopoiesis by local irradiation. Splenic hematopoiesis is not compromised by this treatment; the spleen in fact becomes the sole hematopoietic organ in these animals (16). I reasoned that if CFU-S from 1FU marrow lodged first in the marrow before seeding the spleen, such cells would be killed by local irradiation within the marrow before they could migrate to and colonize the spleen. If this were the case, the number of day 14 colonies should be reduced in strontium-treated hosts when compared with the same marrow assayed in normal hosts. If on the other hand, the requisite steps preceding spleen colony growth occur in the spleen (or other organs except marrow), the AI should be comparable in normal and ^{89}Sr-treated hosts. The results of a representative experiment in Table III show a marked contrast between the AI obtained in the two sets of hosts. Fluorouracil-treated marrow, assayed in irradiated, normal hosts, as expected, had a high AI (~13); in irradiated, ^{89}Sr-treated hosts, the same marrow had an AI of 1.3, a value expected for normal marrow. We assayed normal marrow in the two sets of hosts to show that ^{89}Sr treatment per se did not interfere with spleen colony growth. Colony counts at 8 and 14 d were not significantly different between the two sets of hosts; the AI was ~1 in both cases. Marrow cells harvested from strontium-injected mice and assayed in normal hosts contained no CFU-S, thus demonstrating the efficacy of ^{89}Sr in causing ablation of marrow hematopoiesis. We obtained quantitatively similar results in two other experiments.

Effects of Cell Number on CFU-S Assay. In some experiments (Table I, for example), it was not feasible to assay the same number of cells in both the 8- and 14-d assay animals. When the AI was numerically large, a marrow inoculum that gave a small number of colonies at 8 d could result in confluent spleen colonies

TABLE III

Comparison of Spleen Colony Growth in Normal and ^{89}Sr-injected Hosts

Source of marrow cells	Number of cells injected ($\times 10^{-4}$)	Spleen colonies					
		Normal hosts			^{89}Sr-treated hosts		
		8 d	14 d	Atavism Index (AI)	8 d	14 d	Atavism Index
Normal mice	3	11.8 ± 2.6	11.6 ± 1.9	0.98 ± 0.11*	13.2 ± 1.6	14.5 ± 3.3	1.1 ± 0.21**
1FU mice	30	2.9 ± 0.9	39.0 ± 7.6	13.4 ± 2.7*	3.8 ± 1.2	4.9 ± 1.0	1.3 ± 0.27**
^{89}Sr-treated mice	30	0	0.1 ± 0.3	—	0	0	—
No cells	0	0.2 ± 0.3	0	—	0	0.1 ± 0.3	—

Marrow cells from groups of at least three of the designated donors were pooled and injected into groups of 10–15 (*a*) normal, irradiated (9.5 Gy) hosts and (*b*) irradiated hosts that had been injected with ^{89}Sr (2.0 μCi/gm body weight i.v.) 7 d previously. 1FU marrow is from donors injected 1 d previously with FU (3 mg, i.v.). ^{89}Sr-treated marrow is from donors injected with strontium-89 7 d before marrow harvest. Groups designated * differ significantly ($P < 0.001$) by Student's *t* test; groups marked ** are not significantly different ($P > 0.05$). Spleen colony counts are presented as means ± 1 standard deviation. These data are from a representative experiment; qualitatively similar results were obtained in two others.

TABLE IV

Lack of Effect of Added Irradiated Cells on Spleen Colony Formation by CFU-S From Normal Marrow and 1FU Marrow

Cells injected ($\times 10^{-4}$)				Spleen colonies (±1 SD)		Atavism index
Normal marrow	Irradiated normal marrow	1FU marrow	Irradiated 1FU marrow	Day 8	Day 13	
4	—	—	—	15.3 ± 3.3	19.3 ± 4.1	1.3
4	10	—	—	17.8 ± 2.7	ND	—
4	100	—	—	17.3 ± 3.3	ND	—
4	—	—	10	14.7 ± 3.0	16.3 ± 2.5	1.1
4	—	—	100	16.8 ± 2.0	20.6 ± 4.6	1.2
—	—	40	—	1.2 ± 0.5	28.9 ± 5.8	24
—	—	40	100	1.5 ± 1.1	30.2 ± 3.1	20
—	100	40	—	1.0 ± 0.3	27.0 ± 5.1	27
—	100	—	—	0.1 ± 0.2	ND	—
—	—	—	100	0	0.2 ± 0.3	—

Normal marrow cells and marrow cells from mice 1 d after an injection of 3 mg of FU were either assayed for CFU-S alone or when mixed with irradiated (20 Gy) marrow cells. Cells were irradiated in vitro and mixed with unirradiated cells within 30 min of the time of injection into lethally irradiated (9.5 Gy) hosts. Data are the means of at least 10 spleens per point. ND, not done.

at 14 d. This then raised the question of whether or not additional marrow cells in a transplant influenced the lodging and/or subsequent growth of the injected CFU-S. To answer this question we mixed graded numbers of (*a*) irradiated (20 Gy) normal marrow cells and (*b*) irradiated 1FU marrow cells with both unirradiated normal marrow cells and unirradiated 1FU cells, and assayed them at 8 and 13 d. The irradiated cells were exposed to the ^{137}Cs source in vitro no more than 30 min before they were mixed with untreated cells and injected. The results in Table IV show that the inclusion of as many as 1×10^6 irradiated marrow cells from either normal or FU-treated donors did not alter the number of spleen colonies formed by the nonirradiated cells, nor did they significantly alter the AI. These results suggest that, at least over the cell concentrations used,

TABLE V
Atavism Index of Regenerating Marrow

Marrow cell donors	8 d colonies	Percent of CFU-S in S Phase	14 d colonies	Atavism index
Not treated with hydroxyurea	5.6 ± 1.1	—	5.4 ± 1.3	0.96
Given 50 mg of hydroxyurea 1 h before sacrifice	2.8 ± 0.8	50	3.0 ± 0.5	1.07

12 BDF_1 mice were given 10 Gy of gamma irradiation and immediately injected intravenously with 8×10^6 syngeneic marrow cells. 7 d later, half the mice were injected with 50 mg i.v. of hydroxyurea; the other half were injected with saline. 1 h after those injections, femoral marrow was pooled from the two sets of donors and 1×10^5 cells were injected into groups of 24 lethally irradiated hosts (10 Gy). Spleens were harvested at 8 and 14 d and spleen colonies were counted after the spleens were fixed in Bouins' solution.

spleen colony formation was unaffected by the presence of other normal or 1FU marrow cells.

Rapidly Proliferating CFU-S Don't Represent a Primitive CFU-S Subset. To test the hypothesis that proliferating CFU-S represent a subset of the normal population with regard to spleen colony formation, we determined the fraction of CFU-S that synthesize DNA in marrow undergoing repopulation after sublethal irradiation and the AI of those cells (Table V). 50% of the CFU-S from these marrow donors were killed by a 1-h exposure to hydroxyurea, indicating that most, if not all, were rapidly proliferating. The AI of this CFU-S population and of that population surviving hydroxyurea were both ~1. These results suggest that rapidly proliferating CFU-S contain the same mix of subpopulations found in normal marrow and that proliferative activity per se is not an identifying characteristic of either primitive CFU-S subsets or those with restricted developmental potential.

Discussion

Our results corroborate the findings of Reissmann et al. (17, 18), Hodgson and Bradley (4), and Rosendaal et al. (5–7) and extend their observations on the toxicity of FU to hematopoietic stem cells (CFU-S). We show that FU, either given in vivo or added to bone marrow cells in vitro, (*a*) caused a dose- and time-dependent depletion of the CFU-S population and, more importantly, (*b*) spared CFU-S that were qualitatively different from CFU-S in untreated marrow in that they resulted in spleen colonies that arose later than the colonies usually counted on days 8 or 9. We found that during a recovery period of ~7 d after FU the CFU-S population reassumed the properties of the normal population; i.e., colonies formed by these cells did not change in number between days 8 and 14 of assay. Moreover, during the 7-d postinjection period they rebounded to at least a fourfold higher frequency than in normal marrow. In contrast, the CFU-S population, shortly after FU exposure, especially during the first 2 d, formed few 8-d colonies but often formed a 20-fold greater number of 14-d colonies. The AI thus decreased steadily after FU to a normal value of 1 in ~7 d.

I interpret these changes in the CFU-S population as follows. Spleen colony-

forming cells are a heterogeneous population with different subsets giving rise to colonies at different times after transplantation. Those colonies arising early, at ~8 d, are derived from 'older' (in terms of mitotic history) or more differentiated stem cells, whereas 'younger', or more primitive stem cells, are responsible for colonies arising later, closer to 14 d after transplantation. FU is toxic to the subpopulation forming colonies early after transplantation but is relatively less toxic to cells forming late-appearing colonies. Therefore, immediately after FU injection the AI is high because of the selective loss of day 8 colony-formers. During the next 7 d, the normal mix of CFU-S subpopulations, older and younger, is restored. The older subpopulations ablated by FU recover via proliferation and differentiation of the surviving younger stem cells and at least part of this process must occur in the bone marrow rather than in the spleen.

I have adopted the operational term 'atavism index' (AI) for the ratio of day 14/day 8 colonies because of our results and previous findings showing that CFU-S surviving FU have a greater self-replicative capacity and repopulating ability than CFU-S in normal marrow (4–7). When spleen colonies formed by FU-treated marrow were excised and assayed for CFU-S in a second set of irradiated hosts, substantially more spleen colonies formed than from cells in spleen colonies derived from normal marrow. Moreover, many more progenitors of granulocytes, macrophages, and megakaryocytes were generated in lethally irradiated mice when they were injected with FU-treated marrow than when injected with normal marrow. Thus it appears that the CFU-S spared by FU have significantly greater developmental potential than the normal population and therefore represents a more primitive stem cell population. Hence the term 'atavism index'.

The greater developmental potential of CFU-S after FU argues (*a*) in favor of selective survival of a primitive CFU-S subpopulation(s) after FU and (*b*) against the possibility that the increase between days 8 and 14 in spleen colonies arising from FU-treated cells simply represents a lag period during which sublethal damage caused by FU was repaired in a homogeneous population. If the latter were the case the developmental characteristics of the CFU-S population would not be expected a priori to change as a function of repair.

The cellular composition of spleen colonies arising from transplanted normal marrow has been shown to change with time of growth in the spleen even though the overall numbers do not (10, 11). Colonies appearing at 8 d or earlier were found to be predominately erythroid and often transient (11, 12). Magli et al. (12) found that most of the colonies evident at days 10 or 12 were not visible at days 7 or 8 and that these late-appearing colonies, unlike the earlier, transient colonies, contained primitive cells able to form multilineage colonies in vitro. Therefore, they proposed that the cells giving rise to the early, mostly erythroid colonies were not multipotential and were perhaps analogous to the BFU-E, which, in the same time period in vitro, form erythroid bursts. We believe that cells causing the growth of in vivo colonies that appear after day 8 probably represent the true stem cells and that it is the most primitive subpopulation of those that is spared by FU. The in vitro correlates of cells forming spleen colonies after 8 d may be the CFU-GEMM (granulocyte, erythroid, macrophage, and megakaryocytic CFU) (18). Both cells are multipotential and are capable of

extensive proliferation but at present their mutual identity has not been proven.

A significant result of the present studies is the finding that transplanted CFU-S from FU-treated marrow, but not from normal marrow, must make an obligatory stopover in the bone marrow of hosts before spleen colonization and growth. When we injected FU marrow into irradiated hosts treated 7 d previously with ^{89}Sr, we found that they formed the usual, small number of 8-d spleen colonies. However, on day 14 when in normal hosts there was a 13-fold increase in spleen colonies, we found no significant change in the number of colonies in ^{89}Sr-containing hosts; CFU-S from untreated mice formed nearly identical numbers of spleen colonies in both sets of hosts. Klassen et al. (16) found that the dose of radiation to the spleens of mice injected with 6 μCi/gm body weight of ^{89}Sr was <0.03 Gy per day after the first postinjection week. This low dose did not impair splenic hematopoiesis; in fact, the spleen became the sole hematopoietic organ in ^{89}Sr-treated mice. We used a smaller dose of 2 μCi of ^{89}Sr/gm body weight in the studies reported here. Our findings provide experimental evidence consistent with the notion that the primitive CFU-S subset spared by FU seeds first in the marrow where it undergoes developmental maturation, and perhaps amplification, before spleen colonization. Furthermore, these data suggest that the marrow provides a microenvironment conducive to these development steps whereas the spleen does not. Primitive stem cells may seed into 'niches' (19) or 'stem cell regulatory domains' (20) present only in the bone marrow, whereas 'older' stem cells with diminished proliferative capacity lodge in niches or domains present in both the bone marrow and the spleen. In the adult animal, the reservoir of young stem cells may be restricted to the bone marrow.

Recent studies have shown that the capacity of the stem cell compartment to restore hematopoiesis in irradiated mice is not compromised either during aging of the animal (21) or after 'aging' simulated by repetitive rounds of proliferation caused by repeated hydroxyurea injections (8). In fact, in both studies, old stem cells were more effective than young stem cells in restoring hematopoiesis. Earlier results showing that the repopulating capacity of the stem cell compartment was limited (22) were based on serial transplantation of cells through irradiated hosts. The method of serial transplantation itself was recently shown to cause diminished proliferative capacity rather than the 'aging' caused by repetitive repopulation (23).

In contrast with our results implicating the marrow in stem cell maturation, Rosendaal et al. (5) concluded that colonies arising between 8 and 14 d were due to cells originally seeding in the spleen, but not immediately commencing growth. This conclusion was reached from studies in which they exteriorized and selectively irradiated (8.5 Gy) the spleens of hosts that 1 d previously had received 8.5 Gy of whole-body irradiation and an inoculum of FU-treated marrow. They found that 4 of the expected 22 colonies had subsequently developed by day 11 in these mice. One possibility to account for the discrepancy between our two sets of results is that the 18 'missing' colonies were due to CFU-S that had migrated from the marrow in the 24 h between cell injection and spleen irradiation. The fact that there were any colonies at all in the doubly irradiated spleens indicates that either the spleens were seeded from a nonsplenic site,

possibly the marrow, or, as seems very unlikely, that CFU-S within the spleens survived a dose of 8.5 Gy of ionizing radiation. In our studies, a dose of 20 Gy killed all CFU-S in normal and FU-treated marrow (Table IV). Unfortunately, these authors did not include any times in addition to the 11-d assay point, making it difficult to assess the effect on the AI of reirradiating the spleens.

FU is toxic to cells synthesizing DNA, thus raising the possibility that proliferative quiescent stem cells, those static in G_1 (or G_0) and possibly spared by FU, may represent the source of spleen colonies arising between days 8 and 14. We have shown, however, that proliferating CFU-S do not represent a subpopulation with intrinsically different growth patterns in the spleen than the normal, quiescent CFU-S population (Table V). Marrow regenerating after sublethal irradiation contained a rapidly proliferating CFU-S population of which 50% were in S phase. Both this population and that remaining after the selective killing of cells synthesizing DNA had an AI close to 1. This is consistent with the findings of Ross et al. (8) showing that the proportion of cycling CFU-S does not necessarily reflect the proportion of the CFU-S subset with elevated proliferative capacity.

Summary

Mouse marrow cells were exposed to 5-fluorouracil (FU) either in vivo or in vitro and the effects on the hematopoietic stem cell compartment were studied. The drug was highly toxic to bone marrow cells including the spleen colony-forming unit (CFU-S) population. The small population of stem cells surviving FU, however, caused a different pattern of spleen colony growth when injected into lethally irradiated mice. Whereas numbers of spleen colonies caused by normal marrow cells remained constant during an 8–14 d period after transplantation, spleen colonies derived from FU-treated marrow cells increased by as much as 100-fold during this time. This effect on stem cells was dose dependent both in vitro and in vivo. When FU was given in vivo, the day 14/day 8 ratio of colonies was greatest 1 d after injection and, over the next 7 d, returned to a near-normal value, that is, unity.

A number of studies have shown that the stem cell compartment is heterogeneous with respect to self-replicative capacity and developmental potential. An age structure for the stem cell compartment has been proposed wherein cells with a short mitotic history are more likely to self-replicate than they are to differentiate; hence they are more primitive. 'Older' stem cells with a longer mitotic history are, according to the hypothesis, more likely to differentiate. 5-fluorouracil may be toxic to the older stem cells and selectively spare the more primitive subpopulation. Although the surviving cells may not themselves be able to form spleen colonies, they may give rise to an older cohort of cells more likely to differentiate and form spleen colonies. It is the requisite developmental maturation within the stem cell compartment that may be responsible for the delay in appearance of spleen colonies derived from FU-treated marrow. Our results support this explanation and identify the locus of at least part of this activity as the bone marrow. We found that the FU-treated marrow did not cause an increase in spleen colony numbers between 8 and 14 d in hosts with a long-standing marrow aplasia, due to the incorporation of ^{89}Sr into bone.

I propose that the delayed spleen colony appearance in normal hosts is the result of developmental maturation of the primitive stem cell compartment that survives FU and is responsible for spleen colonies arising around day 14. This maturation, at least initially, occurs in the marrow and leads to the replenishment of the more differentiated CFU-S subsets ablated by FU, which are normally responsible for spleen colonies appearing earlier after transplantation.

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