The Proliferative States of Mouse Granulopoietic Progenitor Cells[1] (34721)

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The hemopoietic system is capable of increasing the supply of functional cells where there is an increased demand for such cells. One way in which this is accomplished is to increase proliferation in undifferentiated precursor cells (1–3). This kind of regulation has been demonstrated for pluripotent hemopoietic stem cells in the mouse. Becker *et al.* (3) used the lethal effects of high specific-activity tritiated thymidine incorporated into the DNA of proliferating cells to estimate the fractions of such cells in the DNA synthesis (S) phase of the cell cycle. Assuming that the proliferative activity of the population is directly related to the fraction in S, these authors found that under normal conditions in the intact adult mouse, most of the colony-forming units (CFU-S) detected by the spleen colony technique proliferate slowly, if at all; whereas, in regenerating hemopoietic tissue, most of the CFU-S proliferate actively. These results are compatible with the view that stem cells may exist in one or the other of two states of proliferation, a resting (G_0) state and a state of rapid proliferation, with the assortment of stem cells between these two states being governed by some unknown regulatory mechanism.

An assay is now available for another class of cells (CFU-C) detected by their capacity to form colonies under suitable conditions in cell culture (4–6). These cells appear to be progenitor cells of the granulocyte series and it is probable that they are early differentiated descendants of the pluripotent stem cell detected by the spleen colony technique (6–8). We have utilized a similar approach to that of Becker *et al.* (3) to determine whether or not the proliferation of CFU-C is regulated in a similar fashion to that of CFU-S. The results obtained indicate that CFU-C are proliferating even in normal adult hemopoietic tissue, but that the proportion of cells that are proliferating rapidly is greater in regenerating marrow than in the marrow of normal intact adult mice.

Materials and Methods. The mice used in these experiments were male F_1 hybrids between C3H/HeOci and C57BL/6JOci ($C3B6F_1$), and were approximately 8 weeks of age. Mice with regenerating marrow were prepared by intravenous injection of 10^7 or 2×10^7 nucleated $C3B6F_1$ marrow cells into isologous animals that had received 950 rads of ^{137}Cs radiation immediately prior to marrow transplantation. The femoral marrows of these animals were harvested 4 or 5 days later.

Marrow suspensions from either irradiated recipients (as stated) or from normal mice were prepared in ice-cold CMRL 1066 (9) without thymidine or coenzyme concentrate. These suspensions were exposed to tritiated thymidine as follows: 1-ml vol of the cell suspensions were placed into 50-ml centrifuge tubes containing a solution of ^{3}HTdR (methyl-*T*-thymidine, 21.9 Ci/mmole, Amersham-Searle) made up in CMRL 1066, minus thymidine and coenzymes, supplemented with 30 μg/ml of L-asparagine. After addition of the cells, the total volume in each tube was 2 ml. The tubes were then incubated with occasional agitation in a water bath at 37°. After 20 min of incubation, uptake of

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^{3}HTdR was stopped by the addition of 30 ml of ice-cold CMRL 1066 containing 100 μg/ml of unlabeled thymidine and 10% fetal calf serum. The tubes were centrifuged at 200*g* for 12 min, the supernatant was pipetted off, and the cells were resuspended in a further 30 ml of unlabeled CMRL 1066 containing thymidine and fetal calf serum. This washing procedure was repeated once, and then the cell concentration in the resulting suspension was determined on the basis of hemocytometer counts. A check on these counts was obtained using a Coulter counter.

Tests for colony-forming ability in cell culture were carried out using the methyl cellulose technique described in detail elsewhere (6). The cells were cultured in 35-mm plastic petri dishes in 1 ml of CMRL 1066 supplemented with 30 μg/ml of L-asparagine and containing 0.8% methyl cellulose, 10% mouse L-cell conditioned medium, 10% fetal calf serum and 2% deionized bovine serum albumin. Each cell suspension was tested at 2 different concentrations (usually 5×10^3 and 2.5×10^4 nucleated cells/culture dish) as a test of the linearity of the relationship between cell number and colony count. The dishes were cultured for 7 days at 37° in a moist atmosphere continuously flushed with 7.5% CO_2 in air.

Colonies were counted using an inverted microscope at 60× magnification. Although most colonies contained in excess of 1000 cells, any colonies containing more than 20 cells were included in the counts. 95% confidence limits for the colony counts were computed on the assumption that the counts were Poisson distributed.

Results. To test whether or not DNA synthesis could be detected in CFU-C derived from normal adult mice, marrow cell suspensions were exposed to ^{3}HTdR *in vitro* for 20 min, as described in materials and methods, and then tested for their content of cells able to form colonies in cell culture. The results of 4 experiments are shown in Table I. The results are expressed as percentages of surviving CFU-C, relative to the control suspension not exposed to ^{3}HTdR. The colony-forming efficiencies for the untreated controls, which were taken to be 100%, are included in Table I. As shown, at either of the two concentrations of ^{3}HTdR used, corresponding to 10 and 200 μCi/ml, approximately 65% of the CFU-C survived the 20-min exposure to ^{3}HTdR. The last two lines of Table I present the results of two experiments carried out on cells from actively regenerating bone marrow. In contrast to the results obtained for normal marrow, only about 20 to 25% of the CFU-C in regenerating bone marrow survived a 20-min exposure to ^{3}HTdR. These results show that detectable inactivation of CFU-C occurred as a result of the 20-min exposure to

TABLE I. Effect of ^{3}HTdR on the Colony-Forming Ability of Mouse Marrow Cells in Culture.[a]

			Percentage survival of colony-forming ability		
Exp. no.	Source of marrow cells	Colonies/10^5 cells control	10 μCi/ml	200 μCi/ml	10 μCi/ml + TdR[b]
1	Normal marrow	608 ± 39[c]	65 ± 5	69 ± 5	106 ± 7
2		231 ± 31	61 ± 8	59 ± 10	86 ± 12
3		302 ± 30	82 ± 11	75 ± 9	110 ± 11
4		458 ± 33	56 ± 6	52 ± 11	95 ± 8
	Mean percentage survival		66	64	99
5	Regenerating marrow	372 ± 40	27 ± 6	19 ± 5	95 ± 11
6		252 ± 33	25 ± 6	18 ± 5	82 ± 12
	Mean percentage survival		26	18	88

[a] Marrow cells were exposed to 21.9 Ci/mmole of ^{3}HTdR *in vitro* for 20 min.
[b] Unlabeled thymidine, 100 μg/ml, was added in addition to the ^{3}HTdR.
[c] 95% confidence limits.

^{3}HTdR, and that the proportion of CFU-C inactivated in regenerating marrow was greater than that in normal marrow.

In order to rule out possible toxicity due to effects other than those resulting from incorporation of ^{3}HTdR into DNA, a sample from each suspension was incubated in a medium containing, in addition to 10 μCi/ml of ^{3}HTdR, an excess of unlabeled thymidine (100 μg/ml). The results obtained are shown in the last column of Table I. The observed protective effect of unlabeled thymidine is evidence against nonspecific toxicity of ^{3}HTdR.

These experiments were designed to detect inactivation of CFU-C resulting from uptake of ^{3}HTdR specifically into CFU-C, with this uptake occurring only during the 20-min exposure *in vitro*. The possibility existed that significant amounts of ^{3}HTdR could be incorporated by other types of cells and thus be carried within these cells into the cultures used to detect colony formation by surviving CFU-C. It seemed possible that radioactivity carried over in this way might be released into the culture medium and inhibit the development of colonies. To rule out such an artifact, controls were added to expts 4 (intact mice) and 6 (regenerating mice) listed in Table I. In these experiments portions of the cells that had been exposed to ^{3}HTdR for 20 min in the usual way were set apart from the rest of the cells and subsequently irradiated with 900 rads of ^{137}Cs gamma rays in order to destroy colony-forming ability. The irradiated cells were then mixed with unirradiated cells to test for an effect on the colony-forming ability of CFU-C in the unirradiated cell suspension. The results of these controls are shown in Table II, and indicate that, if the irradiated cells carried over any ^{3}HTdR into the culture dishes, the amount released into the culture had little or no effect and was certainly insufficient to account for the degree of inactivation indicated in Table I.

Discussion. The proportion of CFU-C inactivated by pulse-labeling with high specific-activity ^{3}HTdR was greater in regenerating marrow (75–80%) than in normal marrow (35%). If it is accepted that any significant loss of colony-forming ability was a consequence of incorporation of ^{3}HTdR into DNA, then these results indicate that 35% of CFU-C were in the DNA synthesis stage (S phase) of the cell cycle in normal animals, and that this percentage increased markedly, to 75–80%, in regenerating marrow. This shift could result either from a shortening of the cycle time of CFU-C relative to the duration of the S phase, or from an increased proportion of the CFU-C being mobilized into active cell cycle with no concomitant change in cycle parameters.

On the basis of experiments in which large doses of ^{3}HTdR were administered *in vivo* and the cells removed 2 hr later, Lajtha *et al.* (10) also concluded that CFU-C turn over rapidly even under conditions where the hemopoietic system is in a steady-state, and there is no unusual demand for new blood cells. In this sense, the CFU-C appear to resemble the "erythropoietin-sensitive" precursors of the erythropoietic cells more close-

TABLE II. Test of Mixtures of Normal Marrow and Irradiated, ^{3}HTdR-Labeled Marrow.

Normal marrow cells (no./dish)	Pretreatment of irradiated cells[a]	Percentage survival	
		Exp. 4	Exp. 6
2.5×10^4	No pretreatment	100 ± 13[b]	100 ± 14
	10 μCi/ml of ^{3}HTdR for 20 min	99 ± 13	84 ± 12
	200 μCi/ml of ^{3}HTdR for 20 min	95 ± 8	89 ± 13
	10 μCi/ml of ^{3}HTdR for 20 min + 100 μg/ml of cold TdR	84 ± 8	87 ± 13
None	No pretreatment	0	0

[a] 2.5×10^4 nucleated marrow cells per dish.

[b] 95% confidence limits.

ly than they do the pluripotent stem cells. Erythropoietin-sensitive cells also appear to be proliferating under normal conditions (11, 10) while the pluripotent stem cells detected by their ability to form colonies in the spleen (CFU-S) proliferate slowly, if at all, under normal conditions (3). These findings are in agreement with the view that CFU-C are early differentiated cells of the granulocytic series (6–8), and that there exist classes of "committed progenitor" cells that exhibit properties not shared by the pluripotent stem cells (6). However, the results obtained above using regenerating marrow, and those obtained by Becker *et al.* (3) indicate that in the presence of an increased demand for new blood cells, both CFU-S and CFU-C are able to respond by increasing the proportion of cells proliferating rapidly. Each of these progenitors, therefore, is a potential site for regulation. It remains to be seen whether or not the same mechanism is operative at both sites.

Summary. Mouse granulopoietic progenitor cells can be detected by their capacity to form colonies in culture (CFU-C). The proliferative state of these cells was studied by determining the degree to which their colony-forming capacity was destroyed following exposure to a pulse of high specific-activity ^{3}HTdR. When marrow was obtained from normal adult mice 35% of CFU-C were inactivated by this procedure. In contrast, 80% of CFU-C were inactivated when cell populations were obtained from regenerating bone marrow. These results are interpreted to mean that CFU-C in normal mice are partitioned into two populations, one proliferating rapidly and the other slowly or not at all. In regenerating marrow the partition is changed, with all or almost all of the cells proliferating rapidly.

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