A Differential Effect of Hydroxyurea on Hemopoietic Stem Cell Colonies *in Vitro* and *in Vivo*[1] (34749)

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Murine hemopoietic tissue has been shown to be capable of forming colonies composed of granulocytic and macrophage cells in semisolid agar cultures in the presence of a colony-stimulating factor (1–4). Initially this agar system was considered to be analogous (1, 4) to the spleen colony technique, used for assessing the pluripotential stem cell compartment of donor bone marrow following marrow transplantation in heavily irradiated hosts (5). Spleen colonies have been shown to arise from a pluripotential transplantable cell which undergoes renewal and differentiation and may be secondarily transplanted (6). Although subcultures of *in vitro* colonies have been reported, the secondary colonies appear to be smaller and less well defined than the primary colonies (7). The majority of the pluripotential cells responsible for transplantation colonization have been shown to be in either G_0 or a prolonged G_1 (8) but the proliferative state of the cells responsible for *in vitro* colonization has not been established.

In order to evaluate the cell cycle status of the bone marrow *in vitro* colony forming cells (CFC) and to further evaluate the relationship between the *in vitro* (agar) colony system and the *in vivo* (spleen) colony technique, hydroxyurea (OHU), a cytotoxic agent lethal for cells in S (9, 10), but not affecting cells in other stages of cycle, was used to treat bone marrow donors prior to the use of their marrow in both systems.

After such treatment we have found approximately a 50% reduction in the number of *in vitro* colony forming cells (CFC) in the bone marrow, but only a 10–15% reduction in the number of bone marrow, pluripotential stem cells or CFU. It would appear, therefore, that these two systems do not measure the same stem cell compartment.

Materials and Methods. Twelve- to 14-week-old female CF_{1s} mice weighing 20–25 g were used. The method for agar culture of mouse bone marrow and the media used were that of Metcalf and Foster (11). The medium was supplemented with L-asparagine (final concentration 20 μg/ml) and DEAE-dextran (final concentration 75 μg/ml). Bone marrow cells were collected using sterile ice-cold TC199 when a transplant was to be performed concurrent with the *in vitro* studies. The colony-stimulating factor was provided by dialyzed conditioned medium prepared according to the method of Bradley and Sumner (2), and used at a dose of 0.1 ml/plate.

Bone marrow cell suspensions were prepared by expressing the tibial bone marrow with ice-cold TC199 or E1010. To do this a tuberculin syringe containing the appropriate medium and a 25-gauge needle was attached to the severed distal end of the tibia and the marrow cells were gently forced into a sterile centrifuge tube by forcing the medium through the tibial cavity. This was done only once per tibia to maintain sterility in the procedure. An even single cell suspension was then obtained by gentle pipetting with a plugged pipette. The bone marrow from at least six mice was collected and pooled 2 hr after they had been treated intravenously with OHU (900 mg/kg) dissolved in 0.25 ml of *N* saline. Marrows from control mice were collected after they had been injected with 0.25 ml of *N* saline. Nucleated cells were counted in these bone marrow cell suspensions.

Aliquots of cell suspensions from the con-

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trol and OHU-treated groups of mice were plated in E 2020-agar medium at doses ranging from 0.5 × 10^5 to 2 × 10^5 cells/plate. Colonies were scored on the eighth day of incubation by two observers. Aliquots from the same suspensions were transplanted into heavily irradiated syngeneic recipients at a dose of 5 × 10^4 cells/animal for spleen colony assay and spleen colonies were counted on day 8 following transplantation. Each suspension was assayed in 10 irradiated recipients. (Total dose 800 R, 62 R/min; 250 kV, 12 mA with 1.0 mm Cu and 1.0 mm Al filtration).

Results. The effect of hydroxyurea on bone marrow in vitro *colony forming cells (CFC).* The results of one typical *in vitro* experiment are given in Fig. 1. These results demonstrate that for any particular bone marrow cell dose plated there is approximately a 50% reduction in the number of colonies obtained from the OHU treated group compared to the control group.

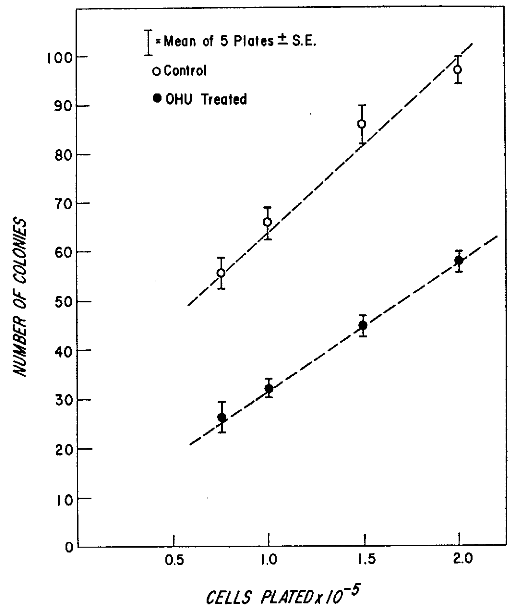

FIG. 1. The number of *in vitro* colonies derived from bone marrow cells of normal and hydroxyurea-treated mice.

TABLE I. The Effect of OHU on Bone Marrow *in Vitro* Colonies.

	Control group		OHU[a] group (900 mg/kg)	
Exp. no.	BM cells plated × 10^{-5}	Mean colony counts	BM cells plated × 10^{-5}	Mean colony counts
52	0.75	56 ± 3[b]	0.75	20 ± 3[b]
	1.0	66 ± 3	1.0	32 ± 2
	1.5	86 ± 4	1.5	45 ± 2
	2.0	97 ± 3	2.0	58 ± 3
64	0.5	29 ± 4	0.5	16 ± 3
	0.75	41 ± 5	0.75	14 ± 4
	1.0	65 ± 3	1.0	18 ± 7
	1.5	68 ± 4	1.5	48 ± 7
	2.0	100 ± 7	2.0	65 ± 13
66	0.5	27 ± 4	0.5	12 ± 4
	1.5	96 ± 5	1.5	70 ± 11
	2.0	120 ± 5	2.0	90 ± 7
71	0.5	8 ± 2	0.5	0
	0.75	18 ± 4	0.75	9 ± 2
	1.0	33 ± 6	1.0	6 ± 2
	1.5	54 ± 7	1.5	33 ± 5
	2.0	73 ± 9	2.0	51 ± 6
81	0.5	42 ± 8	0.5	32 ± 4
	1.0	54 ± 6	1.0	35 ± 6
	1.5	105 ± 8	1.5	76 ± 9

[a] Hydroxyurea.
[b] ± SE.

TABLE II. The Effect of OHU on Bone Marrow *in vitro* Colony Forming Cells (CFC).

	Control group		OHU[a] group (900 mg/kg)		
Exp. no.	Mean cell count/ tibia $\times 10^{-6}$	Mean CFC/ tibia	Mean cell count/ tibia $\times 10^{-6}$	Mean CFC/ tibia	p value
52	12.5	7745 ± 300[b]	10.9	3424 ± 158[b]	<.001
64	9.8	4831 ± 458	6.6	2101 ± 375	<.001
66	8.3	4592 ± 380	6.1	2351 ± 368	<.001
71	10.4	3024 ± 300	8.4	1340 ± 162	<.001
81	9.3	7920 ± 549	11.0	5663 ± 641	<.001

[a] Hydroxyurea.
[b] ± SE.

The composite results of five separate experiments in which bone marrow cells from OHU treated donors and saline treated controls were plated in E 2020-agar medium are given in Table I. As shown, on each occasion OHU reduced the number of colonies for any given cell dose level by ~50%. It should be noted that it is not justifiable to compare absolute numbers of colonies from one experiment to another because of minor variations in the medium or conditioned medium. Accordingly we only compared results between the experimental and control groups within an individual assay.

The total numbers of bone marrow *in vitro* colony forming cells per tibia in each experiment are presented in Table II. Again a consistent reduction by approximately 50% may be seen in the OHU treated group when compared to the control group. The mean CFC per tibia expressed in Table II are calculated from a consideration of the total nucleated cell count per tibia and the numbers of colonies obtained from each dose plated. Then by proportion a series of values for total CFC per tibia can be calculated for each cell dose plated and the mean CFC per tibia estimated from these results.

The effect of hydroxyurea on bone marrow colony forming units (CFU). The mean CFU per tibia of the OHU treated group as compared with values obtained for the control group are shown in Table III. Two hr after treatment with OHU there was a 15–20% reduction in bone marrow CFU. The numbers of CFU in control tibiae were slightly lower than those previously reported from this laboratory. This is thought to reflect less complete evacuation of the tibiae with the modification of our usual technic which was necessary to maintain absolute sterility. It was clear, however, that the magnitude of reduction of colony forming ability by OHU differed significantly between the *in vitro* and *in vivo* systems; there being a much greater reduction in the *in vitro* system.

Discussion. The experiments presented herein permit an estimate of the numbers of

TABLE III. The Effect of OHU on Bone Marrow Colony Forming Units (CFU).

	Control group		OHU[a] group (900 mg/kg)	
Exp. no.	Mean cell count/ tibia $\times 10^{-6}$	Mean CFU/ tibia	Mean cell count/ tibia $\times 10^{-6}$	Mean CFU/ tibia
52	12.5	2450 ± 250[b]	10.9	1918 ± 327[b]
66	8.3	1180 ± 215	6.1	855 ± 180
71	10.4	2360 ± 240	10.2	2040 ± 230
81	9.3	2620 ± 260	11.0	1800 ± 160

[a] Hydroxyurea.
[b] ± SE.

colony-forming cells in cycle in normal mice. Hydroxyurea is a rapidly metabolized agent with a selective lethal effect for cells in DNA synthesis (S) (9, 10). Thus the proportion of cells killed by this agent provides an estimate of the proportion of cells in S. In the case of the cells responsible for *in vitro* colonization ~50% of the cells were killed. If one assumes a DNA synthetic time of 6 hr then the generation time (T_G) would be 12 hr and one would conclude the entire population is in cycle. In contrast only 15–20% of the CFU were affected by OHU. This could reflect either a long T_G with the entire population in cycle, *e.g.*, a 6-hr S and a T_G of 30–36 hr, or a fraction of the population in cycle with the remainder in G_0. From previous studies in this and other laboratories (8, 12), one is led to the conclusion that the latter probably obtains. In any event it is clear that the kinetics of the cells responsible for *in vivo* and *in vitro* colonization differ. The most likely explanation appears to be that a pluripotential cell, the bulk of which are in G_0 (8), is responsible for spleen colonization. In contrast, *in vitro* colonization is accomplished by a cell already committed to myeloid differentiation and one which is normally in cycle. Utilizing the plating efficiency (13, 14) which may be estimated for CFU one can derive the total numbers of CFU in a given organ. Similar calculations, however, cannot be made for the CFC since the growth or plating efficiency is at present unknown. Hence one cannot derive a ratio of committed myeloid to pluripotential cells at the present time.

The morphologic identity of the committed as well as the pluripotential stem cell remains to be established. It seems most likely that it is a functional subset of a mononuclear cell, which might be classified as a lymphocyte under the light microscope. Robinson *et al.* (15) have speculated that it is indeed a lymphocyte that is responsible for colony formation in the soft agar system. Others (16, 17) have suggested that the *in vitro* CFC does in fact differ from the CFU. The present data would appear to be in line with these observations.

Summary. When bone marrow stem cells are assayed simultaneously by the spleen colony technique and the *in vitro* agar system following treatment of donors with hydroxyurea a differential effect was observed. There was found to be approximately a 50% reduction in the number of bone marrow *in vitro* colony forming cells after DNA synthesis inhibition by hydroxyurea and only a slight change in the pluripotential stem cell compartment by the spleen colony technique.

It appears therefore that the *in vitro* colony forming cell derives from a proliferating stem cell compartment, probably myeloid committed, and is distinct from the nonproliferating pluripotential transplantable stem cell.

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