In Vitro Granulocytic Colony-Forming Potential of Bone Marrow from Patients with Granulocytopenia and Aplastic Anemia[1] (35693)

J. E. KURNICK,[2] W. A. ROBINSON, AND CHRISTINE A. DICKEY
(Introduced by D. W. Talmage)

Division of Hematology, University of Colorado Medical Center, Denver, Colorado 80220

Several reports have indicated that bone marrow from normal humans and from patients with various disease states will form colonies in semisolid medium when appropriately stimulated (1–6). In these systems granulocytic colonies arise from single bone marrow cells, the identity of which has not as yet been determined. In our laboratory, normal human bone marrow yields a mean of 65 colonies/2 $\times$ 10^5 nucleated marrow cells plated (3, 4). In murine systems, the colony-forming cell appears to be a committed granulocyte precursor (7–10) but this has not been determined in humans as yet. The present studies were done to determine the colony-forming potential of bone marrow from patients with idiopathic and drug-induced aplastic anemias and granulocytopenias. It has been shown that granulocytic colonies result from the culture of marrows from these disease states. However, the number of colonies is less than produced by cultures of normal human bone marrow.

Methods and Materials. All of the patients studied were seen by the authors at the University of Colorado Medical Center between June, 1969 and August, 1970. The method of human bone marrow culture utilized here has previously been described in detail (3, 4). Ten ml of liquid marrow from a sternal or iliac crest aspirate is obtained in a heparinized syringe and transferred to a sterile tissue culture tube for gravity sedimentation. The marrow-rich plasma is removed with a Pasteur pipette; and the cells are washed three times with McCoy's 5A medium to remove the plasma. After counting in a hemocytometer, 2 $\times$ 10^5 nucleated cells in 1 ml of McCoy's 5A medium, supplemented by 15% fetal calf serum, in 0.3% agar are pipetted onto 1-ml feeder layers containing 1 $\times$ 10^6 normal human WBC in McCoy's 5A medium and 0.5% agar in 35-mm plastic petri dishes. The cultures are allowed to gel at room temperature and are then incubated at 37° in a humidified incubator with a constant flow of 7.5% CO_2 in air. Colonies are visible on plates at days 14–20 of incubation and are counted at that time with a dissecting microscope. For microscopic studies, colonies are removed from the agar with a finely drawn Pasteur pipette and stained with aceto-orcein as previously described (3). Differential cell counts reported were performed on the final cell suspension in McCoy's 5A medium, rather than on smears of the initial marrow aspirate, to obtain counts representative of the material actually plated in the cultures.

Results. Table I shows the number of colonies formed from 2 $\times$ 10^5 nucleated marrow cells from four normal humans, five cases of aplastic anemia, and five cases of isolated granulocytopenia. Marrows from patients with aplastic anemia and granulocytopenia yielded averages of 15 and 26 colonies/2 $\times$ 10^5 cells plated, respectively, compared to an

[1] Supported by grants from the National Institutes of Health, National Cancer Institute (1R01CA11305-2 and CA05058-10) and by the American Cancer Society Grant No. E498.

[2] Present address: Fitzsimons General Hospital, Denver, Colorado 80240.

TABLE I. *In Vitro* Colony-Forming Potential of Human Bone Marrow.

Patient	Diagnosis	Granulocyte colonies/2 × 10^5 bone marrow cells
C. R.	Idiopathic aplastic anemia	25 ± 4.6[a]
W. L.	Idiopathic aplastic anemia	22 ± 1.8
E. R.	Idiopathic aplastic anemia	9 ± 2.1
R. E.	Idiopathic aplastic anemia	8 ± 1.8
V. H.	Chloramphenicol-induced aplastic anemia	11 ± 3.4
Mean		15.0
B. R.	Idiopathic granulocytopenia	19 ± 3.0
E. S.	Idiopathic granulocytopenia	26 ± 3.4
N. P.	Drug-induced granulocytopenia	37 ± 7.5
G. P.	Drug-induced granulocytopenia	31 ± 6.8
B. S.	Drug-induced granulocytopenia	18 ± 3.0
Mean		26.2
A. B.	Normal	68 ± 6.7
E. M.	Normal	43 ± 3.9
A. H.	Normal	58 ±11.7
G. S.	Normal	32 ± 5.6
Mean		50.3

[a] Mean colony count of 5 plates ± standard deviation.

average of 50 colonies from the four normal human bone marrows. The size of colonies produced was not significantly different in any of these three groups, averaging 500–1500 cells at day 20 of incubation.

Morphologic studies were done on colonies at various times of incubation. The progression of cell types was similar in all colonies and was the same as that noted previously with normal human marrow (3). Initially, colonies were composed of large cells with a single nucleus, thought to be myelocytes, and, by day 20 of culture, were composed almost exclusively of cells with the morphologic appearance of mature granulocytes.

Addition of serum or plasma from two patients with aplastic anemia to cultures of normal human bone marrow did not significantly alter the number of colonies produced by the latter, Table II.

Table III shows representative differential cell counts of bone marrow from each of the three groups of patients studied. The predominant cells in the normal human marrow suspensions were mature granulocytes, band forms, and metamyelocytes. This is in contrast to the marrow suspensions of the two groups of patients studied in which the predominant cells were small lymphocytes and erythroblasts. The number of myeloblasts

TABLE II. Effect of Serum and Plasma from Aplastic Patients W. L. and E. R. upon *in Vitro* Colony Formation by Normal Human Marrows.

	No. of colonies/2 × 10^5 bone marrow cells	
	A. H.	G. S.
Marrow alone	58 ± 11.7[a]	32 ± 5.6
+ 0.1 ml of W. L. serum	53 ± 3.7	23 ± 5.2
plasma	48 ± 5.0	25 ± 4.2
+ 0.1 ml of E. R. serum	—	28 ± 4.3
plasma	—	36 ± 3.4

[a] Mean colony count of 5 plates with SD.

TABLE III. 200-Cell Differential Counts on Final Cell Suspensions of 3 Representative Marrows.

	E. M. (normal) (%)	R. E. (aplastic) (%)	N. P. (granulocytopenic) (%)
Small lymphocytes	12.0	30.0	19.5
Large lymphocytes	12.5	4.5	8.5
Myeloblasts	2.0	1.5	5.5
Promyelocytes	4.0	3.5	4.5
Myelocytes	6.5	5.5	2.0
Metamyelocytes, bands, and mature granulocytes	51.5	23.0	1.0
Erythroblasts (all stages)	2.5	21.0	45.5
Other[a]	9.0	11.0	13.5

[a] Includes plasma cells, monocytes, megakaryocytes, reticulum cells, and eosinophils and basophils and their precursors.

and promyelocytes present was similar in all groups.

Discussion. These studies have demonstrated that bone marrows from patients with aplastic anemia and granulocytopenia contain cells capable of giving rise to colonies in semisolid medium. The number of colonies produced, however, is less than that obtained from normal human bone marrow. This effect is apparently not the result of inhibitory factors present in the serum or plasma of these patients, since the marrow cells were washed free of contaminating plasma prior to plating, and their plasma had no effect on colony formation by normal human bone marrow cells. Since most of these patients had hypoplastic marrows, these studies indicate that not only are the relative numbers of colony-forming cells reduced, but the absolute numbers as well.

There is good evidence to suggest that the *in vitro* colony-forming cell is not the pleuripotential stem cell, but is already committed to granulocytic differentiation (7–10). The present studies might suggest that there is decreased feed-in of these cells from a damaged stem cell compartment. Cells morphologically identifiable as early granulocyte precursors should represent descendents of these granulopoietic stem cells. However, the differential counts (Table III) indicate that the relative number of identifiable early granulocyte precursors in the marrows of the aplastic and neutropenic patients is similar to that present in normal human bone marrows. This evidence of normal *in vivo* maturation to a stage presumably more advanced than the granulopoietic colony-forming cell, but with subsequent failure to produce normal numbers of mature granulocytes, either *in vivo* or *in vitro*, suggests that the damage in these disease states may extend into maturing cell lines.

One must be cautious in the interpretation of the data, however, in view of the variable admixture of peripheral blood elements in the plated specimens. There is no good way to assess the relative contributions of bone marrow and peripheral blood cells to the final suspensions plated, since the original marrow sedimented is liberally intermixed with peripheral blood. Because most of the patients in this study had hypoplastic marrows, the relatively decreased numbers of colony-forming cells might simply be a reflection of a greater admixture of mature peripheral blood cells.

Since the colony-forming cell in normal marrows is present in a proportion of less than one per thousand nucleated cells, one would not expect differential counts to yield significant clues as to the identity of the colony-forming cell, as indeed they do not in this study (Table III). Thus the significance of our findings will become clear only upon identification of the *in vitro* colony-forming cell. It is of interest that Paran and colleagues (6) have recently reported that marrows from two patients with congenital neutropenia retained the ability to form normal

numbers of colonies in a similar system. Studies are now under way to determine the change in colony-forming potential of marrows from patients with granulocytopenias induced by chemotherapeutic agents, and during the changing leucodynamic status brought about by recovery from these drug-induced granulocytopenias.

Summary. The colony-forming potential in agar-gel of bone marrows from patients with aplastic anemia and granulocytopenia has been studied. It has been shown that bone marrow cells from these patients retain the ability to produce colonies *in vitro*, but that the number of colonies formed is reduced compared to normal human bone marrow. No inhibitory effect of serum or plasma from patients with aplastic or granulocytopenic states was found upon the colony growth of normal human bone marrow.

1. Senn, J. S., McCulloch, E. A., and Till, J. E., Lancet **2**, 597 (1967).

2. Harris, J., and Freireich, E. J., Blood **35**, 61 (1970).

3. Pike, B. L., and Robinson, W. A., J. Cell. Physiol. **76**, 77 (1970).

4. Robinson, W. A., and Pike, B. L., *in* "Hematopoietic Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 249. Grune & Stratton, New York (1970).

5. Senn, J. S., and McCulloch, E. A., Blood **35**, 56 (1970).

6. Paran, M., Sachs, L., Barak, Y., and Resnitzky, P., Proc. Nat. Acad. Sci. U.S.A. **67**, 1542 (1970).

7. Bennett, M., Cudkowicz, G., Foster, R. S., Jr., and Metcalf, D., J. Cell. Physiol. **71**, 211 (1968).

8. Rickard, K. A., Shadduck, R. K., Howard, D. E., and Stohlman, F., Jr., Proc. Soc. Exp. Biol. Med. **134**, 152 (1970).

9. Rickard, K. A., Shadduck, R. K., Morley, A., and Stohlman, F., Jr., *in* "Hematopoietic Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 238. Grune & Stratton, New York (1970).

10. Chen, M. G., and Schooley, J. C., J. Cell. Physol. **75**, 89 (1970).

Received Feb. 18, 1971. P.S.E.B.M., 1971, Vol. 137.