The Effect of Serum from Patients with Acute Granulocytic Leukemia on Granulocyte Colony Formation *in Vitro*: A Search for Inhibitors[1] (36811)

AROOP MANGALIK[2] AND WILLIAM A. ROBINSON
(Introduced by D. W. Talmage)
Section of Oncology, Division of Hematology, University of Colorado Medical Center, Denver, Colorado 80220

Previous studies have shown that bone marrow and peripheral blood cells from normal humans and patients with acute granulocytic leukemia (AGL) can be stimulated to form granulocytic colonies *in vitro* in the presence of an appropriate stimulus, possibly a granulopoietin (1–6). With few exceptions, however, the colonies formed from the peripheral blood and bone marrow of patients with AGL have been considerably smaller in size than those grown from normal human hematopoietic tissues. It has been shown, however, that such colonies go through morphologic maturation to apparently mature granulocytes in a manner similar to normal human marrow cells (1). The reason why the colonies formed from the hematopoietic cells of these patients are smaller in size and their apparent inability to mature *in vivo* has been unclear. The possibilities that have been suggested are: (a) decreased production of granulopoietic substances in AGL (4), (b) a basic defect in granulocyte precursor cells making them insensitive to the biologic effects of granulopoietic substances, or (c) the presence of inhibitory substances preventing granulopoietic factors from acting on these cells. The first of these possibilities, the lack of granulopoietic substances, does not seem to be operative. While it has been shown that in the early untreated phase of AGL the levels of granulopoietic factors in serum and urine may be low, with treatment, even before remission is obtained, high levels of these substances can be found (4). The fact that many of these cells can be stimulated to divide and form colonies *in vitro* has suggested that they retain the ability to respond in a relatively normal but quantitatively less responsive fashion. The present studies were undertaken to determine whether the third of the possibilities mentioned above might be operative, namely the possibility of inhibitory factors in the serum or blood of these patients. Serum and plasma from patients with acute leukemia before commencing therapy have been investigated for their ability to inhibit granulocyte colony formation by normal human bone marrow cells or leukemic blast cells *in vitro* when stimulated by feeder layers of normal human white blood cells (WBC). In this culture system no inhibitory effect of serum or plasma from these patients was found when compared with serum or plasma from normal humans.

Materials and Methods. The method of tissue culture for colony growth utilized here has been described in detail elsewhere (6). It consists of two cell layers in semisolid agar; the top layer was the bone marrow target cell layer and the bottom layer was a "feeder layer" of normal human peripheral WBC.

Feeder layer preparation. Peripheral blood was collected from normal volunteers in heparinized tubes and allowed to sediment by gravity at room temperature for 1–2 hr. The plasma containing the WBC was then removed and mixed with a 9:1 concentration of McCoy's 5A medium (with 15% fetal calf serum) and 5% agar in a concentration of 1 $\times$ 10^6 cells/ml. One milliliter aliquots were then plated in 35 mm plastic petri dishes and

[1] Supported by grants from the National Institutes of Health, National Cancer Institute (1RO1 CA11305-3 and CA05058-10) and by the American Cancer Society Grant No. ET-4B.

[2] Present address: Division of Hematology, All India Institute of Medical Sciences, New Delhi 16, India.

incubated at 37° prior to use.

Preparation of bone marrow specimens. Aspirate bone marrow specimens collected from the posterior iliac crest of humans without histologic evidence of hematopoietic disorders were used in these experiments. As with the peripheral blood cells these were collected in heparin, allowed to sediment by gravity at room temperature, and the buffy coat-plasma layer separated and centrifuged to remove the plasma. The bone marrow cells were then resuspended in the bone marrow donors' plasma or in the serum or plasma of normal and leukemic subjects in a concentration of 2 $\times$ 10^5 cells/0.1 ml. The bone marrow cells suspended in plasma or serum were then mixed with a 9:1 mixture of McCoy's 5A medium (with 15% fetal calf serum) and 3% agar to a final cell concentration of 200,000 cells/ml of medium. One milliliter aliquots of this were then plated onto the previously prepared feeder layers. Control plates consisted of bone marrow cells plated over blank medium-agar underlays without feeder layer peripheral WBC.

After preparation the plates were incubated at 37° in a fully humidified incubator with a constant flow of 7.5% CO_2 in air. Colony counts were done at days 15–18 of incubation. Only colonies containing 50 or more cells were scored with a dissecting microscope.

For microscopic studies colonies were removed from the agar with a finely drawn Pasteur pipette and stained with aceto-orcein or Giemsa as previously described.

Collection of plasma and serum. Both plasma and serum were studied to rule out the possibility of toxic factors due to anticoagulants or loss during clotting. Plasma was collected from untreated human patients with acute leukemia in vacuum tubes containing 4 drops of 1:1000 heparin (Liquaemin, Sodium "10", Organon Inc., West Orange, NJ) and from normal human volunteers. Clotted blood was also collected for serum. Serum and plasma samples were stored at —20° and in these studies had been stored for 1 day to 4 mo prior to use. All samples were used without manipulation such as dialysis or heating prior to use.

Results. The results of these studies are summarized in Fig. 1. They demonstrate that the addition of serum or plasma from patients with AGL to cultures of normal human bone marrow does not affect the number of colonies formed when compared with samples in which normal human serum or plasma had been incorporated. In both groups of patients there was slight enhancement of colony formation when compared with control plates containing only WBC feeder layers. The size of colonies formed after incorporation of leukemic or normal serum or plasma was not different from that of control plates. Likewise the morphology of colonies was similar in all groups. At 15 days of incubation all colonies appeared to be formed largely of mature granulocytic elements. In particular there was no evidence of decreased maturation or leukemic alterations in colonies from plates to which leukemic sera had been added.

Similar studies have been conducted in which the serum or plasma was added to the WBC feeder underlays in concentrations of 0.05 to 0.15 ml using normal human bone marrow as a target overlay. The results of these studies are similar to those shown above and have indicated no inhibitory or significant stimulatory effect of serum or plasma from leukemic patients when compared with that from normal humans.

Other studies have been done using peripheral blood blast cells from patients with AGL as the target cell layer grown over feeder layers of normal human peripheral WBC. Once again, the results have been similar and have shown no inhibitory or stimulatory effect of leukemic serum or plasma on colony formation when compared with serum or plasma from normal humans.

Discussion. The reason or reasons why colonies grown from the peripheral blood and bone marrow of patients with AGL are significantly smaller in size than those grown from hematopoietic tissue of normal humans remain(s) unknown. The results of the present studies indicate that this is not the result of substances present in the serum or plasma of these patients which prevent the action of granulopoietic factors on colony forming cells *in vitro*. This observation is in line with the findings of Metcalf *et al.*, (7)

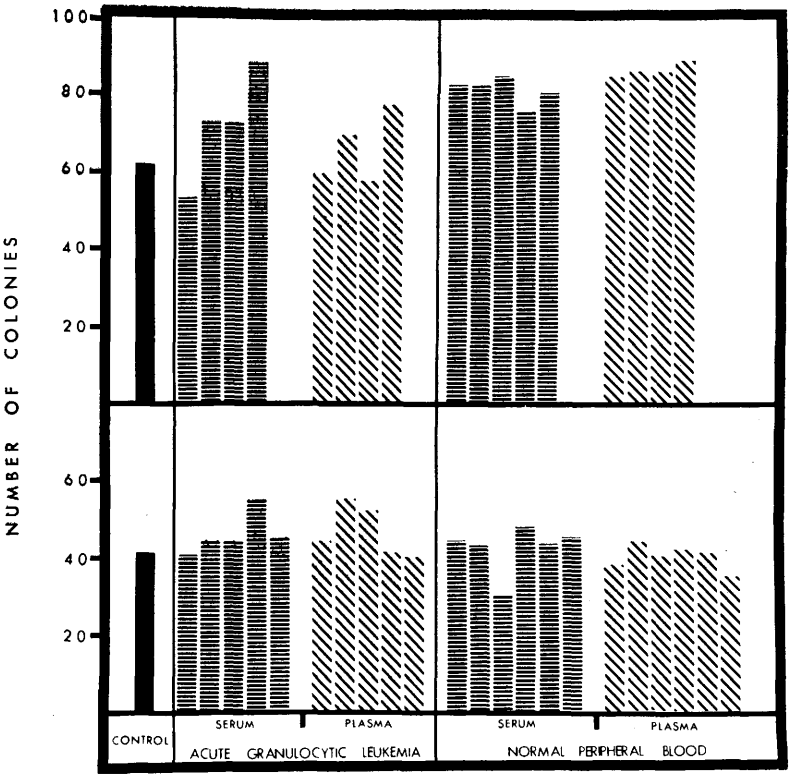

FIG. 1. The effect of serum and plasma from normal humans and patients with acute granulocytic leukemia (AGL) on colony formation by normal human bone marrow cells. The results of two separate experiments are shown. In both, the number of colonies obtained from washed marrow cells resuspended in autologous plasma (control, solid bar) are compared with marrow resuspended in serum (hatched bar) and plasma (slashed bar) from patients with untreated AGL or normal humans. Each bar represents the mean number of colonies grown from 200,000 bone marrow cells on 4 plates.

who noted that levels of inhibitory substances in the serum and plasma of patients with AGL tended to be low during the relapse phase of their disease and normal during remission.

It would appear from the present studies and from those carried out previously that the reason for poor colony formation is the result of an intrinsic defect in the cells of these patients which renders them less responsive to granulopoietic substances. This suggestion must be, however, modified by the possibility that leukemic cells in AGL represent a different population from colony forming cells in normal human hematopoietic tissues. On the basis of cellular separation studies by Moore, Williams and Metcalf (8) this would seem to be the case. It may be that these cells are actually further along in granulocyte differentiation and that it is unreasonable and unwarranted to expect that the colonies arising from them should reach the same numerical proportions as those derived from normal human cells. Numerous attempts in our laboratory to "drive" leukemic cells to bigger and better colony formation by increasing the amount of stimulus present or decreasing the number of target cells have been unfruitful. Incorporation of very large numbers of WBC, up to 16×10^6, in the feeder layers from the usual 1×10^6 used has not, in our hands, increased the number or size of colonies formed. It should also be noted that there is a wide variability in the morphology and kinds of cells found in the peripheral blood of patients with AGL in relapse. While some patients have almost

exclusively blast-like cells, many retain some normal appearing cells and all stages of differentiation between blast and mature granulocytes.

The possibility that leukemic cells contain an altered sensitivity to granulopoietic substances is currently under investigation in this laboratory with the use of cell surface acting agents namely trypsin and concanavalin A (unpublished data). To date there is no evidence that the use of these agents to alter cell surface responsiveness will enhance colony formation by these cells.

The present studies are important and of practical interest to those working in this area. Our initial studies on the maturation and differentiation of target leukemic cells were carried out on cells which had been washed free of plasma prior to use. This was considered necessary because of the possibility of inhibitory substances present in the blood of these patients. This will remain a necessary step in those patients receiving chemotherapeutic or other agents interfering with cellular division. It is probably not necessary for routine studies as indicated here. The amount of serum and plasma used in the present studies is far in excesss of that usually present in unwashed cellular specimens.

The unraveling of the leukemic process at a physiologic level would appear to be well on its way in human forms of this disease. The present studies have added an important and essential piece of evidence in this area.

Summary. The incorporation of serum and plasma from patients with AGL into an *in vitro* culture system failed to influence colony formation by cells of normal bone marrow as well as those from patients with AGL. The significance of these changes and the further approach to the problem have been discussed.

1. Robinson, W., Kurnick, J., and Pike, B. L., Blood **38**, 500 (1971).

2. Greenberg, P., Nichols, W. C., and Schrier, S. L., N. Engl. J. Med. **284**, 1225 (1971).

3. Iscove, N. N., Senn, J. S., Till, J. E., and McCulloch, E. A., Blood **37**, 1 (1971).

4. Robinson, W. A., and Pike, B. L., N. Engl. J. Med. **282**, 1291 (1970).

5. Moore, M. A. S., Metcalf, D., and Williams, N., *in* "*In Vitro*" Culture of Hemopoietic Cells" (D. W. Van Bekkum and K. A. Dicke, eds.), p. 334. Radiobiological Institute TNO, Rijswijk, The Netherlands (1972).

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

7. Metcalf, D., Chan, S. H., Gunz, F. W., Vincent, P., and Ravich, R. B. M., Blood **38**, 143 (1970).

8. Moore, M. A. S., Williams, N., and Metcalf, D., *in* "The Nature of Leukaemia" (P. C. Vincent, ed.), p. 135. Australian Cancer Soc., Sydney (1972).

Received June 26, 1972. P.S.E.B.M., 1972, Vol. 141.