FEEDBACK INHIBITION OF THE DEVELOPMENT OF MACROPHAGE AND GRANULOCYTE COLONIES, II. INHIBITION BY GRANULOCYTES

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Abstract and Summary.—It has been shown that mature normal rat granulocytes produce a substance that inhibits the activity of the inducer required for the development of macrophages (M) and granulocyte (G) colonies from normal hematopoietic cells seeded in soft agar. The granulocyte inhibitor inhibited the activity of the inducer when tested with normal hematopoietic cells from embryonic or adult organs. The inhibitor was not dialyzable, and was obtained in an active form in granulocyte-conditioned medium. The results indicate that the control mechanism that regulates the growth and development of normal macrophages and granulocytes includes a feedback inhibition of the activity of the inducer by inhibitors produced by mature granulocytes and macrophages, presumably at the end of their differentiation process. The inhibition of both M and G colonies by the feedback inhibitors produced by macrophages and granulocytes suggests that both types of colonies may be derived from a common stem cell. A line of primitive myeloid leukemia did not inhibit the activity of the inducer, and the cells of this line were not inhibited by the feedback inhibitors produced by normal granulocytes and macrophages.

The development of an *in vitro* experimental system for the analysis of the control mechanism that regulates the growth and differentiation of single normal hematopoietic cells has previously been described.[1-4] The results of these studies have indicated that a substance produced by various types of cells can induce for single hematopoietic cells the formation of colonies of macrophages and granulocytes; that the G clones contain cells in various stages of differentiation, and this also presumably applies to the M clones; and that mature macrophages produce a dialyzable feedback inhibitor that inhibits the activity of the inducer. The feedback inhibitor produced by macrophages inhibited M colonies more effectively than G colonies and could not be detected in an active form in conditioned medium from mature macrophages.[3]

The present experiments were undertaken to determine (1) whether mature granulocytes produce a feedback inhibitor for the development of M and G colonies; (2) whether this inhibitor is dialyzable and can be obtained in an active form in conditioned medium from granulocytes; (3) whether the inhibitor inhibits M colonies more effectively than G colonies; and (4) whether cells from a myeloid leukemia can inhibit the activity of the inducer and are susceptible to the inhibitor produced by mature normal cells.

Materials and Methods.—Animals: All experiments were carried out with cells from the ICR strain of Swiss mice and Wistar rats.

Cloning of normal hematopoietic cells: The cells used for cloning were taken from embryo liver at about the 17th to 19th day of gestation and from the spleen and bone marrow of

2-month-old animals. The cells were cloned in soft agar (0.33%) on a harder agar base (0.5%) in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 20% inactivated (56°C for 30 min) horse serum. The cell suspensions were made as described.[1-4] Colonies were counted microscopically with an inverted microscope containing a ×5 objective and ×10 eyepiece, generally at 7 days after seeding; and the colonies were classified as macrophages (M), granulocytes (G), blast cells (B), or mixed M and G.[2] Each calculation of the number of cells per colony was based on a count of 30 pooled colonies.

Conditioned medium containing the inducer: This was prepared from mass cultures of spleen cells from 2-month-old animals that were seeded at 5×10^7 cells per 50-mm Petri dish (Falcon Co.) in EM with 10% inactivated horse serum. The medium was harvested 4 days after seeding, centrifuged at 650 *g* for 10–15 min, and stored at −20°C. Unless otherwise stated, the spleen-conditioned medium was used at a concentration of 25%. The conditioned medium was always added to the lower agar layer in the assay for colony formation.

Mature macrophages, granulocytes, and erythrocytes: Peritoneal macrophages were obtained, as in previous experiments,[2, 3] from 2-month-old male mice by intraperitoneal inoculation of 5 ml phosphate-buffered saline (PBS), 4 days after inoculation of 3 ml thioglycolate medium (Difco Labs.).[5] Peritoneal granulocytes were obtained from 2-month-old male rats by intraperitoneal inoculation of 10–20 ml PBS containing 10 units of heparin per ml 4 hr after inoculation of 10 ml of a solution of 2.5% oyster glycogen[6] (Nutritional Biochemicals Corp.) in PBS. The macrophages were used as feeder layers 2 days after seeding 2×10^6 cells per 50-mm Petri dish. To prepare the granulocytes, peritoneal cells were seeded at 5×10^7 cells per 50-mm Petri dish; and after 24 hr the cells that had not attached to the Petri dish, which consisted of more than 95% mature granulocytes, were collected and washed six times with PBS to remove any inducer in the cell suspension obtained from the peritoneal cavity. Feeder layers of granulocytes were prepared by seeding a mixture consisting of 1 ml of a suspension containing (unless otherwise stated) 2×10^7 granulocytes in EM with 20% inactivated horse serum and 0.5 ml of 1% agar medium. Erythrocytes were obtained from 2-month-old male rats. Blood was collected from the heart with a heparinized syringe and centrifuged for 10 min at 650 *g*, the plasma was decanted, and the erythrocytes were suspended in saline. Feeder layers of erythrocytes were prepared as with granulocytes. Although populations containing more than 95% mature macrophages were readily obtained from the peritoneal cells of mice, and populations containing more than 95% mature granulocytes were readily obtained from the peritoneal cells of rats, the methods used did not yield sufficient quantities of homogeneous populations of rat macrophages or mouse granulocytes. The cells used in the present studies were therefore either from mice or from rats, depending on the cell type required in the experiment.

Dialysis membranes were prepared as described,[3] and the membranes on feeder layers were placed on top of a thin layer (1.5 ml) of 0.5% agar medium that had been poured on the feeder layer. Conditioned medium from macrophages was harvested 4 days after the seeding of 2×10^6 cells per 50-mm plate, with a medium change after 2 days, and from granulocytes at 3 days after the seeding of 2×10^7 granulocytes per 50-mm plate.

Myeloid leukemia cells: The myeloid leukemic cells were obtained from a line of primitive myeloid leukemia (P 1081) produced in a DBA mouse after X irradiation with three doses of 100 r by Dr. M. Potter, National Cancer Institute, and cells of this line were kindly supplied by Dr. J. H. Burchanal, Sloan-Kettering Institute of Cancer Research, New York. The cells were grown as mass cultures in EM with 10% inactivated horse serum and cloned in EM with 20% inactivated horse serum in soft agar by the same procedure as that used for the cloning of normal hematopoietic cells. Colonies were counted at 10 days after seeding. Feeder layers of P 1081 cells, irradiated with 2000 r, were prepared as with granulocytes, by the seeding of 2 or 4×10^6 cells per plate.

Results.—The development of colonies from rat hematopoietic cells: Since the normal hematopoietic cells used in the present studies were from rats and from

mice, a comparison was made of the colonies obtained with cells from the two species. The results indicate that the time of colony development and the types of cells in the colonies obtained from rat cells (Fig. 1) were similar to those previously obtained from mouse cells.[2] With both species there were colonies of macrophages, granulocytes, mixed M and G, and blast cells. The number of G, mixed M and G, and B colonies decreased from 5 to 15 days after seeding, whereas the number of M colonies increased during the same period. The average cell number of the G colonies in Figure 1, at five, seven, and ten days, were 770, 1520, and 5840 cells, respectively.

Inhibition of colony formation by granulocyte feeder layers: Results obtained with feeder layers of mature granulcoytes have indicated that these cells can inhibit the development of both M and G colonies. The same results were obtained with hematopoietic cells from embryonic and adult organs (Table 1). The seeding of different numbers of granulocytes as feeder layers has shown that there was an increase in the percentage of G colonies with a decrease in the total number of colonies (Fig. 2), indicating that M colonies were more effectively inhibited than G colonies. This more effective inhibition of M colonies was also seen in the number of cells per colony (Fig. 3). Granulocyte feeder layers exposed to three cycles of freezing at −90°C and thawing at 37°C did not inhibit the formation of colonies. In order to determine whether the inhibition by granulocytes may be due to a nutritional deficiency induced by destruction of an essential constituent of the medium,[7] new medium was added daily to four cylinders per Petri dish that had been placed in the agar above the granulocyte feeder layer. This daily addition of 3.2 ml new medium per plate containing 7.5 ml medium did not change the inhibitory effect of the granulocyte feeder layer.

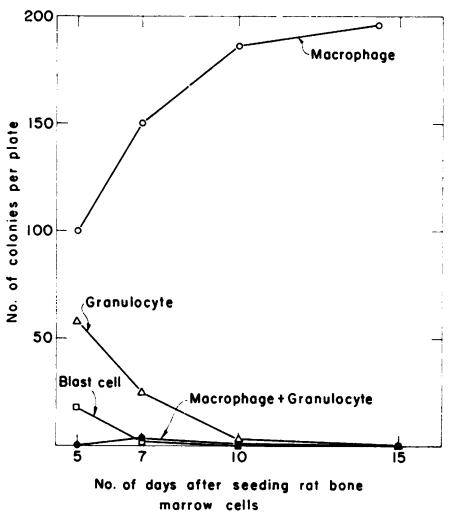

FIG. 1.—Number of macrophage, granulocyte, mixed macrophage and granulocyte, and blast cell colonies at different times after seeding 6 × 10^4 rat bone marrow cells per plate with rat spleen-conditioned medium.

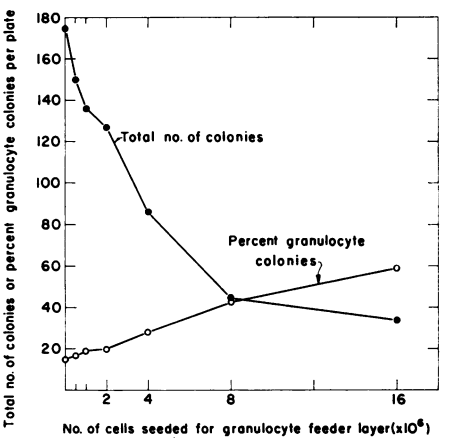

FIG. 2.—Inhibition of the number of colonies by feeder layers with different numbers of mature granulocytes. Rat bone marrow cells were seeded for cloning with rat spleen-conditioned medium at 6 × 10^4 cells per plate.

TABLE 1. *Granulocyte inhibition of colony formation by cells from embryonic liver, adult spleen, and adult bone marrow.*

Granulocyte feeder layer	Cells seeded for cloning	Number of Colonies per Plate M	G	B	Mixed M and G
−	Embryonic liver	98	17	13	5
+	Embryonic liver	0	0	0	0
−	Adult spleen	86	10	10	1
+	Adult spleen	0	0	0	0
−	Adult bone marrow	104	27	3	0
+	Adult bone marrow	0	0	0	0

Rat embryo liver and adult bone marrow cells were seeded for cloning with rat spleen-conditioned medium at 6×10^4 cells per plate and rat adult spleen cells at 6×10^5 cells per plate.

Spleen-conditioned medium from rats or mice can induce the formation of colonies by rat and mouse bone marrow cells (Table 2). Experiments on the species specificity of the inhibition by granulocytes and macrophages have indicated that rat granulocytes and mouse macrophages tested with the inducer from rat or mouse cells, respectively, inhibited the formation of colonies of bone marrow cells from both species (Table 2). The results obtained with a dialysis membrane on the granulocyte feeder layer have indicated that, in contrast to the dialyzable nature of the macrophage inhibitor,[3] the inhibitor produced by granulocytes did not pass through a dialysis membrane (Table 3). Inhibition of M and G colonies was obtained when a dialysis membrane was placed on feeder layers containing both macrophages and granulocytes. This indicates that the presence of granulocytes did not seem to affect the dialyzable nature of the macrophage inhibitor. Feeder layers containing from 1 to 8×10^6 erythrocytes per plate prepared in the same way as the granulocyte feeder layers did not have an inhibitory effect on colony formation by bone marrow cells.

Inhibition of colony formation by granulocyte-conditioned medium: The results of incubating mixtures of granulocyte-conditioned medium with spleen-conditioned medium containing the inducer have indicated that the inhibitory substance

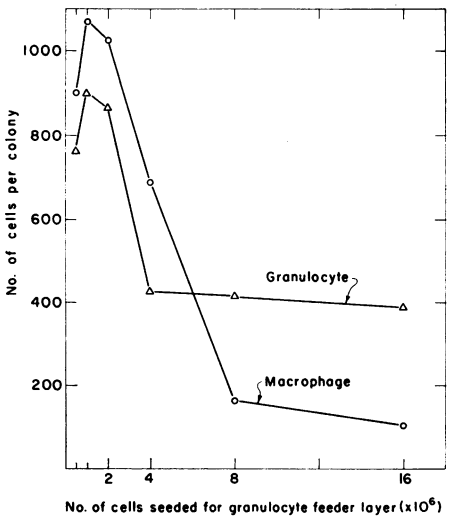

FIG. 3.—Inhibition of the number of cells per colony by feeder layers with different numbers of mature granulocytes. Rat bone marrow cells were seeded with rat spleen-conditioned medium at 6×10^4 cells per plate.

TABLE 2. *Inhibition by rat granulocytes and mouse macrophages of colony formation by rat and mouse bone marrow cells.*

Feeder layer	Source of spleen-conditioned medium	Source of bone marrow cells	Number of Colonies per Plate			
			M	G	B	Mixed M and G
—	Rat	Mouse	42	38	0	3
Rat granulocytes	Rat	Mouse	0	0	0	0
—	Rat	Rat	127	30	2	1
Rat granulocytes	Rat	Rat	3	12	2	0
—	Mouse	Mouse	54	46	3	8
Mouse macrophages	Mouse	Mouse	0	0	0	0
—	Mouse	Rat	83	8	2	0
Mouse macrophages	Mouse	Rat	0	0	0	0

Rat and mouse bone marrow cells were seeded for cloning at 6×10^4 cells per plate.

TABLE 3. *The nondialyzable nature of the inhibitor produced by granulocyte feeder layers.*

Feeder layer	Dialysis membrane	Source of spleen-conditioned medium	Source of bone marrow cells	Number of Colonies per Plate			
				M	G	B	Mixed M and G
—	−	Rat	Rat	127	30	2	1
Rat granulocytes	−	Rat	Rat	3	12	2	0
Rat granulocytes	+	Rat	Rat	110	25	3	1
—	−	Mouse	Mouse	86	9	1	3
Mouse macrophages	−	Mouse	Mouse	0	0	0	0
Mouse macrophages	+	Mouse	Mouse	0	0	0	0

Rat and mouse bone marrow cells were seeded for cloning at 6×10^4 cells per plate.

from granulocytes can be obtained in an active form in conditioned medium. The degree of inhibition with granulocyte-conditioned medium was less than with granulocyte feeder layers and, as with feeder layers, M colonies were more effectively inhibited than G colonies (Table 4). The detection of the inhibitory substance in conditioned medium required incubation with the inducer for at least four days at 37°C before the assay for colony formation. The inhibitory effect of granulocyte-conditioned medium was also found after dialysis for three days against PBS or water (Table 4). This supports the results obtained with feeder layers which indicated that the inhibitory substance was not dialyzable. In agreement with previous results,[3] incubation of macrophage-conditioned medium for four days with spleen-conditioned medium containing the inducer did not result in any detectable decrease of inducing activity.

Stimulation of the growth of myeloid leukemia cells by granulocytes and macrophages: The experiments above have indicated that mature granulocytes, like mature macrophages,[3] produce a feedback inhibitor that inhibits the activity of the inducer required for the formation of M and G colonies from normal hematopoietic cells. It was therefore of interest to determine whether myeloid leukemia cells can inhibit the activity of the inducer and respond to the inhibitor produced by mature normal cells. The results obtained with a line of primitive myeloid leukemia have indicated that feeder layers of these leukemic cells did not inhibit the formation of M or G colonies by the inducer in spleen-conditioned medium, and that the growth of these leukemic cells was not inhibited by feeder

TABLE 4. *Inhibition of colony formation by granulocyte-conditioned medium.*

	Spleen-conditioned medium per plate (ml)	Granulocyte-conditioned medium per plate (ml)	Dialysis of granulocyte-conditioned medium	Number of Colonies per Plate			
				M	G	B	Mixed M and G
Expt. 1	1.2	0	−	147	16	6	5
	1.2	1.2	−	106	13	2	0
	0.6	0	−	169	28	4	2
	0.6	0.6	−	120	15	3	0
	0.6	1.8	−	72	11	5	1
Expt. 2	0.6	0	−	92	6	0	0
	0.6	1.8	+	8	2	0	0

Rat spleen-conditioned medium was incubated with or without rat granulocyte-conditioned medium for 4 days at 37°C before the assay for colony formation. The granulocyte-conditioned medium was dialyzed against water for 3 days with a daily change of water (35 ml conditioned medium against 1 liter water) before the incubation with spleen-conditioned medium. A precipitate formed 1 day after dialysis, and this was removed. Incubation of the resuspended precipitate with spleen-conditioned medium for 4 days did not decrease the colony-forming ability of the spleen-conditioned medium.

Rat bone marrow cells were seeded for cloning at 6×10^4 cells per plate. 1.2 ml spleen-conditioned medium represents a concentration of 25%.

TABLE 5. *Stimulation of the growth of myeloid leukemia cells by normal granulocytes and macrophages.*

Feeder layer	Dialysis membrane	Number of leukemic cell colonies per plate
−	−	19
−	+	20
Granulocytes	−	211
Granulocytes	+	203
Macrophages	−	184
Macrophages	+	173

The leukemic cells were seeded for cloning at 400 cells per plate.

layers of granulocytes or macrophages. In contrast to the inhibition of formation of normal M and G colonies by mature granulocytes and macrophages, the growth of these myeloid leukemic cells was not only not inhibited but was even stimulated by these mature normal cells. This stimulation was detected as an increase in cloning efficiency (Table 5), and the number of cells per colony. The substance that produced this growth stimulation was able to pass through a dialysis membrane. This stimulating effect on the growth of the leukemic cells was also obtained in the mixtures of granulocyte- and spleen-conditioned medium that inhibited the development of normal M and G colonies. The stimulation of tumor cell growth by a dialyzable substance produced by mature macrophages and granulocytes has also been found with lymphoid and erythroid leukemias and with sarcoma cells.[8]

Discussion.—The results of the present experiments have indicated that mature granulocytes, like mature macrophages,[2, 3] produce a substance that inhibits the activity of the inducer required for the formation of M and G colonies. The inhibitory effect of mature macrophages and granulocytes on M and G colonies suggests that both types of colonies may be produced from a common stem cell. The more effective inhibition of M than of G colonies by macrophages and

granulocytes can then be explained in that the formation of M colonies is more susceptible to the inhibitors. Erythrocytes did not inhibit the formation of either M or G colonies, and results which indicate that erythropoietin[9] cannot replace the inducer for M and G colonies have also been obtained.[4] Regarding the nomenclature of the feedback inhibitors, since the macrophage inhibitor has been given the name *balam m*,[3] it is suggested that the granulcocyte inhibitor be given the name *balam g*.

The data obtained on the feedback control mechanism that regulates the growth and development of normal M and G colonies can be used as a basis for a hypothesis on the mechanism of leukemogenesis.[10] This hypothesis suggests that the continued multiplication of cells that occurs in leukemia is due to a block in cell differentiation that brings with it an absence of production of the feedback inhibitor, and that the process of leukemogenesis can also result in the production of cells that are resistant to the feedback inhibitor produced by mature normal cells. The results obtained with the primitive myeloid leukemia have indicated that the cells of this leukemia did not inhibit the activity of the inducer and were not susceptible to the inhibitor produced by mature normal cells. The data obtained with this line of myeloid leukemia thus support the hypothesis. The extension of such studies to other leukemias, including human leukemias, should prove to be of value for a further understanding of the mechanism of leukemogenesis.

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