Mechanism of Action of Serum Factors That Regulate Granulopoiesis In Vitro: Possible Physiologic Role of Serum-Inhibiting Activity

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The purpose of this study was to investigate mechanisms of action and in vivo relationships of serum factors that regulate granulopoiesis in vitro. Granulopoiesis involves interactions between stimulating and proliferating cells, and in order to determine mechanisms of action it is necessary to assess whether an activity acts on the stimulating or on the proliferating cell. Liquid cultures of monocytes, obtained from light-density white blood cells, were used to observe effects on colony-stimulating factor (CSF) production, and soft agar cultures of nonadherent light-density bone marrow cells were used to determine effects on colony-forming cells. Medium molecular weight serum-stimulating activity increased monocyte CSF production but had no effect on colony-forming cells. High molecular weight serum-inhibiting activity had no effect on CSF production but inhibited the action of CSF. Serum-inhibiting activity correlated with the circulating neutrophil concentration. These observations suggest that serum stimulation of granulopoiesis is achieved indirectly by stimulating marrow CSF-producing cells and that growth of the proliferating cell population is stabilized by a direct negative feedback loop.

RECENTLY WE REPORTED that the granulopoietic stimulating activity of human serum is due to a substance(s) requiring the presence of monocytes for its expression. In a large serum survey, none of the normal and only 5% of the patient sera examined stimulated growth in cultures of nonadherent light-density bone marrow cells (NALD BMC), whereas all normal and 95% of patient sera stimulated colony growth when monocytes were included in the cultures. These results suggest that under normal conditions it is unlikely that serum contains a substance that stimulates granulopoiesis directly. Serum contains inhibiting and stimulating activities that affect colony formation. Inhibiting activity is generally regarded as a high molecular weight lipoprotein, and stimulating activity is thought to be a glycoprotein with molecular weight slightly less than that of albumin.

In this study we examined serum-inhibiting and stimulating activity in separate monocyte and NALD BMC cultures to determine their mechanisms of action. In addition, the hypothesis that serum-inhibiting activity is related to the circulating neutrophil concentration was tested.

MATERIALS AND METHODS

Culture medium. CMRL-1066 tissue culture medium, supplemented with 2% of 100X nonessential amino acids, 1% of 100X sodium pyruvate, 1% of 200 mM L-glutamine, and 0.2% of 1000 mEq/ml vitamin B₁₂, was used (Gibco). The tissue culture medium was supplemented with 30% heat-treated (58°C for 120 min) human serum.

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The colony-forming cell culture system consisted of a single 1-ml 0.3% agar (Difco) layer in 35-mm Petri dishes (Falcon 1008) that contained NALD BMC (see below). When specified, either light-density white blood cells (LD WBC) or colony-stimulating factor (CSF) was added (see below). Serum or serum components to be tested were incorporated in this layer. Triplicate cultures were set up and incubated for 7 days at 37°C in a humidified atmosphere containing 5% CO2. Colony formation was assessed on day 7 by scoring aggregates containing more than 20 cells as colonies, using an inverted transmission microscope at 40X; results are expressed as the mean ± 1 SE of triplicate counts. Colony size was observed to increase with increases in colony number.

The monocyte culture system consisted of 2-ml liquid cultures of cells adhering to the bottom of 60-mm Petri dishes. The adherent cell layers were prepared by incubating 4 × 10⁶ LD WBC (see below) in 2 ml per 60-mm Petri dishes (Falcon 1007) for 1.5–2 hr to allow attachment of adherent cells. The plates were then agitated and the nonadherent cells decanted. The adherent cell layers, composed of more than 90% monocytes, were overlaid with 2 ml medium. Substances being examined for effects on monocyte CSF production were included in this over layer. Cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. Duration of incubation is indicated in each experiment. The supernatants from the cultures were stored at +4°C until they could be assayed for CSF at 20% in NALD BMC cultures. CSF content is expressed as “CSF” and indicates the number of colonies that formed in the CSF assay.

NALD BMC. Bone marrow was aspirated from informed normal volunteers using sodium citrate as the anticoagulant. After centrifugation at 2000 g for 10 min, theuffy coat was drawn off with a Pasteur pipette and the cells were washed thrice with heat-treated (58°C for 120 min) plasma. The suspension of nucleated cells was then separated by a single-density cut in human serum albumin (sp. gr. 1.072) to remove neutrophils. The light-density cells were recovered and subjected to an adherence separation to remove monocytes, and 1 × 10⁶ NALD BMC were added to each culture to provide target colony-forming cells.

LD WBC. Blood, drawn from informed normal volunteers into Vacutainers containing sodium citrate, was allowed to settle for 2 hr. The leukocyte-rich plasma was drawn off with a Pasteur pipette. The cells were spun down at 900 g for 10 min, washed thrice with heat-treated (58°C for 120 min) plasma, and depleted of neutrophils using the density separation procedure. The LC WBC fraction, composed of 0.4% ± 0.6% neutrophils, 43% ± 8% lymphocytes, 56.3% ± 8% monocytes, 0.2% ± 0.6% eosinophils, and 0.1% ± 0.3% basophils (means ± 1 SD), was used to provide colony-stimulating cells for specified NALD BMC cultures (2 × 10⁶ cells/culture), to prepare adherent cell layers, and to condition medium with CSF.

CSF was prepared by conditioning medium with LD WBC: 5-ml aliquots of a suspension containing 2 × 10⁶ LD WBC/ml were incubated in 100-mm Petri dishes for 3 days. The supernatant was decanted, millipore filtered (0.45-μm pore size), and stored at +4°C.

Preparation of serum-stimulating and -inhibiting activities. Fresh normal human serum (25 ml) was eluted through a Sephadex G100 column (diameter 50 mm, V₀ 350 ml, V₁ 1090 ml) with saline (8/liter). Eluate with Kᵥ between 0.03 and 0.14 (containing lipoprotein) was collected and used as the source of serum-inhibiting activity. Eluate with Kᵥ between 0.43 and 0.54 (eluting immediately after

Fig. 1. Effect of serum-stimulating activity on colony formation in NALD BMC cultures stimulated with either CSF (○) or LD WBC (□) in the same experiment.
Effect of Serum-Stimulating Activity

Colony formation in NALD BMC cultures. Serum-stimulating activity was included in cultures of NALD BMC stimulated with either CSF or LD WBC. Colony formation was not affected in CSF-stimulated cultures, whereas it was stimulated by serum-stimulating activity in LD WBC-stimulated cultures (Fig. 1). Two experiments of this design were completed, with each producing comparable results.

CSF production in monocyte cultures. Serum-stimulating activity stimulated CSF production when included in otherwise serum-free monocyte cultures (Fig. 2). This observation was made repeatedly, with several independent samples of serum-stimulating activity.

Effect of Serum-Inhibiting Activity

Colony formation in NALD BMC cultures. Serum-inhibiting activity inhibited colony formation, regardless of the nature of the stimulus (Fig. 3 and 4). This effect of serum inhibiting activity was observed in five separate experiments using independent samples of serum-inhibiting activity.
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Fig. 4. Effect of serum-inhibiting activity on colony formation in NALD BMC cultures stimulated with CSF.

CSF production in monocyte cultures. In this experiment, the rate of CSF production by monocytes was observed by setting up replicate monocyte cultures and withdrawing them from the incubator at various times, up to 40 hr. Spontaneous CSF production was ensured by using tissue culture medium supplemented with 30% heat-treated human serum. Serum-inhibiting activity did not inhibit monocyte CSF production (Fig. 5). This observation was made in two additional experiments.

Activity of Human Serum Fractions Assessed in NALD BMC Cultures

First, 5 ml of normal fresh human serum was eluted through a Sephadex G100 column (diameter 2.5 cm., $V_0$ 40 ml, $V_1$ 120 ml) with saline (8 g/liter). Then 5-ml fractions were collected and added at 20% to cultures of NALD BMC stimulated with CSF. Significant inhibiting activity was observed only in the fraction that eluted at 50 ml (Fig. 6). This experiment was repeated four times using LD WBC stimulation; each time, significant inhibitory activity was associated only with serum fractions eluting with lipoprotein.

Fig. 5. Effect of serum-inhibiting activity (20 %) on monocyte CSF production. Replicate monocyte cultures were set up and incubated for various durations. Incubation medium was supplemented with 30% heat-treated human serum; in this medium monocytes produce CSF spontaneously, as indicated by the open circles. Monocyte cultures containing serum-inhibiting activity are indicated by the solid circles.
Relationship Between Serum Colony-Inhibiting Activity and Circulating Neutrophil Concentration

Sera, collected from normal subjects and from "hematologically normal" patients whose neutrophil concentrations were reduced by drugs or elevated by infection, were fractionated on the Sephadex G100 column described above. Fractions eluting at 45, 50, and 55 ml were pooled and assayed at 10% for inhibiting activity in cultures of NALD BMC stimulated with CSF in a single experiment. Inhibiting activity was observed by all of the sera, the magnitude of which correlated with the circulating neutrophil concentration (Fig. 7).

Regulation of Granulopoiesis

Serum was obtained from two patients during drug-induced variations in circulating neutrophil concentration:

Patient I was a 63-yr-old female with advanced carcinoma of the breast who received a single intravenous infusion of cyclophosphamide, methotrexate, and 5-fluorouracil on day 0. Serum was
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Fig. 8. Relationship between circulating neutrophil concentration (□) and whole serum's effect on colony formation (○) assessed at 10% in NALD BMC cultures stimulated by LD WBC. Sera were obtained from two patients during drug-induced episodes of neutropenia. Sera from one patient were also assessed for activity in NALD BMC culture (Fig. 8A).

obtained regularly for 30 days, during which her circulating neutrophil concentration fell to 530 cells/mm³, recovered, and overshot normal values.

Patient 2 was a 25-yr-old female who had severe neutropenia due to phenylbutazone. Two days after admission to the hospital, her fever fell to normal. We then obtained daily serum samples while her circulating neutrophil concentration rose from 960 to 5300 cells/mm³.

The sera were tested at 10% for their effect on colony formation. The tissue culture medium used in this experiment was not supplemented with serum. Colony formation did not occur in cultures that contained NALD BMC only (Fig. 8A). When LD WBC were included in the cultures, colony formation occurred and was observed to vary inversely with the circulating neutrophil concentration (Fig. 8).

Fig. 9. Relationships between serum's effect on colony formation and circulating neutrophil concentration (A) and circulating monocyte concentration (B) assessed in NALD BMC culture stimulated with LD WBC. See Fig. 8 and text for description of sera.
The correlation coefficient between colony formation and circulating neutrophil concentration was $-0.78$ (Fig. 9A). The circulating monocyte concentration also varied during the course of these studies; however, no correlation between colony formation and circulating monocyte concentration was observed (Fig. 9B).

**DISCUSSION**

Granulopoiesis involves interactions between nonproliferating leukocytes, which produce regulatory factors, and proliferating cells, which respond to these factors. Likewise, colony formation by human marrow colony-forming cells involves interactions in which factors produced by nonproliferating cells (CSF by monocytes and colony-inhibiting activity by neutrophils) influence the proliferating cells. Marrow cell cultures also respond to added substances and have been used to assay serum; however, such cultures have the capacity to proliferate spontaneously, indicating that stimulating and proliferating cells are present. Thus assays employing unseparated marrow cells cannot determine whether regulating factors act on proliferating cells or on stimulating cells. To do so requires cultures of proliferating cells free of stimulating cells and vice versa. NALD BMC populations are essentially free of stimulating cells, and cultures of such cells do not form colonies spontaneously but respond sigmoidally to CSF. Thus NALD BMC cultures can be used to assay for CSF and for factors that inhibit CSF-stimulated colony formation. Blood monocytes are generally considered to be a model of the marrow colony stimulating cell, and liquid cultures of monocytes can be used to assess the action of regulatory factors on CSF production.

An additional important consideration of culture systems used to assay for regulatory factors is that the tissue culture medium serum supplement be free of regulatory activity. Serum regulatory factors are thermolabile. Thus tissue culture medium appropriately supplemented with heat-treated serum provides an environment that supports growth and does not interfere with the action of exogenous regulatory factors. Such a supplement was used in this investigation.
Normally, serum does not contain CSF but does exhibit colony-stimulating and -inhibiting activities. Medium molecular weight serum-stimulating activity stimulates monocytic production of CSF (Fig. 2) but has no effect on colony-forming cells (Fig. 1). In vivo relationships of serum-stimulating activity have not yet been evaluated adequately, since the monocyte culture system requires additional technical development to increase its sensitivity. High molecular weight serum-inhibiting activity inhibits colony-forming cells (Figs. 3 and 4) but has no such effect on monocyte CSF production (Fig. 5). The magnitude of its activity bears a relationship to the circulating neutrophil concentration (Fig. 7).

Whole serum obtained from patients during drug-induced neutropenia, assessed in cultures of NALD BMC stimulated with LD WBC, showed an inverse relationship between colony formation and circulating neutrophil concentration (Figs. 8 and 9A). Such a relationship is consistent with the concept that circulating neutrophils produce serum-inhibiting activity.

The results of our studies led us to the following description of the regulation of granulopoiesis (Fig. 10): The granulopoietic system consists of stimulating and proliferating cell populations, which are located in the marrow cavity. Growth of the proliferating cell population is stimulated by CSF, which is produced locally by stimulating cells. The absence of CSF in serum and the rapid clearance of endotoxin-stimulated serum CSF support this concept. Increases in granulocyte production are brought about by serum factor(s) that act to enhance local CSF production. Growth of the proliferating granulocyte progenitor cell population is stabilized by a direct negative feedback loop. This feedback loop consists of circulating granulocytes that produce serum-inhibiting activity, which circulates through the marrow, where it acts directly on the proliferating cells to inhibit their proliferation.

REFERENCES


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