Humoral Stimulators of Granulocyte Production


These studies were designed to determine whether colony stimulating factor (CSF) and diffusible granulocytopenic stimulator (DGS) represented a single substance which stimulates the production of granulocytes in vitro and in vivo. The effect of endogenous CSF upon DGS activity was studied after injection of 40 µg endotoxin. This resulted in both DGS activity and high serum CSF levels, but the time of appearance and duration of the two activities followed different patterns. In particular, CSF peaked and was declining 24 hr following endotoxin at which time DGS was not yet detectable. Then, as serum CSF fell, the DGS activity rose. These data support the concept that the DGS activity detectable with short term Millipore chamber cultures is not due to CSF and suggests the effects of CSF in vitro and DGS in vivo are due to different factors.

Granulocyte production can be stimulated both in vitro and in vivo by humoral agents. Colony stimulating factor (CSF), capable of stimulating granulocyte and macrophage colony formation in vitro is present in various mammalian serums and urines and can be produced by conditioning medium with various tissues. CSF has been prepared in concentrated and partially purified form and has been shown to be a glycoprotein with a molecular weight of 45,000. Diffusible granulocytopenic stimulator (DGS), a humoral substance present in vivo in the body fluid of postirradiated and endotoxin injected mice, has been shown to stimulate granulocyte production in celltight Millipore chambers implanted intraperitoneally. Concentrated or partially purified DGS has not yet been prepared. The purpose of the present studies was to determine whether the biologic activities of CSF and DGS are due to the same or different factors. This was approached by inducing endogenous CSF production with endotoxin and measuring DGS and CSF activity at subsequent intervals.

MATERIALS AND METHODS

Eighteen to twenty-two gram male CBA mice (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. Total leukocyte counts were done electronically in triplicate on vena cava blood anticoagulated with ethylene diamine tetraacetic acid. Tibial marrow...
cellularity was measured in the manner described by Chervenick et al. Differential blood and marrow nucleated cell counts were done with 100 and 500 cell differential counts of Wright's stained cover slip smears, respectively. Salmonella endotoxin (400 μg/ml) was dissolved for CSF was collected under sterile conditions from vena cava blood.

Millipore chambers were constructed as previously described using 13-mm Millipore filters (Millipore Corporation, Bedford, Mass.), pore size 0.45 μm. A Hamilton (Van Waters & Rogers, San Francisco, Calif.) syringe was used to fill the chambers with 50 μl of a suspension of normal mouse hindlimb marrow in Hanks balanced salt solution with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) (HBSS). Each chamber received 1.5-3.0 × 10^6 nucleated cells.

Chambers were implanted within the peritoneal cavities of host mice anesthetized with methoxyflurane through a midline incision under sterile conditions. The incision was closed and for the next 24 hr the host mice moved freely in their cages. The chambers were then retrieved and shaken continuously in 5% Ficoll and 0.5% pronase in HBSS for 50 min. Chambers were then cleaned, inspected for leaks, and their cell content quantified by triplicate electronic count. Differential counts were performed on 500 cell differentials of Wright's stained cover slip smears.

Serum CSF activity was assayed by a modification of the method described by Pluznik and Sachs and Bradley and Metcalf for the in vitro growth of mouse bone marrow. Methyl cellulose was used in place of agar. One hundred thousand mouse marrow cells were suspended in 1.6% methyl cellulose containing CMRL-1066 culture medium (Grand Island Biologicals, Berkeley, Calif.) and 10% horse serum (Flow Laboratories, Inc., Rockville, Md.). To each 0.9 ml of the methyl cellulose, serum, cell mixture, 0.1 ml of the mouse test serum was added. One milliliter of the combined mixture was plated into 10 × 35 mm plastic tissue culture plates (Falcon Plastics, Division of Becton, Dickinson & Co., Cockeysville, Md.) and incubated at 37°C in 7.5% CO₂. After 7 days incubation, colonies (containing > 50 cells) were counted with the aid of an inverted microscope.

RESULTS

**The Measurement of CSF and DGS**

CSF and DGS are both detected by their biologic activity and therefore, the concepts involved in their measurement are outlined below.

Serum CSF activity is assayed in vitro by adding an aliquot of test serum to semisolid culture medium containing normal marrow cells, horse serum, and culture medium. Culture plate containing cells, medium, and serum are then incubated for 7 days. At the end of this period, the colonies formed as a result of the CSF activity in the serum are counted and CSF is expressed as colonies formed/culture plate.

DGS activity is assayed in vivo. Normal marrow cells, within celltight culture chambers, are placed within the abdomens of host mice and are allowed to remain there for 24 hr. Then the chambers are retrieved and their cell content quantified. DGS, when present in the body fluids of a stimulated host, is detected by its ability to diffuse into the chambers and stimulate blast plus promyelocyte (B + P) growth, as compared with chambers implanted in unstimulated control host mice.

**The Effect of Endogenous CSF upon DGS Activity**

In order to evaluate the effect of endogenous CSF upon DGS activity, host mice were given Salmonella endotoxin, 40 μg intravenously. This resulted in changes in the cellularity of blood and bone marrow (Fig. 1), a rise in serum CSF, and in the appearance of DGS activity (Fig. 2). The data for each point
in time were collected in two or more experiments. Serum CSF activity (Fig. 2A) rose promptly after endotoxin injection, reaching peak levels during the first 24 hr and then falling toward normal levels. In some instances detectable activity was measured as long as 96 hr after the endotoxin.

DGS was measured by implanting chambers filled with normal marrow into the abdomens of either endotoxin injected or saline injected (control) host mice. After the 24-hr culture period the chambers were retrieved and the B + P production in the endotoxin hosts' chambers was compared with the

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**Fig. 1.** The blood neutrophil count (A) and tibial content of granulocyte precursors (B) after 40 μg endotoxin. Each point represents the mean value for ten mice. The absolute ranges are depicted by the bars.
controls. Thus, when B + P production in the endotoxin group exceeded the controls, DGS activity was present. Data for each point in time were collected from two or more experiments. The absolute number of blasts and promyelocytes in each chamber ranged between 11,500 and 130,100 cells per chamber. The level of DGS activity followed a different time course (Fig. 2B) than did CSF activity in that CSF peaked during the first 24 hr, but DGS was undetectable. Conversely, as CSF levels fell DGS rose and reached its highest levels. Since each DGS assay represents a single 24 hr cultivation, CSF concentration at a time prior to the cultivation is excluded as having any direct effect upon the cell growth within the chambers.

Fig. 2A. Serum CSF activity after 40 μg endotoxin. Each point represents the CSF activity of pooled serum from two mice. The solid line connects the mean values for each set of observations. 2B. In vivo DGS activity after 40 μg endotoxin. Each point represents the mean value for ten chambers. The absolute range is depicted by the bars.
The Failure of Preincubation of Marrow in Endotoxin Injected Hosts to Inhibit DGS Responsiveness

Further experiments were performed in order to determine whether the lack of DGS activity in the first culture period (5-24 hr) might be due to inhibition of cell growth by the injection of endotoxin. Millipore cultures of marrow were placed in the abdomens of host mice 5 hr after the injection of 40 μg of endotoxin or saline (controls). The chambers were left in the hosts to preincubate for 5 hr, during which time any toxic substance had an opportunity to diffuse into the chambers and damage the marrow cells inside. The chambers were then retrieved and their ability to respond to DGS in 4-day postirradiation hosts7 was measured.

The contents of eight chambers preincubated in endotoxin hosts had B + P values which did not differ significantly from eight chambers preincubated in control mice (Table 1). Thus it appears that the ability of marrow to respond to DGS was not measurably affected by the initial preincubation in endotoxin injected hosts.

DISCUSSION

The regulatory mechanisms which control granulocyte production remain incompletely understood. However, in vivo diffusion chamber studies provide evidence in favor of a humoral control mechanism (DGS activity) which is perhaps analogous to erythropoietin for the erythrocytes.7,8,11 This activity seems to be specific for the neutrophils7 and is apparently separate from neutrophil releasing factor (NRF) also seen after endotoxin injection.8 The nature of DGS is as yet unknown. In these studies, we have attempted to determine whether CSF, which stimulates granulocyte growth in vitro, might in fact be the same substance responsible for DGS activity in vivo. The work of Bradley, Metcalf, Sumner, and Stanley3 and Metcalf and Stanley12 suggests that injected or infused CSF may exert a granulocytopoietic effect in vivo.

The present studies in which DGS and CSF were induced with endotoxin provide evidence to support the concept that endogenous CSF and DGS are different substances acting upon the granulocytes. The pattern of serum CSF rise and fall in the present experiments is similar to that recently reported.13,14 During the early peak of serum CSF activity, no DGS activity was detected even though the 24 hr chamber culture period beginning 5 hr after endotoxin and ending 29 hr after endotoxin was characterized by the highest

<table>
<thead>
<tr>
<th>5 hr preincubation host</th>
<th>24 hr culture host</th>
<th>No. of chambers</th>
<th>Blast + Promyelocytes % mean control value and range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline injected</td>
<td>Normal (control)</td>
<td>10</td>
<td>100% (51%-133%)</td>
</tr>
<tr>
<td>Saline injected</td>
<td>4 days after 370 R</td>
<td>8</td>
<td>283% (104%-510%)</td>
</tr>
<tr>
<td>Endotoxin injected</td>
<td>4 days after 370 R</td>
<td>8</td>
<td>275% (132%-419%)</td>
</tr>
</tbody>
</table>
CSF values. The subsequent separate Millipore cultures showed detectable DGS activity as CSF activity as CSF levels fell. These data indicate that DGS and CSF are both elevated after 40 μg endotoxin, but the time patterns of rise and fall of each differ. Furthermore, when CSF was at its highest level, DGS could not be detected.

The present studies provide evidence to support the concept that the growth of blasts and promyelocytes in Millipore chambers in vivo is not affected in a morphologically recognizable way by CSF during 24-hr cultures. Thus, CSF activity appear to be due to different substances affecting the production of granulocytes in vitro and in vivo.

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REFERENCES

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