Granulocyte Colony Stimulating Factor
I. Response to Acute Granulocytopenia

By Richard K. Shadduck and N. G. Nagabhushanam

The in vitro growth of hemopoietic colonies, in the presence of an active colony stimulating factor (CSF), provides a useful model for evaluation of potential humoral factors controlling granulopoiesis. The present investigations were undertaken to assess the relationship between rapidly induced granulocytopenia in rats and the presence of heightened CSF activity. Sera from control animals showed virtually no activity (0-3 colonies). Significant levels of CSF (40-60 colonies) were demonstrated within 2-6 hr after rabbit antirat neutrophil serum (ANS). The increased activity corresponded with the phase of rapid neutrophil destruction. However, in animals rendered neutropenic by pretreatment with cyclophosphamide, ANS administration was also associated with a prompt rise in CSF levels; the magnitude of this response approximated that seen in animals treated with ANS alone. These results suggest that heightened CSF may accompany destruction of relatively small numbers of neutrophils. The temporal appearance of heightened CSF activity in relation to induced granulocytopenia is consistent with the concept that this factor may be a regulator of granulopoiesis.

The question as to whether granulopoiesis is controlled by humoral mechanisms has received increasing attention in recent years. It is well known that the administration of many substances engenders a neutrophilic leukocytosis. However, in many instances, this appears to be a nonspecific response either to foreign proteins or to contamination with endotoxin. In contrast, specific factors capable of accelerating neutrophil release from marrow storage pools have been demonstrated in response to granulocyte depletion. Although these materials appear to hasten the release of postmitotic maturing granulocytic elements from the marrow, they have not been shown to directly stimulate granulopoiesis.

The present study examines the question as to whether granulopoietic factors are released in response to neutropenia. Serum from neutropenic rats was evaluated for granulopoietic activity by an in vitro mouse marrow culture system. In this soft agar system, colonies of granulocytic elements develop...
from normal marrow cells when incubated in the presence of a colony stimulating factor (CSF). Although this stimulating factor has been demonstrated in various cell feeder layers, certain sera, urinary extracts, and conditioned media obtained from the growth of murine embryonic cells, fibroblasts, and peripheral leukocytes, little information is available as to its production or release in response to induced granulocytopenia.

Rapid depletion of mature granulocytic elements was accomplished by the injection of a potent antineutrophil serum (ANS). At intervals after administration of this antibody, groups of rats were bled and their sera tested for CSF activity. The results indicate marked increases in CSF in association with the phase of rapid neutrophil disappearance.

**Materials and Methods**

Female Sprague-Dawley rats weighing 150–180 g were used for in vivo studies on the effect of antineutrophil sera. Antineutrophil sera were prepared by modifications of the technique described by Lawrence et al. Six hours after the intraperitoneal injection of a beef heart peptone infusion, peritoneal exudates were harvested from 10 to 12 rats in 0.9% sodium chloride containing 10 U heparin per ml. Differential smears obtained at this time indicated that approximately 90% of the cells were mature granulocytes. The cells were washed three times in saline, emulsified in complete Freund's adjuvant, and then injected subcutaneously into each of three to four New Zealand white rabbits. Each injection contained 150–300 × 10⁶ cells. Seven to 14 days after four weekly immunizations, the rabbits were bled by cardiac puncture, the sera separated, pooled, and frozen prior to use. Each serum was heated to 56°C to inactivate complement and then absorbed four times against packed rat red blood cells. This procedure completely removed red cell agglutinins; absorbed serum showed a leukoagglutinin titer against neutrophils from peritoneal exudates to be approximately 1:640. These sera are hereafter termed antineutrophil sera (ANS). Pooled normal rabbit sera were heated and absorbed in a similar manner and are termed NRS.

Groups of 30–40 experimental animals were randomized and given an intraperitoneal injection of 1 ml of either ANS, NRS, or 0.9% sterile saline solution. They were sacrificed at intervals during the next 2–72 hr for the following studies: total leukocyte count, white blood cell differential, bone marrow cellularity, marrow morphology, and serum CSF activity. Further groups of animals were rendered neutropenic by a single intraperitoneal injection of 100 mg/kg cyclophosphamide. Four days subsequent to cyclophosphamide treatment they were injected with either ANS or NRS and sacrificed 4 hr later.

Blood for leukocyte counts was obtained under ether anesthesia by retroorbital puncture; animals were then exsanguinated by cardiac puncture for serum CSF assays. Leukocyte counts were done using a model B Coulter counter after lysing the red cells with an acetic acid-cetrimide diluent. White cell differentials were performed on Wright-Giemsa stained smears; 200 cells were generally evaluated, although 500 cells were examined in severely neutropenic animals. Bone marrow smears were painted on glass slides with a fine camel's hair brush after splitting the femur longitudinally and mixing the marrow with small amounts of isologous serum. Five hundred cell differentials were determined on Wright-Giemsa stained smears. Total marrow cellularity was estimated by flushing the contents of the opposite femur into 1% EDTA saline; suspensions were gently pipetted to disperse all fragments and counted electronically.

Serum CSF activity was assayed using minor modifications of the Bradley and Metcalf technique. Normal bone marrow cells from the tibiae of at least five CF₁ mice were flushed into McCoy's 5A tissue culture medium. The cells were gently pipetted to disperse clumps and enumerated by manual, rather than electronic, counting to ensure that the suspensions contained primarily single cells. Nine parts of single strength McCoy's medium supplemented with 10% fetal calf serum, 5% horse serum, and additional amino acids were added to one part of 3% Bacto agar that had previously been boiled and cooled to 40°C.
Sufficient bone marrow cells were added to yield a concentration of $10^9$ nucleated cells per ml of McCoy's agar. Individual 10 × 35 mm culture plates were prepared by adding 0.1 ml of the rat serum to be tested to 1 ml of the marrow agar suspension; five such plates were prepared for each test serum. Positive control plates contained 0.1 ml of an active CSF harvested from the growth of mouse fibroblast cells (L cells). Negative controls contained only the marrow-agar suspension. Plates were allowed to gel at room temperature and then incubated at 37°C in a humidified atmosphere of 7.5% CO$_2$ for 4 days. Discrete colonies containing eight or more cells were enumerated by two independent observers with the aid of a dissecting microscope ($\times 20$).

**RESULTS**

Granulocyte counts fell to approximately 10% of control observations within 6 hr of ANS injection; values remained low for 48 hr with initial evidence of recovery by 72 hr (Fig. 1). Neutrophil levels initially increased in NRS-treated rats but returned to normal values within 48 hr after injection.

In addition to the changes in circulating cells, bone marrow granulocytic elements decreased by approximately 40% within 6 hr of ANS administration (Table 1). This was due to depletion of cells in the maturation and storage compartments (segs, bands, and metamyelocytes); proliferating granulocyte precursors were numerically unaffected. Increased numbers of myeloblasts and promyelocytes were evident within 24–48 hr after ANS injection. Further maturation to the metamyelocyte and early segmented forms was seen by 72 hr, a finding in accord with the observed increase in peripheral granulocyte levels. NRS treatment did not result in significant changes in marrow granulocyte compartments. Although circulating granulocytes had risen appreciably by 6 hr, this change could not be accounted for by a depletion of marrow storage pools.
Table 1.—Bone Marrow Response to ANS

<table>
<thead>
<tr>
<th>Treatment and No. of Rats</th>
<th>Granulocytic Elements per Femur</th>
<th>Myeloblasts and Promyelocytes</th>
<th>Myelocytes</th>
<th>Metamyelocytes</th>
<th>Bands and Segs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (15)</td>
<td>50.7 ± 2.8</td>
<td>16.8 ± 0.9</td>
<td>5.9 ± 0.5</td>
<td>9.1 ± 1.1</td>
<td>18.9 ± 1.3</td>
</tr>
<tr>
<td>Post-ANS 6 hr (10)</td>
<td>31.1 ± 3.7*</td>
<td>18.3 ± 2.4</td>
<td>6.6 ± 0.8</td>
<td>2.7 ± 0.8*</td>
<td>3.5 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>43.8 ± 2.6</td>
<td>25.2 ± 2.3*</td>
<td>7.4 ± 1.0</td>
<td>4.2 ± 0.4*</td>
<td>7.1 ± 0.5*</td>
</tr>
<tr>
<td>48 hr (10)</td>
<td>49.7 ± 3.4</td>
<td>29.0 ± 3.4*</td>
<td>9.1 ± 1.3*</td>
<td>5.0 ± 0.7*</td>
<td>6.6 ± 1.1*</td>
</tr>
<tr>
<td>72 hr (10)</td>
<td>67.2 ± 7.8</td>
<td>29.6 ± 3.7*</td>
<td>12.2 ± 2.8*</td>
<td>10.4 ± 2.4</td>
<td>14.9 ± 1.5</td>
</tr>
</tbody>
</table>

* Significant deviation from saline controls (p < 0.05).
Values represent mean (± 1SE) number of granulocytic elements per femur (∗10⁶) at indicated times after treatment with 1 ml antineutrophil serum (ANS). Results are pooled values from two replicate experiments.

Normal rat sera obtained from saline-treated control animals did not show appreciable levels of CSF; 0.1 ml generally stimulated zero to one colony. Sera from NRS-treated animals did not differ from the control sera. At no time did any of the sera generate in excess of three colonies; most lacked any demonstrable colony stimulating activity. The possibility that normal rat sera or sera obtained from NRS-treated animals might contain factors inhibitory to colony growth was evaluated by mixing the sera with 0.05 ml of a 1:5 dilution of active CSF. Colony counts were equal to or greater than that obtained with CSF alone, suggesting that the observed lack of activity was not due to inhibitory substances in normal rat sera.

In contrast to the normal sera, those from ANS-treated rats were markedly stimulatory. Sera obtained within 2 hr after ANS administration generated an average of 50 colonies (Fig. 2). Activity remained high throughout the initial 6 hr coincident with a phase of further granulocyte destruction. However, CSF activity returned to baseline levels by 24 hr despite a continued granulocyte deficit through 72 hr. As was the case with normal rat sera, those sera obtained 24–72 hr after ANS injection were devoid of inhibitory properties. Mixtures of 0.1 ml of these sera with 0.05 ml dilute CSF yielded approximately the same number of colonies as seen with CSF alone.

Further in vitro experiments excluded the possibility that the CSF activity was a result of intrinsic properties of the immunized rabbit sera per se. As shown in Table 2, antineutrophil serum did not possess CSF activity either alone or after incubation with normal rat serum. Moreover, when antineutrophil
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Table 2.—In Vitro Activity of Antirat Neutrophil Serum

<table>
<thead>
<tr>
<th>Condition</th>
<th>CSF Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antirat neutrophil serum (ANS)</td>
<td>0</td>
</tr>
<tr>
<td>Normal rat serum (0.09 ml)</td>
<td>0</td>
</tr>
<tr>
<td>ANS + ANS (0.01 ml)</td>
<td>0</td>
</tr>
<tr>
<td>ANS + CSF (0.05 ml)</td>
<td>42 ± 2.6</td>
</tr>
<tr>
<td>CSF (0.05 ml)</td>
<td>91 ± 2.3</td>
</tr>
</tbody>
</table>

CSF values represent mean (±1 SE) number of colonies developed from 10^5 murine bone marrow cells. CSF was derived from L-cell conditioned medium.

serum was mixed with L-cell derived CSF, a diminution in colonies was observed, suggesting that ANS by itself has moderate inhibitory activity.

To test the hypothesis that colony stimulating factor may be directly liberated from destroyed neutrophils, 1 ml of ANS was incubated for 2 hr at 37°C with a suspension of 5 × 10^8 granulocytes obtained from induced peritoneal exudates (Table 3). Granulocytes incubated with antineutrophil serum alone, or in the presence of guinea pig complement, did not generate appreciable colony stimulating activity. Furthermore, the direct in vitro destruction of granulocytes either by repeated freeze-thawing or sonication failed to result in elaboration of this factor. The presence of low molecular weight inhibitory compounds was excluded by repeating this assay after extensive dialysis of the suspension against sterile water. In no instance was significant CSF activity found.

To determine whether CSF could be elaborated in a neutropenic state, further groups of animals were rendered neutropenic by cyclophosphamide and subsequently treated with ANS (Table 4). Four days after the administration of cyclophosphamide the granulocyte count had decreased to 245/cu mm; these sera did not show appreciable levels of CSF. Antineutrophil sera administered to these granulocytopenic animals reduced the granulocyte count to undetectable levels and were associated with CSF levels similar to those observed in animals treated with ANS alone. Bone marrow smears from the cyclophosphamide-treated animals indicated an almost complete absence of band and segmented neutrophils. These observations indicate that ANS is capable of inducing a marked rise in serum CSF activity despite a pre-existent marked deficit of peripheral blood and bone marrow granulocytes.

Table 3.—Effect of In Vitro Granulocyte Destruction

<table>
<thead>
<tr>
<th>Condition</th>
<th>CSF Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes + ANS</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Granulocytes + ANS + C</td>
<td>0</td>
</tr>
<tr>
<td>Granulocytes + saline (freeze-thawed)</td>
<td>0</td>
</tr>
<tr>
<td>Granulocytes + saline (sonicated)</td>
<td>0</td>
</tr>
<tr>
<td>CSF (0.05 ml)</td>
<td>161 ± 8.0</td>
</tr>
</tbody>
</table>

CSF values represent mean (±1 SE) number of colonies developed from 10^5 murine bone marrow cells. Granulocytes were obtained from peritoneal exudates; 5 × 10^8 were incubated with ANS either alone or in presence of guinea pig complement (C) at 37°C for 2 hr. CSF was derived from an L-cell conditioned medium.
Table 4.—Effect of ANS in Neutropenic Animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC</th>
<th>Granulocytes</th>
<th>CSF Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13,200±1,280</td>
<td>1,792±293</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>2,000±293</td>
<td>245±81</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>Cyclophosphamide + ANS</td>
<td>870±134</td>
<td>-0-</td>
<td>41±4.5</td>
</tr>
<tr>
<td>ANS</td>
<td>5,088±563</td>
<td>71±24</td>
<td>39±9.2</td>
</tr>
</tbody>
</table>

Cyclophosphamide-treated animals were sacrificed 4 days after injection; ANS recipients, 4 hr after treatment. WBC and granulocytes are expressed as cells/cu mm. CSF activity represents mean number of colonies developing from $10^5$ murine marrow cells in presence of 0.1 ml of serum from listed sources. Values are mean ± 1 SE.

DISCUSSION

It has been conjectured that, in addition to a granulocyte releasing factor,8,9 granulocytopenia may also induce the elaboration of a substance that accelerates the rate of neutrophil production.19 The presence of this “granulopoietin” has not been universally accepted. The difficulty in defining a granulopoietin result, in part, from the interpretation of in vivo granulocyte responses to injected materials. Since increased proliferation of early granulocytic elements has been consistently observed as a secondary phenomenon following reduction of the marrow storage compartment,17,20 evaluation of certain biologic materials may be severely hampered in the experimental animal.

To obviate the difficulties in interpreting in vivo results, an in vitro technique has been used in these studies. Hemopoietic cells, when plated in a semisolid medium in the presence of certain stimulating factors, will develop into discrete granulocytic colonies. These observations have led to the suggestion that these in vitro stimulating factors may actually represent true granulopoietic substances.12 Disturbing to this notion is the growing number of cell and tissue extracts that possess CSF activity. Included among the materials that have this activity are murine sera,11 human sera,12 urinary extracts,13 and cellular derived substances from kidney,19 embryonic tissue,14 fibroblasts,15 and leukocytes.16 The diverse source of these materials raises the question as to the specificity of CSF.

The present study examined the relationship between granulocytopenia and serum CSF activity. Previous investigations in which granulocytopenia was induced in mice by irradiation have yielded conflicting results. Following whole body exposure to 50–450 R, no significant increase in CSF was detected during the neutropenic phase.21 However, increased activity was observed in animals with a presumably greater degree of neutropenia after 800 R; CSF levels were highest at the nadir of granulocytopenia and varied inversely with the peripheral count.22 In the present study, a specific antineutrophil serum (ANS) was used that produced severe neutropenia in a rapid and predictable fashion. It was anticipated that neutropenia produced in this way would serve as a maximal stimulus for the elaboration of a granulopoietic factor.

As demonstrated by these data, ANS produced an acute depletion of both peripheral blood and bone marrow granulocytes. The rapid destruction of the majority of circulating and storage cells was associated with a marked increase in serum CSF activity. Heightened activity was apparent as early as 2 hr
after ANS; this persisted through 6 hr after injection. However, the rise in 
CSF was transient; levels declined to base-line values within 24 hr in spite 
of the persistence of marked granulocytopenia. These findings are reminiscent 
of the erythropoietin (EP) response to acute hypoxia. In this situation, high 
levels of EP are demonstrated during the initial 6–24 hr of hypoxia, but with 
continued exposure to reduced oxygen tension, EP values return to near nor-
mal levels despite continuation of accelerated red cell production. It has been 
suggested that this phenomenon is due to increased erythropoietin utilization 
by the hyperactive erythroid marrow. Whether a similar mechanism is 
involved with increased utilization of CSF following the initial 6–24-hr period 
of granulocytopenia is not known. However, these observations cannot exclude 
the possibility that increased CSF turnover is occurring during the prolonged 
period of neutropenia.

Recent studies have shown that granulocytes per se are capable of elaborat-
ing a granulopoietic factor. Suspensions of these cells derived from human 
peripheral blood serve to produce a diffusable granulopoietic factor when 
plated in agar underlayers or following prolonged incubation in tissue culture 
medium. This activity appears to be produced, rather than stored by leuko-
cytes. In our study, the in vitro destruction of white cells either by antineutro-
phil serum or by physical means was not associated with detectable CSF activity. 
Further studies in intact animals in which antineutrophil serum was 
administered by cyclophosphamide induced neutropenic recipients suggested 
that this activity could still be elaborated despite a preexistent marked deficit 
of peripheral blood and bone marrow granulocytes.

In this present study, the granulocytopenia induced by alkylating agent 
alone was not associated with a detectable increase in serum CSF activity. 
Separate experiments in which these parameters were monitored over a 14-day 
period after administration of cyclophosphamide indicated a reciprocal rela-
tionship between circulating granulocytes and serum CSF. Increased CSF 
activity was observed on the fifth through seventh days after injection, follow-
ing peripheral neutropenia by approximately 24 hr. The temporal relationship 
was distinct from the present findings in which acute granulocytopenia was 
associated with immediate elaboration of CSF. Further study will be necessary 
to determine whether this difference is a reflection of the rate of granulocyte 
destruction or whether several alternative mechanisms exist for CSF produc-
tion.

The in vitro technique used in these studies has varied somewhat from 
those previously described. Other investigators enumerate colonies of 25–50 
cells, after 7 or 8 days of incubation. Detailed studies of the growth charac-
teristics of in vitro colonies in our hands agree with the findings of Metcalf 
et al. that greater than 95% of the colonies are granulocytic in nature after 
4 days of incubation. However, by the sixth to seventh day, a majority of 
colonies are composed of mononuclear or macrophage cells. This may result 
from actual production of CSF by the granulocytic colonies themselves, allow-
ing for continued proliferation and differentiation of macrophage elements 
on plates with high numbers of granulocytic colonies. Furthermore, in some 
situations low levels of colony stimulating activity can be demonstrated follow-
ing 4 days of incubation, whereas with continued incubation the small number of colonies disappear.\textsuperscript{27} In the present studies, CSF activity was in the order of 30–50 colonies, and repeat observations after 7 days of incubation, in which only large colonies were enumerated, indicated a very close correlation between the 4- and 7-day colony counts (Fig. 3).

The cell population responsible for in vitro colony formation has been contrasted with the pluripotential stem cell that is assayed by spleen colony formation in irradiated mice. In the spleen colony assay,\textsuperscript{28} colonies are composed of erythroid, granulocytic, and megakaryocytic elements.\textsuperscript{29} However, attempts to induce erythroid colony formation in vitro by the addition of erythropoietin have been unsuccessful.\textsuperscript{30} Kinetic studies indicate that the in vitro stem cell is an actively proliferating cell, whereas the pluripotential cell is characterized by a slow rate of cell turnover.\textsuperscript{31,32} These findings have led to the suggestion that the in vitro colony forming cell may represent a granulocyte-committed stem cell analogous to the erythroid-committed stem cell.\textsuperscript{33,34} Since this latter compartment of cells is thought to be responsive to erythropoietin, analogy would suggest that in vitro proliferation and differentiation from the colony forming cell may be in response to granulopoietin.

The finding of increased CSF in response to acute granulocyte depletion is consistent with the concept that this factor is a granulopoietic material. Recent data by the authors suggest that CSF may have active granulopoietic effects in vivo.\textsuperscript{35} Injection of mice with killed pertussis organisms led to high levels of CSF that were followed by a wave of heightened granulopoiesis. However, before conclusions are reached as to the physiologic role of this material, it will be necessary to further show that granulocytic recovery is mediated by this factor and that such material has granulopoietic effects in vivo independent of any neutrophil-releasing activity.

**ACKNOWLEDGMENT**

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