Stimulation of Neutrophil Production in CSF-1–Responsive Clones

By G. Rothstein, S.M. Rhondeau, C.A. Peters, R.D. Christensen, D. Lynch, and S. Gillis

The hematopoietic growth factor CSF-1 has been considered relatively lineage specific for the production of macrophages, whereas GM-CSF elicits a predominance of neutrophils. It is likely that in vivo, individual clones are stimulated by the two CSFs, although the effect of dual stimulation on progenitors and their progeny has not been completely explored. We found that in cultures initiated with low concentrations of CSF-1 or GM-CSF, alone or in combination, production of macrophages predominated. Maximally stimulatory concentrations of CSF-1 elicited a predominance of macrophages, whereas maximal GM-CSF elicited many more neutrophil/macrophage colonies and pure neutrophil colonies. A combination of maximal CSF-1 and GM-CSF elicited the same differentiation as GM-CSF alone. Delayed addition of GM-CSF to cultures initiated with CSF-1 elicited colonies indistinguishable from GM-CSF alone, suggesting that neutrophil production had been switched on by GM-CSF. In mapping studies, colonies initiated by CSF-1 increased or switched on neutrophil production when GM-CSF was added as a second stimulus. These studies show that individual clones are responsive to both CSFs, and that the differentiating influence of GM-CSF predominates over that of CSF-1. In cultures to which only CSF-1 was added, a population of progenitors was sustained that produced neutrophils only after a GM-CSF stimulus. Thus, CSF-1 may participate in maintaining a reserve of progenitors for neutrophils during periods of increased neutrophil demand.

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Supported by National Institutes of Health Grant No. R01-HD22083.

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MATERIALS AND METHODS

Six 8-month-old C57B1/6 NIA mice were obtained from Charles River Laboratory (Stone Ridge, NY). Bone marrow was flushed from both femurs with a Modified Eagle's Medium (MEM) (Flow Laboratories, McLean, VA), and the cells were counted electronically. Cultures of granulocyte/macrophage progenitors were performed in 35-mm plastic dishes in a manner similar to that of Iscove et al.14 The semisolid medium contained 1.3% methylcellulose (Sigma Chemicals, St Louis), MEM (Flow Laboratories), 5 × 10−5 mol/L β-mercaptoethanol, 30% fetal bovine serum (Sigma Chemicals), and 1% irradiated bovine albumin (Sigma Chemicals). Six 10 × 106 marrow cells were cultured per 1-mL dish. Cultures were incubated at 37°C for seven days in a fully humidified 7% CO2 atmosphere. Groups of >50 cells were designated as colonies; groups of eight to 49 cells were designated as clusters. Colony/cluster formation was assessed by examination either with a stereomicroscope or an inverted phase microscope. The morphology of clones was determined by aspirating them from cultures, applying them to glass cover slips, rapidly drying them, and then staining with Wright's stain. Differential counts were performed on 50 to 500 cells depending on the size of colonies, using the criteria of Chervenick et al.15 When differential counts disclosed only one differentiated cell
were performed for macrophages; MAC/NEUT, macrophages plus neutrophils or pure immature cells.

The concentration of the two factors together stimulated a greater percentage. GM-CSF the only differentiated cells were neutrophils or occasion

tal eosinophils. The combination of these concentrations did not augment colony formation above that for CSF-1 alone, but did increase the number of colonies that produced neutrophils and the number of pure neutrophil colonies, as well as increasing the formation of macroscopically visible colonies above that observed with CSF-1 alone.

The delayed addition of plateau GM-CSF to CSF-1-initiated cultures induced differentiation to the neutrophil line, increased the formation of colonies of pure neutrophils, and stimulated the formation of macroscopic colonies. In two additional experiments, the delayed addition of a plateau concentration of CSF-1 to cultures begun with plateau GM-CSF did not alter the total number of colonies, the number of macroscopically visible colonies, or the differentiation of progeny within colonies.

Table 2 shows formation of colonies in which the only differentiated cells were neutrophils with or without occasional eosinophils, macrophages, or a combination of the two. Additional information regarding the actual proportions of macrophages, neutrophils, or immature cells in colonies is shown in Fig 1. The delayed addition of plateau GM-CSF to cultures begun with plateau CSF-1 decreased the proportion of macrophages in colonies, while increasing the proportion of neutrophils and immature cells. In contrast, delayed

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Colonies per Culture</th>
<th>Cellular Composition of Colonies (%)</th>
<th>Macroscopic Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MAC</td>
<td>NEUT</td>
</tr>
<tr>
<td>CSF-1</td>
<td>25.0 ± 2.7</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>18.5 ± 2.1</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>CSF-1 + GM-CSF</td>
<td>24.0 ± 2.1</td>
<td>3</td>
<td>25</td>
</tr>
</tbody>
</table>

The means and SD are shown for groups of four cultures from one of three experiments with the same outcome. Twenty-six to 40 differential counts were performed for each group. The proportion of colonies that were macroscopically visible is also given as a percentage.
addition of CSF-1 did not alter the composition of colonies. As a control, GM-CSF was added on days 3, 4, or 5 to cultures initiated without added CSF. Under these conditions colony formation was sparse, consistent with initial deprivation of added CSF and a short culture period thereafter.

Next, we directly tested the hypothesis that GM-CSF could modify the growth and differentiation of colonies that were already induced by CSF-1. Cultures were established with 88 pm CSF-1/L and on day 3 of culture, the dishes were examined, using an inverted phase microscope. The location of each group of eight cells or more was identified by placing an ink mark on the dish under each clone. Then, five control cultures received 20 sL of α MEM, while five other cultures received 20 μL of GM-CSF to achieve a concentration of 4.8 pm GM-CSF/L. After four additional days of culture, the mapped colonies were counted and smeared for differential counts. Of 70 clones identified in the control (α MEM) cultures, 63 were found after the total culture period (90% survival of mapped clones). Of 61 clones identified on the GM-CSF plates, 57 (93%) survived. The formation of macrophage, neutrophil, or mixed-neutrophil/macrophase colonies is shown in Table 4. It can be seen that 83% of the colonies elicited by CSF-1 alone contained macrophages but no neutrophils or eosinophils. GM-CSF switched differentiation, so that 75% of colonies produced neutrophils, and in 11% the only differentiated cells were neutrophils. Data for the proportions of various cell types within colonies are shown in Table 4. It is shown that the addition of GM-CSF induced an increase in the proportion of neutrophils, and increased the production of immature cells. In addition, 44% of the mapped colonies were macroscopically visible if the delayed stimulus of GM-CSF was applied; only 17% of mapped control colonies were macroscopically visible.

**DISCUSSION**

In intact animals, there is a compensatory increase in granulocytosis following use of neutrophils, so that the reserve of mature granulocytes is replaced. The mechanism by which granulocyte production is up- or down-regulated is not clear; CSF-1 and GM-CSF are candidate "poietins" for directing the proliferation and differentiation of progenitors for neutrophils and macrophages. The two CSFs differ in fundamental ways, interacting with different cellular receptors. Furthermore, the differentiating effect of CSF-1 is relatively lineage-specific for macrophages. In contrast, GM-CSF promotes neutrophil production, and can also stimulate the production of some megakaryocytes, eosinophils, and normoblasts. Consequently, it has been proposed that CSF-1 participates in regulating the production of monocyte/macrophages, while GM-CSF is a regulator of neutrophil production and perhaps other lineages. There also appear to be different regulatory processes for production of the two CSFs. CSF-1 is constitutively produced in biologically active quantities by unstimulated cells in lung, spleen, and other tissues, but GM-CSF appears to be produced in small amounts until stimulated,
when its production increases substantially. Constitutively produced CSF-1, which circulates in the plasma, may be delivered to granulocyte/macrophage progenitors in the steady state, providing sustained or even continuous stimulation by CSF-1. These CSF-1-stimulated clones could then also be stimulated by pulses of GM-CSF. Such an interaction between CSF-1 and GM-CSF may be important in regulating granulocyte/macrophage production.

In the present study submaximally stimulatory concentrations of CSF-1 and GM-CSF predominantly stimulated the formation of macrophages, whether alone or in combination. CSF-1 and GM-CSF together, at low concentrations, exerted a synergistic effect on the size of colonies. These data are compatible with a direct effect of the CSFs on progenitors, but because we studied unseparated marrow, accessory cells such as macrophages or lymphocytes may have contributed to the growth of colonies. Also, we cannot exclude a contributing action by added serum, since enhancement of colony formation by serum has been observed by Koike et al, contributing action by added serum, since enhancement of colony size by serum has been observed by Koike et al,

In maximally stimulatory concentrations, CSF-1 favored the production of macrophages, whereas GM-CSF promoted production of many more neutrophils and immature cells. GM-CSF also induced formation of more macroscopic colonies. These data are consistent with a predominance of the action of GM-CSF over that of CSF-1. It can be speculated that this is a consequence of the ability of GM-CSF to substantially down-regulate the number of CSF-1 receptors; down-regulation of GM-CSF receptors by CSF-1 is much less pronounced. Such down-regulation could induce a relative unresponsiveness to CSF-1, although this has not been directly tested. Another explanation for the ability of GM-CSF to induce CSF-1-responsive clones to produce neutrophils is that CSF-1 may promote the growth of a subset of GM-CSF-responsive progenitors that are relatively dormant until stimulated.

Our observation of enhancement of colony size is consistent with the report of Williams et al. They also noted an increase in the number of colonies in response to a combination of maximally stimulatory concentrations of the CSFs; we did not. In addition, we observed a greater range of maximally stimulatory concentrations of GM-CSF than did Williams et al. However, there are important differences between their experiments and ours. They studied a highly enriched population of progenitors, we studied unfractionated whole marrow populations. In addition, Williams et al studied progenitors from 5-fluorouracil (FU)-treated mice; our marrow was from normal mice. It has been established that the pool sizes, kinetics, and responsiveness of regenerating marrow to stimulators are strikingly different from that of normal marrow.

Additional information is derived from the experiments in which we initiated cultures with one factor, and later added the other factor. Consistent with the predominance of the action of GM-CSF over CSF-1, the delayed addition of CSF-1 had no demonstrable effect on colonies begun with GM-CSF. In contrast, delayed addition of GM-CSF to cultures initiated with CSF-1 stimulated the production of neutrophils and immature cells. The hypothesis that GM-CSF could induce neutrophil production in clones initiated with CSF-1 was tested in mapping experiments, in which GM-CSF-induced neutrophil production in already established CSF-1-responsive clones. The >90% survival of mapped clones excluded the possibility that GM-CSF had acted by selectively eliminating colonies that would have produced macrophages. Thus, the data suggest that CSF-1 either alone or together with a serum or accessory cell factor, sustains a population of progenitors with the potential for producing neutrophils. These data provide an interesting parallel to the studies of Bradley and Hodgson and Bartelmez and Stanley, who proposed that CSF-1 could act synergistically with other factors to promote the growth of...
very primitive progenitors that could then produce macrophages. The present studies demonstrate that individual clones of granulocyte/macrophage progenitors are responsive to both CSF-1 and GM-CSF. However, when the two CSFs are present in maximally stimulatory concentrations, the action of GM-CSF predominates and neutrophil production is favored. It is also concluded that in addition to stimulating the production of macrophages, CSF-1 may participate in promoting the growth of a population of progenitors for neutrophils, which may act as a ready reserve for enhancing neutrophil production during periods of increased demand.

ACKNOWLEDGMENT

The authors thank Kelbey Leach for aid in preparing the manuscript.

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Stimulation of neutrophil production in CSF-1-responsive clones

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