Interferon-γ Stimulates the Survival and Influences the Development of Bipotential Granulocyte-Macrophage Colony-Forming Cells

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NEUTROPHILS and macrophages have a central role in host defence. They have the ability to phagocytose and break down microorganisms and particulate matter, and produce mediators of the inflammatory response such as platelet-activating factor and cytokines. During the course of an infection, the rate of production of these cells from the bone marrow (BM) can be increased, perhaps due to the release of cytokines from specific areas of infection that influence the process of hematopoesis in the BM.1,2 Neutrophils and macrophages are derived from a committed progenitor cell population, the granulocyte-macrophage colony-forming cells (GM-CFC). The proliferation and development of these cells and their progeny can be promoted in vitro by a number of distinct hematopoietic growth factors such as interleukin-3 (IL-3), macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF).3-7 Although these growth factors have been shown to act directly on the neutrophil/macroage progenitor cell population,8,9 the direct effects of other cytokines that mediate the inflammatory response have not been assessed. The effects of cytokines such as the interferons (IFN),10-14 which have been shown to have an antiproliferative effect on mixed populations of BM cells, have not been investigated on highly enriched populations of stem and progenitor cells. One cytokine that is produced by activated T lymphocytes during infection is IFN-γ.15-22 IFN-γ has a range of immunoregulatory activities including macrophage activation, stimulation of cytokine production, and regulation of major histocompatibility complex expression on various cell types.23,24 Although these effects on mature cells are now well defined, the effect of IFN-γ on the development of hematopoietic progenitor cells remains relatively unexplored. Using heterogenous populations as target cells, IFN-γ has been shown to inhibit the proliferation of mast cell precursors,25 and also myeloid progenitor cells from patients with chronic myelogenous leukemia,26,27 hairy cell leukemia,28 or myelodysplastic syndrome.29 IFN-γ has also been reported to promote HL-6030 cells and normal myeloid progenitor cells31,32 to undergo monocytic development. Furthermore, IFN-γ also stimulates proliferation of hematopoietic progenitor cells when combined with IL-3.33 To determine whether such effects extend to direct stimulation of myeloid progenitor cell development, we have explored the effects of IFN-γ on a highly enriched population of GM-CFC.

MATERIALS AND METHODS

Preparation of a highly enriched population of GM-CFC. Murine BM cells highly enriched in GM-CFC were prepared as described previously93 based on the methods developed by Williams et al.36 Briefly, cells from the femora of cyclophosphamide-treated mice were collected and then fractionated using metrizamide density gradient centrifugation.37 The cells were then suspended in Fischer's medium (GIBCO, Middlesex, UK) supplemented with 5% (vol/vol) horse serum (Northumbria Biologicals, Northumberland, UK) and elutriated with a Beckman J2-21M elutriation centrifuge (Beckman Instruments, Palo Alto, CA) at a rotor speed of 2,000 rpm at 22°C. Cells enriched for GM-CFC were pooled and used for the experiments described below. This population has previously been shown to consist of greater than 90% GM-CFC.33,34 Morphology and histochemical analyses were performed as described previously.10 Measurement of [3H]-thymidine incorporation. Elutriated GM-CFC were incubated with appropriate additives for 16 hours as previously described,9 then DNA synthesis was determined using the [3H]-thymidine incorporation assay as described by Whetton et al.38 Colony-forming assays. Soft agar colony-forming assays were performed as described previously.39 Colonies (more than 50 cells) were counted at 7 days. The morphology of the cells was analyzed after May-Grünwald-Giemsa staining of preparations made from individual colonies.40 In such experiments the morphology of
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greater than 50 colonies per experimental condition was determined. Where elutriated cells were preincubated before GM-CFC assays (or intracellular pH [pHi] measurements, see below) they were cultured in Iscove’s medium supplemented with 20% (vol/vol) horse serum plus any other additives.

**Determination of pHi.** pHi measurements were performed using the pH-sensitive fluorescent probe bis(carboxyethyl)carboxyfluorescein (BCECF) as described by Whetton et al. \(^4\)

**Growth factors.** Purified recombinant murine IL-3 and recombinant murine GM-CSF were supplied by Biogen SA (Geneva, Switzerland) as material containing 1.25 \(\times\) \(10^6\) U/mg protein and 2.1 \(\times\) \(10^4\) U/mg protein, respectively. Native M-CSF was purified from L-cell-conditioned medium to stage IV of the method described by Stanley and Heard. \(^5\) Before the experiments, the growth factors were assessed for colony-stimulating activity using normal murine BM cells. Maximum colony formation was observed using 100 U/mL IL-3, 50 U/mL GM-CSF, and 20 U/mL M-CSF. Recombinant murine IFN-\( \gamma \) was prepared and purified as previously described. \(^4,5\)

**RESULTS**

**Antiproliferative effect of IFN-\( \gamma \) on colony formation by purified GM-CFC.** When highly enriched GM-CFC were plated out in increasing doses of IFN-\( \gamma \), irrespective of the growth factor used to stimulate colony formation (ie, M-CSF, IL-3, or GM-CSF), there was a dose-dependent decrease in the number of colonies formed in the presence of IFN-\( \gamma \) (Fig 1). Maximal inhibition was observed at a IFN-\( \gamma \) concentration of 1,000 U/mL (although a concentration of 100 U/mL had a similar effect). In the presence of IL-3, GM-CSF, or M-CSF, the inhibition observed was 43% \(\pm\) 4%, 28% \(\pm\) 6%, and 30% \(\pm\) 5% (mean \(\pm\) standard deviation, \(n > 6\)). When added alone, IFN-\( \gamma \) (1 to 1,000 U/mL) was unable to stimulate the formation of any colonies or clusters from the GM-CFC population described. GM-CSF and IL-3 stimulated the production of mainly neutrophil/macrophage colonies from GM-CFC, while M-CSF promoted the development of predominantly macrophage colonies from the same precursor cell population. To investigate the effect of IFN-\( \gamma \) on the types of cells that developed from GM-CFC in colony-forming assays, colonies were picked out and the morphology of the mature cells determined. Colonies grown in M-CSF in the presence or absence of IFN-\( \gamma \), respectively, were morphologically very similar (>95% macrophages); however, the colonies grown in IFN-\( \gamma \) plus IL-3 were now a mixture of neutrophil/macrophage and macrophage colonies (see Table 1). IFN-\( \gamma \) stimulated an increase in the numbers of macrophages in colonies formed in the presence of IL-3, although, when added alone, no colony (or cluster) formation of any type was observed.

The inhibitory effect of IFN-\( \gamma \) on colony formation was reversible. When GM-CFC were cultured with CSFs \(\pm\) IFN-\( \gamma \) (100 U/mL) for 24 hours, washed, and plated out in soft agar there was no difference in the plating efficiency of the CFCs in the presence of GM-CSF, IL-3, or M-CSF. Furthermore, the morphology of the colonies formed from cells pretreated with IFN-\( \gamma \) in liquid culture were the same as those from control cultures.

To confirm that the IFN-\( \gamma \) was directly affecting GM-CFC, the ability of this cytokine to inhibit CSF-mediated DNA synthesis over an 18-hour period was assessed. In the presence of M-CSF, GM-CSF, or IL-3 there was a marked inhibition of [\( ^3\)H]-thymidine incorporation in GM-CFC (Fig 2A). The degree of inhibition observed with 1,000 U/mL IFN-\( \gamma \) (compared with control cultures) was 49% \(\pm\) 10%, 73% \(\pm\) 4%, and 45% \(\pm\) 10% (mean \(\pm\) standard deviation on six observations) for IL-3–, GM-CSF–, and M-CSF–stimulated [\( ^3\)H]-thymidine incorporation, respectively. The somewhat higher degree of inhibition observed in these assays compared with colony-forming assays may be due to a desensitization of the cells to the IFN-\( \gamma \) over the longer period of the colony-forming assays (see below).

**IFN-\( \gamma \) can promote the survival of GM-CFC.** In the presence of CSFs, IFN-\( \gamma \) inhibited DNA synthesis (see Fig 2A). However, the addition of IFN-\( \gamma \) to the GM-CFC in the

![Fig 1. The effect of IFN-\( \gamma \) on growth factor-stimulated colony formation in soft agar. Concentration of growth factors used were IL-3, 100 U/mL; GM-CSF, 50 U/mL; and M-CSF, 20 U/mL. Results shown here are the mean of greater than six experiments \(\pm\) standard deviations. A colony-forming efficiency of approximately 30% was routinely obtained in the presence of IL-3 (100 U/mL), which resulted in the formation of about 150 colonies per 500 cells plated (if no inhibitory agents were added). A similar plating efficiency was achieved if either GM-CSF or M-CSF were added at optimal concentrations.](www.bloodjournal.org)
Table 1. The Morphology of Colonies Grown in the Presence of Either IL-3 or IL-3 Plus IFN-γ (100 U/mL) Were Compared and the Number of Macrophages and Neutrophils Present Determined

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>&lt;10%</th>
<th>10-50%</th>
<th>50-90%</th>
<th>&gt;90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>7</td>
<td>57</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>IL-3 + IFN-γ</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>45</td>
</tr>
</tbody>
</table>

Colonies consisted of mature neutrophils and macrophages in different proportions (see Cook et al).

absence of CSFs led to a modest but significant increase in DNA synthesis over 24 hours (see Fig 2B). Nevertheless, this effect was not sustained and by 72 hours there was little, if any elevation in [³H]-thymidine incorporation over control incubations, suggesting some desensitization to the effects of IFN-γ. This transient increase in proliferation mediated by IFN-γ was also reflected in the retention of the ability of GM-CFC to form colonies in soft agar. In the absence of the appropriate growth/survival factor(s) in liquid culture, the GM-CFC steadily lost the capacity to form colonies when plated in soft agar. In the presence of IL-3, the colony-forming potential of the cells was maintained (Fig 3). Similarly, even after 72 hours in the presence of IFN-γ there were a significant number of GM-CFC.
interferon-γ effects on gm-cfc

M-CSF

GM-CSF

IL-3

Fig 3. The effect of IFN-γ on the maintenance of colony-forming potential of highly enriched GM-CFC. Cells were incubated with (△) IL-3 (100 U/mL), IFN-γ ([●] 0.1 U/mL; [□] 1 U/mL; [■] 10 U/mL; [■] 100 U/mL; [●] 1,000 U/mL), or (○) no addition. At the times shown the cells were washed and plated out in the growth factors M-CSF (20 U/mL), IL-3 (100 U/mL), and GM-CSF (50 U/mL) to determine the effects of the above agents on the survival of GM-CFC in liquid culture. Results shown are the means ± standard deviations of three experiments.

present, while there were none remaining in control cultures. Medium conditioned by elutriated cells pretreated with IFN-γ (100 U/mL) for 24 hours was unable to stimulate any increase in the survival or [3H]-thymidine incorporation of fresh, elutriated GM-CFC, indicating that CSF production is not stimulated by IFN-γ from the cell population isolated.

IFN-γ acts directly on GM-CFC to elicit a signal associated with proliferation in GM-CFC. To show a direct effect of IFN-γ on GM-CFC we have assessed the ability of this cytokine to stimulate the Na+/H+ antiport to elevate pHi, a cellular signalling system that is associated with proliferation in GM-CFC.10

IFN-γ stimulated an increase in the pHi of the elutriated cells. The effect was observed within 1 minute of the addition of IFN-γ, and was dependent on the dose of the cytokine added. The maximal increase observed was 0.06 ± 0.01 (n = 5, mean ± standard deviation; see Fig 4). This response was equivalent to that seen when other survival/growth factors were added to GM-CFC,16 indicating that the majority of the cells present were responsive to IFN-γ. The dose-response relationship observed was similar to that seen in the IFN-γ-stimulated DNA synthesis experiments described above (see Fig 2A and B). Because IFN-γ-stimulated increases in pHi were totally abrogated by the addition of the amiloride analog 5-methyl N-isopropyl amiloride (5-MNIA; 10 μmol/L), it is probable that the increase in pHi observed is due to the activation of the Na+/H+ antiport.16 IFN-γ did not maintain an increase in pHi because the resting pHi was the same in IFN-γ-treated (100 U/mL) and control incubations after 18 hours. However, this overnight incubation with IFN-γ did inhibit the ability of the mitogen M-CSF to stimulate an increase in pHi. After preincubation (18 hours) with IFN-γ (100 U/mL), followed by washing and preparation of the cells for pHi measurements, M-CSF (20 U/mL) elicited an increase in intracellular pHi of 0.06 ± 0.02 (mean ± SD, n = 5). However, cells incubated overnight in the absence of IFN-γ exhibited an M-CSF-stimulated increase in pHi of 0.12 ± 0.03 (mean ± SD, n = 6). Phorbol ester-stimulated increases in pHi were not affected by pretreating the cells with IFN-γ, evidence that the Na+/H+ antiport itself is not inhibited by the actions of this cytokine and that IFN-γ affects the signal transduction pathway between the M-CSF receptor and the Na+/H+ antiport.

Discussion

IFN-γ is a cytokine produced by T lymphocytes when stimulated with antigen or other agents such as mitogens or alloantigens. A wide range of cells respond to this product of activated T lymphocytes, including B lymphocytes (stim-
one of the major roles of IFN-γ is as an agent that promotes production of other, distinct cytokines and signalling molecules. Previous reports have indicated that IFN-γ has some ability to induce the development of macrophages from myeloid progenitor cells and suppress neutrophilic development.\textsuperscript{31,36,37} Our data also indicate that IFN-γ may influence lineage restriction of GM-CFC, although IFN-γ does not possess macrophage colony-stimulating activity. Yet there is an ability of this cytokine to promote the survival of GM-CFC such that the capacity to form colonies in soft agar in the presence of the appropriate stimuli is maintained. This promotion of survival is unlikely to be due to the paracrine production of hematopoietic growth factors, because the effect is observed at low cell densities and also IFN-γ-treated elutriated cells show no sign of producing the quantities of cytokines that would elicit the responses observed. There are therefore two direct effects of IFN-γ on GM-CFC, an ability to decrease the rate of cell death and also to inhibit the proliferation of GM-CFC.

These two effects of IFN-γ can also be seen in biochemical assays. We have shown that Na\textsuperscript{+}/H\textsuperscript{+} antiport activation is associated with growth factor-stimulated proliferation in GM-CFC. IFN-γ can also transiently stimulate the Na\textsuperscript{+}/H\textsuperscript{+} antiport. The inhibitory effects of IFN-γ do not, however, appear to be transient. The ability of hematopoietic growth factors to stimulate colony formation and increase intracellular pH in GM-CFC are persistently inhibited by IFN-γ.

The developmental consequences of the addition of IFN-γ are perhaps equally surprising in that there is an effect on the type of cell formed from GM-CFC, although IFN-γ does not stimulate colony formation directly. In agreement with previous reports, we have found that IFN-γ induces increased monocytic development at the expense of neutrophilic development.\textsuperscript{31,36,37} Whether this ability to influence the proportion of macrophages and neutrophils produced from GM-CFC has any physiologic significance remains to be determined. It is, however, possible that IFN-γ promotes the formation of macrophages specifically to combat viral infection, a role for which neutrophils are less well suited. Our data suggest that the regulation of survival, development, and lineage commitment of GM-CFC may not be limited to the CSFs, and that other cytokines may profoundly influence these features of the biology of committed neutrophil macrophage CFC. It will be of interest to establish whether other cytokines that mediate the inflammatory response, but have no direct effect on GM-CFC development, can also modulate the lineage restriction and survival of these cells.

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