Effects of Hematopoietic Suppressor Molecules on the in Vitro Proliferation of Purified Murine Granulocyte-Macrophage Progenitor Cells

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ABSTRACT

Highly purified murine granulocyte-macrophage progenitor cells (CFU-GM) were used as target cells to assess the possible direct effects of purified preparations of recombinant human γ-interferon, prostaglandin E, recombinant human heavy chain (acidic) ferritin, and recombinant human granulocyte-macrophage progenitor cells in vitro. Target CFU-GM, with cloning efficiencies of up to 84% and containing 0–3% morphologically recognizable accessory cells at the initiation of the culture period, were plated at a density of 100–150 cells/dish in the presence or absence of pure suppressor molecules. Colony formation was stimulated with either crude pokeweed mitogen-stimulated mouse spleen conditioned medium, pure natural murine macrophage colony-stimulating factor, or pure recombinant murine granulocyte-macrophage colony-stimulating factor. All four suppressor molecules were active in vitro against purified CFU-GM as assessed by their ability to inhibit colony or cluster formation. No apparent difference in the degree of responsiveness to prostaglandin E, γ-interferon, or human heavy chain (acidic) ferritin was noted in the presence of pokeweed mitogen-stimulated mouse spleen conditioned medium, granulocyte-macrophage colony-stimulating factor, or macrophage colony-stimulating factor. In contrast, TNF-α in cultures containing macrophage colony-stimulating factor slightly, but significantly, potentiated colony formation. TNF-α also appeared more active at suppressing colony formation at lower concentrations in pokeweed mitogen-stimulated mouse spleen conditioned medium than in granulocyte-macrophage colony-stimulating factor-stimulated cultures of purified CFU-GM. The results suggest that TNF-α, human heavy chain (acidic) ferritin, γ-interferon, and prostaglandin E can act directly at the progenitor cell level.

INTRODUCTION

The production of mature myeloid cells from primitive hematopoietic stem and progenitor cells in the blood-forming organs appears to involve a balance between inhibitory and stimulatory molecules (reviewed in Refs. 1 and 2). The CSFs are a group of functionally related glycoproteins which stimulate granulocyte and monocyte/macrophage production in vivo and in vitro (1, 2). The actions of the CSFs are thought to be modulated by hematopoietic “suppressor” molecules such as PGE, TNF-α, TNF-β, HF, IFN-γ, IFN-β, and IFN-α and IFN-β under normal conditions, and aberrant responses to these factors in vitro have been observed with cells from patients with leukemia (1, 2). It has recently been demonstrated that the CSFs act directly upon CFU-GM in vitro (3) using highly purified CFU-GM with few or no recognizable accessory cells (lymphocytes, monocytes, fibroblasts, endothelial cells) (4).

In the present studies, we investigated whether the actions of PGE, IFN-γ, TNF-α, and HF could be observed using highly purified murine bone marrow CFU-GM (4). The cellular composition of the target cell population used in these studies has been reported (4) and contains 0–3% accessory cells at the start of the culture period. The need for target CFU-GM with few or no accessory cells is based upon studies which have shown that the suppressor molecules and the CSFs can stimulate the in vitro production of other growth and suppressor factors from accessory cells (1, 2, 5–15) which could mask an effect of these factors on CFU-GM. We have also examined the effects of different colony-stimulating factors on our ability to observe the inhibitory effects of these suppressor molecules. The data suggest that PGE, TNF-α, IFN-γ, and HF can exert their influences directly upon the progenitor cells (CFU-GM).

MATERIALS AND METHODS

Animals. Six- to 8-week-old female C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice were purchased from Cumberland View Farms, Clinton, TN. Purification of Murine CFU-GM. The method for obtaining highly purified murine CFU-GM has been published (4). Briefly, age-matched cohorts of B6D2F1 mice were given an i.p. injection of cyclophosphamide (200 mg/kg; Bristol Myers, Syracuse, NY) 3 days prior to sacrifice. Tibial and femoral marrow cells from 20–40 mice were pooled, washed twice in cold Dulbecco’s phosphate-buffered saline, separated on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient, and further fractionated by counterflow centrifugal elutriation with a Beckman J2-21M elutriation system (Beckman Instruments, Palo Alto, CA) equipped with a Sanderson chamber (Beckman). Cells eluting at a flow rate of 28 ml/min (FR-28) were collected, washed twice in cold Dulbecco’s phosphate-buffered saline, and resuspended in McCoy’s 5A medium (Gibco, Grand Island, NY) with 10% heat-inactivated (56°C, 30 min) FBS (Hyclone, Logan, UT). Viable cell counts were obtained using trypan blue dye exclusion and were uniformly >99%. Cells were stored on ice until needed.

CFU-GM Culture System. One hundred or 150 FR-28 cells were suspended in 1 ml of a mixture of McCoy’s 5A medium with 20% FBS and 0.4% agarose (FMC Corp., Rockland, ME). Colony formation was stimulated with either 10% (v/v) PWMSCM, 100 units of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), kindly provided by Dr. Steven Gillis, Immunex Corporation, Seattle, WA, or 100 units of purified natural murine L-cell CSF-1 (CSF-1, a generous gift of Dr. Richard K. Shadduck, Pittsburgh, PA). These concentrations represent plateau concentrations of PWMSCM, GM-CSF, and CSF-1 (3). Cells were incubated for 7 days in a fully humidified atmosphere of 5% CO2, 5% O2, and 90% N2 at 37°C. Colonies (>50 cells) and clusters (3–49 cells) were scored with a Nikon inverted microscope at ×32.

Suppressor Molecules. Purified natural prostaglandin E (PGE) was purchased from Sigma Chemical Co., St. Louis, MO. Recombinant human TNF-α was a generous gift of Dr. H. Michael Shepard, Genentech Corporation, South San Francisco, CA, and had a specific activity of 5 × 108 units/mg protein (16). Recombinant murine IFN-γ was also kindly provided by Dr. Shepard and had a specific activity of 1.4 × 107 units/mg protein. Purified recombinant human HF was kindly provided.
by Drs. Paolo Arosio and Sonia Levi, Milan, Italy (17). All molecules were diluted to the appropriate concentration in McCoy’s 5A medium with 10% FBS and added to plates on day 0 in a final volume of 100 μl. Control plates were prepared with the diluent only.

Data Analysis. Colony and cluster formation in the presence of diluent (McCoy’s plus 10% FBS) plus the appropriate CSF was used as a control. Data are expressed as the mean (± SD) percentage change from control. A negative percentage change indicates inhibition of colony formation whereas a positive change signifies enhanced colony formation. Triplicate plates were set up at each concentration of test material and 2-4 experiments were performed. Student’s t test was used to determine whether 2 sample means were significantly different.

RESULTS

Characterization of Purified Murine FR-28 CFU-GM. The percentage of cells plated which formed a colony or cluster in the presence of plateau concentrations of PWMSCM, CSF-1, or GM-CSF is illustrated in Table 1. As seen in previous studies (3), PWMSCM stimulates colony or cluster formation by a greater percentage of FR-28 cells than either purified CSF-1 or GM-CSF alone. Thus, CSF-1 and GM-CSF stimulate subpopulations of CFU-GM in FR-28.

Effects of Suppressor Molecules on Purified CFU-GM. Purified FR-28 CFU-GM (100–150 cells) were plated in soft agarose cultures in the presence or absence of various concentrations of PGE, TNF-α, IFN-γ, or HF. CFU-GM growth was stimulated with either PWMSCM, CSF-1, or GM-CSF to assess whether CFU-GM responsive to one growth factor or another would exhibit a differing pattern of responsiveness to these suppressors.

No apparent difference in the response to PGE, was observed in these studies in cultures stimulated by PWMSCM, CSF-1, or GM-CSF (Table 2). At 10^-4 M PGE, approximately 70% inhibition of colony or cluster growth was observed and the suppressive effect was lost at 10^-6 M PGE, (Table 2).

TNF-α demonstrated differences in the pattern of CFU-GM responsiveness when cultures were stimulated with PWMSCM, CSF-1, or GM-CSF. Significant suppression (P at least <0.05) of PWMSCM-stimulated colony and cluster formation was observed with 10–1000 units of TNF-α, with a maximum inhibition of 40–50% (Table 2). A similar degree of suppression with 1000 units of TNF-α in GM-CSF-containing cultures was noted, except TNF-α activity was not significant (P > 0.05) with 10 units (Table 2). In contrast, the effect of TNF-α on CFU-GM growth in CSF-1-stimulated cultures resulted in small but significant (P < 0.05) enhancement of colony and cluster growth with 100 and 1000 units per culture dish (Table 2).

Highly purified FR-28 CFU-GM were inhibited by 10^-8–10^-9 M HF in cultures stimulated by PWMSCM, CSF-1, or GM-CSF (Table 2). At 10^-8 M HF, GM-CSF-stimulated CFU-GM were slightly but not significantly (P > 0.05) more sensitive to the inhibitory effects of HF than PWMSCM- or CSF-1-stimulated CFU-GM.

IFN-α, at 10–100 units, inhibited CFU-GM colony formation in cultures stimulated by PWMSCM, CSF-1, and GM-CSF (Table 2). With GM-CSF, inhibition appeared to be less than that seen with PWMSCM or CSF-1; however, this was not significant (P > 0.05).

DISCUSSION

The study of the regulation of myelopoiesis has been facilitated by the purification and/or cloning of putative regulatory molecules. Until recently, however, it was not possible to collect myeloid progenitor cells of sufficient purity to conduct studies of the direct effects of these molecules on proliferation or differentiation. Using our recently published method for CFU-GM purification (4) we conducted the present studies with target cells of high purity, and either the absence or a low level of contamination by accessory cells, to investigate the direct effects of pure IFN-γ, HF, TNF-α and PGE, on CFU-GM proliferation.

We have previously noted that PGE (18), recombinant human TNF-α (16), recombinant human HF (17), and recombinant murine IFN-γ can suppress colony formation of CFU-GM present in unseparated populations of murine bone marrow. We have now demonstrated that these molecules can suppress the in vitro growth of purified CFU-GM. The fact that the population of cells contained up to 84% CFU-GM, the percentage of accessory cells was zero to 3% in the target cell population (3), and only 100–150 cells were plated per culture strongly suggests that the observed effects with all of the suppressor molecules were mediated directly at the progenitor cell level and did not require the presence of accessory cells at the initiation of the culture period.

Some differences were noted when TNF studies were carried out in cultures stimulated with PWMSCM, CSF-1, or GM-CSF. Most surprising was the apparent enhancement, although slight, of CFU-GM growth when TNF-α and CSF-1 were present throughout the culture period. This is unusual since most previous studies with TNF-α have shown that it inhibits normal CFU-GM growth (16, 19–21). However, these previous studies were not done with purified CFU-GM and the influence of TNF-α or CSF-stimulated cytokine production (5-9, 11, 12, 14, 15) in the cultures is not known. It may also be that a unique subset of CFU-GM has been isolated in FR-28 during our purification procedure. It was also noted that inhibition of CFU-GM grown in PWMSCM was observable at lower concentrations than that observed in GM-CSF-containing cultures. The crude PWMSCM is known to contain IFN-γ (22) which can synergize with TNF-α (16, 19) and thus the combined effects of more than one factor may be occurring in this case.

PGE, HF, and IFN-γ demonstrated no significant difference in the degree of suppression they induced when culturing CFU-GM with either PWMSCM, CSF-1, or GM-CSF (4). It has been reported that PGE, at low concentrations, is specific for macrophage progenitors (18); however, in the present studies, CSF-1-stimulated cultures, which contain almost exclusively macrophage colonies, were no more sensitive to the effects of PGE, at any concentration, than cultures stimulated with PWMSCM or GM-CSF. This may again reflect the use of heterogeneous target cell populations in earlier studies (18) or the use of subpopulations of macrophage progenitors in our studies.

The data reported herein suggest that the inhibitory effects

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Colonies ±</th>
<th>Colonies +</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWMSCM</td>
<td>43 ± 7</td>
<td>73 ± 9</td>
</tr>
<tr>
<td>CSF-1</td>
<td>29 ± 3</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>33 ± 1</td>
<td>54 ± 3</td>
</tr>
</tbody>
</table>

* Unpublished observations.

** Unpublished observations.
also. Thus, many of the recognized regulators of CFU-GM have shown that these molecules act at the progenitor cell level. IFN-7, and PGE, (23). Our previous studies with the CSFs (3) of PGE, IFN-7, HF, and TNF-a may be mediated directly at the progenitor cell level. This substantiates and extends our still containing some accessory cells, which demonstrated formation units (erythrocytes), isolated up to a 44% purity but other studies using human bone marrow CFU-GM and blast- of the progenitor cell level. This substantiates and extends our}

### REFERENCES


### TABLE 2 Effects of pure PGE, TNF-α, HF, and IFN-γ on the growth of purified murine CFU-GM stimulated with either PWMSCM, CSF-1, or GM-CSF

<table>
<thead>
<tr>
<th>Addition to culture</th>
<th>PWMSCM</th>
<th>CSF-1</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGE</strong>&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; M</td>
<td>−68 ± 3%/−57 ± 6%</td>
<td>−76 ± 3%/−59 ± 2%</td>
<td>−70 ± 4%/−65 ± 6%</td>
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<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt; M</td>
<td>−54 ± 2%/−47 ± 5%</td>
<td>−47 ± 5%/−40 ± 5%</td>
<td>−50 ± 6%/−40 ± 6%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>−29 ± 2%/−24 ± 5%</td>
<td>−24 ± 5%/−22 ± 5%</td>
<td>−32 ± 2%/−18 ± 2%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt; M</td>
<td>−19 ± 5%/−15 ± 3%</td>
<td>−19 ± 4%/−18 ± 2%</td>
<td>−21 ± 2%/−12 ± 2%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-2&lt;/sup&gt; M</td>
<td>−6 ± 4%/−11 ± 6%</td>
<td>−3 ± 2%/−11 ± 3</td>
<td>0 ± 1/0 ± 3</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,000 units</td>
<td>−47 ± 2%/−40 ± 1%</td>
<td>+16 ± 4%/+12 ± 4%</td>
<td>−30 ± 1%/−40 ± 4%</td>
</tr>
<tr>
<td>100 units</td>
<td>−29 ± 5%/−27 ± 2%</td>
<td>+15 ± 4%/+9 ± 3%</td>
<td>+33 ± 6%/+21 ± 5%</td>
</tr>
<tr>
<td>10 units</td>
<td>−14 ± 3%/−18 ± 4%</td>
<td>+33 ± 1%/−14 ± 4</td>
<td>−38 ± 5%/−4 ± 4</td>
</tr>
<tr>
<td>1 unit</td>
<td>0 ± 1%/−4 ± 2</td>
<td>0 ± 1%/−4 ± 2</td>
<td>0 ± 1%/−4 ± 2</td>
</tr>
<tr>
<td><strong>HF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; M</td>
<td>−27 ± 3%/−30 ± 2%</td>
<td>−22 ± 7%/−32 ± 1%</td>
<td>−41 ± 6%/−30 ± 2%</td>
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<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt; M</td>
<td>−13 ± 2%/−13 ± 4%</td>
<td>−13 ± 1%/−21 ± 5%</td>
<td>−21 ± 2%/−18 ± 2%</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>−1 ± 1%/−1 ± 2</td>
<td>−4 ± 3%/−1 ± 2</td>
<td>0 ± 1/0 ± 4</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 units</td>
<td>−37 ± 6%/−36 ± 5%</td>
<td>−35 ± 4%/−37 ± 1%</td>
<td>−23 ± 1%/−32 ± 1%</td>
</tr>
<tr>
<td>10 units</td>
<td>−22 ± 6%/−19 ± 5%</td>
<td>−15 ± 8%/−38 ± 1%</td>
<td>−7 ± 2%/−3 ± 1%</td>
</tr>
<tr>
<td>1 unit</td>
<td>−3 ± 4%/−6 ± 3</td>
<td>−6 ± 4%/−3 ± 2</td>
<td>−4 ± 2/±3 ± 1</td>
</tr>
</tbody>
</table>

* Data are shown as the percentage change from medium-containing control cultures of the combined results of 2–4 experiments. Colonies/colonies + clusters are shown as mean ± SD. Suppression (−) or enhancement (+) of growth shown in parentheses.

* Indicates biomolecules used to stimulate colony formation.

* Significantly different from control, P < 0.001.

* Significantly different from control, P < 0.05.

### ACKNOWLEDGMENTS

We wish to thank Stephanie Moore and Judy McConaha for excellent secretarial assistance.

### REFERENCES

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