Further Examination of the Effects of Recombinant Cytokines on the Proliferation of Human Megakaryocyte Progenitor Cells

By Edward Bruno, Ryan J. Cooper, Robert A. Briddell, and Ronald Hoffman

The effect of several recombinant cytokines, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-1α, on megakaryocyte (MK) colony formation by a normal human bone marrow subpopulation (CD34⁺ DR⁺), enriched for the MK colony-forming unit (CFU-MK), was studied using a serum-depleted, fibrin clot culture system. IL-3 and GM-CSF, but not IL-6 or IL-1α, stimulated MK colony formation by CD34⁺ DR⁺ cells. However, the addition of IL-6 to CD34⁺ DR⁺ cultures containing IL-3 resulted in the appearance of CFU-MK-derived colonies, suggesting that IL-6 requires the presence of IL-1α to exhibit its MK colony-stimulating activity (MK-CSA). Addition of neutralizing antibodies to IL-3 and GM-CSF, but not to IL-6 and IL-1α, specifically inhibited the MK-CSA of IL-3 and GM-CSF, respectively. The addition of either anti-IL-6, anti-IL-1α, or anti-IL-3 antisera to cultures containing both IL-6 and IL-1α totally abolished the MK-CSA of the IL-6/IL-1α combination. However, neither anti-IL-3 nor anti-GM-CSF antisera could totally neutralize the additive effect of the combination of IL-3 and GM-CSF on MK colony formation, indicating that these two cytokines act by affecting distinct effector pathways. These results suggest that while IL-3 and GM-CSF can directly affect CFU-MK-derived colony formation, IL-1α and IL-6 act in concert to promote de novo elaboration of IL-3 and thereby promote CFU-MK proliferative capacity.

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Materials and Methods

Bone marrow aspirates were obtained under local anesthesia from the posterior iliac crests of hematologically normal volunteers. Informed consent was obtained from the donors according to guidelines previously established by the Human Investigations Committee of the Indiana University School of Medicine, Indianapolis, which adheres to the principles of the Declaration of Helsinki.

Purification of CFU-MK: Bone marrow cells were diluted 1:1 with Iscove's Modified Dulbecco's Medium (IMDM) containing preservative-free sodium heparin (GIBCO, Grand Island, NY) at 20 U/mL and layered over an equal volume of Ficoll-Paque (specific gravity 1.077 g/cm³; Pharmacia Fine Chemicals, Piscataway, NJ). Density centrifugation was performed at 500g for 25 minutes at 4°C in a Beckman model TJ-R centrifuge (Beckman Instruments, Fullerton, CA). The interface low-density bone marrow (LDBM) mononuclear cells were collected and washed with IMDM containing no defined growth factors. A nonadherent, low-density, T-cell-depleted (NALDT⁻) bone marrow subpopulation was isolated from LDBM according to the methods of Lu et al. The NALDT⁻ cell population contained less than 2% CD14 (Becton Dickinson Monoclonal Center, San Jose, CA) positive and less than 6% CD2 (Becton Dickinson) positive cells as determined by monoclonal antibody staining and flow cytometric analysis.

To further enrich NALDT⁻ bone marrow cells for CFU-MK, we used fluorescence-activated cell sorting according to previously established methods. Cell populations containing a high density of CD34 and a low density of HLA-DR (CD34⁺ DR⁻) were isolated and used in these studies. The purity of sorted cells was determined by reanalyzing aliquots of the sorted subset on the Coulter Epics 753 Flow Cytometer (Coulter Electronics, Hialeah, FL) immediately after the sort was finished. The CD34⁺ DR⁻ cell fraction consisted of greater than 95% CD34 and HLA-DR positive cells. This population has previously been shown to contain more than 98% of the colony-forming unit, granulocyte-macrophage colony-forming unit (CFU-GM).
macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) and to exhibit a 10- to 20-fold increase of CFU-MK cloning efficiency when compared with NALDT- bone marrow cells.8

Purified and recombinant human hematopoietic growth factors and antibodies. Recombinant human IL-6 (specific activity 10^7 U/mg protein as determined by the amount required to augment the production of Ig by the CESS cell line), recombinant human IL-3 (specific activity 3.5 x 10^7 CFU/mg protein), recombinant human GM-CSF (specific activity 2.5 x 10^7 CFU/mg protein), and recombinant human IL-1α (specific activity 10^7 U/mg protein as determined by proliferative effects on D-10 cells) were purchased from the Genzyme Corp (Boston, MA). Rabbit polyclonal antibody to human IL-6 (1 mg/ml, 90% IgG, 0.5 mg of antibody neutralizes approximately 10,000 U [100 ng] of human IL-3 as determined by the 1H-TdR uptake of human bone marrow cells incubated for 96 hours with a preincubated antibody/IL-3 mixture), to human GM-CSF (1 mg/ml, 80% IgG, 1.0 mg of antibody neutralizes 1,000 U of human GM-CSF as determined by radioimmunoassay based on anti-GM-CSF:GM-CSF binding), to human IL-6 (1 mg/ml, 80% IgG, 1.0 mg of antibody neutralizes 10,000 U of IL-6 as determined by radioimmunoassay based on anti-IL-6:IL-6 binding), and to IL-1α (1.25 mg/ml, 90% IgG, 1.25 mg of antibody neutralizes 1,000 U of IL-1 as determined by the phytohemagglutinin-induced thymocyte proliferation assay) were also purchased from the Genzyme Corp.

Treatment of cytokines with polyclonal neutralizing antisera. Recombinant human cytokines were preincubated with neutralizing polyclonal antisera for 2 hours at 37°C before addition to culture. The amount of polyclonal antisera used to neutralize an optimal concentration of each cytokine was supplied in the technical data sheets provided by the Genzyme Corp and the neutralizing ability of each antisera was confirmed in our laboratory. Each cytokine was preincubated with an amount of antisera projected to neutralize a known amount of cytokine added to the particular assay. In addition, either twice or one half the precalculated amount of antisera was tested in culture (data not shown). Based on this data, the lowest concentration of antisera required to neutralize the cytokine tested was used in our studies. The neutralization of the megakaryocyte colony-stimulating activity (MK-CSA) of either IL-1α or IL-6 by polyclonal antisera could not be studied in this assay because neither IL-1α nor IL-6 have inherent MK-CSA against CD34+ DR+ bone marrow cells. However, anti-IL-1α antisera has been previously shown to neutralize numerous biological activities of IL-1α in our laboratory.8 Additionally, anti-IL-6 but not anti-IL-1α antisera was shown to neutralize the proliferative effect of IL-6 on the IL-6 responsive B9 hybridoma cell line (data not shown).

Serum-depleted assay system for megakaryocyte colony formation. CD34+ DR+ cells per milliliter, 5 x 10^5, were assayed for their ability to produce CFU-MK-derived colonies in a serum-depleted fibrin clot culture system as described by Bruno et al.23 Various doses of hematopoietic growth factors, added alone or in combination in the presence or absence of polyclonal neutralizing antisera, were used as a source of MK-CSA. The concentrations of cytokines added to individual assay were determined by data either supplied here or previously by both Bruno et al.23 and Lu et al.24 Cultures were incubated for 12 days at 37°C in a 100% humidified atmosphere of 5% CO₂ in air. After incubation, fibrin clots were fixed in situ in methanol:acetone (1:3) for 20 minutes, washed with 0.01 mol/L phosphate-buffered saline (PBS, pH 7.2), double-distilled water, and then air dried. Fixed plates were stored frozen at -20°C until immunofluorescent staining was performed.

Immunofluorescent identification of human megakaryocytic colonies. 10E5 mouse monoclonal antibody to the human platelet glycoprotein IIb-IIIa complex receptor (graciously supplied by Dr Barry S. Coller, Stony Brook, NY) was used as an immunologic probe for identifying human megakaryocytes, as previously reported.25-27

Cultures were scored in situ to enumerate fluorescein-positive colonies. The 35-mm Petri dishes were inverted, and the base area completely scanned with a fluorescent microscope at х100 (Zeiss standard microscope 18 with IV FL vertical fluorescent illuminator; Carl Zeiss, Inc, Thornwood, NY).

A colony was defined as a cluster of three or more fluorescent cells. Each experimental group was cultured in duplicate or quadruplicate.

Statistical analysis. The results were expressed as the mean ± standard error of the mean (SEM) of data obtained from two or more experiments performed in duplicate or quadruplicate. Statistical significance was determined using the Student's t-test.

RESULTS

The effects of no addition(s), IL-1α, IL-6, GM-CSF, and IL-3 on CFU-MK-derived colony formation by 5 x 10^5 CD34+ DR+ bone marrow cells are shown in Fig 1. No MK colonies appeared in the absence of an exogenous cytokine. IL-3 and GM-CSF, but not IL-1α or IL-6, were capable of promoting significant (P < .05) numbers of MK-CSF and IL-3 promoted MK colony growth over control plates (P < .001). In addition, when the number of MK colonies per CFU-MK colony was enumerated from four individual studies, there was no significant

![Fig 1. Effect of the addition of an optimal concentration of IL-1α, IL-6, GM-CSF, and IL-3 on CFU-MK-derived colony formation by normal human CD34+ DR+ bone marrow cells. Each point represents the mean ± SEM of pooled data from four separate experiments. All assays were performed in duplicate or quadruplicate. Whereas GM-CSF and IL-3 promoted MK colony growth over control plates containing no addition (P < .05), both IL-1α and IL-6 failed to enhance MK colony formation (P > .05) when CD34+ DR+ bone marrow cells were cultured.](https://www.bloodjournal.org)
difference between MK colonies stimulated by either IL-3 (3.6 ± 0.8 cells/colony), GM-CSF (3.6 ± 0.7 cells/colony), or IL-6/IL-1α (3.3 ± 0.5 cells/colony).

The effect of cytokine neutralizing polyclonal antisera on the MK-CSA of their corresponding cytokines on CD34⁺ DR⁺ bone marrow cells was studied next (Table 1). The addition of neither anti-IL-3 nor anti-GM-CSF antisera alone had a stimulatory effect on MK colony formation in the absence of exogenous cytokines. However, the addition of anti-IL-3, but not anti-GM-CSF antisera, totally abolished IL-3-stimulated CFU-MK-derived colony formation. Similarly, GM-CSF-promoted MK colony formation was abolished by the addition of anti-GM-CSF but not anti-IL-3 antisera to CD34⁺ DR⁺ cells. The addition of both IL-3 and GM-CSF to cultures containing CD34⁺ DR⁺ cells resulted in an increase in CFU-MK-derived colony formation over that stimulated by either IL-3 or GM-CSF alone (Table 1). This increase was additive because the combination of the two cytokines approximated the sum of the effects of the addition of each cytokine alone. However, due to the low MK-CSA of GM-CSF alone, the number of MK colonies formed with both IL-3 and GM-CSF was not significantly (P > .05) greater than that of IL-3 alone. The addition of anti-IL-3 antisera to CD34⁺ DR⁺ cultures containing IL-3 plus GM-CSF inhibited CFU-MK-derived colony formation to a level equivalent to that stimulated by GM-CSF alone (Table 1). Similarly, the number of MK colonies resulting from the addition of anti-GM-CSF antisera to CD34⁺ DR⁺ cultures containing IL-3 plus GM-CSF approached that stimulated by IL-3 alone (Table 1).

The addition of neither anti-IL-1α nor anti-IL-6 antisera alone had a stimulatory effect on MK colony formation in the absence of exogenous cytokines (Table 1). MK colony formation stimulated by GM-CSF was not significantly

<p>| Table 1. Effects of the Addition of Recombinant Cytokines and/or Polyclonal Cytokine Neutralizing Antibodies on Megakaryocyte Colony Formation by CD34⁺ DR⁺ Bone Marrow Cells |
|---------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Additions to Culture</strong></th>
<th><strong>Colony Numbers ± SEM</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Anti-IL-3</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Anti-IL-1α</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Anti-GM-CSF</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>GM-CSF (10 U/mL)</td>
<td>2.6 ± 0.4†</td>
</tr>
<tr>
<td>GM-CSF (10 U/mL) + anti-GM-CSF</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>GM-CSF (10 U/mL) + anti-IL-3</td>
<td>2.3 ± 0.6†</td>
</tr>
<tr>
<td>GM-CSF (10 U/mL) + anti-IL-6</td>
<td>3.0 ± 1.2†</td>
</tr>
<tr>
<td>GM-CSF (10 U/mL) + anti-IL-1α</td>
<td>3.0 ± 0.5†</td>
</tr>
<tr>
<td>IL-3 (100 U/mL)</td>
<td>11.3 ± 0.8†</td>
</tr>
<tr>
<td>IL-3 (100 U/mL) + anti-IL-3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-3 (100 U/mL) + anti-GM-CSF</td>
<td>11.4 ± 1.3†</td>
</tr>
<tr>
<td>IL-3 (100 U/mL) + anti-IL-1α</td>
<td>10.9 ± 2.1†</td>
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<tr>
<td>IL-3 (100 U/mL) + anti-IL-6</td>
<td>11.0 ± 1.4†</td>
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<tr>
<td>IL-3 (100 U/mL) + GM-CSF (10 U/mL)</td>
<td>13.3 ± 2.0†</td>
</tr>
<tr>
<td>IL-3 (100 U/mL) + GM-CSF (10 U/mL) + anti-IL-3</td>
<td>2.5 ± 0.6†</td>
</tr>
<tr>
<td>IL-3 (100 U/mL) + GM-CSF (10 U/mL) + anti-GM-CSF</td>
<td>13.3 ± 0.5†</td>
</tr>
</tbody>
</table>

CD34⁺ DR⁺ cells were plated at 5 × 10⁵ cells/mL.

*The results are expressed as mean number of megakaryocyte colonies ± 1 SEM taken from four to six separate studies performed in duplicate or quadruplicate.

†Significant change from control (P < .05).
totally blocked by the addition of either anti-IL-6, anti-IL-1α, or anti-IL-3 antisera (Table 2).

DISCUSSION
Recombinant GM-CSF and IL-3 have been reported by several groups to promote, either alone or in combination, both in vitro and in vivo megakaryocytosis. Effects on both in vitro and in vivo megakaryocytosis by IL-1 have also been observed. IL-6 possesses a large number of biologic activities affecting multiple cellular systems, including the promotion of in vitro MK development. Several groups have further analyzed the effects of IL-6 on both in vitro murine and human megakaryocytosis. Ishibashi et al.53,54 reported that IL-6 functions as a thrombopoietic factor to directly promote murine MK maturation. Other groups have shown that IL-6 functions as a thrombopoietic factor to directly promote murine MK maturation. Other groups have shown that IL-6 promotes in vitro MK colony formation. However, questions have arisen concerning the mechanism by which IL-6 affects MK colony formation. Several investigators have reported that IL-6 alone has no MK-CSA.53,54 However, Koike et al.56 have further suggested that IL-6 requires a factor or factors present in serum to promote murine MK colony formation. However, other groups have reported that IL-6 can act independently on some bone marrow subpopulations, such as NALDT- cells, to promote murine MK colony growth.55 In addition, Lotem et al.56 have conjectured that IL-6 plays an even more critical role in the regulation of the CFU-MK. They have presented data indicating that the induction of murine MK colony formation by IL-6 is actually due to the endogenous production of IL-6 by bone marrow accessory cells.57

In the experiments summarized in this report, the effects of several recombinant cytokines, GM-CSF, IL-3, IL-6, and IL-1α, on human MK colony formation were investigated. A human bone marrow subpopulation enriched for CFU-MK (CD34+ DR+) was used.58 While both IL-3 and GM-CSF alone were capable of promoting CFU-MK-derived colony formation when added to cultures containing CD34+ DR+ cells, both IL-6 and IL-1α alone failed to exhibit MK-CSA. However, the addition of both IL-6 and IL-1α together to CD34+ DR+ cells resulted in a synergistic effect as demonstrated by the appearance of MK colonies. Therefore, these findings suggest that IL-6 cannot act alone in promoting CFU-MK cloning efficiency, but rather requires the presence of IL-1α. However, the very low level of stimulation of CFU-MK proliferation by IL-6/IL-1α may indicate that IL-6 and IL-1α are of limited significance in regulating the proliferative capacity of the human CFU-MK. Our laboratory has previously reported that IL-1α inhibited the MK-CSA of IL-6 when LDBM cells were assayed.59 This seems to contradict the synergistic effect between IL-6 and IL-1α on CFU-MK colony formation by CD34+ DR+ cells reported here. The difference in the IL-6/IL-1α effect between the two studies may be explained by examination of the different target cell populations used in these two reports. The first study reported effects of IL-6/IL-1α on LDBM cells. This heterogenous cell population contains accessory cells, such as T lymphocytes and adherent cells, not present in as high numbers in the CD34+ DR+ bone marrow population used in the present study. IL-1α is known to stimulate the de novo production of such inhibitors of megakaryocytosis as interferon-γ (IFN-γ).60 In addition, Warren and Ralph61 have reported that LDBM cells have a low baseline production of tumor necrosis factor (TNF) that can be increased by the addition of cytokines. Both INFs and TNF are known to synergize in their inhibitory actions on hematopoietic progenitor cells.62 The potential production of INF-γ and possible TNF by IL-1α-treated LDBM cells might, therefore, account for the inhibitory effect of IL-1α on optimal concentrations of IL-6.

The MK-CSA of GM-CSF and IL-3 were each neutralized only by their corresponding antisera. Of special interest was the observation that the MK-CSA of IL-3 was completely abolished by the addition of anti-IL-3 antisera but was not significantly (P > .05) affected by the addition of either anti-GM-CSF, anti-IL-1α, or anti-IL-6 antisera. These findings are in direct conflict with the hypothesis presented by Lotem et al.63 who suggest that IL-3 exerts its MK-CSA by promoting the release of IL-6 from bone marrow auxiliary cells. While the reasons for these discrepancies remain unknown, it should be noted that Lotem et al.64 assayed CFU-MK from unfractioned murine bone marrow cells plated at high density in a serum-containing (10% to 20% horse serum) clonal assay system.65 However, our studies were performed with an enriched bone marrow cell subpopulation (CD34+ DR+ cells) plated at a relatively low density in a serum-depleted culture system. In addition, the failure of some polyclonal antibodies to neutralize cytokine may simply reflect inaccessibility of the cytokine to the neutralizing antibodies due to either compartmentalization in the extracellular matrix or autocrine factors acting on intracellular receptors. Although the present studies suggest that the MK-CSA of IL-3 cannot be attributed to IL-6, IL-6 does, however, appear to play a role in megakaryocytosis by affecting multiple stages of MK development. While IL-6 alone is incapable of promoting CFU-MK colony formation by CD34+ DR+ bone marrow cells, it does interact with IL-1α in supporting the proliferation of MK colonies. However, the MK-CSA of IL-6/IL-1α may reflect,
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in part, de novo elaboration of IL-3, because anti-IL-3 antisera completely blocked the stimulation of CFU-MK colonies by IL-6/IL-3. The accessory cells responsible for such a secondary release of IL-3 have not been identified to date. Migliaccio et al. have also presented data to indicate that the actions of IL-1 and IL-6 on hematopoiesis are primarily indirectly mediated by the production of GM-CSF and/or IL-3 by accessory cells. In addition, the stimulation of MK colonies by IL-6/IL-3e may be due in part to an effect on MK maturation, which could then lead to enhanced detectability. However, the ability of either anti-IL-3 or anti-IL-6 antisera to neutralize the MK-CSA of this cytokine combination clearly demonstrates the necessity for the presence of both components of this combination for this synergistic effect to occur.

Experiments were also performed to examine the nature of the additive effects of IL-3 and GM-CSF on MK colony formation. The addition of neither anti-IL-3 nor anti-GM-CSF antisera to cultures containing both GM-CSF and IL-3 could totally abolish CFU-MK colony formation by this cytokine combination. Such data clearly suggest that both of these cytokines act on the CFU-MK by independent effector pathways or that they directly affect two distinct subpopulations of CFU-MK progenitors present within the CD34+ DR+ bone marrow population.

In this report, IL-3, GM-CSF, IL-1α, and IL-6 have been shown to be capable, either alone or in combination, to affect CFU-MK cloning efficiency. It is likely that additional cytokines possessing this biologic activity will be identified. However, the presently studied cytokines appear to interact in a network-like fashion, resulting ultimately in augmented CFU-MK proliferation.

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