Depletion of macrophages from murine marrow by the use of a monoclonal anti-macrophage antibody resulted in a significant increase in the number of erythroid burst forming units (BFU-E). This increase could be neutralized by the addition back to culture of macrophages or macrophage conditioned medium indicating that the suppression was mediated by soluble factors. To further characterize this effect, the addition to culture, either alone or in combination, of interleukin-1α (IL-1α), tumor necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF) on the growth of BFU-E and the colony-forming unit granulocyte-macrophage (CFU-GM) was examined in macrophage-containing and macrophage-depleted cultures. The addition of IL-1α to culture stimulated the release of both TNFα and GM-CSF and acted synergistically with both cytokines, resulting in a dose-dependent suppression of BFU-E and stimulation of CFU-GM growth. The increase in CFU-GM caused by the addition of IL-1α was mediated by GM-CSF but not by TNFα as the increase was prevented by the addition of a monoclonal anti-GM-CSF antibody but not by anti-TNFα. When either TNFα or GM-CSF was neutralized by monoclonal antibodies the addition of IL-1α resulted in a significant increase in BFU-E growth. The addition of GM-CSF to culture caused a dose-dependent suppression of BFU-E that was mediated by TNFα, as colony number was not reduced when GM-CSF and a monoclonal anti-TNFα antibody were simultaneously added to culture. TNFα-induced suppression of BFU-E only occurred in the presence of macrophages. In macrophage-depleted cultures, a dose-dependent suppression of BFU-E could be induced if subinhibitory concentrations of IL-1α or GM-CSF were simultaneously added with increasing concentrations of TNFα. The effects of IL-1α or GM-CSF and TNFα were markedly synergistic so that the doses required to induce suppression when added simultaneously was only 10% of that required when either were added to culture alone. Suppression of BFU-E by GM-CSF or the combined addition of GM-CSF and TNFα did not require IL-1α because inhibition was not neutralized by the addition of anti-IL-1α antibody. IL-1α and TNFα suppressed the primitive, mostly erythropoietin-independent erythroid progenitors, because no decrease in BFU-E was noted if their addition to culture was delayed beyond the fourth day after culture initiation. These findings indicate that cytokines produced by macrophages exert an important regulatory role in hematopoiesis acting synergistically to primarily suppress erythropoiesis and stimulate myeloid growth. GM-CSF, a potent stimulator of myelopoiesis, simultaneously suppresses erythropoiesis by an interaction with TNFα, whose release it stimulates. IL-1α stimulates the release of, and acts synergistically with both GM-CSF and TNFα to suppress BFU-E and stimulate CFU-GM growth. IL-1α also has the ability to stimulate BFU-E growth when either of these two cytokines are neutralized.

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**Hematopoiesis** involves a multistage cellular proliferation and differentiation of pluripotent stem cell leading to the eventual formation of various mature blood elements. Control of marrow cell proliferation involves complex interactions between hematopoietic cells, accessory cells in the marrow microenvironment, and an array of cytokines that either promote or suppress cell proliferation generally or in a specific cell lineage. Recombinant cytokines produced by macrophages play an important role in the regulation of hematopoietic cell production. Macrophages are known to produce a host of factors that modulate cellular growth and differentiation. These include interleukin-1α (IL-1α) and β, interferon (INFγ), tumor necrosis factor α (TNFα), a variety of mitogens, and a number of myeloid colony-stimulating factors, including the unipotent granulocyte-macrophage colony stimulating factor (GM-CSF), the unipotent macrophage colony stimulating factor (M-CSF) and granulocyte colony stimulating factor (G-CSF).

In relation to erythropoiesis, early studies suggested that macrophages stimulate both the burst forming unit-erythroid (BFU-E) and the colony forming unit-erythroid (CFU-E) growth in vitro by cell to cell interactions and by the release of cytokines, including burst-promoting activity (BPA), erythropoietin (EPO), and an insulin-like growth factor. Cytokines produced by macrophages also suppress erythropoiesis. It is now well accepted that increased macrophage proliferation is the major mechanism accounting for the anemia that accompanies chronic disease and inflammation. This effect appears to be mediated by TNFα.

EPO is a highly lineage-specific factor acting mainly on relatively late-stage erythroid progenitors. It is a stimulatory hormone, promoting growth and differentiation. However, it is also likely that there are negative regulators of the erythroid lineage whose physiologic role is to suppress or inhibit erythropoiesis at times when there are sufficient red blood cells or when a shift to production of other hematopoietic end cells are required. Recent studies have provided strong evidence that this negative regulation is accomplished by macrophages. In vivo studies have shown that the injection of macrophages or macrophage-conditioned medium into mice suppresses CFU-E growth.

From the Geriatric Research Education and Clinical Center (GRECC), John L. McClellan Memorial Veterans Hospital, and the Departments of Medicine, Physiology, and Biophysics, University of Arkansas for Medical Sciences, Little Rock.

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Address reprint requests to K.B. Udupa, PhD, GRECC (182/LR), John L. McClellan Memorial Veterans Hospital, 4300 W Seventh, Little Rock, AR 72205.

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The sequence of events includes the release of IL-1α leading to the release of TNFα, which then participates in the suppression of erythroid growth. The inhibitory effect of macrophages on erythropoiesis involves complex interactions between TNFα, IL-1α, and other cells or cytokines.13,14

In this report we examine the role of macrophages and their cytokines in murine marrow cultured for BFU-E. The use of the BFU-E culture system allows us to examine the effects, not only on BFU-E growth, but also on the production of myeloid colonies, which can be generated in this culture system provided the appropriate stimulatory cytokines are present. We show that IL-1α, TNFα, and GM-CSF act synergistically, affecting both erythroid and myeloid growth in culture.

MATERIALS AND METHODS

Animals. Two- to 3-month-old C57BL/6 female mice were purchased from Charles River Laboratory (Wilmington, MA). Before study, the mice were maintained for at least 4 weeks in our laboratory to minimize recaging or travel stress. They were fed a standard rodent chow and water ad libitum.

Reagents. A monoclonal rat anti-murine antibody against a macrophage antigen (Mac-1) and fluorescein (FITC)-conjugated rabbit anti-rat IgG were purchased from Sera-Lab (Sera-Lab Ltd, Crawley Down, Sussex, England). In some experiments, FITC-conjugated rat anti-murine Mac-1 monoclonal antibody (MoAb) (Coulter Immunology, Hialeah, FL) was also used. Monoclonal hamster anti-murine TNFα, rat anti-murine GM-CSF, hamster anti-murine IL-1α antibodies, recombinant murine TNFα, IL-1α, and an enzyme-linked immunosorbent assay (ELISA) kit to measure murine TNFα were purchased from Genzyme Corp (Boston, MA). Recombinant murine GM-CSF was purchased from Upstate Biotechnology Inc (Lake Placid, NY). Recombinant human EPO was a kind gift from the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). Covalently bound magnetic particles of approximately 1 μm in size to sheep anti-FITC antibody were purchased from Advanced Magnetic Inc (Cambridge, MA).

Macrophage antigen (Mac-1) and fluorescein (FITC)-conjugated rabbit anti-FITC antibody were purchased from Advanced Magnetic Inc (Cambridge, MA).

Marrow cell suspension and Mac-1 positive cell depletion. Mice were killed by cervical dislocation and marrow from the femora and tibiae were flushed with α-medium (Flow Laboratory, McLean, VA) contained in a 1-mL syringe fitted with a 23-guage needle. After preparing single cell suspensions, cell counts were measured using a model ZF Coulter Counter (Coulter Electronics). Their viability was determined to be greater than 95% by trypan blue exclusion staining.

Whole marrow cells were suspended to a concentration of 10 × 10^6/mL in α-medium containing 1:20 rat anti-murine Mac-1 MoAb and incubated at 4°C for 45 minutes. After three washings in cold α-medium, the cells were resuspended in α-medium containing 1:40 FITC conjugated rabbit anti-rat IgG and incubated for a further 45 minutes. The cells were then washed, resuspended in α-medium and incubated with sheep anti-FITC antibody attached to magnetic particles in a ratio of 1:50 (cell:magnetic particle) for 30 minutes at 4°C. Mac-1 positive cells were removed by magnetic separation using a Bio-Mag Separator (Advanced Magnetics). Control marrow cells were treated identically but were not exposed to anti-Mac-1 MoAb.

BFU-E and CFU-GM cultures. For both macrophage-containing and macrophage-depleted marrow cell suspensions, the cell count used for progenitor cell culture was based on the original cell count before removal of macrophages by exposure of the marrow to anti-Mac-1 antibody and magnetic separation. Marrow was cultured for BFU-E using the method described by Ixovce and Sieber.15 One hundred thousand marrow cells were incubated in 1 mL of methylcellulose (0.8%) containing 30% fetal bovine serum, 1% bovine serum albumin, 10^{-6} M L-mercaptoethanol, and 4 U EPO in 35-mm culture dish at 37°C in 5% CO2 in humid air, for 8 days. BFU-E colonies were scored in situ under a low power microscope. Colony-forming unit-macrophage (CFU-M), mixed colony-forming units (CFU-Mix), and colony-forming unit-granulocyte (CFU-G) that grew simultaneously in BFU-E cultures (containing IL-1α, GM-CSF, or combination) were scored using the identifying characteristics described by Metcalf and Moore.16 In this report the total number of colonies are referred to as colony-forming units Granulocyte-Macrophage (CFU-GM).

Preparation of macrophages and macrophage conditioned medium. Resident peritoneal macrophages were obtained from C57BL/6 mice following the injection of 4 mL cold α-medium into the peritoneal cavity and immediately harvesting the fluid, which contained an average of 11.2 ± 2.7 × 10^6 cells/mL. The peritoneal wash fluid was then poured into a 100-mm Petri dish and incubated for 2 hours at 37°C, in 5% CO2. Nonadherent cells were washed out with α-medium and the adherent macrophages were scraped and made into single cell suspension, of which 94.7% ± 3.2% were macrophages. The cell count was determined by Coulter Counter. Macrophage conditioned medium was prepared by culturing 2 × 10^6/mL macrophages in serum-free α-medium for 24 hours at 37°C. The medium was harvested and stored at −20°C until use.

Assay for GM-CSF and TNFα in liquid culture of bone marrow. In some experiments 1 × 10^6 marrow cells/mL were exposed to cytokines or control medium for 7 days after which time the supernatant was removed and assayed biologically for GM-CSF using the method described by Worten et al17 and for TNFα using an ELISA kit for murine TNFα.

RESULTS

Using a fluorescent microscope, an average of 21.9% ± 1.3% of marrow cells were found to be Mac-1 positive and were removed by exposure to a Mac-1 antibody and magnetic separation. This resulted in an 84% ± 5.7% reduction in esterase positive cells and a 60.0% ± 4.2% decrease in the number of macrophages or macrophage-conditioned medium when the macrophage-depleted marrow was cultured for CFU-GM using a maximum stimulatory concentration of lung conditioned medium as a source of GM-CSF.

In macrophage-containing cultures, BFU-E colony number averaged 16.6 ± 1.4/10^5 marrow cells (Fig 1). In macrophage-depleted cultures, BFU-E increased significantly to 23.2 ± 2.3 colonies/10^5 cells, a value that was 39.8% greater than control (P < .05). This increase could be neutralized by the addition back to the depleted culture of either macrophages or macrophage-conditioned medium, which resulted in BFU-E growths of 8.0 ± 1.2 and 17.1 ± 2.3 colonies/10^5 cells, respectively. The former was 55% and the latter virtually identical to the BFU-E growth observed in macrophage-containing cultures (Fig 1). We also found that the addition of macrophage-conditioned medium to macrophage-containing cultures resulted in a significant suppression of BFU-E growth (Fig 1).

These results suggested that macrophages inhibited BFU-E in vitro and that the suppression was mediated by cytokines because the effect could be mimicked by conditioned medium derived from macrophage cultures. Therefore, we examined the effects of the addition to macrophage-
The addition of IL-1α or GM-CSF to BFU-E culture resulted in significant increases in CFU-GM (Table 1). At the concentrations used in this study both IL-1α and GM-CSF primarily stimulated the production of CFU-M colonies, which averaged 23.8 ± 1.3/10⁵ marrow cells (84.7% of total CFU-GM) when 0.2 ng/mL IL-1α was added to macrophage-containing cultures and 21.2 ± 2.7/10⁵ marrow cells (87.2% of total) in macrophage-depleted cultures. In both macrophage-containing and macrophage-depleted cultures the addition of 0.25 ng/mL GM-CSF resulted in the generation of 62.7 ± 4.9 (81.0% of total) and 43.7 ± 3.3 CFU-M/10⁵ marrow cells (80% of the total), respectively. In contrast, no increases in CFU-GM were noted when TNFα alone was added to either macrophage-containing or macrophage-depleted cultures (Table 1).

We then studied the effects of the combined addition of cytokines to macrophage-depleted cultures. In a first experiment, subinhibitory concentrations of either IL-1α (25 pg/mL) or GM-CSF (25 pg/mL), which by themselves caused a minimal and not significant decline in BFU-E growth, were added to the culture together with increasing concentrations of TNFα. The addition of increasing concentrations of TNFα combined with either IL-1α or GM-CSF caused highly significant reductions in BFU-E (P < .001), which was maximal at a TNFα concentration of 125 pg/mL when combined with IL-1α and at a TNFα concentration of 187.5 pg/mL when combined with GM-CSF (Fig 3). The combination of TNFα with either IL-1α or GM-CSF did not result in a increase in CFU-GM above that noted when IL-1α or GM-CSF was added to culture alone. It must be noted that, when combined with either IL-1α or GM-CSF, the concentration of TNFα required to induce suppression of BFU-E was only 10% of that required to result in a similar suppression when the cytokine was added alone to macrophage-containing cultures. We then determined that the combined addition of IL-1α and GM-CSF to culture caused a minimal and not significant reduction in BFU-E and an increase in CFU-GM above that noted when either cytokine alone was added to culture (data not shown).

To further characterize the interactions between IL-1α, TNFα, and GM-CSF on BFU-E and CFU-GM growth, the

Fig 1. BFU-E colony number in macrophage-containing (MC) and macrophage-depleted (MD) cultures. The effects of the addition of macrophage-conditioned medium (CM) to MC and MD cultures and of macrophages (M) to MD cultures are shown. BFU-E growth in MD is significantly (P < .01) greater than MC. Compared with MC alone, BFU-E growth in MD + M and MD + CM is significantly lower than growth in MD (P < .001 and P < .05, respectively). The results are the mean (±SEM) of six experiments.

containing and macrophage-depleted cultures of IL-1α, TNFα, and GM-CSF cytokines that are produced by macrophages and that have been shown to affect BFU-E. The addition to macrophage-containing or macrophage-depleted culture of either IL-1α or GM-CSF alone resulted in a dose-dependent suppression of BFU-E that was maximal at a concentration of 0.8 ng/mL for IL-1α and at a concentration of 0.25 ng/mL for GM-CSF. Maximum inhibition of BFU-E was 55% of control for IL-1α and 60% of control for GM-CSF (Fig 2). BFU-E suppression was approximately equal in macrophage-containing and macrophage-depleted cultures. In contrast to IL-1α and GM-CSF, TNFα required the presence of macrophages to exert an effect on BFU-E (Fig 2). In macrophage-containing cultures, BFU-E number was 71% ± 6.2% of control at a TNFα concentration of 1 ng/mL, and 69% ± 5.4% of control when 2 ng/mL was added. In macrophage-depleted cultures, TNFα caused a minimal and not significant suppression of BFU-E (Fig 2).

Fig 2. Percent decrease in BFU-E colony number in murine marrow cultures to which (A) IL-1α (B) TNFα and (C) GM-CSF were added. (- - -) Macrophage containing cultures; (- - -) macrophage-depleted cultures. The results are the mean (±SEM) of six individual experiments for each group.
various cytokines, either alone or in combination, were added to culture together with MoAbs against murine IL-1α, TNFα, or GM-CSF. Anti-TNFα and anti-GM-CSF alone did not affect BFU-E (Table 2). Anti-GM-CSF significantly reduced and anti-TNFα modestly increased CFU-GM colony number in both macrophage-containing and macrophage-depleted cultures. In both macrophage-containing and macrophage-depleted cultures the combined addition of 0.2 ng/mL IL-1α and either anti-TNFα or anti-GM-CSF caused significant increases (p < .05) in BFU-E, which averaged 151% of control for macrophage-containing and 135.7% of control for macrophage-depleted cultures (Table 2). Anti-GM-CSF neutralized the increase in BFU-E caused by IL-1α addition to culture. However, no decrease in BFU-E occurred when anti-TNFα and IL-1α were simultaneously added to culture.

The addition of anti-TNFα neutralized the inhibitory effects of GM-CSF on BFU-E but had no effect on CFU-GM colony number (Table 3). The suppression of BFU-E and the stimulation of CFU-GM by GM-CSF or GM-CSF, in combination with TNFα, did not require IL-1α, because the addition of anti-IL-1α had no effect on either BFU-E or CFU-GM colony growth. When anti-GM-CSF was added to a culture containing IL-1α and TNFα, BFU-E was higher (p < .05) than that noted in cultures containing the combination of IL-1α and TNFα alone (Table 3). However, the value was still significantly lower than the BFU-E growth in cultures to which no antibodies or cytokine were added (p < .01).

These results indicated that IL-1α- and GM-CSF–induced suppression of BFU-E was mediated by TNFα and that IL-1α–induced stimulation of CFU-GM was mediated by GM-CSF. To confirm this possibility, we measured the ability of IL-1α or GM-CSF either alone or in combination to produce TNFα in liquid cultures of macrophage-containing and macrophage-depleted marrow. As shown in Fig 4, IL-1α and GM-CSF both resulted in a significant increase in TNFα production, which was greatest when both cytokines were simultaneously added to culture. This increase was noted in both macrophage-containing and macrophage-depleted cultures but was invariably greatest in cultures containing macrophages. In this liquid culture system we also measured GM-CSF production following the addition of IL-1α alone to culture. In control cultures GM-CSF production averaged 8.5 ± 0.9 pg/mL compared with 35.2 ± 1.7 pg/mL in cultures to which IL-1α was added (p < .001).

Cytokines that suppress BFU-E growth could exert effects at any point in the generation of BFU-E colonies, from the primitive, EPO-independent erythroid progenitors to the more mature CFU-E, or even the differentiated erythroid cells. To define the site of action of these cytokines we studied the effects of delaying their addition to BFU-E cultures. In this study, 25 pg/mL IL-1α and 62.5 pg/mL TNFα were simultaneously added to culture, either at the time of culture initiation or at 24-hour intervals thereafter. Control cultures received an equal volume of α medium alone. Significant suppression of BFU-E growth was noted if IL-1α and TNFα were added to culture up to 4 days after culture initiation, at which time colony number averaged 52.6% ± 3.8% of control (Fig 5). Suppression of colony growth was significantly less if the cytokines were

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**Table 1. Effects of the Addition of IL-1α, GM-CSF, or TNFα to Murine BFU-E Cultures on CFU-M, CFU-G, CFU-Mix, and Total (CFU-GM) Colony Growth**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Macrophage-Containing Marrow</th>
<th>Macrophage-Depleted Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-M</td>
<td>CFU-G</td>
</tr>
<tr>
<td>α-Medium alone</td>
<td>6.2 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α 25 pg/mL</td>
<td>11.0 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td>200 pg/mL</td>
<td>23.8 ± 1.3</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>GM-CSF 50 pg/mL</td>
<td>26.1 ± 1.9</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>250 pg/mL</td>
<td>62.7 ± 4.9</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>TNFα 62.5 pg/mL</td>
<td>5.0 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>1,000 pg/mL</td>
<td>7.6 ± 1.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Values are the mean (±SEM) of six separate experiments.
added on day 5 (85.0% ± 2.0% of control) and no suppression was noted when the addition was delayed to day 6 or later (Fig 5).

**DISCUSSION**

The possible relationship between erythropoiesis and macrophages dates back to the elegant work of Marcel Bessis in 1958, who, in a system of primitive erythroid precursors. This macrophage, referred to as a nurse cell, was thought to be essential for normal erythroid growth and development. Recent evidence has indicated that macrophages may stimulate erythroid growth and development.2n Although early studies indicated that macrophages may stimulate erythroid growth,21 recent evidence has indicated that they exert inhibitory effects that are not only important in the genesis of various anemias but also play a negative regulatory role in erythropoiesis. The BFU-E culture provides an opportunity, in a system containing high concentrations of EPO, to examine the modulatory effects, on both erythroid and myeloid colony.

In this report we have examined the effects of macrophage depletions on BFU-E growth in murine culture. Using a Mac-1 antibody we were able to remove 80% of marrow macrophages as assessed by reductions in the number of Mac-1 positive cells detected by fluorescence microscopy, by the number of CFU-M and CFU-Mix detected in vitro culture and by the number of esterase positive cells detected on histologic examination of marrow samples. Depletion of macrophages by this technique resulted in a 30% to 40% increase in BFU-E colony growth that could be neutralized by the addition back to culture of either macrophages or macrophage conditioned medium. This indicates that the macrophage effect was mediated by soluble factors.

The BFU-E culture provides an opportunity, in a system containing high concentrations of EPO, to examine the modulatory effects, on both erythroid and myeloid colony

**Table 2. Effects on BFU-E and CFU-GM of the Addition to Culture of IL-1α Alone or in Combination With Murine Monoclonal Anti-TNFα or Anti-GM-CSF Antibodies**

<table>
<thead>
<tr>
<th>Additions</th>
<th>BFU-E/10⁶ Marrow Cells</th>
<th>CFU-GM/10⁶ Marrow Cells</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophage-</td>
<td>Macrophage-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Containing</td>
<td>Depleted</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α-medium (control)</strong></td>
<td>20.0 ± 1.0 (100.0)*</td>
<td>30.8 ± 1.9 (100.0)</td>
<td>10.8 ± 1.2</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>IL-1α (0.2 ng/mL)</td>
<td>13.8 ± 0.8 (66.3)†</td>
<td>20.8 ± 1.2 (67.5)†</td>
<td>28.2 ± 1.3</td>
<td>24.3 ± 2.4</td>
</tr>
<tr>
<td>Anti-GM-CSF</td>
<td>22.8 ± 1.0 (109.6)</td>
<td>31.1 ± 2.9 (101.0)</td>
<td>2.2 ± 0.5</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Anti-TNFα</td>
<td>20.1 ± 1.3 (96.6)</td>
<td>28.9 ± 2.2 (93.8)</td>
<td>14.2 ± 1.3</td>
<td>12.0 ± 1.9</td>
</tr>
<tr>
<td>IL-1α + anti-GM-CSF</td>
<td>31.4 ± 1.6 (151.0)†</td>
<td>41.8 ± 3.6 (135.7)†</td>
<td>13.2 ± 2.5</td>
<td>6.7 ± 1.5</td>
</tr>
<tr>
<td>IL-1α + anti-TNFα</td>
<td>31.2 ± 2.1 (150.0)†</td>
<td>41.5 ± 2.6 (134.7)†</td>
<td>28.9 ± 1.7</td>
<td>26.4 ± 2.9</td>
</tr>
</tbody>
</table>

The concentrations of monoclonal anti-GM-CSF and anti-TNFα added to culture neutralized the inhibitory effects that are not only important in the genesis of various anemias but also play a negative regulatory role in erythropoiesis. The BFU-E culture provides an opportunity, in a system containing high concentrations of EPO, to examine the modulatory effects, on both erythroid and myeloid colony.

**Table 3. Effect of Addition of Small Doses of IL-1α, TNFα, and GM-CSF, Alone or in Combination With Murine MoAbs, on BFU-E and CFU-GM Growth in Macrophage-Depleted Murine Marrow Culture**

<table>
<thead>
<tr>
<th>Additions</th>
<th>BFU-E/10⁶ Marrow Cells</th>
<th>CFU-GM/10⁶ Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (α medium)</td>
<td>29.7 ± 1.0 (100)*</td>
<td>2.7 ± 0.4 (100)</td>
</tr>
<tr>
<td>IL-1α (25 pg/mL)</td>
<td>29.8 ± 1.2 (100.3)</td>
<td>6.8 ± 1.0 (251.9)</td>
</tr>
<tr>
<td>TNFα (62.5 pg/mL)</td>
<td>30.2 ± 1.1 (101.7)</td>
<td>2.3 ± 0.3 (85.2)</td>
</tr>
<tr>
<td>GM-CSF (50 pg/mL)</td>
<td>21.8 ± 1.3 (73.4)†</td>
<td>23.5 ± 1.8 (870.4)</td>
</tr>
<tr>
<td>GM-CSF + anti-TNFα</td>
<td>27.6 ± 2.1 (92.9)†</td>
<td>21.5 ± 0.8 (796.3)</td>
</tr>
<tr>
<td>GM-CSF + anti-IL-1α</td>
<td>20.0 ± 1.3 (67.3)</td>
<td>23.7 ± 0.6 (877.8)</td>
</tr>
<tr>
<td>TNFα + GM-CSF</td>
<td>17.2 ± 2.1 (57.9)</td>
<td>25.7 ± 4.6 (951.9)</td>
</tr>
<tr>
<td>GM-CSF alone</td>
<td>1.3 ± 1.1 (51.5)</td>
<td>27.8 ± 2.6 (1,029.6)</td>
</tr>
<tr>
<td>GM-CSF + anti-IL-1α</td>
<td>14.7 ± 0.7 (49.5)‡</td>
<td>7.8 ± 2.1 (288.9)</td>
</tr>
<tr>
<td>GM-CSF + anti-TNFα</td>
<td>19.2 ± 1.0 (64.6)‡</td>
<td>5.1 ± 1.0 (188.9)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of five individual experiments for each group.

*Values in parenthesis are percent of BFU-E growth in macrophage-containing or depleted marrow to which α medium alone was added.‡Significantly less than control (P < .01).§Significantly greater than control (P < .01). The values are mean (±SEM) of five separate experiments for each group.

Fig 4. TNFα production in liquid culture of (I) macrophage-containing and (II) macrophage-depleted cultures. (1) Cultures to which α medium alone was added; (2) IL-1α alone addition; (3) GM-CSF alone addition; (4) IL-1α + GM-CSF addition to culture. The results are the mean (±SEM) of five individual experiments for each group.
growth of various cytokines produced by macrophages that are known to exert negative effects on erythropoiesis and stimulatory effects on myelopoiesis. We found that the addition of either IL-1α or GM-CSF alone to either macrophage-containing or depleted culture resulted in a dose-dependent suppression in BFU-E while simultaneously stimulating CFU-GM. The addition of IL-1α or GM-CSF stimulated the release of TNFα, which mediated the suppression of BFU-E. We confirmed this by showing that GM-CSF or IL-1α-induced suppression of BFU-E was neutralized by the simultaneous addition of a monoclonal anti-TNFα antibody to culture. These findings, in a murine model, differ from those observed in human studies in which GM-CSF had been shown to increase BFU-E. It may reflect a species difference or may be the result of a different composition of accessory cells or cytokines in the human as compared with the mouse system employed to culture BFU-E.

A dose-dependent suppression of BFU-E by TNFα only occurred in macrophage-containing cultures. However, we could induce suppression of BFU-E in macrophage-depleted cultures if, in addition to TNFα, minimally inhibitory suppression of either IL-1α or GM-CSF were also added. This indicates that TNFα-induced suppression of BFU-E requires the presence of other cytokines and extends the recent observation that suggested that TNFα requires accessory cells to suppress CFU-GM growth in vitro. When added to BFU-E cultures, serial concentrations of TNFα did not increase the number of CFU-GM in either macrophage-containing or depleted culture or further increase CFU-GM when added to culture together with either IL-1α or GM-CSF. In vitro studies have shown that TNFα is capable of stimulating the release of GM-CSF from human fibroblasts and macrophages, and in vivo murine studies have shown that injection of TNFα results in leukocytosis and increases in marrow myeloid cell number. Our in vitro results suggest that, in this culture system, TNFα does not directly stimulate myelopoiesis or result in the release of either IL-1α or GM-CSF. This apparent discrepancy may well be explained by the characteristics or cell composition of the culture system.

These observations extend recent in vivo studies that have suggested that macrophages negatively regulate the production of erythropoiesis by the generation of low concentrations of IL-1α that in turn result in the release of TNFα. We also show that IL-1α stimulates the release of GM-CSF, which results in an increase in CFU-GM colony number that is neutralized by the addition to culture of anti-GM-CSF. It seems likely that these cytokines, produced primarily by macrophages, play a central role in regulating hematopoietic cell proliferation into either myeloid or erythroid cell differentiation. This is supported by the well-known observation that disorders leading to leukocytosis are invariably accompanied by macrophage activation that releases cytokines that stimulate myeloid cell proliferation while simultaneously suppressing erythroid cell growth. In contrast, there is a recent report that indicates that increased erythropoiesis is accompanied by reduced myeloid cell production.

IL-1α does not directly suppress BFU-E even though its addition alone to culture resulted in a dose-dependent suppression of BFU-E production. Low concentrations of both TNFα and GM-CSF were required because the addition of either monoclonal anti-TNFα or anti-GM-CSF not only prevented the negative effects of IL-1α but resulted in a significant increase in BFU-E above that noted in control cultures. Furthermore, IL-1α was not essential for the effects of GM-CSF alone or GM-CSF and TNFα on colony growth because the addition of anti–IL-1α to these cultures did not alter either BFU-E or CFU-GM number. It seems likely that IL-1α may serve as the initial trigger stimulating the release of GM-CSF, TNFα, and perhaps other factors, with which it acts synergistically to modulate hematopoietic cell proliferation. In these studies the release of TNFα by other cytokines appears to be the mechanism whereby suppression of erythroid growth is achieved.

These results provide support for a central role for macrophages in regulating the flow of hematopoietic progenitors into the required progenitor cell compartment. It seems likely that the major role of cytokines produced by macrophages is to primarily suppress erythropoiesis while simultaneously stimulating myeloid cell proliferation and differentiation. However, it is also possible that in certain circumstances, and particularly when demands for erythroid precursors are increased, IL-1α and perhaps other cytokines can act synergistically with EPO to stimulate erythroid cell growth. In this report we show negative regulatory roles in erythropoiesis for IL-1α, TNFα, and GM-CSF. Almost certainly other cytokines produced by macrophages are also involved in the suppression of erythroid growth. For example, a recent report has indicated that IL-1α stimulates T cells to release INFγ, which inhibits CFU-E growth. It is highly likely that erythroid and myeloid
growth in the marrow is continually modulated and balanced by molecules that exert either negative or stimulatory effects. Thus, the inhibitory effects of cytokines on erythroid growth can be overcome if the concentration of EPO is significantly increased.31

Previous studies have shown an inhibitory effect of cytokines on CFU-E growth.32 BFU-E development commences from primitive erythroid progenitors independent of EPO that divide and differentiate into more mature precursors and eventually CFU-E that are EPO dependent.32 Therefore, the inhibitory effects of macrophages and their cytokines could occur anywhere along the pathway of colony development. To determine the site of action of added inhibitory cytokines we undertook a study in which we examined the effects of delaying the addition of IL-1α and TNFs to culture. Suppression only occurred if the cytokines were added within 4 days of culture initiation. This suggests that, in this culture system, the cytokines are primarily acting on the more immature, largely EPO-independent erythroid progenitors and not on the more mature precursors. We cannot adequately explain the lack of inhibition on day 5 or later when large numbers of CFU-E are developing. Possibilities to be considered include inadequate concentration of the inhibitory cytokines, or the lack of needed accessory cells or other factors for TNFs and IL-1α to inhibit CFU-E growth.

The macrophage-depleted culture system for BFU-E has proved useful in defining the complex interactions of IL-1α, TNFs, and GM-CSF on erythroid and myeloid colony growth in vitro. These findings set the stage for future studies in which we will examine the effects of other cytokines, such as interleukin-6 (IL-6), INFγ, and transforming growth factor-β (TGF-β), that may well exert either positive or negative effects on hematopoietic cell growth.

REFERENCES

Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. Blood 70:1700, 1987


The role of macrophages in the regulation of erythroid colony growth in vitro

CQ Wang, KB Udupa and DA Lipschitz