Recombinant Human Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor Show Common Biological Effects and Binding Characteristics on Human Monocytes

By Michael J. Elliott, Mathew A. Vadas, Jeffrey M. Eglinton, Linda S. Park, L. Bik To, Leslie G. Cleland, Steven C. Clark, and Angel F. Lopez

Two human hemopoietic growth factors involved in monocytopoiesis, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were studied for their ability to stimulate blood monocytes and to bind to the monocyte membrane. Both cytokines maintained monocyte/macrophage numbers during long-term culture and increased cell size as compared with controls. Effects on cell numbers were present at low cytokine concentrations (6 to 20 pmol/L), whereas enhanced H-thymidine incorporation was observed only at higher concentrations (>60 pmol/L). Autoradiographic studies showed only 1% to 3% of stimulated monocytes with nuclear grains. These results suggest that the primary mechanism for IL-3 and GM-CSF-induced maintenance of monocyte/macrophage numbers in humans is through an effect on cell survival. Surface receptors for both IL-3 and GM-CSF were studied by using 125I-labeled recombinant human (rh) cytokines and performing Scatchard analyses. Both cytokines showed curvilinear Scatchard plots, and computer analyses favored a two-site binding model. High-affinity binding data for 125I-rhIL-3 (Kd 7.7 to 38.2 pmol/L; receptor number/cell 95 to 580) and for 125I-rhGM-CSF (Kd 4.7 to 38.9 pmol/L; receptor number/cell 8 to 67) show similar binding affinities for the two cytokines but a lower receptor number/cell for 125I-rhGM-CSF. Low-affinity binding characteristics for 125I-rhIL-3 (Kd 513 to 939 pmol/L; receptor number/cell 179 to 5274) and for 125I-rhGM-CSF (Kd 576 to 1.120 pmol/L; receptor number/cell 130 to 667) show a similar pattern for the two cytokines. Specificity of 125I-rhIL-3 and 125I-rhGM-CSF binding to monocytes was established by the ability of the homologous cytokine to inhibit binding and the inability of a range of other cytokines to compete at 100-fold excess molar concentration. It is important, however, that binding of 125I-rhIL-3 was partially inhibited by rhGM-CSF and that rhIL-3 partially inhibited binding of 125I-rhGM-CSF to the monocyte membrane under conditions shown to prevent receptor internalization. The degree of inhibition varied between 25% and 80% in different experiments, and quantitative inhibition experiments showed that 1,000-fold excess concentrations of competitor failed to inhibit binding of the heterologous ligand completely. These results demonstrate that human IL-3 and GM-CSF have similar effects on growth and survival of human monocytes in vitro and suggest that these and other common biological effects may be mediated either through a common receptor or through distinct receptors associated on the monocyte membrane.

Proliferation and differentiation of human myeloid cells is regulated by a group of glycoproteins termed hematopoietic growth factors (HGFs). Three such factors are involved in monocytopoiesis, namely interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF). IL-3 and GM-CSF have similar actions in bone marrow (BM) culture in that they give rise to a variety of daughter cell types, whereas M-CSF induces predominantly macrophage colonies and is referred to as a "lineage-specific" CSF. The role of these factors in the biology of circulating human monocytes and tissue macrophages has been the subject of recent interest. M-CSF influences differentiation of peripheral blood (PB) monocytes in vitro and maintains their respiratory burst activity, whereas both IL-3 and GM-CSF enhance non-antibody-dependent tumor cell killing by monocytes after several days in culture. In addition, monocyte production of cytokines including tumor necrosis factor α (TNFα), M-CSF, and granulocyte-CSF (G-CSF) have been reported in response to both IL-3 and GM-CSF.

The role of HGFs in regulation of tissue macrophage number has been studied largely in mice. M-CSF induced proliferation in up to 90% of murine BM-derived macrophages, and GM-CSF may induce proliferation in both peritoneal exudate macrophages and blood monocytes. In addition, synergistic effects of IL-3 and GM-CSF on M-CSF-induced proliferation have been demonstrated in murine models.

In contrast, the primary effect of M-CSF in human monocyte cultures is one of enhanced survival, with only a subpopulation of cells (1% to 5%) capable of proliferation. The regulatory roles of IL-3 and GM-CSF on monocyte survival and proliferation in humans are largely unknown. Production of both cytokines by activated T cells and the demonstration of IL-3-like and GM-CSF-like activity in bullous pemphigoid blister fluid and synovial fluid in rheumatoid arthritis raise the possibility that these cytokines may help control monocyte/macrophage numbers at the inflammatory site. We present data indicating that...
rhIL-3 and rhGM-CSF are active at picomolar concentrations in supporting the survival of human monocytes and that, in contrast to mice, only a small subpopulation of cells is capable of HGF-induced [3H]-thymidine uptake at high cytokine concentrations.

A second important aspect of HGF–monocyte interaction involves study of surface receptors that may mediate the biological effects observed. High-affinity binding sites for IL-3 on human monocytes have recently been described, and partial inhibition of IL-3 binding by GM-CSF at 37°C was noted. GM-CSF binding to human monocytes has also been described, although no specificity controls have been reported. Remaining unanswered questions concern the relationship between the receptor affinity constants and the concentrations of cytokines required for biological activity, and, in particular, the specificity of binding. We showed that both high- and low-affinity receptors for IL-3 and GM-CSF exist on the monocyte membrane and suggest that the high-affinity receptors are likely to mediate the biological effect of monocyte survival. Performance of quantitative inhibition experiments showed that rhIL-3 competes for binding of [125I]-rhGM-CSF and vice versa, raising the possibility that IL-3 and GM-CSF elicit similar biological effects on human monocytes by binding either to a common receptor or to distinct receptors associated on the cell membrane.

MATERIALS AND METHODS

HGFs. Highly purified rhIL-3 from Escherichia coli and rhGM-CSF and rhM-CSF from COS cells were prepared as described previously. For some experiments, rhIL-3 in the form of an unpurified COS cell supernatant was used, with supernatant from mock-transfected COS cells used as a control. These HGFs were obtained from Genetics Institute (Cambridge, MA).

Because of difficulties in iodinating E coli-derived rhIL-3 to high specific radioactivity, presumably owing to the presence of only one tyrosine residue, binding studies with this cytokine were performed using a modified rhIL-3 produced in yeast with an N-terminal octapeptide containing specific radioactivity, presumably owing to the presence of only one.

Purification of human monocytes. PB from normal volunteers was obtained from the Adelaide Red Cross Transfusion Service, and mononuclear cells were prepared by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). The cells were washed twice with Hank’s balanced salt solution (HBSS) without Ca++ or Mg++ at 150 g to minimize platelet contamination and resuspended in RPMI 1640.

For cell survival experiments, the mononuclear cells were further purified by adherence to serum-coated 35-mm tissue culture dishes (LUX, Naperville, IL) in RPMI 1640 with 10% heat-inactivated pooled human AB serum. After 1-hour incubation at 37°C, nonadherent cells were washed off with at least three changes of medium, and adherent cells were further cultured in situ. For 3H-thymidine experiments, adherent cells prepared as above were detached using a Teflon policeman at 4°C in 0.02% ethylene diaminetetraacetic acid (EDTA) and washed twice in RPMI 1640, and aliquots were placed in 96-well plates (Nunc, Kamstrup, Denmark) at 0.5 × 10^6 or 1 × 10^6 cells per well. Monocyte purity using these methods was 88.8% ± 1.2% (mean ± SEM in five experiments) by staining with Wright’s-Giemsa. The contaminating cells comprised approximately 6% lymphocytes and 5% granulocytes.

For receptor-binding experiments, monocytes were purified by countercurrent elutriation using a modification of the method of Sanderson et al. Mononuclear cells were washed twice in RPMI 1640 at 150 g and resuspended in elutriation medium consisting of HBSS, 0.02% EDTA, and 0.1% heat-inactivated human AB serum. The cells were separated in a Beckman J-GM/E elutriator (Palo Alto, CA) using the Sanderson chamber, with a rotor speed of 2050 rpm and a flow rate of 11.8 mL/min. Cells remaining in the chamber after 30 minutes were collected, washed twice in RPMI, and used immediately. For some experiments, elutriated monocytes from several donors were pooled to achieve the required numbers. These methods resulted in a monocyte purity by Wright’s-Giemsa staining of 91% ± 3.4% (mean ± SEM in five experiments). In addition, >90% of elutriated cells were phagocytic for opsonized zymosan. The contaminating cells comprised approximately 4% lymphocytes and 5% granulocytes, principally basophils. Neutrophils were prepared by density centrifugation on Lymphoprep as previously described.

Monocyte culture. For survival experiments, adherent cells were incubated in 35-mm dishes for the indicated times in 2 mL RPMI supplemented with 10% human AB serum, L-glutamine, penicillin G, gentamicin, and NaHCO3. Batches of serum were selected for their inability to support monocyte cultures without exogenous hematopoietic growth factors added. Culture medium was changed at days 4 and 8 and weekly thereafter, with readdition of any cells in suspension to the fresh medium. HGF was added on day 0 and replaced with each change of medium. Incubations were performed in a 5% CO2 incubator at 37°C. All cultures were performed in triplicate; results are mean ± SEM. Statistical comparisons between different groups were made with the unpaired t test.

Cell number and protein assay. Cell harvests were performed as described above at the indicated times. Cell number was determined by counting in a Coulter counter (Hialeah, FL), and protein was measured by Coomassie blue G250 binding (BioRad, Richmond, CA). Protein measurements are expressed as the mean ± SEM of three replicates per point. Monocyte/macrophage purity was assessed at harvest after cytometric preparations were stained with a modified Wright’s-Giemsa stain (Hema-tek, Ames, IN). Cells were further characterized using nonspecific esterase/chloroacetate (NSE) staining, and with the monocyte/macrophage-specific monoclonal antibody (MoAb) anti-MO2 (Coulter) by an alkaline phosphatase-antialkaline phosphatase technique. This showed 97.1% ± 0.8% (mean ± SEM for eight experiments) of cells with the morphology of mononuclear phagocytes and 95.4% ± 1.3% staining positively for NSE. Labeling with MO-2 revealed positive staining in 93.6% ± 2.5% (mean ± SEM) of cells. Therefore by morphological, histochemical, and adherence criteria, the harvested cell population comprised predominantly cells of the monocyte/macrophage lineage. Measurement of forward-degree scatter as an estimate of cell size was made by flow cytometry with a Coulter EPICS V instrument.

3H-thymidine incorporation. For 3H-thymidine incorporation experiments, 0.5 to 1 × 10^6 adherent cells prepared as described above were aliquoted to each well in 96-well plates and supple-mented with medium plus HGF or control to a total volume of 200 μL. Cultures were pulsed on day 4 with 1 μCi/mL 3H-thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) and harvested 18 hours later onto glass filters with an automated cell harvester (Titertek, Uxbridge, UK). Harvesting was performed under hypoxic conditions and after initial lysis of the cells with 0.02% Triton.
X (Labchem, Sydney, Australia) in water. These procedures ensured that only thymidine incorporated into DNA was subsequently counted. Filter discs were dried and counted in a liquid scintillation counter (Beckman Instruments).

Autoradiography. Four-day-old cells were pulsed for 18 hours with [³H]-thymidine and then detached and used to prepare cytospin trifuge slides. The slides were fixed in methanol/acetic acid (3:1), gelatin-coated, air-dried, and dipped in Ilford K2 emulsion. Slides were developed after 24-hour exposure, and the labeling index was determined by counting the percentage of cells with ≥15 grains per nucleus. A minimum of 400 cells were counted per slide, and results were expressed as the mean ± SEM of duplicate slides per stimulus.

Colon-forming assay. The colony-forming assay was performed in agar culture as previously described⁶ with adherent and nonadherent fractions of mononuclear cells, prepared as described above. rhGM-CSF in different concentrations and human placental conditioned medium (HPCM) prepared as previously described¹⁷ were used as stimuli, and colonies were counted on days 7 and 14.

Measurement of endotoxin. All HGFs used in biological experiments were tested for the presence of endotoxin by the Limulus amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Endotoxin was undetectable (<0.03 ng/mL) at the maximal concentrations of IL-3 and GM-CSF used in these experiments.

Radioiodination of HGFs. Iodination of IL-3 to high specific radioactivity was achieved by using purified rhIL-3 modified by addition of an N-terminal octapeptide with a free tyrosine residue.²² Reaction with I¹²⁵I was as reported previously.²³ rhGM-CSF was iodinated by the two-phase method of Tejedor and Ballesta²⁶ or by the reaction with I¹²⁵I.²⁶

Iodinated protein was separated from free I¹²⁵I by chromatography on a Sephadex G-25 PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated in phosphate-buffered saline (PBS) containing 0.02% Tween 20, and stored at 4°C (¹²⁵I rhIL-3) or at −20°C (¹²⁵I rhGM-CSF) for up to 4 weeks. Before use, the iodinated protein was purified from Tween and nonprotein-associated radioactivity by cation exchange chromatography on a 0.3-mL CM-Sepharose CL-6B column (Pharmacia) and stored at 4°C for up to 5 days. The radiolabeled IL-3 and GM-CSF retained >90% biological activity as judged from titration curves using noniodinated rhIL-3 and rhGM-CSF as controls.²¹ rhIL-3 was tested in an eosinophil activation assay, and rhGM-CSF was tested in an eosinophil or neutrophil activation assay as described previously.²²

Radioreceptor assay. Freshly purified monocytes were suspended in binding medium consisting of RPMI 1640 supplemented with 20 mmol/L HEPES and 0.5% bovine serum albumin (BSA). Typically, 4 × 10⁶ monocytes in 0.15 mL medium were incubated with iodinated ligand in siliconized glass tubes overnight at 4°C on a rotating table. Cell suspensions were overlaid on 0.2 mL fetal calf serum (FCS) at 4°C and centrifuged for 30 seconds at maximum speed in a Beckman Microfuge 12. The tip of each tube was cut off above the visible cell pellet and counted in a Packard Auto-Gamma 5650 (Downer's Grove, IL). Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled cytokine. In the case of I¹²⁵I rhIL-3, preliminary experiments demonstrated complete inhibition of radioligand binding by either modified rhIL-3 (containing the N-terminal octapeptide) or by unmodified rhIL-3. Subsequent experiments were performed using unmodified rhIL-3 as competitor. Specific binding was calculated by subtracting nonspecific from total binding, and free radioligand was the difference between total radioactivity added and that specifically bound.

Cross-competition experiments were all performed overnight at 4°C, either with or without 0.1% sodium azide. To determine whether receptor internalization could occur under these conditions, acid-resistant radioactivity was measured as described previously.²⁶ Aliquots of cells were resuspended in 2 vol 0.5 mol/L acetic acid/0.5 mol/L NaCl, pH 2.0 for 5 minutes at 4°C followed by centrifugation on FCS as described. Total cell-associated radioactivity was measured on the same samples with binding medium (pH 7.2) substituted for the low pH buffer. Dissociable radioactivity was defined as the difference between total specific counts bound and acid-resistant specific counts bound.

For Scatchard analysis of binding data,³¹ free radioactivity was corrected for maximal binding capacity (MBC).²²,²³ and specific radioactivity was determined by self-displacement analysis³² after correction was made for MBC. MBC and specific radioactivity (SR) for the radioligand preparations used in the seven experiments shown in Table 3 were: experiments 1 and 2, MBC 73%, SR 1.9 × 10⁶ cpm/pmol; experiment 3, MBC 85%, SR 2.1 × 10⁶ cpm/pmol; experiments 4 and 5, MBC 50%, SR 0.9 × 10⁶ cpm/pmol; experiment 6, MBC 30%, SR 0.6 × 10⁶ cpm/pmol; and experiment 7, MBC 50%, SR 1.2 × 10⁶ cpm/pmol.

Binding data were analyzed by the EBDA and LIGAND computer programs (Elsevier-Biosoft, Cambridge, England).²⁹ Comparisons of variance about the regression lines for one site and two-site binding models were made by LIGAND using objective statistical measures with weighting for the number of replicates per point (F test, sum of the squares).

RESULTS

rhIL-3 and rhGM-CSF maintain human monocyte numbers in vitro. Figure 1 shows the percent survival over time of monocytes cultured in medium alone and with 20 pmol/L rhIL-3 or rhGM-CSF. Although control cultures showed a gradual decrease in monocyte numbers with time, HGF-treated cultures were maintained at approximately 70% to 80% of initial values. HGF-treated cultures showed significantly higher numbers of cells by unpaired t test at days 6, 8, and 11 of culture (P < .01).

Several experiments were performed with cell harvest at a single time point between 7 and 14 days of culture. The results of three such experiments with rhIL-3 and five experiments with rhGM-CSF are summarized in Table 1. The data show that both cytokines are an effective stimulus for maintenance of monocyte/macrophage numbers and that HGF-treated cells have an increased size as compared with controls as measured by forward-degree scatter on flow cytometry (Table 2).

The specificity of the HGF–monocyte interaction is shown in a representative experiment (Fig 2) in which rhIL-3 and rhGM-CSF, but not rhG-CSF, maintained increased cell numbers (Fig 2A) and protein content per culture (Fig 2B) as compared with controls.

These results suggest that rhIL-3 and rhGM-CSF either enhance monocyte survival or lead to low-grade monocyte proliferation balanced by cell death. To investigate these possibilities, we used [³H]-thymidine incorporation as a measure of monocyte proliferation. Cultures were pulsed with 1 μCi/mL [³H]-thymidine for 18 hours from day 4 of culture, because preliminary experiments demonstrated only low levels of incorporation before day 4. Cell numbers per well were never more than 30% higher in HGF-treated versus untreated wells at days 4 and 5 of culture (Fig 1 and data not shown). Figures 3 and 4 compare representative cell number and [³H]-thymidine experiments at different concentrations of HGFs. Both cytokines influenced monocyte numbers at
concentrations of \( \leq 20 \) pmol/L (Figs 3A and 4A); in contrast, HGF-induced \(^{3}H\)-thymidine incorporation required concentrations of cytokine of at least 60 pmol/L (Figs 3B and 4B). These findings suggest that the primary mechanism for the observed maintenance of monocyte numbers is one of enhanced cell survival.

**rhIL-3 and rhGM-CSF stimulate \(^{3}H\)-thymidine uptake in a subset of monocytes.**

Figure 5 shows a summary of five experiments in which \(^{3}H\)-thymidine uptake was measured at day 4 of monocyte culture. Although the level of responses varied from one individual to another, \(^{3}H\)-thymidine uptake was significantly enhanced in five of five experiments with rhIL-3 and in three of five with rhGM-CSF.

To establish the proportion of cells incorporating \(^{3}H\)-thymidine and to ensure that the thymidine uptake reflected incorporation into DNA, we performed autoradiographic studies. These demonstrated a small increase in the proportion of cells with \( \geq 15 \) nuclear grains in HGF-treated cultures as compared with medium control (Table 2). Of monocytes treated with rhIL-3, 2.15% \( \pm \) 0.42% (mean \( \pm \) SEM of four experiments); and of monocytes treated with rhGM-CSF, 1.7% \( \pm \) 0.07% (mean \( \pm \) SEM of two experiments) showed \( \geq 15 \) grains per nucleus. In contrast, 0.46% \( \pm \) 0.14% of control monocytes (mean \( \pm \) SEM of four experiments) showed \( \geq 15 \) nuclear grains.

Although these experiments were performed using highly purified monocytes, a small proportion of contaminating cells was present. To determine whether contamination with PB-derived colony-forming units (CFU) might account for the observed \(^{3}H\)-thymidine uptake in our adherent cell fractions, we performed semisolid agar culture of adherent and nonadherent fractions of the mononuclear cell population. Measurements of both day 7 clusters and day 14 colonies showed that although some CFU-GM were present in the nonadherent fraction, there was no significant clone formation in the adherent fraction (data not shown).

**Characterization of receptor binding for IL-3 and GM-CSF.**

To determine the characteristics of receptor binding for IL-3 and GM-CSF on monocytes, equilibrium binding data regarding receptor affinity, number, and specificity were gathered. Figure 6 shows the equilibrium binding curve and Scatchard analysis for the binding of \(^{125}I\) IL-3 to fresh human monocytes. Figure 7 shows corresponding data for \(^{125}I\) rhGM-CSF binding. Although the level of nonspecific binding for \(^{125}I\) rhGM-CSF in Fig 7 appears high as compared with the corresponding data for \(^{125}I\) IL-3, this is
fled by elutriation, blood from two donors was purified by adherence (individuals 4 and 5). Both methods of monocyte purification resulted in curvilinear Scatchard plots and a preferred two-site binding model. These binding experiments were conducted for 18 hours at 4°C. The pattern of binding was, however, similar when experiments were performed at 24°C for 1 hour (data not shown). The Scatchard plots for 125I rhGM-CSF binding to monocytes and neutrophils purified from the same donor and using the same range of concentrations of 125I rhGM-CSF are shown in Fig 8. The curvilinear plot and dual binding site model for monocytes (high-affinity Kd 4.7 pmol/L, receptor number/cell 35; low-affinity Kd 991 pmol/L, receptor number/cell 657) contrasts with the straight plot obtained for neutrophils (Kd

Fig 3. Stimulation of purified monocytes by different concentrations of rhIL-3. (A) Number of cells harvested at day 7 of culture. (B) In a separate experiment, 3H-thymidine incorporation in an 18-hour period from day 4 of culture. Results are the mean ± SEM of three replicates (A) and six replicates (B) per point. A result of lower total binding for rhGM-CSF and reflects the lower number of binding sites observed for rhGM-CSF on these cells (Table 3). Scatchard analysis of specific binding data showed a curvilinear plot for each radioligand (Figs 6B and 7B) and statistical comparisons resulted in preference for a two-site binding model (P < .001 for 125I rhIL-3, P ≤ .022 for 125I rhGM-CSF). Binding affinities, the number of high- and low-affinity binding sites per cell, and levels of significance are shown in Table 3 for three experiments with rhIL-3 and four with rhGM-CSF. Although most binding experiments were performed with monocytes puri-

Fig 4. Stimulation of purified monocytes by different concentrations of rhGM-CSF. (A) Number of cells harvested at day 7 of culture. (B) In a separate experiment, 3H-thymidine incorporation in an 18-hour period from day 4 of culture. Results are the mean ± SEM of three replicates (A) and six replicates (B) per point.
172 pmol/L; receptor number/cell 580), and suggests that the low-affinity binding site on monocytes is not an experimental artifact.

To establish the specificity of rhIL-3 and rhGM-CSF binding to monocytes, competition experiments were performed in which a variety of purified recombinant cytokines were used to compete for binding of the radiolabeled ligand (Fig 9). These experiments were conducted overnight at 4°C. Neither the monocyte-active cytokines rm-CSF, rIFN-γ, and rTNF-α, nor rhIL-1 and rhG-CSF tested at 100-fold excess molar concentration were able to compete for 125I rhIL-3 or 125I rhGM-CSF binding. In contrast, 60% of the binding of 125I rhIL-3 to monocytes was competed for by noniodinated rhGM-CSF (Fig 9A) and almost 80% of noniodinated rhIL-3 or rhGM-CSF completely inhibited binding of the homologous ligand, but only 40% of binding of the heterologous ligand, even at 1,000-fold excess molar concentration. In addition, rhIL-3 showed greater efficiency at competing for 125I rhIL-3 and 125I rhGM-CSF binding at low competitor concentrations than did rhGM-CSF (Fig 10A and B). Incubation of 0.1% sodium azide in competition experiments resulted in no change in the pattern of competition observed. Experiments to determine whether receptor internalization could occur under these conditions showed that ≥82% of total specific cell-associated radioactivity was acid-dissociable, both with and without sodium azide added. These findings suggest that the observed cross-competition cannot be explained by receptor transmodulation.

Table 2. Labeling Index of Day 4 Monocytes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>rhIL-3</th>
<th>rhGM-CSF</th>
<th>Medium control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>1.8</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>1.7</td>
<td>1.6</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>ND</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>2.9</td>
<td>ND</td>
<td>0.5</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>2.15 ± 0.42</td>
<td>1.7 ± 0.071</td>
<td>0.46 ± 0.14</td>
</tr>
</tbody>
</table>

Monocytes were cultured for 4 days with or without HGF at a concentration of 600 pmol/L and pulsed for 18 hours with 1 μCi/mL 3H-thymidine. Cells were harvested, and autoradiography was performed as described in Materials and Methods. The results of four experiments with rhIL-3 and medium control and two experiments with rhGM-CSF are shown. Each value is the mean of two determinations in which a minimum of 400 cells was counted. The labeling index was defined as the percentage of cells with more than 15 grains per nucleus.

Fig 6. Percentage of increase in 3H-thymidine incorporation in 4-day-old monocyte cultures. Effects of rhIL-3 and rhGM-CSF at concentrations of 600 pmol/L were compared with no stimulus in five separate experiments. Monocytes (0.5 × 10⁶) purified by adherence were incubated for 4 days in 96-well plates and then pulsed with 1 μCi/mL 3H-thymidine for 18 hours before harvest onto glass filters. Results are the mean ± SEM of six replicates. HGF-treated groups showed significantly higher values by unpaired t test for five of five experiments with rhIL-3 (P ≤ .05) and for three of five experiments with rhGM-CSF (P ≤ .05).

To determine whether higher concentrations of nonradio-labeled heterologous competitor could completely inhibit 125I rhIL-3 and 125I rhGM-CSF binding to monocytes, quantitative inhibition experiments were performed. Figure 10 shows a representative experiment in which a fixed concentration of radioligand (10⁻¹⁰ mol/L) was incubated with increasing concentrations of homologous and heterologous competitor. rhIL-3 and rhGM-CSF completely inhibited binding of the homologous ligand, but only 40% of binding of the heterologous ligand, even at 1,000-fold excess molar concentration.

DISCUSSION

We showed that rhIL-3 and rhGM-CSF, two hematopoietic growth factors that stimulate monocytopoiesis in humans, regulate the number and size of blood monocytes in vitro and bind with high and low affinities to monocyte surface receptors. Of particular interest is the demonstration of partial cross-competition between rhIL-3 and rhGM-CSF for binding to human monocytes, suggesting that the cell surface receptors for the two cytokines are either closely associated on the monocyte membrane or that there exists a common HGF receptor which may mediate the similar biological effects of IL-3 and GM-CSF on monocytes.

Figure 1 shows that rhIL-3 and rhGM-CSF maintain monocyte numbers in culture over an 11-day period, while unstimulated cultures show a gradual decrease in numbers. The specificity of the effect is shown in Fig 2: rhIL-3 and rhGM-CSF but not rhG-CSF resulted in increased mono-
Fig 6. Binding curve (A) and Scatchard analysis (B) for binding of \(^{125}\)I rhIL-3 to purified human monocytes; \(^{125}\)I rhIL-3 was incubated at different concentrations with 2 to 10 \(\times\) 10^5 monocytes at 4\(^{\circ}\)C for 18 hours, and cell-bound radioactivity was separated from unbound radioactivity by centrifugation on FCS at 4\(^{\circ}\)C. Nonspecific binding was determined in the presence of a 100-fold excess molar concentration of unlabeled rhIL-3. Each point is the mean of at least two replicates. Scatchard data were analyzed by EBDA and LIGAND computer programs.

Fig 7. Binding curve (A) and Scatchard analysis (B) for binding of \(^{125}\)I rhGM-CSF to purified human monocytes. Experimental procedures are outlined in Materials and Methods and in the legend to Fig 6.

Table 3. Equilibrium Binding Characteristics for rhIL-3 and rhGM-CSF on Human Monocytes

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Individual</th>
<th>High-Affinity Site</th>
<th>Low-Affinity Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Apparent Kd (pmol/L)</td>
<td>Receptors/Cell</td>
</tr>
<tr>
<td>rhIL-3</td>
<td>1</td>
<td>38.2</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.5</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.7</td>
<td>95</td>
</tr>
<tr>
<td>rhGM-CSF</td>
<td>4</td>
<td>8.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>38.9</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.7</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10.0</td>
<td>67</td>
</tr>
</tbody>
</table>

Radiolabeled ligand prepared as described in Materials and Methods was used at concentrations of up to 3.5 nmol/L with at least 10 dilutions per experiment. Radioligand was incubated with either elutriated monocytes (individuals 1, 2, 3, 6, and 7) or monocytes purified by adherence (individuals 4 and 5) overnight at 4\(^{\circ}\)C, and cell-bound radioactivity was separated from unbound radioactivity by centrifugation on FCS at 4\(^{\circ}\)C. Each point on the binding curve generated was the mean of at least two replicates, with between 2 and 10 \(\times\) 10^5 cells used per replicate. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand. Scatchard data were analyzed by EBDA and LIGAND computer programs. A two-site binding model was statistically superior to a single-site model for all experiments (experiments 1, 2, 3, 6, and 7, \(P < .001\); experiment 4, \(P = .022\); experiment 5, \(P = .006\)).
Fig 8. Scatchard analysis for the binding of $^{125}$I-rhGM-CSF to purified human monocytes (A) and neutrophils (B) prepared from the same donor. The experimental protocol, including maximal concentrations of radioligand used, was the same for the two cell types.

Fig 9. Competition for $^{125}$I-rhIL-3 binding (A) and $^{125}$I-rhGM-CSF binding (B) to purified human monocytes from different donors: 10^{-10} mol/L radioligand was incubated with 4 to 6 x 10^6 monocytes with or without 10^{-6} mol/L competitor cytokine. Specific cpm bound per 10^7 cells without competitor was 6,950 for $^{125}$I-rhIL-3 binding and 580 for $^{125}$I-rhGM-CSF binding. Each point represents the mean ± SD of at least two replicates.

Fig 10. Competition for $^{125}$I-rhIL-3 binding (A) and $^{125}$I-rhGM-CSF binding (B) to human monocytes from different donors: 10^{-10} mol/L radioligand was incubated with 4 to 6 x 10^6 cells and increasing concentrations of homologous and heterologous competitor cytokine. Specific cpm bound per 10^7 cells without competitor added were 7,130 for $^{125}$I-rhIL-3 binding and 1,060 for $^{125}$I-rhGM-CSF binding. Each point represents the mean of at least two replicates.
cyte numbers and protein content per culture as compared with controls. The sensitivity of this interaction is shown in Figs 3A and 4A, in which enhanced survival is evident at low picomolar concentrations of HGF. These findings are consistent with previous reports demonstrating survival effects of HGF on both immature hematopoietic cells, and mature granulocytes at lower concentrations than those needed to induce proliferation or functional change. The greater sensitivity of mature cell survival as compared with functional activation in response to HGF suggests a hierarchy of responses which may be of relevance in inflammation. Thus, production of HGF at the inflammatory site may lead initially to enhanced monocyte and granulocyte survival. With more severe or prolonged inflammation, HGF concentrations may reach levels needed to cause proliferation and functional activation of myeloid cells.

Lipopolysaccharide (LPS) has been reported to enhance human monocyte survival as well, possibly by inducing M-CSF secretion. Our experiments used highly purified recombinant cytokines which contained undetectable levels of LPS (<0.03 ng/mL by Limulus assay) at the highest concentrations used. These findings make it unlikely that our results were an LPS-induced artifact.

The data shown in Figs 3B, 4B, and 5 show that in addition to a survival effect at low dose, rhIL-3 and rhGM-CSF can also enhance 3H-thymidine incorporation in 4-day-old adherent cultures. This effect is only noted, however, at concentrations of ≥60 pmol/L HGF and cannot therefore be responsible for the observed influence of rhIL-3 and rhGM-CSF on cell numbers at lower cytokine concentrations. The autoradiographic data (Table 2) illustrate two further points. First, only a small subpopulation of cells (1% to 3%) had nuclear grains. Second, the localization of grains over the nucleus rather than random distribution over the cells shows that the enhanced 3H-thymidine uptake noted in these cultures was a consequence of uptake into DNA.

The most likely explanation for these findings is that a subpopulation of monocytes is capable of proliferating in response to high concentrations of rhIL-3 or rhGM-CSF. This conclusion is supported by the finding of occasional mitotic figures in HGF-treated but not untreated cultures (data not shown). An alternative hypothesis, namely that HGF simply may have enhanced DNA repair mechanisms in a subpopulation of cells, appears less likely but has not been completely excluded by these experiments.

The results of colony assays performed with adherent and nonadherent fractions of mononuclear cells show that using adherence as a purification step effectively removes CFU-GM from the monocyte population. Identification of occasional myeloblasts and promyelocytes in monocytes prepared by elutriation (data not shown) led us to avoid use of this purification method for survival and 3H-thymidine experiments. These data suggest that the enhanced 3H-thymidine incorporation noted in monocyte cultures treated with high concentrations of rhIL-3 and rhGM-CSF results from uptake by a small subset of monocytes, although uptake by a contaminating cell type other than CFU cannot be entirely excluded. Our findings are in keeping with a recent report of M-CSF-induced human monocyte proliferation in which only a small subpopulation of cells (up to 5%) was capable of division. The findings in humans are in striking contrast with those from murine studies, which have shown up to 90% of murine mononuclear phagocytes proliferating in response to M-CSF. GM-CSF is also an effective proliferative signal in mouse macrophages, both alone and in synergy with M-CSF, but IL-3 has been reported to be active only as a synergizing agent.

Because stimulation of human monocyte survival occurs at low picomolar concentrations of rhIL-3 and rhGM-CSF, it was important to examine monocytes for the presence of high-affinity receptors for rhIL-3 and rhGM-CSF. As shown in Figs 6 and 7, Scatchard analysis of equilibrium binding data for each ligand yielded a curvilinear plot, with a two-site binding model preferred. Table 3 shows that the binding affinity range for both high- and low-affinity sites are similar for the two cytokines, although the number of sites per cell varied between cytokines and experiments. The dual binding site model contrasts with data previously reported by one of us (L.S.P) on the binding of rhIL-3 to human monocytes, which showed only a single affinity binding site with a kd of 1.1 x 10^-10 mol/L. However, several features distinguish the methods used in the two studies. Park et al used 1-day-old leukocyte layers to prepare monocytes and purified the cells on Percoll gradients. In contrast, the studies reported here used cells less than 4 hours old purified by countercurrent elutriation. The influence of methods of purification and cell maturity on monocyte function could therefore explain the differences observed in the two studies.

Previous reports of a single class of binding sites for GM-CSF on human monocytes were based on autoradiographic data or were presented without binding curves or Scatchard plots, making a direct comparison with our work difficult. In addition, the methods used by each of these groups of investigators differed from ours. Low-affinity binding sites have previously been reported for GM-CSF binding to murine cells and for IL-3 on some tumor cell lines, but not on a purified primary cell type in humans. The presence of high- and low-affinity receptors for IL-3 and GM-CSF on monocytes contrasts with the presence of only high-affinity receptors for M-CSF on mouse macrophages. Similarly, although the numbers of IL-3 and GM-CSF receptors on human monocytes are remarkably low, mouse macrophages express about 50,000 M-CSF receptors per cell. These receptors have a role in regulation of circulating M-CSF concentrations, a function which is presumably dependent on their high level of expression. These findings highlight the differences between receptors for M-CSF and those for IL-3 and GM-CSF and suggest fundamentally different biological roles for the two groups of HGF.

Possible experimental artifacts giving rise to nonlinear Scatchard plots were carefully controlled for in these experiments. Non-specific binding was determined for each ligand concentration in the presence of a 100-fold excess of unlabeled ligand. Free ligand concentrations were corrected for maximal binding capacity, and the specific radioactivity of labeled protein was accurately determined by self-displacement analysis. In addition, binding of 125I rhGM-CSF to...
neutrophils and monocytes from the same donor (Fig 8) measured concurrently yielded a straight Scatchard plot and single receptor class for neutrophils but a curvilinear plot and two receptor classes for monocytes. During the course of these experiments, we performed binding studies on monocytes prepared by both elutriation and adherence to plastic and measured binding both at 4°C (for 18 hours) and at 24°C (for 1 hour). Two affinity classes were observed with each protocol.

The low concentrations of rhIL-3 and rhGM-CSF needed to enhance monocyte survival were consistent with an effect through binding to the high-affinity receptors. Although higher concentrations of HGF were needed to enhance monocyte 3H-thymidine uptake, whether this effect is mediated by the low-affinity binding site remains to be established. High concentrations of HGF might be generated at inflammatory sites, and in other microenvironments, allowing direct interaction with low-affinity receptors. However, the exact significance of the low-affinity binding site either in terms of structure or of function is unknown.

Of particular interest in these experiments was the demonstration of cross-competition for 125I rhIL-3 and 125I rhGM-CSF binding by each other. In the experiment shown in Fig 10, up to 40% of ligand binding was inhibitable by the heterologous cytokine and rhIL-3 showed greater efficiency at competing for radioligand binding at low concentrations than did rhGM-CSF. The specificity of this interaction is shown in Fig 9, in which other purified recombinant cytokines failed to act as competitive inhibitors. These include rhM-CSF, rhIFN-γ, and rhTNF-α, all known to have biological effects on human monocytes, and rhG-CSF and rhIL-1 in addition. Previous work on murine BM cells has demonstrated partial inhibition of 35S-murine GM-CSF binding by murine IL-3 at 20 and 37°C. The cross-inhibition was interpreted as resulting from downregulation or modification of the GM-CSF receptor, and these investigators subsequently proposed a model of HGF receptor transmodulation on murine BM cells. Our competition experiments were performed at 4°C either with or without 0.1% sodium azide added. Under these conditions, ≥82% of bound radioactivity was acid-dissociable, indicating that it was surface-bound and not internalized.

These findings suggest that the observed cross-competition for 125I rhIL-3 and 125I rhGM-CSF binding to human monocytes is not a result of receptor transmodulation. Other explanations for the phenomenon may invoke a nonrandom distribution of HGF receptors on the cell membrane, with association between specific IL-3 and GM-CSF binding sites. Alternatively, three types of receptors may exist on monocytes for binding of IL-3 and GM-CSF: one receptor type binding only IL-3, a second specific for GM-CSF, and the third a common receptor, capable of binding both cytokines. Support for these hypotheses comes from the demonstration of competition for 35S rhIL-3 and 125I rhIL-3 binding to KG-1 cells by rhGM-CSF,17,46 Although further insights may result from purification and molecular cloning of the receptors concerned, the existence of associated or common receptors would be consistent with the common ancestry of the IL-3 and GM-CSF genes and with the similar biological effects of the two HGF on monocytes as we and other investigators have demonstrated. Whether all monocytes express both high- and low-affinity binding sites and both unique and competent sites for IL-3 and GM-CSF or whether differential receptor expression occurs on different monocyte subpopulations is unclear. Autoradiographic studies are currently in progress to elucidate this question.

ACKNOWLEDGMENT

We thank Matthew Greenwood for technical assistance, Drs N. Nicola and G. F. Burns for criticizing the manuscript, and Barbara Meakins for secretarial assistance.

REFERENCES

13. Chen BD, Clark CR: Interleukin 3 (IL-3) regulates the in
vitro proliferation of both blood monocytes and peritoneal exudate macrophages: Synergism between a macrophage lineage-specific colony-stimulating factor (CSF-1) and IL-3. J Immunol 137:563, 1986


27. Metcalf D: The hematopoietic colony-stimulating factors. Amsterdam Elsevier/North Holland, 1984, p 1


33. Nicola NA, Metcalf D: Binding of the differentiation-inducer, granulocyte colony-stimulating factor, to responsive but not unresponsive leukemic cell lines. Proc Natl Acad Sci USA 81:3765, 1984


Recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor show common biological effects and binding characteristics on human monocytes

MJ Elliott, MA Vadas, JM Eglinton, LS Park, LB To, LG Cleland, SC Clark and AF Lopez