Granulocyte-Macrophage Colony-Stimulating Factor Enhances Phagocytosis of Bacteria by Human Neutrophils

By Jacob Fleischmann, David W. Golde, Richard H. Weisbart, and Judith C. Gasson

In order to determine whether human granulocyte-macrophage colony-stimulating factor (GM-CSF) can enhance phagocytosis, neutrophils were combined with Staphylococcus aureus (S. aureus), and both the number of bacteria per neutrophil and the percent of neutrophils phagocytizing were assessed in the absence and presence of GM-CSF. Exposure to GM-CSF did not enable neutrophils to ingest unopsonized bacteria. When bacteria were opsonized with serum, both the number of bacteria per neutrophil and the percent of cells phagocytizing were increased by treatment with GM-CSF. Digestion of extracellular organisms by lysostaphin was used to substantiate phagocytosis. These results indicate that another effect of GM-CSF on the mature neutrophil is the enhancement of phagocytosis.

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Neutrophils (PMN). Purified PMN were obtained from heparinized blood of healthy donors by dextran sedimentation and Hypaque-Ficoll density gradient centrifugation.5 Purity of the PMN was >95% by Giemsa stain and viability of neutrophils at each time point, assessed by trypan blue exclusion, was >97%.

Organisms. Staphylococcus aureus ATCC 29213 (SA) was grown on trypticase soy agar (TSA) slants and kept at 4 °C. For individual experiments, the organism was grown overnight in trypticase soy broth (TSB) at 37 °C, washed twice by centrifugation, and suspended in Hank’s balanced salt solution with 5 mmol/L Hepes, at pH 7.4 (HBSS). The desired concentration was attained by monitoring spectrophotometric absorbance at 600 nm, using an appropriate optical density/colony-forming units (CFU) curve.

Serum. Serum was obtained from an AB donor and kept at −70 °C until used. For opsonization, either freshly thawed (NS) or heat-inactivated serum (HIS) (56 °C for 30 minutes) was used. Bacteria (1 × 10⁶) were opsonized at 37 °C for 30 minutes in one mL of serum, washed twice, and resuspended in HBSS.

Lymphokine stimulation of neutrophils. PMN (2 × 10⁶/mL) were incubated in HBSS with 0.01% bovine-serum albumin (BSA) at 37 °C in the presence of either 100 pmol/L or 1.0 nmol/L GM-CSF. For sham stimulation, cells were incubated in the same buffer in the presence of the protein diluent (PBS) with 0.01% BSA only. After either 15 minutes or 120 minutes, the cells were washed with centrifugation and resuspended in HBSS.

Phagocytosis assay. PMN (1 × 10⁶/mL) and SA (1 × 10⁶/mL) were combined in a final volume of one mL in 12 mm × 75 mm sterile, capped plastic tubes and rotated end over end at 37 °C. At appropriate times, 0.2 mL of assay solution was removed and combined with 0.2 mL of HBSS with 0.2 mmol/L N-ethyl maleimide (NEM) to inhibit additional phagocytosis.7 Cytocentrifuge slides were prepared, fixed with methanol, stained with Giemsa, and examined by light microscopy. The slides were evaluated blind by masking the identifying information. Individual PMN were evaluated as having either 0, 1 to 10, 11 to 20, 21 to 30, or >30 bacteria associated with them. A weighted phagocytic index (WPI) was calculated by multiplying the number of PMN in each category by 0, 1, 2, 3, 4, respectively, and dividing the total score by the number of neutrophils examined (usually 100). The percentage of PMN phagocytizing (having one or more bacteria) was also assessed.

Lysostaphin modification. As light microscopy does not differentiate between surface adherence and phagocytosis, lysostaphin was used in one assay to digest extracellular bacteria.8 Cells and bacteria were combined and rotated as above. At appropriate times, 0.2 mL of the mixture were removed, combined with 0.2 mL of HBSS containing 0.2 mmol/L NEM and 40 U/mL of lysostaphin and incubated at 37 °C for 30 minutes. The mixture was then chilled in an ice water bath and cytocentrifuge slides prepared, fixed, stained, and evaluated as above. Prior to assay, 20 U/mL lysosta-

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THE neutrophil or polymorphonuclear leukocyte (PMN) is the major phagocytic cell involved in the first-line defense against invading microorganisms. The neutrophil is also the primary effector cell in acute inflammatory reactions, and participates importantly in autoimmune processes. Recently, human granulocyte-macrophage colony-stimulating factor (GM-CSF), a 22 kd glycoprotein hormone produced by activated T lymphocytes, was shown to have direct effects on the mature PMN. GM-CSF causes neutrophil migration inhibition1 and primes the PMN to increase superoxide anion generation in response to the bacterial chemottractant, N-formyl-methionyl-leucyl-phenylalanine (f-MLP)3 and complement derivatives.3 We have tested the effect of GM-CSF on PMN phagocytosis, a key function of this cell in host defense.

MATERIALS AND METHODS

Granulocyte-macrophage colony-stimulating factor (GM-CSF). Biosynthetic GM-CSF was purified from medium conditioned by COS monkey cells transfected with the GM-CSF cDNA clone in the p91023(B) expression vector.4 Purification was performed as described previously15 employing sequential concentration, lentil lectin affinity chromatography, gel filtration, and reverse-phase high-performance liquid chromatography. The concentration of the homogenous protein was estimated by amino acid analysis, and the purified protein was shown to be free of endotoxin (<0.1 ng/mL) by the limulus amebocyte assay.

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GM-CSF ENHANCES PHAGOCYTOSIS

Phagocytosis

Even in the presence of 0.1 mmol/L NEM, GM-CSF ENHANCES PHAGOCYTOSIS, and stimulation with GM-CSF showed no effect (Table I). Figure 1 shows the combined, described by many free bacteria; cultures with lysostaphin stained slides showed counts of SA from 1 x 10^3 to 0 in 30 minutes, paralleled those of B.

RESULTS

Neutrophils combined with untreated SA were inefficient in phagocytosing bacteria, and stimulation with GM-CSF showed no effect (Table I). Figure 1 shows the combined, normalized data regarding phagocytosis of opsonized SA. Enhancement of phagocytosis was observed after 15 minutes of exposure of PMN to 100 pM GM-CSF (Fig 1A). An even greater increase over control was observed after 120 minutes (P < .05; Fig 1B), a concentration and time period observed to enhance other PMN functions. Raw data from one experiment are shown for comparison (Table I). A tenfold increase in GM-CSF concentration to 100 pM GM-CSF (Fig 1C) did not further enhance phagocytosis. In one experiment with lysostaphin digestion (D) to prove ingestion (see text), increases paralleled those of B.

In order to distinguish between phagocytosis of the bacteria and adherence to the outer surface of the neutrophil membrane, lysostaphin treatment was performed. The organisms were resistant to digestion with lysostaphin, demonstrating the apparently phagocytosed bacteria were in fact intracellular (Fig 1D). Another measurement of phagocytosis is the percent of PMN-ingesting organisms. As shown in Fig 2, after exposure to 100 pM GM-CSF for two hours, a higher percentage of PMN was phagocytic, as compared to control.

Opsonization with normal serum and heat-inactivated serum was compared to determine whether heat-labile complement components were required. Figure 3 shows that priming by GM-CSF enhanced phagocytosis of organisms opsonized with heat-inactivated serum (part B), but was more efficient when both heat-labile and heat-stable components were available (Part A).

Figure 4 shows the data regarding intracellular killing by neutrophils. In this assay, neutrophils are combined with bacteria in a ratio of 1:1, thus obviating any effects of GM-CSF on phagocytosis. There is no difference in killing between GM-CSF-stimulated and -unstimulated cells.

DISCUSSION

In addition to stimulating hematopoietic cell proliferation, colony-stimulating factors affect the functional activity of mature effector-cells. When murine peritoneal macrophages are exposed to GM-CSF, they increase their ability to phagocytize and kill Leishmania tropica. Conditioned media with colony-stimulating activity from several cell types have also been shown to increase tumoricidal capacity.

Table 1. Enhancement of Phagocytosis by GM-CSF

<table>
<thead>
<tr>
<th>Time*</th>
<th>Neutrophils‡</th>
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<th>Bacteria per Neutrophil</th>
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<tr>
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<td>97</td>
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<tr>
<td>32 min</td>
<td>+</td>
<td>13</td>
<td>19 38 19 11</td>
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</tbody>
</table>

*Time before phagocytosis was inhibited.
‡Exposure was to 0.1 mmol/L GM-CSF (+) or diluent (-) for two hours.
†Opsonized with normal AB serum for 30 minutes.

Fig 1. Phagocytosis of S. aureus by PMN exposed to diluent only (control) (b) or exposed to GM-CSF (C) prior to combination with bacteria. WPI (weighted phagocytic index; see Methods) were calculated for each time period (see text) and expressed as a ratio of maximum WPI of control (WPI at 32 minutes). Error bars represent SEM. At 100 pmol/L GM-CSF for 15 minutes (A, n = 2), the increases are not yet significant, but after 120 minutes (B, n = 4), all time points are significant at P < .05 (as calculated by t test for comparison of paired data). Tenfold increase of GM-CSF (C, n = 3) did not further enhance phagocytosis. In one experiment with lysostaphin digestion (D) to prove ingestion (see text), increases paralleled those of B.

Fig 2. Percent of PMN having ingested one or more organisms. PMN were exposed to diluent only (control) (b) or 100 pmol GM-CSF (C) for 120 minutes (n = 4). Error bars represent SEM. At all time points, more GM-CSF-treated PMN contained bacteria; the values at the earliest times tested were significantly different (at 4 min, P < .05, as calculated by student t test).
and intracellular killing of Candida parapsilosis by mononuclear phagocytes. Similar end-cell regulatory activity by GM-CSF has been demonstrated for murine and human PMN. GM-CSF increases antibody-dependent cytotoxicity, inhibits PMN migration, and enhances superoxide anion generation in response to f-MLP and C5a. The present results extend these observations to include PMN phagocytic capacity.

GM-CSF did not induce phagocytosis of unopsonized bacteria, but primed PMN to be more efficient when presented with bacteria already opsonized by serum. These findings are very similar to those of PMN and f-MLP interaction. GM-CSF by itself did not stimulate superoxide anion generation, but primed the cell to increase its response to f-MLP by fourfold. The concentration of GM-CSF (100 pmol/L) and time of exposure (two hours) at which maximal effects were generated were similar in both cases.

Opsonins in serum needed for S. aureus ingestion include antibody and complement components. It appears that GM-CSF is able to enhance phagocytosis mediated by both opsonins. When heat-inactivated serum was used (Fig 3B), GM-CSF enhanced uptake of bacteria, but when both antibody and complement were present (Fig 3A), this enhancement was more marked. The mechanism by which GM-CSF enhances PMN phagocytosis is not known. One possible explanation is modulation of receptors on the cell for the known opsonins, IgG and C3. Indeed, a lymphokine produced by the Mo cell line (the original source of this GM-CSF) has been shown to activate C3 receptors on murine peritoneal macrophages. Intracellular killing of S. aureus was not increased by GM-CSF stimulation (Fig 4), but this organism is highly susceptible to the bactericidal activities of PMN. Augmented microbicidal activity may be demonstrable with other organisms.

GM-CSF has been shown to be produced by activated T lymphocytes and present in areas of inflammation. The biological activities of GM-CSF include inhibition of neutrophil migration, enhanced phagocytosis, and increased production of bactericidal metabolites, suggesting an important role for this mediator in host defense.

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REFERENCES

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