Granulocyte-Macrophage Colony-Stimulating Factor Enhances Selective Effector Functions of Tissue-Derived Macrophages

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a variety of cells at sites of exposure to antigens. GM-CSF has a stimulatory effect on a number of neutrophil functions, but the effect on macrophage function is less clear. We investigated the effect of purified murine recombinant GM-CSF on murine peritoneal macrophage oxidative metabolism, Fc-dependent phagocytosis, anti-Toxoplasma activity, and expression of class II major histocompatibility antigen (Ia). GM-CSF significantly increased phospholipid myristate acetate- and zymosan-elicited H2O2 release by resident and thioglycollate-elicited macrophages after 48 hours in vitro. The effect of recombinant GM-CSF was blocked by polyclonal anti-GM-CSF antibody and was not altered by lipopolysaccharide (0.01 to 1.0 µg/mL). GM-CSF also stimulated Fc-dependent phagocytosis by peritoneal macrophages, although the stimulation of resident macrophages (1.4-fold) was less dramatic than that of thioglycollate-elicited cells (2.1-fold). GM-CSF (at doses up to 100 U/mL) had no effect on macrophage anti-Toxoplasma activity or on expression of Ia. In addition to stimulating macrophage growth, GM-CSF selectively promotes the functional capacity of tissue-derived macrophages.

The functional state of macrophages is regulated by glycoprotein products of T lymphocytes. Optimal conduct of the macrophage's protein effector functions requires a priming or activating signal. The principal macrophage-activating factor (MAF) is interferon-γ (IFN-γ). Based on the assumption that a vital process such as macrophage activation must be regulated by redundant mechanisms, additional MAFs have been studied. The relative importance of noninterferon MAFs is uncertain because several are products primarily of clonally selected lymphocytes or have been investigated in very high doses. In addition, many of the noninterferon MAFs have not been purified to homogeneity or have been labeled as a macrophage activator on the basis of their ability to stimulate a single macrophage function.

Considerable recent interest has been generated in the potential clinical usefulness of the colony-stimulating factors (CSFs), particularly granulocyte-macrophage CSF (GM-CSF), in promoting hematopoiesis and possibly in enhancing granulocyte and macrophage function. GM-CSF has been shown to augment granulocyte oxidative metabolism, cytotoxicity, and chemotaxis. The effects of GM-CSF on macrophage function are less clear. In some studies, GM-CSF stimulated extracellular killing of tumor cells by macrophages to a greater extent than did IFN-γ and promoted intracellular killing of Leishmania donovani by human monocyte-derived macrophages. On the other hand, GM-CSF failed to stimulate oxidative metabolism of human monocyte-derived macrophages. In addition to being produced by T lymphocytes, GM-CSF is also produced by epithelial and endothelial cells. Therefore, its relative importance in regulating macrophage function at sites of antigenic challenge needs to be more clearly defined. We have undertaken the following study to characterize the effects of GM-CSF on tissue-derived macrophages.

Materials and Methods

Animals. Female 6- to 12-week-old BALB/c mice were obtained from the National Institutes of Health breeding colony.

Media and reagents. Dulbecco's modified essential medium (DMEM) was supplemented with glutamine, 2 mmol/L, gentamicin, 25 µg/mL, streptomycin, 25 µg/mL, penicillin, 25 IU/mL, and 2-mercaptoethanol, 0.05 mmol/L (Microbiological Associates, Walkersville, MD). Heat-inactivated (56°C, 30 minutes) fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) was added to the culture media where indicated. Horseradish peroxidase (Sigma Chemical Co, St Louis) was dissolved in 50 mmol/L K2HPO4 (pH 7.0) to a concentration of 5 mg/mL and stored at −70°C prior to use. Phenol red solution (PRS) was composed of NaCl, 140 mmol/L, K2HPO4, 10 mmol/L (pH 7.0), dextrose, 5.5 mmol/L, and phenol red, 0.28 mmol/L, and was stored at 4°C prior to use. Zymosan (Sigma) was prepared by heating a 20-mg/mL solution at 100°C for 15 minutes, washing twice, and suspending to 20 mg/mL in Hank's balanced salt solution (HBSS). Opossum zymosan was prepared fresh daily by incubating 100 mg of the washed zymosan with 2.0 mL of guinea pig serum for 20 minutes at 37°C in a shaking water bath. The opsonized zymosan was washed twice in HBSS and then suspended in PRS to a concentration of 5 × 106 particles/mL. Monoclonal antibody (MKD6) raised against Ia was kindly provided by C. Janeway (Yale University). Purified, recombinant murine GM-CSF was purchased from Genzyme (Boston), and aliquots were stored at −70°C prior to use. For some experiments, native GM-CSF purified from murine T-lymphoma BRM-33-5A4 cells was purchased from Genzyme. A unit of activity was defined as the amount of GM-CSF required to support the growth of a single colony (greater than 50 cells) from 7.5 × 106 murine bone marrow cells in soft agar after 7 days. The specific activity of the GM-CSF preparations was greater than 2.2 × 1010 U/mg and 5 × 109 U/mg before the addition of carrier protein (bovine serum albumin) for the native and recombinant GM-CSF preparations, respectively. The lipopolysaccharide (LPS) from Salmonella minnesota Re595 was purchased from List Biological Laboratories, Inc (Campbell, CA). Highly purified murine recombinant IFN-γ (1.7 × 109 U/mg) was kindly provided by Genentech, South San Francisco, CA. GM-CSF and IFN-γ used in these experiments did not contain detectable activity or expression of Ia.

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(greater than 0.5 ng/U) endotoxin in the limulus amebocyte lysate assay. Neutralizing polyclonal goat antibody for murine GM-CSF was provided by Dr. J. Schreir (DNAX Research Institute, Palo Alto, CA). Murine IgG was purchased from Sigma.

Cell harvest and culture. Thioglycollate-elicited peritoneal exudate cells (PEC) were obtained as described. PEC were suspended to 1 x 10⁶/mL in DMEM with 20% FCS, and 1.0 mL was added to 16-mm wells in a 24-well plate (phagocytosis, H₂O₂ assay, anti-Toxoplasma assay), or 0.2 mL was added to each well of a 96-well plate (IAβ assay). PEC was incubated for three hours at 37°C, 95% humidity, and 5% CO₂ to permit adherence of the macrophages. Nonadherent cells were removed by washing each monolayer twice. The adherent macrophages were cultured in DMEM with 2% FCS for one to three days prior to assay. Resident peritoneal macrophages were obtained and cultured as described earlier for thioglycollate-elicited macrophages except that 7.5 x 10⁶ peritoneal cells were added to 11-mm wells for all but the IAβ assay (2 x 10⁶ cells/well in a 96-well plate). For each assay, a parallel set of macrophages incubated under identical experimental conditions was assayed for cellular protein content. Phagocytosis assay. Fc-dependent phagocytosis was measured as described. In brief, a fresh suspension of sheep erythrocytes (Colorado Serum, Denver) was combined with 0.2 mL of a 51NaCrO₄ solution (Flow Laboratories, McLean, VA) and 0.05 mL of 25NaCl (1.0 mCi/mL; New England Nuclear, Boston) to form 1 mL of 5 x 10⁶ chromium-labeled, opsonized sheep erythrocytes (CrShEA)/mL. The CrShEA suspension was incubated at 37°C for 60 minutes, washed three times with cold HBSS, and resuspended to 5 x 10⁷/mL in DMEM with 10% FCS. After removing the supernatant, approximately 2.5 x 10⁵ CrShEA (0.5 mL) was added to each macrophage monolayer and incubated for 60 minutes at 37°C with 5% CO₂ and 95% humidity. Experimental and control macrophages were assayed in triplicate. Each well was washed three times with HBSS, and extracellular erythrocytes were lysed with 1.0mL ACK-lysing solution for 60 seconds at 37°C. The wells were washed three additional times, and if extracellular erythrocytes were absent by phase microscopic examination, the monolayers were lysed with 0.6 mL of 0.5% sodium dodecyl sulfate (SDS), and radioactivity was measured with a Hewlett-Packard (Palo Alto, CA) Gamma Counter.

H₂O₂ assay. The release of H₂O₂ by adherent macrophages was determined by using the colorimetric method of Pick and Keisari. After 1 to 3 days of incubation under experimental conditions, the macrophage monolayers were washed three times with phenol-free HBSS. One milliliter of warm (37°C) PRS containing horseradish peroxidase (50 µg/mL) was added to each well and incubated at 37°C with phorbol myristate acetate (PMA) (500 ng/mL) or opossum zymosan (5 x 10⁶ particles/mL) as the triggering stimulus. After one (resident macrophages) or two hours (thioglycollate-elicited macrophages) of incubation, the supernatant (0.6 mL) was removed and brought to pH 12.5 with 0.01 mL of 1.0 N NaOH to stop the oxidation reaction. The optical density of 610 nm was read after blanking against alkalinized PRS. A standard curve was prepared with each assay by using dilutions of a stock 10-mmol/L H₂O₂ solution. The H₂O₂ concentration of the stock solution was verified daily by using an extinction coefficient for H₂O₂ of 81 mmol/L⁻¹ cm⁻¹ at 230 nm. The duration of incubation and the PMA and zymosan concentrations were determined empirically to optimize H₂O₂ release. Results from triplicate wells in individual experiments are expressed as mean nanomoles H₂O₂ released after one (resident macrophages) or two hours (thioglycollate-elicited macrophages) per milligram of cell protein. Macrophage Anti-Toxoplasma activity. Intracellular growth of Toxoplasma gondii was determined by the [³H]uracil incorporation assay with slight modification. This method is based on the observation by Pfefferkorn that [³H]uracil is incorporated in much greater amounts by intracellular T gondii than by macrophages or other mammalian cells. The amount of [³H]uracil incorporated correlates directly with intracellular parasite number and inversely with macrophage anti-Toxoplasma activity. RS strain T gondii organisms were maintained by serial passage in human fibroblast cultures (kindly provided by Dr. E. E. Pfefferkorn, Dartmouth Medical School, Hanover, NH). Resident and elicited peritoneal macrophages were cultured for one to two days with GM-CSF, IFN-γ, or control media prior to infection with RN strain T gondii at a parasite-to-host cell ratio of approximately 1:3. Eighteen hours after infection, macrophage monolayers were pulsed with 1 µCi of [³H]uracil (specific activity, 40 Ci/mmol, Moravek Biochemicals, Brea, CA) in serum-free DMEM for six hours. Monolayers were then incubated for 10 mL ice-cold 0.6 N trichloroacetic acid and then incubating for one hour at 4°C. The fixed cells were washed overnight in tap water, dried, and hydrolyzed with 0.2 mL of 3% (vol/vol) perchloric acid for one hour at 80°C. The hydrolysate was added to scintillation fluid (Opti-fluor, Packard, Downers Grove, IL) and assayed in a liquid scintillation counter (Packard Instruments). [³H] uracil incorporation by infected, untreated monolayers ranged from 15,000 to 20,000 cpm for resident macrophages (approximately 3 to 4 x 10⁶ cells) and from 50,000 to 60,000 cpm for elicited macrophages (7 to 8 x 10⁶ cells). Background [³H] uracil incorporation by uninfected macrophages treated with medium alone, GM-CSF, or IFN-γ was consistently less than 500 cpm. The percent change in [³H] uracil incorporation (parasite growth) was calculated with the formula [(B-A)/A] x 100 in which A is the mean counts per minute of control macrophages and B is the mean counts per minute of GM-CSF- or IFN-γ-treated macrophages. Assay of IAβ expression. Macrophage monolayers were washed twice with PBS supplemented with 0.05% NaN₃ and 2% FCS. Monoclonal anti-IAβ antibody (MKD6) was suspended in phosphate-buffered saline (PBS)-azide and incubated with the macrophage monolayers for 20 minutes at 4°C. The monolayers were then washed twice with PBS supplemented with 0.05% NaN₃ and 2.5% (wt/vol) nonfat dry milk. 125I-Protein A (50.2 µCi/µg, New England Nuclear) was diluted in the PBS-milk solution (1 µCi/mL) and was incubated with the monolayers for 30 minutes at 4°C. The monolayers were washed four times with PBS-milk, solubilized with 2% SDS (wt/vol), and counted in a gamma counter. Parallel experimental wells were assayed for protein and results expressed as counts per minute per milligram protein. Use of irrelevant antibody (sheep hemolysin antibody) or deletion of MKD6 antibody yielded counts per minute that were less than 2% of those observed in the presence of MKD6.

Protein assay. To determine cellular protein content, macrophage monolayers were treated with 0.5 N NaOH and protein determined according to Lowry et al.

Data analysis. Student's t test for unpaired samples (two tailed) was used to assess statistical significance. Results of individual experiments are expressed as mean (±SD).

RESULTS

To contrast the effects of GM-CSF on macrophage function with other macrophage activators such as IFN-γ, several macrophage functions were studied. First, recombinant GM-CSF (0.5 to 25 U/mL) was incubated with thioglycollate-elicited macrophages for 48 hours and release of H₂O₂ determined. GM-CSF (5.0 U/mL) produced a mean 3.9-fold (±1.2) increase (n = 8, P < 0.05) in PMA-elicited H₂O₂ release by thioglycollate-elicited macrophages. A further increase in H₂O₂ release at doses of GM-CSF above 5.0
U/mL was not observed. Data from a representative experiment are shown in Fig 1. Under identical experimental conditions, IFN-γ (25 U/mL) produced a similar increase in H₂O₂ release. GM-CSF (5.0 U/mL) increased zymosan-elicited H₂O₂ release 5.7-fold (±2.5) (n = 3, P < .05) by peritoneal macrophages. To determine whether the effect of GM-CSF persists after removal from treated macrophages, GM-CSF (10 U/mL) was incubated with thioglycollate-elicited macrophages for 48 hours. The monolayers were washed and incubated with fresh DMEM with 2% FCS. The stimulated effect of GM-CSF on H₂O₂ release was still present but disappeared by 48 hours after removal from the culture medium.

Since the response of thioglycollate-elicited macrophages to GM-CSF may differ from that of resident peritoneal macrophages, we studied the effect of GM-CSF on resident cells. GM-CSF (1.0 U/mL) increased PMA-elicited H₂O₂ release 2.6-fold (±0.3) (n = 3, P < .05) by resident macrophages after 48 hours. The stimulatory effect of GM-CSF was maximal at concentrations of 5 U/mL. Data from a representative experiment are shown in Fig 1. GM-CSF required at least 24 hours to stimulate H₂O₂ release by both resident and thioglycollate-elicited macrophages. The maximal effect of GM-CSF was observed after 48 hours of incubation.

Since the effect of GM-CSF on macrophage oxidative metabolism may be attributable to other constituents in the preparation, we attempted to block the effect of GM-CSF on macrophage H₂O₂ release with a specific neutralizing polyclonal goat anti-GM-CSF antibody. The effect of GM-CSF was completely blocked by the anti–GM-CSF antibody (data not shown). Nonspecific mouse IgG (1 to 100 ng/mL) had no effect on the stimulatory activity of GM-CSF. Although the GM-CSF was free of detectable endotoxin in the Limulus amebocyte lysate assay, the possibility exists that contaminating LPS is necessary for GM-CSF to stimulate macrophage H₂O₂ release. LPS (10 ng/mL to 1 μg/mL) had no effect, however, on the stimulation of macrophage H₂O₂ release by GM-CSF (data not shown). To be certain that the effect of recombinant GM-CSF was not limited to the recombinant material, the ability of purified, native GM-CSF to increase macrophage H₂O₂ release was tested. The recombinant and native preparations produced similar stimulatory effects on macrophage H₂O₂ release.

The effect of recombinant GM-CSF on thioglycollate-elicited macrophage Fc-dependent phagocytic activity was determined after 48 hours. GM-CSF (10 U/mL) produced a 2.1 (±0.2)-fold increase (n = 4, P < .05) in phagocytic activity. As shown in a representative experiment in Fig 2, the stimulation of phagocytic activity was maximal at 5 U/mL of GM-CSF. The effect of GM-CSF on thioglycollate-elicited macrophage phagocytic activity was consistently 20% to 30% greater than that of IFN-γ under identical experimental conditions. GM-CSF also enhanced Fc-dependent phagocytosis by resident peritoneal macrophages (1.4-fold [±0.2] increase, n = 4, 10 U GM-CSF/mL), although the magnitude of the GM-CSF effect was less striking. IFN-γ produced a similar stimulatory effect on resident IFN-γ.
macrophage Fc-dependent phagocytosis. A representative experiment is shown in Fig 2. Varying the incubation period of GM-CSF with resident macrophages from 24 to 72 hours did not alter the effect of GM-CSF.

Stimulation of macrophage oxidative metabolism has been associated with an enhanced ability to contain T. gondii. Since GM-CSF produced an increase in H$_2$O$_2$ release, we investigated whether GM-CSF inhibits the replication of Toxoplasma within macrophages. At concentrations that consistently enhanced H$_2$O$_2$ release, GM-CSF did not enhance the anti-Toxoplasma activity of either resident or thioglycollate-elicited macrophages (n = 4). A representative experiment is shown in Table 1. The apparent increase in survival of Toxoplasma organisms in GM-CSF-treated macrophages is markedly reduced when corrected for the increase in cell protein. Under similar experimental conditions, IFN-γ significantly enhanced macrophage anti-Toxoplasma activity (Table 1).

To determine whether GM-CSF stimulates expression of class II major histocompatibility complex (MHC) antigens, GM-CSF was incubated with either resident or thioglycollate-elicited macrophages for 24 to 72 hours (n = 4). As shown in Table 2, GM-CSF did not increase the expression of Ia$^b$. GM-CSF had no effect on expression of Ia$^a$ after 24 or 72 hours of incubation in vitro. Under similar experimental conditions, IFN-γ consistently increased the expression of Ia$^a$.

Since GM-CSF was not sufficient to stimulate macrophage anti-Toxoplasma activity or expression of Ia$^a$, we tested whether it could prime the macrophages for subsequent activation with LPS. GM-CSF (10 U/mL) was incubated with thioglycollate-elicited peritoneal macrophages for 24 hours, and the monolayers were washed and then incubated with LPS (0.01 to 0.1 μg/mL) for 48 hours prior to assaying anti-Toxoplasma activity and expression of Ia$^a$. GM-CSF-treated macrophages were no more responsive to LPS than were those pretreated with medium alone. Therefore, GM-CSF does not directly stimulate macrophage anti-Toxoplasma activity or Ia$^a$ expression or prime the cells for subsequent activation by LPS.

**DISCUSSION**

The data presented here strongly support the concept that GM-CSF augments selective functions of tissue-derived macrophages. GM-CSF clearly stimulates the oxidative metabolism and Fc-dependent phagocytic activity of peritoneal macrophages but does not enhance Ia$^a$ expression or anti-Toxoplasma activity. The effect of GM-CSF on macrophage H$_2$O$_2$ release and phagocytic activity occurs after 24 to 48 hours in vitro. GM-CSF increases peritoneal macrophage DNA synthesis after a similar period in vitro but requires at least 72 to 96 hours before an increase in cell number can be documented. Thus, while GM-CSF should be considered an MAF, it does not stimulate as many functions as the prototype MAF-IFN-γ.

GM-CSF enhances macrophage H$_2$O$_2$ release when either soluble (PMA) or particulate (zymosan) stimuli are used. The enhancement of oxidative metabolism is very unlikely to be due to other components in the recombinant GM-CSF preparation since polyclonal antibody to GM-CSF blocked the effect. Furthermore, low levels of LPS do not augment the activity of GM-CSF in stimulating macrophage H$_2$O$_2$ release. These data agree with the studies of Weiser et al who found that LPS did not alter the ability of GM-CSF to promote the killing of L. donovani by monocyte-derived macrophages. While GM-CSF stimulates oxidative metabolism of neutrophils, it does not appear to enhance H$_2$O$_2$ release by blood monocytes. The ability of GM-CSF to stimulate H$_2$O$_2$ release by peritoneal macrophages but not...
blood monocytes suggests that the effects of GM-CSF may differ among monocyte-macrophage populations.

The stimulation of Fc-dependent phagocytic activity of macrophages by GM-CSF suggests that GM-CSF may be important in helping to clear opsonized cells, particles, and microorganisms. Further support for such a role for GM-CSF is the observation that GM-CSF also stimulates the phagocytic activity of neutrophils. The stimulatory effect of GM-CSF on macrophage Fc-dependent phagocytosis is very unlikely to be attributable to low levels of LPS contamination since LPS consistently inhibits peritoneal macrophage Fc-dependent phagocytosis under our experimental conditions. Since other activators of macrophage Fc-dependent phagocytic activity have been demonstrated to enhance expression of Fc receptors and GM-CSF increases Fc rosette numbers on myeloid leukemia cell lines, it is possible that GM-CSF also increases Fc-dependent phagocytosis by increasing Fc receptor expression.

The less dramatic effect of GM-CSF on Fc-dependent phagocytosis by resident peritoneal macrophages suggests that the macrophage population that migrates into the peritoneal cavity following an inflammatory stimulus may be more responsive to GM-CSF than are resident peritoneal macrophages. Such a hypothesis is further supported by our earlier finding that GM-CSF induces DNA synthesis in thioglycollate-elicited but not resident macrophages.

Under the experimental conditions utilized in our studies, GM-CSF does not appear to increase peritoneal macrophage anti-Toxoplasma activity. These findings are similar to those reported by other groups using different monocyte-macrophage populations. Products of oxidative metabolism have been assumed to be vital to the ability of the macrophage to inhibit the replication of Toxoplasma organisms. However, the observation that GM-CSF is able to enhance H2O2 release but not anti-Toxoplasma activity suggests that stimulation of macrophage oxidative metabolism may not be sufficient to effectively inhibit the replication of T gondii. We have observed a similar dissociation between the stimulation of oxidative metabolism and anti-Toxoplasma activity with a low–molecular weight cytokine derived from thymocytes. In addition, Catterall and coworkers have recently provided convincing evidence that human and rat alveolar and peritoneal macrophages efficiently kill Toxoplasma organisms by nonoxidative mechanisms. Although GM-CSF does not enhance anti-Toxoplasma activity, it does induce selective microbialic activities of macrophages. For example, GM-CSF has recently been reported to promote the ability of macrophages to kill L. donovani.

The inability of GM-CSF to enhance the expression of Ia is surprising since GM-CSF appears to increase antigen-presenting functions of splenic macrophages. Our negative findings on Ia expression suggest that GM-CSF may enhance antigen presentation by splenic macrophages through a mechanism different from increasing expression of class II MHC antigens (eg, increased interleukin-1 production) or that its effects on antigen-presenting cells may differ according to their tissue site.

In summary, GM-CSF augments macrophage oxidative metabolism and Fc-dependent phagocytic activity. These data suggest that in addition to stimulating the growth of granulocyte and macrophage precursors GM-CSF selectively activates macrophage functions. The effects of GM-CSF may be influenced by the state of differentiation or tissue site of the macrophage population. These data support additional studies on the ability of GM-CSF to stimulate macrophage functions in vivo.

REFERENCES


31. Pfefferkorn ER: Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. Proc Natl Acad Sci USA 81:909, 1984


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