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Granulocyte-Macrophage Colony-Stimulating Factor Is Not Involved in Production of Reactive Nitrogen Intermediates by or Toxoplasmastatic Activity of Gamma Interferon-Activated Murine Macrophages

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The induction of reactive nitrogen intermediates (RNI) and toxoplasmastatic activity of murine macrophages by recombinant gamma interferon (rIFN-γ) is mediated by an autocrine pathway involving tumor necrosis factor alpha (TNF-α). To investigate whether cytokines other than TNF-α play a role in the activation of these effector functions, granulocyte-macrophage colony-stimulating factor (GM-CSF) was studied. Recombinant GM-CSF (rGM-CSF) could stimulate peritoneal macrophages, since this cytokine stimulated the production of prostaglandin E_2 by these cells. However, rGM-CSF did not induce either the release of RNI by or the toxoplasmastatic activity of macrophages. rGM-CSF in combination with various concentrations of rIFN-γ did not enhance these effector functions more than rIFN-γ alone. Furthermore, neutralization of endogenously produced GM-CSF by monoclonal antibodies did not affect the release of RNI by or the toxoplasmastatic activity of rIFN-γ-activated macrophages. Together these results indicate that GM-CSF is not involved in RNI production by and toxoplasmastatic activity of IFN-γ-activated murine macrophages.

Activation of macrophages to a state of enhanced microbicidal activity by cytokines is essential if a host has to overcome infection with intracellular pathogens, such as *Toxoplasma gondii* (23). Increased microbicidal activity of activated macrophages is achieved by both oxygen-independent and oxygen-dependent mechanisms (6, 20). In mouse macrophages, reactive nitrogen intermediates (RNI), produced during activation of these cells, play an essential role in microbicidal activity against various microorganisms (18, 22).

Recently it has been demonstrated that recombinant gamma interferon (rIFN-γ), which plays a major role in macrophage activation (17), stimulates the production of tumor necrosis factor alpha (TNF-α) by mouse peritoneal macrophages in vitro (12). In turn, TNF-α induces via an autocrine pathway the production of RNI and enhances the toxoplasmastatic activity of these cells (12). In vivo, similar mechanisms have been demonstrated (14). It is uncertain, however, whether TNF-α is the only endogenously produced cytokine involved in the activation of macrophages by rIFN-γ since the addition of neutralizing antibodies to TNF-α does not completely inhibit the production of RNI by or the toxoplasmastatic activity of IFN-γ-activated macrophages (12, 14). It has been reported that several hematopoietic growth factors affect the functions of human and mouse macrophages (2, 7) and that rIFN-γ stimulates the production of granulocyte macrophage colony-stimulating factor (GM-CSF) by human monocytes (11). Recombinant GM-CSF (rGM-CSF) increases hydrogen peroxide production and enhances the intracellular killing of *Trypanosoma cruzi* in human monocytes and monocyte-derived macrophages and in mouse resident and exudate peritoneal macrophages (4, 16, 19). In rIFN-γ-activated human monocytes and mouse macrophages, rGM-CSF enhances the production of TNF-α (9, 10, 21). The aim of the present study was to assess the role of GM-CSF in the activation of murine macrophages by IFN-γ.

Lyophilized rat rIFN-γ produced in Chinese hamster ovary (CHO) cells (24) (kindly provided by P. H. van der Meide, TNO, Rijswijk, The Netherlands) was diluted in pyrogen-free saline containing 1% (vol/vol) fetal calf serum (Flow Laboratories, Rockville, Md.) and stored at −70°C. The amino acid composition of rat rIFN-γ is 87% homologous to that of mouse rIFN-γ (5), and the interferons have similar biological activities (15). Recombinant mouse TNF-α (rTNF-α), a gift from P. de Waele (Innogenetics, Ghent, Belgium), which was produced in *Escherichia coli* and which had a specific activity of 1.2×10^8 U/mg of protein, was diluted in pyrogen-free saline containing 0.01% (vol/vol) bovine serum albumin and stored at −70°C. Mouse rGM-CSF with a specificity of 3×10^7 U/mg of protein, generously provided by Sandoz (Vienna, Austria), was diluted in pyrogen-free saline containing 1% (vol/vol) fetal calf serum and stored at −70°C. Rat monoclonal antibodies against murine GM-CSF (IgG2a) (Genzyme, Cambridge, Mass.) were diluted in pyrogen-free saline containing 1% (vol/vol) fetal calf serum, stored at −70°C, and used at concentrations of 1 to 20 μg/ml. Rat IgG1, generously provided by H. Bazin (University of Louvain, Brussels, Belgium), was used as a control at concentrations of 1 to 20 μg/ml. All reagents contained less than 0.1 ng of lipopolysaccharide (LPS) per ml, as determined with the *Limulus* lysate assay. Peritoneal macrophages, harvested from female CBA/J mice (IFFA-Credo, Saint Germaine-sur-L'Abresle, France) by lavage as described previously (25), were suspended to a concentration of approximately 10^6 macrophages per ml of RPMI 1640 (Flow Laboratories) containing 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 100 U of penicillin per ml, and 50 μg of streptomycin per ml (hereafter called medium) and incubated in a 24-well culture plate at 37°C and 7.5% CO_2 (Costar, Cambridge, Mass.) for 2 h. Nonadherent cells were removed by washing, and the adherent cells were incubated in fresh medium for 24 h with or without cytokines or antibodies. The amount of RNI generated by these cells, as measured by the amount of NO_2^- in the

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TABLE 1. Release of NO_2^- by and toxoplasmastatic activity of activated macrophages[a]

Cytokine (amt)	n	Release of NO_2^- (nmol)[b]	n	Fold increase of intracellular proliferation of *T. gondii*[c]
None	10	2.6 ± 2.1	3	7.6 ± 1.8
rGM-CSF (10 ng)	6	2.7 ± 1.8	3	6.3 ± 1.1
rGM-CSF (80 ng)	6	2.6 ± 1.8	3	6.8 ± 0.9
rIFN-γ (0.1 U/ml)	3	21.4 ± 2.8		
rIFN-γ (0.1 U/ml) + rGM-CSF (10 ng)	3	24.6 ± 4.1		
rIFN-γ (0.1 U/ml) + rTNF-α (10^4 U/ml)	3	31.4 ± 3.0		
rIFN-γ (0.1 U/ml) + rTNF-α (10^4 U/ml) + rGM-CSF (10 ng)	3	33.6 ± 3.1		
rIFN-γ (1 U/ml)	6	44.6 ± 3.4	3	4.2 ± 0.8
rIFN-γ (1 U/ml) + rGM-CSF (10 ng)	3	41.3 ± 3.8	3	3.9 ± 0.2
rIFN-γ (100 U/ml)	10	66.0 ± 4.4	3	3.1 ± 1.9
rIFN-γ (100 U/ml) + rGM-CSF (80 ng)	3	59.7 ± 4.4	1	3.5
rIFN-γ (100 U/ml) + anti-GM-CSF (1 to 20 μg/ml)	5	72.0 ± 5.5	3	2.4 ± 0.4

[a] Mouse peritoneal macrophages (10^6/ml) were incubated with various cytokines alone and in combinations for 24 h at 37°C, and the NO_2^- release in the supernatant was determined. Then the cells were infected for 30 min with 10^6 protozoa per ml and incubated for 18 h in fresh medium. After 18 h, the number of protozoa was counted and compared with that at the start of the assay. Values are means ± standard errors.

[b] NO_2^- released per 10^6 cells.

[c] Fold increase in the number of protozoa per 100 macrophages.

medium, was assessed with Griess reagent (1). Great care was taken to use solutions without any preservatives, since preliminary experiments revealed that low concentrations of $NaNH_3$ present in anti-GM-CSF preparations caused inhibition of the RNI release by macrophages (results not shown). Infection of the macrophages with *T. gondii* and the subsequent increase in the number of protozoa in these macrophages 18 h later were assessed as described earlier (13). Production of GM-CSF by macrophages at various intervals after administration of rIFN-γ was assessed by enzyme-linked immunosorbent assay (Endogen Inc., Boston, Mass.). The prostaglandin E_2 (PGE_2) content of the culture medium after a 24-h incubation of the macrophages with the cytokines was determined by radioimmunoassay (Amerlex-M magnetic separation; Amersham International plc, Amersham, United Kingdom). The percent increase in PGE_2 production by macrophages incubated with cytokines compared with that found for macrophages incubated with medium alone was calculated. Data obtained were analyzed for statistical significance by means of the Mann-Whitney U test.

Mouse peritoneal macrophages incubated with 10 or 80 ng of rGM-CSF per ml did not generate more RNI than control macrophages (Table 1). Incubation of macrophages with various concentrations of rIFN-γ stimulated RNI generation in a dose-dependent fashion (Table 1), with significantly different amounts of RNI being found after incubation with 0.1, 1.0, and 100 U of rIFN-γ per ml ($P < 0.05$). Since incubation of macrophages with a suboptimal concentration (0.1 U/ml) of rIFN-γ and 10^4 U of rTNF-α per ml resulted in the synergistic release of RNI compared with incubation of macrophages with rIFN-γ alone (12), the question of whether rGM-CSF enhances RNI production after (sub)optimal stimulation of macrophages by rIFN-γ arose. Incubation of macrophages with various concentrations of rIFN-γ together with 10 or 80 ng of rGM-CSF per ml did not result in an enhanced generation of RNI relative to that found for macrophages incubated with rIFN-γ alone (Table 1). The addition of 10 ng of rGM-CSF to the combination of 0.1 U of rIFN-γ per ml and 10^4 U of rTNF-α per ml also did not affect RNI production by the macrophages (Table 1).

Macrophages incubated with 100 U of rIFN-γ per ml released GM-CSF: 5 pg of GM-CSF per ml at 5 h of incubation, a maximal release of 67 pg of GM-CSF per ml at 9 h, and only 5 pg of GM-CSF per ml at 20 h. A similar time course for GM-CSF release was found when macrophages were stimulated with 10 ng of LPS per ml: 606 pg/ml at 5 h, 1,100 pg/ml at 9 h, and 77 pg/ml at 20 h after incubation. Control macrophages did not release detectable amounts of GM-CSF. Since macrophages release GM-CSF after stimulation with rIFN-γ, the addition of 1 to 20 μg of neutralizing antibodies against GM-CSF per ml 1 h before and during incubation of macrophages with rIFN-γ was studied. The results showed no inhibition of RNI generation by macrophages activated by 100 U of rIFN-γ per ml (Table 1), and anti-GM-CSF alone did not induce generation of RNI by macrophages (data not shown).

To investigate whether GM-CSF is required for the toxoplasmastatic activity of mouse peritoneal macrophages, these cells were incubated with rGM-CSF; incubation with rGM-CSF did not result in a decrease of the intracellular proliferation of *T. gondii*, while incubation with 1 and 100 U of rIFN-γ per ml significantly inhibited this intracellular proliferation ($P \leq 0.05$ relative to control macrophages) (Table 1). The addition of rGM-CSF to rIFN-γ did not increase the toxoplasmastatic activity compared with that induced by incubation with rIFN-γ alone ($P > 0.1$) (Table 1). Although macrophages release a small amount of GM-CSF upon stimulation with rIFN-γ, neutralizing antibodies against GM-CSF did not affect the rIFN-γ-induced toxoplasmastatic activity ($P > 0.1$) (Table 1).

Macrophages incubated with 80 ng of rGM-CSF per ml produced large quantities of PGE_2 compared with those produced by control macrophages (Fig. 1). Macrophages incubated with 100 U of rIFN-γ per ml exhibited only a slight increase in PGE_2 production compared with that of control cells, and the addition of IFN-γ to GM-CSF had no additive effect on PGE_2 production (Fig. 1). When 80 ng of rGM-CSF per ml was added to 10 ng of LPS per ml, PGE_2 production by peritoneal macrophages was enhanced compared with that by cells incubated with LPS alone. In the present study, PGE_2 production was not due to LPS contamination of the rGM-CSF, because LPS was not detectable in this preparation.

The findings of the present study indicate that neither endogenous GM-CSF nor exogenous GM-CSF plays a role in the production of RNI by or the toxoplasmastatic activity of mouse peritoneal macrophages activated by rIFN-γ. This conclusion is based on the observations that the addition of rGM-CSF alone or in combination with various concentrations of rIFN-γ or with rIFN-γ and rTNF-α did not induce or

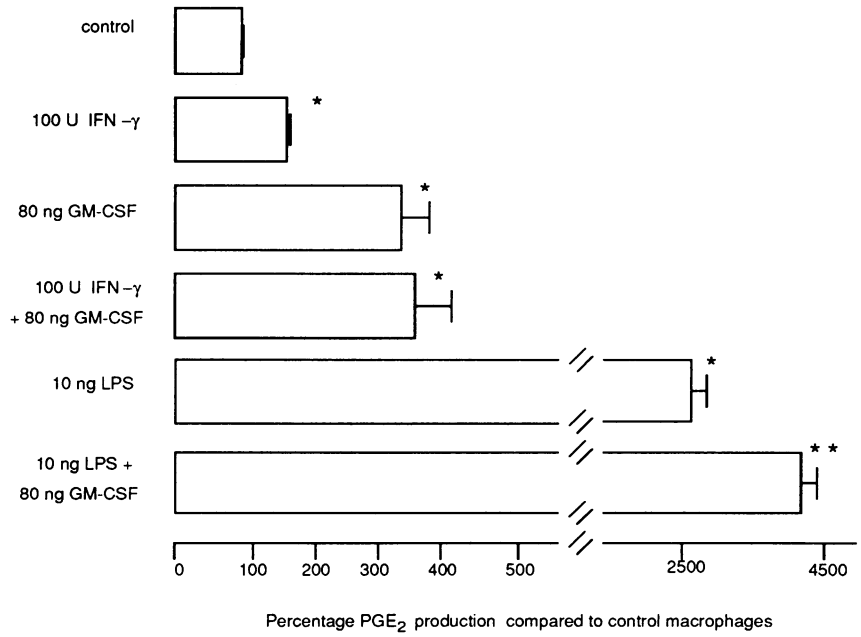

FIG. 1. PGE_2 production by mouse peritoneal macrophages. Mouse peritoneal macrophages (10^6/ml) were incubated with rIFN-γ, rGM-CSF, and combinations of these cytokines for 24 h at 37°C. After 24 h of incubation, PGE_2 release in the supernatant was measured, and it is expressed as percent relative to control macrophages. Values are the means and standard deviations of three measurements within one representative experiment. *, significantly ($P < 0.05$) enhanced PGE_2 release relative to that by control macrophages. **, significantly ($P < 0.05$) enhanced PGE_2 release relative to that by LPS-stimulated macrophages.

enhance RNI generation by or the toxoplasmastatic activity of macrophages compared with that induced by the cytokines alone. These results extend previous findings that rGM-CSF does not induce toxoplasmastatic activity in mouse peritoneal macrophages (4). Neutralization of endogenously produced GM-CSF did not inhibit the release of NO_2^- by or the induction of the toxoplasmastatic activity of macrophages activated by rIFN-γ, which provides additional evidence that GM-CSF is not involved in the activity of IFN-γ. However, rGM-CSF did induce an enhanced production of PGE_2 by the peritoneal macrophages, which demonstrates that this cytokine can interact with mouse peritoneal macrophages and induce a response. These results are in agreement with earlier findings that rGM-CSF enhances the production of PGE_2 during stimulation of mouse peritoneal macrophages with IFN-γ and LPS (10). In our experiments, rGM-CSF alone increased PGE_2 production by the macrophages, while incubation of the cells with both rGM-CSF and LPS enhanced the production of PGE_2 compared with incubation with LPS alone. The role of PGE_2 in macrophage activation is unclear; PGE_2 is generally considered to be an inhibitory factor (8), although it has been reported that PGE_2 does not inhibit macrophage activation induced by IFN-γ plus LPS (3). We found that the increased production of PGE_2 by macrophages stimulated with rGM-CSF plus rIFN-γ coincided with an increased production of RNI and a decreased proliferation of *T. gondii*.

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