

RECOMBINANT HUMAN GRANULOCYTE/MACROPHAGE
COLONY-STIMULATING FACTOR ACTIVATES
INTRACELLULAR KILLING OF *LEISHMANIA DONOVANI* BY
HUMAN MONOCYTE-DERIVED MACROPHAGES

BY WEISHUI Y. WEISER,* ANTHONY VAN NIEL,* STEVEN C. CLARK,‡
JOHN R. DAVID,* AND HEINZ G. REMOLD*

*From the *Department of Medicine, Harvard Medical School, and the Department of
Rheumatology and Immunology, Brigham and Women's Hospital,
Boston, Massachusetts 02115; and the ‡Genetics Institute, Cambridge, Massachusetts 02140*

Macrophages, the final effector cells in many cellular immune responses, are able to destroy a variety of intracellular pathogens, such as viruses, fungi, chlamydia, rickettsiae, and leishmania (1, 2), as well as neoplastic cells (3). It has been demonstrated that human monocyte-derived macrophages can be activated by conditioned medium from lectin-stimulated human peripheral blood lymphocytes to kill *Leishmania donovani* (4), and the substance responsible for this activity has been shown to be IFN- γ (5).

However, several macrophage-activating factors distinct from IFN- γ have been detected (6–15). The molecular cloning of monokines and lymphokines including human granulocyte/macrophage colony-stimulating factor (GM-CSF)¹ (16) makes it possible to test these homogeneous factors for their capacity to activate macrophages.

Colony-stimulating factors are defined by their capacity to stimulate the clonal expansion of individual progenitor cells resulting in the generation of distinct colonies (17), which in the case of GM-CSF consist of granulocytes and macrophages (18). Recombinant GM-CSF (rGM-CSF) was found to have all the effects on human bone marrow cells of GM-CSF produced by T cells (16), to alter the physiology of mature neutrophils (19) and eosinophils (20), and to activate macrophages for extracellular killing of tumor cells (21).

The data reported here show that rGM-CSF, which shares no sequence homology with IFN- γ (16), is also a potent activator of cultured human monocyte-derived macrophages and induces the intracellular killing of *L. donovani*. The antileishmanial effect of both rGM-CSF and IFN- γ does not depend on LPS as an additional signal. However, unlike rIFN- γ , which requires 48–72 h to activate macrophages to kill *L. donovani*, the antileishmanial effect of rGM-CSF is sooner and reaches maximal activation at 36 h.

This work was supported by grants AI-22801, T32 AI-07061, AI-22532, AI-22530, CA-41063, and AM-35907 from the National Institutes of Health.

¹ Abbreviation used in this paper: GM-CSF, granulocyte/macrophage colony-stimulating factor.

Materials and Methods

Parasites. Promastigotes of *L. donovani* were obtained from the World Health Organization, Geneva, Switzerland, and were maintained in vitro at 26°C in tissue culture flasks containing Schneiders's *Drosophila* medium (Gibco Laboratories, Grand Island, NY), 20% heat-inactivated FCS, and 5 ng/ml gentamycin (M.A. Bioproducts, Walkersville, MD). The parasites were subcultured by weekly passage at 1:10 dilution. To obtain amastigotes of *L. donovani*, 10⁷ promastigotes were injected via the intracardiac route to hamsters. Spleen from infected animals were removed ~8 wk after infection. Amastigotes were isolated from homogenates of infected spleens according to the method of Chang and Hendricks (22) using Percoll gradient centrifugation.

Cells. Human peripheral blood was obtained from healthy volunteers. Mononuclear cells isolated by Ficoll-Hypaque density centrifugation were suspended at 2.5 × 10⁶ cells/ml in medium 199 (M.A. Bioproducts) containing 15% heat-inactivated FCS, 2 mM L-glutamine (Gibco Laboratories), and 5 ng/ml gentamycin, and seeded on top of four glass coverslips in a 35-mm petri dish (Falcon Labware, Oxnard, CA). All the FCS used for the study was from the same lot (No. 11N5261), which was prescreened and selected from many lots for the ability to support in vitro culture of cells and parasites. After 2 h of incubation at 37°C the nonadherent cells were removed by washing, and the remaining cells were incubated for 10–12 d in a 5% CO₂ atmosphere to obtain monocyte-derived macrophages. The cultures were examined every 3–4 d and the culture medium described above was replenished at the same time. Only monolayers containing cells with marked increase in size and cytoplasmic spreading were chosen as effector cells for experiments, whereas cultures containing poorly differentiated cells were discarded. After 10 d, the monolayers consisted of 95–97% macrophages, as determined by morphology and esterase staining.

In experiments where LPS-free condition was required, extreme caution was taken to ensure the absence of LPS. Human mononuclear cells were processed in solutions and medium prepared with LPS-free water (Travenol Laboratories, Inc., Deerfield, IL) and cultured in LPS-free medium supplemented with 15% autologous serum. The absence of LPS of all cultures was ensured before use by monitoring LPS at each step with the Limulus Amebocyte Lysate Assay (M.A. Bioproducts). Both rGM-CSF and IFN-γ were also examined for the presence of LPS before use. Occasionally, 0.3–0.5 ng/ml of LPS was found in rIFN-γ preparations; therefore, a stock of IFN-γ (Melo Laboratory, Inc., McLean, VA) free of detectable (<0.01 ng/ml) was used in LPS-free studies.

Recombinant Human GM-CSF and Recombinant Human IFN-γ. rGM-CSF, isolated by COS cell expression screening of cDNA from Mo cells for GM-CSF activity using KG-1 human myeloid leukemia cells and fresh human bone marrow cells (16), was provided by Genetics Institute, Cambridge, MA. The specific activity of purified rGM-CSF was 10⁷ U/mg protein, whereas the titer of crude rGM-CSF was 80,000 U/ml. Crude GM-CSF was used for most of one study, unless indicated otherwise. Crude rIFN-γ was also provided by Genetics Institute. The titer of rIFN-γ was 10⁶ U/ml. Various dilutions of rGM-CSF or rIFN-γ were added to monocyte-derived macrophage preparations after the cells had been in culture for 10–12 d.

Infection of Monocyte-derived Macrophages. Promastigotes of *L. donovani* at the stationary growth phase were used to infect macrophages at parasite-to-cell ratio of ~10:1 in all experiments. In experiments using amastigotes, freshly isolated amastigotes were used to infect macrophages at a ratio of ~8:1. After 2 h of incubation at 37°C, the uningested parasites were removed by washing, and the cultures were reincubated in fresh medium. Coverslips were removed at 2, 24, 48, and 72 h, washed in PBS, and stained with Diff-Quick stain (American Scientific Products, McGaw Park, IL); the number of intracellular parasites was determined by microscopy. From each coverslip, three to four randomly selected areas were examined; each area consisted of 100 cells.

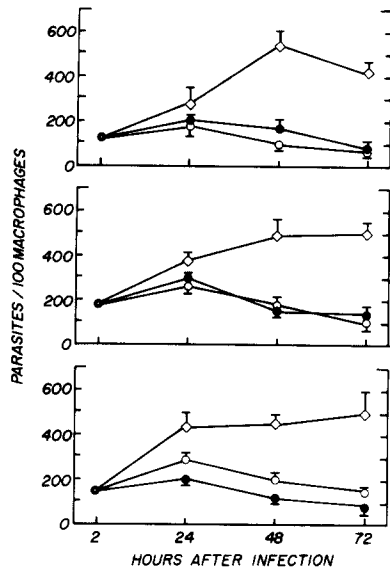


FIGURE 1. The effect of rGM-CSF and rIFN- γ on the intracellular fate of *L. donovani* in human monocyte-derived macrophages. The monocyte-derived macrophages were treated with rGM-CSF, 4 ng/ml (●) or rIFN- γ , 1,000 U/ml (○), or received only fresh medium (control, ◇) for 72 h before infection with *L. donovani*. After 2 h of incubation at 37°C, the uningested parasites were removed by washing and the cultures were reincubated in fresh medium containing no rGM-CSF or rIFN- γ . Coverslips were removed at 2, 24, 48 and 72 h, washed, and stained. The number of intracellular parasites was determined by microscopy. The starred circle represents the overlaps of open circle, closed circle, and diamond. Three experiments are shown; each is expressed as the mean \pm SEM of three quantifications.

Results

Killing of Intracellular *Leishmania Donovanii* by rGM-CSF or rIFN- γ -activated Monocyte-derived Macrophages. It has been shown that 1-d-old human monocytes displayed effective promastigocidal activity and eradicated >90% of ingested parasites 24 h after infection (4). However, monocyte-derived macrophages (monocytes cultured for >7 d) that could be activated to exhibit antileishmanial activity, displayed little or no leishmanicidal activity towards promastigotes of *L. donovani* and none towards amastigotes. Instead, these cells readily supported the replication of both parasite forms. Therefore, monocyte-derived macrophages were used as the effector cells in the present study. IFN- γ has been shown to activate human mononuclear phagocytes cultured for 10–20 d to kill intracellular *L. donovani* (5), with optimal results being achieved after 48–72 h of exposure. To determine if rGM-CSF can activate macrophages for antileishmanial activity and to compare the effect of pretreatment with rGM-CSF or rIFN- γ on cultured monocyte-derived macrophages, rGM-CSF (4 ng/ml, the optimal dose) or rIFN- γ (1,000 U/ml) was added once to 10-d-old cultures of monocyte-derived macrophages for 72 h before infection with the parasites. The actual replication of the parasites in rGM-CSF- and rIFN- γ -treated cultures was compared with cultures receiving medium alone.

As can be seen in Fig. 1, the uptake of parasites after 2 h was similar for each group, indicating no differential uptake of parasites among lymphokine-treated cells. In the control cultures, monocyte-derived macrophages readily supported the replication of *L. donovani*. However, in macrophages pretreated with rGM-CSF or rIFN- γ , the growth of the parasites was inhibited.

We initiated our studies of the *Leishmania*-macrophage interaction using the promastigote because the hemoflagellate is the form first encountered by the phagocytic cells. The amastigote, however, to which the promastigote rapidly transforms within the phagolysosomes, is responsible for persistent tissue infec-

TABLE I
Enhancement of Antileishmanial Activity by rGM-CSF and rIFN- γ

Time after infection*	Number of amastigotes per 100 macrophages [†]					
	Control		rGM-CSF (4 ng/ml)		rIFN- γ (1,000 U/ml)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
<i>h</i>						
2	173 \pm 10	217 \pm 15	186 \pm 20	220 \pm 17	166 \pm 5	213 \pm 8
24	181 \pm 12	233 \pm 16	167 \pm 11	219 \pm 9	161 \pm 14	212 \pm 14
48	240 \pm 18	276 \pm 19	175 \pm 8	199 \pm 13	156 \pm 13	168 \pm 21
72	377 \pm 20	496 \pm 24	249 \pm 18	352 \pm 19	200 \pm 16	253 \pm 16

* Monocyte-derived macrophages were pretreated with rGM-CSF (4 ng/ml) or rIFN- γ (1,000 U/ml) for 72 h. The cells were then infected with amastigotes of *L. donovani*. Coverslips from treated cultures and control cultures (cultures received no lymphokine) were removed at 2, 24, 48, and 72 h after infection and were stained.

[†] The number of intracellular amastigotes present in lymphokine-treated as well as control cultures at each time point was determined by counting the respective number of parasites in four randomly selected areas (each area consisted of 100 macrophages) of every coverslip and expressed as mean \pm SEM of four countings. All the experiments within each group were analyzed together by the two-way analysis of variance. Statistically significant increase in antileishmanial activity was found in cultures treated with GM-CSF as well as IFN- γ ($p < 0.1$).

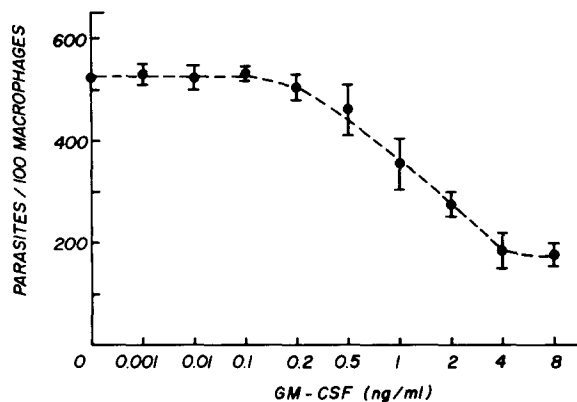


FIGURE 2. The dose response of purified rGM-CSF. The abscissa shows the concentration of rGM-CSF in ng/ml, the ordinate indicates the number of parasites per 100 macrophages 48 h after infection. rGM-CSF was purified from transfected COS-1 cell supernatants by Ultrogel AcA54 gel filtration and reverse-phase high-pressure liquid chromatography on Vydac C4 columns (16). The data are presented as the mean \pm SEM of four separate quantifications of one experiment. Four experiments using nonpurified rGM-CSF showed a similar dose-response curve.

tion. Therefore, amastigotes were also used to infect monocyte-derived macrophages treated with rGM-CSF or rIFN- γ . The antileishmanial effect of these two lymphokines was evaluated (Table I). Again, there was a significant decrease of amastigotes in macrophages treated with either lymphokine, although the antileishmanial effect was more pronounced when promastigotes were used.

The Fig. 2 shows the antileishmanial effect of increasing concentrations of purified rGM-CSF on monocyte-derived macrophages infected with *L. donovani*. Killing of *L. donovani* was observed at a concentration as low as 0.5 ng/ml rGM-CSF, and the optimal effect was reached at 4 ng/ml. The antileishmanial effect of rGM-CSF was entirely abolished by treatment of rGM-CSF with anti-rGM-CSF antiserum but not with anti-rIFN- γ mAb (Fig. 3). However, it is of note that no antileishmanial activity was observed in monocyte-derived macrophages treated with human recombinant macrophage-colony stimulating factor (ob-

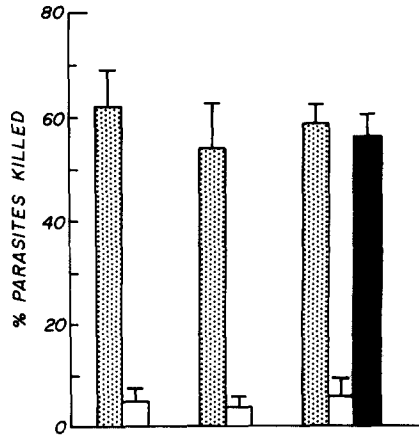


FIGURE 3. Effect of anti-rGM-CSF or anti-IFN- γ antibody on the antileishmanial activity of rGM-CSF. rGM-CSF (4 ng/ml) was incubated with protein A-Sepharose-bound sheep anti-human rGM-CSF antiserum (40 ng/ml) or with protein A-Sepharose-bound anti-IFN- γ mAb (4,000 neutralizing units/ml) overnight at 4°C. The slurry was removed by centrifugation at 300 g for 10 min. 4 ng/ml rGM-CSF (▨), rGM-CSF treated with anti-rGM-CSF antibody (□), or rGM-CSF with anti-IFN- γ antibody (■) were added to cultured macrophages 72 h before infection with *L. donovani*. The percentage of parasites killed was assessed 48 h after infection and calculated as described in Table I. Three experiments are shown; each is expressed as the mean \pm SEM of three quantifications.

tained from Genetics Institute), another cytokine produced by using the same COS cell expression system as rGM-CSF (data not shown).

It has been reported that the macrophage-activating activity of IFN- γ is dependent on the presence of small amounts of bacterial LPS as a second signal (21, 23, 24). Therefore, to assess whether exogenous LPS is required for antileishmanial activity of rGM-CSF or IFN- γ , human monocyte-derived macrophages were processed and cultured under stringent LPS-free conditions as described in Materials and Methods. rGM-CSF and IFN- γ , both free of detectable amounts of LPS, were used to activate monocyte-derived macrophages with and without the addition of 10 ng or 50 ng/ml of LPS. As illustrated in Table II, the antileishmanial activity of both rGM-CSF and IFN- γ was found not to be affected by the addition of either 10 ng or 50 ng of LPS. Furthermore, both lymphokines were able to function in a LPS-free environment since culture medium removed at the termination of the experiment (72 h after infection) contained no detectable amount of LPS (<0.01 ng/ml). In addition, LPS alone showed no antileishmanial activity.

Time Course of rGM-CSF. The antileishmanial effect of pretreating cultured monocyte-derived macrophages for different times with 4 ng/ml rGM-CSF or with 1,000 U/ml rIFN- γ was measured 48 h after infection (Fig. 4). Already at 6 h of pretreatment, activation was seen with rGM-CSF, whereas no effect was seen with rIFN- γ . Optimal activation with rGM-CSF occurred at 36 h of pretreatment with rGM-CSF whereas activation by rIFN- γ was maximal only after 72 h of preincubation. The latter is in agreement with previous reports by Murray and Cartelli (4). These studies clearly demonstrate that rGM-CSF activates cultured human monocytes quicker than rIFN-gamma.

Are the Antileishmanial Effects of rGM-CSF and rIFN- γ Additive? Our experiments show that even when optimal concentrations of rIFN- γ or rGM-CSF are used, all intracellular parasites are not eradicated. Some residual parasites remain insensitive to the treatment of macrophages with either lymphokine. These observations bring up the question: can the residual parasite population be killed by treating monocyte-derived macrophages with a combination of rGM-CSF and rIFN- γ , especially in view of the reports that IFN- γ but not GM-CSF induced

TABLE II
Failure of LPS to Alter Macrophage Antileishmanial Activity Induced by GM-CSF and IFN- γ

Treatment*	Number of parasites/100 macrophages [†]			
	At 2 h		At 48 h	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None	188 \pm 14	134 \pm 11	551 \pm 40	473 \pm 34
GM-CSF				
0.5 ng/ml	178 \pm 7	150 \pm 10	457 \pm 44	383 \pm 23
0.5 ng/ml + 10 ng/ml LPS	191 \pm 21	124 \pm 9	446 \pm 38	388 \pm 37
0.5 ng/ml + 50 ng/ml LPS	169 \pm 15	127 \pm 12	455 \pm 27	355 \pm 27
4 ng/ml	181 \pm 16	149 \pm 13	209 \pm 43	194 \pm 32
4 ng/ml + 10 ng/ml LPS	195 \pm 21	144 \pm 8	193 \pm 49	208 \pm 14
4 ng/ml + 50 ng/ml LPS	166 \pm 17	132 \pm 17	253 \pm 32	213 \pm 38
IFN- γ				
1,000 U/ml	176 \pm 13	128 \pm 14	248 \pm 51	241 \pm 29
1,000 U/ml + 10 ng/ml LPS	183 \pm 15	151 \pm 9	276 \pm 39	232 \pm 34
1,000 U/ml + 50 ng/ml LPS	162 \pm 14	119 \pm 11	231 \pm 24	222 \pm 22
LPS, 10 ng/ml	201 \pm 13	143 \pm 18	529 \pm 29	485 \pm 51
LPS, 50 ng/ml	177 \pm 14	129 \pm 11	583 \pm 41	462 \pm 28

* Monocyte-derived macrophages were treated as indicated above for 72 h. The cells were then infected with promastigotes of *L. donovani* for 2 h. Coverslips were removed at 2 h and 48 h after infection.

[†] The number of intracellular parasites per 100 macrophages at 2 h and at 48 h after infection was determined as described in Table I. All the experiments within each group were analyzed by the two-way analysis of variance. No statistical significance was found to reject the null hypothesis of equal activity of activated samples untreated or further treated with LPS. Therefore, it is concluded that no significant enhancement of activity is detected by adding LPS to GM-CSF or IFN- γ -treated cultures.

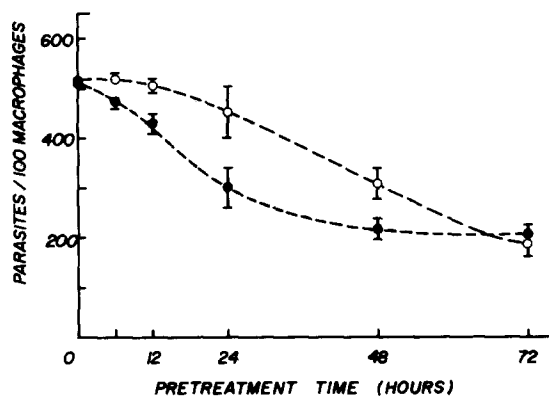


FIGURE 4. The antileishmanial effect of pretreatment time with rGM-CSF or rIFN- γ on the cultured macrophages. The abscissa shows the pretreatment time. The ordinate shows the number of parasites per 100 macrophages assessed 48 h after infection. rGM-CSF, 4 ng/ml (●) and rIFN- γ , 1,000 U/ml (○) were used. The data are expressed as mean of three experiments \pm SEM. The data derived from cultures treated with GM-CSF or IFN- γ at each time point were analyzed by the analysis of variance. The p values for the time points presented are as follows: 6 h, $p < 0.005$; 12 h, $p < 0.005$; 24 h, $p < 0.025$; 48 h, $p = 0.025$; 72 h, $p < 0.25$. As evidenced by the p values, there exists statistically significant difference between pretreatment of monocyte-derived macrophages with GM-CSF and IFN- γ at 6, 12, 24, and 48 h, but not as 72 h.

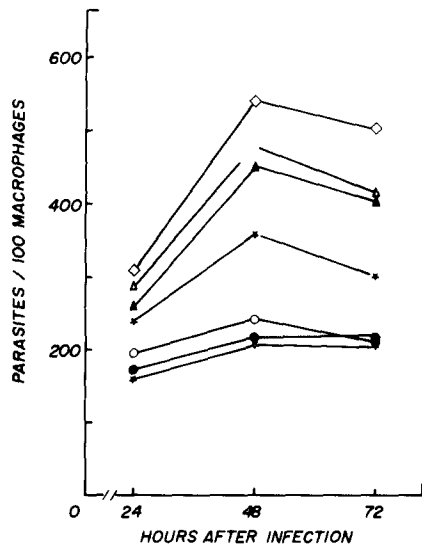


FIGURE 5. Antileishmanial activity of combined treatment of monocyte-derived macrophages with rGM-CSF and rIFN- γ . Cells were treated with medium alone (\diamond), 200 U/ml rIFN- γ (Δ), 0.5 ng/ml rGM-CSF (\blacktriangle), 0.5 ng rGM-CSF and 200 U/ml rIFN- γ (\star), 1,000 U/ml rIFN- γ (\circ), 4 ng/ml rGM-CSF (\bullet), or 4 ng/ml rGM-CSF and 1,000 U/ml rIFN- γ (\star) for 72 h before infection with *L. donovani*. Number of intracellular parasites were was 2, 24, 48, and 72 h after infection. The data represent the mean of three experiments.

human macrophages to secrete H_2O_2 (25) and that promastigotes of *L. donovani* were found to be exquisitely susceptible to H_2O_2 (4). Results of three separate experiments using different concentrations of rGM-CSF and rIFN- γ concomitantly are shown in Fig. 5. As can be seen, pretreatment of monocyte-derived macrophages with a combination of rGM-CSF and rIFN- γ at suboptimal doses (0.5 ng/ml and 200 U/ml) results in an additive killing effect of *L. donovani*, when compared with pretreatment with either lymphokine alone. However, pretreatment with rGM-CSF and rIFN- γ at optimal doses (4 ng/ml and 1,000 U/ml) showed no significant enhanced killing of parasites.

Discussion

These results demonstrate that human rGM-CSF activates cultured human monocyte-derived macrophages to exhibit antileishmanial activity towards both promastigotes and amastigotes of *L. donovani*. The activation of the macrophages by rGM-CSF is maximal after 36 h of incubation, whereas, activation by rIFN- γ is maximal at 72 h. The activation of intracellular killing by rGM-CSF or by IFN- γ does not require LPS as a second stimulus; the antileishmanial effect of both lymphokines containing no detectable LPS is not potentiated by the addition of LPS. Furthermore, treatment with LPS alone showed no effect on the intracellular killing of *L. donovani*. In this context, Grabstein et al. (21) found that the capacity of GM-CSF to activate macrophages to lyse A375 target cells was independent of stimulation with suboptimal amounts of LPS, but the capacity of IFN- γ to activate macrophages, on either hand, required the addition of suboptimal amounts of LPS to achieve lysis of the same cells. Furthermore, LPS alone (1 ng or 10 ng/ml) was able to induce nonspecific tumoricidal activity.

Other studies have also addressed the process of macrophage activation by GM-CSF. In regard to antileishmanial activity, it was demonstrated that conditioned medium from mouse spleen cells containing GM-CSF activity was able to enhance ingestion and killing of *Leishmania tropica* by mouse macrophages (26);

however, in this study homogeneity of GM-CSF could not be demonstrated in order to exclude the action of IFN- γ . On the other hand, Ralph et al. (27) also using conditioned medium from mouse spleen cells reported that GM-CSF obtained by gel chromatography of conditioned medium does not play a role in the induction of tumoricidal and microbicidal activity of mouse macrophages. The reason for the latter finding could be the presence of an inhibitor of GM-CSF in the conditioned medium or insufficient pretreatment time of macrophages, or that mouse GM-CSF indeed lacks macrophage-activating activity. The antimicrobial activity of GM-CSF has recently been extended to *Trypanosoma cruzi* and *Salmonella typhimurium* (13, 15). In these studies, treatment of rGM-CSF was found to activate human peripheral blood monocytes and macrophages as well as mouse peritoneal macrophages for the inhibition of intracellular replication of trypomastigotes of *T. cruzi* (13, 15), and daily GM-CSF therapy significantly increased the survival of mice challenged with a lethal dose of *S. typhimurium* (13).

Recently, the contribution of highly active NK cells, which are present in small numbers in monocyte preparations, to extracellular cytotoxicity has gained increasing attention (28). This is important because target cells, including the A375 cell line, previously thought to be exclusively sensitive to monocyte killing were found to be highly susceptible to NK cell killing. We have circumvented this problem by assessing intracellular killing associated exclusively with macrophages.

It is not yet clear how GM-CSF induces antileishmanial activity in macrophages upon infection with leishmania. Human mononuclear phagocytes have been shown to use both oxygen-dependent and -independent mechanisms to kill ingested Leishmania (4). In the oxygen-dependent mechanism, H₂O₂ has been identified as the key leishmanicidal oxygen intermediate (29, 30). Whereas IFN- γ has the capability to induce mononuclear phagocytes to use both oxygen-dependent and -independent mechanisms to achieve leishmanicidal activity (4), GM-CSF, on the other hand, did not enhance H₂O₂ release from macrophages upon triggering with PMA (25). However, it is not entirely impossible that macrophages treated with GM-CSF are rendered more susceptible to be triggered for oxidative burst during parasite entry than untreated macrophages, since oxidative burst could also be readily triggered by the entry of promastigotes of *L. donovani* (4). Alternatively, GM-CSF might operate through an oxygen-independent mechanism to achieve antileishmanial activity. In a recent report by Passwell et al. (31), mAb to IFN- γ , which abrogated the increase in production of H₂O₂ induced by supernatants from mitogen-stimulated human peripheral blood mononuclear cells, resulted in only partial inhibition of leishmanicidal capacity of these lymphokines on human monocytes. It is also possible that GM-CSF activates macrophages to release TNF and that some of the biological effects of GM-CSF might be amplified through the release of TNF. By Northern blot analysis, rGM-CSF has recently been shown to induce expression of the TNF gene in U937 cells and in normal human monocytes (32). Furthermore, rGM-CSF, but not rIFN- γ was found to induce human monocyte-derived macrophages to express mRNA for TNF- α (33). Also, rTNF has been demonstrated to inhibit intracellular multiplication of trypomastigotes of *T. cruzi* in murine macrophages

(10). The role of TNF in the antileishmanial action is currently being investigated.

The experiments reported here show that homogeneous human rGM-CSF devoid of other lymphokines induces intracellular killing of *L. donovani* by monocyte-derived macrophages. The role of GM-CSF in vivo as a macrophage-activating factor and its functional relationship to IFN- γ remains to be determined. GM-CSF might be produced rapidly after an antigenic stimulus, or exposure to endotoxin or IL-1, because injection of various *Salmonella* antigens or endotoxin into mice caused a 50–100-fold increase of serum CSF levels, maximal at 3–9 h (34), and administration of 100 ng of human IL-1 to mice resulted in the appearance of high serum titers of CSF at 3 and 6 h (35). Therefore, GM-CSF might be important whenever early activation of macrophages is necessary, whereas IFN- γ might function as a long-term macrophage-activating factor. Since simultaneous administration of suboptimal doses of rGM-CSF and rIFN- γ to macrophages induces an additive antileishmanial effect, it may be beneficial to combine these two lymphokines therapeutically to promote hematopoietic cell proliferation and to activate macrophages.

Summary

Recombinant granulocyte/macrophage colony-stimulating factor (rGM-CSF) obtained from cloned complementary Mo cell DNA and expressed in COS-1 cells activates cultured peripheral blood monocyte-derived macrophages in vitro to become cytotoxic for intracellular *L. donovani*. The antileishmanial effect of rGM-CSF, which can be completely neutralized by anti-rGM-CSF antiserum, is maximal after 36 h preincubation with the cultured macrophages, compared with that of rIFN- γ , which reaches its maximum at 72 h of preincubation. The antileishmanial effect of GM-CSF as well as IFN- γ is independent of detectable amounts of LPS and is not augmented by the addition of 10 or 50 ng/ml of LPS. Simultaneous administration of suboptimal doses of rGM-CSF and rIFN- γ to monocyte-derived macrophages results in greater antileishmanial activity by these cells than administration of either lymphokine alone, although no enhancement of antileishmanial activity is observed when optimal doses of these two lymphokines are applied together.

The authors wish to thank Dr. Heinz Weiser, Center for Astrophysics, Cambridge, Massachusetts for data analysis.

Received for publication 12 January 1987 and in revised form 22 June 1987.

References

1. Nacy, C. A., C. N. Oster, S. L. James, and M. S. Meltzer. 1984. Activation of macrophages to kill Rickettsiae and Leishmania: dissociation of intracellular microbicidal activities and extracellular destruction of neoplastic and helminth targets. *Contemp. Top. Immunobiol.* 13:146.
2. Murray, H. W. 1984. Macrophage activation: enhanced oxidative and antiprotozoal activity. *Contemp. Top. Immunobiol.* 13:97.
3. Adams, D. O., and T. A. Hamilton. 1984. The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2:283.

4. Murray, H. W., and D. M. Cartelli. 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. *J. Clin. Invest.* 72:32.
5. Murray, H. W., B. Y. Rubin, and C. D. Rothermel. 1983. Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human mononuclear phagocytes. Evidence that interferon-gamma is the activating lymphokine. *J. Clin. Invest.* 72:1506.
6. Weiser, W. Y., and F. B. Bang. 1977. Blocking of in vitro and in vivo susceptibility to mouse hepatitis virus. *J. Exp. Med.* 146:1467.
7. Buchmuller, Y., W. Y. Weiser, and H. Remold. 1985. Partial purification and characterization of a human lymphokine which induces antileishmanial activity in mouse macrophages. *Cell. Immunol.* 90:242.
8. Lee, J. C., L. Rebar, P. Young, F. W. Ruscetti, N. Hanna, and G. Poste. 1986. Identification and characterization of a human T cell line-derived lymphokine with MAF-like activity distinct from interferon-gamma. *J. Immunol.* 136:1322.
9. Hoover, D. L., D. S. Finbloom, R. M. Crawford, C. S. Nacy, M. Gilbreath, and M. S. Meltzer. 1986. A lymphokine distinct from interferon-gamma that activates human monocytes to kill *Leishmania donovani* in vitro. *J. Immunol.* 136:1329.
10. De Titto, E. H., J. R. Catterall, and J. S. Remington. 1986. Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi*. *J. Immunol.* 137:1342.
11. Rose, R. M., A. S. Wasserman, W. Y. Weiser, and H. G. Remold. 1986. An acidic lymphokine distinct from interferon-gamma inhibits the replication of herpes simplex virus in human pulmonary macrophages. *Cell. Immunol.* 97:397.
12. Shparber, M., and C. F. Nathan. 1986. Autocrine activation of macrophages by recombinant tumor necrosis factor but not interleukin-1. *Blood.* 68:86A. (Abstr.)
13. Grabstein, K., S. Reed, K. Shanebeck, and P. Morrissey. 1987. Induction of macrophage microbicidal activity by granulocyte-macrophage colony stimulating factor (GM-CSF). *Lymphokine Res.* 6:1707A. (Abstr.)
14. Meltzer, M. S., R. M. Crawford, D. S. Finbloom, J. Ohara, and W. E. Paul. 1987. BSF-1: A macrophage activating factor. *Lymphokine Res.* 6:1719A. (Abstr.)
15. Pihl, D., K. Grabstein, and S. Reed. 1987. Inhibition of *Trypanosoma cruzi* in mouse macrophages and in human monocytes and macrophage by recombinant interferon-gamma (rIFN-gamma) and granulocyte-monocyte colony stimulating factor (rGM-CSF). *Fed. Proc.* 46:1756A. (Abstr.)
16. Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, D. P. Luxenberg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. Wang, and S. C. Clark. 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science (Wash. DC).* 228:810.
17. Metcalf, D. 1977. Hematopoietic Colonies. In *Vitro Cloning of Normal and Leukemic Cells*. Springer-Verlag New York, Inc., New York.
18. Burgess, A. W., and D. Metcalf. 1980. The nature and action of granulocyte-macrophage colony stimulating factor. *Blood.* 56:947.
19. Gasson, J. C., R. H. Weisbart, S. E. Kaufman, S. C. Clark, and R. M. Hewick. 1984. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. *Science (Wash. DC).* 226:1339.
20. Silberstein, D. S., W. F. Owen, J. C. Gasson, J. F. Dipersio, D. W. Golde, J. C. Bina, R. Soberman, K. F. Austen, and J. R. David. 1986. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 137:3290.
21. Grabstein, K. H., D. L. Urdal, R. J. Tushiuski, D. Y. Mochizuki, V. L. Price, M. A.

- Cantrell, S. Gillis, and P. Conlon. 1986. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science (Wash. DC)*. 232:506.
22. Chang, K. P., and L. D. Hendricks. 1985. Laboratory cultivation and maintenance of *Leishmania*. In *Leishmaniasis*. K. P. Chang and R. S. Bray, editors. Elsevier Science Publishing Co., Inc., New York, 214–230.
 23. Schultz, R. M. 1980. Macrophage activation by interferons. *Lymphokine Reports*. 1:63.
 24. Pace, J. L., and S. D. Russell. 1981. Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interactions between lymphokine and lipopolysaccharide. *J. Immunol.* 126:1863.
 25. Nathan, C. F., T. J. Prendergast, M. E. Wiebe, E. R. Stanley, E. Platzer, H. G. Remold, K. Welte, B. Y. Rubin, and H. W. Murray. 1984. Activation of human macrophages. Comparison of other cytokines with interferon-gamma. *J. Exp. Med.* 160:600.
 26. Handman, E., and A. W. Burgess. 1979. Stimulation by granulocyte-macrophage colony-stimulating factor of *Leishmania tropica* killing by macrophages. *J. Immunol.* 122:1134.
 27. Ralph, P., C. A. Nacy, M. S. Meltzer, N. Williams, I. Nakoinz, and E. J. Leonard. 1983. Colony-stimulating factors and regulation of macrophage tumoricidal and microbicidal activities. *Cell. Immunol.* 76:10.
 28. Koren, H. S., and R. B. Herberman. 1985. The current status of human monocyte mediated cytotoxicity against tumor cells. *J. Leukocyte Biol.* 38:441.
 29. Murray, H. W. 1981. Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* 153:1302.
 30. Murray, H. W. 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J. Immunol.* 129:351.
 31. Passwell, J. H., R. Shor, E. Gazit, and J. Shoham. 1986. The effect of Con A-induced lymphokines from the T-lymphocyte subpopulations on human monocyte leishmaniacidal capacity and H₂O₂ production. *Immunology*. 59:245.
 32. Cannistra, S. A., A. Rambaldi, D. R. Spriggs, F. Herrmann, D. Kufe, and J. D. Griffin. 1987. Human granulocyte-macrophage colony-stimulating factor induces expression of the tumor necrosis factor gene by the U937 cell line and by normal human monocytes. *J. Clin. Invest.* 79:1720.
 33. Vermeulen, M. W., and H. G. Remold. 1987. Different program of gene expression in human macrophage activation. *Lymphokine Res.* 6:1419A. (Abstr.)
 34. Metcalf, D. 1971. Acute antigen-induced elevation of serum colony stimulating factor (CSF) levels. *Immunology*. 21:427.
 35. Neta, R., S. N. Vogel, J. J. Oppenheim, and S. D. Douches. 1986. Cytokines in radioprotection. Comparison of the radioprotective effects of IL-1 to IL-2, GM-CSF and IFN-gamma. *Lymphokine Res.* 5:105.