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Production of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) by Monocytes and Large Granular Lymphocytes Stimulated with *Mycobacterium avium-M. intracellulare*: Activation of Bactericidal Activity by GM-CSF

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Treatment of monocytes with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to enhance their antimycobacterial activity in an in vitro assay. Furthermore, *Mycobacterium avium-M. intracellulare* was found to induce the production of this hemopoietic growth factor. Human peripheral blood mononuclear cells were fractionated by plastic adherence and Percoll density centrifugation, and each population of cells was stimulated with mycobacteria. GM-CSF was produced by both monocytes and large granular lymphocytes (LGL) but not T lymphocytes. The phenotype of the GM-CSF-producing LGL was found to be $CD2^+$, $CD16^+$, and HLA-DR^+ but negative for T-cell and monocyte markers. Kinetic studies demonstrated that GM-CSF appeared in the supernatant fluids within 2 days of culture of either monocytes or LGL and continued to be produced up to 7 days of incubation. Northern (RNA) blot analysis of RNA from both cell types demonstrated the expression of GM-CSF message within 24 h of stimulation. From these studies, LGL and monocytes are capable of responding to *M. avium-M. intracellulare* by producing factors that augment normal immune functions, including the antibacterial capability of monocytes.

Infection with *Mycobacterium avium-M. intracellulare* is one of the most common mycobacterium-related diseases in patients with AIDS (7, 15, 28). *M. avium-M. intracellulare* is a ubiquitous saprophyte commonly found in soil, water, house dust, and plants, and it demonstrates low pathogenicity in humans unless a predisposing condition exists that allows tissue invasion to occur. In AIDS patients infected with *M. avium-M. intracellulare*, aggregated foamy macrophages containing innumerable acid-fast bacteria can be found in many tissues of the body. Little is known of the factors that influence *M. avium-M. intracellulare* infectivity and the mechanisms for the normally high resistance against this microorganism. It has been demonstrated that *M. avium-M. intracellulare* can survive within normal macrophages and multiply until the host cell is killed (15). However, normal immune responses appear to be sufficient for controlling *M. avium-M. intracellulare* infection, since disease is usually found only in immunocompromised conditions.

Colony-stimulating factors (CSFs) are glycoproteins that are characterized by their ability to induce the proliferation and differentiation of hemopoietic progenitor cells (19). It has also become evident that these CSFs can enhance the effector functions of mature myeloid lineage cells. For example, human monocyte cytotoxicity can be activated by granulocyte-macrophage CSF (GM-CSF) and interleukin-3 (IL-3) (5, 12), apparently via induction of tumor necrosis factor release. With regard to other cytokines, GM-CSF augments IL-1 production (20) and can indirectly boost release of IL-2 by stimulating antigen-processing cells (21).

CSFs are also active in enhancing antimicrobial activities of phagocytes. GM-CSF and macrophage CSF have been shown in separate reports to enhance macrophage function against *Leishmania tropica* (13), *Candida albicans* (16, 26), and *M. avium* complex (3). GM-CSF also affects the functional activity of granulocytes by inhibiting migration (11), stimulating phagocytosis of *Staphylococcus aureus* (10), augmenting oxidative metabolism (27), and enhancing the in vitro survival of human polymorphonuclear leukocytes and eosinophils (2).

Although *M. avium-M. intracellulare* is a known intracellular parasite, the fact remains that this organism is not highly pathogenic in the host. Factors governing host responses to opportunistic *M. avium-M. intracellulare* infections therefore need to be further studied. Natural killer (NK) cells have been postulated to be important in early host defenses against viruses and tumor cells, and we have therefore set out to determine whether NK cells may also contribute to host resistance mechanisms against opportunistic pathogens such as *M. avium-M. intracellulare*. NK cells, or large granular lymphocytes (LGL), are known to produce a variety of cytokines, including tumor necrosis factor (9), gamma interferon (17), IL-2 (17), and IL-1 (24). Using Percoll density gradient fractionation and negative selection by specific antibodies plus complement, we attempted to determine whether LGL and monocytes could be sources of mycobacterially induced GM-CSF, which in turn might activate monocytes to kill the intracellular bacterium.

MATERIALS AND METHODS

Culture of bacteria. Two *M. avium-M. intracellulare* isolates were used in this study; both were obtained from the Tampa Branch of Florida State Laboratories. For the induction of GM-CSF, we used an avirulent laboratory isolate that had been identified as serotype 8. The bacteria were maintained in Middlebrook 7H9 broth and were passaged weekly.

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Mycobacteria were obtained from 5- to 7-day cultures, washed twice in sterile phosphate-buffered saline (PBS), vigorously vortexed, and passaged several times through a 30-gauge needle to obtain a predominantly single-cell suspension of bacteria. The concentration of *M. avium-M. intracellulare* was adjusted to 10^8/ml in sterile PBS and was confirmed by plating on Middlebrook 7H11 agar plates. A virulent clinical isolate, identified as *M. avium-M. intracellulare* by the GenProbe detection system, was used for in vitro infection assays. In these experiments, the bacteria were used after growth in a second or third subculture. The first subculture was suspended in Middlebrook 7H9 broth containing 10% glycerol, and aliquots were stored at −80°C. Cultures from frozen suspensions were prepared by inoculating Middlebrook 7H10 agar plates and incubating the plates for 2 to 3 weeks. For each assay, bacteria were scraped off the plates, suspended in sterile PBS, and prepared as described above.

Preparation of human PBMC. Leukocyte buffy coats, obtained from normal volunteers at the Southwest Florida Blood Bank, were diluted 1:2 in PBS and centrifuged over Ficoll-Hypaque solution. The peripheral blood mononuclear cells (PBMC) at the interphase were collected, washed twice with PBS, and suspended in RPMI 1640 medium containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, Va.), 2 mM L-glutamine, 10 U of penicillin per ml 100 μg of streptomycin per ml, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, and 5×10^{-5} M 2-mercaptoethanol (complete medium). Recombinant human GM-CSF (specific activity, 4×10^7 CFU/mg of protein) was a very generous gift from Steven C. Clark (Genetics Institute, Cambridge, Mass.). All media contained less than 0.1 ng of endotoxin per ml, as determined by the *Limulus* lysate assay.

Preparation of monocytes. PBMC were incubated on gelatin-coated tissue culture flasks for 1 h at 37°C to allow adherence of monocytes (14). Nonadherent cells were recovered by vigorous washing of the flasks with warm medium, and the adherent cells were removed by vigorous pipetting after the addition of cold PBS. Cells were then washed and resuspended in complete medium.

Discontinuous Percoll density gradient centrifugation. LGL were separated from T cells by the use of a discontinuous Percoll density gradient (25). Nonadherent cells were further depleted of adherent cells and B cells by incubation on nylon wool columns for 30 min at 37°C. The cells passing through the columns were then placed on a six-step discontinuous density gradient with a range of 40 to 52.5% Percoll. After centrifugation at $550 \times g$ for 30 min at room temperature, the bands of lymphocytes were collected and examined for morphology on Giemsa-stained cytocentrifuged slides.

Serological depletion studies. To determine the phenotype of the LGL responsible for GM-CSF production, 4×10^6 washed LGL per treatment were pelleted in a 5-ml plastic tube. A previously determined optimal dilution of each monoclonal antibody was then added to the cell pellet and incubated for 20 min at 37°C. Low-toxic rabbit complement was then added to the antibody-treated cells at a 1/10 dilution and incubated for an additional 45 min to effect lysis. Cells were examined for viability by trypan blue exclusion, washed twice in RPMI medium, and resuspended to the volume corresponding to the cell concentration of untreated LGL (2×10^6 cells per ml) without adjusting for nonviable cells to avoid enrichment of interfering cell populations in the experiments.

Induction of GM-CSF. Leukocytes were cultured in 24-well tissue culture dishes at a concentration of 2×10^6 cells per ml with 1 ml per well. Bacteria were added at the indicated ratios, and the cocultures were incubated for 1 to 7 days at 37°C. Supernatant fluids were collected and passed through a 0.22-μm-pore-size filter to eliminate bacteria and cell debris. Samples were either assayed immediately or stored at −70°C. Live bacteria were used for induction of GM-CSF to simulate as closely as possible in vivo conditions. As a control for the number of bacteria, however, antibiotics were included in the medium to prevent bacterial growth from occurring during the stimulation period.

Assay for GM-CSF. The presence of CSF in supernatants was assayed by using the human Mo7e cell line, which was established from the peripheral blood of a patient with acute megakaryocytic leukemia (1). This cell line was a generous gift from Steven C. Clark (Genetics Institute) and is maintained in Dulbecco modified Eagle medium supplemented with 20% fetal calf serum and antibiotics. Recombinant human IL-3 or GM-CSF is a required growth factor and is added at a final concentration of 8 U/ml for continuous culture. For assay, Mo7e cells were washed twice with PBS and resuspended at a concentration of 10^5/ml in Dulbecco modified Eagle medium without growth factors, and 0.1 ml of the cell suspension was added to each well of a 96-well U-bottom microtiter plate in which supernatant fluids were serially diluted in a final volume of 0.1 ml. Triplicate cultures for each dilution were incubated for 48 h at 37°C and then pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol) per well. The cultures were incubated an additional 18 h, harvested, and counted in a beta scintillation counter. A standard curve obtained by using recombinant human GM-CSF was included with each assay and was used to calculate units of CSF activity in supernatant fluids.

Infection of monocytes. Freshly isolated monocytes in antibiotic-free medium were plated in 96-well flat-bottom microtiter dishes at a concentration of 5×10^4 cells per well in a volume of 50 μl. Various concentrations of GM-CSF were added directly to each well and incubated for 24 h before the addition of *M. avium-M. intracellulare*. Bacteria were added in a volume of 25 μl per well in quadruplicate wells at a bacteria-to-monocyte ratio of 1:1. Control wells of *M. avium-M. intracellulare* were also prepared as described above but were cultured in the absence of monocytes. Plates were then incubated at 37°C in a humidified 5% CO_2 incubator for 3 days, which was determined to result in optimal antimycobacterial activities of monocytes (4). To enumerate the bacteria, monocytes were lysed by the addition of 10 μl of 1.0 N NaOH and 20 μl of 5% Tween 80 solutions. Then 10 μl of 1.0 N HCl was added to neutralize pH, and the bacteria were enumerated by a radiolabel assay described below. Control wells of *M. avium-M. intracellulare* alone were also treated as described above. There was no direct effect of GM-CSF on *M. avium-M. intracellulare* at any of the concentrations used in all cases.

Radioassay for *M. avium-M. intracellulare* growth. To each well of NaOH-Tween-HCl-treated cultures was added 100 μl of Middlebrook 7H9 broth medium (GIBCO) containing [2-^{3}H]glycerol (specific activity, 10 Ci/mmol; ICN Radiochemicals, Costa Mesa, Calif.) at a concentration of 10 μCi/ml. The plates were incubated for an additional 3 days at 37°C to allow for incorporation of the radiolabel into the proliferating bacteria. Bacteria were then harvested by first adding 50 μl of 5.25% sodium hypochlorite solution (bleach) and then processing through a Skatron harvestor with distilled water onto glass filter paper. Radioactivity of each well was assessed by an LKB beta scintillation counter. The

mean of quadruplicate cultures was determined, and the standard error of the mean was usually within 10% of the mean. Student's t tests were performed to assess whether differences existed between control and GM-CSF-treated monocytes. The percent of growth inhibition of *M. avium-M. intracellulare* was calculated as GI = [1 − (cpm of *M. avium-M. intracellulare* with monocytes/cpm of *M. avium-M. intracellulare* alone)] × 100.

Northern (RNA) blot analysis. To detect the presence of specific mRNA, Northern blot analysis was performed on leukocytes stimulated with *M. avium-M. intracellulare* at a ratio of 10 bacteria per leukocyte. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (6). Approximately 2×10^7 to 3×10^7 cells were lysed by a guanidinium thiocyanate solution followed by a phenol-chloroform-isoamyl alcohol mixture. RNA was purified by precipitation with isopropanol, washed with 70% ethanol, and dissolved in water. Then 20- to 30-μg samples of RNA were denatured in a glyoxal-dimethyl sulfoxide mixture (18) and fractionated on a 0.8% agarose gel in 10 mM sodium phosphate buffer (pH 7.0) with circulation. Fractionated RNA was then transferred to Nytran filter paper (Schleicher & Schuell, Keene, N.H.) by capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), UV irradiated to ensure complete binding of RNA to nylon, and then stained with methylene blue-acetate (18) to determine the presence and integrity of transferred RNA. Prehybridization was performed at 45°C for 2 h, and hybridization was done at the same temperature for 18 h. Prehybridization solution consisted of 5× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, 200 μg of salmon sperm DNA per ml, 50% deionized formamide, 10 mM piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES; pH 6.5), and 10 mM EDTA (22). Hybridization was performed in the same solution after the addition of a random-primed GM-CSF cDNA probe labeled with [α-^{32}P]dCTP at a concentration of 3×10^6 cpm/ml. Human GM-CSF cDNA was released from the pXM vector, a generous gift from Steven C. Clark (Genetics Institute), by digestion with *Xho*I. The 0.8-kb insert was labeled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; New England Nuclear, Boston, Mass.), using a random-primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

After hybridization, the membrane was washed for 5 min in 5× SSC–0.2% SDS–1 mM EDTA at room temperature, twice for 30 min in 1× SSC–0.2% SDS–1 mM EDTA at 42°C, and once for 30 min in the same buffer at 60°C. The membrane was then exposed to film with an intensifying screen at −70°C for 5 days.

RESULTS

Effect of GM-CSF on the antimycobacterial activity of monocytes. GM-CSF has been shown to activate macrophage activity against a variety of targets, ranging from tumor cells (5, 12) to fungi (16, 26) and intracellular parasites (13). A recent report by Bermudez and Young (3) also described the activation of human culture-derived macrophages by GM-CSF to inhibit growth of *M. avium* complex bacteria. Before determining whether *M. avium-M. intracellulare* can induce GM-CSF, we examined the effect of this cytokine on the antibacterial activity of freshly isolated monocytes toward *M. avium-M. intracellulare*. Recombinant human GM-CSF was added to monocytes seeded in microtiter wells at the indicated concentrations, and mycobacteria were then added at a final ratio of 1:1. In the absence of GM-CSF, negative growth inhibition was noted in all cases (Fig. 1), indicating that intracellular growth occurred. However, the presence of GM-CSF activated the growth-inhibitory capability of the monocytes from all three donors in a dose-dependent manner. As little as 1 U of GM-CSF per ml added to monocytes resulted in a decrease of bacterial growth, with increasing effects seen at higher doses of the cytokine. There was no direct effect of GM-CSF seen on the growth of *M. avium-M. intracellulare* alone at any of the doses used (data not shown). Thus, recombinant human GM-CSF was found to stimulate the ability of normal monocytes to restrict growth of *M. avium-M. intracellulare*.

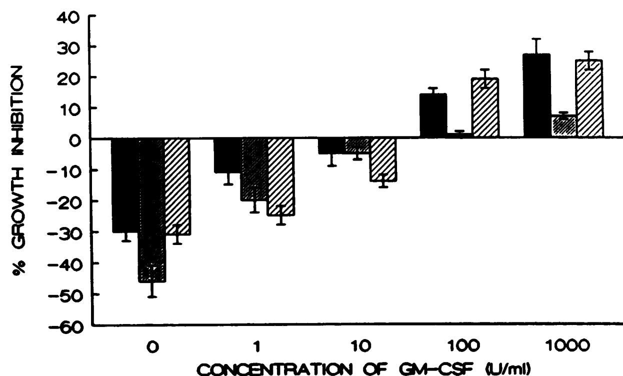

FIG. 1. Effect of GM-CSF on the ability of monocytes to kill *M. avium-M. intracellulare*. Monocytes were pretreated with the indicated concentrations of GM-CSF 24 h prior to inoculation with mycobacteria at a ratio of 1:1. Monocytes and bacteria were cocultured for 3 days, lysed, and pulsed with [^{3}H]glycerol for an additional 3 days prior to harvesting. Numbers represent percent growth inhibition ± standard error of the mean of mycobacteria compared with counts per minute of control wells in which mycobacteria were cultured alone. The value for control wells of mycobacteria was 47,441 ± 3,778 cpm. Values for all treatments of monocytes with GM-CSF were statistically different ($P < 0.05$) from growth inhibition of mycobacteria by untreated monocytes. Symbols: ■, donor 1; ▨, donor 2; ▨, donor 3.

Induction of CSF by *M. avium-M. intracellulare* and identification of the CSF-producing cell population. To define which cells from normal peripheral blood responded to this bacterium by producing hemopoietic growth factors, various subpopulations of peripheral blood leukocytes were isolated by adherence, to yield monocytes, and by Percoll fractionation, to separate LGL from T lymphocytes. The cell subpopulations were then similarly stimulated with *M. avium-M. intracellulare* for 3 days, and their supernatants were collected and assessed for CSF content by bioassay using Mo7e cell proliferation. As shown in Fig. 2, fractionation of peripheral blood leukocytes into various subsets indicated that monocytes responded to mycobacterial stimulation by production of 42 U of CSF activity per ml. Also, cells from Percoll fractions 2 and 3, which contained the LGL subpopulation of lymphocytes, produced 28 and 52 U of CSF activity per ml, respectively. However, CSF was not found in the supernatants of T cells, which were isolated from Percoll fractions 4 through 7. Thus, both monocytes and LGL were found to produce CSF activity after direct stimulation by *M. avium-M. intracellulare*.

Kinetics of CSF production. The experiments described above showed that *M. avium-M. intracellulare* was capable of inducing CSF from LGL and monocytes within 3 days of culture. To determine the time course of growth factor

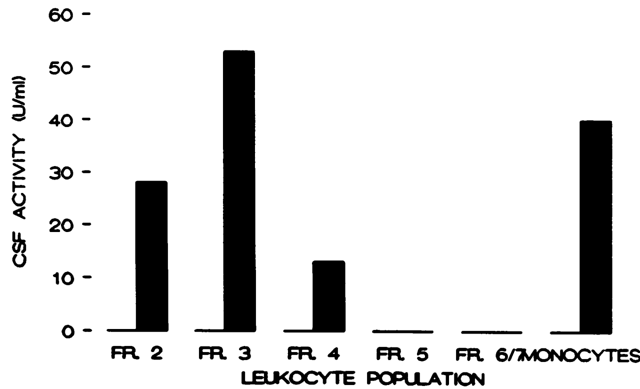

FIG. 2. Production of GM-CSF from Percoll-fractionated lymphocytes. The indicated cell populations were cultured for 3 days in the presence of *M. avium-M. intracellulare* at a ratio of 10 bacteria per leukocyte. The supernatant fluids were collected and assayed for the presence of CSF activity. Symbol: ▩, with mycobacteria. All controls produced no detectable CSF activity.

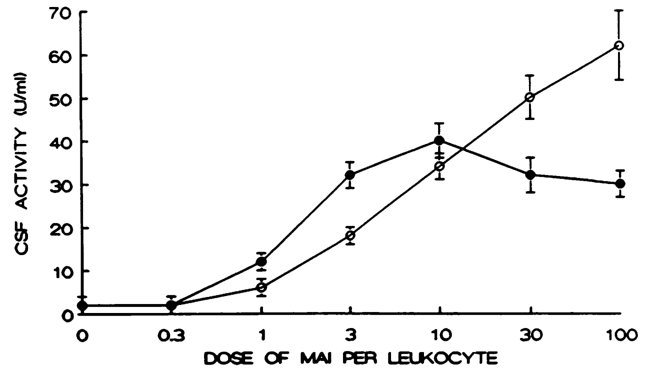

FIG. 4. Dose response of CSF production to *M. avium-M. intracellulare*. LGL (●) or monocytes (○) were incubated in medium alone or in the presence of the indicated numbers of *M. avium-M. intracellulare* (MAI) for 3 days. Supernatants were then harvested and assessed for CSF activity.

production, LGL and monocytes were cocultured with mycobacteria for 1 to 7 days before the supernatants were assayed for CSF. As shown in Fig. 3, little CSF activity was seen in the supernatants of either cell type until day 2 of culture. From that time, CSF continually increased throughout the 7 days of incubation. No CSF was seen in culture fluids of unstimulated LGL or monocytes.

Dose dependence of CSF production. To determine whether a dose response to the number of stimulating mycobacterial cells existed, LGL and monocytes were cultured with various ratios of bacteria to leukocyte, ranging from 0.3:1 to 100:1 (Fig. 4). As few as one bacterial cell per leukocyte was found to induce detectable levels of CSF. Optimal stimulation was found at a ratio of 10 bacterial cells per LGL, resulting in 41 U/ml after 3 days of incubation. As *M. avium-M. intracellulare* concentrations increased to 30:1, less CSF was produced from LGL, with levels falling to 30 U/ml. The dose response of monocytes to *M. avium-M. intracellulare* stimulation demonstrated that increasing numbers of bacteria, up to 100:1, resulted in higher levels of

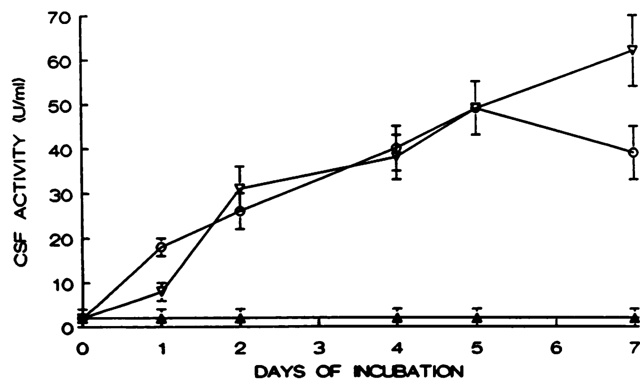

FIG. 3. Kinetics of CSF production. LGL or monocytes were incubated alone or in the presence of *M. avium-M. intracellulare* at a ratio of 10 bacteria per leukocyte for the indicated times. Supernatants were then harvested and assessed for CSF activity. Symbols: |, monocytes; ○, monocytes plus mycobacteria; ▲, LGL; ▽, LGL plus mycobacteria.

CSF, with no inhibition of CSF production like that seen with LGL at the higher concentrations of bacteria.

Neutralization of CSF with antibodies. Since Mo7e cells could respond to both GM-CSF and IL-3, it was important to differentiate the type of CSF that was produced by *M. avium-M. intracellulare*-stimulated LGL and monocytes. For this experiment, supernatants that contained CSF activity were incubated with excess amounts of neutralizing antibody preparations and then assayed for residual growth factor activity. As shown in Table 1, treatment of LGL supernatants with sheep anti-recombinant human GM-CSF neutralized 97.3% of CSF activity, indicating that *M. avium-M. intracellulare* induced GM-CSF from LGL. On the other hand, rabbit anti-recombinant human IL-3 minimally affected CSF activity in the LGL supernatants. The small decrease in activity by anti-IL-3 may be attributed to some cross-reaction of this preparation with GM-CSF. This can be seen by its ability to neutralize the proliferative activity of recombinant human GM-CSF by 14.6%. Similar results were noted for neutralization of *M. avium-M. intracellulare*-stimulated monocyte supernatants by both antibodies. Thus, because of the almost complete neutralization of CSF activity by anti-GM-CSF and the lack of neutralization by anti-IL-3, the growth factor produced by stimulated LGL and monocytes was identified as GM-CSF.

Phenotype of CSF-producing LGL. Since both monocytes and LGL were found to respond to mycobacteria by release of GM-CSF, it was important to confirm that cells of LGL phenotype, not contaminating monocytes or T cells, were responsible for this cytokine production. LGL were serologically depleted of various subpopulations by incubation with monoclonal antibodies and complement to lyse the indicated phenotype. After complement depletion, the remaining LGL were stimulated with mycobacteria for 3 days at a ratio of 3:1. Treatment of LGL with anti-CD15 monoclonal antibodies, which deplete monocytes and macrophages, had only a slight effect on the production of CSF activity (Table 2). Further treatment with anti-CD4 and anti-CD8, which lyse T helper and T suppressor/cytotoxic lymphocyte subsets, respectively, did not affect CSF production. However, elimination of HLA-DR$^+$ LGL resulted in a loss of CSF activity. Finally, treatment of LGL with anti-CD16, which identifies NK cells, also eliminated CSF production.

Northern blot analysis of *M. avium-M. intracellulare*-stimulated leukocytes. To definitively identify the cytokine pro-

TABLE 1. Neutralization of CSF activity from *M. avium-M. intracellulare*-stimulated LGL with specific antisera[a]

Sample	cpm ± SEM (% inhibition) of samples after treatment with:		
	Medium	Anti-GM-CSF	Anti-IL-3
Medium	225 ± 31	198 ± 3	165 ± 16
Recombinant GM-CSF (30 U/ml)	5,265 ± 159	269 ± 8 (98.6)	4,414 ± 55 (15.4)
Recombinant IL-3 (30 U/ml)	4,036 ± 140	3,453 ± 240 (14.6)	197 ± 16 (99.2)
LGL + mycobacteria	2,576 ± 88	261 ± 13 (97.3)	2,055 ± 45 (19.6)
Monocytes + mycobacteria	1,999 ± 60	242 ± 37 (97.5)	1,701 ± 69 (13.4)

[a] Samples were incubated at ambient temperature for 30 min with an excess amount of the indicated antibodies and then assessed for residual CSF activity as detected by proliferation of the Mo7e cell line. Supernatants were collected from LGL or monocytes stimulated with mycobacteria at a ratio of 10 bacteria per leukocyte for 2 days. Data are representative of two experiments that were performed with similar results.

duced by LGL and monocytes, Northern blot analyses were performed on total RNA extracted from stimulated cells. For these studies, LGL or monocytes were stimulated with *M. avium-M. intracellulare* for the indicated times, and their RNA was isolated and probed for the expression of GM-CSF mRNA. As shown in Fig. 5, in the absence of stimulation, LGL did not express mRNA for GM-CSF. Additionally, 2 h of incubation with *M. avium-M. intracellulare* was insufficient to induce detectable message. As shown, however, 6 h was required for the induction of GM-CSF mRNA in LGL, with some expression still noted after 24 h of stimulation with *M. avium-M. intracellulare*. On the other hand, monocytes expressed GM-CSF mRNA after only 2 h of bacterial stimulation, with levels decreasing by 24 h (Fig. 6). Thus, mRNA for GM-CSF was expressed by stimulation of LGL and monocytes with mycobacteria, with monocytes responding as early as 2 h after stimulation and LGL requiring a longer induction period. These kinetics are consistent with the earlier presence of GM-CSF protein in the supernatant fluids of *M. avium-M. intracellulare*-stimulated monocytes.

DISCUSSION

Using a sensitive radiolabel assay that measures [^{3}H]glycerol uptake in proliferating *M. avium-M. intracellulare*, this study provides definitive evidence that the survival of *M. avium-M. intracellulare* in human monocytes can be greatly reduced by treatment with GM-CSF. Intracellular growth of mycobacteria was retarded in monocytes treated with as little as 1 U of the cytokine per ml, and doses higher than 10 U/ml were found to activate monocytes to kill the intracellular parasite, similar to the results presented by Bermudez and Young (3). On the basis of this information, further studies were performed to determine whether *M. avium-M. intracellulare* could induce GM-CSF in PBMC of normal donors. We found that LGL and monocytes, but not small, mature T cells, were able to produce GM-CSF when directly stimulated with mycobacteria. As few as one bacterial cell per leukocyte could elicit sufficient levels of this cytokine to activate monocyte antimycobacterial activity. Neutralization of CSF activity by anti-GM-CSF demonstrated that this factor was produced by both cell types. While anti-IL-3 antibodies were found to decrease LGL- and monocyte supernatant-induced proliferation of Mo7e cells by about 20%, the decrease was not significantly different from the neutralization of recombinant human GM-CSF by this preparation, demonstrating that some cross-reactivity existed. Although LGL have been shown to be capable of producing IL-3, only a combination of phorbol diester and calcium ionophore was effective in stimulating such production (8). In our hands, only GM-CSF was produced by *M. avium-M. intracellulare*-stimulated LGL.

Characterization of the LGL population responsible for CSF production revealed that CD16$^+$ HLA-DR$^+$ cells were required. During studies to identify phenotype and during Northern blot analyses, all LGL were depleted of CD15$^+$ cells to eliminate possible monocyte contamination prior to GM-CSF induction. The requirement for HLA-DR$^+$ LGL for CSF production indicates two possibilities. First, a small

TABLE 2. Phenotype of CSF-producing LGL by serologic depletion[a]

Treatment of LGL (% viability)	GM-CSF (U/ml) in medium supplemented with mycobacteria (% of control)
Complement only (99)	64 ± 6 (100)
Anti-CD15 (94)	63 ± 5 (98)
Anti-CD15/CD4/CD8 (51)	53 ± 5 (83)
Anti-CD15/HLA-DR (86)	11 ± 2 (17)
Anti-CD15/CD16 (73)	21 ± 2 (33)

[a] LGL were treated with the indicated antibodies plus complement and then incubated in medium alone or with *M. avium-M. intracellulare* at a ratio of 10:1 for 3 days. Data are representative of three experiments that were performed with similar results. In all cases, values for LGL incubated with medium alone were <1 U of GM-CSF per ml.

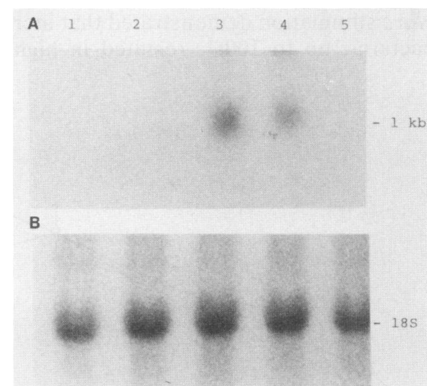

FIG. 5. Northern blot analysis of total RNA from *M. avium-M. intracellulare*-stimulated LGL. Total cellular RNA was isolated from LGL cultured in the presence or absence of 10 mycobacterial cells per LGL at the indicated times, and Northern blot analysis was performed on 20 μg of RNA. The filter was hybridized with a ^{32}P-labeled cDNA probe for human GM-CSF (A). The methylene blue-stained filter showing the 18S rRNA (B) indicates that equivalent amounts of RNA were loaded per lane. Lanes: 1, LGL in medium alone (2 h); 2, LGL plus mycobacteria (2 h); 3, LGL plus mycobacteria (6 h); 4, LGL plus mycobacteria (24 h); 5, LGL in medium alone (24 h).

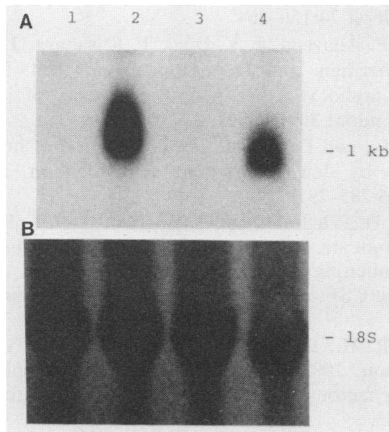

FIG. 6. Northern blot analysis of total RNA from *M. avium-M. intracellulare*-stimulated monocytes. Total cellular RNA was isolated from monocytes cultured in the presence or absence of 10 mycobacterial cells per monocyte at the indicated times, and Northern blot analysis was performed on 30 μg of RNA. The filter was hybridized with a ^{32}P-labeled cDNA probe for human GM-CSF (A). The methylene blue-stained filter showing the 18S rRNA (B) indicates that equivalent amounts of RNA were loaded per lane. Lanes: 1, monocytes in medium alone (2 h); 2, monocytes plus mycobacteria (2 h); 3, monocytes in medium alone (24 h); 4, monocytes plus mycobacteria (24 h).

population of HLA-DR$^+$ LGL may exist that would present antigen or otherwise stimulate HLA-DR$^-$ LGL, responsible for NK activity, to produce GM-CSF. Second, it is possible that a HLA-DR$^+$ LGL population that itself possess no spontaneous NK function may directly respond to *M. avium-M. intracellulare* by GM-CSF release and develop into lymphokine-activated killer (LAK) cells. Cuturi et al. (8) induced NK cells, which were expanded in vitro with B-lymphoblastoid cell stimulation, to produce GM-CSF. These initially HLA-DR$^-$ NK cells were found to become >98% HLA-DR$^+$. However, Pistoia et al. (23) depleted their NK population of HLA-DR$^+$ cells and demonstrated that GM-CSF was spontaneously produced. Further studies are required to resolve the role of HLA-DR$^+$ cells in GM-CSF production, but the present data suggest that an HLA-DR$^+$ NK or LAK precursor may be responsible for this activity.

For these studies, a virulent isolate of *M. avium-M. intracellulare* was used to determine the effect of GM-CSF on antimycobacterial activity of monocytes since the avirulent mycobacteria were readily controlled by untreated monocytes. Further studies had shown that similar levels of GM-CSF were induced by both isolates (data not shown), indicating that endogenous production of this cytokine by infected monocytes did not alter the virulence of the bacteria used in this study. However, it is tempting to speculate that GM-CSF released by infected monocytes, as well as by *M. avium-M. intracellulare*-stimulated LGL, provide resistance factors for uninfected cells. Thus, the importance of GM-CSF induction during microbial infection may lie in its ability to activate phagocytic cells to contain the microorganism. Our observation that GM-CSF is readily induced by *M. avium-M. intracellulare* from LGL and monocytes, coupled with its ability to mobilize monocyte function, suggests that it may be a key cytokine that plays an important role in keeping this usually nonpathogenic organism under the control of LGL and phagocytes in a normal host.

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