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Response to Stimulation with Recombinant Cytokines and Synthesis of Cytokines by Murine Intestinal Macrophages Infected with the *Mycobacterium avium* Complex

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Current evidence suggests that the gut is the chief portal of entry for organisms of the *Mycobacterium avium* complex (MAC) in AIDS patients. Bacterial invasion of intestinal mucosa presumably occurs through epithelial cells, and M cells in the Peyer's patches, where the bacteria have contact with immunocompetent cells such as macrophages and T and B lymphocytes. As mucosal macrophages are probably the first line of defense against MAC, we examined their ability to inhibit intracellular growth of MAC when properly stimulated. Mouse intestinal macrophages were purified, infected with MAC 101, serovar 1, and MAC 86-2686, serovar 16, and subsequently stimulated with recombinant tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), or macrophage colony-stimulating factor (M-CSF). Viable intracellular bacteria were quantitated at 24 h after infection and again after 4 days of infection. Stimulation with TNF-α, IFN-γ, and GM-CSF, but not M-CSF, was associated with mycobacteriostatic and/or mycobactericidal activity in macrophages. Treatment with 10^3 U of TNF-α, GM-CSF, and IFN-γ per ml at 24 h prior to infection with MAC resulted in a significant enhancement in killing of MAC at 4 days after infection, compared with that observed for macrophages exposed to cytokines after infection. When stimulated with lipopolysaccharide or live MAC, intestinal macrophages had produced significantly less TNF-α and transforming growth factor β than had splenic and peritoneal macrophages, although the levels of production of interleukin 6 and interleukin 10 among the three populations of cells were similar. Intestinal macrophages can be stimulated with cytokines to inhibit the intracellular growth of MAC, but they have differentiated abilities to produce cytokines which can modulate the anti-MAC immune response.

Organisms of the *Mycobacterium avium* complex (MAC) are the most common bacteria causing infection in patients with AIDS. In this population MAC is associated with bacteremia and disseminated disease (15, 16, 29).

Current evidence suggests that the majority of AIDS patients acquire MAC infection following colonization of the gastrointestinal tract. At autopsy, large numbers of organisms are found in the intestinal mucosa and submucosa of patients with MAC disseminated infection, and a few studies had suggested that intestinal colonization precedes the onset of bacteremia (16, 19, 22).

MAC organisms are intracellular bacteria that invade and replicate within macrophages (10). In the intestine, the majority of the resident macrophages are located in the terminal ileum, more specifically in the Peyer's patches. Intestinal macrophages may come in contact with MAC very early in the infection, and their ability to phagocytize and inhibit the intracellular growth of the bacteria probably influences the progression of disease (4).

The purpose of this investigation was to determine whether intestinal macrophages can phagocytize and be stimulated by recombinant cytokines to inhibit the intracellular growth of MAC. We also determined whether intestinal macrophages are capable of synthesizing and releasing cytokines when stimulated with live MAC.

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MATERIALS AND METHODS

Mycobacteria. MAC strains 101 (serovar 1) and 109 (serovar 4) were isolated from the blood of AIDS patients. Strain 86-2486 (serovar 14) and strain 86-2686 (serovar 16) were obtained from Robert Good (Centers for Disease Control and Prevention, Atlanta, Ga.). While MAC 101 and MAC 109 are capable of multiplying within macrophages, strains 8602686 and 8602486 usually have their intracellular growth inhibited by macrophages from the majority of donors. Bacteria were cultured on Middlebrook 7H10 agar (Difco, Detroit, Mich.) for 10 days at 37°C. Transparent colonies were then resuspended in Hanks' solution and adjusted to 10^7 bacteria per ml by using a McFarland standard. To prevent the formation of clumps, the bacterial suspension was vortex agitated for 2 min and allowed to rest for 15 min. Only the top half of the bacterial suspension, containing dispersed bacteria as determined by microscopic observation, was used to infect macrophages.

Cytokines. Recombinant murine tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) were gifts from Genentech (South San Francisco, Calif.). TNF-α had activity of 3×10^8 U/mg of protein, and IFN-γ had activity of 2×10^8 U/mg of protein. Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was a gift from Immunex (Seattle, Wash.), and macrophage colony-stimulating factor (M-CSF) was kindly provided by Chiron (Emeryville, Calif.). GM-CSF had activity of 3×10^8 U/mg of protein, and M-CSF had activity of 2×10^7 U/mg of protein. Concentrations of endotoxin in the cytokine preparations were measured by using the Limulus Amebocyte Assay (Sigma Chemical Co., St. Louis, Mo.) and were found to be less than 0.01 ng/ml. Heat-inactivated cytokines were used as controls in all the assays.

Mice. C57BL/6 (bg^+/bg^-) mice used in these experiments were obtained from Jackson Laboratories (Bar Harbor, Maine). The animals were submitted to 1 week of quarantine, and they were between 4 and 6 weeks old at the time of the experiment.

Intestinal, peritoneal, and splenic macrophages. Intestinal macrophages were isolated from C57BL/6 mice as previously described (9, 20, 21, 24) with small modifications. Briefly, mice were anesthetized by intraperitoneal injection of nembutol. Immediately after this the animals were sacrificed, and the abdominal cavity was carefully opened. Two inches (5.08 cm) of the terminal ileum portion of the intestine was identified, isolated, and further dissected free from underlying musculature. To remove epithelial cells, the intestinal segment was washed twice with Hanks' buffered salt solution (HBSS), minced into small pieces in 10 ml of HBSS containing 0.75 nmol of EDTA per liter, 500 μg of clindamycin

(Upjohn Co., Kalamazoo, Mich.) per ml, and 100 μg of amikacin (Sigma Chemical) per ml, and incubated at 37°C for 1 h. The supernatant was then removed, and the pieces of intestinal segments were resuspended in 10 ml of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma) and 2 mM L-glutamine containing 500 μg of clindamycin per ml, 100 μg of amikacin per ml, and 16 U of collagenase (Sigma Chemical) per ml, and this suspension was incubated at 37°C for 2 h. Preliminary studies have shown that the use of amikacin and clindamycin at these concentrations is sufficient to sterilize the intestinal segment (unpublished data). The cell suspension containing macrophages, epithelial cells, and lymphocytes (approximately 25% of the number of macrophages) was washed twice with RPMI 1640 to remove antibiotics and then transferred into 15-ml plastic centrifuge tubes. A volume of Histopaque (Sigma Chemical) was added at a 1:2 ratio to each tube. The samples were centrifuged at 1,600 × *g* for 40 min. Mononuclear cells were removed, resuspended in RPMI 1640 supplemented with 5% FBS, and centrifuged at 600 × *g* for 10 min. The pellet, rich in macrophages and lymphocytes (approximately 41% of the number of macrophages), was resuspended in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine. To ensure that no antibiotic which could interfere with the experimental assay was present intracellularly, 10^5 macrophages submitted to the process described above were lysed with sterile water and the lysate was incubated with 10^5 MAC 101 organisms for 24 and 48 h. The number of viable bacteria did not differ between the control (absence of macrophage lysate) and the experimental group (in the presence of macrophage lysate). An average of $5 \times 10^8 \pm 2 \times 10^8$ macrophages was obtained from each mouse. After the cells were counted, the cell suspension was adjusted to 5×10^5 cells per ml and 1 ml was added to each well of a 24-well tissue culture plate (Costar) for enrichment of macrophages. Only preparations containing more than 95% viable cells were used for the assays. Approximately 30% of the cells from the three populations of macrophages were lost in the first day in culture. After the second day, 5% ± 1.5% detached from the plastic every day. There was no differential detachment among monolayers treated with different cytokines. Peritoneal and splenic macrophages were purified as previously reported (3, 5) and plated into a tissue culture plate (Costar) at 5×10^5 cells per well. Preparations of intestinal, splenic, and peritoneal macrophages were evaluated for purity by their ability to ingest neutral red and by the ability to bind anti-Mac-3 antibody (American Type Culture Collection, Rockville, Md.). Preparations containing less than 93% macrophages were not used in the reported assays. Macrophage monolayers were incubated for 24 h before being used in the assays.

Establishment of infection and stimulation of intestinal macrophages. Intestinal macrophages (approximately 10^5 cells per monolayer) cultured for 24 h in RPMI 1640 with 5% FBS and 2 mM glutamine were exposed to 10^6 bacteria at 37°C for 4 h, without antibiotics. Three replicated wells were used for each experimental condition. Macrophage monolayers were then washed three times with HBSS to remove extracellular bacteria. This procedure has been shown to be effective in removing at least 99.9% of the extracellular bacteria (8). A few monolayers were lysed to establish the time zero (baseline) bacterial load after infection. Infected macrophage monolayers were then cultured in the presence of RPMI 1640 supplemented with 5% FBS. Treatment with 10 to 10^3 U of recombinant TNF-α, IFN-γ, M-CSF, or GM-CSF per ml was then administered to identify infected monolayers. The concentrations of the cytokines were established to be nontoxic for the monolayer in preliminary experiments (data not shown). Cytokines and supernatant were replenished daily. In addition, the numbers of macrophages in the monolayers were carefully monitored by the method described by Nakagawara and Nathan (23). Furthermore, the percents infected cells were established daily for the three populations of macrophages. Thus, approximately 72% ± 6% of the peritoneal macrophages, 74% ± 5% of the intestinal macrophages, and 75% ± 4% of the splenic macrophages were infected after 4 days (untreated monolayers). After 18 h the number of viable intracellular bacteria was determined after lysis of the macrophage monolayers. Macrophages were lysed by incubation with 500 μl of sterile deionized water for 10 min and 500 μl of 0.025% sodium dodecyl sulfate (SDS) for 10 additional min. Afterward, 500 μl of 20% albumin in HBSS was added to the cell lysate to neutralize the SDS. The suspension was then vortex agitated for 1 min, serially diluted in sterile water, and plated onto Middlebrook 7H10 agar plates. The plates were incubated at 37°C for 10 days, and the number of CFU was determined. Each experiment was repeated at least three times.

Because the response of infected macrophages to cytokines can differ if macrophages are stimulated prior to the infection, macrophage monolayers were also treated with TNF-α, GM-CSF, M-CSF, or IFN-γ at 24 h prior to infection with either MAC 101 or MAC 86-2686, and treatment was continued for 4 days.

Superoxide anion production. Intestinal macrophages were evaluated to establish their degree of activation by determining their ability to release superoxide anions (O_2^-) as previously described (8).

Cytokine production. Intestinal, peritoneal, and splenic macrophages were obtained as previously described. To comparatively measure the levels of cytokines (TNF-α, interleukin 10 [IL-10], transforming growth factor β [TGF-β], and IL-6) secreted by three populations of macrophages, intestinal, peritoneal, and splenic macrophage monolayers (10^5 cells per well) were infected with 10^6 MAC organisms belonging to strain 86-2486, 101, or 109 and incubated at 37°C for 18 h. Five hundred nanograms of *Escherichia coli* 0111B6 lipopolysaccharide (LPS) (List Biograd Laboratories, Inc., Campbell, Calif.) was added to the control wells without bacteria. After 24 h, the supernatant was removed from each well and filtered through a 0.2-μm-pore-size Millipore filter (Gelman Sciences, Ann Arbor, Mich.). Three replicated samples were obtained for each experimental condition. The samples were then stored at −20°C. Concentrations of cytokines in supernatants (TNF-α, IL-6, and IL-10) were determined by enzyme-linked immunosorbent assay (ELISA) (murine TNF-α ELISA purchased from Biosource International, Camarillo, Calif.; murine IL-6 ELISA from Genzyme, Cambridge, Mass.; and murine IL-10 ELISA from Endogen, Boston, Mass.) and read on an ELISA reader at 490 nm. The concentration of TGF-β was determined by using a biologic assay with Mu1Lv mink lung cells as previously described (2).

TABLE 1. Production of superoxide anions (O_2^-) by intestinal, peritoneal, and splenic macrophages following the purification procedure

Population of macrophages[a]	Amt of O_2^- (nmol/10^5 cells) produced at[b]:		
	2 h	24 h	48 h
No cells	0.33 ± 0.06	0.34 ± 0.06	0.37 ± 0.07
Intestinal	14.6 ± 2.6	3.7 ± 0.9	3.5 ± 0.5
Peritoneal[c]	12.4 ± 2.0	3.2 ± 0.8	3.4 ± 0.7
Peritoneal[d]	16.1 ± 3.2	3.6 ± 0.9	3.4 ± 0.5
Splenic	11.7 ± 1.7	3.4 ± 0.6	3.1 ± 0.4

[a] Macrophages were treated with 0.1 μg of phorbol myristate acetate per ml for 1 h. Supernatant was then obtained, and O_2^- was measured as previously described (7).
[b] Results are means ± SD for three different experiments. Times represent hours after purification.
[c] Not submitted to the same process of purification as intestinal macrophages.
[d] Submitted to the same process of purification as intestinal macrophages.

Use of neutralizing antibodies. To determine the influence of TGF-β, IL-10, and IL-6 on the ability of MAC to survive within intestinal macrophages, a purified preparation of intestinal macrophages was infected with 10^6 MAC 101 organisms (10 bacteria:1 macrophage), in the presence of 0.1 ml of rabbit anti-TGF-β antibody (R and D System; enough to neutralize 10^3 U of active TGF-β) or 0.1 ml of rabbit anti-mouse IL-10 polyclonal antibody (enough to neutralize 10^4 U of IL-10) (3) or anti-murine IL-6 antibody (Genzyme; enough to neutralize 10^3 U of IL-6). Antibodies did not affect phagocytosis of MAC organisms by macrophages or the viability of the monolayer (data not shown). Extracellular bacteria were removed after 4 h, and the medium and the antibodies were replenished. The infection was monitored for 4 days, with medium and antibodies being replenished daily. After this period monolayers were lysed and viable intracellular bacteria were quantitated as described above. Antibodies by themselves had no effect on cell viability (data not shown).

Statistical analysis. Each experiment was repeated three times. Values were obtained for triplicate wells, and means ± standard deviations (SD) were calculated. The significance of the experimental results versus those for controls at identical time points was tested by Student's *t* test.

RESULTS

Evaluation of intestinal macrophages. Two major questions regarding working with intestinal macrophages existed. The first concerned the degree of activation of intestinal macrophages after being submitted to the purification process. The second question related to the presence of endotoxin in the preparations. To address these questions, we evaluated superoxide anion production by intestinal, peritoneal, and splenic macrophages at 2, 24, and 48 h after purification. Table 1 shows that following the purification process, intestinal, peritoneal, and splenic macrophages responded similarly to stimulation with phorbol ester. By 24 h all three populations of cells failed to show signs of activation.

Regarding the presence of endotoxin in the intestinal macrophage preparations, we examined all preparations of intestinal as well as splenic and peritoneal macrophages by using the Limulus Amebocyte Assay (Sigma). Only preparations of macrophages with undetectable levels of endotoxin were used in the assays (55% of the preparations). Because of the limitations of the assays used, some of the assays were run with polymyxin B (Sigma) as a control. No difference was observed

TABLE 2. Effects of recombinant cytokines on MAC-infected intestinal macrophages

Treatment (time of measurement)[a]	Concentration (U/ml)	No. of organisms (mean ± SD)	
		MAC 101	MAC 86-2686
None (time zero)		$(2.1 \pm 0.3) \times 10^5$	$(1.1 \pm 0.4) \times 10^5$
None (24 h)		$(5.4 \pm 0.4) \times 10^5$	$(1.3 \pm 0.3) \times 10^5$
TNF-α	10	$(3.1 \pm 0.3) \times 10^5$	$(8.3 \pm 0.3) \times 10^4$
	10^2	$(9.4 \pm 0.2) \times 10^{4b}$	$(6.1 \pm 0.2) \times 10^{4b}$
	10^3	$(4.0 \pm 0.3) \times 10^{4b}$	$(3.1 \pm 0.3) \times 10^{4b}$
GM-CSF	10	$(2.6 \pm 0.4) \times 10^{5b}$	$(7.1 \pm 0.3) \times 10^4$
	10^2	$(8.5 \pm 0.4) \times 10^{4b}$	$(5.6 \pm 0.4) \times 10^{4b}$
	10^3	$(4.1 \pm 0.3) \times 10^{4b}$	$(3.3 \pm 0.5) \times 10^{4b}$
M-CSF	10	$(5.3 \pm 0.3) \times 10^5$	$(1.7 \pm 0.4) \times 10^5$
	10^2	$(5.0 \pm 0.4) \times 10^5$	$(1.6 \pm 0.3) \times 10^5$
	10^3	$(4.5 \pm 0.3) \times 10^5$	$(1.0 \pm 0.4) \times 10^5$
IFN-γ	10	$(5.4 \pm 0.4) \times 10^5$	$(1.0 \pm 0.3) \times 10^{5b}$
	10^2	$(2.1 \pm 0.3) \times 10^{5b}$	$(9.3 \pm 0.4) \times 10^{4b}$
	10^3	$(9.0 \pm 0.4) \times 10^{4b}$	$(8.7 \pm 0.4) \times 10^{4b}$

[a] Cells were infected with MAC, and then 4 h later they were treated with recombinant cytokines for 24 h.
[b] $P < 0.05$ compared with the control at 24 h.

between assays with or without the presence of polymyxin B (data not shown).

Response to stimulation with recombinant cytokines. To determine whether intestinal macrophages could be stimulated to inhibit the growth of intracellular MAC, a pure population of murine intestinal macrophages was infected with MAC 101 or MAC 86-2686. At 4 h after infection, macrophage monolayers were washed to remove extracellular bacteria and control monolayers were lysed to establish the initial inoculum ($t = 0$). Monolayers were then treated with recombinant cytokines (TNF-α, 10 to 10^3 U/ml; IFN-γ, 10 to 10^3 U/ml; GM-CSF, 10 to 10^3 U/ml; and M-CSF, 10 to 10^3 U/ml) for both 24 h and 4 days. As shown in Table 1, in contrast to treatment of the time zero control, treatment of infected intestinal macrophages with either recombinant TNF-α or IFN-γ was associated with mycobacteriostatic and/or mycobactericidal activity as determined by the number of viable bacteria at 24 h ($P < 0.05$). Similarly, treatment of intestinal macrophages with GM-CSF resulted in inhibition of growth and killing of MAC 101 and 86-2686 ($P < 0.05$). In contrast, treatment with M-CSF resulted in a small, although not statistically significant, inhibition of MAC 86-2686 but had no effect on intracellular MAC 101 (Table 2). Similar results were observed when the period of exposure of infected monolayers to cytokines was extended to 4 days (Table 3).

Macrophage monolayers were also treated with TNF-α (10^3 U/ml), GM-CSF (10^3 U/ml), M-CSF (10^3 U/ml), or IFN-γ (10^3 U/ml) at 24 h prior to infection. Since stimulation of macrophages can influence phagocytosis, we determined the uptake of MAC 101 and MAC 86-2686 by macrophages at both 1 and 4 h after infection. Pretreatment with cytokines did not influence the uptake of MAC by macrophages. Thus, pretreatment with TNF-α resulted in phagocytosis of $5.3 \times 10^4 \pm 0.4 \times 10^4$ MAC 101 organisms and $4.9 \times 10^4 \pm 0.3 \times 10^4$ MAC 86-2686 organisms after 1 h and $1.7 \times 10^5 \pm 0.3 \times 10^5$ MAC 101 organisms and $1.4 \times 10^5 \pm 0.3 \times 10^5$ MAC 86-2686 organisms after 4 h compared with $5.1 \times 10^4 \pm 0.3 \times 10^4$ and $1.3 \times 10^5 \pm 0.4 \times 10^5$ control organisms after 1 and 4 h, respectively.

TABLE 3. Effects of recombinant cytokines on the ability of murine intestinal macrophages to inhibit intracellular growth of MAC after 4 days

Treatment (time of measurement)[a]	Concentration (U/ml)	No. of organisms (mean ± SD)	
		MAC 101	MAC 86-2686
None (time zero)		$(1.5 \pm 0.4) \times 10^5$	$(1.7 \pm 0.3) \times 10^5$
None (4 days)		$(3.8 \pm 0.3) \times 10^6$	$(4.4 \pm 0.3) \times 10^5$
TNF-α	10	$(9.1 \pm 0.4) \times 10^{5b}$	$(1.2 \pm 0.4) \times 10^5$
	10^2	$(5.2 \pm 0.5) \times 10^{5b}$	$(5.0 \pm 0.2) \times 10^{4b}$
	10^3	$(8.3 \pm 0.3) \times 10^{4b}$	$(11 \pm 0.3) \times 10^{4b}$
GM-CSF	10	$(8.0 \pm 0.5) \times 10^{5b}$	$(9.3 \pm 0.3) \times 10^4$
	10^2	$(4.1 \pm 0.3) \times 10^{5b}$	$(4.1 \pm 0.4) \times 10^{4b}$
	10^3	$(9.2 \pm 0.4) \times 10^{4b}$	$(1.7 \pm 0.3) \times 10^{4b}$
M-CSF	10	$(3.7 \pm 0.3) \times 10^6$	$(4.6 \pm 0.3) \times 10^5$
	10^2	$(3.0 \pm 0.4) \times 10^6$	$(3.9 \pm 0.2) \times 10^5$
	10^3	$(3.1 \pm 0.3) \times 10^6$	$(3.8 \pm 0.3) \times 10^5$
IFN-γ	10	$(2.9 \pm 0.4) \times 10^6$	$(2.3 \pm 0.5) \times 10^{5b}$
	10^2	$(8.4 \pm 0.3) \times 10^{5b}$	$(7.5 \pm 0.5) \times 10^{4b}$
	10^3	$(5.7 \pm 0.2) \times 10^{5b}$	$(2.0 \pm 0.4) \times 10^{4b}$

[a] Cells were infected with MAC, and then 4 h later they were treated with recombinant cytokines for 4 days (medium and cytokines were replenished daily).
[b] $P < 0.05$ compared with the untreated control at 4 days.

Pretreatment with GM-CSF was associated with uptake of $4.7 \times 10^4 \pm 0.3 \times 10^4$ MAC 101 organisms and $4.1 \times 10^4 \pm 0.3 \times 10^4$ MAC 86-2686 organisms after 1 h and $2.1 \times 10^5 \pm 0.3 \times 10^5$ MAC 101 organisms and $1.7 \times 10^5 \pm 0.4 \times 10^5$ MAC 86-2686 organisms after 4 h compared with $5.1 \times 10^4 \pm 0.3 \times 10^4$ and $1.3 \times 10^5 \pm 0.4 \times 10^5$ control organisms after 1 and 4 h, respectively.

Following treatment with IFN-γ, the uptake of MAC by macrophages was $5.1 \times 10^4 \pm 0.5 \times 10^4$ MAC 101 organisms and $6.1 \times 10^4 \pm 0.3 \times 10^4$ MAC 86-2686 organisms after 1 h and $2.7 \times 10^5 \pm 0.4 \times 10^5$ MAC 101 organisms and $1.8 \times 10^5 \pm 0.2 \times 10^5$ MAC 86-2686 organisms after 4 h compared with $5.1 \times 10^4 \pm 0.3 \times 10^4$ and $1.3 \times 10^5 \pm 0.4 \times 10^5$ control organisms after 1 and 4 h, respectively.

The inhibition and/or killing of MAC by macrophages treated with TNF-α, GM-CSF, and IFN-γ prior to the infection was significantly greater than the inhibition and/or killing of MAC by macrophages treated after infection ($P < 0.05$)

TABLE 4. Effects of pretreatment of murine intestinal macrophages with recombinant cytokines on the survival of intracellular MAC

Treatment (time of measurement)[a]	Concentration (U/ml)[b]	No. of organisms (mean ± SD)	
		MAC 101	MAC 86-2686
None (time zero)		$(2.1 \pm 0.3) \times 10^5$	$(1.6 \pm 0.3) \times 10^5$
None (4 days)		$(3.9 \pm 0.4) \times 10^6$	$(5.1 \pm 0.3) \times 10^5$
TNF-α	10^3	$(4.7 \pm 0.4) \times 10^{4c}$	$(2.5 \pm 0.5) \times 10^{4c}$
GM-CSF	10^3	$(6.3 \pm 0.3) \times 10^{4c}$	$(5.2 \pm 0.5) \times 10^{4c}$
M-CSF	10^3	$(3.3 \pm 0.3) \times 10^6$	$(3.9 \pm 0.4) \times 10^5$
IFN-γ	10^3	$(3.1 \pm 0.2) \times 10^{5c}$	$(6.7 \pm 0.5) \times 10^{4c}$

[a] Macrophage monolayers were incubated with recombinant cytokines for 24 h prior to infection.
[b] Cytokines were added daily for 4 days.
[c] $P < 0.05$ compared with the untreated control at 4 days.

TABLE 5. Production of TNF-α by murine intestinal, splenic, and peritoneal macrophages submitted to stimuli

Stimulus	Concn of TNF-α (pg/ml) in 24 h (mean ± SD)[a]		
	Intestinal	Peritoneal	Splenic
No treatment	Undetectable	Undetectable	Undetectable
LPS (0.5 μg/ml)	178 ± 12	246 ± 18[b]	296 ± 15[b]
MAC 86-2486[c]	129 ± 8	164 ± 10[b]	192 ± 6[b]
MAC 101	46 ± 9	98 ± 7[b]	207 ± 6[b]
MAC 109	37 ± 6	42 ± 6	131 ± 11[b]

[a] Concentration in the supernatant. Production of the cytokine was adjusted for 10^5 cells.
[b] $P < 0.05$ relative to intestinal macrophages.
[c] Infection rate, 10^6 bacteria per 10^5 cells.

(Table 4). Heat-inactivated cytokines had no effect on *M. avium* growth (data not shown).

Production of cytokines. To investigate further their function, we examined the ability of intestinal macrophages to synthesize TNF-α and IL-6 and compared the results with those for peritoneal and splenic macrophages. Macrophage monolayers were infected with strain 101 of MAC (10^6 bacteria per ml) or were treated with LPS (0.5 μg/ml). After 24 h, cultured supernatant was obtained and the concentrations of TNF-α and IL-6 were determined by ELISA.

As shown in Table 5, both infection with MAC and treatment with LPS resulted in greater production of TNF-α by splenic and peritoneal macrophages than by intestinal macrophages. In contrast, production of IL-6 by intestinal macrophages following both infection with MAC and treatment with LPS was similar to the production of IL-6 by peritoneal and splenic macrophages submitted to the same stimuli (Table 6; $P > 0.05$ for all the comparisons between intestinal macrophages and splenic or peritoneal macrophages).

We then determined if exposure of intestinal, splenic, and peritoneal macrophages in vitro to MAC 101, 109, or 86-2486 was associated with production of IL-10 and TGF-β. As shown in Tables 7 and 8, MAC 101 and 109, but not 86-2486, induced production of IL-10 by intestinal, peritoneal, and splenic macrophages, and only MAC 101 and 109, but not 86-2486, were capable of inducing detectable levels of TGF-β production by the three populations of macrophages. Furthermore, TGF-β production was significantly smaller after MAC infection of intestinal macrophages than after infection of splenic and peritoneal macrophages.

Treatment with antibodies. To determine whether autocrine production of IL-10, TGF-β, and IL-6 had an important influence on the ability of MAC to survive within mucosal intestinal macrophages, gut macrophage monolayers (10^5 cells) were infected with MAC 101 (10^7 bacteria) in the presence of neutralizing antibodies for TGF-β, IL-10, or IL-6. As shown in Fig. 1, infection of intestinal macrophages in the presence of anti-IL-10 antibody and/or anti-TGF-β was associated with a significantly greater ability of macrophages to control intracellular infection ($P < 0.05$ for anti-TGF-β, anti-IL-10, or a combination of both antibodies compared with the control group). In contrast, anti-IL-6 antibody had no effect on the ability of MAC to survive intracellularly. Neither anti-IL-10 antibody, anti-TGF-β antibody, nor anti-IL-6 antibody had any effect on the phagocytosis of MAC strains by intestinal macrophages (data not shown).

TABLE 6. Production of IL-6 by murine intestinal, splenic, and peritoneal macrophages submitted to stimuli

Stimulus	Concn of IL-6 (pg/ml) in 24 h (mean ± SD)[a]		
	Intestinal	Peritoneal	Splenic
No treatment	Undetectable	Undetectable	Undetectable
LPS (0.5 μg/ml)	410 ± 34	389 ± 30	410 ± 26
MAC 86-2486[b]	126 ± 21	126 ± 27	142 ± 9
MAC 101	164 ± 14	136 ± 14	163 ± 12
MAC 109	196 ± 15	171 ± 16	184 ± 11

[a] Concentration in the supernatant. Production of the cytokine was adjusted for 10^5 cells.
[b] Infection rate, 10^6 bacteria per 10^5 cells.

TABLE 7. Production of IL-10 by murine intestinal, splenic, and peritoneal macrophages after infection with MAC

Infection[a]	Concn of IL-10 (pg/ml) in 24 h[b]		
	Intestinal	Peritoneal	Splenic
None	Undetectable	Undetectable	Undetectable
MAC 101	125 ± 16	96 ± 14	147 ± 32
MAC 109	115 ± 20	89 ± 21	116 ± 18
MAC 86-2486	<10	<10	<10

[a] Infection with 10^6 bacteria per 10^5 cells for 24 h.
[b] Concentration in the supernatant. Production of the cytokine was adjusted for 10^5 cells. Results are means ± SD for three different experiments.

DISCUSSION

Organisms of MAC are associated with bacteremia and disseminated infection in patients with AIDS (15, 16, 29). In the majority of AIDS patients, MAC infection appears to follow colonization of the gastrointestinal tract (16, 18). In contrast, in non-AIDS populations, MAC infection usually is associated with colonization and infection of the respiratory tract (29).

Recent epidemiologic data have suggested that colonization of the intestinal tract by MAC precedes invasion of the mucosa and submucosa and, therefore, that immune-competent cells present in the intestinal mucosa and submucosa might constitute a first line of defense against MAC. The immune reactivity of circulating lymphocytes or monocytes may not reflect events mediated by cells in the tissues, more specifically in the intestinal tract; nonetheless, human peripheral blood and murine peritoneal macrophages have been used almost exclusively as a source of cells for immunologic studies of MAC infection.

In an attempt to elucidate the nature of the local gastrointestinal immune response to MAC, we examined the ability of intestinal macrophages to perform a number of functions that would be required following exposure to a pathogen, e.g., pro-

TABLE 8. Production of TGF-β by intestinal, splenic, and peritoneal macrophages infected with MAC

Infection[a]	Concn of TGF-β (pg/ml) in 24 h[b]		
	Intestinal	Peritoneal	Splenic
None	Undetectable	Undetectable	Undetectable
MAC 101	96 ± 12[c]	246 ± 21	152 ± 36
MAC 109	73 ± 18[c]	204 ± 19	209 ± 25
MAC 86-2486	Undetectable	66 ± 8	69 ± 16

[a] Infection with 10^6 bacteria per 10^5 cells for 24 h.
[b] Concentration in the supernatant. Production of the cytokine was adjusted for 10^5 cells. Results are means ± SD for three different experiments.
[c] $P < 0.05$ for the comparison with TGF-β production by peritoneal and splenic macrophages.

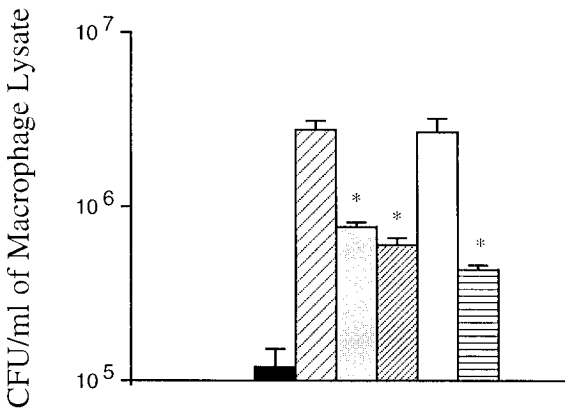

FIG. 1. Effects of anti-TGF-β, anti-IL-10, and anti-IL-6 antibodies on the ability of MAC to survive within macrophages. Antibodies were added before infection at a concentration sufficient to neutralize 10^3 U of TGF-β per ml and 10^4 U of IL-10. *, $P < 0.05$ compared with the control at day 4. ■, time zero; ▨, control at 4 days; ▩, anti-TGF-β; ▨, anti-IL-10; □, anti-IL-6; ▤, anti-TGF-β and anti-IL-10.

duction of cytokines as well as response to stimulation with recombinant cytokines.

Stimulation of intestinal macrophages with recombinant murine IFN-γ, GM-CSF, and TNF-α, but not M-CSF (used at up to 10^3 U/ml), resulted in mycobacteriostatic activity. Previous studies in our laboratory have shown that M-CSF is effective in priming human monocyte-derived macrophages (at a concentration of 10^2 U/ml) to subsequent stimulation with TNF-α in vitro, but further studies could not demonstrate the same ability in vivo by using the beige mouse model of MAC infection (data not shown).

IFN-γ has been associated with the induction of anti-MAC activity in macrophages in vitro (6, 25). However, this activity appears to be strain dependent (6, 7, 14, 25, 26). Previous work in our laboratory showed that the intracellular growth of strain 101 of MAC could not be inhibited by human monocyte-derived macrophages stimulated with IFN-γ, while the present work, using murine intestinal macrophages, showed inhibition of growth of the same strain after 24 h as well as 4 days of exposure of macrophage monolayers to IFN-γ. These findings can reflect the abilities of different populations of macrophages and different systems (human versus mouse macrophages as well as blood monocyte-derived macrophages versus intestinal macrophages) to respond to stimulation with IFN-γ. In support of our findings, a recent study by Appelberg and colleagues (1) suggests a role for IFN-γ in the host defense against MAC in three different mouse strains. As a potential explanation, human macrophage infection with disease-causing strains of MAC induced a rapid release of TGF-β, which has been associated with unresponsiveness of macrophages to IFN-γ (2), whereas intestinal macrophages, when infected with MAC strains, produced significantly less TGF-β than peritoneal and splenic macrophages. In contrast to TGF-β production, production of IL-10 following MAC infection was at similar levels for intestinal, peritoneal, and splenic macrophages. IL-10 suppresses macrophage function (17), in particular the production of cytokines TNF-α and GM-CSF, and it has been shown to impair the host immune defense against MAC (3, 13). Although the production of IL-10 could explain the impaired synthesis of TNF-α by infected intestinal macrophages, this does not seem to be the case because intestinal macrophages also produced smaller amounts of TNF-α compared with splenic and peritoneal macrophages, when stimulated with LPS.

Infection of gut macrophages in the presence of antibodies to TGF-β or IL-10 resulted in decreased survival of intracellular bacteria compared with that observed for the untreated control, suggesting that the release of TGF-β and IL-10 by infected macrophages might have a role in the mechanisms of mycobacterial survival. TGF-β is a macrophage suppressor factor (27, 28) that was demonstrated to be released by human macrophages infected with MAC (2, 12). In fact, studies in our laboratory using purified proteins from MAC have shown that a protein of 33 kDa is the major mycobacterial antigen responsible for the induction of TGF-β (2). Therefore, there is sufficient evidence to suggest that TGF-β has a role in the ability of MAC to survive within macrophages, and the present study has extended the previous observation to intestinal mucosal macrophages.

IL-10 is a key cytokine that is secreted primarily by mononuclear phagocytes and also by the TH_2 subset of T lymphocytes (17). IL-10 causes macrophage deactivation (17) and blocks IFN-γ release by the TH_1 subset of T lymphocytes (17). The role of IL-10 in infectious diseases is not well known, but recently we, as well as Denis and Ghadirian, demonstrated that neutralization of IL-10 in mice infected with MAC resulted in increased resistance to the systemic MAC infection (3, 13). In the present study, we showed that infection of intestinal macrophages with a disease-associated MAC strain led to the release of a large amount of the cytokine in contrast to infection with MAC 86-2686, suggesting that stimulation of secretion of IL-10 may be related to the pathogenesis of MAC disease.

In contrast to peritoneal and splenic macrophages, intestinal macrophages secreted a decreased amount of TNF-α when infected with MAC. It seems plausible to hypothesize that because mucosal intestinal macrophages are constantly exposed to endotoxin in the intestinal tract, over the years they have developed a mechanism of tolerance by producing decreased amounts of TNF-α when exposed to endotoxin. Taking into account the previous observations of a number of researchers establishing a role for TNF-α in the host response against mycobacteria (1, 5, 6, 11), it is possible that the blood monocytes that are attracted to the site of intestinal infection would have an important role as TNF-α producers. Conversely, the ability of intestinal macrophages to secrete IL-6 did not differ from the ability of peritoneal and splenic macrophages to do so when infected with MAC.

Noteworthy is the amount of TNF-α secreted by macrophages when infected with MAC. The observation that MAC 86-2486 (*Mycobacterium intracellulare*), a less virulent strain of MAC (16), induced significantly greater synthesis and secretion of TNF-α than MAC 101 and MAC 109 (both *M. avium*) might be related to the ability of these latter strains to cause infection. TNF-α has been shown to be important in the host's immune defense against MAC (6, 11), and variation in the abilities of several populations of macrophages to secrete TNF-α in response to infection with different strains of MAC has been recently correlated with virulence (26).

In summary, our results suggest that intestinal macrophages as the first line of defense against MAC can secrete as well as be stimulated with recombinant cytokines to inhibit the growth of intracellular MAC. Further studies are needed to determine whether infection with human immunodeficiency virus type 1 or with other opportunistic organisms can impair the ability of intestinal macrophages to suppress the growth of intracellular MAC.

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