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Infection with *Mycobacterium avium* Induces Production of Interleukin-10 (IL-10), and Administration of Anti-IL-10 Antibody Is Associated with Enhanced Resistance to Infection in Mice

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Organisms of the *Mycobacterium avium* complex are associated with disseminated infection in patients with AIDS. The mechanisms that account for the survival of the intracellular bacteria are unknown. We document here that infection of C57BL/6 black mice with *M. avium* 101 triggered interleukin-10 (IL-10) production. The synthesis of IL-10 peaked after 2 weeks of infection and remained elevated throughout the period of infection. Treatment of *M. avium*-infected peritoneal macrophages with recombinant IL-10 suppressed the stimulatory effect of tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor. To confirm the possible role of IL-10 in the infection in vivo, mice were infected with *M. avium* 101 and simultaneously received treatment with neutralizing anti-IL-10 antibody. After 4 weeks the animals were harvested and the numbers of viable bacteria were quantitated in the liver, spleen, and blood. The liver and spleen of animals receiving anti-IL-10 antibody had 2 to 3 log units fewer bacteria than did those of control animals. These results suggest a role for IL-10 in the pathogenesis of *M. avium* infection.

Organisms of the *Mycobacterium avium* complex are the main cause of bacterial infection in patients with AIDS (13, 18). *M. avium* is a facultative intracellular organism which invades and multiplies within macrophages (12).

Infection with disease-associated strains of *M. avium* is associated with suppression of the synthesis of tumor necrosis factor alpha (TNF) by macrophages and impairment of the response of infected macrophages to stimulation with recombinant TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1a, 7), two cytokines which have been shown to inhibit the intracellular growth of *M. avium* (5, 6). Suppression is observed 3 to 4 days after the infection in vitro and has also been observed in vivo.

A complex combination of factors, including cytokines, appears to be involved in the process of suppression of the host defense associated with *M. avium* infection. Natural killer cells and macrophages-monocytes have been shown to secrete interleukin-6 (IL-6) when exposed to *M. avium* in vitro (9). Secretion of IL-6 has been shown to down-regulate TNF receptors in macrophages, which can lead to an impaired response to stimulation (4). Others have shown that IL-6 may be a growth factor for some strains of *M. avium* (23). In addition, recent observation suggests that transforming growth factor beta (TGF-β_1) is secreted in large quantities by *M. avium*-infected macrophages (1), with consequent inhibitory activity toward the macrophage.

IL-10 was initially described as cytokine synthesis inhibitory factor, an activity produced by murine type 2 helper T cells which suppress cytokine production by type 1 helper T cells (14, 19). IL-10 inhibits the secretion of IL-1, IL-3, TNF-α, GM-CSF, and gamma interferon (IFN-γ) by THP-1 cells and macrophages. In addition, IL-10 has inhibitory activity over many of the macrophage functions. For example, it inhibits monocyte class II expression and intracellular and extracellular killing of parasites (21, 24). Recent work by Bogdan et al. (10) showed that a number of bactericidal activities such as superoxide anion production and H_2O_2 production in macrophages are inhibited by the presence of IL-10. We have performed the present studies to determine whether IL-10 is secreted during *M. avium* infection in mice and to evaluate its possible role in the regulation of infection.

M. avium 101 (serovar 1) was isolated from the blood of a patient with AIDS. Mycobacteria were cultured in Middlebrook agar 7H10 medium (Difco Laboratories, Detroit, Mich.) for 10 days at 37°C. Transparent colonies were resuspended in Hanks' balanced salt solution and washed twice, and the suspension was adjusted to 3×10^9 bacteria per ml by using a McFarland standard. A sample obtained from the bacterial suspension was plated for confirmation of the number of bacteria. Before being used to infect animals, the final suspension was vortex agitated for 2 min to prevent clumping. Strain 101 is the most highly virulent strain for mice of all the strains we have tested in animal studies, and it is associated with a reproducible level of infection in mice (8).

Experiments were performed with 6- to 7-week-old female mice (C57BL/6 bg^-/bg^+ mice; Jackson Laboratory, Bar Harbor, Maine). This strain of mouse has been used previously as a model of chronic disseminated *M. avium* infection (3). Infection persists in this model for up to 6 months.

C57BL/6 mice were anesthetized by chloroform inhalation and given 0.2 ml containing 3×10^8 organisms by gavage in five doses on alternate days (for a total of 10 days). After 4

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weeks the numbers of viable bacteria in the liver, spleen, and appendix were quantitated (2). To measure the concentration of IL-10 produced by splenic cells, we monitored the animals for 5 weeks (20).

Recombinant murine IL-10 was kindly provided by DNAX, Palo Alto, Calif. It had a specific activity of 5×10^6 U/mg of protein as measured by the provider. Recombinant murine TNF-α was a gift from Genentech, South San Francisco, Calif., and had a specific activity of 2×10^7 U/mg of protein. Recombinant murine GM-CSF was kindly provided by Immunex Corp., Seattle, Wash., and had a specific activity of 2×10^7 U/mg of protein.

Anti-IL-10 antibody production was induced in rabbits by subcutaneous injection of 10^2 U of recombinant IL-10 for 3 consecutive weeks. The animals were then bled, and the sera were tested for specificity and neutralizing capacity. The antibody (immunoglobulin G) was partially purified by ammonium sulfate precipitation. The antibody tested was specific for IL-10 and did not react with IL-1, IL-2, IL-3, IL-4, IL-6, TGF-β, TNF, GM-CSF, or IFN-γ (by an immunodot assay with 10, 10^2, 10^3, and 10^4 U/ml). A 0.1-ml volume of antibody neutralized 1,000 U of IL-10.

Anti-IL-10 antibody (5 mg) was administered intraperitoneally on days 0, 5, 10, 15, and 20 after infection. The animals were monitored for 4 weeks and then harvested.

The livers and spleens of mice were removed aseptically at the termination of the experiment. Organs were weighed, and Middlebrook medium was added to yield a 10% (wt/vol) suspension after homogenization of the organs with a handheld glass homogenizer. Serial 10-fold dilutions were plated in duplicate on Middlebrook 7H11 agar supplemented with oleic acid, albumin, glucose, and catalase (Difco). After incubation for 10 to 14 days at 37°C under 15% CO_2, the colonies were counted and the number of CFU per gram of tissue was calculated.

Blood was collected at the end of the experiment; 100 ml was drawn from the tail vein and was inoculated into 4 ml of BACTEC 12B radiometric medium (Johnston Laboratories, Towson, Md.). The number of bacteria present in the blood was determined by using the T100 method as previously described (10).

Spleen cells were obtained from normal controls and infected mice as previously reported (3). Briefly, spleens were obtained weekly and minced into small fragments with sterile scissors. These fragments were subsequently reduced to a fine cell suspension with the use of a sterile needle. The suspension was passed through a 19-gauge needle and gently centrifuged. Cells were adjusted to 2×10^5 cells and plated in 24-well plates (Costar, Cambridge, Mass.).

IL-10 was measured by using the IFN-γ inhibition assay (24). Spleen cells from normal controls and infected animals were aseptically removed at different time points after infection, washed, and adjusted to 10^6 cells ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (endotoxin tested; Sigma). The cells were incubated in 24-well plates (Costar). Supernatants from triplicate cultures were tested for IL-10 activity after depletion of IFN-γ with antibody covalently bound to Affi-Gel (Bio-Rad Laboratories). Verification of IFN-γ depletion was determined by using an enzyme-linked immunosorbent assay (ELISA) for mouse IFN-γ (Genzyme, Cambridge, Mass.).

The IL-10 assay was carried out with the BD7.7 cell line, maintained by stimulation with irradiated allogeneic mouse spleen cells and IL-2. Resting cells (7 days poststimulation) were added to dilutions of the supernatants at 10^5 cells per well with irradiated allogeneic stimulator cells (10^6 per well) in 1 ml of medium. After 24 h of incubation, supernatants were collected and filtered in a 22-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) for determination of IFN-γ by ELISA. In some wells, anti-IL-10 was used to ensure the specificity of the assay.

Peritoneal macrophages were obtained from the peritoneal cavities of healthy mice as previously described (3). The macrophages were washed, and the number of cells was adjusted to 10^6/ml. Then 1 ml of peritoneal cells was plated per well in a 24-well culture plate (Costar) and allowed to adhere for 2 h at 37°C under 5% CO_2 in the presence of RPMI 1640 supplemented with 10% heat-inactivated, endotoxin-tested fetal bovine serum. About 50% of the macrophages detached from the monolayer during the experiment. After 2 h of incubation, the monolayers were washed with warmed (37°C) Hanks' balanced salt solution and infected with *M. avium* at 10 bacteria per cell. After an infection period of 4 h, the extracellular bacteria were removed by three washes.

The inhibition of growth of intracellular bacteria was concluded when the number of bacteria in stimulated monolayers was smaller than in control monolayers at the same time point after infection, comparing monolayers with a similar number of intracellular bacteria at time zero after infection. Killing was considered to have occurred when the number of bacteria at day 4 was smaller than the number at time zero. The number of bacteria per monolayer was counted 4 h after infection (implantation inoculum) and 4 days after infection. To lyse macrophages, 0.5 ml of cold (4°C) sterile water was added to each well and incubated for 10 min at room temperature. Then 0.5 ml of another lysing solution containing 1.1 ml of 7H9 medium and 0.4 ml of 0.25% sodium dodecyl sulfate (SDS) in phosphate buffer was added to each well for another 10 min. The wells were vigorously scraped with a rubber policeman, and the macrophage lysates were resuspended in 0.5 ml of 20% bovine serum albumin in sterile water to neutralize the SDS effect. The suspension was then vortex agitated for 1 min for complete lysis of macrophages. The macrophage lysate suspension was sonicated for 5 s (power output, 2.5 W/s) to disperse the bacterial clumps and permit reliable pour plate quantitation. As a control for osmotic stability, mycobacteria without macrophages were subjected to the same procedure and quantitative colony counts were determined by pour plate methods. The bacteria were 100% viable. To ensure that macrophages were totally disrupted, samples were examined by Giemsa staining.

The macrophage lysate suspension was plated onto 7H10 agar. The plates were allowed to dry at room temperature for 15 min and were then incubated at 37°C under 5% CO_2 and moist air for 2 weeks. The results are reported as mean CFU per milliliter of macrophage lysate suspension, obtained after 14 days in culture. Duplicate plates were prepared for each well.

The results were analyzed by comparison at the same time point by Student's *t* test.

To quantitate IL-10 protein, spleen cells from mice were cultured during the course of infection and stimulated ex vivo with concanavalin A (5 μg/ml) for 24 h (five animals for each time point). As shown in Table 1, IL-10 was not detected on the day of infection but its concentration was elevated (103 ± 47 ng) 1 week following infection. The concentration peaked 2 weeks following infection and remained elevated for the last 4 weeks of the experiment. A neutralizing antibody was used to verify that the cytokine

TABLE 1. Production of IL-10 by spleen cells of infected mice

Time (days) after infection	IL-10 concn[a] (pg/ml) in:	
	Control mice	Infected mice
0	ND[b]	ND
7	ND	103 ± 47
14	ND	634 ± 148
21	15 ± 7	563 ± 112
28	21 ± 11	674 ± 162
35	11 ± 6	498 ± 116

[a] Mean and standard deviation. The concentration was measured as IFN-γ-inhibiting activity. Sensitivity of the assay, 10 pg of IL-10 per ml.
[b] ND, not detected.

synthesis inhibitory factor produced by splenic cells was IL-10. The cytokine synthesis inhibitory factor activity was determined by using 10 U and a 1/20 dilution of spleen cell supernatant from mice infected with *M. avium* for 21 days. The IFN-γ-inhibiting activity was neutralized by anti-IL-10 antibody.

To determine whether IL-10 has any influence in the survival of *M. avium* within macrophages, we infected murine peritoneal macrophages with *M. avium* 101 (serovar 1). The macrophage monolayers were then treated with IL-10 (10^2 U/ml) alone or IL-10 in combination with TNF (10^3 or 10^4 U/ml) or GM-CSF (10^2 or 10^3 U/ml), concentrations known to induce mycobacteriostatic or mycobactericidal activity in macrophages (5, 7). The concentration of IL-10 was chosen on the basis of previous data, which showed that 10^2 U/ml has maximal effect in this assay (data not shown).

As shown in Fig. 1, treatment with IL-10 either simultaneously with or 4 h prior to TNF treatment significantly inhibited the TNF-mediated mycobacteriostatic and mycobactericidal activity in macrophages ($P < 0.05$). Likewise, treatment with IL-10 in combination with GM-CSF was also associated with significant inhibition of the effect of GM-CSF on infected macrophages ($P < 0.05$) (Fig. 2). If IL-10 was added to infected monolayers after 24 h of stimulation with TNF or GM-CSF, its inhibitory effect on the TNF- and GM-CSF-mediated mycobacteriostatic and mycobactericidal activity was only partial (it inhibited 20% ± 4% of the TNF-mediated mycobacteriostatic activity and 24% ± 10% of the GM-CSF-mediated mycobacteriostatic activity).

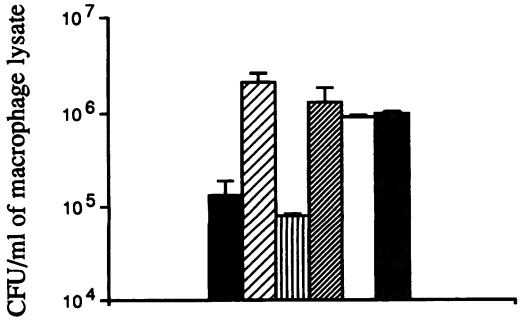

FIG. 1. Effect of recombinant murine IL-10 on TNF-mediated mycobacteriostatic and mycobactericidal activity in macrophages. IL-10 (10 U/ml) was added to the culture simultaneously with or 4 h before TNF (10^3 U/ml). Symbols: ■, control day 0; ▨, control day 4; ▥, TNF; ▨, IL-10; □, IL-10 and TNF added simultaneously; ▩, IL-10 added 4 h before TNF.

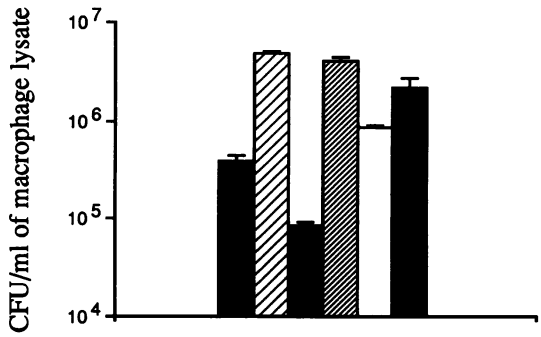

FIG. 2. Effect of recombinant murine IL-10 on GM-CSF-mediated mycobacteriostatic and mycobactericidal activity in macrophages. IL-10 (10 U/ml) was added to the culture simultaneously with or 4 h before GM-CSF (10^2 U/ml). Symbols: ■, control day 0; ▨, control day 4; ▩, GM-CSF; ▨, IL-10; □, IL-10 and GM-CSF added simultaneously; ▩, IL-10 added 4 h before GM-CSF.

To determine whether IL-10 had any influence in the host response against *M. avium* in vivo, we infected mice by the intestinal route, a model that resembles *M. avium* disease in patients with AIDS, in the presence of anti-IL-10 antibody.

Mice were infected orally on days 1, 3, 5, 7, and 9, and anti-IL-10 antibody was administered intraperitoneally on days 0, 5, 10, 15, and 20. The mice were monitored for 4 weeks and harvested. Thirty mice divided in two groups of 15 were used in the anti-IL-10 antibody and the control experiments. Six animals received a nonrelevant antibody (rabbit immunoglobulin G). One mouse in the control group developed bacteremia, whereas no bacteremia was detected in the anti-IL-10 antibody group and in the nonspecific immunoglobulin G group ($P > 0.05$). Treatment with anti-IL-10 antibody was associated with significantly fewer bacteria in the liver and the spleen compared with the controls. Therefore, although control mice had $1.2 \times 10^2 \pm 0.8 \times 10^2$ viable bacteria in the liver, mice treated with anti-IL-10 antibody had $4.0 \times 10^4 \pm 0.6 \times 10^4$ viable bacteria ($P < 0.01$) and mice treated with nonrelevant antibody had $2.4 \times 10^7 \pm 0.6 \times 10^7$ viable bacteria.

Treatment with anti-IL-10 antibody was associated with a significant reduction in the number of viable bacteria in the spleen from $3.2 \times 10^7 \pm 0.7 \times 10^7$ (control) to $1.0 \times 10^5 \pm 0.6 \times 10^5$ (anti-IL-10 antibody) ($P < 0.01$). Mice receiving nonrelevant antibody showed no difference from control mice ($3.6 \times 10^7 \pm 0.6 \times 10^7$ bacteria). Nonrelevant immunoglobulin G had no effect on the level of bacteremia.

Murine IL-10 cytokine synthesis inhibitory factor is produced by the Th2 cells and macrophages and inhibits the synthesis of cytokines (particularly IFN-γ) by type 1 helper T cells and macrophages (15, 22).

Little is known about the role of IL-10 in the complex mechanism of host defense. Recent work by Silva et al. (24) demonstrated that IL-10 is secreted during infection with *Trypanosoma cruzi* and antagonizes the effect of IFN-γ on macrophages. Likewise, Heinzel et al. showed the synthesis of IL-10 mRNA during murine infections with leishmania (17). We report here that infection with *M. avium* is associated with synthesis and secretion of IL-10 in vivo and that treatment with recombinant IL-10 impairs murine macrophage function, as evidenced by the lack of response to stimulation with recombinants TNF and GM-CSF. Furthermore, the use of neutralizing antibody against

IL-10 significantly enhanced resistance to infection with *M. avium*.

Infection of monocytes and macrophages with *M. avium* is associated with the production of GM-CSF, IL-6, TGF-β, IL-1, and IL-10 (1, 4, 9). Recent studies showed that IL-6, TGF-β, and IL-10 can suppress macrophage and type 1 helper T-cell function (15, 22). All three molecules are capable of blocking the effects of stimulatory cytokines, such as TNF-α, and GM-CSF, on macrophages and therefore have significant roles in pathogenesis of the infection. Data obtained from experiments with animals and with human cells in vitro suggest that prolonged infection with *M. avium* is associated with inhibition of the synthesis of TNF-α by macrophages (6, 16), which could be due to the production of IL-10 by type 2 helper T cells and macrophages.

IFN-γ is an effective inhibitor of the in vitro replication of several bacteria and parasites in macrophages. It is produced in the course of *M. avium* infection in mice but is not effective in inducing mycobactericidal or mycobacteriostatic activity in macrophages in vitro. Recent observations demonstrated that the inability of *M. avium*-infected human macrophages to respond to stimulation with recombinant IFN-γ is dependent in part on the secretion of TGF-β (1). In addition, the antagonistic effect of IL-10 can play an important role in the kinetics of cytokine response following infection with *M. avium*.

Evidence is accumulating that to survive within macrophages, *M. avium* uses a number of overlapping mechanisms which depend on the ability of the organism to control the expression of growth factors or cytokines that mediate cellular immune response. Antigens present in the bacterial cell wall, such as lipoarabinomannan, glycopepidolipid, and proteins, have been shown to be capable of triggering cytokine production by mammalian cells (11, 25). When subjected to determined stress conditions such as phagocytosis and acidic pH, *M. avium* secretes proteins that are associated with the synthesis of TGF-β (7) and can have a role in the induction of IL-10. Preliminary studies with purified proteins from *M. avium* have suggested this property, indicating that the induction of "suppressive" cytokines can be advantageous to the bacterium. Further studies with experimental models should be valuable in analyzing the immunologic activities of IL-10 as well as its functional significance in the host response to *M. avium* infection.

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