INFECTION AND IMMUNITY, Feb. 1991, p. 570–574
0019-9567/91/020570-05$02.00/0
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Vol. 59, No. 2

Immunomodulation of Mouse Macrophage Killing of *Mycobacterium avium* In Vitro

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Received 29 August 1990/Accepted 14 November 1990

When C57BL/6 mice were infected intravenously with *Mycobacterium avium*, bacterial growth continued within the spleen until more than 10^8 CFU/g of tissue were attained. This contrasted with *Mycobacterium bovis* BCG infections where growth declined after 2 weeks. In vivo *M. avium*-infected splenic macrophages were harvested from chronically infected mice and cultured in vitro for 4 days at 37°C. The number of viable mycobacteria within the resulting macrophage monolayers decreased when cultured in the presence of autologous sensitized T cells and an exogenous source of interleukin-2 (recombinant interleukin-2; 50 U/ml) compared with untreated controls ($P < 0.05$). Incubation of the infected macrophages with autologous T cells and soluble *M. avium* antigens also significantly reduced the number of viable organisms. These results indicate that the mycobactericidal activity of *M. avium*-infected macrophages can be enhanced in a way that may have important therapeutic implications for patients infected with this opportunistic pathogen.

The *Mycobacterium avium* complex includes a number of opportunistic human pathogens which, until recently, were rarely associated with the development of systemic disease. This situation changed dramatically with the emergence of the AIDS epidemic in the United States (27). AIDS patients infected with *M. avium* die earlier than those infected with other opportunistic pathogens (15). Because of the drug resistance exhibited by most *M. avium* complex strains, alternative immunotherapeutic measures need to be developed for the treatment of these patients (6, 10).

Recent studies indicate that the bactericidal ability of *M. avium*-infected monocytes can be activated by treatment with interleukin-2 (IL-2) and tumor necrosis factor (TNF) (1, 2). Other investigators showed that IL-2 injections alone can augment host resistance to *Mycobacterium bovis* BCG and *Mycobacterium lepraemurium* infections (4, 16). Soluble mycobacterial antigens have been used to stimulate T cells from anergic lepromatous leprosy patients in vitro, restoring T-cell responsiveness to the *M. leprae* antigens and suggesting their possible role as immunostimulatory reagents (23).

In the present study, splenic macrophages were infected in vivo with *M. avium* to avoid technical problems associated with in vitro infection studies (5) which in earlier reports produced variable results (2, 3, 19, 24). The immunomodulatory effect of recombinant IL-2 (rIL-2), lipopolysaccharide (LPS), concanavalin A (ConA), or soluble *M. avium* antigens was examined with respect to their ability to reduce the number of viable *M. avium* present in splenic macrophages harvested from chronically infected mice. The addition of rIL-2 or soluble *M. avium* antigens brought about a significant reduction in the number of viable mycobacteria recovered from these stimulated cells 4 days later.

MATERIALS AND METHODS

Bacteria. *M. avium* (TMC 724) and *M. bovis* BCG Pasteur (TMC 1011) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y. They were grown in Proskauer and Beck medium supplemented with sodium pyruvate (0.5%), glucose (1%), and Tween 80 (0.05%), without albumin additive, and harvested in mid-logarithmic growth phase (at day 7) (7). The organisms were stored at −70°C in 1-ml ampoules. Before use, an ampoule of the bacteria was thawed rapidly, sonicated for 10 s to disperse any clumps of bacteria, and diluted to the desired concentration in sterile saline. Either 10^4 (low-dose inoculum) or 10^7 (high-dose inoculum) of *M. avium* or 10^7 BCG was given intravenously via a lateral tail vein. The inoculum size was determined beforehand by plating 10-fold serial saline dilutions of a number of frozen ampoules on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.). The bacterial colonies were counted after incubation of the plates in sealed plastic bags at 37°C for 21 days (8). In this way, a known number of viable organisms from frozen stock cultures could be used for inoculation. The inoculum dose was also checked by determining the number of viable organisms within infected target organs 1 day after infection (8).

CF antigens. Culture filtrate (CF) antigens were prepared from mid-logarithmic-growth-phase cultures in which the cells were removed by centrifugation at 10,000 rpm (16,270 × *g*) for 20 min at 4°C and the supernatant fluid sterile was filtered through a 0.45-μm-pore-size membrane. Proteins in the *M. avium* CF antigens were precipitated overnight with 80% ammonium sulfate at 4°C and exhaustively dialyzed against 0.05 M phosphate-buffered saline (PBS), pH 7.4, after which the protein content was determined by use of the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) against a bovine serum albumin standard. Samples were stored at −20°C until needed.

***M. avium* SS.** *M. avium* soluble sonic extracts (SS) were prepared from mycobacteria suspended in PBS and heated at 56°C for 30 min to kill the organisms before they were ultrasonically disrupted at 0°C. The *M. avium* SS supernatant was collected after ultracentrifugation at 100,000 × *g* for 2 h at 4°C and then filtered through a 0.45-μm-pore-size filter. The protein content was determined as described above, and portions were stored at −20°C until needed.

Mice. Eight-week-old male C57BL/6 mice were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, N.Y. They were maintained under barrier conditions and given sterile food and acidified water (pH 3) ad libitum. Drinking water was acidified to prevent environ-

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mental contamination of water bottles. To monitor the growth of the bacterial inoculum in mice, target organs were removed aseptically from groups of randomly selected animals at each harvest time point and the organs were homogenized separately in cold sterile saline before plating 10-fold dilutions on Middlebrook 7H11 agar (8).

Lymphokine assays. TNF and gamma interferon (IFN-γ) activity levels, in culture supernatants from unstimulated spleen cells harvested from infected mice, were assayed by use of the methods described by Havell (12, 13). Spleen cells (10^7/ml) were incubated for 16 h in complete medium at 37°C and 5% CO_2 in 24-well flat-bottomed plates (Corning Glass Works, Corning, N.Y.), after which the supernatants were collected. TNF activity (both TNF-α and -β) was determined by measuring the cytotoxic activity of culture supernatants on L929B cell monolayers pretreated with actinomycin D. Interferon neutralization of antiviral activity with anti-IFN-α, -IFN-β, and -IFN-γ antibody indicated that the IFN activity present in culture supernatants was due primarily to IFN-γ although some IFN-α and IFN-β activity was also present.

In vitro assays on infected spleen cells. Spleens from infected mice were removed aseptically, and the cells were gently dispersed by being pressed through a stainless-steel screen into RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 5×10^{-5} M 2-mercaptoethanol, without antibiotics (complete medium). Nucleated cells were counted and standardized to 10^7/ml. A 1-ml volume of the cell preparation was added to each well of 24-well culture dishes (Corning) and incubated in humidified air overnight. Nonadherent cells were then removed by gently rinsing the wells with RPMI 1640 medium. The percentage of macrophagelike cells (MP) in cultures prepared in this manner was evaluated by means of nonspecific esterase staining (alpha-naphthyl acetate esterase demonstration kit; Sigma Chemical Co., St. Louis, Mo.), by engulfment of opsonized sheep erythrocytes followed by hypotonic shock to remove the external erythrocytes, and finally for the presence of acid-fast-staining bacteria within the phagocytic cells. By these criteria, adherent cell cultures from 30-day *M. avium*-infected mice contained 90 to 96% MP.

Monolayers of adherent cells were prepared from spleens of mice infected with *M. avium* 30 days earlier, and sensitized splenic lymphocytes harvested from the same group of mice were added to some of the monolayers. The cultures were incubated for 4 days in complete medium in the presence of rIL-2 (final concentration, 50 U/ml; kindly provided by E. I. du Pont de Nemours & Co., Glenolden, Pa.). This concentration of rIL-2 was similar to that contained in lymphokine preparations used in preliminary experiments and in previous reports from this laboratory (24). Other cultures received 10 ng of LPS (*Escherichia coli*; Sigma) per ml as a macrophage stimulant (11), 1 μg of ConA per ml as a T-cell mitogen, or *M. avium* SS or CF antigen (1 μg of protein per ml). All MP assays were done in triplicate.

Adherent cells and viable intracellular mycobacteria were enumerated by use of methods described elsewhere (24). Briefly, the culture medium was removed and the monolayer was washed twice with PBS, followed by the addition of 250 μl of sterile naphthol blue black (Sigma) at 0.5 mg/ml in an aqueous solution of 0.1 M citric acid and 1% Triton X-100. This solution lyses adherent cells, releasing the intracellular mycobacteria and the MP nuclei. The number of MP per well was determined by counting, with a hemacytometer, the blue-stained kidney-shaped MP nuclei released by cell lysis. Viable bacteria were enumerated by transferring a 0.1-ml volume of the resulting MP lysate suspension into 0.9 ml of 0.2 M $NaPO_4$ buffer. This mixture was sonicated briefly (5 s) to disperse clumps of mycobacteria before plating 10-fold saline dilutions onto Middlebrook 7H11 agar. CFU were counted after the plates were incubated at 37°C for 21 days.

T-cell subset depletion studies. Depletion of T-cell subsets was accomplished in vitro by incubation of spleen cells with the monoclonal antibodies GK 1.5 (anti-L3T4) and/or TIB 210 (anti-Lyt-2), at a concentration of 500 μg/10^7 cells, on ice for 30 min, after which a 1:10 dilution of nontoxic rabbit complement (Accurate, Westbury, N.Y.) was added and the mixture was incubated for 45 min at 37°C. The cells were washed, resuspended in fresh medium, and added to infected adherent cells. The effectiveness of the depletion step (>90% depletion) was substantiated by means of fluorescent-antibody staining with fluorescein isothiocyanate-labeled anti-L3T4 or Lyt-2 antibody (14).

^{51}Cr-release assay. An assay was performed to monitor the release of ^{51}Cr from labeled MP due to lysis by cytotoxic cells by using previously described methods (17, 26). Briefly, 10^5 MP from mice chronically infected with *M. avium* were allowed to adhere to wells of 96-well U-bottomed plates (Corning) by overnight incubation in complete medium at 37°C in an atmosphere of 5% CO_2. Nonadherent cells were then removed by washing the wells three times with 0.2 ml of medium before the adherent cells were labeled with approximately 1 mCi of ^{51}Cr-labeled sodium chromate (Amersham, Arlington Heights, Ill.) in 25 μl of medium for 1 h. Wells were then washed three times with medium after which 10^6 viable effector cells were added. Effector cells included sensitized T cells (from chronically *M. avium*-infected C57BL/6 mice), normal C57BL/6 T cells, or allogeneic (BALB/c) cytotoxic cells. Allogeneic cytotoxic cells were prepared by incubating 5×10^7 BALB/c spleen cells with an equal number of normal irradiated (3,000 rad) C57BL/6 splenic stimulator cells for 5 days at 37°C and 5% CO_2, after which the number of viable cells was standardized to 10^7/ml (17). The assay microcultures were incubated for 6 to 60 h, after which the radioactivity in 100 μl of supernatant was determined in a gamma counter (Compugamma; LKB Instruments, Gaithersburg, Md.). The percentage of specific lysis was determined from the formula $(T - S)/(M - S) \times 100$, where T is the counts per minute in test wells, S is the counts per minute in wells with no added T cells or cytotoxic cells (spontaneous release), and M is the counts per minute in wells containing labeled adherent cells treated with 1% Triton X-100 (maximum release).

RESULTS

Growth of *M. avium* in intravenously infected mice. Groups of *M. avium*-infected C57BL/6 mice showed a prolonged logarithmic growth phase within the spleen (Fig. 1) and liver (data not shown). Regardless of the inoculum size, the *M. avium* counts in the spleen continued to increase. This contrasts with the growth behavior of a similar inoculum of *M. bovis* BCG in which the number of viable organisms in the spleen declined sharply after 2 weeks of infection (Fig. 1).

Lymphokine production during the course of the infection. In an effort to determine whether an immune response to the *M. avium* was occurring in the heavily infected mice, spleens were harvested at intervals throughout the infection period

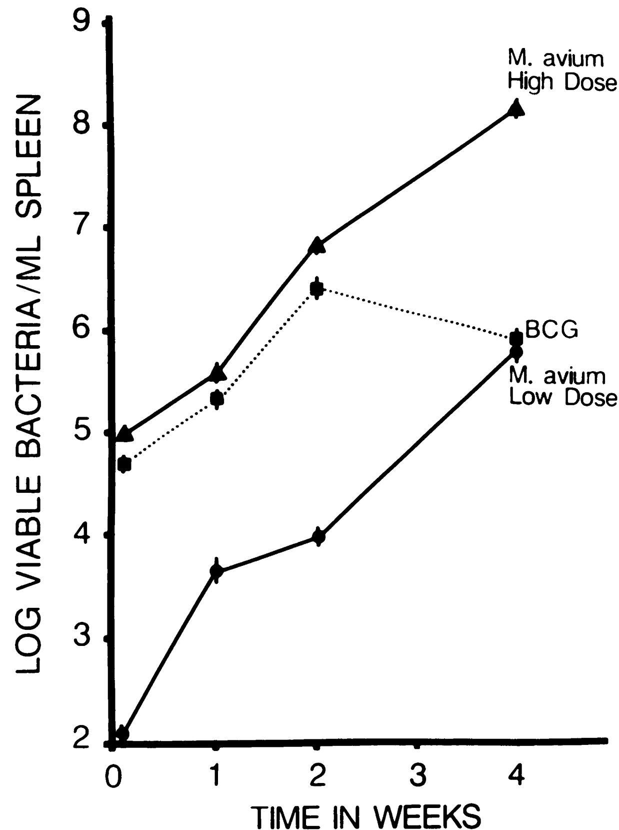

FIG. 1. Growth of *M. avium* at high dose (10^7), *M. avium* at low dose (10^4), and BCG (10^7) administered intravenously (n = 5).

and constitutive lymphokine production by the spleen cells was monitored (Table 1). Significant levels of TNF activity were detected at 1, 2, and 4 weeks postinfection. Levels of IFN-γ activity were increased at 2 and 4 weeks in both the *M. avium-* and BCG-infected macrophages. Cell cultures prepared from 1-day-infected control mice did not contain detectable levels of TNF or IFN-γ. It was concluded that sensitized, lymphokine-secreting T cells were generated in response to both infectious agents.

Enhanced anti-*M. avium* activity by macrophages in vitro. Splenic macrophages (i.e., MP) were harvested from *M. avium*-infected mice at 30 days postinfection. The effect of various treatments on the ability of these macrophages to reduce the viability of the intracellular *M. avium* was determined. When the monolayers were incubated with sensitized T cells and rIL-2 for 4 days, the number of viable *M. avium* recovered from the wells was significantly reduced (P < 0.02) compared with control wells containing infected MP

TABLE 2. Reduction of viable *M. avium* by infected mouse macrophages after treatment in vitro

Addition of sensitized T cells[a]	Treatment[b]	No. of *M. avium* after treatment (log CFU/10^5 MP)	P^b
–		4.98 ± 0.17	NS
–	rIL-2	4.65 ± 0.18	NS
+		4.79 ± 0.08	Control
+	rIL-2	2.81 ± 0.33	<0.02
+	LPS	4.56 ± 0.18	NS
+	ConA	4.53 ± 0.07	NS
+	*M. avium* SS	3.69 ± 0.11	<0.01
+	*M. avium* CF antigens	4.36 ± 0.15	<0.05

[a] Sensitized T cells were collected from high-dose *M. avium*-infected mice at 30 days postinfection.
[b] Treatments included incubation with rIL-2 (50 U/ml), *M. avium* SS and CF antigens (1 μg/ml), ConA (1 μg/ml), and LPS (10 ng/ml).
[b] Student's t test was used to calculate significant differences of mean ± standard error. NS, Not significant; n = 3.

and T cells only (Table 2). However, no significant effect was observed when the MP were incubated with rIL-2 alone (Table 2) or with T cells harvested from normal mice (Table 3).

Incubation of infected MP and sensitized T cells with *M. avium* SS or *M. avium* CF antigens also resulted in a significant reduction of viable organisms recovered from these cultures 4 days later compared with the controls (P < 0.01 and P < 0.05, respectively). When these monolayers were incubated with a macrophage stimulant (LPS) or mitogen (ConA), no significant reduction in *M. avium* viability was observed. The number of MP recovered per well after 4 days of incubation was not significantly reduced compared with the untreated control wells, indicating that the reduction of viable organisms in the treated wells was not due to lysis or detachment of the macrophages from the monolayer. This is consistent with the inability to detect substantial numbers of viable *M. avium* in the supernatant fluid taken from these cultures.

Mice chronically infected with *M. avium* were used as a source of infected MP to prepare monolayers which were examined for changes in the number of viable mycobacteria over time. Enumeration of viable organisms from the monolayers after overnight adherence (time zero) and again after 2 and 4 days of incubation indicated that the number of viable bacteria did not change substantially over this period. The mean (± standard error) log CFU/10^5 MP was 4.89 ± 0.11 at time zero, 4.89 ± 0.15 after 2 days of incubation, and 4.79 ± 0.22 after 4 days of incubation.

Role of T-cell subsets in enhancement of antimycobacterial activity. Experiments were performed to determine the contribution of L3T4$^+$- and Lyt-2$^+$-sensitized T cells on the

TABLE 1. Constitutive secretion of IFN-γ and TNF by macrophages from mice after 1, 2, or 4 weeks of infection, cocultured with T cells

Infective dose	Amt secreted (U/ml) on:							
	Day 1		Wk 1		Wk 2		Wk 4	
	TNF[a]	IFN-γ	TNF	IFN-γ	TNF	IFN-γ	TNF	IFN-γ
10^7 *M. avium*	<4	<4	64	<4	64	32	64	16
10^7 BCG	<4	<4	16	<4	32	16	64	8

[a] TNF and IFN-γ values are reciprocals of duplicate end-point titers. Twofold differences are not significant (P < 0.05).

TABLE 3. Effect of immune T-cell subset depletion on the ability of high-dose *M. avium*-infected macrophages to reduce the number of viable intracellular organisms during 4 days of incubation

MP cultured with:[a]	Addition of rIL-2 (50 U/ml)	No. of *M. avium* after treatment (log CFU/10^5 MP)	P^b
Sensitized T cells	−	4.79 ± 0.08	Control
Sensitized T cells	+	2.81 ± 0.33	<0.02
Sensitized T cells − $L3T4^+$ cells	+	3.81 ± 0.27	<0.05
Sensitized T cells − $Lyt\text{-}2^+$ cells	+	4.12 ± 0.25	<0.05
Sensitized T cells − $Lyt\text{-}2^+$ and $L3T4^+$ cells	+	4.60 ± 0.05	NS
Normal T cells	+	4.96 ± 0.33	NS

[a] Sensitized T cells were collected from spleens of high-dose *M. avium*-infected mice at 30 days postinfection; normal T cells were from spleens of normal C57BL/6 mice.
[b] Student's *t* test was used to calculate significant differences of means ± standard error. NS, Not significant; $n = 3$.

anti-*M. avium* activity of the infected MP. A significant decrease ($P < 0.05$) in the number of viable organisms recovered from infected monolayers was seen after depletion of either $L3T4^+$ or $Lyt\text{-}2^+$ cells (Table 3). However, when both T-cell subsets were removed, there was no significant anti-*M. avium* response.

Lysis of labeled MP in culture. A ^{51}Cr-release assay was performed on the infected macrophage monolayer to determine whether significant numbers of MP were lysed during incubation with the sensitized T cells and rIL-2. Percent specific release of ^{51}Cr from labeled MP incubated with allogeneic cytotoxic cells for 6 h was 22.5% (Table 4). However, only 7.9% specific release was detected when labeled MP were incubated with T cells and rIL-2, indicating that little lysis of the macrophages occurred under these test conditions. Even when the incubation period was increased 10-fold, there was little increase in the release of ^{51}Cr (Table 4). The cytotoxic T-cell controls induced nearly 50% specific release during this time. Spontaneous release for all assays and time points was between 250 and 320 cpm.

DISCUSSION

This study confirms the inability of the innately susceptible C57BL/6 mouse to limit the growth of *M. avium* within the spleen, despite evidence that immunologically activated T cells were present within these mice. Experiments were designed to examine the role of immune T cells in promoting anti-*M. avium* macrophage activities. At 30 days postinfection with *M. avium*, mice were sacrificed and their splenic macrophages were cocultured with autologous T cells and rIL-2, resulting in substantial killing of *M. avium* (nearly 100-fold reductions; Table 2), without a significant loss of adherent cells from the monolayers over the 4-day incubation period. The presence of an exogenous source of IL-2 in the macrophage and T-cell cultures was essential for this bactericidal activity to occur (Table 2). IL-2 has been shown to have direct effects on macrophage functions (3, 20), but, in the present study, killing of *M. avium* occurred only when sensitized T cells were incubated with the infected MP (Table 2). This argues against a direct effect of IL-2 on the macrophage anti-*M. avium* activity and indicates the importance of coculturing the infected MP with sensitized T cells during in vitro experiments, if significant antimycobacterial activity is to be observed. This point was confirmed when sensitized T cells were replaced with normal control T cells and no anti-*M. avium* response was detected (Table 3).

Depletion experiments in which $L3T4^+$ and/or $Lyt\text{-}2^+$ sensitized T cells were removed from the T-cell suspension indicated that both T-cell subsets must be removed to ablate the antimycobacterial activity (Table 3). This indicates that both $L3T4^+$ and $Lyt\text{-}2^+$ sensitized T cells have the ability to enhance the bactericidal activity of the infected macrophages provided that rIL-2 is also present.

The reduction in the number of viable mycobacteria recovered from the infected MP after the addition of rIL-2 to the cultures is consistent with other reports in which IL-2 injections enhanced the immune response seen in mice chronically infected with *M. avium*, BCG, and *M. lepraemurium* (1, 4, 16). Stimulation of *M. leprae*-specific T cells from human lepromatous leprosy patients was also induced by IL-2 treatment (22).

The availability of IL-2 in sufficient quantities to stimulate the sensitized T cells appears to play a pivotal role in the immune response to chronic mycobacterial infections (4, 22, 25). Possible explanations for the enhanced mycobactericidal activity of infected macrophages after IL-2 treatment may involve the ability of this lymphokine to augment the expression of major histocompatibility complex antigens, which may be suppressed in the mycobacteria-infected macrophages (18). Treatment with exogenous IL-2 may also reverse the effects of specific suppressor lymphocytes which are reportedly generated in animals with heavy mycobacterial infections (4, 22).

The addition of *M. avium* SS or CF antigens to the in vitro cultures in the presence of sensitized T cells also significantly reduced the number of viable *M. avium* recovered after 4 days of incubation (Table 2). The addition of specific mycobacterial antigens may improve the ability of infected MP to present the T-cell stimulatory antigens to reactivate resistance mechanisms. The activated macrophages may

TABLE 4. Percent ^{51}Cr-specific release from labeled, *M. avium*-infected, adherent cells

Culture condition[a]	Addition of rIL-2 (50 U/ml)	% Specific release at:[b]	
		6 h	60 h
Sensitized T cells	−	6.4	8.3
Sensitized T cells	+	7.9	10.3
Normal T cells	−	10.7	7.9
Normal T cells	+	8.8	3.5
Allogeneic cytotoxic cells	−	22.5	48.6

[a] Sensitized T cells were from high-dose *M. avium*-infected mouse spleens at 30 days postinfection; normal T cells were from normal C57BL/6 mice; allogeneic cytotoxic cells were from BALB/c spleen cells cultured with irradiated (3,000 rad) normal C57BL/6 splenic stimulator cells for 5 days.
[b] Effector-to-target-cell ratio, 10:1; $n = 3$.

rapidly lose their bactericidal activity in the absence of continued stimulation of the sensitized T cells, thus explaining the effect observed when soluble *M. avium* antigens were added to the cultures (21, 23).

Cytolytic T cells have been implicated in the expression of protective immune responses to mycobacterial infections (9). The antimycobacterial activity seen in the infected macrophages incubated in the presence of immune T cells may be due to cytolysis of the infected MP with the release of the intracellular organisms into the culture medium. However, no evidence for such cytolytic activity against ^{51}Cr-labeled macrophages was obtained (Table 3) nor were there substantial numbers of free mycobacteria in the culture supernatant fluid during the incubation period. This suggests that cytolysis of the infected macrophages was not a major factor.

Elucidation of the mechanisms involved in the enhancement of mycobactericidal activity by infected MP is an important step in developing more effective therapeutic procedures for treating *M. avium* complex-infected individuals. Such therapies are urgently needed to reverse the decreased survival times in *M. avium*-infected AIDS patients (15). The present study suggests that mice heavily infected with *M. avium* may be treated therapeutically with IL-2 and soluble *M. avium* antigens, possibly together with TNF, to reduce their infective burden (1, 2). These experimental procedures may eventually be adapted to the treatment of human *M. avium* complex infections (6, 10).

ACKNOWLEDGMENTS

We thank Michael Zemany and Kay Granger for technical assistance and Mary Durett for secretarial assistance. This work was supported by Public Health Service grants AI-27156, administered by the U.S.-Japan Cooperative Medical Sciences Program, and HL-19774, both from the National Institutes of Health.

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