Growth of *Mycobacterium avium* in Activated Macrophages Harvested from Inbred Mice with Differing Innate Susceptibilities to Mycobacterial Infection

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The growth of *Mycobacterium avium* in macrophages obtained from *Mycobacterium bovis* BCG-infected mice was compared with that in macrophages from uninfected mice. BCG vaccination resulted in substantial macrophage activation, measured as increased acid phosphatase and superoxide anion production, as well as enhanced leishmanicidal activity. However, the activated macrophages were only able to reduce the rate of intracellular growth by *Listeria monocytogenes* and *M. avium* in vivo and did not express detectable levels of mycobactericidal activity in vitro. Exposure of the macrophage monolayers to concanavalin A-stimulated spleen cell supernatant fluid and lipopolysaccharide did not further enhance the ability of the BCG-activated macrophages to control the intracellular replication of the *M. avium*. Macrophages from BCG-infected C57BL/6 (BCG*) mice were quantitatively better able to control the intracellular replication of the *M. avium* challenge than were similar phagocytes obtained from BCG* (A/J) mice. These findings have important implications with respect to the expression of acquired resistance to these atypical mycobacterial infections.

Many nontuberculous mycobacteria are opportunistic human pathogens, capable of causing disease in normal adults only when there is some other underlying disease which potentiates the severity of the mycobacterial infection (23). The prevalence of chronic lung infections (caused by *Mycobacterium kansasii* and members of the *Mycobacterium avium* complex) has not declined within the United States in a manner comparable to that of *Mycobacterium tuberculosis* lung infections (13). Although these organisms are less virulent than *M. tuberculosis*, they can still persist within the lung for long periods of time (4, 5). One explanation for this persistence is their ability to resist the microbicidal capacity of activated macrophages, which are generated as a result of the cell-mediated immune response (14, 15). A number of studies have been made of the interactions occurring between the tubercle bacillus and these activated macrophages (6, 9, 17), but there is generally much less known about the response of the atypical mycobacteria to these cells, especially when tested in vitro (8).

Earlier studies have indicated substantial differences between the growth rates for *M. avium* when introduced into normal macrophages which have been harvested from naturally susceptible C57BL/6 and resistant A/J inbred strains of mice (21). The present study shows that immunologically activated macrophages taken from these same mice are incapable of killing the *M. avium*, although they did reduce their rate of intracellular replication to some extent.

**MATERIALS AND METHODS**

**Bacteria.** *M. avium* (TMC 702) and *Mycobacterium bovis* (BCG Pasteur, TMC 1011) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y. They were grown in modified Sauton medium with added Tween 80 as described previously and stored in 1-ml ampoules at −70°C until required (3). The frozen suspension was thawed and briefly sonicated to break up any clumps before being diluted to the desired concentration in 0.05% Tween 80-saline. The number of viable bacteria in each preparation was checked by plating serial dilutions on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) and counting the resultant colonies after 21 days of incubation in sealed plastic bags at 37°C.

*Listeria monocytogenes* EGD (serotype 1/2a) was grown in tryptic soy broth (Difco), harvested in the midlogarithmic growth phase, and stored in 1-ml samples at −70°C until required. The intravenous 50% lethal dose was approximately 10⁵ bacteria for the AB6 mice, determined after 10 days by the Reed Muensch method (3).

Mice. Specific-pathogen-free C57BL/6 (naturally susceptible) and A/J and AB6 (A × C57BL/6) F₁ male (naturally resistant) mice between 6 and 10 weeks old were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, N.Y. They were raised under barrier conditions and fed sterile mouse chow and acidified water ad libitum (15).

Demonstration of nonspecific resistance in BCG-infected mice. A/J and C57BL/6 mice were infected with 10⁶ CFU of BCG suspended in 0.2 ml of phosphate-buffered saline, which was injected via a lateral tail vein (3). Twenty-one days later, the vaccinated mice (along with naive, age-matched controls) were challenged intravenously with either *M. avium* (10⁶ or 10⁷ CFU per mouse) or *L. monocytogenes* (10⁵ CFU per mouse) in 0.2 ml of phosphate-buffered saline. The subsequent growth of the challenge infection within the spleen and liver was followed by plating 10-fold dilutions of the organ homogenates on either tryptic soy agar (*L. monocytogenes*) or on Middlebrook 7H11 agar (*M. avium*), the latter being supplemented with 1 µg of 2-thiophencarboxylic acid hydradize per ml of 7H11 agar to inhibit BCG growth (3). Bacterial colonies were enumerated after incubation at 37°C in a humidified atmosphere for 21 days for *M. avium* and 24 h for *L. monocytogenes*.

**Infection of macrophages in vitro.** Monolayers of peritoneal macrophages were prepared from mice infected with 10⁶ CFU of BCG injected intravenously 21 days earlier and elicited by an intraperitoneal injection (3 days before collect-
ing the washouts) of 2 ml of sterile 3.8% casein (Eastman Kodak Co., Rochester, N.Y.) plus 10° heat-killed BCG organisms per ml. The peritoneal exudate cells were sus-
pended at a concentration of 5 x 10^5 cells per ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 20% fetal calf serum (GIBCO), 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer solution (Whittacker Biopro-
ducts, Walkerville, Md.). Samples (1 ml) of the cell suspen-
sion were added to replicate cover slips in 24-well cluster plates and incubated for 2 h at 37°C in 6% CO_2-enriched air. Nonadherent cells were removed by washing with PBS, and 1 ml of fresh complete medium containing 10^6 CFU of mycobacteria was then added. After 4 h incubation at 37°C, the unengested parasites were removed by vigorously washing the cover slips with phosphate-buffered saline before transferring them to fresh medium in new 24-well plates. At this time, and again after 7 days of incubation at 37°C in 6% CO_2-enriched air, three cover slips were processed to determine the number of viable intracellular bacteria per 10^3 adherent macrophages. To each cover slip, 0.25 ml of 0.05% napthol blue black in 0.1 M citric acid plus 1% Triton X-100 (12) was added; after agitation, a count was made of the blue-stained macrophage nuclei. In addition, 0.1 ml of the lysisate was removed from each well and transferred into 0.9 ml of 0.1 M Na_2HPO_4, briefly sonicated to break up any clumps of bacteria, and plated on Middlebrook 7H11 agar to determine the number of viable _M. avium_. The counts are expressed as the number of viable bacteria per 10^3 adherent macrophages. The number of times the intracellular myco-
bacteria had doubled in number over the 7 day incubation period was calculated as follows: replication index = Log([CFU/10^5 macrophages at 7 days]/[CFU/10^5 macrophages at 4 h]).

**Demonstration of macrophage activation in vitro.** Three parameters of macrophage activation were determined for the macrophages used in the present experiments. Macrophage monolayers prepared from both normal and BCG-
infected mice were assayed simultaneously for acid phosphatase activity, superoxide anion production, and leishmanicidal capacity.

**Acid phosphatase activity.** Monolayers were prepared in 75-cm² tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif.). After 4 h of incubation at 37°C in 6% CO_2-enriched air, nonadherent cells were removed by three washes with sterile phosphate-buffered saline. Distilled water (3 ml) containing 1% Triton X-100 was then added to each flask, and the washes were frozen and thawed three times. The resultant cell lysates were removed and assayed for acid phosphatase activity by using _p_-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) as the substrate (2). Results are expressed as nanomoles of _p_-nitrophenol produced per milligram of protein per 30-min period. Protein was assayed by using the bicinchoninic acid assay against a bovine serum albumin standard curve (19).

**Superoxide anion production.** Monolayers were prepared in 24-well cluster plates without cover slips. After 4 h of incubation at 37°C to allow adherence, the monolayers were washed, and 1 ml of complete medium containing 10^6 CFU of nitroblue tetrazolium (Sigma) per ml was added to each well. Phorbolmyristate acetate (Sigma) was added at a final concentra-
tion of 50 ng/ml.

Monolayers were incubated for 60 min at 37°C in 6% CO_2-enriched air. For each experimental group, three wells were processed to assess the average number of adherent cells in the monolayer, and three other wells were processed to assess the amount of reduced nitroblue tetrazolium present (18). Superoxide anion production was expressed as nanomoles of nitroblue tetrazolium reduced per 10^6 macro-
phages per 60 min.

**Leishmanicidal assay.** Macrophage monolayers were pre-
pared on cover slips in 24-well cluster plates. After 2 h of incubation at 37°C, the monolayers were washed to remove nonadherent cells, and 1 ml of complete medium containing 5 x 10^6 _Leishmania mexicana amazonensis_ (strain Maria) promastigotes (kindly provided by J. O. Hill, Trudeau Insti-
tute) was added to each well. After 4 h of incubation at 32°C, unengested parasites were removed by washing, and fresh complete medium was added. At this time, and again after 3 days of infection, three replicate cover slips were fixed and stained with 6% Giemsa (Polysciences, Inc., Warrington, Pa.). The number of intracellular amastigotes within 100 macrophages was assessed microscopically for each mono-
layer and the percentage difference between amastigote numbers at 4 h and 3 days was assessed.

**Lymphokine production.** Normal AB6 F₁ mice were in-
fected intravenously with 10^6 CFU of BCG. After 21 days the spleens were removed and dissociated by passage through a stainless steel sieve into RPMI medium plus 5% fetal calf serum. Erythrocytes were lysed with buffered ammonium chloride (155 mM NH_4Cl, 10 mM KHCO_3, 0.1 mM EDTA), and clumps of cells were removed by sedimentation. The resulting single-cell suspension was diluted in RPMI medium plus 5% fetal calf serum to a concentration of 2 x 10^6 cells per ml. The cell suspension (50 ml) was dispensed into 75-cm² tissue flasks, and concanavalin A (Sigma) was added to give a final concentration of 2 _μg/ml_. The cultures were incubated at 37°C in 6% CO_2-enriched air for 3 days, the cells were removed by centrifugation (150 x g for 15 min), and the supernatant fluid was sterilized by filtration. Stimulated spleen cell supernatants (lymphokine) were stored at -20°C until required.

**Statistics.** Collected data were analyzed by using analysis of variance and the Student _t_ test for a difference between two of several independent means with the Statview 512+ program (Brainpower Inc., Calabasas, Calif.) run on a MAC plus microcomputer.

**RESULTS**

**Growth of _M. avium_ in normal and BCG-infected mice.** A/
J and C57BL/6 mice were infected with about 10^6 CFU of strain BCG Pasteur and 21 days later were challenged (along with age-matched, uninfected mice) with either 10^4 or 10^5 CFU of _M. avium_. Growth of the _M. avium_ challenge injection in the spleen and liver is shown in Fig. 1. The BCG-vaccinated C57BL/6 mice limited the growth of the _M. avium_ challenge more effectively than did the BCG-infected A/J mice. As a result, 10 days after administering the low dose, 10^4 CFU of _M. avium_ challenge, a significant reduction in the bacterial load was seen in both the spleens and livers of the BCG-vaccinated C57BL/6 mice ( _P_ < 0.05). Furthermore, this reduction continued throughout the experiment, the numbers within the BCG-vaccinated C57BL/6 mice remaining 100- to 1,000-fold lower than those in the normal controls (Fig. 1A). On the other hand, the BCG-infected A/
J mice were relatively much less protected against the subsequent challenge, at least judging from the bacterial load seen in the livers of the normal mice compared with those in the BCG-vaccinated A/J mice on day 60. Some protection ( _P_ < 0.05) was observed in the spleen 20 days into the low _M. avium_ challenge infection (Fig. 1A), but both groups of...
vaccinated mice receiving the larger (10^7 CFU) M. avium challenge exhibited similar viable counts (Fig. 1B); the difference in the A/J mice was not sufficient to be significant. On the other hand, the heavily challenged C57BL/6 mice exhibited significant differences (P < 0.05) from day 10 of the challenge.

Growth of L. monocytogenes in normal and BCG-vaccinated mice. The above experiment suggested that BCG-infected A/J mice did not express effective levels of acquired resistance against the subsequent M. avium challenges. However, the innately resistant host developed some activated macrophages in response to the BCG infection, since the BCG-infected A/J mice challenged intravenously with a 5 times the 50% lethal dose of L. monocytogenes showed a substantial reduction in the growth of the L. monocytogenes in both the liver and spleen over a 7-day period (Fig. 2). In the normal control mice, the L. monocytogenes multiplied progressively in vivo, resulting in the death of all of the A/J mice. On the other hand, the C57BL/6 mice readily limited this challenge to sublethal proportions so that most of the animals survived. BCG vaccination afforded increased anti-L. monocytogenes protection to both the A/J and the C57BL/6 mice, resulting in a 1,000-fold reduction in the bacterial load in both the liver and spleen, thereby protecting all of the A/J mice from death.

Quantitative estimates of macrophage activation in vitro. Resistance to a Listeria challenge was one measure of nonspecific macrophage activation (14). Other parameters of macrophage activation, such as acid phosphatase activity, were also increased substantially (approximately twofold) in BCG-infected A/J and C57BL/6 mouse macrophages compared with those observed in normal controls (Table 1). On the other hand, superoxide anion production (in response to phorbolmyristate acetate stimulation) was enhanced in the C57BL/6 macrophages but not in cells harvested from BCG-infected A/J mice. Such activated macrophages were able to kill leishmanias; the C57BL/6 cells were more active in this regard (Table 1). Macrophages harvested from normal A/J and C57BL/6 mice were consistently unable to kill leishmanias.

Growth of mycobacteria in macrophage monolayers prepared from normal and BCG-infected mice. Having established that macrophages obtained from A/J and C57BL/6

FIG. 1. Growth of 10^6 (A) or 10^7 CFU (B) of M. avium in C57BL/6 (●) or A/J (○) mice. Mice had either been infected with 10^6 CFU of BCG Pasteur 21 days earlier (——) or were age-matched untreated control mice (---). Vertical bars represent the standard errors of the means for five replicate determinations.

FIG. 2. Growth of 10^6 viable L. monocytogenes in C57BL/6 (●), or A/J (○) mice. Mice had been infected with 10^6 CFU of BCG Pasteur 21 days before the Listeria challenge (●, ○) or were age-matched untreated mice (●, ○). Vertical bars represent the standard errors of the means for four determinations.
TABLE 1. Parameters of activation in peritoneal macrophages obtained from normal and BCG infected C57BL/6 and A/J mice

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Acid phosphatase activity</th>
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<tbody>
<tr>
<td>A/J control</td>
<td>51.4 ± 10.7</td>
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<tr>
<td>A/J BCG</td>
<td>97.6 ± 11.1</td>
</tr>
<tr>
<td>C57BL/6 control</td>
<td>44.1 ± 8.1</td>
</tr>
<tr>
<td>C57BL/6 BCG</td>
<td>87.6 ± 6.3</td>
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</tbody>
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<tr>
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<th>O2⁻ production</th>
<th>Growth of Leishmania (%)</th>
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<tbody>
<tr>
<td>A/J control</td>
<td>28.4 ± 1.9</td>
<td>+5</td>
</tr>
<tr>
<td>A/J BCG</td>
<td>39.0 ± 8.3</td>
<td>-37</td>
</tr>
<tr>
<td>C57BL/6 control</td>
<td>36.0 ± 8.7</td>
<td>+59</td>
</tr>
<tr>
<td>C57BL/6 BCG</td>
<td>92.7 ± 3.1</td>
<td>-76</td>
</tr>
</tbody>
</table>

Elicited peritoneal macrophages were obtained from uninfected mice (control) or mice infected with 10⁶ CFU of BCG 21 days previously (BCG). Nanomoles of p-nitrophenol produced per milligram of protein per 30 min (mean ± standard error; n = 3). Nanomoles of nitroblue tetrazolium reduced per 10⁶ macrophages per 60 min (mean ± standard error; n = 3). Percent difference in intracellular amastigote numbers after a 3-day incubation period.

FIG. 3. Replication of M. avium in elicited macrophages obtained from normal (■) or BCG-infected (■) C57BL/6 and A/J mice. See Materials and Methods for an explanation of the replication index. Vertical bars represent the standard errors of the means for seven separate experiments.

FIG. 4. Replication of BCG Pasteur in elicited macrophages obtained from normal (■) or BCG-infected (■) C57BL/6 and A/J mice. Vertical bars represent standard errors for five experiments.

FIG. 5. Replication of M. avium in elicited macrophages obtained from normal (■) or BCG-infected (■) C57BL/6 and A/J mice. Lipopolysaccharide (LPS; 50 ng/ml, final concentration) or lymphokine (50%, final concentration) were (+) or were not (−) added to macrophages. Vertical bars represent the standard errors of the means for three separate experiments.

DISCUSSION

The present study confirms the fact that macrophages harvested from infected mice are substantially activated, both in terms of acid phosphatase and superoxide anion production, as well as inactivation of Listeria and Leishmania species (7). However, these same activated macrophages seem incapable of killing a standardized challenge inoculum of M. avium (or even BCG), although they could reduce their rates ofintracellular growth substantially (Fig. 3 and 4). The BCG-activated macrophages taken from the susceptible C57BL/6 mice were quantitatively more effective at slowing the intracellular growth of these mycobacteria, compared with cells harvested from the naturally resistant mice (A/J). This difference correlated well with enhanced superoxide anion production after phorbolmyristate acetate stimulation.
as well as a greater degree of leishmanicidal activity. On the other hand, acid phosphatase levels in BCG-activated macrophages from either C57BL/6 or A/J mice were comparable in activity, emphasizing the divergence in functional capacities exhibited by macrophages activated with live BCG vaccine (22). However, the present studies generally support the contention that activated macrophages produced by resistant strains of mice are functionally defective, at least in some activities (11).

The demonstration that BCG-activated macrophages can restrict the growth of M. avium in an in vitro test environment extends earlier reports of such activity in other mycobacteria (1, 6, 9, 17). However, lymphokine-activated macrophages showed no demonstrable bacterial activity in vitro, a finding in sharp contrast to earlier reports with M. tuberculosis (6, 20). This difference may simply reflect the experimental models used in these tests, or it may relate to the greater resistance shown by M. avium to inactivation by activated macrophages in general (14).

Lipopolysaccharide added to these cells in trace quantities is said to provide a second signal which transforms the primed macrophage into one with bactericidal qualities (10). Thus, the mycobactericidal (or at least mycobacteriostatic) activity of the BCG-activated macrophages could be enhanced by prior lipopolysaccharide treatment of the cells. The results obtained in the present study do not support this lipopolysaccharide trigger hypothesis since, if anything, lipopolysaccharide treatment of stimulated mycobacterial growth within the BCG-activated macrophages (Fig. 5), suggesting that some sort of antagonistic action may occur after lipopolysaccharide treatment of the BCG-stimulated macrophage (16).

The best that the BCG-activated macrophage could achieve was the inhibition of growth by the M. avium challenge within the phagocyte. This relatively slight reduction in growth by the M. avium (Fig. 3) contrasted with the active killing of a secondary M. avium infection population in vivo (Fig. 1). Inefficiency of growth reduction by the BCG-activated macrophage seems dependent upon the genetic makeup of the host, so that activated macrophages harvested from the naturally resistant A/J mice failed to inhibit the growth of the M. avium challenge substantially, whereas similar phagocytes obtained from the naturally susceptible mouse (C57BL/6) could restrict the growth of the M. avium population to some degree. The reason for this apparent paradox remains unclear and deserves further examination.

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LITERATURE CITED