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Interaction of *Mycobacterium avium* with Environmental Amoebae Enhances Virulence

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Environmental mycobacteria are a common cause of human infections. Recently, contaminated domestic water supplies have been suggested as a potential environmental source of several mycobacterial diseases. Since many of these mycobacterial species replicate best intracellularly, environmental hosts have been sought. In the present study, we examined the interaction of *Mycobacterium avium* with a potential protozoan host, the water-borne amoeba *Acanthamoeba castellanii*. We found that *M. avium* enters and replicates in *A. castellanii*. In addition, similar to that shown for mycobacteria within macrophages, *M. avium* inhibits lysosomal fusion and replicates in vacuoles that are tightly juxtaposed to the bacterial surfaces within amoebae. In order to determine whether growth of *M. avium* in amoebae plays a role in human infections, we tested the effects of this growth condition on virulence. We found that growth of *M. avium* in amoebae enhances both entry and intracellular replication compared to growth of bacteria in broth. Furthermore, amoeba-grown *M. avium* was also more virulent in the beige mouse model of infection. These data suggest a role for protozoa present in water environments as hosts for pathogenic mycobacteria, particularly *M. avium*.

Microorganisms in environmental biofilms must effectively interact with each other to insure survival. These microenvironments are important in the growth and survival of many bacterial species (24). The interaction of bacterial species with protozoa, in particular, has significant advantages (8), including resistance to bactericidal agents (8, 9, 50). These interactions have led to endosymbiotic relationships between bacteria and amoebae that closely resemble the pathogenesis of chronic bacterial infections in mammalian cells (1, 65). These similarities may result from parallels in phagocytosis (2, 19, 51), bactericidal mechanisms (26, 27), and surface receptors (3) of amoebae and macrophages. Have the virulence mechanisms of bacterial pathogens evolved through interaction with protozoa in the environment? Do protozoa provide an environmental host for the bacterial pathogens that currently cause disease in humans? Previous studies have shown that pathogenic bacteria, including *Legionella pneumophila* (39, 47, 63, 69, 75), *Vibrio cholerae* (74), *Listeria monocytogenes* (52, 53), *Chlamydia pneumoniae* (31), and *Edwardsiella tarda* (48), can survive and replicate in protozoa. This relationship has been amply shown in the interaction of *L. pneumophila* with *Acanthamoeba castellanii* (4, 39, 69) in which the amoeba has been used as a virulence model (58). Furthermore, the involvement of this interaction in the pathogenic life cycle of *L. pneumophila* has been suggested (10, 21).

The bacterial species of the *Mycobacterium avium* complex are the most common cause of systemic bacterial infection in patients with AIDS (11, 40). Recently, *M. avium* infections have been associated with bacterial colonization of domestic water supplies (34, 60, 76, 77, 79). This observation is reminiscent of the epidemiological picture of *L. pneumophila* (61, 71). In addition to *M. avium*, a number of other mycobacterial diseases have been associated with the presence of water-borne bacterial species (35, 46). Granulomas associated with swimming pools, fish tanks and other water activities are frequently due to *Mycobacterium marinum* infection (32, 38, 66). *Mycobacterium kansasii*, which can be isolated from a number of domestic and environmental water supplies (54), primarily causes pulmonary infections (62) but may also cause chronic extrapulmonary disease (29, 45). Other mycobacteria that are associated with infections from water supplies include *M. intracellulare*, *M. scrofulaceum*, *M. chelonae*, and *M. fortuitum* (35). Thus, water supplies present a potential source of a number of mycobacterial infections.

Since there are epidemiological aspects of *M. avium* infections that resemble *L. pneumophila* infection, we examined the possibility of an interaction of mycobacteria with the water-borne amoeba, *A. castellanii*. Previous studies have demonstrated that *M. leprae* (43) and *M. avium* (49) may survive in the amoeba *A. castellanii*. For this reason we examined the ability of *M. avium* not only to survive but also to inhibit phagosome-lysosome fusion and to replicate within *A. castellanii*. We extended these studies to examine the effects of this growth environment on virulence in epithelial cell and macrophage in vitro models as well as in the beige mouse model of infection. Amoeba-grown *M. avium* displays enhanced entry into epithelial cells and is more virulent in the macrophage and mouse models of infection. These studies indicate that protozoa may provide an environmental reservoir for pathogenic mycobacteria. Since *M. avium* grown in this environment is more virulent, these data support a role for protozoa in the production of disease.

MATERIALS AND METHODS

Strains and growth conditions. *M. avium* 101 (serovar 1) was isolated from the blood of an AIDS patient. Previous studies determined that this strain causes disseminated infection in mice (13, 17). 7H9-grown *M. avium* was cultured on Middlebrook 7H10 agar (Difco) for 10 days, and isolated transparent colonies were washed and resuspended in Middlebrook 7H9 broth (Difco) for 5 days. Prior to the assay, the bacteria were washed in Hank's buffered salt solution

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(HBSS) and passed throughout an 18-gauge needle 10 times. The suspension was placed in a 15-ml polypropylene conical tube (Costar) and vortex agitated for 2 min. The suspension was then allowed to settle for 15 min, and the top 1 ml was removed and used for infections. To confirm that this suspension was well dispersed, a sample of it was stained by the Ziehl-Nielssen technique (37) and examined by light microscopy. Amoeba-grown *M. avium* was introduced into *A. castellanii* as described below for different periods of time and harvested by lysis of the amoebae, and the resulting suspension was used in invasion and virulence assays. *Mycobacterium smegmatis* mc^2 155 was obtained from the laboratory of William Jacobs, Jr. (Albert Einstein College of Medicine, New York, N.Y.), and *M. smegmatis* 11727 was obtained from the American Tissue Culture Collection, Rockville, Md.). *M. marinum* was a clinical isolate (strain M [68]) obtained from the skin of a patient. *M. fortuitum* was a clinical isolate also obtained from a patient. All bacteria were cultured at 37°C except *M. marinum*, which was cultured at 32°C. *M. marinum*, *M. fortuitum*, and *M. smegmatis* were cultured in 7H9 broth (Difco) before use.

Bacterial viability was determined for each assay by using the LIVE-DEAD assay (Molecular Probes, Eugene, Oreg.). The viabilities of representative samples of *M. avium* from each growth condition were approximately 80%. In addition to direct examination, the bacterial inoculum was plated onto 7H10 agar for quantitation of CFU. To determine the viability of intracellular bacteria, infected macrophages and *A. castellanii* were lysed, and the LIVE-DEAD assay was performed with the lysate.

Cell lines and culture conditions. *A. castellanii* Neff (ATCC 30234) was grown to 90% confluency at 23°C in the dark in 75-cm^2 tissue culture flasks (Falcon) containing PYG broth (59). Amoebae were suspended before use by rapping the flask sharply by hand, and the number of cells were determined as described previously (59).

The colon carcinoma cell line HT-29 (ATCC HTB38), a well-differentiated cell line with marked characteristics of human intestinal epithelial cells, was maintained in RPMI 1640 medium with galactose 1% (GIBCO Laboratories, Detroit, Mich.) supplemented with 2 mM L-glutamine and heat-inactivated 5% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.). For the assays, 10^5 HT-29 cells were seeded in each well of a 24-well tissue culture plate (Costar) and grown to 80% confluency. The cell line was used between passages 56 and 65 in all assays.

Human monocyte-derived macrophages were purified as previously described (16, 25). The number of macrophages in the monolayer (approximately 5×10^5) was monitored daily, to determine whether differential detachment occurred among the experimental groups in comparison with uninfected monolayers. Monolayers containing 5×10^5 macrophages were culture in RPMI 1640 supplemented with 2 mM L-glutamine and 5% heat-inactivated fetal bovine serum.

Microscopic techniques. Electron microscopy was used to examine the intracellular compartment of *M. avium* within *A. castellanii*. Amoebae that had been infected with *M. avium* for various periods of time were suspended by using a rubber policeman, fixed, and prepared for electron microscopy by previously described techniques (18, 64). Basically, the samples were fixed in 2% gluteraldehyde–1% OsO_4 for 2 h and post-fixed with 0.5% uranyl acetate overnight at 4°C. The resulting pellets were then embedded and sectioned for electron microscopy as described previously (21).

In order to quantitate the frequency of lysosomal fusion with mycobacterial vacuoles in amoebae, the cells were prelabeled with thorium dioxide by the method described previously for macrophages (5, 41). Basically, 12 μl of 2% thorium dioxide (Polysciences) in phosphate-buffered saline (PBS) per well was added directly to 10^6 amoebae in 1 ml of PYG in a 24-well tissue culture dish 24 h prior to infection. At the time of bacterial infection, the cells were washed once with *A. castellanii* buffer to remove excess thorium dioxide and infected with *M. avium* at an MOI of 10 in 1 ml of *A. castellanii* buffer for 5 minutes at 37°C. The monolayer was then washed once with *A. castellanii* buffer and suspended in 1 ml of *A. castellanii* buffer for various periods of incubation at 37°C.

Infection of *A. castellanii*. Plastic-adherent *A. castellanii* monolayers (approximately 10^6 amoebae/well) in 24-well tissue culture plates were infected with mycobacteria at a multiplicity of infection (MOI) of 10, as described previously for *L. pneumophila* (21). After 30 min of incubation at 37°C, the monolayer was washed with *A. castellanii* buffer (59) to remove extracellular bacteria. To kill any remaining extracellular or adherent bacteria, the monolayers were treated with 100 μg of amikacin per ml for 2 h as described previously (15). This treatment was sufficient to reduce the numbers of extracellular bacteria by greater than 90% without affecting intracellular viability. The monolayers were then washed and resuspended in 1 ml of *A. castellanii* buffer for various times before determining the number of intracellular CFU.

To recover intracellular bacteria from amoebae, the *A. castellanii* monolayer was lysed with 0.5% sodium dodecyl sulfate (SDS) in PBS at various time points. The lysate was then passed through a 27-gauge needle five times to ensure breakage of the amoebae. The lysate was centrifuged at $5{,}000 \times g$ for 5 min, the bacterial pellet was resuspended in 1 ml of PBS, and dilutions were plated to determine CFU.

The percentage of infected *A. castellanii* amoebae was determined by light microscopy following careful fixation of the monolayer with heat and staining of the intracellular bacteria by the Kinyoun method (44). The percentage of viable amoebae was determined by Trypan blue exclusion (15, 16). Twenty fields with at least 20 amoebae/field were examined. To further verify viability of infected amoebae, samples were observed by phase-contrast microscopy for motility and cytoplasmic streaming.

Invasion assays. Invasion assays of HT-29 cells and human macrophages were carried out as previously reported (15, 42). Briefly, 10^6 7H9- or amoeba-grown *M. avium* organisms were added to an HT-29 or macrophage monolayer and entry was allowed to occur at 37°C in 5% CO_2 for 1 h. The infected HT-29 monolayers were treated with 100 μg of amikacin per ml for 2 h as previously described (15). Monolayers were washed three times with HBSS to remove extracellular bacteria and then lysed by being washed once with 0.5 ml of sterile water followed by 0.5 ml of 0.025% SDS in 7H9 broth for 10 min. The resulting suspension was then vigorously pipetted 10 times to ensure cell lysis. The activity of the SDS was subsequently reduced by adding 0.5 ml of 20% bovine serum albumin (Sigma) in phosphate buffer. The lysate was serially diluted and plated onto 7H10 agar for quantitation of intracellular CFU.

Growth in macrophages. Plastic-adherent human monocyte-derived macrophages (5×10^5 cells/well) were infected at an MOI of 10 in triplicate wells by an invasion assay. After invasion, the cells were suspended in 1 ml of RPMI 1640 for 3 days at 37°C in 5% CO_2. The ability of 7H9- and amoeba-grown *M. avium* to replicate in these cells was assessed by the fold increase in the number of CFU present at the time of entry and after 3 days of intracellular growth.

Mouse infections. In order to examine the virulence of amoeba-grown and 7H9-grown *M. avium* in mice, we used a previously characterized virulence model for *M. avium* (13). Six different *M. avium* 101 preparations were used for these studies: (i) *M. avium* grown in 7H9, (ii) *M. avium* grown within *A. castellanii* for 3 days, (iii) *M. avium* grown in 7H9 mixed with a lysate of *A. castellanii*, (iv) *A. castellanii* (10^6 amoebae) infected with 10^6 *M. avium* organisms for 3 days, (v) 10^5 *A. castellanii* amoebae infected with 10^6 *M. avium* organisms for 3 days, and (vi) 10^3 *A. castellanii* amoebae infected with 10^6 *M. avium* organisms for 3 days. For each preparation the inoculum was equilibrated to 10^6 bacteria at the time of inoculation. Preparations i and ii were obtained as described for invasion assays. Preparation iii was made by mixing 10^6 bacteria with a lysate of 10^6 amoebae. Amoebae were lysed in the same manner as described for preparation of intracellular bacteria except that uninfected amoebae were used. Preparations iv to vi were made by infecting the designated number of amoebae with 10^6 bacteria for 3 days. The infected amoebae were then suspended by rapping the flask vigorously and centrifuged at $1{,}000 \times g$ for 5 min, and the resulting pellet was suspended in 1 ml of PBS.

M. avium virulence was examined in the oral model of infection (13). C57BL/6 bg^+/bg^+ mice were infected by gavage with *M. avium* (10^6 bacteria), and after 2, 14, and 28 days, bacteria in the livers and spleens were quantitated as previously described (13). In order to compare the ability of amoeba-grown *M. avium* to colonize the intestinal mucosa to that of 7H9-grown *M. avium*, an intestinal segment from the terminal ileum was obtained 18 h following infection. The tissue was washed extensively and homogenized, and the number of CFU/g of tissue was determined by plating as described previously (13).

Statistical analyses. All in vitro experiments were carried out in triplicate and repeated three times. The in vivo experiments were carried out with 16 mice per experimental group. Significance of the results was analyzed by analysis of variance. P values of <0.05 were considered significant.

RESULTS

Intracellular growth of mycobacteria. The ability of *M. avium* to replicate within *A. castellanii* was compared to those of *M. fortuitum*, *M. marinum* and *M. smegmatis*. As shown in Fig. 1, we found that *M. avium* was able to replicate well in the presence of *A. castellanii* at both 37 and 32°C. *M. fortuitum* was found to have a similar ability to replicate within amoebae at both temperatures but at a lower growth rate (Fig. 1). *M. marinum*, however, was found to replicate only at 32°C and was killed at higher temperatures (Fig. 1). We tested the ability of *M. avium* to replicate in amoebae at other temperatures and found that replication occurs at temperatures as low as 24°C (data not shown). In addition, we examined the ability of 29 other *M. avium* strains (both clinical and environmental samples) to grow within *A. castellanii*. We found that 24 of the 29 strains grew well within *Acanthamoeba* while the remaining three strains grew but more slowly (data not shown). Furthermore, two of the rapidly growing strains were found to replicate significantly faster than strain 101. Thus, the ability to grow within amoebae is not a unique characteristic of *M. avium* 101. The generation time for *M. avium* in log phase was calculated to be approximately 14 h for all three growth environments used in these studies in amoebae, macrophages, and 7H9. The nonpathogenic species, *M. smegmatis*, was not able to survive within the amoebae. Although *M. smegmatis* was

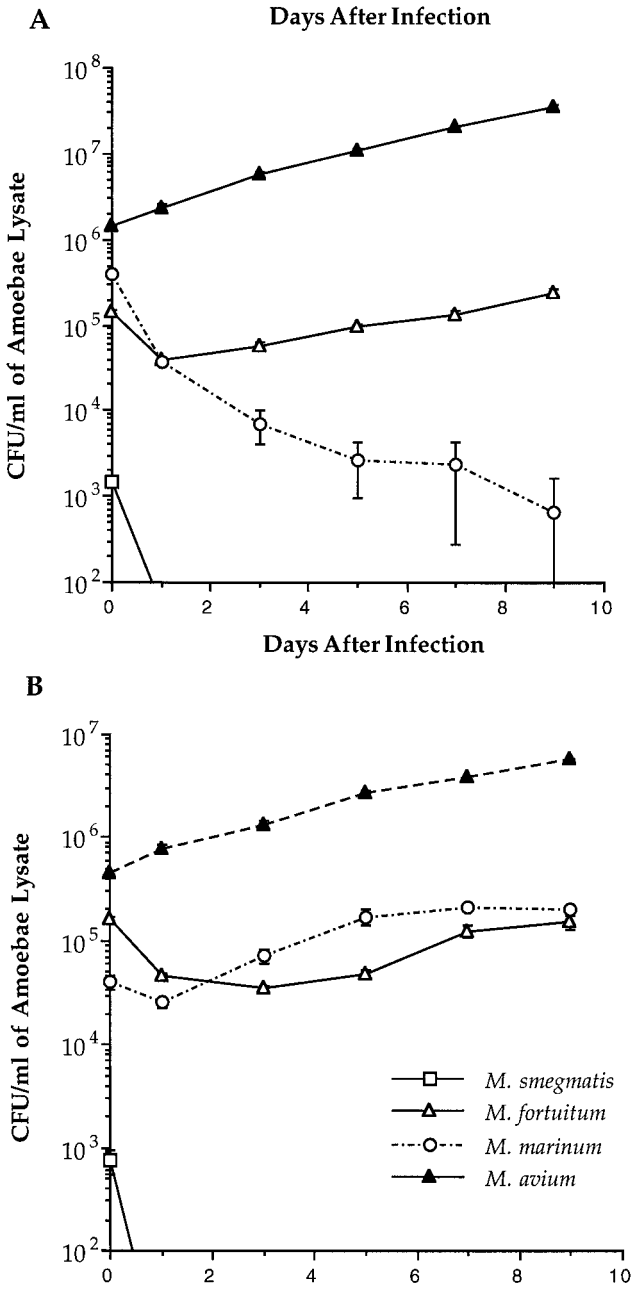

FIG. 1. Growth of *M. avium* (strain 101), *M. marinum*, *M. fortuitum*, and *M. smegmatis* in *A. castellanii* at 37°C (A) and at 32°C (B). Time zero was taken at 2.5 h after infection, immediately after amikacin treatment. Datum points are the means of triplicate wells, and the standard errors are represented by error bars (often overlapping the datum points). The results for a typical experiment are shown. The symbols are described in panel B.

capable of infecting *A. castellanii*, the number of intracellular bacteria decreased to nearly zero within 2 days after infection, independent of the temperature of incubation (Fig. 1).

Amikacin at 100 μg/ml, the concentration used in these studies, was not found to have any cytotoxic activity for amoebae (data not shown). In order to ensure that amoebae do not internalize sufficient amikacin to inhibit bacterial growth during this assay, we determined the effects of a 2-h treatment with 100 μg of amikacin per ml on the number of intracellular bacteria after 1, 3, and 5 days of growth in amoebae. No significant difference was observed between the number of intracellular bacteria in the presence or absence of amikacin after 2 h at 37°C (data not shown). In addition, none of the mycobacterial species used in these studies replicated at similar levels in PYG broth, *A. castellanii* buffer alone, or *A. castellanii* buffer plus lysed amoebae compared to replication in the presence of viable amoebae (data not shown).

TABLE 1. Characterization of *M. avium* vacuole in amoebae

Time after entry	No. of bacteria/ vacuole[a]	No. of vacuoles/ cell[a]	% Spacious vacuoles
0	1.5 ± 0.66	3.3 ± 0.91	19
5 min	1.7 ± 1.1	1.1 ± 0.33	52
15 min	1.4 ± 0.59	1.5 ± 0.64	73
1 h	2.2 ± 1.3	1.4 ± 0.62	38
1 day	1.3 ± 0.94	1.8 ± 0.76	17
3 days	2.4 ± 1.9	1.1 ± 0.29	22
5 days	9.5 ± 9.2	1.5 ± 0.85	38

[a] Data are the means ± standard errors of three counts from 50 amoebae that contain at least one bacterial vacuole each for each time point. Results are from a representative experiment.

Vacuole morphology and trafficking. Infection of *A. castellanii* with *M. avium* was monitored for 5 days by transmission electron microscopy (Table 1). We found that *M. avium* is taken up into individual vacuoles that appear to coalesce into a single large spacious vacuole during the first 15 min after infection (Fig. 2A and B and Table 1). Within 30 min after infection, the bacteria are found within vacuoles that are tightly juxtaposed to the electron-transparent zone surrounding them (Fig. 2C). The majority of *M. avium* vacuoles maintain this morphology throughout the course of infection of the amoebae from day 2 (Fig. 2) to at least day 15 (data not shown) of intracellular growth. However, a significant proportion of the vacuoles that are observed continue to have the spacious morphology after 3 days (Table 1).

Interestingly, the average number of intracellular bacteria per cell remains approximately 15 bacteria per cell from 3 days (Table 1) to 15 days after infection (data not shown). Since the total number of CFU continues to increase during this time, these data suggest that the percentage of infected cells rather than the number of intracellular bacteria per cell increases. In order to test this hypothesis we examined the change in the percentage of infected amoebae over the course of infection (Table 2). We observed that, as expected, the percentage of infected amoebae increases over time. Since the percentage of viable amoebae decreases more rapidly in the infected amoeba cultures than in the uninfected amoeba cultures (Table 2), one method for spread of *M. avium* to additional amoebae could be through killing of infected amoebae.

The ability of *M. avium* to replicate in amoebae, whereas *M. smegmatis* is readily killed, may be at least partially due to prevention of lysosomal fusion. In order to examine this possibility, we chose to compare the frequencies of lysosomal fusion of *M. avium* and *M. smegmatis* in amoebae. Since no specific markers exist for lysosomal proteins in *A. castellanii* we chose to examine lysosomal fusion using thorium dioxide, a lysosomal marker that should function regardless of cell type. We found that thorium dioxide was taken up well by *A. castellanii* and was found in vacuoles with characteristics similar to those of lysosomes in other cell types. The frequencies of lysosomal fusion with the *M. avium* and *M. smegmatis* vacuoles

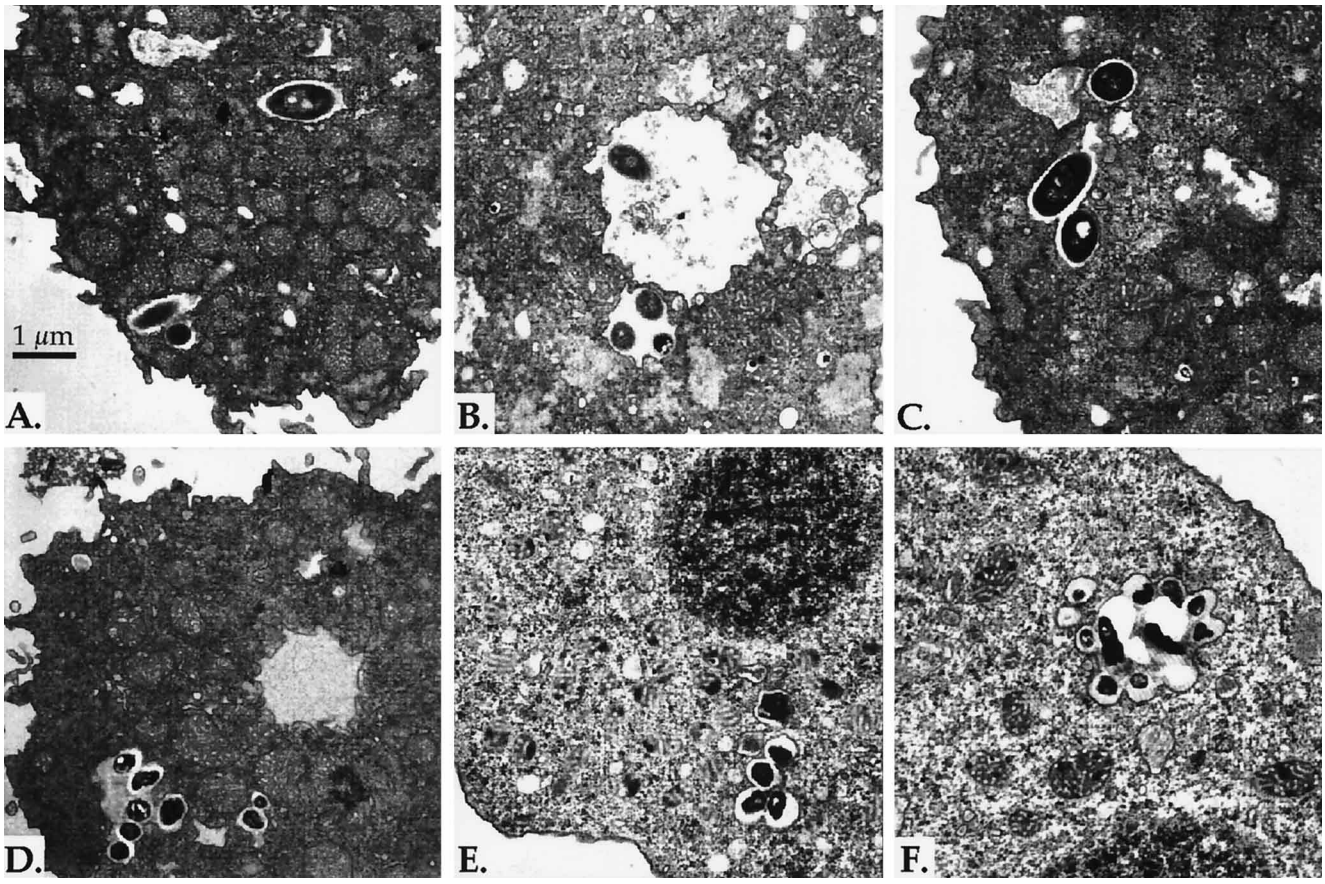

FIG. 2. Transmission electron micrographs of *M. avium* within *A. castellanii* at 5 min (A), 15 min (B), 1 h (C), 2 days (D), 3 days (E), and 5 days (F) following infection. The 1-μm bar shown in panel A applies to all panels.

were examined immediately after entry to 2 h following infection (Table 3; Fig. 3) by using thorium dioxide. Fusion of the *M. smegmatis* vacuole occurred as early as 5 min after entry (Fig. 3B), reaching approximately 90% fusion within 30 min (Table 3). At 30 min after entry the majority of the bacteria in the *M. smegmatis* infections were partially degraded (data not shown). In contrast, fusion of thorium-containing vesicles and the *M. avium* vacuole was never greater than 22% (Table 3). Thus, the survival of *M. avium* in amoebae may be due at least partially to the prevention of lysosomal fusion.

Effect of growth in amoebae on entry. Since *M. avium* is thought to replicate primarily intracellularly during disease, the ability to enter host cells is likely to be a key component of pathogenesis. To determine whether growth within an environmental amoeba might be associated with increased virulence, we examined the ability of amoeba-grown *M. avium* to enter host cells. We isolated *M. avium* that had been grown in amoebae for 1, 2, 3, or 5 days and carried out invasion assays with *A. castellanii* monolayers. As shown in Fig. 4A, *M. avium* grown intracellularly for 3 or more days invades *A. castellanii* with an 8- to 10-fold-greater efficiency than *M. avium* grown in 7H9 broth ($P < 0.01$). To rule out the possibility that growth in amoebae selects for invasive mutants rather than induction of an invasive phenotype, we carried out an assay in which bacteria retrieved from amoebae (after 5 days of infection) were grown in 7H9 broth for 5 days and subsequently used to infect another *A. castellanii* monolayer. No significant difference in the ability to invade amoebae was observed between *M. avium* grown first within amoebae and subsequently in 7H9 broth and *M. avium* grown only in 7H9 broth (data not shown). This observation is consistent with the hypothesis that the *M. avium* population grown in amoebae underwent a phenotypic change.

To determine whether growth within *A. castellanii* increased the ability of *M. avium* to invade host cells other than amoebae, we compared the entry into HT-29 epithelial cells and macrophages by amoeba- and 7H9-grown *M. avium*. As shown in Fig. 4B and C, intracellular growth in amoebae conferred upon *M. avium* an increased ability to invade both epithelial cells and macrophages. This phenomenon was not restricted to *M.*

TABLE 2. Percentage of infected and viable amoebae

Day after infection	% of amoebae		
	Infected[a]	Viable	
		Infected	Uninfected
1	31 ± 6	94 ± 5	97 ± 2
3	44 ± 5	81 ± 5	95 ± 2
5	48 ± 7	71 ± 6	95 ± 3
10	63 ± 5	66 ± 15	94 ± 3

[a] *A. castellanii* (10^6 amoebae per monolayer) was infected with *M. avium* at a ratio of 10 bacteria:1 amoeba. The percentage of infection was established after counting 200 cells in 10 different fields.

TABLE 3. Fusion of *M. avium* vacuole with lysosomes in amoebae

Time (min) after entry	% Fusion[a]	
	M. smegmatis	*M. avium*
0	28 ± 5	22 ± 3
5	70 ± 10	16 ± 2
15	78 ± 7	20 ± 4
30	86 ± 9	16 ± 1
60	94 ± 7	12 ± 2
120	92 ± 5	14 ± 2

[a] Percentage of bacterial vacuoles containing thorium. Results are the means ± standard errors for three counts of vacuoles within 50 amoebae on different sections of the same preparation. Data are from a representative experiment. Other than for time point 0 (fixation was done immediately after the addition of the bacteria and washing), the percentages of fusion are significantly different between *M. avium* and *M. smegmatis* for each time point ($P < 0.05$).

avium 101, since comparable results were obtained with other strains of *M. avium* (data not shown).

Virulence in vitro and in vivo. To examine the virulence of *M. avium* in an in vitro model of infection we infected human monocyte-derived macrophages with *M. avium* that had been grown in 7H9 broth and *A. castellanii* for 1, 3, and 5 days (Fig. 5). The number of intracellular CFU were quantitated immediately after infection and 3 days after infection to determine the ability of the bacteria from each growth environment to replicate intracellularly. We found that, in addition to invading macrophages at a higher frequency, *M. avium* grown within *A. castellanii* for periods of time equal to or greater than 3 days was capable of replicating inside macrophages with a greater efficiency than *M. avium* grown in 7H9 broth (Fig. 5). This phenomenon was not due to differences in the viability of the bacteria grown under different conditions since the viabilities of *M. avium* grown in *A. castellanii*, macrophages, and 7H9 broth are similar (Table 4).

To determine whether this enhanced virulence in the in vitro macrophage model is applicable to in vivo models, we examined the virulence of *M. avium* grown under different conditions in the C57BL/6 beige mouse model using the oral route of inoculation. *M. avium* was grown within amoebae for 3 days in 7H9 broth alone and in 7H9 broth in the presence of an amoebae lysate for 3 days at 37°C. We found that *M. avium* grown within amoebae was able to colonize the intestine (Fig. 6) and to replicate in both the liver and the spleen (Table 5) of beige mice significantly better than *M. avium* grown in broth. We also grew *M. avium* in different numbers of amoebae (10^6 to 10^4 amoebae per 10^6 bacteria), and rather than lysing the amoebae to harvest the bacteria as in previous experiments, the amoebae and bacteria were suspended and inoculated together. We found that the presence of higher numbers of viable amoebae in these preparations correlated with increased ability to colonize the intestine (Fig. 6) and to replicate in the liver and the spleen (Table 5). These data suggest that growth of *M. avium* in and interaction of *M. avium* with amoebae can enhance virulence.

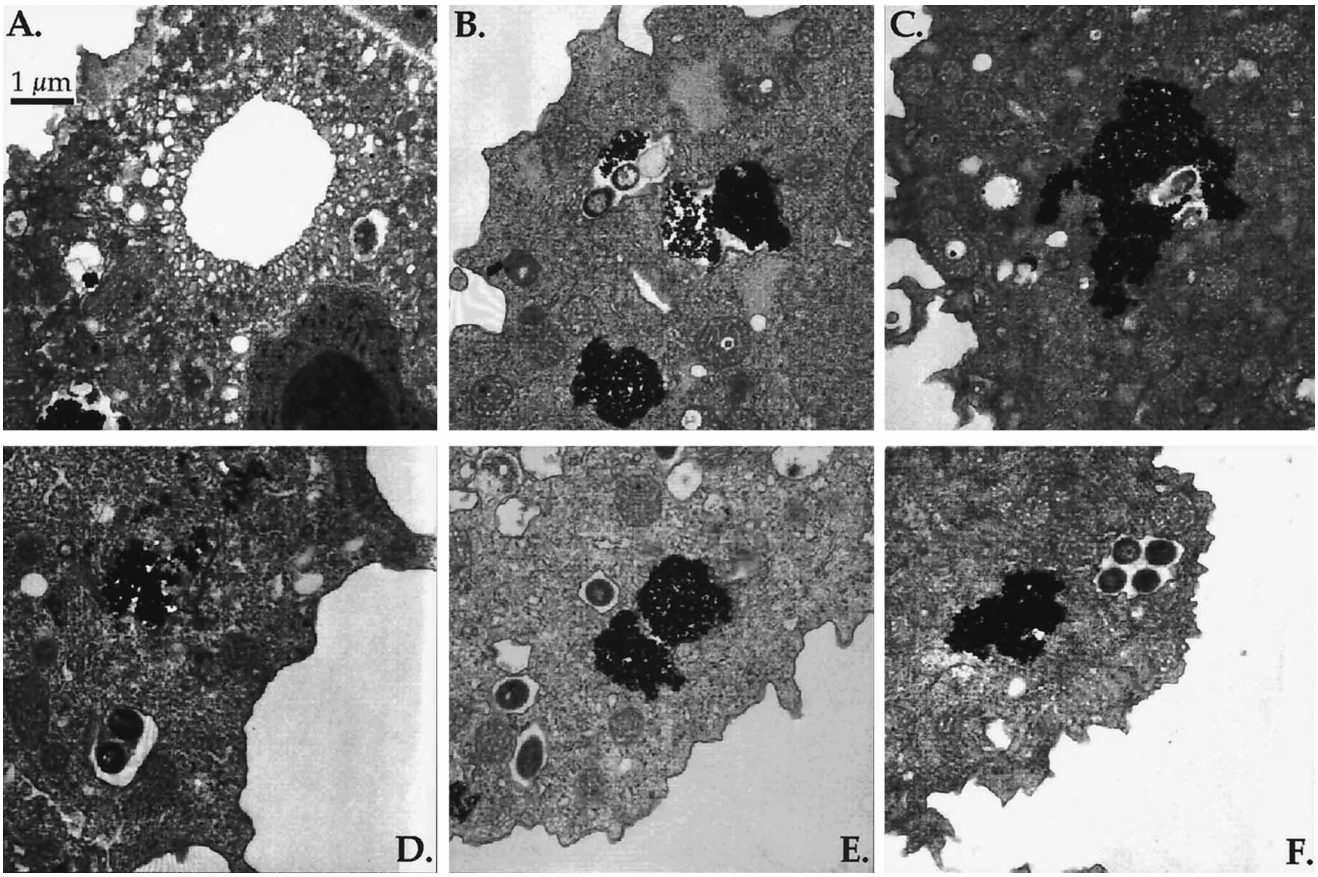

FIG. 3. Fusion of lysosomes containing thorium with *M. smegmatis* (A to C) and *M. avium* (D to F). The time points for the electron micrographs are immediately (A), 5 min (B and D), 15 min (C and E), and 30 min (F) after the addition of bacteria. The 1-μm bar shown in panel A applies to all panels.

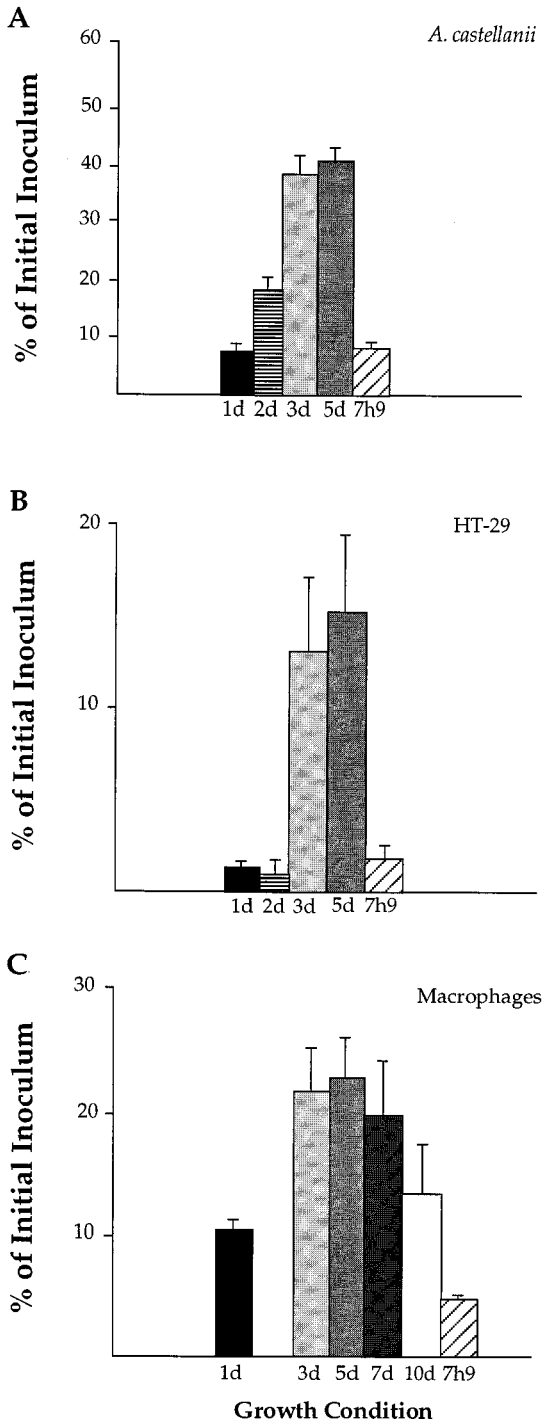

FIG. 4. Entry into *A. castellanii* (A), intestinal epithelial cell line HT-29 (B), and macrophages (C) by *M. avium* grown within *A. castellanii* for different periods of time. The percentage of inoculum at 1 day (1d) represents the invasive ability of *M. avium* strain 101 grown in amoebae for 1 day. These data were compared to those for 7H9-grown controls (7H9). Data are the means of triplicate wells, and the standard errors are represented by the error bars. Results shown are for a typical experiment.

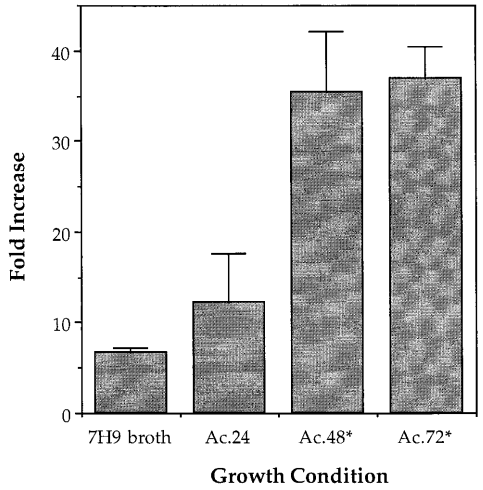

FIG. 5. Growth of intracellular *M. avium* in human peripheral blood monocyte-derived macrophages after growth in 7H9 broth or *A. castellanii* for 24 (Ac.24), 48 (Ac.48), or 72 (Ac.72) h. Growth is expressed as the fold increase in intracellular bacteria after 3 days in macrophages. Data are the means of triplicate wells, and the standard errors are represented by the error bars. Results shown are for a typical experiment. *, significant difference in results between this growth condition and growth in 7H9 broth ($P < 0.05$).

DISCUSSION

Both environmental amoebae (55) and *M. avium* (36) are ubiquitous organisms. Hot and cold water supplies have been shown to carry pathogenic bacteria (34, 76); however, warm water supplies are most frequently associated with bacterial infections, including *M. avium* (30, 34) and *Legionella* (23, 57). Although *M. avium* was found to replicate in amoebae at temperatures as low as 24°C, the highest growth rate was found to be near 37°C, the preferred growth temperature in laboratory medium (78). This observation may be due to a combination of the optimal growth temperature of *M. avium* and decreased bactericidal activity of the amoebae at this temperature (4). If growth of *M. avium* in water environments occurs primarily within protozoa, the fact that *M. avium* has temperature dependent growth in amoebae may explain why *M. avium* infections are more frequently associated with warm water supplies.

Interestingly, *M. avium* replicated more efficiently at both 32 and 37°C than both *M. fortuitum* and *M. marinum*. *M. marinum*, which has a growth optimum of approximately 32°C in laboratory medium (7, 78), was found to replicate in amoebae only at 32°C. These data are consistent with previous observations on the effects of temperature upon growth of *M. marinum* in macrophages, CHO cells (68), HeLa cells (73), and mice and various poikilothermic species (22). *M. fortuitum*, which has previously been shown to replicate in HeLa cells 37°C (72), was found to replicate both at 37°C and, to a lesser extent, at 32°C

TABLE 4. Viability of *M. avium* after growth under different conditions

Day after infection	% Viable bacteria in[a]:		
	A. castellanii	Macrophages	
		Growth in 7H9	Growth in amoebae
1	76 ± 5	79 ± 7	84 ± 4
3	82 ± 6	84 ± 9	83 ± 5
5	85 ± 7	83 ± 7	89 ± 6
10	80 ± 5	86 ± 5	88 ± 4

[a] Both *A. castellanii* and macrophages were lysed with 0.25% SDS, and viability was determined by the LIVE-DEAD assay. Data represent the means of triplicate wells ± standard errors. Data are from a representative experiment.

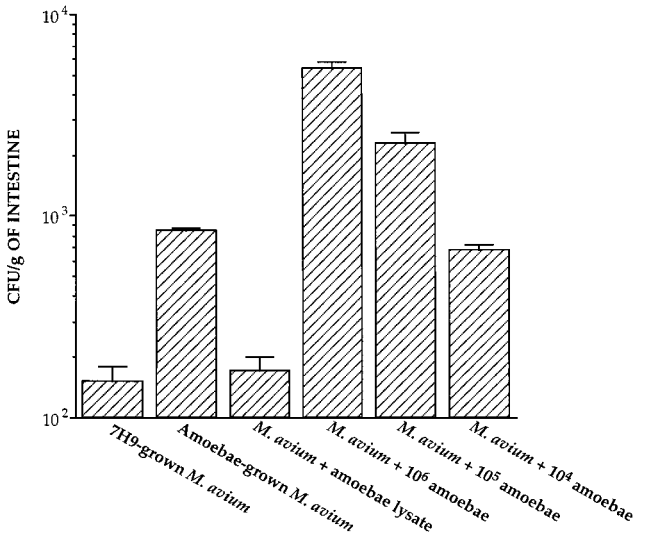

FIG. 6. Infection of intestinal mucosa by *M. avium*. Data are the means of duplicate platings of tissues from 16 mice, and the standard errors are represented by error bars. Inoculum sizes are as follows: for *M. avium* in broth, 2×10^6; for *M. avium* plus *A. castellanii* lysate, 1×10^6; for *M. avium* obtained from *A. castellanii*, 9.1×10^5; for *M. avium* plus *A. castellanii* (10^6 amoebae), 7.9×10^5; for *M. avium* plus *A. castellanii* (10^5 amoebae), 8.4×10^5; and for *M. avium* plus *A. castellanii* (10^3 amoebae), 6.2×10^5. Inoculation with each preparation was administered orally by gavage.

in amoebae. In addition, the nonpathogenic *M. smegmatis* strain was readily killed within amoebae at both temperatures. In tissue culture cells *M. smegmatis* normally persists, although it does not replicate (14). Thus, the amoebae appear to kill nonpathogenic mycobacteria very well. The ability of mycobacteria to replicate in amoebae correlated well with the virulence of the particular species tested. These data suggest that *A. castellanii* may provide a useful virulence model for studies of mycobacterial pathogenesis.

The ultrastructural characteristics of *M. avium* growth in amoebae were similar to those observed previously in HEp-2 cells (15) and murine macrophages (28, 33, 70). Intracellular survival was found to correlate with differences in intracellular trafficking. Thus, the ability of *M. avium* to survive intracellularly may be partially due to inhibition of lysosomal fusion in a similar manner to that observed in macrophages (28, 33). The presence of spacious vacuoles is a novel characteristic of the intracellular compartment for *M. avium* in amoebae. The fact that vacuoles of this type have not been observed in other host cells may be due to the transient nature of these vacuoles at early time points after entry. However, it is more likely that this observation relates to differences in the mechanisms of phagocytosis utilized by amoebae. The actual significance of this morphological difference is not yet understood. The role of spacious vacuoles, particularly if they are specific to the interaction of *M. avium* with amoebae, warrants further investigation.

Growth of *M. avium* in amoebae resulted in enhanced entry into amoebae, HT-29, and macrophages. These results are similar to those obtained after intracellular growth of *M. avium* in macrophages (12). This similarity suggests that *M. avium* grown in amoebae may also enter macrophages by a complement-independent mechanism of uptake similar to that of *M. avium* grown in macrophages (12). Since this phenotypic change is transient, the most likely explanation is that an alteration in the expression of genes involved in entry occurs during growth in the amoebae. Previous studies have provided evidence that a number of physiological changes occur in *M. avium* during intracellular growth (12, 67). Up-regulation of any of the factors suggested to be directly involved in entry (14) could explain this phenotype, or it may be due to as-yet-unidentified entry factors. Although up-regulation is the most intuitive explanation for this phenotype, down-regulation or modification of a gene product or products could clearly be an explanation as well.

Amoeba-grown bacteria are more virulent in the beige mouse model of infection. The observed increase in virulence may not be due to the same phenotypic changes observed in the in vitro models of infection. Growth in amoebae may have pleiotropic effects on the bacteria, and thus, the increased virulence in mice may be due to effects on other aspects of the host immune defense mechanisms. Since growth in amoebae enhanced the ability of *M. avium* to colonize the intestine, this may be one mechanism of increasing virulence. The inoculation of viable amoebae with *M. avium* also increased colonization of the intestine and the ability of *M. avium* to replicate in the liver and the spleen. This observation could be due to enhancement of the ability of *M. avium* to cross the intestinal epithelium. Amoebae in this assay should have the ability to transverse the intestinal epithelium through a mechanism similar to that used by *A. castellanii* to cause meningitis (20, 56) and could carry bacteria along with them.

Since growth of *M. avium* in *A. castellanii* results in increased virulence in animal models, it is important to consider the relevance of this observation to human infections. This amoeba

TABLE 5. Replication of *M. avium* in liver and spleen after oral infection

Experimental group[a]	CFU/g of tissue					
	Liver			Spleen		
	2 days	2 wk	4 wk	2 days	2 wk	4 wk
M. avium (amoeba grown)	2.4×10^{b}	4.7×10^{2b}	6.1×10^{3b}	9.2×10^{2b}	1.4×10^{4b}	3.1×10^{5b}
M. avium + 10^6 amoebae	3.9×10^{2b}	5.6×10^{3b}	1.1×10^{4b}	3.9×10^{4b}	3.7×10^{5b}	1.0×10^{6b}
M. avium + 10^5 amoebae	1.6×10^{b}	2.1×10^{2}	4.9×10^{3b}	5.0×10^{3b}	3.1×10^{4b}	4.2×10^{5b}
M. avium + 10^3 amoebae	$<10^{c}$	7.8×10	1.8×10^{3}	7.2×10^{2}	9.8×10^{3}	2.7×10^{5b}
M. avium alone	<10	1.5×10^{2}	9.5×10^{2}	1.6×10^{2}	2.8×10^{3}	1.6×10^{4}
M. avium + *A. castellanii* lysate	<10	3.7×10	9.1×10^{2}	2.1×10^{2}	1.9×10^{3}	2.2×10^{4}

[a] An inoculum of 10^6 bacteria was used for all experimental groups. *M. avium* (amoeba grown), amoeba-grown *M. avium* obtained by lysing *A. castellanii*-infected *M. avium* after 3 days; *M. avium* plus amoebae, 7H9-grown *M. avium* plus the indicated number of viable amoebae were administered in an oral inoculum. Data represent the means for duplicate platings of tissues from 16 mice.

[b] Significantly different ($P < 0.05$) from the result for 7H9-grown *M. avium* alone.

[c] Below the detectable level of 10 CFU/g of tissue.

and other similar protozoa are found in almost all water supplies and have been associated with infections by other bacteria that may utilize the amoeba as an environmental host (6, 61). There is recent evidence that *M. avium* infections may be associated with the presence of *M. avium* in domestic water supplies (34, 76, 77, 79). These data in combination with our observation that the amoebae represent a potential environmental host, thereby enhancing the virulence of *M. avium*, suggest that there may be an association between this growth environment and human infections. However, in order to test this hypothesis, we must determine whether there is a correlation between the presence of amoebae with *M. avium* in water supplies and infections in humans. These data also suggest that further examination of the genes regulated by growth of *M. avium* in amoebae may result in a better understanding of the factors responsible for *M. avium* entry and intracellular replication. Thus, the environmental amoeba, *A. castellanii*, represents a new model for the study of mycobacterial virulence mechanisms.

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