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T-Cell-Mediated Immunity in Persistent *Mycobacterium intracellulare* Infections in Mice

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Growth of mouse-virulent *Mycobacterium intracellulare* D673 and TMC 1405 in the lung was affected by T-cell depletion in susceptible C57BL/6 mice. Significant differences also occurred between the growth patterns seen in congenitally athymic (*nu/nu*) mice and their *nu/+* littermates. Treatment of the mice with an immunosuppressive regimen of cyclosporin A (75 mg/kg of body weight per day subcutaneously) provided further evidence of the importance of T cells in controlling growth of *M. intracellulare* in the normal host. Adoptive transfer experiments indicated the presence of a T-cell-mediated specific protective immunity against a subsequent *M. intracellulare* challenge when transfer was carried out 3 weeks after immunization of the donor host. At this time, cross-protective immunity was also observed against a virulent *M. tuberculosis* challenge. There was no difference in the rate of growth by *M. intracellulare* as challenge in *Mycobacterium bovis* BCG-activated or normal peritoneal macrophages from C57BL/6 mice tested in vitro during a 7-day period. However, *M. tuberculosis* growth rates were decreased substantially in the BCG-activated macrophages. These studies suggest that mice infected with *M. intracellulare* do not eliminate the infection, because this organism can resist the bactericidal activity of the T-cell-activated macrophage better than *M. tuberculosis* can.

A number of atypical mycobacteria are opportunistic human lung pathogens (16) that cause disseminated disease only when there is some other underlying illness, such as pneumoconiosis or chronic bronchitis, or when the patient has been immunosuppressed because of cancer chemotherapy or exposure to the human immunodeficiency virus (3). Acquired resistance to pulmonary tuberculosis has long been recognized as a T-cell-mediated response, the growth of the organisms being controlled after the activation of blood monocytes as they enter the developing tubercle (10, 11). Much less is known about the immune response developed against virulent members of the *Mycobacterium avium* complex which seem to survive and grow within lungs with little or no sign of acquired resistance on the part of the host (2). As a result, the *M. avium*-infected host may not develop detectable levels of delayed type hypersensitivity, and this persistent anergy has been shown experimentally to be mediated by suppressor cells within the heavily infected spleen (15). Virulent members of the *M. avium* complex appear to be remarkably resistant to the killing action of hydrogen peroxide, which is known to be released by immunologically activated macrophages during the expression of cellular immunity to some intracellular pathogens (7, 12). Considerable differential susceptibility by different substrains of these mycobacteria after exposure to activated macrophages in vitro has been reported (6, 13). However, there is still relatively little information about the host-parasite events which lead to the establishment of these chronic atypical mycobacterial infections within the tissues.

The present study shows that T-cell depletion can affect the in vivo growth of virulent members of *M. avium* complex. Thus, it seems likely that a T-cell-mediated antituberculous response is involved in limiting the growth of these organisms in the normal immunocompetent host. However, despite this immune response, virulent *M. intracellulare* strains appear to be almost totally resistant to the bactericidal action of *Mycobacterium bovis* BCG-activated macrophages, compared with the response observed with the *M. tuberculosis*-infected controls. This resistance may explain the continued persistence of *M. intracellulare* infections in both normal and BCG-vaccinated mice.

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MATERIALS AND METHODS

Animals. Specific-pathogen-free innately susceptible C57BL/6 mice were bred at the Trudeau Animal Breeding Facility, Saranac Lake, N.Y. Groups of mice, 7 to 9 weeks old at the time of their infection, were fed sterilized commercial mouse chow and acidified water ad libitum (4). Congenitally athymic (*nu/nu*) mice and their littermates (*nu/+*) bred on a BALB/c background were maintained under barrier conditions and were fed autoclaved food and water. BALB/c mice, 4 weeks old, were surgically thymectomized, and 1 week later, some of them were lethally irradiated (900 rads) and infused intravenously with 10^7 syngeneic bone marrow cells. These mice and a group of sham-thymectomized controls were infected 5 to 6 weeks later (10).

Bacteria. Strains of *M. intracellulare* (D673, high virulence; TMC 1405, medium virulence), *M. tuberculosis* Erdman (TMC 107), and *M. bovis* BCG (TMC 1011) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y. The organisms were grown in modified Sauton liquid medium, incubated at 37°C, and harvested during the late logarithmic growth phase before being stored in 1-ml ampoules at −70°C (5). Viable counts were carried out in randomly selected vials whose contents were thawed at 37°C, homogenized briefly, and plated on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.). The plates were incubated for 3 weeks at 37°C in sealed plastic bags before the colonies were counted.

Immunosuppressive regimen. Cyclosporin A (CsA) was kindly provided by J. F. Borel, Sandoz Ltd., Basel, Swit-

zerland. The powder was dissolved at 60°C in sterile olive oil at a concentration of 20 mg/ml. Mice received daily 0.1-ml injections of this solution given subcutaneously over the periods indicated below. Controls received an equivalent volume of olive oil only (14).

Experimental infection protocol. The contents of a frozen ampoule of *M. intracellulare* were thawed at 37°C, briefly sonicated, and diluted appropriately in sterile saline (2). Aerogenic challenges were carried out in a Middlebrook chamber (Tri-R Instruments, New York, N.Y.) by using a suspension known to deposit approximately 10^3 to 10^4 CFU into the lung over a 30-min period (2). Five randomly selected mice were sacrificed after 24 h, and the lung homogenates were plated on 7H11 agar to check the inoculum size (4).

Enumeration of bacterial populations in vivo. Spleens and lungs were removed aseptically from five randomly selected mice at monthly intervals and were homogenized separately in sterile cold saline. The number of viable bacteria was determined by plating serial 10-fold saline dilutions on Middlebrook 7H11 agar. In some experiments, the 7H11 agar was supplemented with 2 μg of 2-thiophenecarboxylic acid hydrazide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml to inhibit the growth of the BCG (2).

Adoptive transfer of antituberculous resistance. Spleens were removed, and single-cell suspensions were prepared in RPMI 1640 medium (GIBCO Diagnostics, Detroit, Mich.) containing 2% heat-inactivated fetal calf serum. Suspensions containing 5×10^7 cells were depleted of macrophages by adherence to plastic petri dishes (4030; Miles Laboratories, Inc., Naperville, Ill.) during incubation at 37°C in 5% CO_2-enriched air for 60 min (12). Nonadherent cells were harvested, washed, and suspended in fresh RPMI 1640 medium. Cell viability was determined by trypan blue dye exclusion and was always >90%. One spleen equivalent of cells (approximately 5×10^7 to 8×10^7 cells), with or without prior treatment with anti-Thy 1.2 antibody plus complement (14), was infused intravenously into each of the age- and sex-matched syngeneic recipients that had been rendered T cell deficient by exposure to 500 rads of whole-body gamma irradiation 24 h prior to the transfer. The mice were challenged intravenously with either 10^5 CFU of *M. tuberculosis* Erdman or 10^7 CFU of *M. intracellulare* D673 or TMC 1405 about 16 h later. Carry-over of viable *M. intracellulare* was checked by means of viable counts carried out on each spleen cell suspension. Less than 1% of the cells were infected with bacteria per recipient (10^5 CFU). The number of viable *M. tuberculosis* cells was determined 14 days postchallenge, while the *M. intracellulare* counts were carried out after 30 days.

Growth of *M. intracellulare* in macrophage monolayers in vitro. Macrophages were harvested from the peritoneal cavities of mice which had been injected intraperitoneally with 2 ml of sterile 3.8% casein about 3 days earlier (13). The cells were suspended at a concentration of 5×10^5/ml in RPMI 1640 medium which was supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2). Samples of each cell suspension (1 ml) were placed on glass cover slips (13 mm) held in 24-well culture plates (3047; Becton Dickinson Labware, Oxnard, Calif.) and incubated at 37°C in 5% CO_2-enriched air for 1 h to allow adherence of the macrophages. Replicate monolayers were washed thoroughly with warm culture medium to remove the nonadherent cells and incubated for 4 h with 1 ml of a bacterial suspension containing approximately 10^6 viable bacteria. Non-cell-associated bacteria were removed by repeatedly washing the monolayers with phosphate-buffered saline, and the cover slips were then transferred to new 24-well culture plates and reincubated in complete medium for up to 7 days in 5% CO_2-enriched air (13).

Immediately after infection and again at daily intervals, medium was removed from selected cover slips and replaced with 0.25 ml of a nucleus-counting solution consisting of 0.1 M citric acid, 1% Triton X-100, and 0.05 g of naphthol blue black per 100 ml, adjusted to pH 2.2 with 1 mM sodium hydroxide (9). The plates were kept at room temperature for

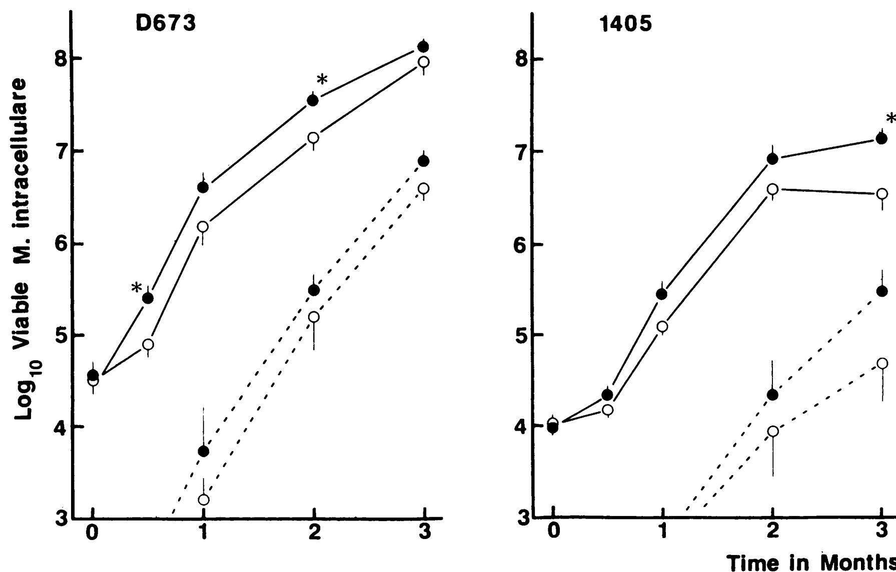

FIG. 1. Effect of T cell depletion on growth of *M. intracellulare* D673 and TMC 1405 in the lung (——) and dissemination into the spleen (----). Thymectomized, irradiated, and bone-marrow-reconstituted (●) and sham-thymectomized (○) mice were aerogenically infected with approximately 10^4 CFU of bacteria. Data are expressed as mean ± standard error of the mean (n = 4 or 5). Symbol: *, $P < 0.05$.

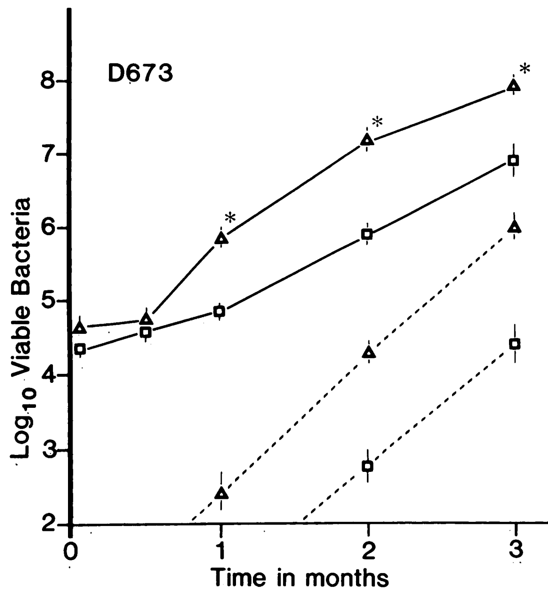

FIG. 2. Growth of *M. intracellulare* D673 in lungs (——) and dissemination into the spleen (– – –). BALB/c mice, *nu/nu* (△) and *nu/+* (□), were aerogenically infected with approximately 10^4 CFU of bacteria. Data are expressed as mean ± standard error of the mean (n = 4). Symbol: *, $P < 0.005$.

15 min, and the number of nuclei per cover slip was determined microscopically. To enumerate viable bacteria in these preparations, 0.1 ml of this solution was neutralized with 0.9 ml of 0.1 M $NaHPO_4$, and samples were plated onto 7H11 agar. At each time point, three cover slips were counted and the number of viable bacteria per 10^5 macrophages was calculated. In some experiments, a second set of three cover slips was fixed in 10% formaldehyde made up in ethanol and stained sequentially with auramine O and acridine orange (Sigma Chemical Co., St. Louis, Mo.) to determine the percentage of macrophages containing intracellular mycobacteria (13). Activated macrophages which had been harvested from the peritoneal cavities of mice immunized intravenously 3 weeks earlier with 10^7 CFU of BCG were also tested. These mice were injected intraperitoneally with 2 ml of 3.8% casein plus 10^8 heat-killed BCG 3 days prior to harvest of the macrophages, and the activated macrophages were collected as before. The replication index for the challenge inoculum was calculated by dividing the number of viable bacteria counted after 7 days by that observed at 4 h (13).

RESULTS

Growth of *M. intracellulare* in T-cell-depleted mice. Mice infected aerogenically with 10^4 CFU of *M. intracellulare* D673 or TMC 1405 showed substantial growth within the lungs that spread to the spleens of the innately susceptible C57BL/6 mice (Fig. 1). Both bacterial strains induced persistent and ultimately fatal infections in the T-cell-depleted C57BL/6 mice, but the C57BL/6 sham-thymectomized control mice did not succumb to this challenge. The rate of dissemination of the infection from the lungs to the spleen was accelerated in both strains of T-cell-depleted mice. However, the actual differences in the bacterial counts seen at 3 months were statistically significant only in the less virulent TMC 1405 strain.

When congenitally athymic nude (*nu/nu*) mice were tested in a second study, the amount of *M. intracellulare* growth seen in both the lungs and the spleen was substantially greater than in the immunocompetent (*nu/+*) controls (Fig. 2). This suggests that the level of T-cell depletion at the time of challenge may be an important factor in determining the severity of the resulting atypical mycobacterial infection.

Effect of CsA treatment on *M. intracellulare* infection. The severity of the *M. intracellulare* infection in mice subjected to CsA treatment was then examined. A group of C57BL/6 mice were infected aerogenically with 10^4 CFU of *M. intracellulare* D673 or TMC 1405, and a daily regimen of CsA (75 mg/kg of body weight per day subcutaneously) was implemented 2 months later. Growth of both *M. intracellulare* strains was increased by this immunosuppressive treatment (Table 1), resulting in an almost 10-fold-higher viable count for the TMC 1405 within the lungs after only 30 days of CsA treatment. The D673 increases were again less substantial during the course of this experiment.

Adoptive transfer of acquired resistance, using *M. intracellulare*-infected donor mice. Spleen cells were harvested 21, 60, and 90 days after a group of B6D2 mice received 10^7 CFU of *M. intracellulare* D673 or TMC 1405. Single-cell suspensions were depleted of adherent cells, along with most of the residual mycobacteria, and their ability to transfer antituberculous resistance to sublethally irradiated syngeneic recipients was determined (Table 2). No significant reduction was observed in the spleens of mice that were immunized with *M. intracellulare* 3 months previously. However, substantial levels of resistance did occur when the transfer was carried out 3 weeks after immunization.

Spleen cells harvested from the immunized mice were then examined for their ability to transfer cross-protective resistance to *M. tuberculosis*. BCG-vaccinated mice were used as positive controls. Reductions (approximately 10-

TABLE 1. Effect of CsA treatment on *M. intracellulare* infection[a]

Strain	Treatment	Log_{10} viable bacteria in lung (mean ± SEM) on day[b]: 60	75	90
D673	Olive oil	7.35 ± 0.16	7.79 ± 0.12	8.05 ± 0.17
	CsA	ND	7.94 ± 0.14 (0.15)	8.40 ± 0.12 (0.35)
TMC 1405	Olive oil	6.72 ± 0.13	6.65 ± 0.18	6.97 ± 0.13
	CsA	ND	7.30 ± 0.19 (0.65)[c]	7.96 ± 0.17 (0.99)[d]

[a] C57BL/6 mice were infected aerogenically with 10^4 CFU of *M. intracellulare* D673 or TMC 1405 on day 0. CsA (75 mg/kg per day) was injected subcutaneously from day 61 to 89.
[b] n = 4 or 5. Values in parentheses indicate replication indices. ND, Not done.
[c] $P < 0.05$.
[d] $P < 0.005$.

TABLE 2. Transfer of specific acquired immunity to *M. intracellulare* in C57BL/6 mice[a]

Type of immunization and cell source	Log_{10} bacteria in spleens (mean ± SEM) on day[b]:		
	21	60	90
Aerogenic			
10^4 D673	6.85 ± 0.04	6.41 ± 0.04	6.33 ± 0.03
Control	6.66 ± 0.02 (−0.20)	6.05 ± 0.08 (−0.36)	6.44 ± 0.10 (0.11)
10^4 TMC 1405	5.57 ± 0.06	5.80 ± 0.03	5.70 ± 0.13
Control	5.55 ± 0.15 (0.02)	5.51 ± 0.19 (−0.29)	5.32 ± 0.10 (−0.38)
Intravenous			
10^7 D673	5.98 ± 0.07	7.49 ± 0.20	ND
Control	6.38 ± 0.08 (0.40)[c]	7.19 ± 0.08 (−0.30)	
10^7 TMC 1405	5.21 ± 0.09	6.01 ± 0.09	ND
Control	5.41 ± 0.07 (0.20)	5.62 ± 0.13 (−0.39)	

[a] Donors were immunized either aerogenically or intravenously with *M. intracellulare* D673 or TMC 1405 on day 0. Recipients were challenged intravenously with 10^7 CFU of *M. intracellulare* D673 or TMC 1405, and the number of viable bacteria in the spleens was determined 30 days later. Controls were treated with anti-Thy 1.2 antibody plus complement.

[b] $n = 4$ or 5. Values in parentheses indicate replication indices. ND, Not done.

[c] $P < 0.005$.

fold) in the number of viable *M. tuberculosis* cells seen in the recipient of the BCG-immune T-cells were significant compared with the number of cells in controls that received an equivalent number of spleen cells treated with anti-Thy-1.2 antibody plus complement. The *M. intracellulare*-immune T cell expressed a significant level of protection against the subsequent *M. tuberculosis* challenge, and this protection was seen on days 21 and 60 after immunization (Table 3).

In vitro growth of *M. intracellulare* in macrophage monolayers. Attempts to explain why the vaccinated mice were unable to provide significant protection against a subsequent *M. intracellulare* challenge led to a comparison of the bactericidal activity of macrophage monolayers prepared from BCG-vaccinated and control C57BL/6 mice.

The growth of *M. tuberculosis* was substantially depressed in the BCG-activated macrophages. However, rates of growth for both strains of *M. intracellulare* in these macrophage monolayers were much less affected, and after 4 days of incubation, the amounts of growth in the activated macrophages were little different from those seen in the control preparations. The replication indices for these preparations were compatible with the same conclusion (Fig. 3). This experiment was repeated three times, and the result was confirmed. Both mouse-virulent strains of *M. intracellulare* (D673 and TMC 1405) proved to be markedly more resistant to the action of BCG-activated macrophages than was the virulent *M. tuberculosis*.

DISCUSSION

The importance of the T-cell defenses in limiting the severity of a persistent *M. intracellulare* infection can be inferred from the enhanced growth seen in the T-cell-depleted and nude mice (Fig. 1 and 2), an observation confirmed by CsA treatment of chronically infected mice (Table 1). Both tuberculin hypersensitivity and acquired antituberculous resistance are severely depressed by such treatments, apparently because the ability of the effector T cells to produce the necessary lymphokines in vivo is affected (14).

In both *M. tuberculosis*- and BCG-infected mice, the bacterial infection is controlled, and eventually reduced, by a specific T-cell-mediated immune response (1, 10). However, in the corresponding *M. intracellulare*-infected animals, the bacterial counts did not decline with time, although splenic T cells harvested from these mice were able to transfer acquired resistance to syngeneic recipients (Table 2). This finding appears to be consistent with earlier observations of minimal protective immunity against a secondary challenge with *M. intracellulare* (8). This limited response could be due to the release of insufficient amounts of protective antigen(s) by the slow-growing *M. intracellulare* in the primary lesion. *M. intracellulare* may resist the digestive ability of the antigen-presenting cells, making it difficult for the host defenses to develop adequate T-cell responses. Another possibility could be the induction of suppressor cells (both T cells and macrophages) by the excessive amounts of mycobacteria within the heavily infected spleen. This would reduce lymphokine production

TABLE 3. Transfer of cross-protective immunity to *M. tuberculosis* in C57BL/6 mice

Cell source[a]	Log_{10} bacteria in spleen (mean ± SEM) on day[b]:	
	21	60
BCG (10^7 i.v.)	5.98 ± 0.07	6.02 ± 0.13
Control	6.91 ± 0.11 (0.98)[c]	7.01 ± 0.11 (0.99)[c]
D673 (10^7 i.v.)	5.51 ± 0.12	5.20 ± 0.09
Control	6.24 ± 0.17 (0.73)[d]	5.90 ± 0.05 (0.70)[c]
TMC 1405 (10^7 i.v.)	5.45 ± 0.08	5.46 ± 0.07
Control	6.40 ± 0.06 (0.95)[c]	5.96 ± 0.06 (0.50)[e]

[a] Donors were immunized intravenously (i.v.) on day 0. Recipients were challenged intravenously with 10^5 CFU of *M. tuberculosis* Erdman, and the number of viable *M. tuberculosis* in spleens was determined 14 days later. Controls were treated with anti-Thy 1.2 antibody plus complement.

[b] $n = 4$ or 5. Values in parentheses indicate replication indices.

[c] $P < 0.001$.

[d] $P < 0.05$.

[e] $P < 0.005$.

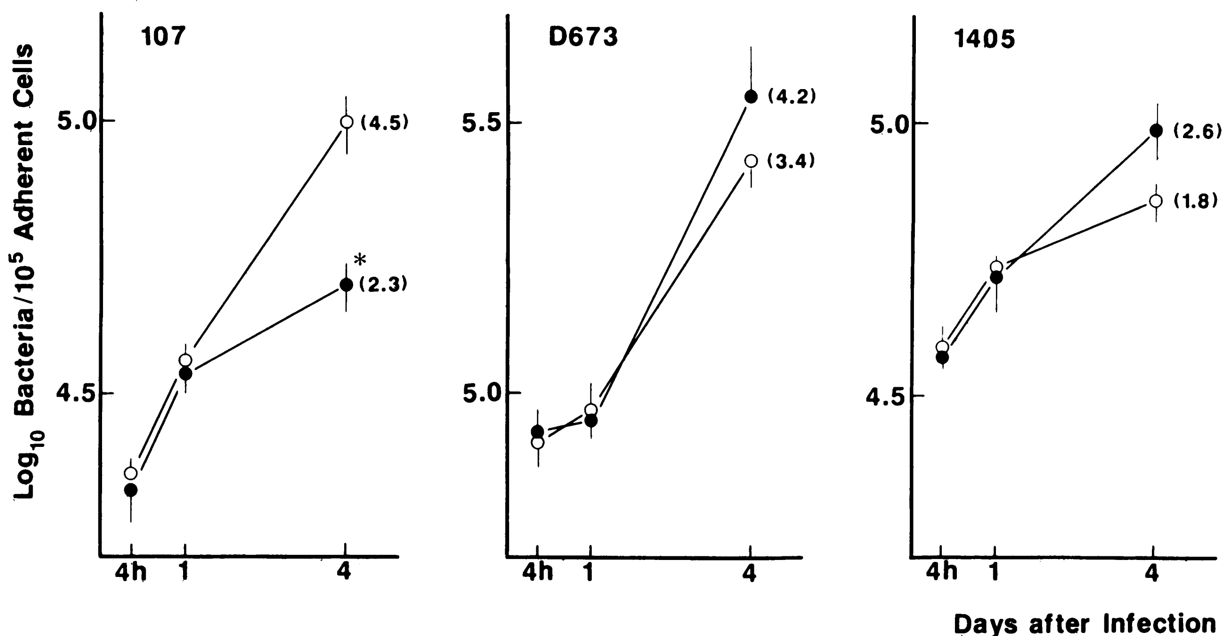

FIG. 3. In vitro growth of *M. tuberculosis* Erdman (TMC 107) and *M. intracellulare* (D673 and TMC 1405) in the peritoneal macrophages from nonimmunized (○) and BCG-immunized (●) C57BL/6 mice. Data are expressed as mean ± standard error of the mean (n = 3). Symbol: *, $P < 0.05$.

affecting the ability of the host to activate the blood monocytes as they enter the tubercle (15). Such an explanation seems unlikely, however, in view of the ability of *M. intracellulare*-infected mice to provide cross-protection against an *M. tuberculosis* challenge (Table 3). *M. intracellulare* appears to induce an adequate T-cell-mediated response capable of providing some protection against the heterologous challenge, and the reason these cells cannot express specific immunity is still unclear. However, the heterologous challenge may be partially inhibited by nonspecifically activated macrophages. It is clear that the growth of atypical mycobacteria is not greatly affected by these cells expressing an intrinsic resistance to immunologically activated macrophages (12). There have been reports of differential susceptibility to alpha interferon-activated macrophages (6), and *M. intracellulare* has also been reported to be more resistant to the action of hydrogen peroxide than *M. tuberculosis* (7). Whatever the nature of this difference, the fact remains that mice infected with atypical mycobacteria are unable to eliminate the heavy bacterial load within the chronic tissue granulomas. Substantial T-cell responsiveness may be present in the lymphoreticular organs draining the heavily infected tissues, yet these cells seem to have little effect on the outcome of the chronic infection within the lungs and spleen.

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