

## *Mycobacterium marinum* Persists in Cultured Mammalian Cells in a Temperature-Restricted Fashion

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**We have explored the relatively rapidly growing animal and human pathogen *Mycobacterium marinum* as an experimental model for mycobacterial pathogenesis. *M. marinum*, which has a lower temperature for optimal growth than does *Mycobacterium tuberculosis*, has a much shorter generation time and can be safely studied in ordinary laboratory facilities and examined in multiple animal infection models. We have established an in vitro assay for its interaction with eukaryotic cells and shown that it persists in these cells in a temperature-specific fashion that correlates with its ability to cause disease in vivo at lower temperatures. Additionally, preliminary evidence that *M. marinum* causes a chronic disease with some features resembling tuberculosis in frogs of the species *Rana pipiens* is presented.**

The study of mycobacterial disease has been confounded by the extremely slow growth of *Mycobacterium tuberculosis* and the need for strict isolation (P3) facilities to prevent laboratory infection (3, 10, 23). To circumvent the problems associated with the study of pathogenesis using *M. tuberculosis*, we have explored a surrogate mycobacterial model system. For this purpose, we selected *Mycobacterium marinum*, a species closely related to members of the *M. tuberculosis* complex, as judged by 16S rRNA sequence analysis (17). *M. marinum*, a relatively rapidly growing species, has a generation time of 4 h versus over 20 h for *M. tuberculosis*. *M. marinum* grows optimally at 25 to 35°C and poorly or not at all at 37°C (1, 4). It was first isolated from dying saltwater fish in which a tuberculosis-like systemic disease was found (1). Consistent with its optimal growth at lower temperatures, inoculation into mice causes ulceration of the cooler body surfaces but no disseminated disease (4). In contrast, disseminated disease has been produced experimentally in 50 poikilothermic species, as well as in mice whose body temperatures were lowered to 25°C and in chick embryos maintained at 33°C but not at 37°C (4). Conversely, a strain of *M. marinum* adapted to optimal growth at 37°C caused disseminated systemic disease when injected into mouse footpads or tail vein, with a disease pattern similar to that of tuberculosis (5). Furthermore, prior footpad inoculation with this strain was found to protect mice from *M. tuberculosis* disease. All of the studies described above suggest that the species spectrum of *M. marinum* pathogenicity is related to its low temperature optimum for growth.

An attractive feature of *M. marinum* is that it is a well-recognized human pathogen but, consistent with its optimal growth temperatures, causes only localized nodular and ulcerated lesions on the cooler surfaces of the extremities (14, 24). The disease is called swimming pool granuloma or aquarium tank granuloma and occurs in individuals exposed to contaminated water. The histopathology of the disease resembles that of *M. tuberculosis* (9). However, in humans, as in other warm-blooded animals, dissemination to systemic organs oc-

curs extremely rarely, even in the immunocompromised population (13, 16, 22). Hence, *M. marinum* has the unique advantage of being a naturally occurring human pathogen which can be safely handled with basic bacteriological precautions and without P3 facilities.

Here, we report our studies on the interactions of *M. marinum* with cultured animal cells and the development of a laboratory animal model for chronic granulomas.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Three isolates of *M. marinum* were used: strain M, a human patient isolate, and strain C, an American Type Culture Collection isolate, both obtained from the clinical laboratories of Moffitt Hospital at the University of California, San Francisco, and strain K, a human patient isolate from the clinical laboratory of the Stanford University School of Medicine. Strain C was used as a reference strain by the clinical laboratory. Strains M and K were isolated from tissue samples of human lesions characteristic of *M. marinum* infection, typed by the clinical laboratories, and found to be *M. marinum* as judged by several criteria, including primary isolation at 30°C and photochromogenicity. All of the studies reported here were done using isolate M, except for the animal studies, in which all three isolates were used. The selection of these strains was based on preliminary light microscopy experiments which showed that they all associated with eukaryotic cells. The selection of strain M for the majority of the experiments was based on our finding that it is susceptible to more antibiotics than the others and thus has the potential to be easier to use in subsequent genetic and molecular biological studies. Furthermore, our preliminary data (see Results) suggested that strain M gave more extensive histopathological disease in frog tissues.

The original isolates were restreaked for single colonies, one of which was grown at 33°C in liquid 7H9 medium (Difco, Detroit, Mich.) supplemented with 0.5% glycerol, 10% oleic acid-albumin-glucose complex, 0.01% cycloheximide, and 0.25% Tween 80 for 7 to 9 days. The cultures were then frozen at -70°C in 50% glycerol in 1-ml aliquots. Before use, a drop of the frozen culture was inoculated into 10 ml of liquid growth medium and grown without shaking for 5 to 7 days. This

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culture was kept at 4°C, and 1 ml was used to inoculate 10-ml cultures which were used for the experiments after they had been grown for 3 days, corresponding to a density of  $5 \times 10^7$  to  $5 \times 10^8$  bacteria per ml. Cultures of *Mycobacterium smegmatis* mc<sup>2</sup>155 (10) were grown in the same way from a frozen aliquot at 37°C. Viable counts were obtained by plating appropriate dilutions in phosphate-buffered saline (PBS) on solid 7H9 bacteriological medium supplemented with 0.5% glycerol, 10% oleic acid-albumin-glucose complex, and 0.01% cycloheximide. Colonies were counted after 5 to 7 days for *M. marinum* and 2 to 3 days for *M. smegmatis*.

**Eukaryotic cell lines.** The mouse macrophage cell line J774A.1 (ATCC TIB67) was maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (high glucose) containing 584 mg of glutamine (Gibco, Bethesda, Md.) per liter supplemented with 10% fetal bovine serum (Gibco) and 1 mM sodium pyruvate. Chinese hamster ovary (CHO-K1) cells (ATCC CCL 61) and HEp-2 cells (ATCC CCL 23) were maintained in RPMI 1640 (Cell Culture Laboratories, Cleveland, Ohio) supplemented with 100 mM L-glutamine (Mediatech, Washington, D.C.) and 5% fetal bovine serum.

**Invasion and persistence assay.** Eukaryotic cells were seeded into 24-well microdilution plates at a density of  $2 \times 10^4$  cells per well in 1 ml of medium 18 to 24 h prior to use. Bacterial cultures were diluted into fresh tissue culture medium to achieve a multiplicity of infection (MOI) of approximately 1 for J774A.1 cells and approximately 10 for the epithelial cell lines. This medium was used to replace the one in which the cells had been grown overnight. The infection was allowed to proceed for 3 h at either 33 or 37°C, and the wells were then washed twice with Dulbecco's modified Eagle medium in the case of J774A.1 cells and PBS in the case of the epithelial cell lines. The cells were then incubated in 1 ml of tissue culture medium containing 200 µg of amikacin (Sigma Chemicals, St. Louis, Mo.) per ml for 2 h at 37°C. The cell monolayers were again washed twice, and those being assessed for initial cell-associated bacteria were lysed as described below. Those being assessed for intracellular bacterial persistence were incubated at 33 or 37°C in tissue culture medium containing 20 µg of amikacin per ml. This medium was replaced either daily or every other day with fresh medium containing 20 µg of amikacin per ml. Prior to analysis of intracellular bacterial counts, the cell monolayer was washed twice and a sample of the medium was removed for assessment of residual extracellular bacterial counts. (In all experiments, the number of residual extracellular bacteria was, at most, 5% that of the intracellular bacteria at both 33 and 37°C.) The monolayer was then lysed with 0.1 ml of 1% Triton X-100 for 15 min. The lysed monolayer was brought up to a total volume of 1 ml with PBS and plated at appropriate dilutions for intracellular bacterial cell counts. Intracellular bacteria were expressed as raw numbers for the persistence assay. For assessment of initial cell association, bacterial numbers were expressed as a percentage of the inoculum.

**Assay for cytopathic effect.** J774A.1 cells were infected with bacteria and maintained as in the persistence assay for 7 days at both 33 and 37°C. The medium was removed, and the monolayers washed twice with PBS, fixed in 1 ml of methanol for 10 min, and then stained with 1 ml of Giemsa stain (EM Diagnostic Systems, Inc., Gibbstown, N.J.) diluted 20-fold in water for 30 min. The stain was removed, and the dishes were washed three times with PBS prior to visual assessment.

**Fixation of cells for electron microscopy.** Cells were grown in Permax 35-mm-diameter dishes (Nunc, Inc., Naperville, Ill.), infected with bacteria, and maintained as described for the invasion and persistence assay. Fixation was performed as

described elsewhere (6). Briefly, prior to fixation the monolayers were washed once in PBS and fixed for 90 min with 2% glutaraldehyde (Polysciences, Warrington, Pa.) and 1% OsO<sub>4</sub> (Polysciences) in 0.1 M sodium phosphate buffer, pH 7.4, at 4°C. Samples were then postfixed in 0.5% uranyl acetate overnight or 1% uranyl acetate for 1 h at 4°C. Following dehydration with a series of solutions containing ascending ratios of ethanol and water mixtures, samples were embedded in Polybed-812 (Polysciences). Sections were made, stained with uranyl acetate and lead citrate, and examined in a model 201c electron microscope (Philips Electronic Instruments Company, Eindhoven, The Netherlands). For analysis, three to four grids containing sections cut from different regions of the sample were examined. Approximately 100 cells were examined for each sample. In the case of samples in which no intracellular bacteria were seen, the analysis was extended to examine approximately 300 cells.

**Maintenance, inoculation, and examination of frogs.** Male or female young adult frogs of the species *Rana pipiens* were housed in the biohazard suite of the Department of Laboratory Animal Medicine of Stanford University, where the ambient temperature was approximately 25°C. They were inoculated with the three isolates of *M. marinum* suspended in 0.5 ml of either PBS or 7H9 liquid medium by intraperitoneal injection. Frogs were killed by an intraperitoneal injection of 100 mg of tricaine in 1 ml of saline, and their tissues were examined for gross pathology. Portions of their tissue were put into 10% formalin in PBS for histopathological examination, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Separate portions of their tissue were placed in 5 ml of PBS in a sterile plastic bag and gently washed free of adherent bacteria with a Stomacher Lab-Blender 80 (Tekmar, Cincinnati, Ohio). Two hundred microliters of the homogenate was plated on 7H9 solid medium containing 50 µg of ampicillin and 25 µg of gentamicin. Plates were assessed for the presence of colonies after incubation at 33°C for 7 to 10 days.

## RESULTS

***M. marinum* and *M. smegmatis* associate with macrophages at similar levels.** The optimal growth of *M. marinum* at temperatures lower than 37°C led us to assay cell association levels with macrophages and epithelial cells at both 33 and 37°C. At 3 h after infection, levels of *M. marinum* association with J774A.1 and CHO-K1 cells were similar at 33 and 37°C (Fig. 1A and B). We also wanted to determine if the level of association of the nonpathogen *M. smegmatis* was different from that of *M. marinum* in the case of J774A.1 cells. *M. smegmatis* initially associated with J774A.1 cells at a level only slightly lower than that of *M. marinum* (Fig. 1C). We also compared levels of association of *M. marinum* and *M. smegmatis* with J774A.1 cells at 3 h postinfection, using nearly identical MOIs. In a representative experiment in which the MOI was 2.8, the level of cell association for *M. marinum* was 37.7% (standard error of the mean [SEM], 10.7%) at 37°C and 23.7% (SEM, 1.7%) at 33°C. In the same experiment, in which the MOI for *M. smegmatis* was 2.9, its level of cell association was 13.2% (SEM, 1.3%) at 37°C and 11.4% (SEM, 1.0%) at 33°C.

***M. marinum* replicates in eukaryotic cells in vitro selectively at 33°C.** Because of the predilection of *M. marinum* to cause systemic disease in animals at lower temperatures, we examined its long-term interactions with eukaryotic cells in vitro at 33 and 37°C. The J774A.1 macrophage cell line and two epithelial cell lines, CHO-K1 and HEp-2, were infected with *M. marinum* and then maintained at 33 and 37°C, respectively,

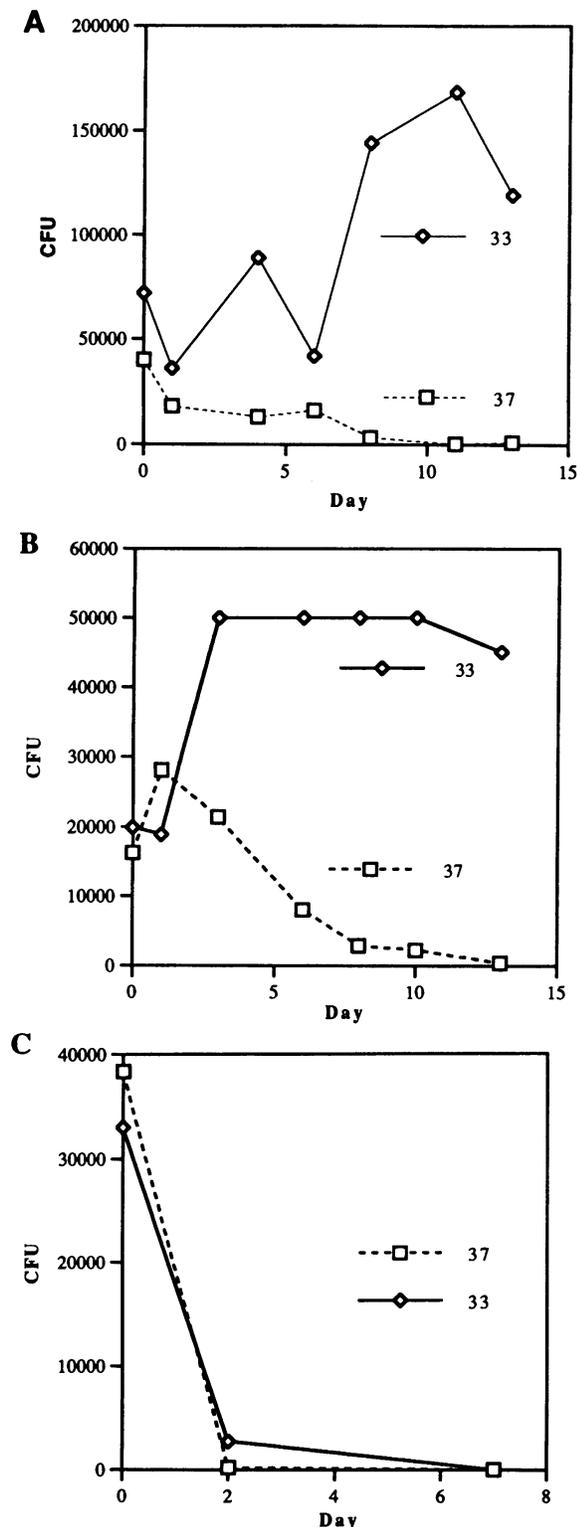


FIG. 1. Persistence of *M. marinum* in tissue culture cells. (A) Representative of three individual experiments showing the colony counts of *M. marinum* at various time points after infection of J774A.1 cells at 33 and 37°C. A MOI of 0.6 was used. (B) Representative of two independent experiments showing the colony counts of *M. marinum* at various time points after infection of CHO cells at 33 and 37°C. A MOI of 20 was used. (C) Representative of three independent experiments showing the colony counts of *M. smegmatis* at various time points after

for up to 14 days. As shown in a representative experiment in Fig. 1, the initial levels of cell association at both temperatures were similar, but thereafter, a profound difference emerged in the pattern of *M. marinum* intracellular persistence and replication. *M. marinum* did not replicate in J774A.1 cells at 37°C, and the viable count steadily declined (Fig. 1A). In contrast, at 33°C, there was a 2.3-fold increase in the number of intracellular bacteria by day 11. After 12 days, the number of intracellular *M. marinum* cells declined and by 14 days was nearly undetectable (Fig. 1A and data not shown). This decline in intracellular bacteria was associated with a sudden increase in the number of bacteria in the extracellular medium, suggesting that infected J774A.1 cells were being lysed once the intracellular bacterial burden had reached a certain level. A similar pattern of *M. marinum* intracellular replication at 33 but not at 37°C was noted in the cultured epithelial cell lines, CHO-K1 (Fig. 1B) and HEP-2 (data not shown). We also examined the growth of *M. marinum* in the cell culture medium at the two temperatures in the absence of eukaryotic cells. It was found to grow at 37°C, albeit more slowly, with a longer lag phase than at 33°C. This adaptation to slower growth at 37°C is typical for *M. marinum* strains following primary isolation at the lower temperature.

In marked contrast to the pattern observed with *M. marinum*, the nonpathogen *M. smegmatis*, which grows equally well at 33 and 37°C (data not shown), did not persist in J774A.1 cells at either temperature and was undetectable by day 2 of the assay (Fig. 1C). This implies that the ability of *M. marinum* to replicate selectively at the lower temperature is due not to impaired defenses of the macrophages at 33°C but rather to the properties of the bacteria themselves. To further ascertain that this differential effect was not due to impaired macrophage function, the opportunistic pathogen *Mycobacterium fortuitum*, which also grows equally well at 33 and 37°C (data not shown), was tested in the same persistence assay. *M. fortuitum* persisted equally in J774A.1 cells at both temperatures for up to 7 days after infection (data not shown). Furthermore, in the case of both *M. smegmatis* and *M. fortuitum* infection, the eukaryotic cells looked equally healthy at both 33 and 37°C.

**Selective intracellular replication of *M. marinum* at 33°C is confirmed by transmission electron microscopy.** J774A.1 cells were examined by transmission electron microscopy at 3 h and 3, 7, 9, and 14 days after infection with *M. marinum*. At a MOI of 10, 2 to 20 bacteria were observed in approximately 10% of the cells at 3 h at both 33 and 37°C (Fig. 2a and c). By 3 days, there was evidence of *M. marinum* intracellular replication at 33°C, whereas virtually no bacteria were seen in several hundred J774A.1 cells maintained at 37°C (data not shown). At 7 and 9 days, there was marked increase in the number of intracellular *M. marinum* cells in the infected cells maintained at 33°C (Fig. 2b and data not shown). In addition, the number of infected J774A.1 cells had also increased to between 30 and 50%, suggesting that there is some cell-to-cell spread during the course of the in vitro infection (Fig. 2b). Consistent with the results of the colony counts from the persistence assay, no *M. marinum* cells were seen in approximately 300 cells examined at either 7 or 9 days at 37°C (Fig. 2d and data not shown). At 14 days, no bacteria were detectable at either temperature (data not shown). This result corroborated those from the

infection of J774A.1 cells at 33 and 37°C. A MOI of 2.9 was used. In each case, the day 0 time point reflects bacterial counts after 3 h of infection.

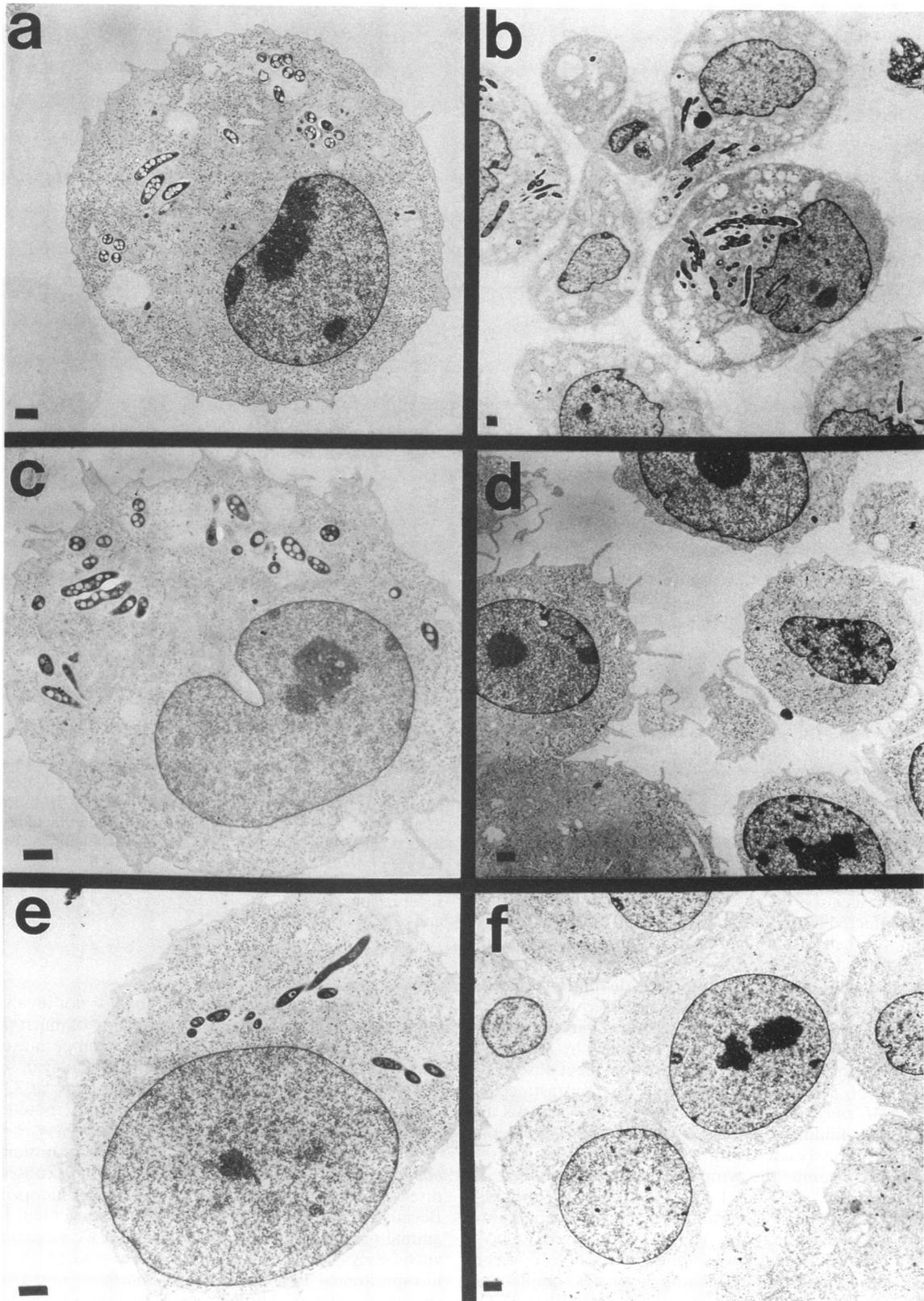


FIG. 2. Transmission electron micrographs of *M. marinum* and *M. smegmatis* infection of J774A.1 cells. The MOI was 5 for *M. marinum* and 1.1 for *M. smegmatis*. (a and b) *M. marinum* at 3 h and 7 days after infection, respectively, in cells maintained at 33°C; (c and d) *M. marinum* at 3 h and 7 days after infection, respectively, in cells maintained at 37°C; (e and f) *M. smegmatis* at 3 h and 3 days after infection, respectively, in cells maintained at 33°C. Bars represent 1  $\mu$ m.

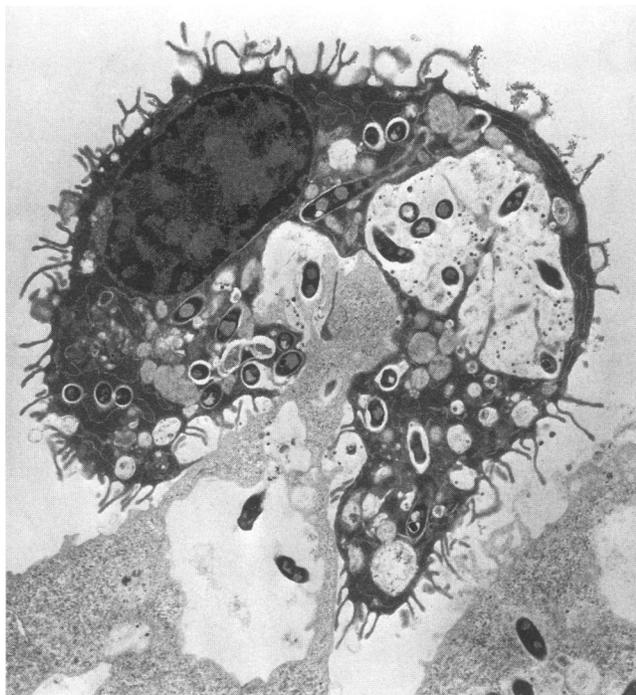


FIG. 3. Transmission electron micrograph showing cytopathic effect of *M. marinum* in J774A.1 cells infected with *M. marinum*. The MOI was 10, and the cells were maintained at 33°C for 7 days.

quantitative persistence assay, in which very few bacteria were seen at 14 days. The interaction of *M. smegmatis* with J774A.1 cells at both 33 and 37°C was also examined. *M. smegmatis* was initially internalized by J774A.1 cells at both temperatures at levels comparable to those of *M. marinum* (Fig. 2e and data not shown). However, by days 3 and 7, no intracellular *M. smegmatis* cells were observed in hundreds of cells examined at either 33 or 37°C (Fig. 2f and data not shown).

***M. marinum* infection of J774A.1 macrophages is associated with a cytopathic effect.** Examination of infected J774A.1 cells by transmission electron microscopy revealed that many of the cells heavily infected by *M. marinum* were either dying or dead. A representative cell with a high bacterial burden is shown in Fig. 3; it is dead, as evidenced by its small, pyknotic nucleus pushed to one side and by the complete disruption of cell organelles. Interestingly, it is being phagocytosed by a neighboring macrophage which also contains some bacteria. This latter phenomenon has been observed occasionally and may partially account for the progressive cytopathic effect observed in the course of *M. marinum* infection. In contrast, cells infected with *M. smegmatis* showed no discernible changes in morphology. Our findings demonstrate that *M. marinum* replication in cells is associated with a cytopathic effect. These findings led us to examine the cytopathic effect of *M. marinum* at a macroscopic level in J774A.1 cells. At a MOI of 1 or less, no cytopathic effects were discernible by the eye or by light microscopy. However, when the MOI was increased to between 10 and 65, cell death was apparent by 4 to 5 days postinfection, and by 7 days, the cell monolayer was completely destroyed. The effect was found only at 33°C and not at 37°C and was easily discerned by Giemsa staining of the plates (Fig. 4A and B). No cytopathic effect was observed when cells were incubated with the supernatant in which the *M. marinum* had been grown, for 3 h (Fig. 4C and D) or even for 24 h (data not

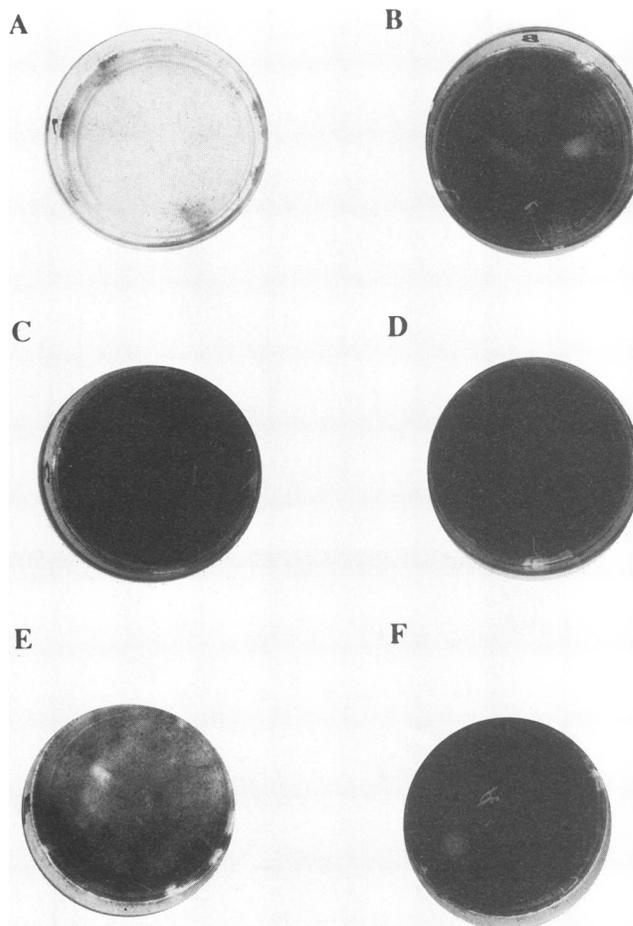


FIG. 4. Giemsa staining showing cytopathic effect of *M. marinum* in J774 cells. Tissue culture dishes were stained with Giemsa stain after *M. marinum* and *M. smegmatis* infection of J774A.1 cells for 7 days at 33 and 37°C. The MOI of *M. marinum* was 12, and that of *M. smegmatis* was 25. The supernatant of *M. marinum* was obtained from a liquid culture of *M. marinum* corresponding to a MOI of 120. (A and B) *M. marinum* at 33 and 37°C, respectively; (C and D) *M. marinum* supernatant at 33 and 37°C, respectively; (E and F) *M. smegmatis* at 33 and 37°C, respectively.

shown), suggesting that a secreted toxin is not involved in this effect. As expected, *M. smegmatis* infection of macrophages did not lead to a cytopathic effect at either temperature (Fig. 4E and F). The slight patchiness of the monolayer seen when J774A.1 cells were infected with *M. smegmatis* at 33°C in this experiment (Fig. 4E) was not consistently noted in other experiments.

***M. marinum* induces chronic systemic granulomas in *R. pipiens*.** It has been reported that *M. marinum* causes systemic disease and death in a number of poikilothermic species (1, 4). Because it was important to establish a convenient laboratory animal model using the same strain that we were using for in vitro assays and genetic studies, we studied the effect of intraperitoneal inoculation of *M. marinum* into frogs of the species *R. pipiens*. Three frogs were injected with isolate M (the one chosen for our in vitro and genetic studies), four were injected with isolate K, and six were injected with isolate C. In the case of strain M, one of the frogs was inoculated with  $1.3 \times 10^6$  bacteria and the remaining two were injected with  $1.3 \times$

$10^7$  bacteria. For isolate K, two of the frogs received  $1.5 \times 10^6$  bacteria and the remaining two got  $1.5 \times 10^7$ . In the case of C, three of the frogs got  $4.5 \times 10^6$  bacteria while the other three got  $4.5 \times 10^6$  bacteria. In addition, two frogs were inoculated with PBS. In contrast to previous reports (1, 4), none of the frogs succumbed to disease by 7 weeks postinoculation. At this time, one of the frogs inoculated with the higher dose of each of the three strains and one that had been injected with PBS were killed and their tissues were examined. Macroscopic examination of their organs revealed no change in liver, spleen, kidney, lungs, gut, and mesentery and, in the case of the one male frog, testes. In the frog which had received isolate M, microscopic examination of the liver, kidney, and spleen revealed the presence of extensive granulomas (Fig. 5a-d, g, and h), particularly in the spleen, where the tissue was almost completely replaced by granulomas (Fig. 5g and h). Interestingly, the granulomas were localized to the pleural lining of the lung tissue (Fig. 5e and f). The granulomas were composed of epithelioid macrophages as well as sparse acid-fast bacilli (Fig. 5i and j, respectively), features typical of the chronic lesions of *M. tuberculosis* disease. Disease was also present in the frogs which had received isolates K and C, but it was much less pronounced than in the one inoculated with isolate M (data not shown). Culture of the livers, spleens, kidneys, lungs, and testes yielded growth of *M. marinum* in all the infected frogs but not in the control frogs.

At 6 months, the remaining frogs were killed and the livers and spleens of five of the frogs infected with *M. marinum* isolates (two with C, two with M, and one with K) as well as from the remaining control frog were sampled for growth of acid-fast bacilli. Again, *M. marinum* was recovered from all of the infected frogs but not from the control frog. Histopathological examination of the livers and spleens from all of the frogs was done. None of the K- and C-infected frogs showed evidence of disease, despite the presence of *M. marinum* in their tissues. Of the two frogs which had been inoculated with isolate M, only the one that had received  $1.3 \times 10^7$  bacteria showed evidence of disease. Disease was as severe as that found in the frog which had received  $1.3 \times 10^7$  bacteria of strain M and that had been examined at 7 weeks. The other frog, which had received  $1.3 \times 10^6$  bacteria, had no evidence of granulomas despite the continued presence of *M. marinum* in its organs.

## DISCUSSION

We have developed an in vitro assay for invasion and persistence of *M. marinum* in eukaryotic cells. Of particular interest is the demonstration that it replicates in macrophage and epithelial cells in a fashion consistent with its disease pattern in humans and other animals. This result suggests that our persistence assay is a valid in vitro correlate of its in vivo pathogenicity. We have also shown that its selective persistence at the lower temperature of 33°C is due not to impaired host cell defenses at that temperature but rather to the optimal growth of *M. marinum*. It is noteworthy that the progression of infection of J774A.1 cells is strikingly similar to that of a virulent strain of *M. tuberculosis*, H37Rv, as assessed by both intracellular replication and cell-to-cell spread (15).

The selective persistence of *M. marinum* at lower temperatures both in vitro and in vivo is likely a reflection of its optimal growth at the lower temperature. *M. marinum* has a much longer generation time at 37°C (14.3 h) than at 33°C (4.6 h) in 7H9 broth (4). All of the strains that we have examined, including five human isolates and one fish isolate, grow at 37°C, albeit more slowly than at 33°C.

The in vitro cytopathic assay that we have developed correlates well with the replication of *M. marinum* within macrophages. Our initial results indicate that this effect is not mediated by a soluble cytotoxin, such as the cytolysin recently identified in *M. tuberculosis* (12). Rather, our electron microscopic studies show suggest that intracellular bacterial replication to a critical level kills the eukaryotic cells. Such cytopathic effects have been noted for other pathogenic mycobacteria by light microscopy, particularly in the case where *M. fortuitum* was allowed to grow in HeLa cells (19). Foci corresponding to cytopathic effects of *M. tuberculosis* growing intracellularly in tissue culture cells have also been reported (7). We do not know if such in vitro cytopathic effects per se are relevant to the pathogenicity of mycobacteria. However, the correlation of this effect with the intracellular replication of *M. marinum* in our assay makes it the basis for a simple and rapid genetic screen for invasion- and persistence-deficient mutants. Furthermore, the results of this assay lend hope that a plaque assay can be developed for *M. marinum*.

All mycobacterial species studied, pathogenic and nonpathogenic, can be internalized both by epithelial (HeLa) cells and by macrophages (18-21). The distinguishing feature of the pathogenic species appears to be their unique ability to survive and replicate in HeLa cells and macrophages (15, 18-20). Our results also show that despite similar levels of entry, the natural human and animal pathogen *M. marinum* and the opportunistic human pathogen *M. fortuitum* survive in macrophages while the nonpathogen *M. smegmatis* does not. This study corroborates the results of Shepard (18-20) as well as those of McDonough et al. (15) that intracellular replication and persistence rather than entry into macrophages reflect the pathogenicity of mycobacteria. The mechanism of intracellular persistence is virtually unknown. Recently, a gene fragment from *M. tuberculosis* which permits transformed *Escherichia coli* to be internalized by HeLa cells and to exhibit increased survival and replication in macrophages at 24 h after infection was isolated (2).

Like other mycobacteria, *M. marinum* is also able to enter both macrophages and epithelial cells, although our preliminary data suggest that the level of entry into epithelial cells is lower. This may suggest the existence of multiple routes of entry into eukaryotic cells, perhaps involving diverse pathways and receptors. Such diverse entry mechanisms exist for other intracellular organisms, such as *Toxoplasma gondii* (11), and in this case, the mode of entry has been shown to impact on intracellular survival. Our data show that *M. marinum* is able to replicate in a similar fashion both in J774A.1 macrophages which express complement receptors and in two epithelial cell lines which do not. This finding suggests that more than one pathway of entry may be conducive to intracellular survival. It will be of great interest to carry out more-detailed studies of intracellular localization of *M. marinum* in different cell types in the presence and absence of additional cellular factors such as complement. The ease of working with the system that we have developed makes it an ideal one in which to test the role of various pathways of entry and their impact on intracellular localization and replication.

The animal model that we have established for *M. marinum* using *R. pipiens* has some distinctive features. Previous studies report an acute ulcerative disease in frogs and other poikilothermic animals when either a fish or a human isolate of *M. marinum* was injected intraperitoneally (1, 4). In most cases, the animals died within a few weeks of infection and numerous acid-fast bacilli were found in their tissues. However, tissue histopathology was not reported. In contrast, we found a chronic disease with very few intralesional organisms that

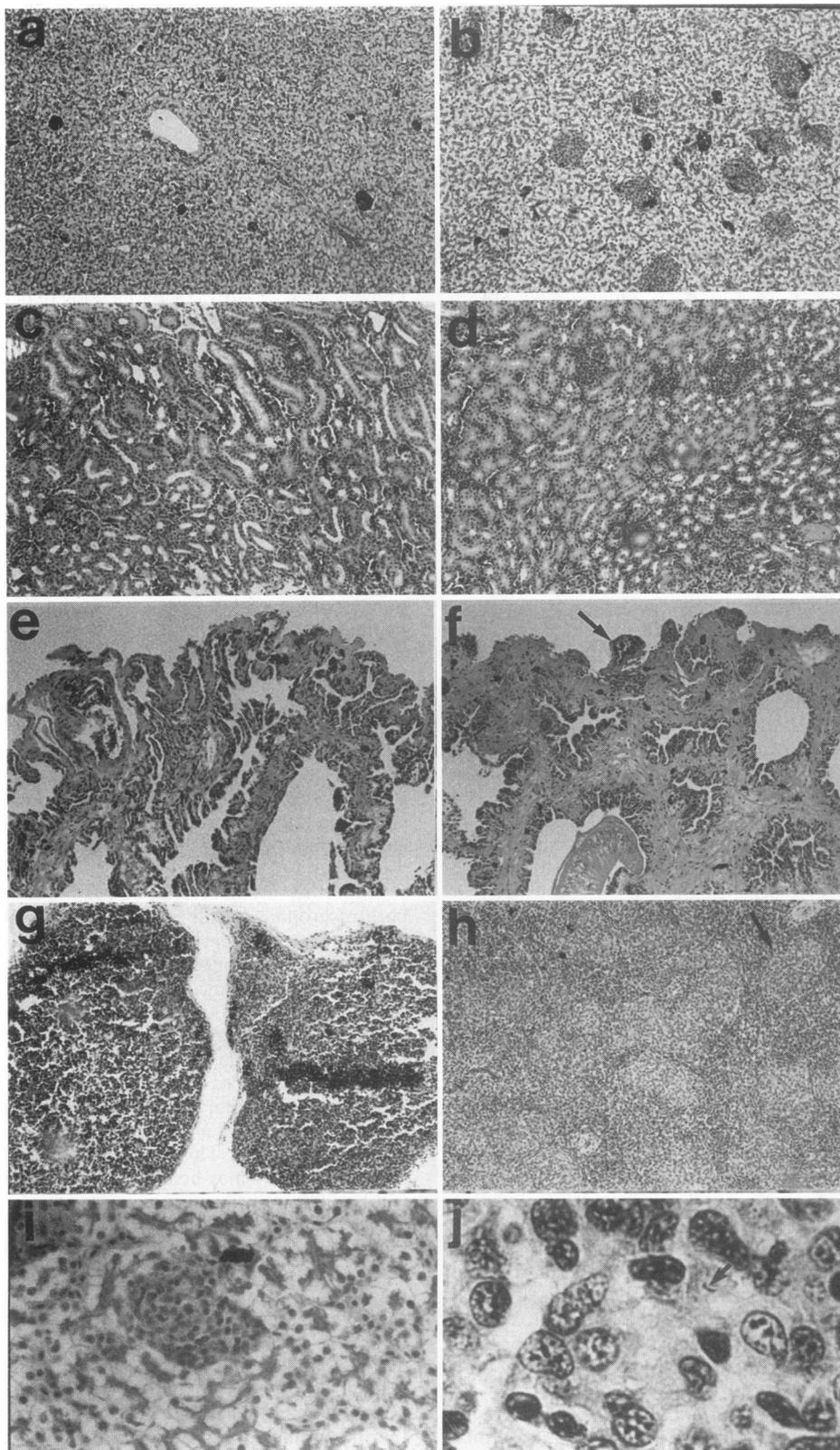


FIG. 5. Histopathology of tissues obtained from frogs infected with *M. marinum* for 7 weeks. (a to i) Tissues stained with hematoxylin and eosin. Magnification:  $\times 10$  (a to h),  $\times 40$  (i), and  $\times 100$  (j). (a and b) Normal and diseased liver, respectively; (c and d) normal and diseased kidney, respectively; (e and f) lung tissue showing normal and diseased pleura, respectively (arrow points to a representative granuloma); (g and h) normal and diseased spleen, respectively (arrow points to a representative granuloma); (i) granuloma in diseased liver; (j) tissue from diseased spleen stained with carbol fuchsin (arrow points to an acid-fast bacillus within a granuloma).

resembles *M. tuberculosis* disease. Indeed, the histopathology of the lesions that we found resembles that of the peripheral lesions caused by *M. marinum* in humans which have been confused with *M. tuberculosis* disease (9, 14). *M. marinum* has been previously recognized as the causative agent of a chronic wasting disease in fish, taking up to 1 year to kill them (8). It is possible that the type of disease seen in frogs is influenced by the pathogenicity of individual strains, the host response, and the inoculum. Nevertheless, our results suggest that the strain that we have chosen for our work may serve as a suitable model for mycobacterial disease as judged by its chronic course, the typical histopathology with chronic granulomas, and the sparsity of acid-fast bacilli in the lesions. We are currently assessing the pathogenicity of *M. marinum* in a greater number of animals.

In addition to the studies reported here, we have been successful in establishing basic genetic tools in *M. marinum*, including genetic transformation and random transposon mutagenesis (15a). We hope to isolate mycobacterial virulence factors by coupling these genetic tools with the *in vitro* persistence and cytopathic assays and the laboratory animal disease model. Our results so far demonstrate that this largely overlooked pathogenic mycobacterial species may indeed serve as an excellent model system for mycobacterial pathogenesis.

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