

## Fish Monocytes as a Model for Mycobacterial Host-Pathogen Interactions†

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***Mycobacterium marinum*, a relatively rapid-growing fish and human pathogen, has become an important model for the investigation of mycobacterial pathogenesis. *M. marinum* is closely related to the *Mycobacterium tuberculosis* complex and causes a disease in fish and amphibians with pathology similar to tuberculosis. We have developed an in vitro model for the study of *M. marinum* virulence mechanisms using the carp monocytic cell line CLC (carp leukocyte culture). We found that fish monocytes can differentiate between pathogenic and nonpathogenic mycobacterial species. Interestingly, *M. marinum* enters fish monocytes at a 40- to 60-fold-higher rate than *Mycobacterium smegmatis*. In addition, *M. marinum* survives and replicates in fish monocytes while *M. smegmatis* is killed. We also found that *M. marinum* inhibits lysosomal fusion in fish monocytes, indicating that these cells may be used to dissect the mechanisms of intracellular trafficking in mycobacteria. We conclude from these observations that monocytic cells from fish, a natural host for *M. marinum*, provide an extremely valuable model for the identification and characterization of mycobacterial virulence determinants in the laboratory.**

Tuberculosis, caused by *Mycobacterium tuberculosis*, is currently the number one cause of death worldwide from a single infectious agent (11, 28). Genetic analysis of the molecular mechanisms of pathogenesis by *M. tuberculosis* is ongoing, but the few putative virulence determinants that have been identified are not well understood and specific inactivation of many of these genes by allelic exchange has not been accomplished. The fact that few specific mutations have been constructed in *M. tuberculosis* may be at least partially due to the fact that homologous recombination is more efficient in rapidly growing mycobacterial species (49, 50). Due to the low growth rate of *M. tuberculosis*, difficulty of manipulation, risk to research personnel, and cost of running a biosafety level three facility, model systems that would allow a better understanding of the causes of tuberculosis are of great interest (8, 12). Recently, it has become apparent that *Mycobacterium marinum* has all of the necessary characteristics of an ideal model organism for genetic analysis of *M. tuberculosis* pathogenesis. *M. marinum* has a generation time of 4 h compared to 20 h for *M. tuberculosis* (17, 34), it is a biosafety level two organism, human disease caused by *M. marinum* presents almost exclusively as lesions on the extremities (4, 18, 20), and construction of specific mutations by homologous recombination is relatively straightforward (52, 53). For these reasons, there is heightened interest in *M. marinum* as a model for the study of mycobacterial pathogenesis (6, 51, 54, 73).

Since *M. marinum* is a natural pathogen of poikilothermic organisms (17), the fish (70) and frog (54) animal models should offer the opportunity to closely approximate natural mycobacterial infections in the laboratory. *M. marinum* is pre-

dominantly an aquatic organism that causes systemic tuberculous infections in fish and amphibians (17, 27, 46). This bacterium was first isolated from dying saltwater fish in 1926 (3) and later identified as a human pathogen (40, 47). Humans are infected by exposure to contaminated water or infected fish (34, 38). The primary reason that *M. marinum* only causes lesions on the extremities in humans is thought to be its optimal growth temperature of 25 to 35°C (17). Despite its preference for lower temperatures, the histopathology of *M. marinum* infections in both fish and humans is characterized by granuloma formation (20, 74) with similarities to those seen in human tuberculosis (20, 32). Furthermore, *M. marinum* is very closely related to the *M. tuberculosis* complex by 16S rRNA and DNA-DNA homology (58, 71). These characteristics make *M. marinum* a facile and relevant model for mycobacterial pathogenesis.

It has been shown that *M. marinum* can persist (7) and replicate (44, 51) in murine macrophages, as well as in a number of epithelial cell lines (62, 64), while the nonpathogenic mycobacterial species *Mycobacterium smegmatis* cannot (10, 51). Most of these experiments, however, were carried out at sub-optimal growth temperatures for the host cells which might affect bacterium-host cell interactions. In addition to the above cell lines, environmental protozoa have been used as in vitro models for *M. marinum* (14). Although protozoa grow well at the optimum temperatures for *M. marinum* studies, a close association between *M. marinum* and protozoa in the environment has not been well established. Since fish are natural hosts for *M. marinum*, fish monocytes should allow us to examine host-pathogen interactions in cells from a natural host at the proper temperature.

In the present study, we characterize a carp monocytic cell line known as CLC (carp leukocyte culture), established from the common carp *Cyprinus carpio* (30). This cell line has an optimum growth temperature of 28°C, well within the preferred temperature range for *M. marinum*. We found that *M.*

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*marinum* enters efficiently and replicates in CLC cells while the nonpathogenic species, *M. smegmatis*, enters poorly and is killed intracellularly. *M. marinum* is also able to block lysosomal fusion in CLC cells, which makes them a suitable model for examination of intracellular trafficking in mycobacteria. This is the first report of an in vitro model for the study of *M. marinum* virulence using monocytic cells obtained from fish, a natural host. This model is convenient, easily reproducible, sensitive, and likely to be important for the identification and characterization of mycobacterial virulence determinants.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *M. marinum* strain M, a clinical isolate obtained from the skin of a patient (51), was used in this study. *M. marinum* was grown at 33°C in 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.5% glycerol, 10% albumin-dextrose complex, and 0.25% Tween 80 for 7 to 10 days. *M. smegmatis*, strain mc<sup>2</sup>155 (66) cultures were grown in the same manner but only for 3 days at 37°C. The number of viable bacteria was determined for each assay using the LIVE-DEAD assay (Molecular Probes, Eugene, Oreg.) and by plating dilutions for CFU on 7H9 agar (Difco). All inocula used were >99% viable.

**Cell lines and culture conditions.** The mouse macrophage cell line J774A.1 (ATCC TIB67) was maintained at 37°C and 5% CO<sub>2</sub> in high glucose Dulbecco's modified eagle medium (Dulbecco's MEM) (Gibco, Bethesda, Md.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 2 mM L-glutamine. The adherent carp monocytic/macrophage cell line CLC (European Collection of Cell Cultures no. 95070628) was maintained at 28°C and 5% CO<sub>2</sub> using high glucose MEM (Gibco) supplemented with 10% essential amino acids (Gibco), 10% heat-inactivated fetal bovine serum (Gibco), and 2 mM L-glutamine.

**Adherence and entry assays.** Entry and adherence assays were carried out with 24-well tissue culture plates (Costar) as described previously (15, 16). J774A.1 cells were seeded at a density of 10<sup>6</sup> cells per well 18 to 24 h prior to use. CLC cells were seeded in the same manner, at a density of 5 × 10<sup>5</sup> cells per well. The medium was replaced before use, and bacteria were added to achieve a multiplicity of infection (MOI) of 10. The infection was allowed to proceed for 30 min at 28°C for CLC and 33°C for J774A.1. The cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium plus amikacin (200 µg/ml; Sigma Chemicals, St. Louis, Mo.) for 2 h. The cells were then washed once with PBS and lysed using 1 ml of 0.1% Triton X-100 (Sigma) for 10 min. Dilutions were plated to determine the number of intracellular CFU. Adherence assays were carried out in a similar manner except that bacteria were added to the cells and immediately washed five times with PBS prior to lysis. Adherence assays using fixed cells were carried out as previously described (15). Cells were fixed in 3.7% formaldehyde for 10 min at room temperature and washed three times with PBS prior to addition of bacteria. Bacteria were cocultured with fixed cells for 30 min, and then the cells were washed and lysed and the number of CFU was determined. Triton X-100 had no effect on the viability of *M. marinum* and *M. smegmatis*, and all strains used displayed comparable rates of killing by amikacin.

**Intracellular growth assays.** Intracellular growth assays were carried out in a similar manner to entry assays, but after amikacin treatment, fresh medium plus amikacin (30 µg/ml) was added. The cells were incubated at the appropriate temperature and then lysed and plated as described above at different time points. Survival is expressed as the percentage of CFU present at each time point ( $T_x$ ) compared to the number present at time zero ( $T_0$ , or 2.5 h), i.e., % survival = (CFU at  $T_x$ /CFU at  $T_0$ ) × 100.

**Microscopy.** For acid-fast stains, CLC and J774A.1 cells were seeded as described above on coverslips in 24-well tissue culture plates. Bacteria were added to achieve an MOI of 10. The infection was allowed to proceed for 5 min at 28 or 33°C, after which the cells were washed two times with PBS and the media were replaced with fresh medium plus amikacin (30 µg/ml). This time point represents time zero. Cells were reincubated at 28 or 33°C, and the medium was removed at appropriate time points. The cells were then fixed with methanol, washed once with PBS, and stained by the Ziehl-Neelsen technique (37), using carbol-fuchsin and malachite green (Sigma). Cells were examined using a Nikon TE300 light microscope with differential interface contrast optics.

Transmission electron microscopy was used to examine the ultrastructure and trafficking of *M. marinum* and *M. smegmatis* vacuoles in CLC cells. CLC monocytes were infected at an MOI of 10 for 5 min at 28°C, washed two times with

PBS, and incubated at 28°C in MEM plus amikacin (30 µg/ml). After incubation for various times, the cells were suspended in medium with a rubber policeman, pelleted by centrifugation for 2 min at 740 × g at 25°C, fixed, and prepared for electron microscopy as previously described (14). The samples were suspended in 2% glutaraldehyde plus 1% OsO<sub>4</sub> for 2 h and postfixed with 0.5% uranyl acetate overnight at 4°C. The cells were embedded and sectioned as described previously (14). In order to quantitate the frequency of lysosomal fusion with mycobacterial vacuoles in CLC cells, they were prelabeled with thorium dioxide (24 µg/ml; Polyscience) for 18 to 24 h prior to infection as described previously (14). The cells were then fixed and prepared for electron microscopy.

**Statistical analyses.** Data presented are the means and standard deviations of triplicate samples from a representative experiment. All experiments were repeated at least three times, unless otherwise noted. Significance was determined by analysis of variance using the Student *t* test. *P* values of <0.05 were considered significant.

## RESULTS

**Fish monocytes are bactericidal for nonpathogenic mycobacteria.** We examined the ability of pathogenic (*M. marinum*) and nonpathogenic (*M. smegmatis*) mycobacteria to survive and replicate in the fish monocytic cell line, CLC. As shown in Fig. 1A, we found that *M. marinum* replicates well in fish monocytes. *M. marinum* CFU increase 10-fold within 24 h. In contrast, over 90% of the *M. smegmatis* organisms are killed within the first 24 h. These data are comparable to those obtained with J774A.1 murine macrophages (Fig. 1B), a cell line that has been widely used as an in vitro model for mycobacterial infections (43, 55, 56, 76). As expected, *M. marinum* replicates in J774A.1 macrophages, while *M. smegmatis* is killed. Though *M. smegmatis* has a generation time (1.1 h) that is 3.65-fold that of *M. marinum* (3.8 h) in broth media, *M. smegmatis* has a negative generation time in both cell lines while *M. marinum* doubles in ~4.9 h within CLC cells. Interestingly, there is a greater difference between the growth of *M. marinum* and *M. smegmatis* in fish monocytes than in murine macrophages. This difference is about twofold at 4 h and increases throughout the experiment. These data suggest either that *M. marinum* is better adapted to parasitize fish monocytes than murine macrophages or that fish monocytes are more bactericidal than murine macrophages for nonpathogenic mycobacteria or that a combination of these factors is responsible.

**Ultrastructure of the mycobacterial vacuoles in fish monocytes.** The ultrastructure of the mycobacterial vacuole in fish monocytes was monitored for 2 h postinfection by transmission electron microscopy. We found that both *M. marinum* and *M. smegmatis* reside within vacuoles that conform to the shape of the bacteria, which are surrounded by a typical electron-transparent zone (Fig. 2). Quantitation of infected cells by electron microscopy (Table 1) indicates that there is a significant difference ( $P < 0.05$ ) between the number of cells infected with *M. marinum* and *M. smegmatis*, the number of vacuoles per infected cell ( $P < 0.03$ ), and the number of bacteria per vacuole ( $P < 0.01$ ).

***M. marinum* inhibits lysosomal fusion in fish monocytes.** The ability of *M. marinum* to replicate, while *M. smegmatis* is killed, in fish monocytes, may be at least partially due to the ability of the former to prevent fusion of the bacterial vacuoles with lysosomes. In order to examine this possibility, we quantitated the frequencies of lysosomal fusion with the *M. marinum* and *M. smegmatis* vacuoles in CLC cells (Fig. 3). Fusion of *M. smegmatis* vacuoles with lysosomes is greater than 75% by

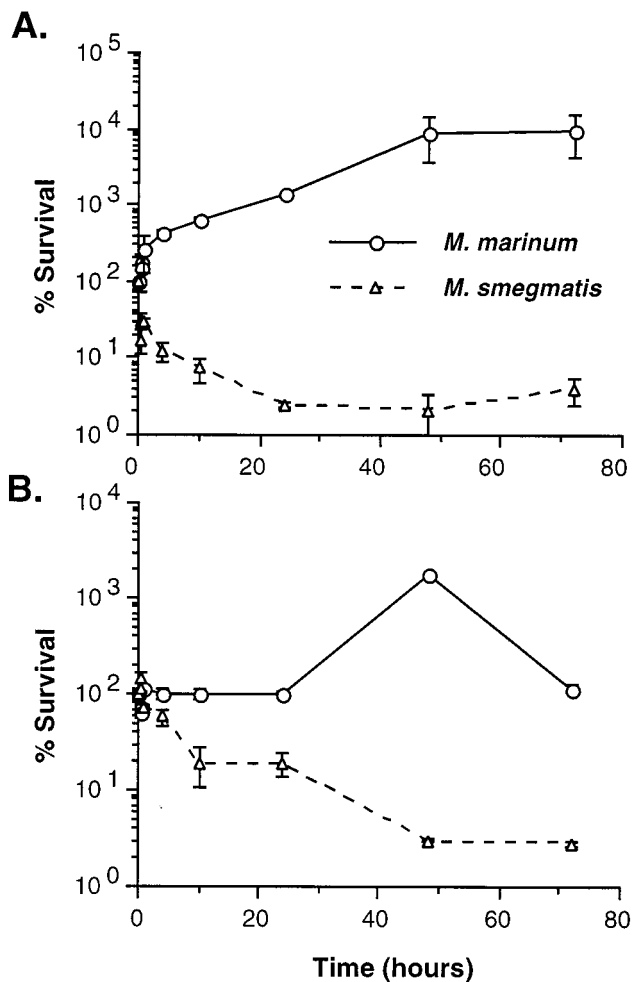


FIG. 1. Survival and replication of *M. marinum* and *M. smegmatis* in the fish monocyte cell line CLC (A) and the murine macrophage cell line J774A.1 (B). The survival rate was arbitrarily set to 100% at time zero for each strain. Data points and error bars represent the means and standard deviations, respectively, of assays done in triplicate from a representative experiment.

1 h (Table 1), compared to 18% for *M. marinum*. *M. marinum* vacuoles display significantly lower levels of fusion with lysosomes than *M. smegmatis* at all time points ( $P < 0.01$ ). At 2 h postinfection it was difficult to find intact *M. smegmatis* in infected cells, as most bacteria were in the process of being degraded (Fig. 3C). On the other hand, at the same time point most bacteria were found in unfused vacuoles in cells infected with *M. marinum* (Fig. 3F). These data suggest that the survival of *M. marinum* in CLC cells is at least partially due to the prevention of lysosomal fusion, similar to previous observations of murine macrophages (7).

**Entry of *M. marinum* into fish monocytes.** Since entry into human and murine monocytes is thought to play an important role in pathogenesis (29), we wished to characterize the entry of mycobacteria into CLC cells. The ability of *M. marinum* to enter CLC cells was compared to that of *M. smegmatis* at 30 min postinfection. As shown in Fig. 4A, we found a significant difference in the entry of *M. marinum* into CLC cells relative to

*M. smegmatis* ( $P < 0.01$ ). *M. marinum* enters fish monocytes at a 60-fold-higher rate than *M. smegmatis*. Similar results were obtained in murine macrophages (Fig. 4B). These data were confirmed by staining infected monolayers by the Ziehl-Neelsen technique and counting acid-fast bacilli within infected cells. Typically, *M. marinum*-infected CLC and J774A.1 cells contain more bacilli than those infected with *M. smegmatis* (data not shown). Quantitation of the number of acid-fast bacilli within infected CLC monocytes (Table 2) indicates that *M. marinum* infected cells at a higher rate than *M. smegmatis* ( $P < 0.01$ ). In addition, more vacuoles per cell ( $P < 0.01$ ) and more bacteria per vacuole ( $P < 0.03$ ) are observed with *M. marinum* than *M. smegmatis*. By microscopy, *M. marinum* enters fish monocytes at 27- and 40-fold-higher levels than *M. smegmatis* after 1 and 2 h, respectively. Similar results were obtained with the murine macrophage cell line J774A.1 (Table 3). The results obtained by microscopy are comparable to those obtained by standard invasion assays. Since acid-fast stains do not differentiate between viable and nonviable bacteria, the differences in entry between *M. marinum* and *M. smegmatis* are not solely due to differences in intracellular survival.

**Adherence of *M. marinum* to fish monocytes.** To determine whether the differences we observed in entry are at least partially due to differences in adherence, we compared the levels of adherence of *M. marinum* and *M. smegmatis* to CLC cells. *M. marinum* displays a fourfold-higher rate of adherence to fish monocytes than does *M. smegmatis* ( $P < 0.01$ ) (Fig. 5A). Similar but somewhat greater differences between these strains are observed with J774A.1 murine macrophages ( $P < 0.01$ ) (Fig. 5C). We repeated the adherence assays with formaldehyde-fixed cells to prevent the possibility that the effects on adherence are due to uptake or intracellular survival. Adher-

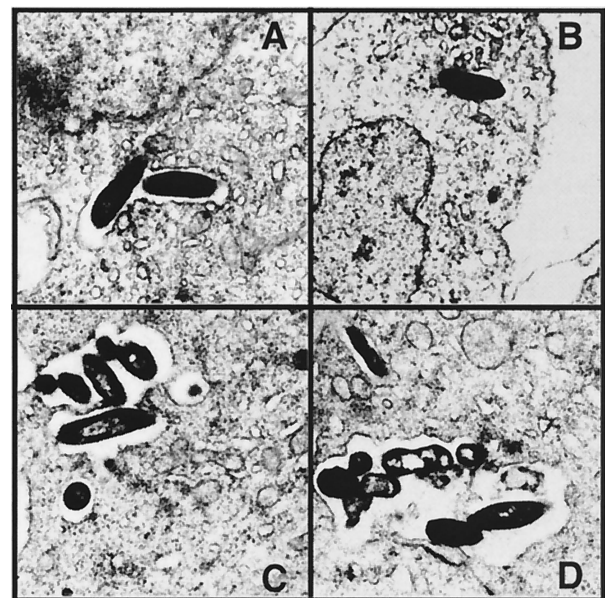


FIG. 2. Transmission electron micrographs of *M. marinum* (C and D) and *M. smegmatis* (A and B) at 1 h (A and C) and 2 h (B and D) postinfection of the fish monocyte cell line CLC. Magnification,  $\times 10,000$ .

TABLE 1. Mycobacterial infections in fish monocytes

Time (min) after entry	% Infected cells <sup>a</sup>		No. of vacuoles/cell <sup>b</sup>		No. of bacteria/vacuole <sup>c</sup>		% Fusion <sup>d</sup>	
	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>
60	25 ± 1.0	9 ± 1.0	1.31 ± 0.01	1.10 ± 0.04	1.56 ± 0.12	1.00 ± 0.00	18 ± 5	75 ± 8
120	30 ± 2.0	5 ± 1.0	1.39 ± 0.01	1.19 ± 0.01	1.91 ± 0.09	1.01 ± 0.01	23 ± 6	88 ± 53

<sup>a</sup> Percentage of cells containing at least one bacterium. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

<sup>b</sup> Number of bacterial vacuoles in each cell. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

<sup>c</sup> Number of bacteria in each vacuole. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

<sup>d</sup> Percentage of lysosomal fusion, calculated by counting bacterial vacuoles containing thorium. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

ence assays in fixed CLC (Fig. 5B) and J774A.1 (Fig. 5D) cells show significant ( $P < 0.01$ ) but smaller differences in adherence of *M. marinum* and *M. smegmatis* than those seen with live cells. These data suggest that fish monocytes are a useful in vitro model for examination of *M. marinum* mechanisms of adherence, entry, trafficking, and intracellular replication.

DISCUSSION

We have developed an in vitro model for the study of mycobacterial virulence using *M. marinum* and fish monocytes. An important feature of this model is that it provides the opportunity to study the pathogenesis of a mycobacterial species in cells from a natural host. Since *M. marinum* infects more than 150 species of salt- and freshwater fish and replicates in monocytic cells (3, 27, 74), results obtained in this in vitro model should closely resemble those during natural infections. Though murine macrophages have been used extensively as in

vitro models for mycobacterial infections, differences exist between their microbicidal mechanisms and those of human macrophages (25, 26, 33, 59, 60). Mice are also naturally resistant to tuberculosis (48), raising the issue of differences in host-pathogen interactions between this model and humans. Human cell lines are available for mycobacterial pathogenesis studies; however, no fully differentiated adherent human macrophage cell line exists. In order to study mycobacterial host cell interactions in adherent macrophages, human monocytes must be treated with phorbol esters (41, 61, 72), lipopolysaccharide (1, 45), or interferon gamma plus tumor necrosis factor alpha (1, 45). Such treatments can complicate analysis of data obtained, since they have pleiotropic effects on macrophages (31, 39, 69, 77). Thus, the use of fish monocytes, in combination with the previously developed systems, should improve our ability to evaluate potential mycobacterial virulence determinants.

Fish monocytes are capable of differentiating between

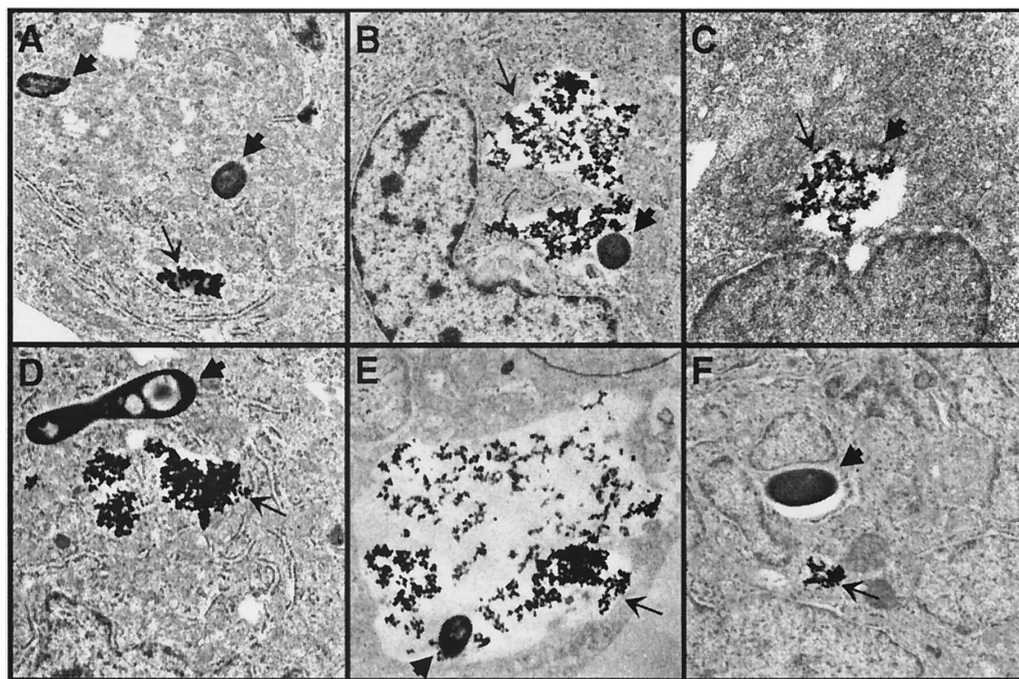


FIG. 3. Transmission electron micrographs of fused (B, C, and E) and nonfused (A, D, and F) *M. marinum* (D to F) and *M. smegmatis* (A to C) vacuoles with thorium dioxide-labeled lysosomes, at 1 h (A, B, D, and E) and 2 h (C and F) postinfection of the fish monocytic cell line CLC. Thick arrows point to bacteria or bacterial debris (C), while thin arrows point to thorium dioxide-containing vacuoles. (C) *M. smegmatis* bacterium in the process of being degraded. Magnification, ×10,000.

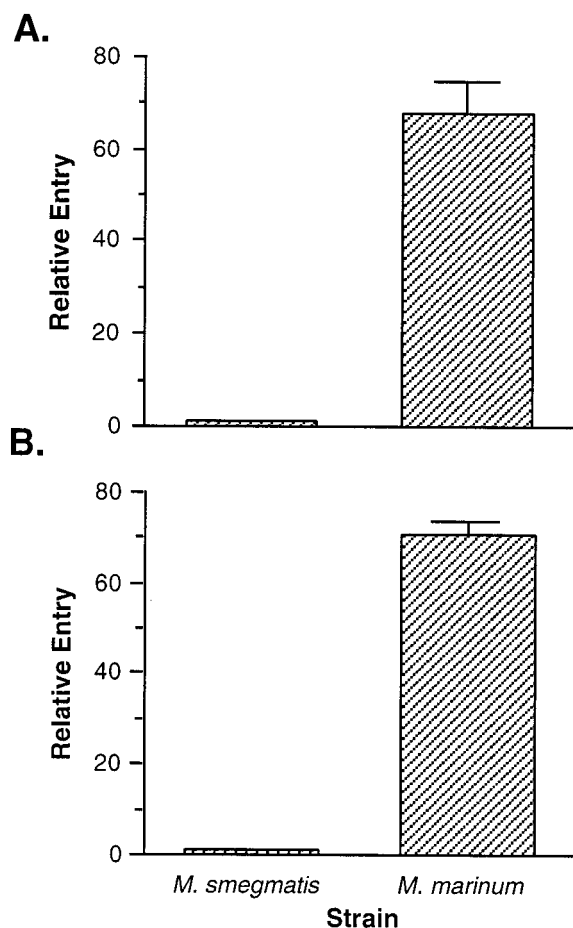


FIG. 4. Entry of *M. marinum* into the fish monocytic cell line CLC (A) and the murine macrophage cell line J774A.1 (B) relative to *M. smegmatis* at 30 min postinfection. Entry of *M. smegmatis* was arbitrarily set at 1. Data points and error bars represent the means and standard deviations, respectively, of assays done in triplicate from a representative experiment.

pathogenic and nonpathogenic mycobacteria. There is a much greater difference between the growth of *M. marinum* and *M. smegmatis* in fish monocytes than there is between these pathogens in murine macrophages. In support of the high bactericidal ability of fish monocytes, we have also found that they are able to kill an *M. marinum oxyR* mutant (E. Pagán-Ramos and V. Deretic, unpublished data) that persists in murine macrophages (S. El-Etr and J. Cirillo, unpublished data). This suggests that fish monocytes are more sensitive to subtle differ-

ences in virulence within the same species. Generally, all mycobacterial species studied are internalized by macrophages and epithelial cells (36, 62–64). However, only pathogenic species survive and replicate efficiently in mammalian cells (62–64). Like other pathogenic mycobacteria, *M. marinum* survives and replicates in macrophages and epithelial cells (44, 51, 62, 64). Previous studies of these cells were conducted between 28 and 33°C, temperatures permissive to the growth of *M. marinum*. The bactericidal ability of J774A.1 macrophages does not appear impaired at 33°C, as judged by their ability to kill nonpathogenic mycobacteria. However, we cannot exclude the possibility that the lower temperature is at least partially responsible for the smaller differences we observed in the intracellular replication of pathogenic and nonpathogenic mycobacteria in murine macrophages.

The ultrastructural characteristics of the *M. marinum* vacuole in fish monocytes are similar to those previously seen in murine (51) and freshly explanted trout (13) macrophages. As seen in other pathogenic mycobacterial species (19, 23, 67, 75), *M. marinum* can circumvent the host endocytic pathway blocking fusion of its phagosome with lysosomes (7, 13) and reside in vacuoles that do not acidify (7). Similarly, *M. marinum* multiplies in typical vacuoles and blocks phagosome-lysosome fusion within fish monocytes. *M. smegmatis*, on the other hand, is not able to prevent the fusion process, which may contribute to its inability to survive. Results obtained with fish monocytes, therefore, mimic the observations made with *M. tuberculosis* and *M. avium* in mammalian macrophages. This observation makes CLC cells suitable for dissection of the mechanisms used by mycobacteria to prevent lysosomal fusion. Though specific lysosomal markers for fish monocytes are not available, lysosomes can be labeled with ferritin (2) or thorium dioxide. Frequencies of lysosomal fusion can also be assessed by assaying for acid phosphatase (57) and fluid phase endosomal tracers like Texas red-ovalbumin (68), which should function regardless of the cell type.

Since *M. smegmatis* is less efficient than *M. avium* at entering the laryngeal epithelial cell line HEp-2 (10), it is not surprising that similar results were obtained in our studies. This indicates that fish monocytes differentiate between the entry and adherence mechanisms of pathogenic and nonpathogenic mycobacteria. Comparable levels of entry were obtained with fish monocytes and murine macrophages, suggesting that similar mechanisms are used by mycobacteria to enter both cell types. This is further supported by studies implicating complement and fibronectin receptors in entry of mycobacteria into murine macrophages (29) and by data showing that entry of *M. mari-*

TABLE 2. Entry into fish monocytes

Time (min) after entry	% Infected cells <sup>a</sup>		No. of vacuoles/cell <sup>b</sup>		No. of bacteria/vacuole <sup>c</sup>		Infectivity index <sup>d</sup>	
	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>
60	17.0 ± 0.22	5.5 ± 0.12	1.30 ± 0.02	1.08 ± 0.00	8.35 ± 0.15	1.14 ± 0.03	27.25 ± 0.22	1.0 ± 0.10
120	20.7 ± 0.12	5.9 ± 0.11	1.72 ± 0.04	1.14 ± 0.10	8.89 ± 0.09	1.19 ± 0.04	39.56 ± 0.12	1.0 ± 0.10

<sup>a</sup> Percentage of cells containing at least one bacterium in a field. Results are the means ± standard deviations for three counts of 25 fields.

<sup>b</sup> Number of bacterial vacuoles in each cell. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

<sup>c</sup> Number of bacteria in each vacuole. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.

<sup>d</sup> The infectivity index is calculated as follows: (% infected cells × number of vacuoles per cell × number of bacteria per vacuole for *M. marinum*)/(% infected cells × number of vacuoles per cell × number of bacteria per vacuole for *M. smegmatis*).

TABLE 3. Entry into murine macrophages

Time (min) after entry	% Infected cells <sup>a</sup>		No. of vacuoles/cell <sup>b</sup>		No. of bacteria/vacuole <sup>c</sup>		Infectivity index <sup>d</sup>	
	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>	<i>M. marinum</i>	<i>M. smegmatis</i>
60	17.4 ± 0.91	7.0 ± 0.21	1.96 ± 0.04	1.18 ± 0.02	6.23 ± 0.17	1.32 ± 0.05	19.49 ± 0.90	1.0 ± 0.90
120	24.3 ± 0.20	9.1 ± 0.60	1.97 ± 0.10	1.14 ± 0.20	9.85 ± 0.29	1.12 ± 0.12	40.58 ± 0.29	1.0 ± 0.60

<sup>a</sup> Percentage of cells containing at least one bacterium in a field. Results are the means ± standard deviations for three counts of 25 fields.  
<sup>b</sup> Number of bacterial vacuoles in each cell. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.  
<sup>c</sup> Number of bacteria in each vacuole. Results are the means ± standard deviations for two counts of 50 cells in different sections of the same preparation.  
<sup>d</sup> The infectivity index is calculated as follows: (% infected cells × number of vacuoles per cell × number of bacteria per vacuole for *M. marinum*) / (% infected cells × number of vacuoles per cell × number of bacteria per vacuole for *M. smegmatis*).

*num* into rainbow trout macrophages is enhanced in the presence of complement (13). The difference between *M. marinum* and *M. smegmatis* in adherence to murine macrophages is higher than to fish monocytes. Since both cell types differentiate equally well between the entry of these species, this observation suggests that fish monocytes primarily differentiate pathogenic and nonpathogenic mycobacteria at a step in the entry process subsequent to adherence: signal transduction, phagocytosis, or initial intracellular survival.

Improved animal models would greatly facilitate studies focusing on mycobacterial pathogenesis and vaccines (9, 35).

Rabbits (24) and guinea pigs (5, 65) usually develop an acute disease, resulting in death within a few weeks, while many mouse strains are naturally resistant to tuberculosis and do not develop typical granulomas (21, 48). A number of animal models have been described for *M. marinum* infections (22, 54, 70). In addition, the technology for production of zebra fish germ line chimeras from embryo cell cultures has recently become available (42). Since fish are natural hosts for *M. marinum*, it is now possible to produce specific transgenic zebra fish to study the genetic basis of host susceptibility and resistance to *M. marinum* disease. Thus, the animal models available for *M.*

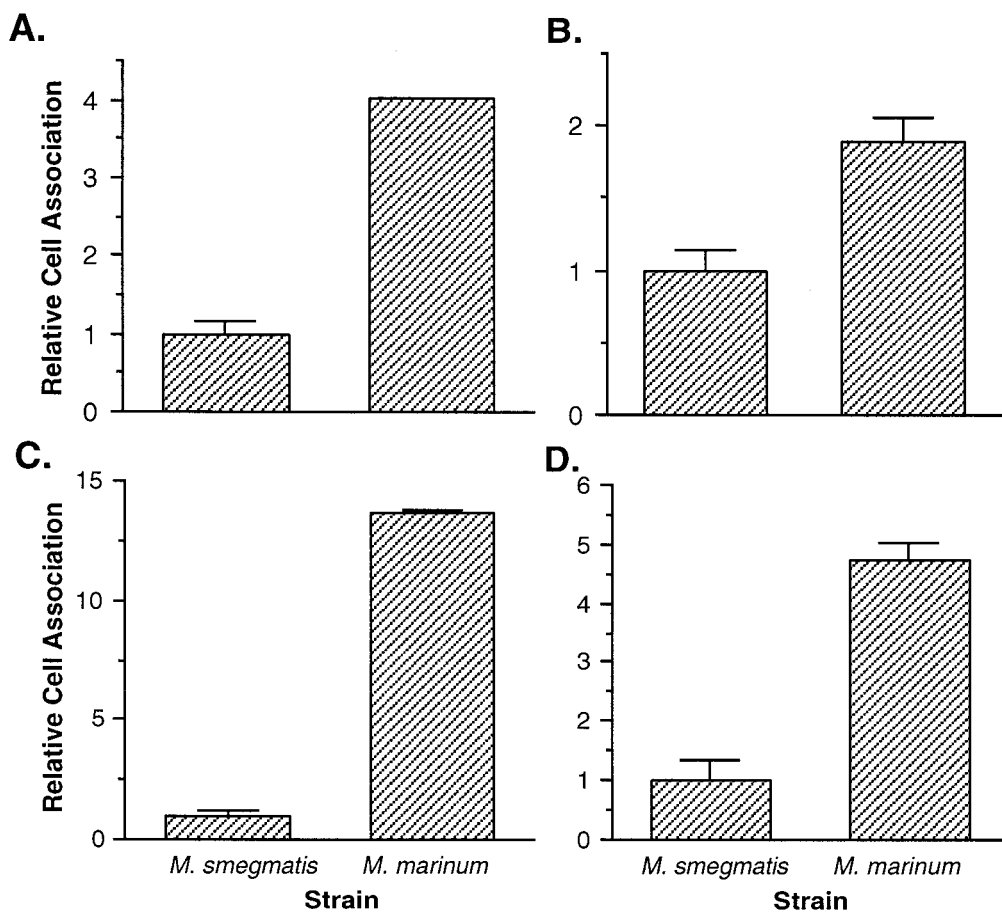


FIG. 5. Adherence of *M. marinum* to the fish monocytic cell line CLC (A and B) and the murine macrophage cell line J774A.1 (C and D) relative to *M. smegmatis*. Assays were carried out in live (A and C) and formaldehyde-fixed (B and D) cells. Adherence of *M. smegmatis* was arbitrarily set to 1. Data points and error bars represent the means and standard deviations, respectively, of assays done in triplicate from a representative experiment.

*marinum* pathogenesis studies are plentiful, are relevant to natural infections, and should allow genetic analysis of the host components involved.

Fish monocytes provide a valuable tool for screening *M. marinum* strains prior to conducting virulence studies with animals. In addition, they allow pathogenesis studies in a relevant *in vitro* model at the proper temperature. The CLC fish monocytic cell line used in the present study displays all of the necessary characteristics for examination of the molecular mechanisms of mycobacterial host-pathogen interactions. This model system should allow rigorous analysis of the genetic basis of *M. marinum* pathogenesis and the identification of novel mycobacterial virulence determinants. However, although *M. marinum* is closely related to *M. tuberculosis*, it is likely that some differences in their mechanisms of pathogenesis exist. Thus, it is necessary that, once potential virulence determinants are identified and characterized, their role be confirmed in *M. tuberculosis* itself. Even with this caveat, the multiple advantages offered by the CLC monocytic cell line suggest that pathogenesis research in *M. marinum* will yield vast benefits in the coming years.

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