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Differential Trafficking of Live and Dead *Mycobacterium marinum* Organisms in Macrophages

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We characterized the *Mycobacterium marinum* phagosome by using a variety of endocytic markers to follow the path of the bacteria through a mouse macrophage cell line. Using a laser confocal microscope, we found that the majority of viable *M. marinum* cells were in nonacidic vacuoles that did not colocalize with the vacuolar proton ATPase (V-ATPase), the calcium-independent mannose-6-phosphate receptor (CI-M6PR), or cathepsin D. In contrast, heat-killed organisms and latex beads were in acidic vacuoles which contained the V-ATPase, the CI-M6PR, and cathepsin D. A population of vesicles that contained live *M. marinum* labeled with the lysosomal glycoprotein LAMP-1, but the percentage of vacuoles that labeled was lower than for heat-killed organisms or latex beads. When testing live and heat-killed *Mycobacterium tuberculosis*, we found levels of colocalization with LAMP-1 and cathepsin D comparable to those for the *M. marinum* isolate. We conclude that *M. marinum*, like *M. tuberculosis*, can circumvent the host endocytic pathway and reside in an intracellular compartment which is not acidic and does not fuse with lysosomes. In addition, we describe a system for sampling a large population of intracellular organisms by using a laser confocal microscope.

Mycobacterium marinum is a widespread aquatic organism that causes fatal infections in fish and amphibians (4). It is also an important human pathogen, frequently causing chronic skin infections of the extremities referred to as swimming pool granuloma (13, 18, 19, 22). A range of symptoms are exhibited, from epidermal lesions to infection of deeper tissues, also called *M. marinum* arthritis (1, 26). *M. marinum* may also cause disseminated infections in immunocompromised patients (14, 25, 38).

Consistent with the prevalence of human *M. marinum* disease in the extremities, the optimal growth temperature of *M. marinum* is 25 to 35°C, though some isolates grow well at 37°C (4, 6). Taxonomically, *M. marinum* is classified as a slow-growing member of the genus *Mycobacterium* (28, 35). *M. marinum* has a relatively short generation time of 4 h, compared to 20 h for *Mycobacterium tuberculosis*, and has been shown to be very closely related to *M. tuberculosis* by DNA-DNA homology and 16S RNA sequence studies (39). In a recent study, *M. marinum* was exploited as a model for mycobacterial pathogenesis (27). In this study, it was demonstrated that at permissive temperatures, *M. marinum* could persist in cultured macrophages and cause a systemic granulomatous disease in frogs. Other studies have indicated that pathogenic mycobacteria, including *M. marinum*, survive and replicate in HeLa cells and macrophages (21, 30–32).

The mechanisms which enable mycobacteria to avoid killing and degradation by the host macrophage have been the subject of much research. Several endosomal and cellular markers have been used to dissect the trafficking pathways of *Mycobacterium avium* and *M. tuberculosis* through the macrophage (8, 36, 40). These studies, along with a number of earlier studies (9–11, 15), suggest that phagosomes containing mycobacteria do not fuse with lysosomes and that the mycobacterium-containing phagosome is only mildly acidified. Other studies, however, suggested that, at least in some cases, mycobacterial species can avoid the endosomal network by escaping from the vacuole into the cytoplasm (21, 23). Although the majority of previous work places mycobacteria in a nonacidified membrane-bound vacuole, there is still controversy about this issue.

The lack of phagosome acidification and subsequent fusion with lysosomes in *M. tuberculosis*- and *M. avium*-containing vesicles has been attributed to the exclusion of the vesicular proton ATPase (V-ATPase) from the membranes of these vesicles (36, 40). The membranes of vesicles containing mycobacteria, however, did contain the lysosomal glycoprotein (LGP) LAMP-1, indicating that although the V-ATPase is not present, these vesicles continue to fuse with vesicles containing LAMP-1. Another study, however, indicated that low to intermediate levels of LAMP-1 associated with *M. tuberculosis*-containing vacuoles (8). Lysosomal enzyme markers, including cathepsin D and acid phosphatase, as well as the late endosomal/prelysosomal marker, the mannose-6-phosphate receptor, were absent in these vesicles, indicating that the mycobacteria resided in vacuoles that were endosomal, rather than lysosomal, in nature.

In a recent study, dual-label spectrofluorometry was used to differentiate between the intracellular fate of live and dead *M. avium* (24). Phagosomes containing live *M. avium* did not traffic through the endocytic pathway and fuse with lysosomes, whereas vacuoles containing heat-killed *M. avium* colocalized with lysosomal markers. This finding correlates with an earlier study which indicated that live *M. avium* did not fuse with acid phosphatase-containing vacuoles, i.e., lysosomes, whereas *M. avium* killed with gamma radiation did (10).

In this study, we characterized the *M. marinum* phagosome by using a variety of endocytic markers to follow the path of *M. marinum* through a mouse macrophage cell line. This enabled us to compare the trafficking phenotype of *M. marinum* to those of other pathogenic mycobacterial species. To address whether viability affects the trafficking patterns of these mycobacteria, we differentially labeled live and heat-killed organ-

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isms to determine whether *M. marinum* organisms are trafficked through the macrophage differently depending on their viability. In addition, we compared results obtained for *M. tuberculosis* trafficking in our model system to results obtained with other methods.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. marinum* 1218R was originally obtained from the American Type Culture Collection (ATCC 927), as were *M. tuberculosis* H37Ra (ATCC 25177) and *M. avium* (ATCC 19075). The *M. marinum* 1218R isolate has been shown to be virulent in both the frog and guinea pig models (34). Individual isolates of *M. marinum* 1218R were streaked for single colonies on 7H10 medium (Difco, Detroit, Mich.) supplemented with 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (OADC; Difco) and grown at 32°C for 5 to 7 days. A single colony was inoculated into 7H9 medium (Difco) supplemented with 10% OADC and 0.2% Tween 80 and allowed to incubate at 32°C for 10 to 14 days. The cultures were then frozen in 0.5-ml aliquots at −70°C in 7H9 medium with 10% OADC. This culture was designated passage 2. Aliquots were added as needed for experiments into 30 ml of liquid growth medium and grown without shaking for 7 to 10 days. These cultures were designated passage 3. Passage 3 *M. marinum* used for experiments was in stationary phase, having been in medium for more than 10 but less than 30 days. *M. tuberculosis* H37Ra and *M. avium* were cultured under similar conditions, but grown and maintained at 37°C.

Macrophage cell line. The mouse macrophage cell line RAW 264.7 (ATCC TIB71) was maintained at 37°C in 5.0% CO_2 in Dulbecco's modified Eagle medium (Gibco, Bethesda, Md.) supplemented with 10% fetal bovine serum (Gibco). RAW macrophages are fully capable of phagocytosing bacteria and latex beads. Further RAW cells are able to kill nonvirulent mycobacteria in vitro (unpublished data).

Reagents and chemicals. The Baclight Live/Dead kit, calcein, and ethidium homodimer 1 (EtH-1) were obtained from Molecular Probes (Junction City, Oreg.). Acridine orange (AO) was obtained from Sigma Chemical Company (St. Louis, Mo.). Tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG), fluorescein isothiocyanate-conjugated goat anti-mouse IgG, tetramethyl rhodamine isothiocyanate-conjugated rabbit anti-rat IgG, and fluorescein isothiocyanate-conjugated goat anti-rat IgG were obtained from Accurate Biochemicals (Westbury, N.Y.). Monoclonal antibody against human cathepsin D was obtained from Triton Laboratories (Alameda, Calif.). Rat-derived antibody to the LGP LAMP-1 was generously provided by Michelle Rathman, Stanford University, Stanford, Calif. Monoclonal antibody 2G11 against bovine cation-independent mannose-6-phosphate receptor (CI-M6PR) and monoclonal antibody 3.2-F1, which recognizes the 73-kDa subunit of the bovine coated vesicle V-ATPase, were graciously provided by Susan Pfeffer, Stanford University, and Michael Forgac, Tufts University Medical School, Boston, Mass., respectively, and used as previously described (16).

Fluorescent labeling of mycobacteria. A 0.5-ml aliquot of a stationary-phase *M. marinum* or *M. tuberculosis* culture was spun down in a microcentrifuge for 5 min and resuspended in 1.0 ml of water. The suspension was then passed through a 25-gauge needle at least 10 times to break up clumps. Five microliters of calcein dye (1.0 mg/ml) in H_2O was added to the bacterial suspension to label live bacteria. This suspension was incubated in the dark at room temperature for 20 min. The bacteria were then spun down and resuspended in phosphate-buffered saline (PBS) to a concentration of approximately 10^7/ml and passed through a 25-gauge needle to break up clumps of bacteria. To kill bacteria, mycobacterial cultures were spun down in a microcentrifuge for 5 min, resuspended in H_2O, passed through a 25-gauge needle, and incubated at 80°C for 30 min. Heat-killed bacteria were labeled with EtH-1 as instructed by the manufacturer, diluted in PBS to approximately 10^7/ml, and passed through a 25-gauge needle. UV treatment of bacteria involved placing a bacterial suspension (10^7 cells/ml) in PBS into a tissue culture dish and exposing the culture for 10 min, uncovered, to UV light in a model 1800 UV Stratalinker (Stratagene, La Jolla, Calif.). UV-treated bacteria were labeled with calcein. The Molecular Probes Baclight Live/Dead kit was used as instructed by the manufacturer to label both live and dead bacteria simultaneously. After labeling, bacteria were diluted to approximately 10^7/ml in PBS and passed through a 25-gauge needle. Loss of viability was checked for the heat-killed and UV-treated bacteria by plating bacterial suspensions on 7H10 agar. No CFU were detected in either group after heat or UV treatment.

Population studies. An unlabeled, stationary-phase passage 3 culture of *M. marinum* was added to RAW macrophage monolayers in 24-well tissue culture plates at a multiplicity of infection (MOI) of between 1:1 and 10:1. An aliquot of the original inoculum was then labeled with the Baclight Live/Dead kit as described above, and bacteria were visualized on an MRC 1000 laser confocal microscope (Bio-Rad, Hercules, Calif.). The percentages of live and dead bacteria were ascertained by counting at least 100 organisms in at least five random fields.

At 4, 24, 48, and 72 h and 5 days postinfection, monolayers in eight infected wells were washed three times with PBS, and each was lysed with 0.1 ml of 0.1% sodium dodecyl sulfate. The lysate was retained, and wells were washed with an additional 0.9 ml of H_2O. The wash solutions were added to the respective lysate solutions for a total volume of 1.0 ml. The live/dead kit reagents were combined as instructed by the manufacturer and added to four of the lysate-wash mixtures. After labeling, the bacteria were microcentrifuged for 5 min, and the supernatant was discarded. The four pellets were combined into approximately 0.1 ml of 50% glycerol in PBS and passed through a 25-gauge needle at least 10 times. A 20-μl aliquot of this solution was placed onto a glass slide, covered with a coverslip, and sealed with nail polish. Bacteria were visualized on a laser confocal microscope, and percentages of live and dead bacteria were quantitated as described above.

To quantitate the number of intracellular bacteria in parallel with the viability studies, the remaining four lysates were each diluted into PBS and plated on 7H10 agar at several different concentrations. CFU were enumerated for each dilution at each time point. As CFU are a reflection of viable and replicating bacteria present, numerical values for live and dead bacteria over time were calculated by using CFU and the relative percentage of live bacteria determined with the live/dead labeling studies. The total number of dead organisms = [CFU/(% live/100)] − CFU.

Infection of monolayers for confocal studies. RAW cells were seeded into six-well tissue culture plates containing 18-mm-diameter sterile glass coverslips at 5.0×10^5 macrophages per well in a total volume of 3.5 ml. Macrophages on coverslips were infected 24 to 48 h after initial seeding. Twenty microliters of fluorescent live (calcein) or dead (EtH-1) labeled mycobacteria was added to each well to obtain an MOI of between 1:1 and 10:1 and allowed to settle for 2 h at 4°C. The number of bacteria added was checked by dilution plate counts. One-micrometer-diameter green latex beads (Polysciences, Inc., Warrington, Pa.) were added at an equivalent MOI and incubated in the same way. Latex beads are a control for normal endocytic trafficking and lysosomal acidification (19, 29). After bacteria and beads were allowed to settle, the plates were transferred to a 32°C, 5.0% CO_2 incubator. Settling the bacteria and beads onto the monolayer at 4°C allowed for a synchronous infection of the macrophages. The latex beads were constantly being taken up by an ever-expanding monolayer of RAW macrophages throughout the course of the experiments. It was, therefore, difficult to determine that the addition of beads was synchronized to a well-established initial point of infection. After 4 h at 32°C, the medium was removed and fresh tissue culture medium containing amikacin (100 μg/ml) was added to all samples to kill extracellular organisms. At 24 h, the concentration of amikacin was reduced to 20 μg/ml.

AO assay. AO at a final concentration of 5.0 μg/ml was added to each sample 30 min prior to each measurement time point. Medium was then removed, and the coverslips were washed three times with PBS. Coverslips were immediately inverted onto a glass slide and visualized on a Bio-Rad MRC 1000 laser confocal microscope. All samples were visualized no more than 30 min after removal of AO, as the dye will dissipate from the vacuole soon after labeling.

Antibody labeling. At each measurement time point, coverslips were removed from tissue culture wells, washed three times with PBS, and fixed for 20 min with 4% paraformaldehyde in PBS. Coverslips were then washed three times with PBS and permeabilized for 20 min with 0.1% saponin in PBS. This solution was removed, and coverslips were blocked with 0.1% saponin–2.0% bovine serum albumin in PBS (SBP) for 20 min. Primary antibodies (to LAMP-1, V-ATPase, cathepsin, or the CI-M6PR) were added at appropriate dilutions in SBP and allowed to incubate overnight at 4°C. Coverslips were then washed three times with PBS and blocked for 20 min with SBP. Fluorescently labeled secondary antibodies were then added at appropriate dilutions in SBP and allowed to incubate for 4 h at 37°C (for LAMP-1 and CI-M6PR experiments) or overnight at 4°C (for cathepsin D or V-ATPase experiments). After incubation with secondary antibodies, coverslips were washed three times with PBS and mounted in 50% glycerol in PBS. Coverslips were sealed onto slides with nail polish and stored at 4°C until confocal visualization.

Image analysis and colocalization determination. Images were accumulated and analyzed by using COMOS software (Bio-Rad), and green and red images were merged and formatted on Confocal Assistant software (Bio-Rad). Each bacterium or latex bead was visually scored for colocalization with AO or antibodies to different endocytic markers by ascertaining whether fluorescence was in a discrete vesicular pattern around the bacteria or beads. At least 50 individual beads or bacteria were scored for colocalization in at least five random fields for each experiment, unless otherwise noted. Percentages shown are the average of at least two experiments.

RESULTS

Infection of RAW murine macrophages with *M. marinum*. It has already been established that *M. marinum* is able to survive and replicate in macrophages (27). To determine the viability of intracellular *M. marinum*, we used differential live/dead fluorescence staining of intracellular *M. marinum* over the course of a 5-day infection. Approximately 80 to 90% of the inoculating culture labeled with the calcein (or live) stain, whereas approximately 15 to 20% of these bacteria labeled with EtH-1 (or dead) stain (Table 1). At each time point after addition of unlabeled bacteria to the macrophage monolayers, bacteria were diluted for CFU determinations. An aliquot of bacteria

TABLE 1. Percentages and total numbers of dead *M. marinum* organisms over a 5-day infection of RAW murine macrophages

Time (h) post-infection[a]	% Live	% Dead	Total live (CFU)	Total dead[b]
0	83.6	16.4	1.0×10^6	2.0×10^5
24	85.8	16.6	3.6×10^5	7.2×10^4
48	83.4	15.0	3.7×10^5	6.5×10^4
72	78.8	21.2	3.5×10^5	9.4×10^4
120	85.1	14.9	1.3×10^6	2.3×10^5

[a] The MOI was 5:1 in this representative experiment. The experiment was repeated three times.
[b] Total dead was calculated as follows: total dead = (CFU/(% live/100) − CFU.

harvested at each time point was also labeled with the live/dead kit to determine the viability of the intracellular population of organisms. The percentages of live and dead bacteria did not vary significantly from those for the infecting population over a 5-day period (Table 1). In persistence assays done in parallel with the viability studies, *M. marinum* organisms were shown to persist and replicate within the macrophages over the same 5 days. The total number of dead bacteria increased slightly over time to 2.3×10^5 at the 5-day time point. Although not a significant difference, this number exceeded the total number of dead organisms added to the monolayers in the original infection. These data suggest that throughout the 5-day infection of RAW macrophages by *M. marinum*, a subpopulation of organisms was being killed.

Previous studies of mycobacterial vesicle acidification and lysosomal markers done with immunoelectron microscopy distinguished between damaged and intact bacteria based on the morphological appearance of the bacteria in vesicles (2, 3). To more accurately assess the viability of infecting *M. marinum*, we used a live/dead assay system which is based on membrane permeability. Calcein, a green nuclear dye, is membrane permeable and will fluoresce only in actively metabolizing bacteria. To label dead bacteria, we used EtH-1, which is membrane impermeable and will label only the nuclear material of bacteria that have died and acquired enough membrane damage to allow entry of the stain. Differential live and dead staining is reliable in that bacteria are easily scored as to viability up to 72 h after infection. A limitation of this method is that although bacteria retain the calcein (green) stain while replicating, the fluorescence is diluted through each replication cycle. The heat-killed bacteria retain their fluorescence over time, but we have observed that at later time points, these bacteria are prone to degradation, making it somewhat difficult to find enough dead bacteria to represent a significant population. Bacteria exposed to UV label well with calcein, as they retain residual metabolism. These bacteria, however, are unable to replicate and do not yield any CFU when plated on permissive media.

Preliminary studies indicated that prelabeling of infecting bacteria with these two fluorescent stains did not affect the infectivity, growth, or persistence of *M. marinum* in RAW macrophages (data not shown). These dyes can also be added differentially, allowing the study of one population of infecting bacteria at a time. One hundred percent of the heat-killed bacteria labeled red.

Vesicles containing live *M. marinum* do not acidify and exclude the V-ATPase. It has been postulated for some time that mycobacteria are able to survive in the macrophage, in part, because they are located in nonacidic vacuoles (9). AO was used to determine the acidification status of *M. marinum*-containing vacuoles. AO is a pH-sensitive dye that produces yellow-green fluorescence at neutral pH. When it accumulates within acidic vesicles, it stains the vacuole bright red or orange (37). In a recent study by Baintner, AO was shown to stain the nucleic material of cells yellow-green and the transport and digestive vacuoles red or orange (5). The color shift from yellow-green to red can easily be detected and separated on a laser confocal microscope into green and red at excitation wavelengths 488 and 568 nm, respectively.

As seen in Fig. 1A, an uninfected macrophage can be labeled with AO, and there is little or no overlap of the two colors. This makes it possible to distinguish acidified vesicles (red) from background fluorescence (green) in the cell. When macrophages were infected with live *M. marinum* and stained with AO at 4, 8, and 24 h postinfection, very few bacteria (20 to 30%) were found in acidified compartments (Table 2; Fig. 1B). In contrast, 60 to 90% of the latex bead controls were found in acidified vesicles at all time points. We were unable to determine whether heat-killed bacteria were colocalizing with the AO-labeled vesicles, as heat-killed bacteria labeled with EtH-1 fluoresce red and could not be distinguished from the vesicles themselves. We did, however, test UV-treated *M. marinum*. At 4 h, only 30% of the UV-treated bacteria colocalized with the acidified vesicles. These results were similar to those found with live *M. marinum* at the same time point. The percentage of UV-treated organisms in acidified compartments increased over time so that 60% of the UV-treated bacteria were in acidified compartments by 24 h (Table 2). Thus, at later time points, UV-treated bacteria and latex beads show nearly identical levels of colocalization with AO. These results indicate that most live *M. marinum* bacteria are in vesicles that do not acidify, whereas with UV-treated organisms, delivery to an acidified vacuole is delayed.

We also used *Salmonella typhimurium* as a positive control and *M. avium* as a negative control for vacuolar acidification. We found that a high percentage (67%) of *S. typhimurium* bacteria colocalized with AO-labeled vesicles 4 h postinfection. Conversely, *M. avium* was primarily in vesicles that were not acidic, and by 24 h postinfection, only 24% of the live *M. avium* bacteria were colocalizing with the AO-labeled vacuoles.

M. avium and *M. tuberculosis* have been shown to exclude the V-ATPase from the vacuoles in which they reside (36, 40). Presumably, the lack of V-ATPase in mycobacterial vacuoles results in a static pH in the compartment, which does not facilitate fusion of the phagosome with lysosomes. To test whether *M. marinum* resides in vesicles that contain the V-ATPase, we infected monolayers of RAW cells for 2, 4, and 24 h with live, heat-killed, and UV-treated fluorescently labeled *M. marinum* and scored infected monolayers for bacterial colocalization with V-ATPase. As shown in Fig. 2, live *M. marinum* colocalized with the V-ATPase at very low levels (15 to 30%) compared to heat-killed *M. marinum* (75 to 80%).

Discriminating between late endosomal and lysosomal membrane markers. Late endosomal vesicles are enriched for the CI-M6PR, but this marker is excluded from lysosomes (20). LGPs, such as LAMP-1, are found in lysosomal membranes but are also included in endosomes (12).

To ascertain whether *M. marinum*-containing vacuoles are enriched for the CI-M6PR or LGPs, infected RAW macrophages were stained by indirect immunofluorescence with the anti-bovine CI-M6PR monoclonal antibody 2G11 or the anti-LAMP-1 antibody 1D4B, respectively. As seen in Fig. 3, laser confocal visualization of *M. marinum*-containing vesicles over a period of 72 h indicated very low levels of CI-M6PR colocalization with live *M. marinum* (e.g., 15% at 72 h postinfection). Over the same infection period, the LAMP-1 endosomal/lysosomal marker colocalized with live *M. marinum* at intermedi-

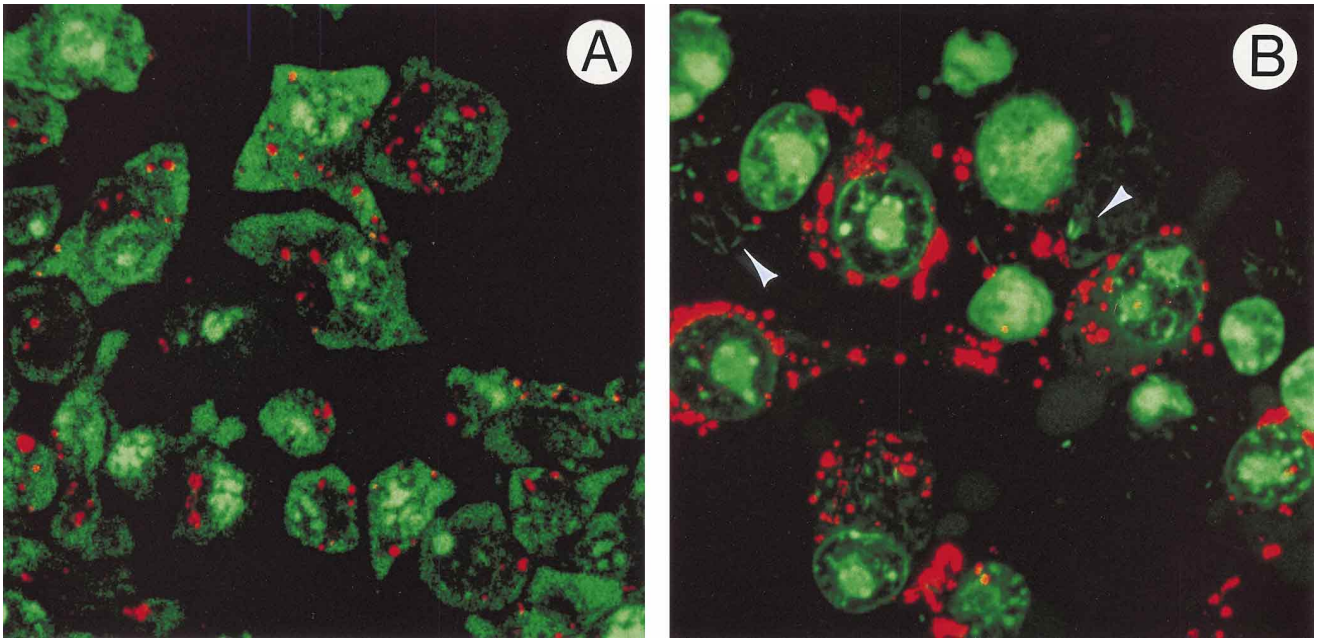

FIG. 1. Vacuolar acidification. At each time point, AO was added to wells containing uninfected RAW macrophages (A) or macrophages infected with live *M. marinum* 1218R (B) 30 min prior to the end of the time point. Coverslips were washed three times with PBS, inverted onto a glass slide, and visualized on a laser confocal microscope. Arrows indicate live *M. marinum* in nonacidified vesicles. Magnification is ×1,020.

ate (30 to 40%) levels (Fig. 3 and 4). Heat-killed *M. marinum*, however, colocalized with the CI-M6PR and LAMP-1 markers at consistently higher levels that were comparable to those for the latex bead controls (Fig. 3). These results indicate that the majority of vesicles containing live *M. marinum* exclude the CI-M6PR marker and contain low to intermediate amounts of the LAMP-1 membrane proteins. In contrast, heat-killed organisms are trafficked to vesicles containing high levels of these markers.

Live *M. marinum* does not colocalize with cathepsin D. Cathepsin D is a soluble lysosomal acid protease that is a marker for phagosomal fusion with lysosomes (8). We found that over a 72-h period of infection, live *M. marinum* was predominantly in vesicles that lacked cathepsin D (Fig. 4 and 5). For example, at 72 h, only 12% of the live bacteria colocalized with cathepsin D. In contrast, 63% of latex bead controls and 57% of heat-killed *M. marinum* were in vesicles that stained positively for cathepsin D. Our data suggest that whereas heat-killed *M. marinum* is trafficked to lysosomal compartments, the majority of live organisms are in phagosomes that do not acquire the lysosomal marker cathepsin D.

Studies with *M. tuberculosis* H37Ra. To directly compare results obtained with our method for following trafficking patterns of mycobacteria through macrophages, we used *M. tuberculosis* H37Ra in our system. When observing H37Ra colocalization with the LAMP-1 marker, we found that 70 to 80% of the heat-killed organisms colocalized with LAMP-1 over a 72-h infection period, whereas only 40 to 50% of the live organisms colocalized with this marker at the same time points (Table 3). Live *M. tuberculosis*, like live *M. marinum*, exhibits intermediate levels of colocalization with LAMP-1, not significantly different from the levels of colocalization demonstrated for latex beads (Table 3). In measuring colocalization of H37Ra with the lysosomal enzyme cathepsin D, we found a reduced level of cathepsin D colocalization with live *M. tuber-*

TABLE 2. Percentages of *M. marinum* in acidified vacuoles in an infection of RAW murine macrophages

Prepn	Mean % *M. marinum* in acidified vacuoles ± SD ($n \geq 2$) at indicated time (h) postinfection		
	4	8	24
Latex beads	88.8 ± 8.2	79.2 ± 19.6	62.4 ± 31.6
Live *M. marinum*	21.0 ± 9.5	20.9 ± 3.5	26.7 ± 10.6
UV-treated *M. marinum*	29.4 ± 7.6	38.5 ± 13.2	60.2 ± 1.5

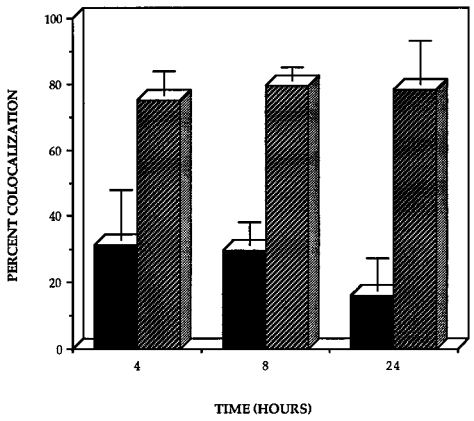

FIG. 2. Percentages of live and heat-killed *M. marinum* colocalizing with the V-ATPase. At each time point, infected monolayers on coverslips were washed, fixed, and incubated with antibody to the V-ATPase. Secondary antibody conjugated with fluorescent markers was added and incubated with the monolayers. Samples were washed and visualized on a laser confocal microscope. Values indicate the percentages of live (black bars) and heat-killed (striped bars) *M. marinum* colocalizing with the V-ATPase. Each value is the mean percentage (± standard deviation) of colocalization for at least two experiments.

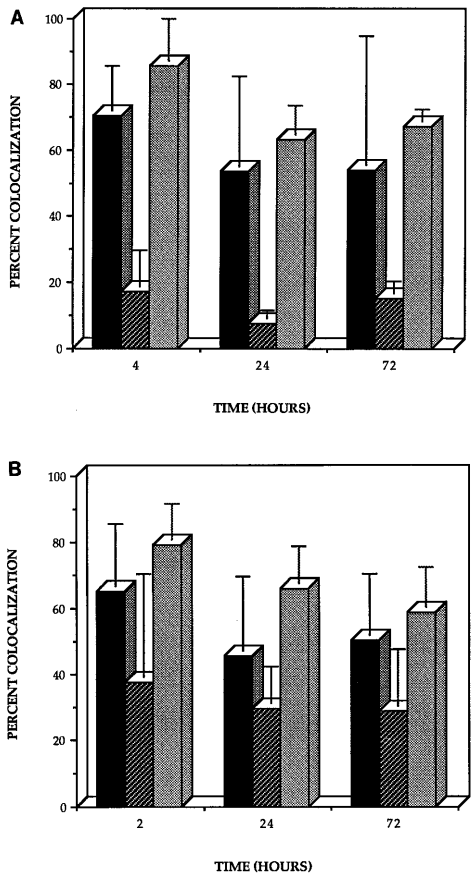

FIG. 3. Colocalization of latex beads or live or heat-killed *M. marinum* with the CI-M6PR and LAMP-1. At each time point, infected monolayers on coverslips were washed, fixed, and incubated with antibody to the CI-M6PR (A) or LAMP-1 (B). Secondary antibody conjugated with fluorescent markers was added and incubated with the monolayers. Samples were washed and visualized on a laser confocal microscope. Values indicate the percentages of latex beads (black bars), live *M. marinum* (striped bars), or heat-killed *M. marinum* (grey bars) colocalizing with the CI-M6PR (A) or LAMP-1 (B). Each value is the mean percentage (± standard deviation) of colocalization for at least two experiments.

culosis compared to heat-killed organisms or latex beads (Table 4). These results are similar to results described for *M. marinum* in this study as well as similar to results described elsewhere for the *M. tuberculosis* Erdman strain (8).

DISCUSSION

Intracellular pathogens have a number of strategies for surviving in a normally hostile environment. These pathogens can escape from their vesicles into the cytoplasm of the host cell (e.g., *Trypanosoma cruzi* and *Listeria monocytogenes*), be resistant to the lysosomal compartment itself (e.g., *Coxiella burnetii*), or reside in a compartment that does not become lysosomal in nature (e.g., *Legionella pneumophila*) (17). Pathogenic mycobacterial species are believed to survive by use of the latter strategy, although the mechanisms by which these bacteria avoid phagosome-lysosome fusion appear to be different from those of other organisms studied (7, 29, 33). Our data show that live *M. marinum*, like other pathogenic mycobacterial species, reside in a compartment which is not acidified and does not contain lysosomal enzymes.

We propose that *M. marinum* is an excellent model for the study of mycobacterial pathogenesis. In addition to having a strong phylogenetic similarity to *M. tuberculosis* (39), *M. marinum* is capable of replication and persistence in cultured macrophage cell lines (27). We have described the trafficking phenotype of *M. marinum* through the endocytic pathway of macrophages and found it to be very similar to that of *M. tuberculosis* and *M. avium* described in other studies (8, 36, 40). In addition, *M. marinum* is amenable to laboratory manipulation, as it yields colonies on permissive media in 5 to 6 days (compared to 10 to 14 days for *M. tuberculosis*) and does not require biosafety level 3 precautions for growth or experimental techniques. Ease of *M. marinum* manipulation and the phylogenetic and phenotypic similarities between *M. marinum* and *M. tuberculosis* make *M. marinum* a valuable tool for the study of mycobacterial pathogenesis.

Our data for *M. marinum* are in agreement with studies showing that pathogenic mycobacteria primarily reside in nonacidified, nonlysosomal environments. Using the weakly basic dye AO, we found that the majority of live *M. marinum* organisms observed were not in acidified vesicles in RAW macrophages. We also used live and heat-killed *M. marinum* in experiments to study whether these organisms were in vesicles containing the V-ATPase. Live *M. marinum* colocalized with the V-ATPase marker at significantly lower levels than did the heat-killed bacteria. This lack of association of *M. marinum* with the V-ATPase marker may lead to a reduced fusion of *M. marinum* phagosomes with lysosomes.

There was a clear difference between the ability of live and dead *M. marinum* to exclude the CI-M6PR from the membranes of vesicles in which they resided. Vesicles containing live *M. marinum* lacked the CI-M6PR endocytic marker, indicating that compartments containing live *M. marinum* rarely reach the late endosomal stage of maturation. Heat-killed *M. marinum*, on the other hand, was located in compartments that contained this marker at levels similar to those in the latex bead controls. The dead bacteria appear, at this stage, to be in phagosomes that progress through the endocytic pathway to fuse with lysosomes. Although LAMP-1 was found in relatively high levels in the *M. marinum*-containing vesicles, cathepsin D was absent from most vesicles containing live organisms. This finding indicates that although LAMP-1 is present, no fusion with lysosomes that contain degradative enzymes occurs. This suggests that the vesicle which contains *M. marinum* is endosomal, rather than lysosomal, in nature and obtains LAMP-1, but not the CI-M6PR, from late endosomes.

The LAMP-1 endocytic marker was found to be present in a relatively high percentage of vacuoles containing live *M. marinum* or *M. tuberculosis*. The percentage of vesicles containing heat-killed bacteria that colocalized with the LAMP-1 marker was, however, significantly higher. LAMP-1 is considered a lysosomal marker, but it is also present on the surface of the macrophage as well as in vesicles being transported from the Golgi apparatus to endosomes (12). Within 24 h of infection, most *M. marinum* organisms reside within separate and discrete vesicles (34). As these bacteria grow and divide, they require more membrane surface area within the host cell. We do not believe that intermediate levels of LAMP-1 labeling seen with live organisms indicate that *M. marinum* or *M. tuberculosis* resides in lysosomal compartments. Rather, we hypothesize that the bacterial vacuole acquires more membrane and associated membrane constituents, including LAMP-1, during bacterial replication within the host cell.

In experiments testing acidification of *M. marinum*-containing vacuoles, we found that most UV-killed *M. marinum* organisms early in the infection course were not found in acidified vesicles. Over time, however, these bacteria were trafficked to acidified compartments. Since UV treatment would not be

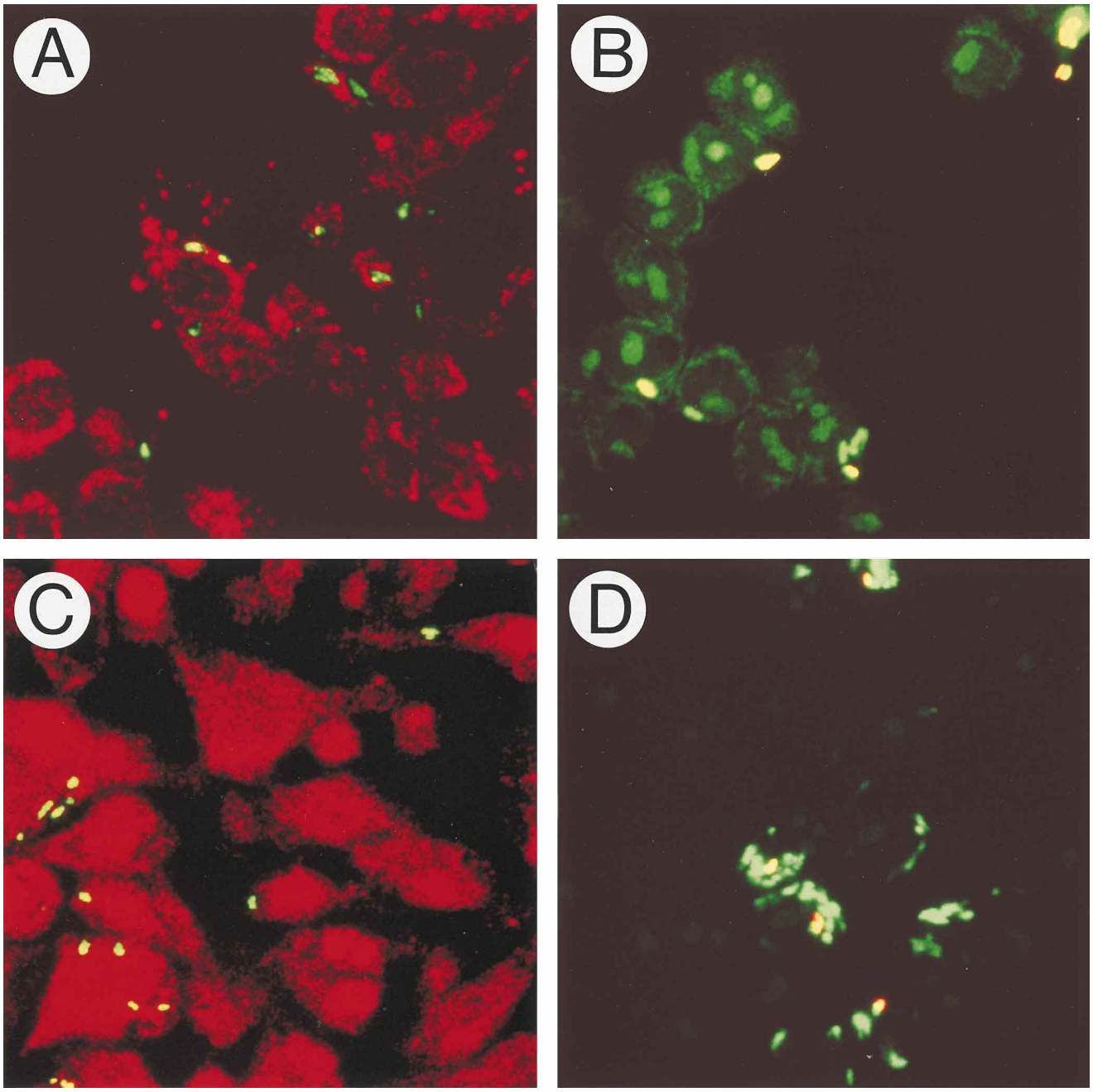

FIG. 4. Colocalization of live or heat-killed *M. marinum* with LAMP-1 or cathepsin D. At 4 h postinfection, monolayers on coverslips were washed, fixed, and incubated with antibody to LAMP-1 (A and B) or cathepsin D (C and D). Secondary antibody conjugated with fluorescent markers was added and incubated with the monolayers. Samples were washed and visualized on a laser confocal microscope. (A) Live *M. marinum* (green) colocalizing with LAMP-1 (red); (B) heat-killed *M. marinum* (red) colocalizing with LAMP-1 (green); (C) live *M. marinum* (green) colocalizing with cathepsin D (red); (D) heat-killed *M. marinum* (red) colocalizing with cathepsin D (green). Magnification is ×776.

expected to cause major changes in bacterial cell wall composition, it is doubtful that differences in the UV-treated *M. marinum* cell wall are responsible for the trafficking of the UV-treated bacteria to acidified compartments over time. Rather, these data suggest that the eventual trafficking of UV-treated organisms to acidified compartments may be related to the inability of the organism to produce some active and/or protective component over time.

In comparing the trafficking phenotype of *M. marinum* to those previously described with other pathogenic mycobacteria, we obtained results similar if not identical to those reported by other groups. In agreement with the findings of the Russell group (36, 40), live *M. marinum*, like other pathogenic mycobacteria, is able to reside in vacuoles that exclude the V-ATPase. Our data also correlate with previous studies showing that pathogenic mycobacteria reside in compartments that lack the CI-M6PR marker (8). Our results indicating that *M. marinum* is in phagosomes that do not contain cathepsin D are similar to those described by Clemens and Horowitz for the *M. tuberculosis* phagosome (8). The lack of association of live *M.*

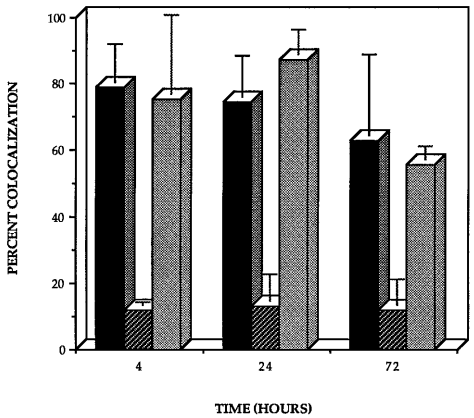

FIG. 5. Colocalization of latex beads or live or heat-killed *M. marinum* with cathepsin D. At each time point, infected monolayers on coverslips were washed, fixed, and incubated with antibody to cathepsin D. Secondary antibody conjugated with fluorescent markers was added and incubated with the monolayers. Samples were washed and visualized on a laser confocal microscope. Values indicate the percentages of latex beads (black bars), live *M. marinum* (striped bars), or heat-killed *M. marinum* (grey bars) colocalizing with cathepsin D. Each value is the mean percentage (± standard deviation) of colocalization for at least two experiments.

marinum with cathepsin D and the CI-M6PR indicates that these organisms rarely progress beyond the late endosomal stage into the phagolysosomal compartment, which would lead to degradation and death of the organism. In Fig. 6, a hypothetical scheme outlining the pathway of *M. marinum* through the host cell is presented.

In using a confocal microscope to sample a large number of *M. marinum* organisms infecting RAW murine macrophages, our results were very similar to results previously reported with other assay systems and with other pathogenic mycobacteria. A recent report by Zimmerli et al. directly compared immunoelectron microscopy to fluorescence microscopy for determining the LAMP-1 contents of vesicles and found that the results of the two methods correlated (41). We believe that our data strengthen previous immunoelectron microscopy results in much the same way.

Confocal microscopy can offer a number of experimental advantages. One can distinguish between live and dead organisms with fluorescent labeling, whereas immunoelectron microscopy can only differentiate between damaged and intact bacteria based on the morphology of the organisms observed (2, 3). In addition, the observation and analysis of *M. marinum*-infected macrophages is not confounded by any complex fixation required for electron microscopy. Confocal microscopy also makes quantitative studies more feasible in that this method enables the visualization of a larger number of bacterium-macrophage interactions than does electron microscopy.

TABLE 3. Percent colocalization of *M. marinum* 1218R and *M. tuberculosis* H37Ra with LAMP-1

Time (h) post-infection	Mean % colocalization with LAMP-1 ± SD ($n \geq 2$)				
	Beads	Live *M. marinum*	Live *M. tuberculosis*	Heat-killed *M. marinum*	Heat-killed *M. tuberculosis*
4	65.2 ± 19.0	37.8 ± 30.9	47.5 ± 6.4	79.4 ± 10.6	70.0 ± 11.2
24	45.4 ± 22.7	29.7 ± 11.1	43.3 ± 13.6	65.9 ± 11.2	81.0 ± 2.8
72	50.5 ± 18.4	28.8 ± 17.1	57.4 ± 17.8	58.7 ± 12.1	72.1 ± 5.0

TABLE 4. Percent colocalization of *M. marinum* 1218R and *M. tuberculosis* H37Ra with cathepsin D

Time (h) post-infection	Mean % colocalization with cathepsin D ± SD ($n \geq 2$)				
	Beads	Live *M. marinum*	Live *M. tuberculosis*	Heat-killed *M. marinum*	Heat-killed *M. tuberculosis*
4	78.7 ± 11.6	11.8 ± 1.0	31.3 ± 11.7	75.2 ± 24.1	35.5 ± 41.7
24	74.5 ± 12.4	13.2 ± 8.1	24.8 ± 18.6	87.4 ± 7.4	56.3 ± 12.9
72	62.7 ± 24.6	12.1 ± 7.6	41.5 ± 7.8	55.6 ± 4.0	62.5 ± 5.0

The data are therefore more likely to be representative of the population being observed. A growing number of different fluorescent cellular markers are now available along with fluorescently labeled primary and secondary antibodies to host cell constituents. These greatly expand the opportunities for using confocal microscopy routinely for determining the trafficking patterns of bacteria.

It is clear that our confocal microscopy trafficking results for *M. tuberculosis* H37Ra correlate with previous findings as to the colocalization of live *M. tuberculosis* with cathepsin D and LAMP-1 (8). It is also of interest that heat-killed *M. tuberculosis* organisms appear to progress through the macrophage to be degraded in lysosomes at significantly higher percentages than do their live *M. tuberculosis* counterparts. This differential trafficking of live and dead organisms has been postulated to occur for mycobacterial species observed in previous studies (2, 8, 10, 24) and correlates with the findings described here for *M. marinum*. This is the first time, however, that live and dead organisms could be definitively distinguished within a single trafficking assay system.

When quantitating the number of bacteria that reside in

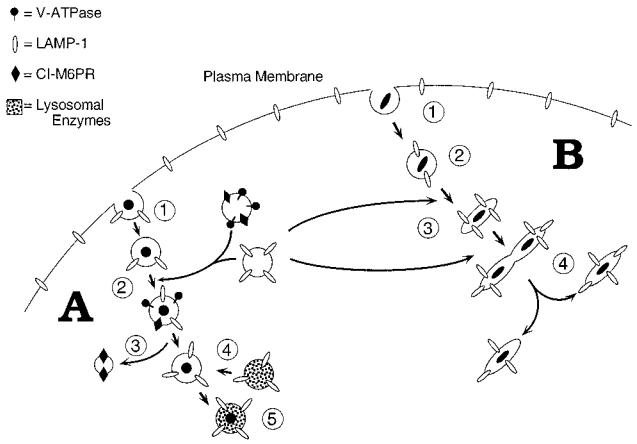

FIG. 6. Proposed trafficking scheme of live and dead *M. marinum* in the macrophage. (A) When a dead *M. marinum* or inert particle (such as a latex bead) is phagocytosed (1), the phagosome acquires LAMP-1, the CI-M6PR, and the V-ATPase (2). The V-ATPase will partially acidify the interior of the phagosome. The CI-M6PR is trafficked back to the Golgi apparatus (3), and this late endosome will fuse with a lysosome containing lysosomal enzymes (4) and additional LGPs, such as LAMP-1. This results in the generation of a phagolysosome (5) within which organisms are killed and dead organisms and other materials are degraded. (B) In the case of live *M. marinum*, organisms are phagocytosed (1) but do not acquire the V-ATPase or the CI-M6PR (2). Instead, these *M. marinum*-containing vesicles appear to acquire modest amounts of LAMP-1 (3) in an active exchange process that may be driven by the need for the bacterium to acquire more membrane as it replicates within the macrophage (4). Our data indicate that the majority of vesicles containing live *M. marinum* do not progress into the late endosomal stage and therefore are not degraded in a phagolysosome.

vesicles containing different endocytic markers, we found a certain amount of heterogeneity between phagosomes even within the same macrophage. It has been postulated that this may be due to a difference in the degree of clumping of bacteria that are in different phagosomes (7). In this study, only bacteria that were in individual vesicles were counted, as clumped bacteria are difficult to score for colocalization with cellular fluorescent markers. It has also been postulated that the viability of infecting bacteria may lead to a heterogeneity of results. We have addressed that possibility in this study and found that whereas there are significant differences in the way that live and heat-killed bacteria are trafficked through the endocytic network, there is also heterogeneity among the population of vacuoles containing live or heat-killed organisms. It is possible that even within a live, clonal population of *M. marinum*, there is heterogeneity in the way phagosomes containing individual bacteria interact with different endocytic markers. It should also be noted that our data indicate a subpopulation of dead organisms that is present throughout our 3-day infection period. This would indicate that a proportion of live *M. marinum* organisms are not surviving within the macrophage. We will be able to address the possibility of two or more populations of infecting bacteria within the same inoculum when we can separate out genetic determinants that dictate passage of mycobacteria through host cells.

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