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Effects of Cytochalasin D and Methylamine on Intracellular Growth of *Legionella pneumophila* in Amoebae and Human Monocyte-Like Cells

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A cloned and axenically cultured strain of *Hartmannella vermiformis* was used as a model to study intracellular multiplication of *Legionella pneumophila* in amoebae. The growth of *L. pneumophila* in both *H. vermiformis* and a human monocyte-like cell line (U937) was investigated with cytoskeletal and metabolic inhibitors. *L. pneumophila* replicated only intracellularly in these cellular models, and electron microscopy showed ultrastructural similarities in the initial phases of multiplication. Treatment of amoebae with an inhibitor of microfilament-dependent phagocytosis (cytochalasin D, 0.5 or 1.0 μg/ml) did not inhibit intracellular growth of *L. pneumophila*; however, intracellular multiplication was inhibited by treatment of U937 monocytes with the same concentrations of cytochalasin D. Methylamine (10 to 100 mM), an inhibitor of adsorptive pinocytosis, inhibited the replication of *L. pneumophila* in amoebae in a dose-dependent manner. All doses of methylamine tested (10 to 50 mM) inhibited growth of *L. pneumophila* in U937 monocytes. Cytochalasin D and methylamine had no effect on the multiplication of *L. pneumophila* in culture medium or on the viability of amoebae or U937 monocytes. Intracellular replication of *L. pneumophila* in *H. vermiformis* may be accomplished by a cytochalasin D-independent mechanism, such as adsorptive pinocytosis. In contrast, both cytochalasin D- and methylamine-sensitive mechanisms may be essential for the intracellular multiplication of *L. pneumophila* in U937 monocytes.

Legionella pneumophila is a facultative intracellular bacterial parasite of human monocytes and macrophages (17, 18, 23, 42) and the most frequent etiological agent of Legionnaires disease (6, 31, 41). The bacterium also multiplies intracellularly within protozoa, including ciliates and amoebae (2, 8, 16, 24, 31, 36). Free-living amoebae isolated from potable and nonpotable water containing *L. pneumophila* (15, 38) have been shown to support the intracellular multiplication of this bacterium, and amoebae isolated from river water sediments have been shown to contain legionellae (14). A strain of amoebae, *Hartmannella vermiformis*, has been isolated and cloned from waters associated with an epidemic strain of *L. pneumophila* (3, 38). This strain of *H. vermiformis* was established in axenic culture (10) and deposited with the American Type Culture Collection (ATCC 50237). Monoxenic cultures of this strain of *H. vermiformis* are known to support the intracellular multiplication of *L. pneumophila* (8).

Several investigators have reported similarities in *L. pneumophila* infection of protozoa and human monocytes and macrophages, and virulence for protozoa and human phagocytes appears to be related (18, 21, 31). Ultrastructural similarities in the intracellular multiplication of *L. pneumophila* in protozoa and human alveolar monocytes have also been noted (2, 15, 17, 24).

Phagocytosis appears to be one mechanism by which *L. pneumophila* infect and multiply within human monocytes (17, 25). Elliott and Winn (7) supported this hypothesis by inhibiting *L. pneumophila* uptake and intracellular multiplication in guinea pig alveolar macrophages through treatment with cytochalasin D. Phagocytosis and intracellular survival of opsonized *L. pneumophila* in cultured human monocytes have been shown to be mediated in part through complement receptors (26). Monoclonal antibodies against monocyte CR1 and CR3 receptors inhibited intracellular multiplication by abrogating *L. pneumophila* adherence and phagocytosis. In contrast, nonopsonized *L. pneumophila* have been shown to infect human monocytes and macrophages (17, 27) and amoebae (2, 9, 15, 24, 28, 31, 35). This phenomenon suggests that mechanisms other than complement-mediated phagocytosis are important in the uptake of *L. pneumophila* by both human phagocytes and amoebae.

Amoeba models should prove useful in understanding the invasion of legionellae in other host cells for two reasons. First, multiplication in the amoeba model is not complicated by the intricate interactions of the host cytokine pathways and serum proteins (e.g., complement and antibody), and second, the amoeba model appears to be especially relevant because of the close relationship of these organisms with *Legionella* spp. in the environment. This study examined the effects of the phagocytosis inhibitor cytochalasin D and the adsorptive pinocytosis inhibitor methylamine on the intracellular multiplication of *L. pneumophila* in amoebae and human monocytes. This was done by using static coincubation models of *L. pneumophila* with an axenic culture of the amoeba *Hartmannella vermiformis* and an untransformed human monocyte-like cell line (U937).

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MATERIALS AND METHODS

Bacterial cultivation. Virulent *L. pneumophila* (serogroup 1, strain RI-243) was initially isolated from a cooling tower implicated in an outbreak of legionellosis. This strain was passaged less than four times on buffered charcoal-yeast extract (BCYE) agar before being harvested and suspended in defibrinated sheep blood at −70°C. When needed, aliquots of bacteria were thawed and cultured on BCYE agar for 4 days at 35°C in a humidified 2.5% CO_2 atmosphere. Bacterial growth was harvested in Pucks saline F (PSF), pH 7.0 (30), and adjusted to an OD_{540} of 0.90 with a Beckman model 24 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Suspensions were serially diluted in PSF to a concentration of approximately 10^4 CFU/ml before inoculation into cultures of amoebae or U937 monocytes. The number of *Legionella* CFU from all cultures and cocultures was determined by triplicate plate counts on BCYE agar incubated as described above.

Amoeba cultivation. *H. vermiformis* (strain CDC-19, ATCC 50237) was isolated as described previously (3) and cloned and identified by Thomas K. Sawyer, Rescon Associates, Inc. The amoebae were stored in 7.5% dimethyl sulfoxide in fresh culture medium under liquid nitrogen until needed, at which time they were thawed and cultivated in ATCC culture medium 1034 in 75-cm^2 tissue culture flasks at 35°C (10). The medium was changed at 3-day intervals for 9 days to establish a subconfluent monolayer of cells. Prior to use in experimental assays, amoebae were harvested by centrifugation (200 × *g*) and resuspended in assay medium (1:1 with ATCC medium 1034 without fetal bovine serum and PSF, pH 6.5). Trophozoites were enumerated with a hemacytometer and diluted in assay medium to a cell density of 10^6/ml before use in experimental assays. Viability was assessed by examination of cytoplasmic streaming with light microscopy.

Human monocyte cultivation. A human histiocytic lymphoma cell line (U937) was obtained from the American Type Culture Collection and maintained as nonadherent monocytelike cells in a static suspension (34). Cells were grown in 25-cm^2 tissue culture flasks in RPMI 1640 medium (pH 8.0) with L-glutamine (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HFBS). Cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. Before use in experimental assays, U937 cells were cultured to the late logarithmic growth phase (approximately 10^7 cells per ml), collected by centrifugation (200 × *g*), resuspended in fresh RPMI-HFBS, and diluted to a viable-cell density of 10^6/ml. Viability was assessed by trypan blue exclusion.

Cell models for intracellular multiplication. Intracellular multiplication of *L. pneumophila* in amoebae and U937 cells was defined as an increase in numbers of an inoculum of *L. pneumophila* in static coincubations with amoebae or U937 monocytes. Controls were used to show that viable amoebae and monocytes were required for growth of *Legionella* organisms, and cocultures were monitored by transmission electron microscopy to demonstrate that all growth was intracellular.

The *L. pneumophila*-amoeba cocultures were prepared by inoculation of *H. vermiformis* into a 25-cm^2 tissue culture flask containing assay medium to achieve a final cell density of 10^5/ml. Amoebae were allowed to adhere for 10 min, at which time *L. pneumophila* were inoculated into the flask at a final concentration of approximately 10^3 CFU/ml. Cocultures were incubated at 35°C for 7 days, and aliquots of cells were removed at 0, 3, and 7 days for culture on BCYE agar. Control cultures of *L. pneumophila* in assay medium without amoebae were prepared in a similar manner.

Parabiotic chambers (Bellco Glass Inc., Vineland, N.J.) were used to demonstrate that no extracellular growth of *L. pneumophila* occurred in assay medium in the presence of viable amoebae. Bacteria (10^3 CFU/ml) and amoebae (10^5/ml) were inoculated separately into two chambers containing assay medium separated by a 0.22-μm-pore-size polycarbonate filter (Nuclepore Corp., Pleasanton, Calif.). The chamber containing amoebae was mixed with a magnetic stir bar (50 rpm), and the apparatus was incubated at 35°C. Bacteria were then enumerated over a 7-day period as described above. At the end of the 7-day incubation, membrane permeability was tested by adding crystal violet (407 Da) to the chamber containing amoebae (7).

The *L. pneumophila*-monocyte cocultures were prepared by inoculation of U937 cells to a final cell density of 10^5/ml into 25-cm^2 tissue culture flasks containing fresh RPMI 1640 medium with 10% HFBS. Bacteria were inoculated to a final concentration of 10^3 CFU/ml, the cocultures were incubated for 7 days, and bacteria were enumerated as described above. All assays were performed in triplicate.

Effects of cytochalasin D and methylamine on intracellular growth. A stock solution of cytochalasin D (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was diluted in assay medium (final pH, 6.25) or RPMI-HFBS (final pH, 8.0) to a concentration of 1.0 or 0.5 μg/ml. A stock solution of methylamine (500 mM) (Sigma) was diluted into assay medium (final pH, 6.30) or RPMI (pH 7.5) to achieve 100, 50, and 10 mM concentrations. Aliquots of assay medium containing inhibitors were then filter sterilized (0.22-μm pore size) and warmed to 35°C in separate 25-cm^2 tissue culture flasks before the addition of amoebae or U937 monocytes.

Amoebae were diluted to 10^5/ml with assay medium containing cytochalasin D (1.0 or 0.5 μg/ml) or methylamine (100, 50, and 10 mM) in tissue culture flasks. The cells were incubated for 1 h at 35°C, inoculated with a 1-ml suspension of 10^4 *L. pneumophila*, and incubated for 7 days. Aliquots of these suspensions containing whole amoebae cells were removed at 0, 3, and 7 days to determine the number of CFU of *L. pneumophila*. Control cultures of the amoebae in assay medium without inhibitors were incubated and inoculated with *L. pneumophila* in a similar manner.

U937 monocytes were diluted 1:10 with 9 ml of fresh RPMI-HFBS containing cytochalasin D (1.0 or 0.5 μg/ml) or methylamine (50 or 10 mM) in tissue culture flasks and incubated for 1 h. A 1-ml suspension of 10^4 *L. pneumophila* was then inoculated into each flask, which were then cultured as described above. Control flasks of *L. pneumophila* with U937 monocytes in fresh RPMI-HFBS without inhibitors were prepared in a similar manner.

The effect of each inhibitor on the survival of *L. pneumophila* in cell culture medium and growth of *L. pneumophila* in buffered ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-yeast extract (BAYE) broth was tested. Bacteria were inoculated into assay medium or RPMI-HFBS containing cytochalasin D (1.0 μg/ml) or 100 mM methylamine to achieve a 10^3-CFU/ml concentration and plated to determine the number of CFU at 0, 3, and 7 days. Legionellae were also grown in and inoculated into BAYE broth (pH 6.75) with and without cytochalasin D (1.0 μg/ml) or 100 mM methylamine to achieve a 10^7 CFU/ml concentration, incubated on a rotary shaker (150 rpm) at 35°C, and incubated and enumerated as above. Experiments with BAYE broth were performed to determine whether the bacteria could multiply in

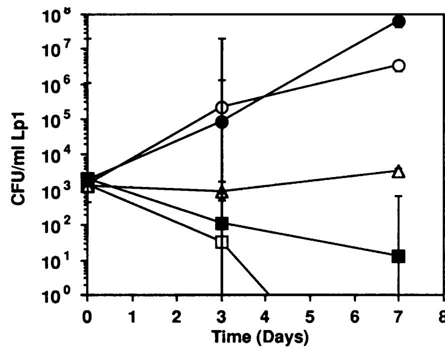

FIG. 1. Intracellular multiplication of *L. pneumophila* (Lp1) in *H. vermiformis* and U937 monocyte cell models. Shown are CFU of *L. pneumophila* in coculture with *H. vermiformis* in assay medium (○), alone in assay medium (□), in assay medium separated from viable *H. vermiformis* by a 0.22-μm-pore-size filter (△), in coculture with U937 monocytes incubated in RPMI-HFBS (●), and in RPMI-HFBS without monocytes (■). Bars represent the standard error of the mean of three replicate experiments.

the presence of these inhibitors under favorable conditions. All tests were performed in duplicate.

Electron microscopy. Amoebae and U937 monocytes from coincubations were fixed for transmission electron microscopy by the addition of a 2% glutaraldehyde–0.1 M collidine buffer to tissue culture flasks after 1 and 3 days of incubation. The flasks were incubated for 1 h at 4°C, at which time fixed cells were decanted and washed three times by centrifugation in 0.2% collidine buffer. Fixed cells were resuspended in molten 2% agarose (45°C), centrifuged into a pellet, allowed to cool, and cut into 1-mm^3 sections. Agar pieces were postfixed with 1% OsO_4 for 45 min, stained with methanolic uranyl acetate for 2 h, and dehydrated in a graded ethanol series culminating in absolute acetone. Dehydrated cells in agar were infiltrated with propylene oxide and Maraglas plastic, polymerized at 60°C overnight, thin sectioned, and mounted on copper mesh grids. Sections were poststained with lead citrate and viewed with a Philips transmission electron microscope at 40 kV.

Statistical analysis. All tests for significance were performed with one-factor analysis of variance and Fisher's protected least-squares difference with the Statview program (BrainPower Inc., Calabasas, Calif.) and a Macintosh Plus computer (Apple Computer Inc., Cupertino, Calif.).

RESULTS

Multiplication of legionellae in amoebae and monocytes. Figure 1 shows that the number of *Legionella* CFU increased 1,000-fold over the initial concentration in static coculture with *H. vermiformis* but failed to multiply in assay medium without the amoebae. The majority of these amoebae were encysted by the third day of incubation, while approximately 10% persisted as trophozoites throughout the 7-day coincubation with legionellae. The parabiotic chamber

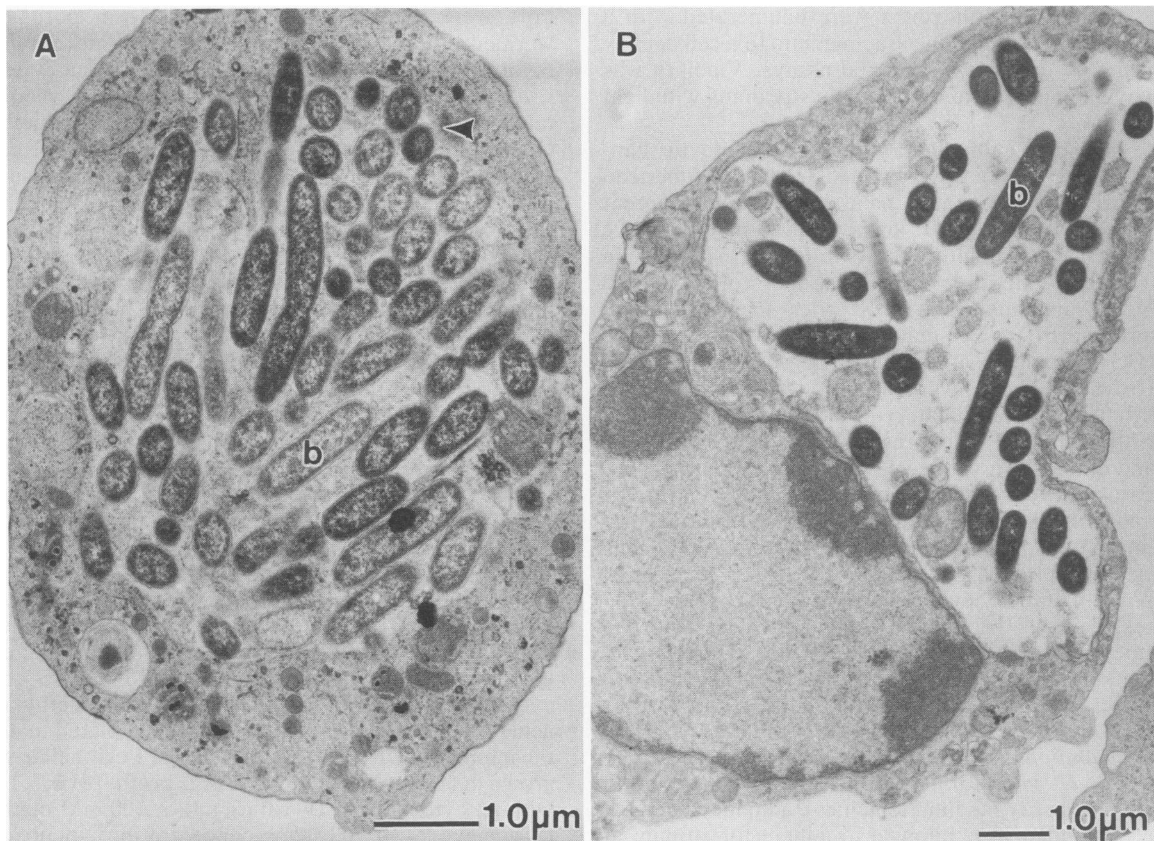

FIG. 2. Transmission electron photomicrographs of *H. vermiformis* and U937 monocytes infected with *L. pneumophila*. (A) Amoeba cell from 3-day coculture heavily infected with bacteria (b). Note loss of vacuole membrane integrity (arrow). (B) U937 monocyte from 3-day coculture with single loose-fitting vacuole containing bacteria (b).

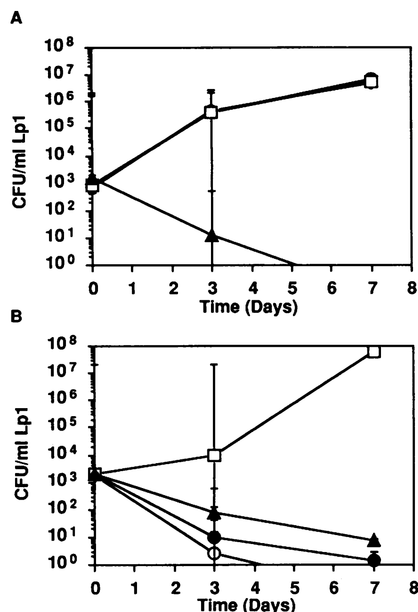

FIG. 3. (A) Effects of cytochalasin D on *L. pneumophila* (Lpl) coincubated with *H. vermiformis* treated with cytochalasin D at 0.5 μg/ml (○) or 1.0 μg/ml (●), with untreated amoebae in assay medium (□) (data points for treatments overlap), and without amoebae in assay medium containing cytochalasin D (1.0 μg/ml) (▲). (B) Effects of cytochalasin D on *L. pneumophila* (Lpl) coincubated with U937 monocytes treated with cytochalasin D at 0.5 μg/ml (●) or 1.0 μg/ml (○), with untreated monocytes in RPMI-HFBS (□), and without monocytes in RPMI-HFBS containing cytochalasin D (1.0 μg/ml) (▲). Bars represent the standard error of the mean of three replicate experiments.

experiment demonstrated that legionellae did not grow in the presence of metabolic by-products of viable amoebae in assay medium. The membrane was permeable to crystal violet (>407 Da). Although legionellae remained viable for more than 7 days, there was no significant increase ($P > 0.05$) in CFU during this period (Fig. 1).

L. pneumophila numbers increased greater than 10,000-fold in coculture with U937 monocytes, but it did not multiply in RPMI-HFBS without monocytes (Fig. 1). Many of the monocytes were lysed after 7 days; however, some larger cells were observed to be filled with bacteria.

Transmission electron microscopy of *H. vermiformis* in coculture with *L. pneumophila* for 3 days showed that legionellae multiplied within large vacuoles of the amoeba (Fig. 2A). Legionellae also appeared to multiply within large vacuoles of U937 monocytes after 3 days of coincubation (Fig. 2B).

Effects of cytoskeletal and metabolic inhibitors on intracellular growth of *L. pneumophila*. Preliminary experiments indicated that higher concentrations of methylamine (250 mM) in assay medium caused encystation of amoebae within 24 h, and 250 and 100 mM methylamine in RPMI-HFBS caused rounding and eventual death of U937 cells after 24 h. Lower concentrations of methylamine were not toxic to either the amoebae or the U937 monocytes after 7 days, as shown below.

Cytochalasin D had no effect on the intracellular multiplication of legionellae in *H. vermiformis* (Fig. 3A). There was no significant difference ($P > 0.01$) in the growth of legionellae in coculture with amoebae treated with 0.5 or 1.0 μg of cytochalasin D per ml or in the positive control culture of untreated amoebae. Legionellae persisted longer in assay medium containing 1.0 μg of cytochalasin D per ml (Fig. 3A) than in assay medium alone (Fig. 1), indicating that cytochalasin D in the assay medium was not toxic to the bacteria. Concentrations of 5, 10, and 50 μg of cytochalasin D per ml were evaluated with *H. vermiformis* (data not shown). Legionellae grew intracellularly in the presence of 5 and 10 μg/ml; 50 μg of cytochalasin D per ml inhibited growth of legionellae, but this concentration appears to kill amoebae within 24 h.

Legionellae did not grow within U937 cells that were treated with 0.5 or 1.0 μg of cytochalasin D per ml but did multiply to greater than 10^7 CFU in the absence of cytochalasin D (Fig. 3B). Bacteria persisted for 7 days in RPMI-HFBS medium containing 1.0 μg of cytochalasin D per ml without monocytes, and 90% of the U937 monocytes exposed to 1.0 μg of cytochalasin D per ml in RPMI-HFBS for 3 days were viable, as determined by trypan blue exclusion.

Methylamine exhibited a dose-dependent effect on the multiplication of legionellae in *H. vermiformis*. Legionellae increased 1,000-fold in the absence of this inhibitor, but a 100 mM concentration of methylamine completely inhibited intracellular multiplication of bacteria in the amoebae (Fig. 4A). A 50 mM concentration of methylamine reduced the increase in legionellae to less than 100-fold the number in treated amoebae, and 10 mM methylamine, although significantly inhibitory ($P < 0.01$), was only slightly so after 7 days of incubation. Methylamine (100 mM) in the assay medium was not toxic to legionellae or amoebae, as evidenced by the

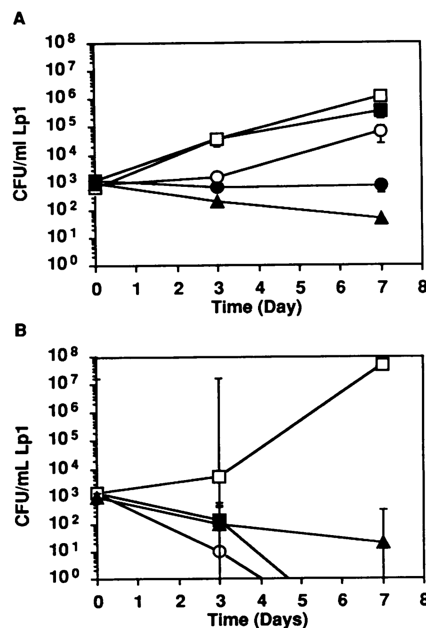

FIG. 4. (A) Effects of methylamine on *L. pneumophila* (Lpl) coincubated with *H. vermiformis* treated with 100 mM methylamine (●) or 50 mM methylamine (○), with amoebae and 10 mM methylamine in the assay medium (■), with untreated amoebae in assay medium (□), or without amoebae and with 100 mM methylamine in the assay medium (▲). (B) Effects of methylamine on *L. pneumophila* (Lpl) coincubated with U937 monocytes treated with 50 mM methylamine (○) or 10 mM methylamine (■), with untreated monocytes in RPMI-HFBS (□), and without monocytes in 100 mM methylamine (▲). Bars represent the standard error of the mean of three replicate experiments.

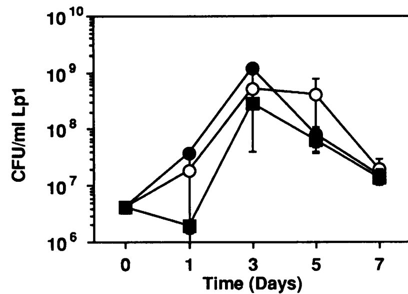

FIG. 5. Effects of cytochalasin D and methylamine on the extracellular growth of *L. pneumophila* (Lp1) in BAYE broth. Symbols: ○, BAYE broth without inhibitors; ●, BAYE broth with 1.0 μg of cytochalasin D per ml; ■, BAYE broth with 100 mM methylamine. Bars represent the standard error of the mean of two replicate experiments.

persistence of viable bacteria (Fig. 4A) and the observation of cytoplasmic streaming in amoebal trophozoites after 3 and 7 days.

L. pneumophila failed to multiply in U937 monocytes treated with 10 or 50 mM methylamine (Fig. 4B), and bacteria were not detected after 3 and 4 days, respectively. Legionellae in control cultures of untreated U937 monocytes grew to 10^7 CFU. Bacteria persisted for 7 days in RPMI-HFBS medium containing 100 mM methylamine without monocytes. Approximately 85% of the U937 monocytes exposed to 50 mM methylamine were viable after 3 days as indicated by trypan blue exclusion.

Legionellae multiplied >100-fold in BAYE broth after 3 days with and without cytochalasin D (1.0 μg/ml) or 100 mM methylamine and showed a typical decline in numbers after 7 days in all cultures (Fig. 5). The pH of BAYE broth was not altered by the addition of cytochalasin D or methylamine.

DISCUSSION

L. pneumophila multiplied when coincubated with axenic *H. vermiformis* and U937 monocytes but were unable to grow in assay medium or RPMI-HFBS without these cells (Fig. 1). Legionellae failed to multiply in assay medium when separated from viable amoebae by a 0.22-μm-pore-size membrane (Fig. 1); therefore, bacterial multiplication in amoebae was dependent on an intracellular environment and not on soluble nutrients secreted by amoebae. Similarly, viable *L. pneumophila* have been shown to persist in, but not grow in, RPMI-HFBS when separated from viable macrophages (7). Transmission electron microscopy confirmed that multiplication of bacteria was due to intracellular multiplication in amoebae and U937 monocytes (Fig. 2A and B).

Cytoskeletal and metabolic inhibitors have been useful in describing bacterial invasion of different host cells due to the conserved nature of eukaryotic cell biology. Cytochalasin D is an inhibitor of actin filaments that are involved in plasma membrane invagination during phagocytosis (5). Cytochalasins have been used extensively in describing microfilament-dependent phagocytosis of bacterial pathogens in nonprofessional- and professional-phagocytic-cell models (7, 13, 28, 39, 40). The use of cytochalasins B and D has demonstrated that actin filaments also play a significant role in amoeba locomotion and phagocytosis (29, 33). Methylamine is an inhibitor of transglutaminase, a plasma membrane enzyme involved in the aggregation of ligand-receptor complexes, and is required for receptor-mediated pinocytosis in fibroblasts and macrophages (20, 35). This inhibitor has been used to inhibit adsorptive pinocytosis of Semliki Forest virus in BHK-21 cells and infection of McCoy cells by *Chlamydia trachomatis* (21, 32).

Previous studies have demonstrated that *L. pneumophila* do not actively penetrate or passively enter macrophages (37) but are ingested by a type of microfilament-dependent phagocytosis which can be inhibited with cytochalasin D (1.0 μg/ml) (7). We also found that cytochalasin D inhibited intracellular multiplication of *L. pneumophila* in the U937 monocyte model (Fig. 3B). In contrast, cytochalasin D had no effect on the intracellular multiplication of *L. pneumophila* in *H. vermiformis* (Fig. 3A). There are several reports of the use of cytochalasins to inhibit actin polymerization in amoebae (29, 33). These studies used cytochalasins at concentrations of up to 50 μg/ml. We also tested these concentrations of cytochalasin D with *H. vermiformis*. Growth of legionellae was inhibited only in the presence of concentrations of cytochalasin D which appear to kill the amoebae (50 μg/ml). Whether the bacteria failed to grow because of microfilament inhibition or death of the host cell is unclear. Some legionellae may enter amoebae by phagocytosis even in the presence of these concentrations of cytochalasin D.

Methylamine (100 and 50 mM) inhibited intracellular multiplication of *L. pneumophila* in both *H. vermiformis* and U937 monocytes (Fig. 4A and B). The pH in these assays was not sufficiently different to affect the intracellular growth of *L. pneumophila*. Legionellae persisted in assay medium and RPMI-HFBS containing cytochalasin D (1.0 μg/ml) or 100 mM methylamine (Fig. 3A and B and 4A and B) and multiplied extracellularly in BAYE medium containing the same inhibitor concentrations (Fig. 5). These tests indicate that these inhibitors are not toxic to *L. pneumophila* and have no effect on bacterial multiplication under favorable conditions.

Our findings indicate that the intracellular multiplication of *L. pneumophila* in *H. vermiformis* and U937 monocytes occurs in part by a microfilament-independent process, such as pinocytosis. However, intracellular multiplication in U937 monocytes appears to require an additional microfilament-dependent mechanism. A simple assumption would be that *L. pneumophila* infect U937 monocytes via two independent mechanisms. It would not be surprising to find that *L. pneumophila* have developed a more efficient mechanism, i.e., a single event, for the invasion of amoebae as a means of ensuring their survival in the environment (12, 13). Additional studies are required to further define the attachment and entry mechanisms of *L. pneumophila* in amoebae.

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