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Interactions between *Naegleria fowleri* and *Legionella pneumophila*

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Using electron microscopy we documented some of the intracellular events that occur in *Naegleria fowleri* suspended in Page amoeba saline after ingestion of *Legionella pneumophila*. Photomicrographs showed intracellular vacuoles containing bacteria in the process of binary fission that was accompanied by alignment of mitochondria and ribosome-like structures along the vacuole membrane. Although these intracellular events are remarkably similar to that seen in *Legionella* replication within human monocytes, we could not demonstrate an increase in the number of bacteria by CFU or dark-field microscopy. However, when the *Naegleria* cells were allowed to ingest *Legionella* cells while suspended in amoeba culture medium, the number of bacteria increased, and this was contingent upon the presence of viable amoebae.

The bacterium *Legionella pneumophila* occurs primarily in aquatic habitats and has been responsible for sporadic episodes of human infection (6–8). It is considered to be a facultative intracellular parasite, and it multiplies intracellularly in human blood monocytes and apparently in alveolar macrophages (3, 15). A prominent feature is the sequence of cytoplasmic events in the host cell after infection. Phagocytized *Legionella* cells are enclosed in vacuoles which are encircled by one or more mitochondria apposed to the vacuolar membrane. Subsequently, ribosomes line the margin of the bacteria-filled vacuole (13). This has also been observed in leukocytes from victims of Legionnaires disease (10).

L. pneumophila is relatively widespread and may be found in waters which have not been associated with Legionnaires disease (6, 7, 22). When epidemic outbreaks do occur, however, evidence suggested in some instances the source of infection to be warmed water in air-conditioning cooling towers (8). The difficulty associated with culturing *Legionnela* isolates in the laboratory has led to speculation that this bacterium might benefit from or require interaction with other procaryotic or eucaryotic microbes to sustain itself in nature. There is now accumulating evidence to support this proposal. Tison et al. (21) observed that *L. pneumophila* could use algal extracellular products as a source of carbon and energy and suggested that this association could explain its widespread distribution. In addition, Tyndall and Domingue (23) reported that *L. pneumophila* could be propagated in association with the amoebae *Naegleria lovaniensis* and *Acanthamoeba royreba*. Most recently, Holden et al. (11) conclusively showed intraamoebal multiplication in the amoeba *Acanthamoeba castellanii* Neff.

An intriguing aspect of these reports is the possibility that cytoplasmic events associated with the intracellular multiplication of *L. pneumophila* in human cells might be common to bacterial multiplication in amoebae. To investigate this possibility, we cultured *L. pneumophila* with the amoeba *Naegleria fowleri*. This particular thermophilic amoeba is the causative agent of primary amoebic meningoencephalitis, and pathogenic *N. fowleri* has been isolated most often from cooling towers, thermal effluents, hot springs, or other waters with naturally or artifically elevated temperatures (2, 5, 20).

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MATERIALS AND METHODS

Cultivation of *N. fowleri* and *L. pneumophila*. The Lee strain of pathogenic *N. fowleri* was cultured at 37°C in 75-cm^2 tissue culture flasks containing Chang SCGYEM medium (4) without the antibiotic supplement. At the late exponential or early stationary stage the flasks were chilled on ice to dislodge the monolayer of amoebae adhering to the flask. The culture was centrifuged (1,000 × *g* for 10 min) at room temperature (22°C), and amoebae were suspended in Page amoeba saline (PAS) containing 120 mg of NaCl per liter (16) or Chang medium. After hemacytometer counts, the cell density was adjusted to 5 × 10^5 *Naegleria* cells per ml. A 50-ml volume of the amoeba suspension was then pipetted into 75-cm^2 tissue culture flasks. *L. pneumophila* (serogroup 1), a direct environmental isolate from a cooling tower, was maintained at 37°C with weekly passages on buffered charcoal-yeast extract agar (17). The bacterial inoculum was prepared by flooding the agar plate with PAS and adjusting the bacterial suspension to an optical density of 0.16 (660 nm) with a Varian Cary 219 spectrophotometer. A 1-ml sample of this suspension was added to amoebae in culture medium or amoebae suspended in PAS and incubated at 37°C. The *Escherichia coli* inoculum was grown in brain heart infusion broth (37°C) and likewise adjusted in PAS.

Interaction between *Naegleria* and *Legionella* cells. During incubation of amoebae with bacteria, the number of amoebae was determined by hemacytometer counts. The number of *Legionella* CFU was determined by serially diluting 0.1 ml from the suspension in deionized water and plating onto buffered charcoal-yeast extract agar. Plates were incubated at 37°C. These cells had typical *Legionella* morphology as evidenced by the cut-glass appearance of colonies with a slightly pink color around the margin of the colony. Results are based on three separate experiments with uniform results.

Electron microscopy. Amoebae that had been incubated in PAS with *Legionella* cells were fixed by adding an equal volume of sodium cacodylate-buffered (0.1 M, pH 7.4) 3% glutaraldehyde to the cell suspension at room temperature (22°C). After a 1- to 3-h period of fixation, the suspensions were centrifuged at 1,000 × *g* for 10 min, and the pellets were suspended in fresh sodium cacodylate buffer. For electron microscopy, *N. fowleri* and *L. pneumophila* were immobilized in agar. Pellets of fixed and washed cells were mixed with a small quantity of 7% agar at 60°C in a small

FIG. 1. Multiplication of *L. pneumophila* in association with *N. fowleri* in amoeba culture medium. Symbols: ○, *L. pneumophila* and *N. fowleri*; ×, *L. pneumophila* only or with lysed *Naegleria* cells.

tube. After cooling, the solidified agar containing the cells was removed, minced into 1-mm^2 pieces, and placed in cacodylate buffer. Specimens were postfixed in 1% sodium cacodylate-buffered osmium tetroxide for 1 h, dehydrated in a graded series of ethanols, and embedded in Polybed 812 (Polysciences). Semithin sections (1 to 15 μm) were cut on glass knives and stained with a mixture of methylene blue and azure II followed by basic fuchsin. Thin sections (60.0 to 80.0 nm) were cut on a diamond knife and stained with uranyl acetate followed by lead citrate (18). Stained specimens were examined and photographed with a Phillips 300 electron microscope at 80 kV.

RESULTS

L. pneumophila was incubated with *N. fowleri* suspended in amoeba growth medium (Chang SCGYEM), and after 6 days the number of bacterial CFU increased from 10^6 to 2.8 × 10^8 *Legionella* cells per ml (Fig. 1). When viewed by dark-field microscopy, extracellular bacteria were free in culture fluid and were not clumped or enclosed in membrane vesicles. If *L. pneumophila* was incubated alone or with lysed amoebae in this medium, the number of CFU declined to 10^4 bacteria per ml after 6 days. The number of amoebae increased from an initial concentration of 5 × 10^5 to approximately 10^6 amoebae per ml during the first 48 h and remained at that level throughout the course of the experiment and was comparable to the control with *N. fowleri* only.

Conversely, when *L. pneumophila* was incubated with *N. fowleri* suspended in PAS, destruction of amoebae was apparent after 48 h, and by 96 h only 1% of the original number of amoebae could be observed. Destruction of amoebae did not occur when *E. coli* was used instead of *L. pneumophila* or if the *Naegleria* cells were incubated alone. When these *L. pneumophila* infected amoebae were viewed by electron microscopy, notable changes were observed in the protozoan cell morphology. Intact intracellular *Legionella* cells were observed 3 h after coincubation, and the bacteria were enclosed in vacuoles. Mitochondria were in proximity to the vacuoles and on occasion were apposed to the vacuole membrane (Fig. 2). After 12 h the number of intracellular bacteria began to increase, and in some instances they occupied most of the cytoplasmic space. Intracellular bacteria were enclosed in membrane-bound vacuoles, and on occasion some bacteria were in the process of binary fission. As the bacteria-filled vacuole occupied a greater portion of the cell, the nucleus and other cellular constituents were pushed to the margin of the cytoplasmic membrane (Fig. 3). Bacteria were not observed in the nucleus. Of particular interest was that the sides of the vacuoles containing *Legionella* bacteria were invariably lined with spherical structures approximately 18 nm in diameter that resembled host-cell ribosomes (Fig. 4). Despite the accumulation of bacteria in the amoeba with the appearance of some bacteria in the process of binary fission, the number of *Legionella* cells decreased from 10^6 to 10^3 bacteria per ml after 6 days, as evidenced by a reduction in CFU and lack of observable bacteria by dark-field microscopy. However, the number of *Legionella* cells remained relatively constant when incubated alone in the PAS solution.

DISCUSSION

Results of this investigation show that *L. pneumophila* is readily taken into the amoeba *N. fowleri* and that this protozoan can provide an intracellular environment conducive to multiplication of *L. pneumophila*. However, this is contingent upon the experimental conditions. The failure of *Legionella* CFU to increase when incubated with *N. fowleri* in PAS alludes to the importance of the host cell and its environment as a limiting factor. Under these conditions intracellular *Legionella* cells may initiate cell division and induce a variety of changes in amoebae (as shown by electron microscopy), but the *Naegleria* cells apparently could not sustain bacterial growth and multiplication. Presently this anomaly cannot be explained, but it is likely that

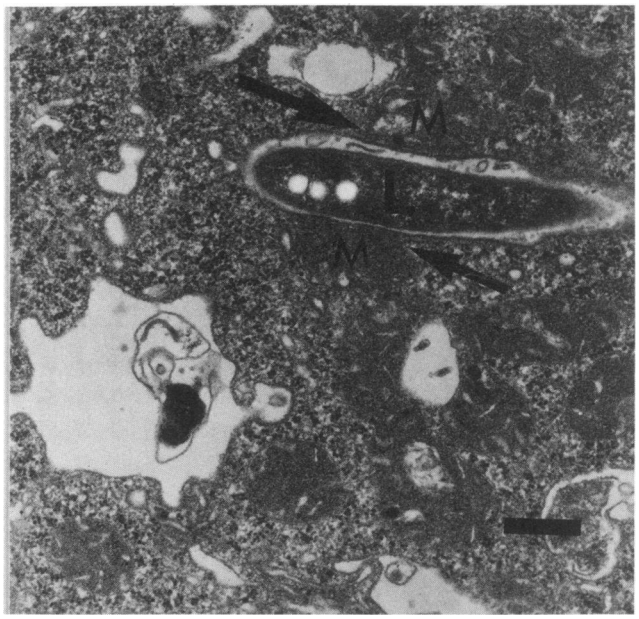

FIG. 2. Single *Legionella* (L) bacterium enclosed in a cytoplasmic vacuole within a *Naegleria* cell. Note the mitochondria (M) closely apposed to the vacuole membrane (arrows). Bar = 0.5 μm.

amoebae require nutritionally replete conditions and perhaps additional factors to promote an increase in *Legionella* CFU. In saline, the ingestion of *Legionella* cells by *Naegleria* cells might be expected to decrease the number of CFU if the bacteria are aggregated and fail to multiply. This along with the possible accumulation of toxic products or lytic enzymes from dead amoebae could serve to decrease the number of *Legionella* cells. Clearly, however, based on the absence of bacteria by dark-field microscopy and the lack of CFU, the bacteria failed to increase in number despite ingestion by amoebae.

In previous studies documenting the intracellular multiplication of *Legionella* cells in human monocytes (15) or in amoebae incubated in culture medium (11, 23), the host cells were disrupted by artificial means to facilitate liberation of bacteria, and an increase in *Legionella* CFU was observed 24 h after coincubation. For our investigation, when the amoebae host cells were incubated in culture medium and were not artifically disrupted, the CFU in culture supernatants did not increase until 72 h. Although intracellular *L. pneumophila* cells may initiate cell division soon after coincubation, they may not be liberated from amoebae for some time. It is likely that the bacteria are sequestered within the amoebae and that their liberation is dependent upon death of the amoeba. In saline, the destruction of amoebae by *Legionella* cells and not by *E. coli* suggests a cytotoxic effect that was not apparent when *Legionella* and *Naegleria* cells were coincubated in culture medium. Previous studies have suggested the production of a cytotoxic factor by *L. pneumophila* (9). Using amoebae, Rowbotham (19) observed that on agar plates, *Legionella* cells were phagocytized by *Naegleria* and *Acanthamoebae* cells, and the bacteria exerted a cytopathic effect on the protozoans. However, Tyndall and Domingue (23) reported that when *L. pneumophila* was incubated with *Naegleria* and *Acanthamoeba* cells in amoeba culture medium no deleterious effect on amoebae was observed. This difference was attributed in part to the fact they were using a low-virulent *Legionella* bacterium. More recently Anand et al. (1) incubated *L. pneumophila* with *Acanthamoeba palestinensis* in Neff amoeba culture medium and observed an increase in *Legionella* CFU, and the total number of amoebae did not change significantly.

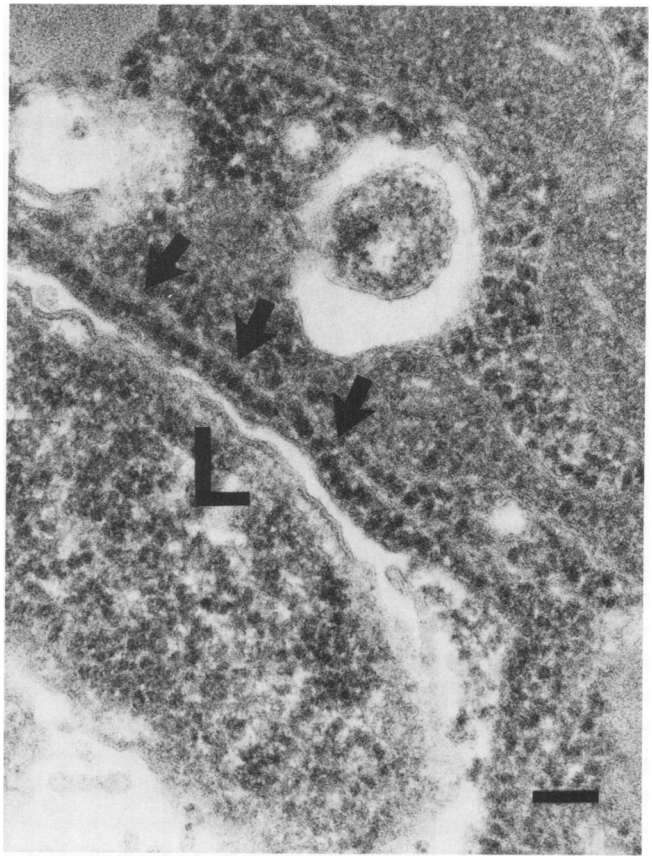

FIG. 4. A portion of a *Legionella* (L) cell adjacent to the vacuole membrane. The undulate bacterial membrane is apparent. Note the presence of large numbers of closely packed ribosome-like particles lining the cytoplasmic surface of the vacuole membrane (arrows). Bar = 100 nm.

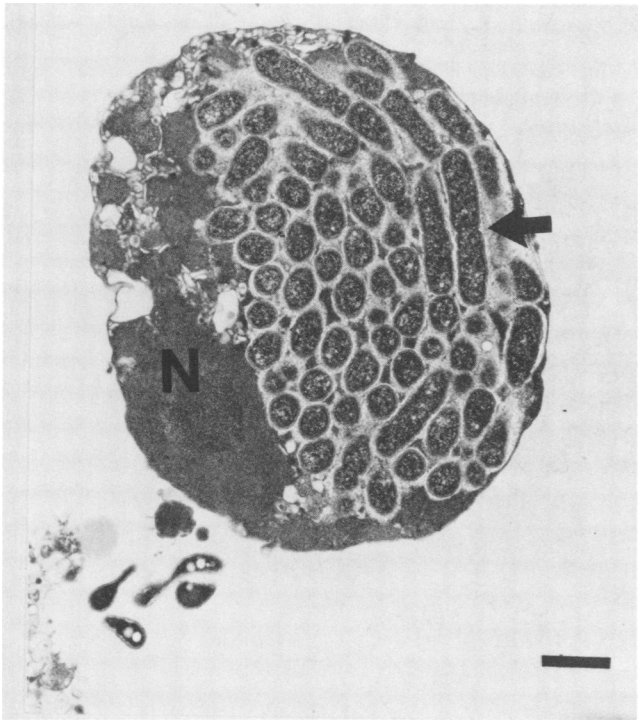

FIG. 3. *L. pneumophila*-filled vacuole occupying a significant portion of the cytoplasm with a bacterium in binary fission (arrow). Note that the nucleus (N) has been displaced and is compressed against the cytoplasmic membrane. Bar = 1.0 μm.

In our investigation, destruction of amoebae was apparent only in saline and not in culture medium. A likely explanation is that a portion of infected amoebae may have been killed by interaction with *Legionella* cells (followed by escape of bacteria), but because of multiplication of lightly infected or noninfected amoebae it was not apparent. This would help explain the disparate observations in previous studies. It is possible that under growing conditions amoebae may be able to neutralize a toxin or components of amoeba culture medium could inhibit the toxicity of *Legionella* cells.

When we suspended *Naegleria* cells in saline and allowed them to ingest *Legionella* cells, conspicuous intracellular events were observed within the amoebae. The appearance of mitochondria apposed to the membrane-enclosed *Legionella* cells followed by ribosomes along the margin of the

bacteria-filled vacuoles parallels that observed in *L. pneumophila*-infected human monocytes by Horwitz and Silverstein (13, 15). At this time it is not known if the appearance of these structures is a specific response to this bacterium or is of a more general nature. However, it has been suggested that *L. pneumophila* is unique among bacterial pathogens in promoting the formation of ribosome-lined vacuoles in monocytes (15).

The similarity in intracellular events in both *L. pneumophila*-infected *Naegleria* cells and human monocytes has ramifications for understanding the biology and possibly the ability of this bacterium to retain virulence in the environment for susceptible humans. Recently, Horwitz has reported that in human monocytes *L. pneumophila* inhibits phagosome-lysosome fusion (12) and that phagocytosis of the bacterium occurs by a novel mechanism involving a pseudopod coil (14). Our study suggests future investigations to determine if these events also occur in amoebae and utlimately if Legionnaires disease could be the result of unwitting human intervention into the life cycle of this facultative intracellular parasite, which in various aquatic habitats may normally replicate within freshwater amoebae.

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