APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1996, p. 2022–2028 Vol. 62, No. 6
0099-2240/96/$04.00+0

The Phagosome Containing *Legionella pneumophila* within the Protozoan *Hartmannella vermiformis* Is Surrounded by the Rough Endoplasmic Reticulum

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Received 30 November 1995/Accepted 20 March 1996

Legionella pneumophila **is an intracellular parasite of protozoa and human phagocytes. To examine adaptation of this bacterium to parasitize protozoa, the sequence of events of the intracellular infection of the amoeba *Hartmannella vermiformis* was examined. The previously described uptake phenomenon of coiling phagocytosis by human monocytes was not detected. At 1 h postinfection with wild-type strain AA100, mitochondria were observed within the vicinity of the phagosome. At 2.5 h postinfection, numerous vesicles surrounded the phagosomes and mitochondria were in close proximity to the phagosome. At 5 h postinfection, the bacterium was surrounded by a ribosome-studded multilayer membrane. Bacterial multiplication was evident by 8 h postinfection, and the phagosome was surrounded by a ribosome-studded multilayer membrane until 15 h postinfection. The recruitment of organelles and formation of the ribosome-studded phagosome was defective in an isogenic attenuated mutant of *L. pneumophila* (strain AA101A) that failed to replicate within amoebae. At 20 h postinfection with wild-type strain AA100, numerous bacteria were present in the phagosome and ribosomes were not detected around the phagosome. These data showed that, at the ultrastructural level, the intracellular infection of protozoa by *L. pneumophila* is highly similar to that of infection of macrophages. Immunocytochemical studies provided evidence that at 5 h postinfection the phagosome containing *L. pneumophila* acquired an abundant amount of the endoplasmic reticulum-specific protein (BiP). Similar to phagosomes containing heat-killed wild-type *L. pneumophila*, the BiP protein was not detectable in phagosomes containing the mutant strain AA101A. In addition to the absence of ribosomes and mitochondria, the BiP protein was not detected in the phagosomes at 20 h postinfection with wild-type *L. pneumophila*. The data indicated that the ability of *L. pneumophila* to establish the intracellular infection of amoebae is dependent on its capacity to reside and multiply within a phagosome surrounded by the rough endoplasmic reticulum. This compartment may constitute a rich source of nutrients for the bacteria and is probably recognized as a cellular compartment. The remarkable similarity of the intracellular infections of macrophages and protozoa by *L. pneumophila* strongly supports the hypothesis that adaptation of the bacterium to the intracellular environment of protozoa may be the mechanism for its ability to adapt to the intracellular environment of human alveolar macrophages and causes pneumonia.**

The causative agent of Legionnaires' pneumonia, *Legionella pneumophila*, is a facultative intracellular bacterium. This bacterium is ubiquitous in the aquatic environment, where it resides as a parasite of protozoa (22, 36). Transmission to humans occurs through inhalation of contaminated aerosols generated by numerous man-made devices, such as shower heads (9, 19, 37). The ability of *L. pneumophila* to cause pneumonia is dependent on its capacity to multiply within alveolar macrophages.

Although *L. pneumophila* can parasitize mammalian cells and protozoa, the uptake mechanisms of *L. pneumophila* by both host cells are different (3, 30). The intracellular pathway of *L. pneumophila* in human phagocytic cells has been well characterized. Following uptake by human phagocytes, the bacterium resides in a phagosome that does not undergo acidification or phagolysosomal fusion (13, 24, 25). Mitochondria and other host cell organelles are recruited around the phagosome (25). Within the first few hours of the intracellular infection, the phagosome containing *L. pneumophila* is surrounded by the rough endoplasmic reticulum (RER) (38, 39). Since mutants of *L. pneumophila* that fail to establish this replicative phagosome also fail to replicate intracellularly, the formation of this specialized phagosome is believed to be essential for intracellular survival and replication of *L. pneumophila* within mammalian macrophages (7, 27, 32, 38). The capacity of *L. pneumophila* to infect mammalian cells and protozoa and the failure of mutants to multiply within both host cells (3, 12, 17) indicate common mechanisms of interaction with both host cells. Understanding the interactions between *L. pneumophila* and its natural protozoan host would provide insights into its adaptation to parasitize protozoa and its evolution to infect human alveolar macrophages.

Although replication of *L. pneumophila* within a ribosome-studded protozoan phagosome is well documented (16, 17, 34), there have been no studies to examine the development of the protozoan phagosome at several stages of the course of infection. To start dissecting the intracellular pathway of *L. pneumophila* within protozoa and its interaction with the host cell, the ultrastructural manifestations at several stages of the intracellular infection were examined. Immunocytochemical studies were performed to examine the nature of the phagosome containing *L. pneumophila* within one of its protozoan hosts, *Hartmannella vermiformis* (40, 41). In this report, it is shown that the intracellular pathway of *L. pneumophila* in protozoa is very similar to that of mammalian phagocytes.

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Immunocytochemical evidence is provided to show that similar to mammalian cells (38, 39), the phagosome containing *L. pneumophila* matured to become surrounded by the RER. The phagosome containing an isogenic mutant of *L. pneumophila* that did not replicate within *H. vermiformis* failed to be surrounded by the RER. These data indicate that the ability of *L. pneumophila* to survive and replicate within *H. vermiformis* is dependent on its capacity to be surrounded by the RER.

MATERIALS AND METHODS

Bacterial and protozoan strains and media. *L. pneumophila* serogroup 1 strain AA100 is a clinical virulent isolate that has been described previously (1–3). Strain AA101A is an isogenic mutant of AA100 that was derived by passage on Mueller-Hinton agar, as described previously (33). Compared with the wild-type strain, strain AA101A failed to replicate within macrophages or within protozoa. *L. pneumophila* strains were grown on BCYE agar plates at 37°C for 48 h.

H. vermiformis CDC-19 (registered with the American Type Culture Collection as strain 50237) was isolated from a water sample obtained during an outbreak of Legionnaires' pneumonia (9, 12, 20). The amoebae were grown in American Type Culture Collection medium 1034 at 35°C, as described elsewhere (19).

Infection of *H. vermiformis* with *L. pneumophila*. Infection of *H. vermiformis* with *L. pneumophila* has been described previously (3). Briefly, triplicate cultures of *H. vermiformis* containing 10^5 CFU/ml were infected with approximately 10^3 CFU of *L. pneumophila* per ml. At several time intervals, triplicate aliquots were withdrawn from each flask and cultured on BCYE agar plates for bacterial enumeration. *L. pneumophila* does not replicate extracellularly in the growth medium whether or not amoebae are present (19, 21, 30), and thus, any increase in the number of bacteria is due to intracellular multiplication.

Transmission electron microscopy. Monolayers of *H. vermiformis* were infected with *L. pneumophila* at a multiplicity of infection of 10. At several time intervals, aliquots of the infected and uninfected monolayers were fixed for transmission electron microscopy with a solution of 1% OsO_4 (2 parts) and 2.5% glutaraldehyde (1 part) in 0.1 M cacodylate buffer (pH 7.4), stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer (pH 6.3), dehydrated with ethanol, and embedded in Epon (Shell Chemical Co.), as described previously (23, 25). The sections were stained with lead citrate and uranyl acetate (35) and examined with a Hitachi H7110/STEM electron microscope at 75 kV.

Sample preparation for immunocytochemistry. Infected monolayers of *H. vermiformis* were harvested at several time intervals, washed with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde–0.1% glutaraldehyde in PBS for 2 h at 4°C. The cells were pelleted by low-speed centrifugation, washed with and resuspended in PBS, and dehydrated. The cells were infiltrated and low-temperature embedded (6) in Lowicryl K4M (Polysciences, Inc., Warrington, Pa.) by a progressive low-temperature technique as described in detail elsewhere (11). Sections, 80 to 90 nm, were cut on a Reichart (Vienna, Austria) Ultracut 4E ultramicrotome with a diamond knife (Diatome, Fort Washington, Pa.) and collected onto 400-mesh nickel grids (Ted Pella Inc., Redding, Calif.). The grids were floated on microdrops (26 to 60 ml) for the following procedures except where extensive washing was involved. Sections collected on grids were quenched with 0.5 M ammonium chloride (pH 5.5) for 1 h and then transferred directly for blocking in 10% normal goat serum in PBS supplemented with 1% bovine serum albumin (BSA) for 15 min at room temperature (6). Grids prepared in this manner were placed on drops of a 1:200 dilution of rabbit anti-BiP specific antiserum (StressGen, Inc., Victoria, British Columbia, Canada) overnight in a humidity chamber at 4°C, washed by passage at room temperature over a series of 7 microdrops of PBS with 1% BSA, and reacted for 1 h in a humidity chamber at room temperature with a 1:100 dilution of goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles (Ted Pella) in PBS with 1% BSA. The grids were then washed exhaustively three times in distilled water and air dried at room temperature prior to being stained with 5% uranyl acetate for 10 min. Sections were examined with a Hitachi H7000/STEM electron microscope equipped with a Lab6 electron source, on-line frame store and advanced image processing (Fisons Co.).

RESULTS

Formation of the replicative phagosome of *L. pneumophila* in *H. vermiformis*. The uptake and intracellular fate of *L. pneumophila* in the amoeba *H. vermiformis* was examined by transmission electron microscopy. Aliquots of monolayers of *H. vermiformis* infected with *L. pneumophila* were withdrawn at several time intervals and processed for transmission electron microscopy. Regular phagocytosis by *H. vermiformis* was observed within 30 min of the infection (Fig. 1A). In contrast to uptake of this strain by the macrophage-like U937 cells by coiling phagocytosis (15a), no coiling phagocytosis by *H. vermiformis* was detected (26). At 1 h postinfection, the bacteria were localized in a phagosome and few mitochondria were observed within the vicinity of the phagosome (Fig. 1B). At 2.5 h postinfection, 51 of 65 (78%) of the phagosomes examined were surrounded by numerous vesicles, and mitochondria were seen in close proximity to the phagosome (Fig. 1C). At 5 h postinfection, the phagosomal membrane was composed of multiple layers and was studded with ribosomes in 61 of 75 (81%) of the phagosomes examined (Fig. 1D). The ribosome-studded multilayer membrane was detected around 28 of 35 (80%), 25 of 35 (71%), and 21 of 35 (61%) of the phagosomes at 8, 12, and 15 h postinfection, respectively (Fig. 1E, F, and G). However, at 15 h postinfection (Fig. 1G), the phagosomal membrane was partially surrounded by the ribosome-studded membrane. At 8 h postinfection and later, there was no detectable preferential localization of mitochondria around the phagosomes, but at these stages, the phagosomes occupied a major portion of the host cell. At 20 h postinfection, numerous bacteria were present in the phagosome (Fig. 1H), but the ribosomes were not detected around the phagosome at this late stage of the infection. Although localization of the ribosome-studded membrane around the phagosomes of *L. pneumophila* in macrophages was not examined at later stages of the infection, our data showed an identity or a remarkable similarity in the early development of the replicative phagosome of *L. pneumophila* in protozoa to that of mammalian cells.

An isogenic mutant of *L. pneumophila* (strain AA101A) was isolated by passage of strain AA100 onto Mueller-Hinton agar plates, as described previously (18, 33). Characteristically, this passage results in the isolation of attenuated variants of *L. pneumophila* that fail to infect phagocytic cells, protozoa, and guinea pigs (3, 17, 18, 33). In vitro, strain AA101A had the same growth kinetics as that of the wild-type strain (data not shown). In contrast to the wild-type strain AA100, the mutant AA101A failed to replicate within *H. vermiformis* and was gradually killed by the amoebae (Fig. 2). Strain AA101A was killed by the amoebae and not by the medium since the decline in the number of bacteria was minimal in control flasks that did not contain amoebae (Fig. 2). Compared with the wild-type strain, the mutant also failed to replicate within U937 macrophage-like cells (data not shown). The mutant failed to form the multilayer membrane around the phagosome and to recruit other host cell organelles, and none of the phagosomes was studded with ribosomes (data not shown). These data indicated that formation of the replicative phagosome surrounded by the RER was essential for survival and replication of the bacteria. These findings are similar to observations derived from macrophages infected by mutants of *L. pneumophila* (7, 27, 32, 38).

The phagosome containing *L. pneumophila* is surrounded by the RER. Swanson and Isberg have recently shown that the replicative phagosome of *L. pneumophila* in macrophages is surrounded by the RER (38, 39). The similarity in the replicative phagosomes containing *L. pneumophila* in macrophages and protozoa suggested that it is most likely that the protozoan phagosome was surrounded by the RER. To test this hypothesis, we used a monospecific rabbit polyclonal antiserum against the endoplasmic reticulum (ER)-specific heat shock protein 78 or BiP (8, 29). This protein is involved in a chaperonin function of proteins across the membrane of the ER and is specific for this compartment of the eukaryotic cell (10, 31). The BiP protein was not detected in the phagosomes containing wild-type *L. pneumophila* AA100 during the first 2 h of the infection. At this stage, the phagosomal membrane

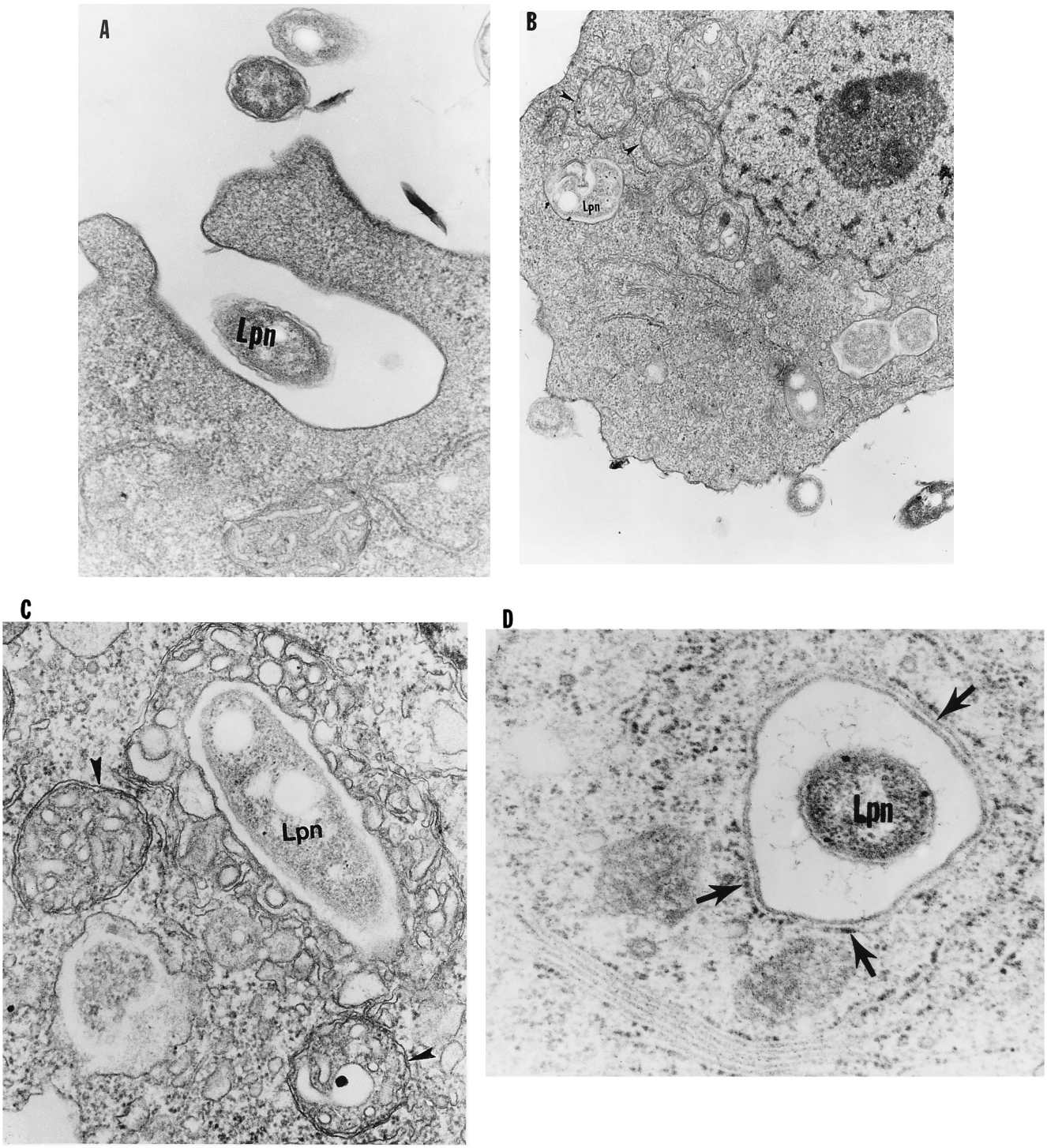

FIG. 1. Transmission electron microscopy of *H. vermiformis* infected with *L. pneumophila* (Lpn) AA100 at several time intervals, namely, 30 min (A), 1 h (B), 2.5 h (C), 5 h (D), 8 h (E), 12 h (F), 15 h (G), and 20 h (H). Arrowheads indicate mitochondria, and arrows indicate the ribosome-studded multilayer phagosomal membrane. Magnifications: A, ×30,000; B, ×20,000; C, ×50,000; D, ×50,000; E, ×15,000; F, ×15,000; G, ×20,000; and H, ×10,000.

appears to be composed of a single smooth layer (Fig. 1B). An intense accumulation of the BiP protein was detected on the membrane and within the lumen of the phagosome containing *L. pneumophila* at 5 h postinfection (Fig. 3A), which is the time of the infection at which ribosomes were also seen to be attached to the phagosomal membrane (Fig. 1C). This phenotype was observed in 49 of 68 (72%) of the phagosomes examined. These data indicated that similar to mammalian cells, the protozoan phagosome containing *L. pneumophila* is surrounded by the RER (38, 39). Gold particles were detected in certain vesicles in the cytoplasm of infected (Fig. 3A) and uninfected amoebae, which indicated the presence of BiP in

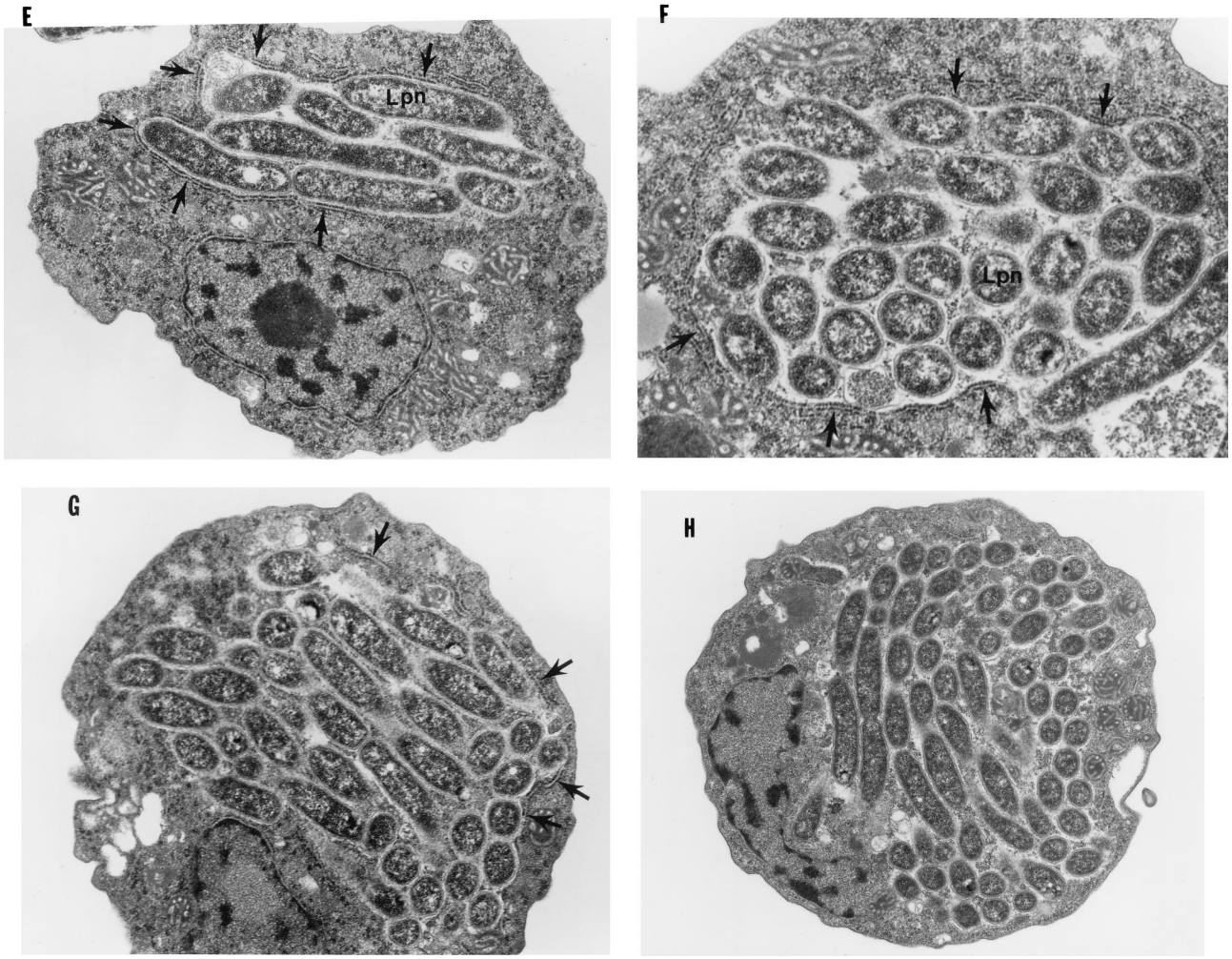

FIG. 1—*Continued.*

transport vesicles or a nonspecific binding of the antiserum to these vesicles. The BiP protein was not detected in the phagosome at 20 h postinfection (data not shown), at which time numerous bacteria were seen within the phagosome. At this stage of the course of infection, the phagosome is surrounded by a ribosome-free membrane. Control sections probed with the goat anti-rabbit conjugate without the primary anti-BiP antiserum showed no binding of gold particles (data not shown).

The BiP protein was never detected in the phagosomes containing the isogenic mutant of *L. pneumophila* AA101A at all time intervals tested (0.5, 1, 2, 5, and 12 h) after infection (Fig. 3B). This is despite the fact that some gold particles were detected in few vesicles in the protozoan cytoplasm (Fig. 3B), which may be due to nonspecific binding of the anti-BiP antiserum or to the presence of BiP in transport vesicles. There was no detectable intracellular AA101A at 12 h postinfection, presumably as a result of protozoan degradation. The BiP protein was not detected in the phagosomes containing heat-killed wild-type *L. pneumophila* (data not shown). These data showed that the ability of *L. pneumophila* to survive and replicate within its phagocytic protozoan host is dependent on its capacity to be surrounded by the RER.

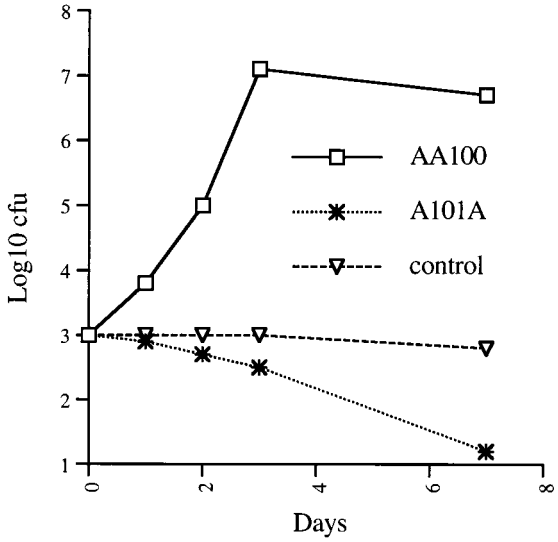

FIG. 2. Kinetics of multiplication of *L. pneumophila* AA100 (wild type) and AA101A (isogenic mutant) within *H. vermiformis*. The control graph indicates the number of viable AA101A organisms in the absence of *H. vermiformis.*

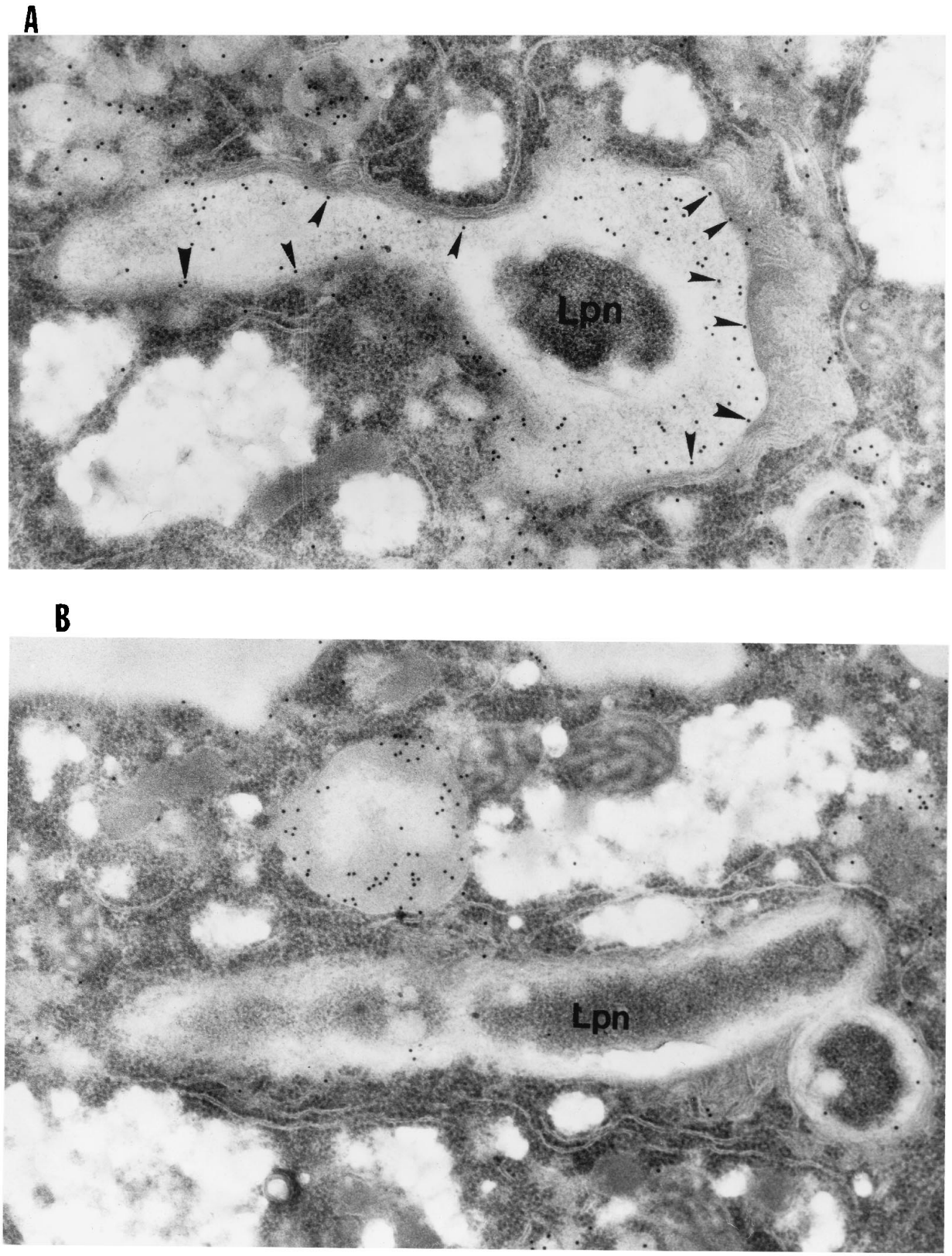

FIG. 3. Immunoelectron microscopy of the phagosome containing *L. pneumophila* (Lpn) AA100 (A) or AA101A (B) at 5 h postinfection probed with anti-BiP antiserum. The abundance of gold particles (arrowheads) indicates the ER-specific BiP protein. Magnification, ×50,000.

DISCUSSION

The intracellular microenvironment in which intracellular bacteria reside is not well understood. Although the facultative intracellular bacterium *L. pneumophila* requires a complex medium for growth in vitro, the nutritional requirements are provided to the phagosome containing *L. pneumophila* within human phagocytes and freshwater protozoa. The adaptation of *L. pneumophila* to the intracellular environment is evident by the shorter generation time of intracellular bacteria compared with that of bacteria grown in vitro in rich medium (28). In this report, it is shown that adaptation of *L. pneumophila* to replicate within protozoa is associated by its capacity to form a phagosome that is surrounded by the RER.

Surrounding of the replicative phagosome of *L. pneumophila* by the RER is similar to the intracellular localization of the intracellular bacterium *Brucella abortus* and the simian virus 40 (4, 29). Considering the rich nutritional microenvironment of the ER, the formation of the ER-surrounded phagosome may be essential for providing nutrients to the bacterium. Swanson and Isberg (38) have recently shown a twofold increase in the number of RER-surrounded phagosomes of *L. pneumophila* by prior starvation of macrophages for amino acids (5, 14, 15). They hypothesized that *L. pneumophila* stimulates autophagy in phagocytic cells to obtain metabolites or to induce degradation of host cell proteins, which increases the local supply of small peptides and proteins to the replicative phagosome.

In addition to similarity in the ultrastructural phagosomal pathway of *L. pneumophila* in protozoa and mammalian phagocytes, there is also similarity in the presence of the RER-specific protein (BiP) in the phagosomal membrane in these evolutionarily divergent hosts (25, 28, 38). The presence of the RER around the phagosome of both host cells suggests that the failure of mammalian phagocytes and protozoa to kill *L. pneumophila* may be due, in part, to recognition of the ER-surrounded phagosome as a cellular compartment. However, earlier events prior to recruitment of the ER must also play a role in protecting the bacteria.

The RER was detected around the majority of the phagosomes containing *L. pneumophila* at 5 to 15 h postinfection but disappeared at 20 h postinfection, as shown by the absence of ribosome-studded membrane and of BiP. These data indicated that establishment of the replicative phagosome during early stages of the infection is a critical step in the establishment of the intracellular infection and that the RER is probably required for bacterial growth until the late stages of the infection. Whether this ribosome-studded multilayer membrane is also present around the phagosome containing *L. pneumophila* in macrophages at late stages of the infection is still to be determined.

The partial disappearance of the RER from the phagosome at 15 h postinfection and its complete disappearance at 20 h of the protozoan infection may be due to a gradual degradation of the ribosome-studded layer of the phagosomal membrane. It is also possible that maintenance of normal physiology of the host cell is gradually lost at late stages of the infection as a result of the metabolic and physical burden of the expanding phagosome on the host cell. Alternatively, it may be due to a gradual budding off of vesicles from the phagosome or to a physical failure of the RER to expand around the growing phagosome.

Many investigators have speculated that adaptation of legionellae as a parasite of freshwater protozoa has allowed this bacteria to infect and adapt to mammalian cells. My observations along with those of others (16, 17, 34) strongly support this hypothesis and show a remarkable similarity between macrophages and protozoa in the development and biochemical composition of the replicative phagosome of *L. pneumophila* at several stages of the infection. Since there may be few differences between the intracellular environment of mammalian phagocytic cells and that of the primitive phagocytic protozoa, it is most likely that the only thing legionellae needed to infect humans was just to gain access, which our civilized living conditions and technology have already provided.

ACKNOWLEDGMENTS

Y. Abu Kwaik is supported by Public Health Service grant 1R29AI38410-01 and by University of Kentucky Research Fund grant 762.

I thank Robert J. Jacob, Janice Pennington, and Mary Gail Ingel for their technical help and suggestions. I thank Susan Straley for her comments on the manuscript.

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