Signal Transduction in the Protozoan Host *Hartmannella vermiformis* upon Attachment and Invasion by *Legionella micdadei*

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Received 28 April 1998/Accepted 5 June 1998

The intracellular pathogens *Legionella micdadei* and *Legionella pneumophila* are the two most common *Legionella* species that cause Legionnaires' disease. Intracellular replication within pulmonary cells is the hallmark of Legionnaires' disease. In the environment, legionellae are parasites of protozoans, and intracellular bacterial replication within protozoans plays a major role in the transmission of Legionnaires' disease. In this study, we characterized the initial host signal transduction mechanisms involved during attachment to and invasion of the protozoan host *Hartmannella vermiformis* by *L. micdadei*. Bacterial attachment prior to invasion of *H. vermiformis* by *L. micdadei* is associated with tyrosine dephosphorylation of multiple host cell proteins, including a 170-kDa protein. We have previously shown that this 170-kDa protein is the galactose *N*-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin receptor that mediates attachment to and invasion of *H. vermiformis* by *L. pneumophila*. Subsequent bacterial entry targets *L. micdadei* into a phagosome that is not surrounded by the rough endoplasmic reticulum (RER). In contrast, uptake of *L. pneumophila* mediated by attachment to the Gal/GalNAc lectin is followed by targeting of the bacterium into an RER-surrounded phagosome. These results indicate that despite similarities in the *L. micdadei* and *L. pneumophila* attachment-mediated signal transduction mechanisms in *H. vermiformis*, the two bacterial species are targeted into morphologically distinct phagosomes in their natural protozoan host.

Along with *Streptococcus pneumoniae* and *Haemophilus influenzae*, *Legionella* species are some of the most common etiologic agents of bacterial pneumonia (10). *Legionella pneumophila* and *Legionella micdadei* are the two most common *Legionella* species that are associated with the majority of outbreaks of Legionnaires' disease (29). Upon inhalation, both of these *Legionella* species are phagocytosed by alveolar cells (30). After internalization, both *Legionella* species replicate within pulmonary cells, and this intracellular replication is believed to be the hallmark of Legionnaires' disease (15, 22). While *L. pneumophila* is targeted into a rough endoplasmic reticulum (RER)-surrounded phagosome that does not fuse with lysosomes, *L. micdadei* is targeted into a RER-free phagosome that is thought to fuse to lysosomes in human monocytes (16). Interestingly, within the RER-surrounded phagosome, *L. pneumophila* undergoes dramatic alterations in gene expression, which are thought to be required for bacterial adaptation to this intracellular niche (2–4, 6, 7). Gene expression by intracellular *L. micdadei* has never been examined.

Outbreaks of Legionnaires' disease occur through droplet transmission of the bacteria from an environmental water source, predominantly air conditioning cooling towers and showerheads (16). In the environment, *Legionella* species are ubiquitous and are parasites of at least 13 species of amoebae and ciliated protozoans (16). Invasion by and intracellular replication of *L. pneumophila* in protozoans play major roles in the infectivity and transmission of the Legionnaires' disease

bacteria to humans (16). We have recently shown that *L. pneumophila* possesses genetic loci that are required for survival and replication within both human macrophages and protozoans, and these loci have been designated *pmi* (protozoan and macrophage infectivity) loci (19, 20). However, there are other distinct bacterial loci that are uniquely required for intracellular replication within macrophages but not within protozoans, and these loci have been designated *mil* (macrophage-specific infectivity) loci (19, 21).

Uptake of *L. pneumophila* by monocytes occurs by microfilament-dependent coiling and conventional phagocytosis mechanisms, while uptake of *L. micdadei* occurs only by conventional phagocytosis (30, 35). In contrast to uptake of *L. pneumophila* by macrophages, entry of the bacterium into the protozoan host *Hartmannella vermiformis* occurs by different mechanisms and is not inhibited by the microfilament disrupting agent cytochalasin D (5, 20, 21, 24, 27). Recently, we demonstrated the presence of a 170-kDa galactose *N*-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin in *H. vermiformis* (24, 34). We have recently shown that type IV pili, which are filamentous appendages expressed on the bacterial surface, are involved in bacterial adherence to mammalian and protozoan cells (32). Whether the type IV pili are the ligands for the Gal/GalNAc lectin is not known.

Most studies have used *L. pneumophila* as a model pathogen for studying Legionnaires' disease, and the mechanism of entry of *L. micdadei* into *H. vermiformis* and the intracellular environment in which *L. micdadei* resides within the host cell are not well-defined. Hence, we sought to address these issues, which may be important for understanding the intracellular life cycle and pathogenesis of *L. micdadei* in its protozoan host, *H. vermiformis*.

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MATERIALS AND METHODS

Bacterial strain and culture. Virulent strain AA100 of *L. pneumophila* is a virulent clinical isolate that has been described previously (3). The virulent strain *L. micdadei* Rivera was obtained from Barry Fields and Janet Purckler, Centers for Disease Control and Prevention (Atlanta, Ga.). The strains were grown on buffered charcoal yeast extract (BCYE) agar plates.

Protozoan culture. *H. vermiformis* CDC-19 (= ATCC 50237) has been cloned and grown in axenic culture and has been used as a model organism to study the pathogenesis of *L. pneumophila* (1, 17). This strain was isolated from a water source during an outbreak of nosocomial Legionnaires' disease in a hospital in South Dakota (11, 17). The amoebae were maintained in American Type Culture Collection axenic culture medium 1034 supplemented with 10% fetal calf serum at 37°C (17).

Infection protocol. *H. vermiformis* was harvested and infected with *L. micdadei* as previously described (24, 34). Briefly, amoebae were incubated overnight in culture flasks in serum-free axenic medium. Then amoebae were harvested by centrifugation and resuspended in fresh serum-free axenic medium. Aliquots containing 2×10^7 amoebae/ml were infected with 10^9 *L. micdadei* cells. After several intervals of coinoculation at 37°C, amoebal cell lysates were prepared for immunoblot analysis as described below.

To examine the ability of some sugars to block tyrosine dephosphorylation of amoebal proteins upon contact with *L. micdadei*, *H. vermiformis* cells were preincubated prior to infection in serum-free axenic medium in the presence of different sugars. Incubation was for 15 min on ice and was followed by coinoculation for 20 min at 37°C. At the end of the coinoculation period, *H. vermiformis* cell lysates were prepared as described below.

Transmission electron microscopy. Monolayers were infected with *L. micdadei* or *L. pneumophila* by using a multiplicity of infection of 100 for 1 h, followed by extensive washing of extracellular bacteria with American Type Culture Collection axenic medium 1034 supplemented with 10% fetal calf serum. Ultrathin sections were prepared as described previously (21). Briefly, infected amoebae were fixed with 3.5% glutaraldehyde and then with 1% OsO_4 , dehydrated with ethanol, and embedded in Eponate 12 resin (Ted Pella, Redding, Calif.). Ultrathin sections were stained with uranyl acetate and then with lead citrate and were examined with a model H-7000/STEM electron microscope (Hitachi Inc., Tokyo, Japan) at 75 kV.

Preparation of cell lysates and Western blotting. After incubation of *H. vermiformis* with *L. micdadei*, infection was stopped by using cold stop buffer containing $1 \times$ phosphate-buffered saline (pH 7.2) and the phosphatase inhibitors NaF (5 mM) and Na_3VO_4 (1 mM) (Sigma Chemical Co., St. Louis, Mo.) (34). To examine the effects of methylamine on tyrosine phosphorylation, amoebae were pretreated with methylamine, and cells were also infected in the presence of this inhibitor, which blocks entry of the bacteria (see below). The cells were washed three times with stop buffer and pelleted by low-speed centrifugation at $735 \times g$ for 2 min. The supernatant containing bacteria was discarded, and the amoebae were lysed with cold 1% Triton X-100 lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4 , 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml) for 30 min on ice (34). The detergent-soluble and -insoluble fractions were separated by centrifugation at $16,000 \times g$ for 30 min at 4°C in a microcentrifuge. Proteins from detergent-soluble fractions were resolved on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels under reducing conditions. The blots were transferred onto Immobilon-P membranes (Millipore, Bedford, Mass.) in a transfer cell (Bio-Rad, Hercules, Calif.) for 1.5 h with 0.2 M Tris–0.025 M glycine buffer containing 20% (vol/vol) methanol. After the proteins were transferred, the membranes were incubated for 30 min in a blocking buffer containing 1.5% bovine serum albumin. The membranes were then probed with a 1:5,000 dilution of horseradish peroxidase-conjugated recombinant anti-phosphotyrosine antibody RC20H (Transduction Laboratories, Lexington, Ky.) for 30 min at room temperature. After extensive washing, the blots were developed with an enhanced chemiluminescence kit (DuPont NEN, Boston, Mass.) according to the manufacturer's instructions.

Inhibition of *L. pneumophila* uptake by sugars. *H. vermiformis* was infected with *L. micdadei* as described below (23). To analyze the effects of different sugars on invasion of *H. vermiformis* by *L. micdadei*, infection experiments were performed in triplicate in the presence of the following sugars: galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), glucose, mannose, and lactose (Sigma). Solutions of these sugars were prepared in assay medium and stored at 4°C (1).

The effects of sugars on invasion of *H. vermiformis* by *L. micdadei* were studied by examining the growth kinetics of *L. micdadei* in *H. vermiformis* in the presence of sugars. *H. vermiformis* cells were preincubated for 15 min with various sugars at a concentration of 100 mM prior to infection with *L. micdadei*. At several times after infection (1, 2, 3, 4, and 5 days) amoebae were lysed by adding a mild detergent (0.04% Triton X-100) (20). Lysis of the amoebae was monitored microscopically and was complete within 1 min. This treatment had no effect on the viability of bacteria (data not shown). Dilutions were plated onto BCYE plates for colony enumeration.

Invasion of amoebae in the presence of methylamine. Uptake of *Legionella* strains by amoebae was studied by using gentamicin protection invasion assays (24, 31). Briefly, *H. vermiformis* was resuspended in axenic medium at a concentration of 10^7 cells/ml. The amoebae were incubated for 1 h in the presence of

FIG. 1. Attachment to and invasion of *H. vermiformis* by *L. micdadei* induces reversible tyrosine dephosphorylation of several host cell proteins. *H. vermiformis* cell extracts were prepared from uninfected cells (lane 1) or cells infected for 1, 5, 15, and 30 min (lanes 2 through 5), subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and probed with anti-phosphotyrosine antibody. Lane 6 contained cell extracts from uninfected cells incubated at 37°C for 30 min. Lane 7 contained extracts from *H. vermiformis* infected for 30 min; then the extracellular bacteria were washed off and the amoebae were incubated for 15 min. Arrowheads indicate the positions of proteins that were markedly tyrosine dephosphorylated. The second arrowhead from the top indicates the position of the 170-kDa Gal/GalNAc lectin (34). The results are representative of the results of at least two independent experiments. KD, kilodaltons.

100 mM methylamine (Sigma), an inhibitor of receptor-mediated endocytosis, prior to infection (13, 27). Following infection with 10^9 *L. micdadei* cells for 1 h, the extracellular bacteria were killed with 50 μg of gentamicin per ml, and the intracellular bacteria were released following lysis of the amoebae with 0.04% Triton X-100. Lysis of the amoebae was monitored microscopically and was complete within 1 min. Different dilutions of bacteria were plated onto BCYE plates for colony enumeration (34).

RESULTS

Tyrosine dephosphorylation of *H. vermiformis* proteins after infection by *L. micdadei*. In this study we examined the changes in host protein tyrosine phosphorylation status during attachment to and invasion of the protozoan host *H. vermiformis* by *L. micdadei*. Amoebae were coinoculated with *L. micdadei* for different periods of time, and cell lysates were immunoblotted with anti-phosphotyrosine antibody (Fig. 1). Infection of *H. vermiformis* with *L. micdadei* resulted in prominent time-dependent tyrosine dephosphorylation of several host cell proteins, including 200- to 205-, 170-, 150-, 69-, and 55-kDa proteins (Fig. 1, arrowheads).

In order to further examine the specificity of the initial signaling events, several control experiments were performed (Fig. 2). *H. vermiformis* cells were coinoculated with dead bacteria (Fig. 2, lane 3) or latex beads (lane 4) for 30 min. Neither of these treatments resulted in any detectable changes in tyrosine phosphorylation of proteins in *H. vermiformis*. In addition, supernatants from *L. micdadei* cultures (lane 5) or culture supernatants obtained after infection (lane 6) had no effect on the pattern of protein phosphorylation in *H. vermiformis*. These results indicated that contact with live bacteria is required to induce host tyrosine dephosphorylation events and that the secretory products of *L. micdadei* were unable to mediate this effect.

***L. micdadei*-induced protein tyrosine dephosphorylation in *H. vermiformis* is reversible.** Next, we examined whether attachment to and invasion of *H. vermiformis* by *L. micdadei* resulted in a permanent change in the tyrosine phosphorylation status of cellular proteins or whether the alteration in host cell processes was reversible. *H. vermiformis* was coinoculated

FIG. 2. Tyrosine dephosphorylation of *H. vermiformis* proteins induced by *L. micdadei* is specific and requires attachment of the bacteria to the Gal/GalNAc lectin. *H. vermiformis* cell extracts were prepared from uninfected cells (lane 1) or cells infected for 30 min with live (lane 2) or dead (lane 3) bacteria. Amoebal extracts were also prepared after stimulation with latex beads (lane 4), bacterial culture supernatants (lane 5), or supernatant collected from an infection (lane 6) as additional controls. Lanes 7 through 9 contained host cell extracts prepared from infections in the presence of Gal, GalNAc, and mannose, respectively. The arrowhead indicates the position of the 170-kDa Gal/GalNAc lectin (34). The results are representative of the results of at least two independent experiments. KD, kilodaltons.

with *L. micdadei* for 30 min, and extracellular bacteria were washed away after this incubation period. *H. vermiformis* cells were subsequently incubated for an additional 15 min, and the patterns of tyrosine-phosphorylated proteins were determined in immunoblots probed with anti-phosphotyrosine antibody. The levels of all of the tyrosine-dephosphorylated proteins returned to the normal levels observed with uninfected cells (data not shown). Overall, these observations indicated that attachment and invasion by *L. micdadei* resulted in tyrosine dephosphorylation of multiple proteins in *H. vermiformis*. This bacterium-induced tyrosine dephosphorylation in *H. vermiformis* was reversible and dependent on the continuous presence of bacteria.

Invasion of *H. vermiformis* by *L. micdadei* can be blocked by Gal and GalNAc. Previous work in our laboratory showed that a 170-kDa β 2 integrin-like Gal/GalNAc lectin is present in *H. vermiformis* (Fig. 2, arrowhead) (34). This lectin mediates attachment of *L. pneumophila* and undergoes tyrosine dephosphorylation during this process (34). Invasion and intracellular replication of *L. pneumophila* in *H. vermiformis* were effectively blocked by the Gal and Gal/GalNAc monomers (34). To examine the functional significance of the 170-kDa lectin of *H. vermiformis* in the invasion of *L. micdadei*, blocking experiments were performed in the presence of Gal and Gal/GalNAc. Amoebae were preincubated with Gal or Gal/GalNAc for 15 min prior to infection with *L. micdadei* (Fig. 3). Mannose, lactose, and glucose were used as negative controls to verify the specificity of the sugars (data not shown). The presence of GalNAc had a dramatic effect on invasion by *L. micdadei*. Gal had a less dramatic effect but still sufficiently blocked the invasion of bacteria. Mannose, glucose, and lactose had no effect on bacterial invasion, and the number of intracellular bacteria at the end of the infection period in the presence of these sugars was very similar to the number in the untreated control (data not shown). The inhibition of bacterial invasion by Gal and Gal/GalNAc was dose dependent (data not shown), and these sugars were not toxic to *H. vermiformis*

FIG. 3. Inhibition of invasion of *H. vermiformis* by *L. micdadei* in the presence of different sugar monomers. The growth kinetics of *L. pneumophila* in cocultures with *H. vermiformis* were determined in the absence or presence of sugars at a concentration of 100 mM. At several times after infection, the numbers of bacteria in the cocultures were determined following growth on agar plates. The bacteria did not replicate extracellularly in the cocultures, and thus the increase in the number of bacteria was due to intracellular replication.

(34). These results indicated that the 170-kDa Gal/GalNAc lectin was involved in attachment to and invasion of *H. vermiformis* by *L. micdadei*.

Attachment of *L. micdadei* to *H. vermiformis* is required to induce protein tyrosine dephosphorylation of host cell proteins. We examined whether bacterial attachment to the Gal/GalNAc lectin was required to cause changes in the pattern of protein tyrosine phosphorylation in *H. vermiformis*. In these experiments, *H. vermiformis* cells were incubated with *L. micdadei* in the presence of Gal, Gal/GalNAc, or mannose (Fig. 2, lanes 7 through 9). Incubation of *H. vermiformis* with these sugars does not alter the profile of tyrosine phosphorylated proteins in resting amoebae (34). *H. vermiformis* cell lysates were prepared after 20 min of infection and were analyzed by using immunoblots probed with the anti-phosphotyrosine antibody. The presence of Gal (Fig. 2, lane 7) or Gal/GalNAc (Fig. 2, lane 8) completely blocked bacterium-induced tyrosine dephosphorylation of the 170- and 69-kDa proteins (Fig. 2, lanes 7 and 8). These proteins have been identified as the Gal/GalNAc lectin (170 kDa) (34) and paxillin (69 kDa) (unpublished data). As expected, mannose failed to block tyrosine dephosphorylation of proteins in *H. vermiformis* (Fig. 2, lane 9). These results indicated that contact of *L. micdadei* with the Gal/GalNAc lectin on *H. vermiformis* was required to mediate early changes in the tyrosine phosphorylation of host cell proteins.

Invasion of *H. vermiformis* by *L. micdadei* is not required to induce protein tyrosine dephosphorylation. Since bacterial attachment was essential for induction of tyrosine dephosphorylation of amoebal proteins (Fig. 2), we examined whether entry of *L. micdadei* into *H. vermiformis* was also required to mediate these effects. Methylamine has been shown to block the entry of *L. pneumophila* into *H. vermiformis* (27). We examined whether methylamine blocks invasion by *L. micdadei* by using the gentamicin protection assay (see above). In this assay, following a period of infection, extracellular bacteria were killed with the antibiotic gentamicin, while intracellular bacteria were protected and subsequently enumerated (34). The data showed that like inhibition of invasion of *H. vermiformis* by *L. pneumophila*, invasion by *L. micdadei* was inhibited 99% in the presence of 100 mM methylamine (data not shown).

FIG. 4. Tyrosine dephosphorylation of *H. vermiformis* proteins induced by *L. micdadei* does not require entry of the bacteria. *H. vermiformis* cell extracts were prepared from uninfected cells in the absence (lane 1) or presence (lane 2) of methylamine. Lane 3 contained cell lysates prepared after 30 min of infection of *H. vermiformis* with *L. micdadei* in the presence of methylamine. The arrowheads indicate the positions of the proteins that were tyrosine dephosphorylated. The second arrowhead from the top indicates the position of the 170-kDa Gal/GalNAc lectin (34). The results are representative of the results of at least two independent experiments. KD, kilodaltons.

Next, we examined whether inhibition of invasion of *H. vermiformis* by *L. micdadei* blocked bacterium-induced tyrosine dephosphorylation. Incubation of resting *H. vermiformis* with methylamine did not affect the pattern of protein tyrosine phosphorylation (Fig. 4, lanes 1 and 2). Interestingly, inhibition of protozoan invasion by *L. micdadei* did not block bacterium-induced tyrosine dephosphorylation of *H. vermiformis* proteins. These results indicated that attachment but not entry of *L. micdadei* was sufficient to induce protein tyrosine dephosphorylation in *H. vermiformis*.

***L. micdadei* is surrounded by a smooth phagosome in *H. vermiformis*.** In *H. vermiformis*, *L. pneumophila* is targeted into an RER-surrounded phagosome (1). Transmission electron microscopy showed that after invasion of *H. vermiformis* by *L. micdadei*, the bacteria were targeted into RER-free phagosomes (Fig. 5). None of the approximately 100 phagosomes examined that contained *L. micdadei* was surrounded by RER; in contrast, 83% of the phagosomes containing *L. pneumophila* were surrounded by RER (1, 20). By 18 h postinfection, the amoebae were heavily infected with both *Legionella* species. These data showed that despite the fact that *L. pneumophila* and *L. micdadei* had similar mechanisms for utilizing the Gal/GalNAc lectin in bacterial attachment and invasion, these two species were targeted into phagosomes that were morphologically distinct, at least at the ultrastructural level.

DISCUSSION

This study focused on the initial signaling events that are activated upon attachment to and invasion of *H. vermiformis* by the Legionnaires' disease bacterium *L. micdadei*. We recently identified the Gal/GalNAc lectin of *H. vermiformis* as a potential receptor utilized by *L. pneumophila* for attachment to and invasion of protozoans (34). In this study we examined the role of the Gal/GalNAc lectin in the attachment to and invasion of *H. vermiformis* by *L. micdadei*. While entry of several pathogenic bacteria, including *Salmonella typhimurium* and *Shigella flexneri*, induces host tyrosine phosphorylation events (18), uptake of *L. micdadei* by *H. vermiformis* is associated with rapid

and reversible tyrosine dephosphorylation of host cell proteins. These effects of *L. micdadei* on *H. vermiformis* are similar to the effects induced by *Yersinia pseudotuberculosis* during invasion of macrophages, which are mediated by a bacterial tyrosine phosphatase (28). Dephosphorylation of cellular proteins prevents uptake of *Yersinia* cells (18). In contrast, tyrosine dephosphorylation of *H. vermiformis* proteins appears to be associated with attachment and uptake of *L. micdadei*. These observations indicate that there is a novel mechanism for uptake of *L. micdadei* by *H. vermiformis*.

The Gal/GalNAc lectin of *Entamoeba histolytica* is antigenically similar to the human $\beta 2$ integrin and is involved in cytoskeleton-mediated capping and shedding of antibody and complement from the protozoan surface in order to evade the immune system of the human host (8, 9). Utilization of the Gal/GalNAc lectin on the protozoan host and its tyrosine dephosphorylation upon bacterial attachment and invasion may provide a selective advantage for *L. micdadei* in the regulation of its uptake process. In contrast to the induction of tyrosine phosphorylation of the receptor and other proteins upon engagement of receptors with their ligands (12), attachment of *L. micdadei* to the Gal/GalNAc lectin was associated with tyrosine dephosphorylation of the lectin and other host proteins. We offer three hypotheses to explain this observation. First, bacterium-induced tyrosine dephosphorylation of the lectin may disrupt communication of the lectin with the underlying cytoskeleton in order to avoid shedding of bacteria from the protozoan surface. This hypothesis is supported by our recent observations that several lectin-associated proteins are dissociated upon attachment of *L. pneumophila* to *H. vermiformis* (unpublished data). Some of these dissociated proteins may represent important cytoskeletal proteins involved in cell shape, motility, and chemotaxis in amoebae. In addition, four protozoan cytoskeletal proteins, including paxillin, vinculin, and pp125^{FAK}, undergo tyrosine dephosphorylation upon attachment to *L. pneumophila*, indicating that disruption of the cytoskeleton occurs (33). Second, dephosphorylation of *H. vermiformis* proteins upon attachment to *L. micdadei* may down-regulate host signals in order to prevent activation of phagocytic signals and microbicidal mechanisms that result in degradation of engulfed bacteria. Subversion of the bactericidal protozoan host cell processes by *L. micdadei* may be supported by observations made with neutrophils (14). Suppression of host cell functions, like superoxide anion production, chemotaxis, and bactericidal activity, by *L. micdadei* was observed when human neutrophils were allowed to attach to and ingest bacteria (14). Third, dephosphorylation of *H. vermiformis* proteins, including the Gal/GalNAc lectin, by *L. micdadei* may increase the efficiency of bacterial uptake. Overall, attachment to and invasion of *H. vermiformis* by *L. micdadei* mediated by the Gal/GalNAc lectin may be required to accomplish more than one of the strategies proposed above to ensure efficient uptake and subsequent targeting of the bacteria into a safe phagosome in amoebae.

The Gal/GalNAc lectin of *H. vermiformis* is important for invasion by *L. micdadei*, as determined by blocking experiments performed with Gal and Gal/GalNAc. Recent studies in our laboratory have demonstrated the importance of the 170-kDa Gal lectin in the invasion of *H. vermiformis* by *L. pneumophila* (24, 34). The sugars Gal and Gal/GalNAc block invasion of *H. vermiformis* by *L. pneumophila* (24, 34). It is interesting to note that the two most frequent causative agents of Legionnaires' disease, *L. micdadei* and *L. pneumophila*, utilize a common host cell receptor to invade *H. vermiformis*.

Intracellular pathogens like *L. pneumophila* and *Mycobacterium tuberculosis* utilize the CR3 receptor to enter human

FIG. 5. *L. micdadei* and *L. pneumophila* are targeted into morphologically distinct phagosomes in *H. vermiformis*: transmission electron micrographs of *H. vermiformis* infected with *L. micdadei* (A and C) or *L. pneumophila* (B and D). The micrographs were obtained 4 h postinfection (A and B) or 18 h postinfection (C and D). The arrows in panel B indicate the RER-surrounded phagosome. b, bacteria. (A and B) Bars = 0.5 μ m. (C and D) Bars = 1 μ m.

macrophages, which prevents activation of oxidative bursts and other microbicidal mechanisms (36, 37). Uptake of many intracellular pathogens through the Fc receptor (instead of CR3) targets the internalized bacteria into lysosomes instead of a replicative niche (25, 26). Although *L. pneumophila* and *L. micdadei* utilize the Gal/GalNAc lectin for attachment and invasion of their protozoan host, *H. vermiformis*, the intracellular fates of the two bacterial species are different, at least at the ultrastructural level. Accordingly, a phagosome that contains *L. pneumophila* but not *L. micdadei* is surrounded by RER. These observations are similar to observations made with a phagosome containing both of these *Legionella* species in macrophages and epithelial cells (unpublished data). These results suggest that additional mechanisms (other than utilizing the Gal/GalNAc lectin) may be involved in the trafficking *L. micdadei* to the RER-free replicative phagosome. It is possible that *L. micdadei* resides in a relatively nascent phagosome, while *L. pneumophila* exists in a more mature phagosome that is surrounded by RER.

In summary, this study and our earlier report (34) revealed novel but similar signaling mechanisms that are triggered in the protozoan host *H. vermiformis* following attachment and invasion by the two *Legionella* species that have been most commonly implicated in outbreaks of Legionnaires' disease. Despite the similarities in what appears to be receptor-mediated signaling events, the two species are targeted into different compartments in the protozoan host. Additional studies should help explain the exact strategies utilized by *Legionella* spp. to enter and invade two evolutionarily distant hosts, human cells and environmental free-living amoebae.

ACKNOWLEDGMENTS

Y.A. was supported by Public Health Service award R29AI38410. O.S.H. was supported by predoctoral National Research Service award 5T32CA09509.

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