Heterogeneity in the Attachment and Uptake Mechanisms of the Legionnaires' Disease Bacterium, *Legionella pneumophila*, by Protozoan Hosts

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Invasion and intracellular replication of *Legionella pneumophila* within protozoa in the environment plays a major role in the transmission of Legionnaires' disease. Intracellular replication of *L. pneumophila* within protozoa occurs in a rough endoplasmic reticulum (RER)-surrounded phagosome (Y. Abu Kwaik, Appl. Environ. Microbiol. 62:2022–2028, 1996). Since the subsequent fate of many intracellular pathogens is determined by the route of entry, we compared the mechanisms of attachment and subsequent uptake of *L. pneumophila* by the two protozoa *Hartmannella vermiformis* and *Acanthamoeba polyphaga*. Our data provide biochemical and genetic evidence that the mechanisms of attachment and subsequent uptake of *L. pneumophila* by the two protozoan hosts are, in part, different. First, uptake of *L. pneumophila* by *H. vermiformis* is completely blocked by the monovalent sugars galactose and *N*-acetyl-D-galactosamine, but these sugars partially blocked *A. polyphaga*. Second, attachment of *L. pneumophila* to *H. vermiformis* is associated with a time-dependent and reversible tyrosine dephosphorylation of multiple host proteins. In contrast, only a slight dephosphorylation of a 170-kDa protein of *A. polyphaga* is detected upon infection. Third, synthesis of *H. vermiformis* proteins but not of *A. polyphaga* proteins is required for uptake of *L. pneumophila*. Fourth, we have identified *L. pneumophila* mutants that are severely defective in attachment to *A. polyphaga* but which exhibit minor reductions in attachment to *H. vermiformis* and, thus, provide a genetic basis for the difference in mechanisms of attachment to both protozoa. The data indicate a remarkable adaptation of *L. pneumophila* to attach and invade different protozoan hosts by different mechanisms, yet invasion is followed by a remarkably similar intracellular replication within a RER-surrounded phagosome and subsequent killing of the host cell.

The Legionnaires' disease bacterium, *Legionella pneumophila*, is a common etiologic agent of bacterial pneumonia (13, 23, 38, 53). Upon transmission to individuals through aerosols generated in the environment, the bacteria invade and replicate within pulmonary macrophages and epithelial cells (20, 31, 39).

In the environment, *L. pneumophila* is a parasite of at least 13 species of amoebae and ciliated protozoa (reviewed in reference 24). In many outbreaks of Legionnaires' disease, the bacteria and protozoa have been isolated from the same water source, and the isolated protozoa have been shown to support intracellular multiplication of the bacterial isolate (10, 16, 26, 37). Additionally, in many confirmed cases of Legionnaires' disease, the bacterium could only be isolated by its capacity to multiply within protozoa (8, 18, 28, 47).

Rowbotham has postulated that the infectious particle involved in transmission of Legionnaires' disease is *L. pneumophila*-infected amoebae (44). Several lines of evidence indicate that protozoa play major roles in the continued presence of *L. pneumophila* in the environment as well as in the infectivity of the bacteria to humans. Intracellular replication of *L. pneumophila* within protozoa increases bacterial resistance to harsh environmental conditions, which may allow the bacteria to survive extracellularly for prolonged periods of time in the environment (6, 11, 12). Interestingly, it has been recently

shown that viability and infectivity of nonculturable *L. pneumophila* can be "resuscitated" by intracellular replication within protozoa (45). Moreover, intracellular multiplication within protozoa enhances the infectivity of *L. pneumophila* to human-derived cells (21). These observations may explain why Legionnaires' disease is not transmitted between individuals and why transmission occurs despite the presence of low numbers of *L. pneumophila* from the source of aerosol (14, 22, 40).

The hallmark of *L. pneumophila* infection of humans is the intracellular survival and replication of the bacteria within macrophages in a rough endoplasmic reticulum (RER)-surrounded phagosome (2, 24, 31). The intracellular infection of protozoa is similar to that of macrophages at the ultrastructural and molecular levels (1, 29). In addition, diaminopimelic acid auxotrophs of *L. pneumophila* are defective in intracellular growth within mammalian and protozoan cells (30a). During intracellular replication, changes in bacterial gene expression are manifested (2–4, 6, 7, 48). Although protein synthesis by the RER is not required for intracellular bacterial replication (2), the role of the RER in intracellular survival is not known.

The mechanisms involved in intracellular trafficking leading to survival and replication of the bacterium within mammalian and protozoan cells are not yet known. Several lines of evidence indicate that the fate of some intracellular pathogens is determined by the route of entry and the specific host cell receptor involved, which may trigger different host cell signal transduction mechanisms (9, 32, 35). We have recently shown that uptake of *L. pneumophila* by the protozoan *Hartmannella vermiformis* is mediated by the Gal/GalNAc lectin receptor and

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is associated with tyrosine dephosphorylation of this receptor (52). In this report, we extended our studies on the host cell processes involved in uptake of *L. pneumophila* to another protozoan host, *Acanthamoeba polyphaga*, and compared its host cell response to that of *H. vermiformis*. The attachment and subsequent cross talk between *L. pneumophila* and its hosts may play a role in the intracellular fate of the bacterium.

Uptake of *L. pneumophila* by the protozoan *H. vermiformis* has been proposed to occur through a microfilament-independent but receptor-mediated mechanism (36), but the processes involved in bacterial uptake by other protozoan hosts have not been reported. This paper describes the investigation of the different mechanisms utilized for the attachment and uptake of *L. pneumophila* by two protozoa, *H. vermiformis* and *A. polyphaga*.

MATERIALS AND METHODS

Bacterial strains. The virulent AA100 strain of *L. pneumophila* has been described previously (3, 5). The *L. pneumophila* mutants used in this study (GF162, GG104, GB112, GM128, GO128, GP65, GQ262, and GT251) were generated by mini *Tn10::kan* transposon mutagenesis of the AA100 strain (29, 30). Southern hybridizations were used to confirm that these mutants contained distinct insertions in their chromosomes (29). *L. pneumophila* were grown on buffered charcoal yeast extract (BCYE) agar plates. Growth of the insertion mutants was on BCYE medium with 20 μ g of kanamycin (Sigma Chemical Co., St. Louis, Mo.)/ml.

Protozoan culture. Axenic *A. polyphaga* was obtained from B. S. Fields (Centers for Disease Control and Prevention, Atlanta, Ga.) and cultured as adherent cells in peptone-yeast-glucose medium (15). *H. vermiformis* CDC-19 (ATCC 50237) has been cloned and grown in axenic culture as a model for the study of the pathogenesis of *L. pneumophila* (25). This strain has been isolated from a water source of an outbreak of nosocomial Legionnaires' disease in a hospital in South Dakota, and its presence in the potable water sites correlated with the presence of the epidemic strain of *L. pneumophila* (16, 25). *H. vermiformis* was maintained in ATCC culture medium 1034 (5, 25).

Detection of tyrosine-phosphorylated proteins in *H. vermiformis* and *A. polyphaga* upon contact with *L. pneumophila*. Amoebae were incubated overnight in culture flasks in serum-free axenic medium or PYG medium for *H. vermiformis* and *A. polyphaga*, respectively. The amoebae were harvested by centrifugation and were resuspended in the corresponding fresh medium. Aliquots of $\sim 2 \times 10^7$ amoebae/ml were infected by 10^9 *L. pneumophila*. At several time intervals of coinoculation at 37°C, amoebal cell lysates were prepared for Western blotting as described below.

Preparation of cell lysates and Western blotting. After incubation of amoebae with *L. pneumophila*, infections were stopped by using cold stop buffer (1X PBS, pH 7.2) containing the phosphatase inhibitors NaF (5 mM) and Na_3VO_4 (1 mM) (Sigma Chemical Co.). Cells were washed three times with cold stop buffer and pelleted by low-speed centrifugation at $735 \times g$ for 2 min. The supernatant containing bacteria was discarded, and amoebae were lysed by using 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 μ g of leupeptin/ml, and 2 μ g of aprotinin/ml). The soluble and insoluble fractions were separated by centrifugation at $16,000 \times g$ for 30 min at 4°C in a microcentrifuge. Proteins from soluble fractions were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis under reducing conditions. The transfer of proteins onto Immobilon-P membranes (Millipore, Bedford, Mass.) was performed in a Bio-Rad transfer cell (Bio-Rad, Hercules, Calif.) for 1.5 to 2 h with 0.2 M Tris–0.025 M glycine buffer containing 20% (vol/vol) methanol. After transfer of proteins, membranes were incubated for 30 min in a blocking buffer containing 1.5% bovine serum albumin. Membranes were probed with antiphosphotyrosine antibody conjugated to horseradish peroxidase (RC-20) according to the manufacturer's recommendations (Transduction Laboratories, Lexington, Ky.). The blots were developed by using an enhanced chemiluminescence kit (DuPont NEN, Boston, Mass.) according to the manufacturer's instructions. The specificity of the RC-20 antibody for protozoan phosphotyrosine-containing proteins was confirmed by Western blots probed with another antiphosphotyrosine antibody, clone 4G10 (Upstate Biotechnology Inc., Lake Placid, N.Y.) followed by a horseradish peroxidase-P conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) (data not shown).

Inhibition of *L. pneumophila* uptake by sugars. Infection of amoebae with *L. pneumophila* was performed exactly as described previously (5). To analyze the effects of different sugars on invasion of amoebae by *L. pneumophila*, infections were performed in triplicate in the presence of a 100 mM concentration of the following sugars: galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), glucose, lactose, and mannose (Sigma). Sugar solutions were prepared in the medium used to maintain each of the amoebae.

The effects of the sugars on invasion were investigated by gentamicin protec-

tion invasion assays. In these assays, *A. polyphaga* or *H. vermiformis* was suspended in its respective medium at a concentration of $\sim 10^7$ /ml. Amoebae were incubated in triplicate in the presence of different sugars for 15 min prior to infection. *L. pneumophila* was added to a final concentration of 10^8 /ml. The samples were incubated at 37°C for 4 h followed by the addition of 50 μ g of gentamicin/ml for 1 h to kill extracellular bacteria. Intracellular bacteria are protected from this antibiotic. The amoebae were washed with medium and lysed by the addition of a mild detergent (0.04% Triton X-100). Lysis of the amoebae was monitored microscopically and was complete within 1 min. This treatment had no significant effect on the viability of the bacteria (data not shown). Dilutions were plated on BCYE plates for colony enumeration. The percent invasion was calculated by dividing the number of CFU in the presence of each sugar by the number of CFU in the absence of the sugar.

The effects of the sugars on invasion of amoebae by *L. pneumophila* were also determined by examination of the growth kinetics of *L. pneumophila* within *H. vermiformis* and *A. polyphaga* in the presence of each sugar. Amoebae were incubated in the presence of each sugar for 15 min prior to infection with *L. pneumophila*. At several time intervals after infection (1, 3, 5, and 7 days), amoebae were lysed as described above, and samples were diluted and plated on BCYE plates for colony enumeration. The percent invasion was calculated as described above.

Uptake of *L. pneumophila* by amoebae in the presence of inhibitors of cytoskeletal integrity. Infection of amoebae with *L. pneumophila* was performed exactly as described previously (5). To analyze the effects of inhibitors of cytoskeletal function on the uptake of *L. pneumophila*, amoebae were incubated in the presence of each inhibitor for 1 h prior to infection. The inhibitors used were 2 μ M cytochalasin D (microfilaments) and 10 μ M colchicine (microtubules) (Sigma). Following a 3-h infection, extracellular bacteria were killed by the addition of 50 μ g of gentamicin/ml, and intracellular bacteria were plated for enumeration on BCYE plates.

Infection of *A. polyphaga* by *L. pneumophila* in the presence of cycloheximide. To assess the effect of cycloheximide on the uptake of *L. pneumophila* by *A. polyphaga*, $\sim 10^7$ *A. polyphaga* was pretreated for 3 h with 200 μ g of cycloheximide/ml to inhibit protein synthesis. Amoebae were infected with $\sim 10^8$ *L. pneumophila* for 4 h in the presence of cycloheximide. Cycloheximide has no detectable effect on the viability or gene expression of *L. pneumophila* (5). Subsequently, nonadherent bacteria were removed by extensive washing, and extracellular bacteria were killed by gentamicin, as described above. Intracellular bacteria were recovered after lysis of the amoebae and were plated on BCYE for colony enumeration, as described above.

Attachment of *L. pneumophila* mutants to *H. vermiformis* and *A. polyphaga*. *H. vermiformis* was infected with eight different mini *Tn10::kan* insertion mutants that are defective in attachment to human-derived U937 macrophages (29). To assess the ability of the mutant strains to adhere to *A. polyphaga* and *H. vermiformis*, 5×10^5 amoebae were infected for 20 min in triplicate with 10^7 bacteria of each mutant. Infections were carried out in the presence of 10 mM methylamine to prevent internalization of the bacteria (36). The amoebae were washed three times to remove unattached bacteria and were subsequently lysed with a mild detergent (0.04% Triton X-100). Dilutions were plated on BCYE. Adherence of the mutants was measured by comparison to the adherence of the wild-type strain, AA100, to amoebae in the same assay. To ensure that the inhibitors were effective in the inhibition of uptake, control monolayers infected in the presence of the inhibitor were treated with gentamicin to kill extracellular bacteria. The data showed that methylamine inhibited uptake by *A. polyphaga* and *H. vermiformis* by approximately 98% (data not shown).

RESULTS

Effects of sugars on the ability of *L. pneumophila* to invade *A. polyphaga* and *H. vermiformis*. We used two strategies to investigate the roles of several sugars in blocking the attachment and invasion of amoebae by *L. pneumophila*. First, a gentamicin protection invasion assay was utilized to measure invasion of amoebae in the presence of various sugars (see Materials and Methods). Our data showed that the presence of specific sugars had different effects on bacterial uptake by *A. polyphaga* and *H. vermiformis*. Incorporation of 100 mM Gal or GalNAc in the infection assay had a dramatic effect on invasion of *H. vermiformis* by *L. pneumophila* (Fig. 1A). These sugar monomers were able to block uptake of *L. pneumophila* by *H. vermiformis* by ~ 70 and 89%, respectively. Gal and GalNAc are not toxic to amoebae, and their inhibition of uptake of *L. pneumophila* is specific, dose dependent, and reversible (52). Other sugars (glucose, mannose, and lactose) at a concentration of 100 mM had no detectable effect on the uptake of *L. pneumophila* by *H. vermiformis* (Fig. 1A and data not shown).

FIG. 1. Effects of different sugar monomers on invasion of *H. vermiformis* (A) and *A. polyphaga* (B) by *L. pneumophila* by gentamicin protection assays. Invasion of amoebae by *L. pneumophila* was performed in the absence or presence of various sugars. The percent invasion was determined by a comparison between the number of intracellular bacteria in the presence of sugars compared to that in untreated cultures. Values are the means of triplicate samples, and error bars represent standard deviations.

In contrast, Gal and GalNAc had slight effects on the uptake of *L. pneumophila* by *A. polyphaga* (Fig. 1B). These data showed that uptake of *L. pneumophila* by *H. vermiformis* but not by *A. polyphaga* is dramatically blocked by Gal and GalNAc.

Second, a growth kinetics assay was performed in the presence of the above sugars. The data confirmed our results obtained from the gentamicin protection invasion assays. Over the 7-day infection, 100 mM Gal or GalNAc caused a dramatic reduction or a complete inhibition in the growth kinetics of *L. pneumophila* within *H. vermiformis* (Fig. 2A). The inhibition of uptake of *L. pneumophila* by *H. vermiformis* in the presence of Gal or GalNAc is specific, reversible, and dose dependent (52). Other sugars (glucose, mannose, and lactose) at a concentration of 100 mM had no detectable effect on the uptake of *L. pneumophila* by *H. vermiformis* (Fig. 1A and data not shown).

In contrast, parallel experiments performed with *A. polyphaga* showed that the presence of Gal or GalNAc caused a slight reduction in the growth kinetics of *L. pneumophila* (Fig. 2B). These data confirmed the distinct difference in the effects of Gal and GalNAc on the uptake of *L. pneumophila* by *H. vermiformis* and *A. polyphaga*.

Protein tyrosine phosphorylation in *A. polyphaga* and *H. vermiformis* upon attachment to *L. pneumophila*. Tyrosine phosphorylation of host proteins has been shown to be important in the uptake of many intracellular pathogens (27, 33, 41–43, 49). We have recently shown protein tyrosine dephosphorylation in the protozoan *H. vermiformis* upon attachment and invasion by *L. pneumophila* (52). In this report, we extended our studies to another protozoan host, *A. polyphaga*, and compared its host cell response to that of *H. vermiformis*. First, we examined host cell signaling events in *H. vermiformis* and *A. polyphaga* upon contact with their bacterial parasite, *L. pneumophila*. We utilized Western blots of amoebal proteins to examine the status of tyrosine phosphorylation of host proteins in resting amoebae and following contact with *L. pneumophila*. Examination of *H. vermiformis* cell lysates showed several proteins that were tyrosine phosphorylated in resting amoebae (Fig. 3A, lane 1) that underwent a time-dependent and reversible de-

phosphorylation upon contact with *L. pneumophila*. Dephosphorylation of several *H. vermiformis* proteins, including those with apparent molecular masses of 190, 170, 130, and 70 kDa, was prominent and evident as early as 5 min (Fig. 3A, lane 2), and was complete by 15 min (Fig. 3A, lane 3), confirming our previous observations (52).

To compare the host cell response in *H. vermiformis* to that of another protozoan host, we also examined the pattern of protein tyrosine phosphorylation in resting *A. polyphaga* and upon attachment and invasion by *L. pneumophila*. Many tyrosine-phosphorylated proteins were detected in resting *A. polyphaga* (Fig. 3B). The pattern of tyrosine-phosphorylated proteins in *A. polyphaga* was distinctly different from that in *H. vermiformis*. However, in contrast to *H. vermiformis*, there was only a slight tyrosine dephosphorylation of a 170-kDa protein in *A. polyphaga* upon contact with *L. pneumophila*. These data demonstrated major differences in triggering the biochemical events involved in the attachment, and possibly the subsequent uptake, of *L. pneumophila* by the two amoebae.

Effects of inhibitors of cytoskeletal integrity on uptake of *L. pneumophila* by amoebae. To further characterize the differences in the mechanisms of uptake of *L. pneumophila* by *H. vermiformis* and *A. polyphaga*, the effects of two inhibitors of cytoskeletal function (cytochalasin D and colchicine) on uptake of *L. pneumophila* were examined by gentamicin protection invasion assay. After pre-incubating the amoebae with each inhibitor for 3 h, infections were performed for 3 h. Subsequently, extracellular bacteria were killed, and the number of intracellular bacteria was determined. The data showed no detectable effect by either inhibitor on the uptake of *L. pneumophila* by *A. polyphaga* (Fig. 4). Similar results were obtained from *H. vermiformis* infected with *L. pneumophila* in the presence of these inhibitors (data not shown). These data indicated that the uptake mechanisms of *L. pneumophila* by the two amoebae are independent of microfilaments and microtubules. Our data are consistent with previous observations that the uptake of *L. pneumophila* by *H. vermiformis* is independent of the microfilaments (36).

Effect of inhibition of protozoan protein synthesis on uptake of *L. pneumophila*. The uptake of *L. pneumophila* by *H. vermi-*

FIG. 2. Growth kinetics of *L. pneumophila* in cocultures with *H. vermiformis* or *A. polyphaga* in the absence or presence of different sugars. The bacteria do not replicate extracellularly in the coculture, and thus, the increase in the number of bacteria is due to intracellular replication. Values are the means of triplicate samples, and error bars represent standard deviations.

FIG. 3. Effects of attachment of *L. pneumophila* to *H. vermiformis* and *A. polyphaga* on tyrosine phosphorylation of host proteins. (A) Cell extracts were prepared from uninfected *H. vermiformis* (lane 1), from *H. vermiformis* infected with *L. pneumophila* for 5 min (lane 2) or for 15 min (lane 3), or from uninfected cells incubated for 15 min (lane 4). Lane 5 represents samples prepared from *H. vermiformis* infected for 15 min followed by removal of extracellular bacteria and further incubation for 15 min. (B) Extracts were prepared from uninfected *A. polyphaga* (lane 1) or from *A. polyphaga* infected with *L. pneumophila* for 5 min (lane 2) or for 15 min (lane 3). Lane 4 represents samples prepared from infection with dead *L. pneumophila*. Western blots were probed with antiphosphotyrosine antibody (see Materials and Methods). Arrows indicate the position of the 170-kDa protein.

formis and macrophages is known to differ in the requirement for host protein synthesis. Our previous work showed that uptake of *L. pneumophila* by macrophages is independent of protein synthesis of the host cell (5). In contrast, synthesis of new host proteins is required for uptake of the bacterium by *H. vermiformis* (5). We extended these observations to the uptake of *L. pneumophila* by *A. polyphaga*. Our data showed that inhibition of *A. polyphaga* protein synthesis had no detectable effect on uptake of *L. pneumophila* (Fig. 4). In contrast, and consistent with our previous observations, inhibition of *H. vermiformis* protein synthesis completely inhibited the uptake of *L. pneumophila* (data not shown) (5). These data further substantiated our observations on the differences in the uptake mechanisms of *L. pneumophila* by *A. polyphaga* and *H. vermiformis*.

Attachment of mutants of *L. pneumophila* to *A. polyphaga* and *H. vermiformis*. We have recently isolated a group of mutants, generated through transposon mutagenesis, that are defective in attachment to *A. polyphaga* and to U937 human macrophage-like cells (29, 30). We examined the ability of these mutants to attach to *H. vermiformis* and compared it to their attachment to *A. polyphaga*. In these assays, attachments were assessed in the presence of an inhibitor of uptake to prevent internalization of the bacteria (see Materials and Methods). Our data showed that the 10 mutants were severely defective in attachment to *A. polyphaga* but 4 of them (GP65, GM128, GT251, and GM224) attached at higher levels to *H. vermiformis* (Fig. 5). Interestingly, six of the mutants were severely defective in attachment to both *A. polyphaga* and *H. vermiformis*, suggesting that some of the bacterial ligands involved in attachment to both protozoa may be similar. These data indicated that *L. pneumophila* possesses multiple ligands involved in distinct attachment to *H. vermiformis* or *A. polyphaga*, and some of these may be involved in attachment to both protozoa.

DISCUSSION

The replication of *L. pneumophila* within free-living amoebae is believed to be an integral component in the transmission of Legionnaires' disease (24). It has been hypothesized that the intracellular infections of mammalian and protozoan cells by *L. pneumophila* are mediated by similar mechanisms (1, 19, 24). We have recently shown that 89 distinct insertion mutants

of *L. pneumophila* exhibited similar defective phenotypes (severe to modest) in their cytotoxicity and intracellular replication within U937 macrophages and *A. polyphaga* (29), thus providing genetic evidence that many of the mechanisms uti-

FIG. 4. Effects of disruption of *A. polyphaga* microtubules (by colchicine) and microfilament (by cytochalasin D) and inhibition of protein synthesis (by cycloheximide) on invasion by *L. pneumophila*. The percent invasion was determined by dividing the number of intracellular bacteria recovered following the invasion period after killing of extracellular bacteria by the number of intracellular bacteria in untreated samples. Values are the means of triplicate samples, and error bars represent standard deviations.

FIG. 5. Attachment levels of mutants of *L. pneumophila* to *A. polyphaga* and *H. vermiformis*. The number of attached bacteria is represented by the percentage of attached bacteria compared to that of the wild-type, strain AA100, in the presence of an inhibitor of uptake in both amoebae. Values are the means of triplicate measurements, and the error bars represent standard deviations. Some of the error bars cannot be seen due to their small values.

lized by *L. pneumophila* to parasitize the two evolutionarily distant hosts are similar. In contrast, processes involved in the uptake of *L. pneumophila* by mammalian macrophages have been shown to differ from those by *H. vermiformis* (5, 36, 52), although the mechanisms of uptake by amoebae are not known.

Previous studies have shown that uptake of *L. pneumophila* by *H. vermiformis* is not inhibited by cytochalasin D, an inhibitor of microfilament-dependent endocytosis (36). In this report, we provide evidence that the attachment and uptake of *L. pneumophila* by the two protozoa, *A. polyphaga* and *H. vermiformis*, occur by different mechanisms, adding further complexity to the host-parasite interaction process. First, uptake of *L. pneumophila* by *H. vermiformis* is completely blocked by the monovalent sugars Gal and GalNAc, but these sugars partially blocked *A. polyphaga*. Second, attachment of *L. pneumophila* to *H. vermiformis* is associated with a time-dependent and reversible tyrosine dephosphorylation of multiple host proteins. In contrast, only a slight dephosphorylation of a 170-kDa protein of *A. polyphaga* is detected upon infection. Third, synthesis of *H. vermiformis* proteins but not of *A. polyphaga* proteins is required for uptake of *L. pneumophila*. Fourth, we have identified *L. pneumophila* mutants that are severely defective in attachment to *A. polyphaga* but which exhibit minor reductions in attachment to *H. vermiformis* and, thus, provide a genetic basis for the difference in mechanisms of attachment to both protozoa.

The dramatic inhibition of uptake of *L. pneumophila* by *H. vermiformis* compared to that by *A. polyphaga* in the presence of Gal or GalNAc indicates that the receptors utilized by *L. pneumophila* to attach to the two amoebae may be different or have different affinities. We have recently identified a 170-kDa Gal/GalNAc lectin as one of the tyrosine-phosphorylated

proteins in resting *H. vermiformis* that undergoes dephosphorylation upon attachment and invasion by *L. pneumophila* (52). Whether the slightly dephosphorylated 170-kDa protein in *A. polyphaga*, as a result of bacterial infection, is related to the *H. vermiformis* lectin is still to be determined.

Therefore, *A. polyphaga* possesses another receptor or a similar receptor but with lower affinity to which *L. pneumophila* attaches during the initial steps of interaction. The severe defect in attachment of the *L. pneumophila* mutant strain GM224 to *A. polyphaga*, but its normal attachment to *H. vermiformis*, provides strong genetic evidence for the presence of different receptors on both protozoa for attachment of *L. pneumophila*. Since these mutants are also defective in intracellular replication (29, 30), it is unlikely that expression of the recently described pili of *L. pneumophila* is defective in any of these mutants (46). Future characterization of the defective ligand in the mutant GM224 will allow us to characterize the host cell receptor involved in attachment.

Attachment of *L. pneumophila* to *H. vermiformis* is associated with tyrosine dephosphorylation of multiple host proteins. In contrast, there is only a slight reduction in the level of tyrosine phosphorylation of a 170-kDa protein (which may be related to the Gal/GalNAc lectin of *H. vermiformis*) in *A. polyphaga* upon attachment to *L. pneumophila*. This host cell response may correlate with the relative inhibition of bacterial uptake by Gal and GalNAc sugars, which was less pronounced in *A. polyphaga*. These data showed a crucial difference in the initial steps involved in attachment and subsequent cross talk between *L. pneumophila* and two of its protozoan hosts, *A. polyphaga* and *H. vermiformis*. Since there are at least 13 species described to be environmental hosts for *L. pneumophila* (24), it would be interesting to examine the remarkable adaptation and the differential complexity of the interaction of this intracellular parasite with its numerous environmental protozoan hosts.

The fate of some intracellular parasites may be determined by the specific ligand-receptor interaction and subsequent signal transduction involved in uptake by the host cell (9, 32, 35). Our observations of tyrosine dephosphorylation of *H. vermiformis* proteins, including the Gal/GalNAc receptor, upon contact and uptake of *L. pneumophila* are rather intriguing since conventional phagocytosis is associated with tyrosine phosphorylation of host cell proteins (9, 52). Interestingly, attachment of many bacterial pathogens to host cell receptors and their exploitation of host cell processes is associated with tyrosine phosphorylation of host cell proteins (27). We speculate that attachment of *L. pneumophila* to *H. vermiformis* is associated with disruption of the classical phagocytic process. Additionally, cross talk between *L. pneumophila* and *H. vermiformis* activates host cell signaling pathways that will trigger new protein synthesis in *H. vermiformis*, which is required for subsequent uptake of *L. pneumophila* (5). In contrast, adherence of *L. pneumophila* to *A. polyphaga* induces only minor changes in the host tyrosine phosphorylation, indicating that the initial cross talk does not induce the same set of biochemical signaling events seen in *H. vermiformis*.

Many facultative intracellular pathogens exploit host signal transduction pathways to their own advantage (27). This exploitation includes induced cytoskeletal rearrangement and subsequent internalization of the bacterium. *L. pneumophila* invasion of both *H. vermiformis* and *A. polyphaga*, however, remains unaffected by inhibitors of the cytoskeleton that are commonly exploited by other bacterial pathogens (27). Both of these inhibitors have been found to perform their functions in protozoa in ways that are similar to those in mammalian cells (34, 36, 50, 51). Therefore, our observations confirm previous

reports that the mechanisms of invasion of amoebae by *L. pneumophila* are different from those utilized by other intracellular pathogens to invade their mammalian host cells (5, 27, 36).

L. pneumophila is transmitted only from environmental sources, where protozoa play a major factor in transmission of Legionnaires' disease (24). Intracellular replication within protozoa increases the number of *L. pneumophila* in the environment (24), resuscitates viability and infectivity of nonculturable bacteria (45), increases bacterial resistance to harsh environmental conditions (6, 11, 12), and enhances bacterial infectivity to human cells and A/J mice (17, 21). Thus, understanding the mechanisms of uptake by protozoa will facilitate the design of measures to prevent uptake of *L. pneumophila* by protozoa, providing effective preventive approaches for controlling the transmission of Legionnaires' disease.

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REFERENCES

- Abu Kwaik, Y. 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. *Appl. Environ. Microbiol.* **62**:2022–2028.
- Abu Kwaik, Y. Induced expression of the *Legionella pneumophila* gene encoding a 20-kilodalton protein during intracellular infection. *Infect. Immun.*, in press.
- Abu Kwaik, Y., B. I. Eisenstein, and N. C. Engleberg. 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. *Infect. Immun.* **61**:1320–1329.
- Abu Kwaik, Y., and N. C. Engleberg. 1994. Cloning and molecular characterization of a *Legionella pneumophila* gene induced by intracellular infection and by various *in vitro* stress stimuli. *Mol. Microbiol.* **13**:243–251.
- Abu Kwaik, Y., B. S. Fields, and N. C. Engleberg. 1994. Protein expression by the protozoan *Hartmannella vermiformis* upon contact with its bacterial parasite *Legionella pneumophila*. *Infect. Immun.* **62**:1860–1866.
- Abu Kwaik, Y., L.-Y. Gao, O. S. Harb, and B. J. Stone. 1997. Transcriptional regulation of the macrophage-induced gene (*gspA*) of *Legionella pneumophila* and phenotypic characterization of a null mutant. *Mol. Microbiol.* **24**:629–642.
- Abu Kwaik, Y., and L. L. Pederson. 1996. The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol. Microbiol.* **21**:543–556.
- Adeleke, A., J. Pruckler, R. Benson, T. Rowbotham, M. Halablab, and B. S. Fields. 1996. *Legionella*-like amoebal pathogens—phylogenetic status and possible role in respiratory disease. *Emerg. Infect. Dis.* **2**:225–229.
- Allen, L. H., and A. Aderem. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med.* **184**:627–637.
- Barbaree, J. M., B. S. Fields, J. C. Feeley, G. W. Gorman, and W. T. Martin. 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. *Appl. Environ. Microbiol.* **51**:422–424.
- Barker, J., M. R. W. Brown, P. J. Collier, I. Farrell, and P. Gilbert. 1992. Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Appl. Environ. Microbiol.* **58**:2420–2425.
- Barker, J., H. Scaife, and M. R. W. Brown. 1995. Intraphagocytic growth induces an antibiotic-resistant phenotype of *Legionella pneumophila*. *Antimicrob. Agents Chemother.* **39**:2684–2688.
- Bates, J. H., G. D. Capmpbell, and A. L. Baron. 1992. Microbial etiology of acute pneumonia in hospitalized patients. *Chest* **101**:1005–1012.
- Bhopal, R. S., R. J. Fallon, E. C. Buist, R. J. Black, and J. D. Urquhart. 1991. Proximity of the home to a cooling tower and risk of non-outbreak Legionnaires' disease. *Br. Med. J.* **302**:378–383.
- Bozue, J. A., and W. Johnson. 1996. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infect. Immun.* **64**:668–673.
- Breiman, R. F., B. S. Fields, G. N. Sanden, L. Volmer, A. Meier, and J. S. Spika. 1990. Association of shower use with Legionnaires' disease: possible role of amoebae. *JAMA* **263**:2924–2926.
- Brieland, J. K., J. C. Fantone, D. G. Remick, M. LeGendre, M. McClain, and N. C. Engleberg. 1997. The role of *Legionella pneumophila*-infected *Hartmannella vermiformis* as an infectious particle in a murine model of Legionnaires' disease. *Infect. Immun.* **65**:5330–5333.
- Britles, R. J., T. J. Rowbotham, D. Raoult, and T. G. Harrison. 1996. Phylogenetic diversity of intra-amoebal legionellae as revealed by 16S rRNA gene sequence comparison. *Microbiology* **142**:3525–3530.
- Cianciotto, N. P., and B. S. Fields. 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc. Natl. Acad. Sci. USA* **89**:5188–5191.
- Cianciotto, N. P., J. K. Stamos, and D. W. Kamp. 1995. Infectivity of *Legionella pneumophila mip* mutant for alveolar epithelial cells. *Curr. Microbiol.* **30**:247–250.
- Cirillo, J. D., L. S. Tompkins, and S. Falkow. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect. Immun.* **62**:3254–3261.
- Dennis, P. J., A. E. Wright, D. A. Rutter, J. E. Death, and B. P. Jones. 1984. *Legionella pneumophila* in aerosols from shower baths. *J. Hyg.* **93**:349–353.
- Fang, G. D., M. Fine, and J. Orloff. 1990. New and emerging etiologies of community-acquired pneumonia with implications for therapy, a prospective multicenter study of 359 cases. *Medicine (Baltimore)* **69**:307–316.
- Fields, B. S. 1996. The molecular ecology of legionellae. *Trends Microbiol.* **4**:286–290.
- Fields, B. S., T. A. Nerad, T. K. Sawyer, C. H. King, J. M. Barbaree, W. T. Martin, W. E. Morrill, and G. N. Sanden. 1990. Characterization of an axenic strain of *Hartmannella vermiformis* obtained from an investigation of nosocomial legionellosis. *J. Protozool.* **37**:581–583.
- Fields, B. S., G. N. Sanden, J. M. Barbaree, W. E. Morrill, R. M. Wadowsky, E. H. White, and J. C. Feeley. 1989. Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. *Curr. Microbiol.* **18**:131–137.
- Finlay, B. B., and P. Cossart. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**:718–725.
- Fry, N. K., T. J. Rowbotham, N. A. Saunders, and T. M. Embley. 1991. Direct amplification and sequencing of the 16S ribosomal DNA of an intracellular *Legionella* species recovered by amoebal enrichment from the sputum of a patient with pneumonia. *FEMS Microbiol. Lett.* **83**:165–168.
- Gao, L.-Y., O. S. Harb, and Y. Abu Kwaik. 1997. Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant hosts, mammalian and protozoan cells. *Infect. Immun.* **65**:4738–4746.
- Gao, L.-Y., O. S. Harb, and Y. Abu Kwaik. Submitted for publication.
- Harb, O. S., J. K. Brieland, and Y. Abu Kwaik. Submitted for publication.
- Horwitz, M. A. 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. *J. Exp. Med.* **158**:1319–1331.
- Horwitz, M. A. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. Exp. Med.* **158**:2108–2126.
- Iretton, K., B. Payraastre, H. Chap, W. Ogawa, H. Sakaue, M. Kasuga, and P. Cossart. 1996. A role for phosphoinositide 3-kinase in bacterial adhesion. *Science* **274**:780–782.
- Isenberg, G., U. Aebi, and T. D. Pollard. 1980. An actin-binding protein from *Acanthamoeba* regulates actin filament polymerization and interactions. *Nature* **288**:455–459.
- Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* **249**:641–646.
- King, C. H., B. S. Fields, E. B. Shotts, Jr., and E. H. White. 1991. Effects of cytochalasin D and methylamine on intracellular growth of *Legionella pneumophila* in amoebae and human monocyte-like cells. *Infect. Immun.* **59**:758–763.
- Kurtz, J. B., C. L. R. Bartlett, U. A. Newton, R. A. White, and N. L. Jones. 1982. *Legionella pneumophila* in cooling tower systems. *J. Hyg.* **88**:369–381.
- Marston, B. J. 1995. Epidemiology of community-acquired pneumonia. *Infect. Dis. Clin. Pract.* **4**:S232–S239.
- Mody, C. H., R. Paine III, M. S. Shahrabadi, R. H. Simon, E. Pearlman, B. I. Eisenstein, and G. B. Toews. 1993. *Legionella pneumophila* replicates within rat alveolar epithelial cells. *J. Infect. Dis.* **167**:1138–1145.
- O'Brein, S. J., and R. S. Bhopal. 1993. Legionnaires' disease: the infective dose paradox. *Lancet* **342**:5–6.
- Rosenshine, I., V. Duronio, and B. B. Finlay. 1992. Tyrosine protein kinase inhibitors block invasion-promoted bacterial uptake by epithelial cells. *Infect. Immun.* **60**:2211–2217.
- Rosenshine, I., S. Ruschkowski, V. Foubister, and B. B. Finlay. 1994. *Salmonella typhimurium* invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. *Infect. Immun.* **62**:4969–4974.
- Rosenshine, I., S. Ruschkowski, M. Stein, D. J. Reinscheid, S. D. Mills, and B. B. Finlay. 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J.* **15**:2613–2624.
- Rowbotham, T. J. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* **33**:1179–1183.
- Steinert, M., L. Emody, R. Amann, and J. Hacker. 1997. Resuscitation of

- viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **63**:2047–2053.
46. Stone, B. J., and Y. Abu Kwaik Submitted for publication.
47. Stout, J. E., J. Joly, M. Para, J. Plouffe, C. Ciesielski, M. J. Blaser, and V. L. Yu. 1988. Comparison of molecular methods for subtyping patients and epidemiologically linked environmental isolates of *Legionella pneumophila*. J. Infect. Dis. **157**:486–495.
48. Susa, M., J. Hacker, and R. Marre. 1996. De novo synthesis of *Legionella pneumophila* antigens during intracellular growth in phagocytic cells. Infect. Immun. **64**:1679–1684.
49. Tang, P., I. Rosenshine, and B. B. Finlay. 1994. *Listeria monocytogenes*, an invasive bacterium, stimulates MAP kinase upon attachment to epithelial cells. Mol. Biol. Cell **5**:455–464.
50. Udezulu, I. A., and G. J. Leitch. 1987. A membrane-associated neuraminidase in *Entamoeba histolytica* trophozoites. Infect. Immun. **55**:181–186.
51. Vazquez-Prado, J. and I. Meza. 1992. Fibronectin “receptor” in *Entamoeba histolytica*: purification and association with the cytoskeleton. Arch. Med. Res. **23**:125–128.
52. Venkataraman, C., B. J. Haack, S. Bondada, and Y. Abu Kwaik. 1997. Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires’ disease bacterium, *Legionella pneumophila*. J. Exp. Med. **186**:537–547.
53. Woodhead, M. A., J. T. Macfarlane, J. S. McCracken, D. H. Rose, and R. G. Finch. 1987. Prospective study of the aetiology and outcome of pneumonia in the community. Lancet **i**:671–674.