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The Role of *Legionella pneumophila*-Infected *Hartmannella vermiformis* as an Infectious Particle in a Murine Model of Legionnaires' Disease

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***Legionella pneumophila* is a bacterial parasite of many species of freshwater protozoa and occasionally an intracellular pathogen of humans. While protozoa are known to play a key role in the persistence of *L. pneumophila* in the environment, there has been limited research addressing the potential role of *L. pneumophila*-infected protozoa in the pathogenesis of human infection. In this report, the potential role of an *L. pneumophila*-infected amoeba as an infectious particle in replicative *L. pneumophila* lung infection was investigated in vivo with the amoeba *Hartmannella vermiformis*, a natural reservoir of *L. pneumophila* in the environment. *L. pneumophila*-infected *H. vermiformis* organisms were prepared by coculture of the amoebae and virulent *L. pneumophila* cells in vitro. A/J mice, which are susceptible to replicative *L. pneumophila* lung infection, were subsequently inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* organisms (10^6 amoebae containing 10^5 bacteria), and intrapulmonary growth of the bacteria was assessed. A/J mice inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* organisms developed replicative *L. pneumophila* lung infections. Furthermore, *L. pneumophila*-infected *H. vermiformis* organisms were more pathogenic than an equivalent number of bacteria or a coinoculum of *L. pneumophila* cells and uninfected amoebae. These results demonstrate that *L. pneumophila*-infected amoebae are infectious particles in replicative *L. pneumophila* infections in vivo and support the hypothesis that inhaled protozoa may serve as cofactors in the pathogenesis of pulmonary disease induced by inhaled respiratory pathogens.**

Legionella pneumophila, the causative agent of Legionnaires' disease, is a bacterial parasite of many species of protozoa and occasionally a pathogen of humans (2, 3, 17, 19, 22, 24, 28, 31, 35). Pulmonary infection of humans with *L. pneumophila* usually occurs following exposure to contaminated potable and nonpotable water systems. The portal of entry is the lung, via inhalation of *L. pneumophila*-containing aerosols or microaspiration of contaminated water (10–14). Following inhalation, the bacteria invade and replicate within host mononuclear phagocytic cells, primarily alveolar macrophages. The intracellular infection of alveolar macrophages that is characteristic of Legionnaires' disease is remarkably similar in its ultrastructural features to *L. pneumophila* infection of protozoal organisms (1, 18, 19, 21, 28, 31, 33, 34).

Since the discovery of the bacterium-protozoon interaction, there has been much speculation, but limited research, on the relevance of *L. pneumophila*-infected protozoa to human infection (31). In the environment, protozoa maintain *L. pneumophila* in natural aquatic and potable water systems, as they both provide a niche for bacterial replication and serve as a vehicle to protect *L. pneumophila* during the process of water treatment (4). *Hartmannella vermiformis*, the most common protozoon isolated from potable water supplies in the United States, is permissive towards growth of *L. pneumophila* and is frequently isolated in water implicated as the source of the bacteria in outbreaks of Legionnaires' disease (4, 15, 16). Furthermore, amoebae can be aerosolized (30), and antibodies to amoebal species, including *H. vermiformis*, are common in asymptomatic persons, indicating exposure (29). While *L. pneumophila* lung infection secondary to exposure to aerosolized contaminated water supplies has been well documented, person-to-person transmission of the bacteria has not been demonstrated, despite the presence of viable bacteria in sputum. This observation suggests that other factors, including the extent of exposure and/or coinhalation of organisms which potentiate *L. pneumophila* replication, may be required for and/or facilitate replicative *L. pneumophila* lung infection. With regard to potential cofactors, it has been theorized that as few as one inhaled or aspirated *L. pneumophila*-infected protozoon may constitute an infectious particle for Legionnaires' disease (31). However, this hypothesis has not been rigorously tested in vivo.

In the present study, the potential role of *L. pneumophila*-infected *H. vermiformis* as an infectious particle in legionellosis was investigated with a previously described A/J mouse model of replicative *L. pneumophila* lung infection (7). Unlike mononuclear phagocytic cells from other inbred mouse strains, macrophages from A/J mice are permissive toward growth of the bacteria (36, 37). Consequently, we have previously demonstrated that A/J mice develop replicative *L. pneumophila* lung infection in response to intratracheal inoculation with virulent *L. pneumophila* cells ($\leq 10^6$ bacteria per mouse), with maximal intrapulmonary growth of *L. pneumophila* at 48 h postinoculation (7). In the current study, we demonstrate that *L. pneumophila*-infected *H. vermiformis* organisms induce replicative *L. pneumophila* lung infection in vivo and are more pathogenic than an equivalent number of bacteria or a coinoculum of *L. pneumophila* and uninfected *H. vermiformis* organisms. These results demonstrate that *L. pneumophila*-infected *H. vermiformis* organisms are infectious particles in replicative

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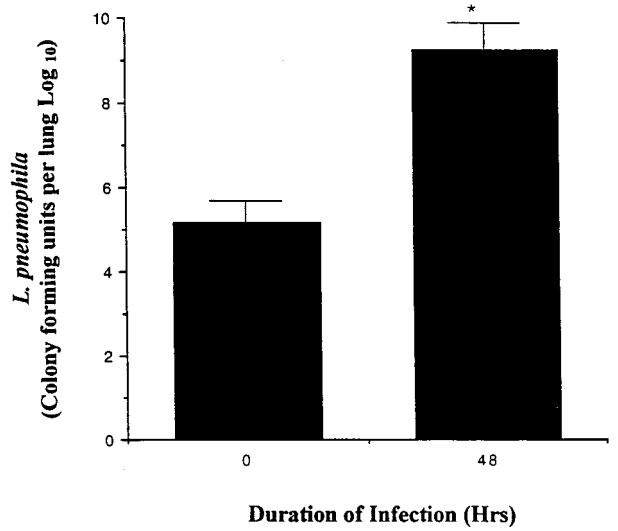

FIG. 1. *L. pneumophila*-infected *H. vermiformis* organisms induce replicative *L. pneumophila* lung infection in intratracheally inoculated A/J mice. A/J mice were inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae containing 10^5 bacteria per mouse, harvested after 6 h of coculture). At 0 and 48 h postinoculation, the mice were euthanized, the lungs were excised and homogenized, and *L. pneumophila* CFU in the lung were determined by culture of lung homogenates as previously described (7). Data are expressed as the number of *L. pneumophila* CFU per lung and are representative of the mean ± standard error of the mean (SEM) of 9 to 18 mice per treatment group. *, significant increase in *L. pneumophila* CFU per lung homogenate at 48 h postinoculation; Student's *t* test, $P < 0.05$.

L. pneumophila lung infection and support the hypothesis that inhaled protozoa may serve as cofactors in the pathogenesis of inflammatory lung disease induced by inhaled respiratory pathogens.

A virulent strain of *L. pneumophila* serogroup 1 (strain AA100), a rederivation of a primary clinical isolate from the Wadsworth Veterans Administration Hospital, Wadsworth, California, was provided by Paul Edelstein. The bacteria were maintained and passaged on buffered charcoal-yeast extract medium (BCYE; Becton Dickinson, Cockeysville, Md.). The amoeba *H. vermiformis* CDC-19 (ATCC 50237), originally isolated from a water sample obtained during an investigation of nosocomial legionellosis, was maintained axenically as confluent monolayers in trophozoites in T-75 flasks in ATCC culture medium 1034 supplemented with 10% fetal bovine serum as previously described (16).

For preparation of *L. pneumophila*-infected *H. vermiformis*, confluent monolayers of *H. vermiformis* (containing approximately 5×10^7 amoebae per flask) were inoculated with *L. pneumophila* AA100 at a multiplicity of infection of 100:1 for 1 h. After 1 h of coculture, the monolayers were washed to remove nonadherent bacteria and incubated in media containing gentamicin (100 μg/ml) for 2 h to kill extracellular bacteria. Antibiotic-containing medium was subsequently removed and replaced with antibiotic-free medium, and *L. pneumophila*-infected-amoeba monolayers were incubated for a total of 6 h. One hour prior to harvest, the monolayers were treated again with gentamicin (100 μg/ml) to kill extracellular bacteria. *L. pneumophila*-infected amoebae were subsequently removed from the flasks, resuspended in phosphate-buffered saline (4×10^7 amoebae per ml), and used in vivo within 30 min of preparation. (Results of our preliminary experiments demonstrated that *L. pneumophila* replicated within *H. vermiformis*, resulting in lysis of the amoebae within 18 to 20 h postinoculation [data not shown].) Female 6- to 8-week-old A/J mice (Jackson Laboratories, Bar Harbor, Maine), which are susceptible to replicative *L. pneumophila* lung infections (7), were subsequently inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae per mouse, harvested after 6 h of coculture) (7). At 0 and 48 h postinoculation, the mice were euthanized, the lungs were excised and homogenized, and *L. pneumophila* CFU were determined by culture of lung homogenates on modified BCYE (BCYE selective agar with polymyxin B-anisomycin-cefamendole; Becton Dickinson). The mice were euthanized at 48 h postinoculation as we have previously demonstrated that intrapulmonary growth of *L. pneumophila* AA100 in A/J mice inoculated with bacteria alone is maximal at 48 h postinoculation (7). As is shown in Fig. 1, while 10^5 bacteria were recovered from lung homogenates of A/J mice euthanized immediately after intratracheal inoculation with infected amoebae (10^6 *H. vermiformis* per mouse), more than 10^9 bacteria were recovered from lung homogenates of inoculated mice at 48 h postinfection. Similar in vivo experiments were conducted with *L. pneumophila*-infected *H. vermiformis* harvested after 4 and 12 h of coculture, yielding similar results (data not shown).

The relative pathogenicity of *L. pneumophila*-infected *H. vermiformis* harvested after 6 h of coculture was subsequently assessed. A/J mice were inoculated intratracheally with either *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae containing 10^5 bacteria harvested after 6 h of coculture) or *L. pneumophila* alone (10^5 bacteria per mouse) or were coinoculated with *L. pneumophila* (10^5 bacteria per mouse) and uninfected amoebae (10^6 *H. vermiformis* per mouse). At 24 to 72 h post-

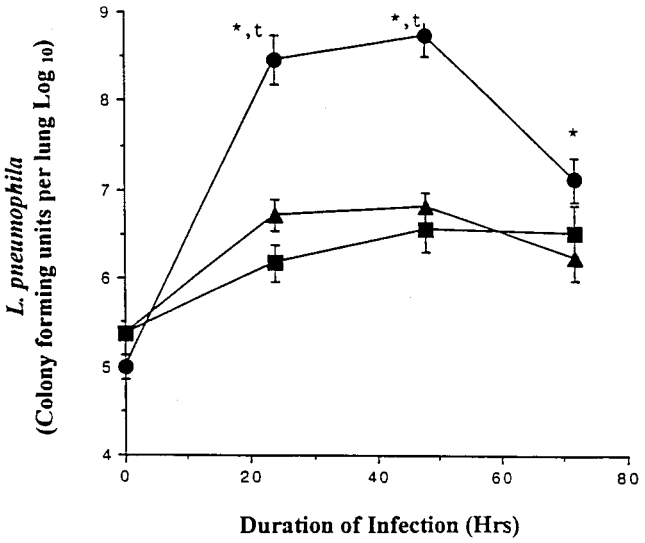

FIG. 2. *L. pneumophila*-infected *H. vermiformis* organisms are more pathogenic than an equivalent number of bacteria or a coinoculum of *L. pneumophila* and uninfected amoebae. A/J mice were inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae containing 10^5 bacteria per mouse, harvested after 6 h of coculture [●]) or with *L. pneumophila* alone (10^5 bacteria per mouse [▲]) or were coinoculated with *L. pneumophila* and uninfected *H. vermiformis* (10^5 bacteria and 10^6 amoebae per mouse [■]). At 24, 48, and 72 h postinoculation, the mice were euthanized, the lungs were excised and homogenized, and the number of *L. pneumophila* CFU per lung was determined by culture of lung homogenates as previously described (7). Data represent the mean ± SEM of three separate experiments (6 to 12 mice per treatment group). *, significant difference in intrapulmonary growth of *L. pneumophila* compared with that of mice inoculated with bacteria alone; Mann-Whitney test, $P < 0.05$; t, significant difference in intrapulmonary growth of *L. pneumophila* compared with that of mice coinoculated with *L. pneumophila* and *H. vermiformis*; Mann-Whitney test, $P < 0.05$.

TABLE 1. Effect of intratracheal inoculation of *L. pneumophila*-infected *H. vermiformis* on IFN-γ and TNF-α levels in bronchoalveolar lavage fluid of A/J mice at 24 h postinoculation[a]

Inoculum	Level (pg/ml of bronchoalveolar lavage fluid) of:	
	IFN-γ	TNF-α
Phosphate-buffered saline	22 ± 13	52 ± 14
L. pneumophila	45 ± 24	1,468 ± 180
H. vermiformis	225 ± 69	11,972 ± 5,243
L. pneumophila and *H. vermiformis*	152 ± 26*	21,226 ± 4,691*
L. pneumophila-infected *H. vermiformis*	163 ± 38*	25,037 ± 6,626*

[a] A/J mice were inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae containing 10^5 bacteria per mouse, harvested after 6 h of coculture) or with *L. pneumophila* (10^5 bacteria per mouse) and/or *H. vermiformis* (10^6 amoebae per mouse). At 24 h postinoculation, the mice were sacrificed and the lungs were lavaged. IFN-γ and TNF-α levels were assessed in bronchoalveolar lavage fluid by ELISA and bioassay, respectively (7–9). Results represent the mean ± SEM of activity in four to six mice per treatment group. *, significant difference in cytokine activity in bronchoalveolar lavage fluid compared with that in mice inoculated with *L. pneumophila* alone; Mann-Whitney test, $P < 0.05$.

inoculation, the mice were euthanized, the lungs were excised and homogenized, and the numbers of *L. pneumophila* CFU per lung homogenate were compared. As is shown in Fig. 2, while mice in all treatment groups developed replicative *L. pneumophila* lung infections, intrapulmonary growth of *L. pneumophila* was significantly greater in mice inoculated with *L. pneumophila*-infected *H. vermiformis* than in mice inoculated with an equivalent number of bacteria or coinoculated with *L. pneumophila* and uninfected *H. vermiformis*.

The mechanism by which *L. pneumophila*-infected *H. vermiformis* potentiated intrapulmonary growth of the bacteria was subsequently investigated. Cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), have been shown to play key roles in resolution of replicative *L. pneumophila* infections as they induce mononuclear phagocyte cell activation, resulting in restriction of intracellular replication of *L. pneumophila* and elimination of the bacteria from infected cells (5–8, 20, 27). However, exaggerated intrapulmonary levels of proinflammatory cytokines, including TNF-α, have also been demonstrated to induce lung injury in other inflammatory lung diseases, including adult respiratory distress syndrome (25, 32). We have previously demonstrated that both IFN-γ and TNF-α are induced in *L. pneumophila*-infected A/J mice within 24 h postinoculation and are critical for resolution of legionellosis in this murine model system (7, 8). Because *L. pneumophila*-infected *H. vermiformis* potentiated intrapulmonary growth of *L. pneumophila* within 48 h postinoculation, we conducted studies to determine if this was due to *H. vermiformis*-induced inhibition of IFN-γ and/or TNF-α. A/J mice were inoculated intratracheally with *L. pneumophila*-infected *H. vermiformis* (10^6 amoebae containing 10^5 bacteria per mouse harvested after 6 h of coculture) or were inoculated with *L. pneumophila* (10^5 bacteria per mouse) and/or uninfected *H. vermiformis* (10^6 amoebae per mouse). At 24 h postinoculation, the mice were sacrificed and the lungs were lavaged with phosphate-buffered saline (1.6 ml per mouse) as previously described (9). IFN-γ and TNF-α were assessed in bronchoalveolar lavage fluid by enzyme-linked immunosorbent assay (ELISA) and bioassay, respectively (7–9). As is shown in Table 1, intrapulmonary levels of IFN-γ and TNF-α were significantly enhanced in A/J mice inoculated with *L. pneumophila*-infected *H. vermiformis* compared with levels in mice inoculated with the bacteria alone. These results demonstrate that enhanced intrapulmonary growth of *L. pneumophila* in mice inoculated with *L. pneumophila*-infected *H. vermiformis* is not mediated by amoeba-induced inhibition of IFN-γ and/or TNF-α. Furthermore, intrapulmonary levels of IFN-γ and TNF-α were similarly enhanced in mice inoculated with *L. pneumophila*-infected *H. vermiformis* or coinoculated with an equivalent number of bacteria and uninfected *H. vermiformis*. This suggests a proinflammatory effect of *H. vermiformis* that is not related to *L. pneumophila* replication. The potential relationship between exaggerated local production of TNF-α induced by inhaled *H. vermiformis* and lung injury during replicative *L. pneumophila* lung infection remains to be investigated.

In summary, results of these experiments demonstrate that *L. pneumophila*-infected amoebae are infectious particles in a murine model of legionellosis and are more pathogenic than an equivalent number of bacteria alone or a coinoculum of *L. pneumophila* and uninfected amoebae. While these results support the hypothesis that *L. pneumophila*-infected amoebae may be infectious particles in human legionellosis, the role of *L. pneumophila*-infected protozoa in the pathogenesis of human outbreaks of Legionnaires' disease is currently unknown and difficult to assess. We have previously demonstrated that *H. vermiformis* organisms are identifiable in the lung only during the initial phase of replicative *L. pneumophila* lung infection (at <72 h postinoculation) in mice coinoculated with *H. vermiformis* (9). Diagnostic tests on human patients suspected of having Legionnaires' disease are conducted considerably later in the course of infection, after inhaled amoebae would have been cleared from the lung. Studies addressing seroconversion to positivity for antibodies to amoebae in patients with Legionnaires' disease have not been performed and may be confounded by preexisting antibodies in many humans (29). Other respiratory pathogens, including *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, have been associated with protozoa (23, 26). However, as with *L. pneumophila*, the potential role of the bacterium-protozoon interaction in the pathogenesis of lung disease induced by these agents has not been investigated. Replicative lung infection in A/J mice inoculated with *L. pneumophila*-infected protozoa may provide a model system to investigate an exciting new area of biology and medicine, the effects of commensal or parasitic relationships of bacteria with other microorganisms in the natural environment on the pathogenesis of human disease.

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