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Intrapulmonary *Hartmannella vermiformis*: a Potential Niche for *Legionella pneumophila* Replication in a Murine Model of Legionellosis

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The potential role of inhaled protozoa as a niche for intrapulmonary replication of *Legionella pneumophila* was investigated in vivo with mutant strains of *L. pneumophila* which have reduced virulence for the amoeba *Hartmannella vermiformis*. *L. pneumophila* AA488 and AA502 were derived from wild-type strain AA100 after transposon mutagenesis. These mutants have reduced virulence for *H. vermiformis* but are fully virulent for mononuclear phagocytic cells. A/J mice, which are susceptible to replicative *L. pneumophila* lung infections, were inoculated intratracheally with *L. pneumophila* AA100, AA488, or AA502 (10^6 bacteria per mouse) or were coinoculated with one of the *L. pneumophila* strains (10^6 bacteria per mouse) and uninfected *H. vermiformis* (10^6 amoebae per mouse). The effect of coinoculation with *H. vermiformis* on intrapulmonary growth of each *L. pneumophila* strain was subsequently assessed. In agreement with our previous studies, coinoculation with *H. vermiformis* significantly enhanced intrapulmonary growth of the parent *L. pneumophila* strain (AA100). In contrast, intrapulmonary growth of *L. pneumophila* AA488 or AA502 was not significantly enhanced by coinoculation of mice with *H. vermiformis*. These studies demonstrate that *L. pneumophila* virulence for amoebae is required for maximal intrapulmonary growth of the bacteria in mice coinoculated with *H. vermiformis* and support the hypothesis that inhaled amoebae may potentiate intrapulmonary growth of *L. pneumophila* by providing a niche for bacterial replication.

Legionella pneumophila is a bacterial parasite of many species of freshwater protozoa and an occasional intracellular pathogen of humans (2, 3, 21, 24, 26, 27, 29, 32, 36, 42). Infection of humans with *L. pneumophila* usually occurs after bacteria from natural aquatic sources contaminate potable and nonpotable water systems. The portal of entry is the lung, via inhalation of aerosols or microaspiration of contaminated water (10, 14–16). Following inhalation, the bacteria invade and replicate within host mononuclear phagocytic cells, primarily within 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, 23, 24, 26, 32, 36, 40, 41).

Since the discovery of the bacterium-protozoan interaction, there has been much speculation but limited research directed towards elucidating the relevance of protozoa to human *L. pneumophila* infection (36). Protozoa play a key role in the persistence of *L. pneumophila* in natural aquatic water systems, since growth of *L. pneumophila* in the environment in the absence of these organisms has rarely been documented (22, 37, 43). Protozoa may also serve as a vehicle to protect *L. pneumophila* through the process of water treatment and/or serve as a reservoir for growth of the bacteria in potable water systems (4). Amoebae which are permissive for growth of *L. pneumophila* are frequently found in water implicated as the source of the bacteria in outbreaks of Legionnaire's disease (4, 10, 11, 19, 20). It is not known whether the presence of amoebae is a prerequisite for an outbreak, since contamination of potable water systems with protozoa is exceedingly common (23, 36, 42).

Using an animal model of replicative *L. pneumophila* lung infection in A/J mice inoculated intratracheally with virulent bacteria, we have recently demonstrated that protozoa may have a more direct effect on the pathogenesis of replicative *L. pneumophila* lung infection. Specifically, we have shown that intrapulmonary growth of *L. pneumophila* in A/J mice is potentiated by coinoculation with *Hartmannella vermiformis*, an amoebal species which is permissive for growth of the bacteria and a natural reservoir of *L. pneumophila* in the environment (7). These results support the hypothesis that coincident inhalation of protozoa may enhance the severity of Legionnaire's disease. However, the mechanism(s) by which intrapulmonary *H. vermiformis* organisms potentiate *L. pneumophila* replication in vivo has not been thoroughly investigated.

In the present study, the potential role of intrapulmonary *H. vermiformis* as a niche for *L. pneumophila* replication was assessed with mutant strains of *L. pneumophila* which have reduced virulence for *H. vermiformis* and a previously described murine model of replicative *L. pneumophila* lung infection in A/J mice (8). We demonstrate that bacterial virulence for *H. vermiformis* is required for maximal intrapulmonary growth of *L. pneumophila* in A/J mice coinoculated with the amoebae. These results support the hypothesis that intrapulmonary *H. vermiformis* organisms likely potentiate growth of *L. pneumophila* in vivo by their ability to provide a niche for *L. pneumophila* replication.

A virulent strain of *L. pneumophila* (AA100) originally isolated from an outbreak of Legionnaires' disease was used in all assays (8). The bacteria were maintained and passaged on buffered charcoal-yeast extract (BCYE) agar (18). The amoeba *H. vermiformis* CDC-19 (ATCC 50237), originally isolated from a water sample obtained during an investigation of nos-

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ocomial legionellosis, was maintained axenically as confluent monolayers of trophozoites in T-75 flasks in ATCC culture medium 1034 supplemented with 10% fetal bovine serum as previously described (19).

To identify *L. pneumophila* mutants with reduced virulence for *H. vermiformis*, phosphatase-positive (Pho^+) mutant strains of *L. pneumophila* were generated by transposon insertion mutagenesis of *L. pneumophila* AA100, with the previously described alkaline phosphatase fusion-generating transposon mTn*10phoA* (28). The results of these experiments identified approximately 4,000 mutagenized legionellae, yielding 206 Pho^+ *L. pneumophila* mutant bacterial strains. The ability of each Pho^+ mutant *L. pneumophila* strain to grow in U937 cells (a human monocytic cell line which is permissive for *L. pneumophila* growth [12, 33]) and *H. vermiformis* was subsequently assessed. Briefly, differentiated U937 cells were inoculated with the parent bacterial strain (AA100) or with a Pho^+ strain of *L. pneumophila* at a multiplicity of infection (MOI) of 20:1 for 2 h at 37°C. *L. pneumophila*-infected U937 cell monolayers were then washed and incubated for an additional 2 h in media containing gentamicin (50 μg/ml) to kill extracellular bacteria. The cells were subsequently washed and either immediately hypotonically lysed or incubated for an additional 24 to 72 h (37°C, 5% CO_2). Following incubation, the culture supernatant was removed, the cells were hypotonically lysed, and the corresponding cell supernatant and lysate were pooled. An aliquot of each sample was serially diluted in water and cultured on BCYE. The number of *L. pneumophila* CFU per sample, indicative of growth of Pho^+ *L. pneumophila* mutants in U937 cells, was determined and compared to that of the parent *L. pneumophila* strain (AA100). To assess the virulence of each *L. pneumophila* strain for *H. vermiformis*, axenically maintained *H. vermiformis* monolayers were inoculated with each mutant *L. pneumophila* strain at an MOI of 1:1. Immediately after inoculation, an aliquot of the culture supernatant was removed and serially diluted, and *L. pneumophila* CFU in the inocula were determined. *L. pneumophila*-infected *H. vermiformis* monolayers were then incubated for 24 to 72 h, at which time an aliquot of the culture supernatant was removed and cultured on BCYE. The number of *L. pneumophila* CFU in culture supernatant, indicative of growth of Pho^+ *L. pneumophila* mutants in *H. vermiformis*, was determined and compared to that of the parent *L. pneumophila* strain (AA100). As shown in Fig. 1, these screening methods identified three Pho^+ *L. pneumophila* strains, designated AA488, AA501, and AA502, which are as virulent as *L. pneumophila* AA100 for cultured mononuclear phagocytic cells but have significantly reduced virulence for *H. vermiformis.*

Virulence of *L. pneumophila* AA488, AA501, and AA502 in vivo was subsequently assessed with a previously described murine model of legionellosis in intratracheally inoculated A/J mice (8). For preparation of in vivo inocula, each *L. pneumophila* strain was grown on BCYE agar for 48 h and resuspended in phosphate-buffered saline (4×10^7 bacteria per ml). Female 6- to 8-week-old A/J mice (Jackson Laboratories, Bar Harbor, Maine), which are susceptible to replicative *L. pneumophila* lung infections (8), were subsequently inoculated intratracheally with the parent bacterial strain (AA100) or with one of the three mutant *L. pneumophila* strains (10^6 bacteria per mouse) (8). At 48 h postinoculation, the mice were humanely euthanized, the lungs were excised and homogenized, and *L. pneumophila* CFU per lung were determined by culture of lung homogenate on bacteriological media (8, 17). (The mice were euthanized at this time point because the results of our previous studies have demonstrated that intrapulmonary growth of *L. pneumophila* AA100 in intratracheally inoculated A/J mice

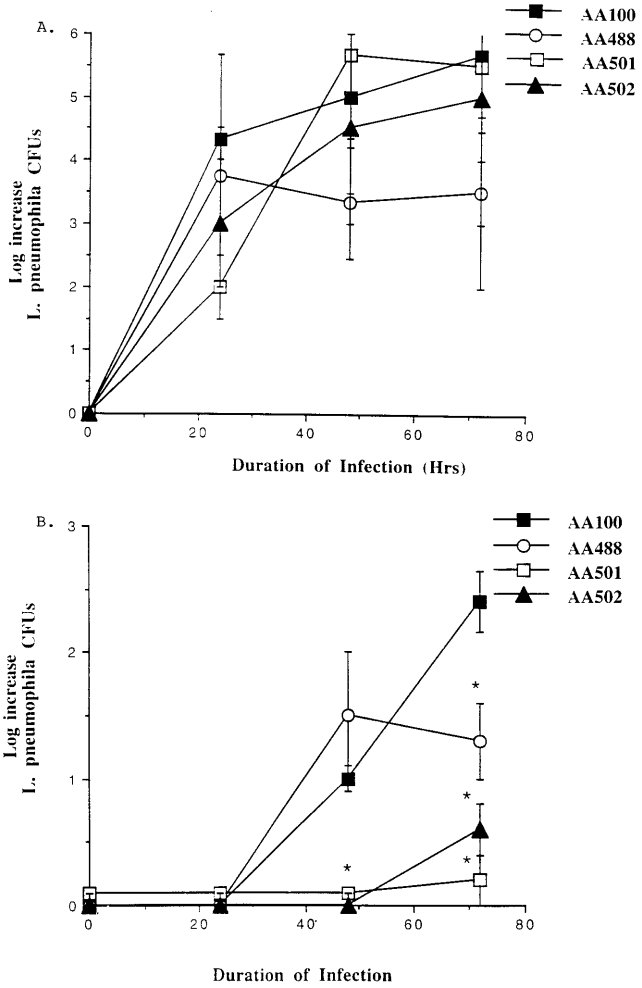

FIG. 1. Kinetics of growth of Pho^+ *L. pneumophila* strains for mammalian cells and amoebae. (A) Growth of Pho^+ *L. pneumophila* strains in differentiated U937 cells was monitored as previously described and compared to that of the parent bacterial strain (AA100). (B) Growth of Pho^+ *L. pneumophila* strains in the amoeba *H. vermiformis* was determined as previously described and compared to that of the parent bacterial strain (AA100). Data for both panels A and B represent the mean log increase in CFU from duplicate wells of a minimum of two separate experiments. *, significant difference in growth compared with that in *L. pneumophila* AA100 (Student's *t* test [$P < 0.05$]).

is maximal at 48 h postinoculation [8].) As shown in Fig. 2, while there was no significant difference between intrapulmonary growth of either *L. pneumophila* AA488 or AA502 and the parent bacterial strain (AA100), significantly fewer *L. pneumophila* CFU were recovered from lung homogenates of mice inoculated intratracheally with *L. pneumophila* AA501. These results demonstrate that *L. pneumophila* AA501 has reduced virulence in vivo. Consequently, this strain was eliminated from further analysis.

In subsequent experiments, the growth kinetics of *L. pneumophila* AA488, AA502, and AA100 in the lung were compared. A/J mice were inoculated intratracheally with either *L. pneumophila* AA488, AA502, or AA100 (10^6 bacteria per mouse). At 24 to 72 h postinoculation, the mice were humanely euthanized and *L. pneumophila* CFU in lung homogenates were determined by culture of lung homogenate on bacteriological media (8, 17). As shown in Fig. 3, the levels of intrapulmonary growth of *L. pneumophila* AA488 and AA502 were similar to that of *L. pneumophila* AA100. These results dem-

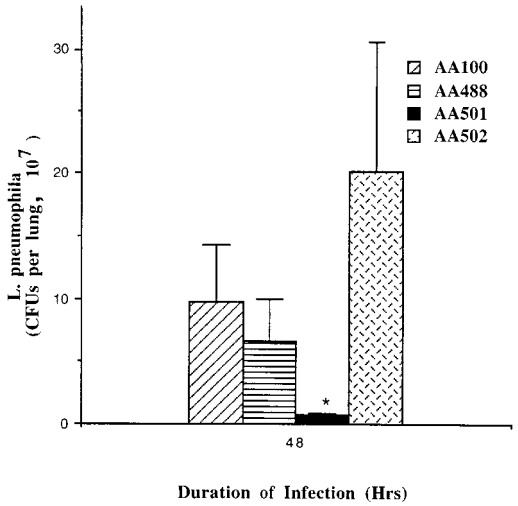

FIG. 2. Intrapulmonary growth of Pho$^+$ *L. pneumophila* strains in A/J mice at 48 h postinoculation. A/J mice were inoculated intratracheally with a Pho$^+$ *L. pneumophila* strain (AA488, AA501, or AA502) which is virulent for U937 cells but has reduced virulence for *H. vermiformis* in vitro (10^6 bacteria per mouse). At 48 h postinoculation, the mice were euthanized, and the lungs were excised and homogenized. *L. pneumophila* CFU per lung were subsequently determined by culture of lung homogenates on bacteriological media. Results represent the mean ± standard error of 8 to 22 mice per treatment group. *, significant difference in growth compared with that in mice inoculated with *L. pneumophila* AA100 (Mann-Whitney test [$P < 0.05$]).

onstrate that *L. pneumophila* AA488 and AA502 are fully virulent in vivo.

The effect of coinoculation with *H. vermiformis* on the intrapulmonary growth of each *L. pneumophila* strain was subsequently assessed. A/J mice were inoculated intratracheally with the parent strain of *L. pneumophila*, AA100 (which is virulent for both mononuclear phagocytic cells and *H. vermiformis* [10^6 bacteria per mouse]), or with Pho$^+$ *L. pneumophila* AA488 or AA502 (strains which are fully virulent for mononuclear phagocytic cells in vivo but have reduced virulence for *H. vermiformis* [10^6 bacteria per mouse]), or they were coinoculated with these bacterial strains (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse) as previously described (7, 8, 38). At 48 h postinoculation, the mice were humanely euthanized and the lungs were excised and homogenized. *L. pneumophila* CFU per lung were determined by culture of lung homogenates on bacteriological media. Bacterial growth was assessed at 48 h postinoculation, because we have previously demonstrated that growth of *L. pneumophila* in mice coinoculated with *H. vermiformis* and strain AA100 is maximal at this time point (7). In agreement with the results of our previous studies (7), coinoculation of A/J mice with *H. vermiformis* significantly enhanced intrapulmonary growth of the parent strain of *L. pneumophila* (AA100) (Table 1). In contrast, coinoculation with *H. vermiformis* did not result in a significant increase in intrapulmonary growth of *L. pneumophila* AA488 or AA502 compared to that in mice inoculated with the same strain of bacteria alone. These results demonstrate that *L. pneumophila* virulence for *H. vermiformis* is required for maximal enhancement in intrapulmonary growth of the bacteria in mice coinoculated with the amoebae and support the hypothesis that intrapulmonary *H. vermiformis* may enhance growth of *L. pneumophila* in the lung by providing a niche for bacterial replication.

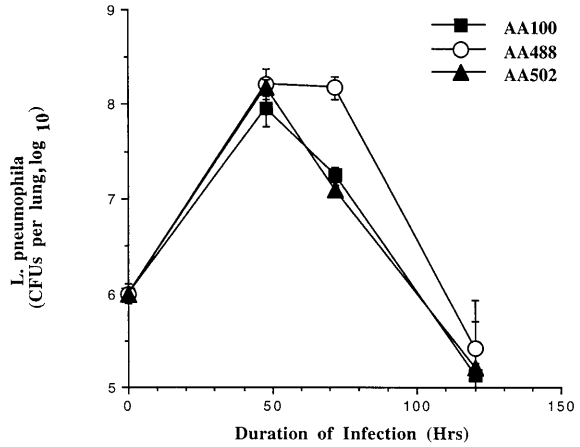

FIG. 3. Kinetics of intrapulmonary growth of Pho$^+$ *L. pneumophila* strains in A/J mice. A/J mice were inoculated intratracheally with *L. pneumophila* AA488, AA502, or AA100 (10^6 bacteria per mouse). At 24 to 72 h postinoculation, the mice were euthanized, and the lungs were excised and homogenized. *L. pneumophila* CFU per lung were subsequently determined by culture of lung homogenates on bacteriological media. Results represent the mean ± standard error of 3 to 22 mice per treatment group.

TABLE 1. Effect of *H. vermiformis* on intrapulmonary growth of *L. pneumophila* strains at 48 h postinoculation

Inoculum	*L. pneumophila* CFU/lung 48 h postinoculation[a]
AA100	$(9.68 \pm 4.63) \times 10^7$
AA100+ amoebae	$(2.10 \pm 0.58) \times 10^{9b}$
AA488	$(6.51 \pm 3.33) \times 10^7$
AA488+ amoebae	$(1.97 \pm 1.0) \times 10^8$
AA502	$(2.01 \pm 1.21) \times 10^8$
AA502+ amoebae	$(1.32 \pm 0.73) \times 10^9$

[a] Results represent mean ± standard error of 7 to 22 mice per treatment group.
[b] Significant increase in intrapulmonary growth of *L. pneumophila* in mice coinoculated with *H. vermiformis* compared with that in mice inoculated with the same strain of bacteria alone (Mann-Whitney test [$P < 0.05$]).

However, our results also demonstrate that there were small increases (i.e., three- and sixfold, respectively) in the growth of *L. pneumophila* in mice coinoculated with *H. vermiformis* and *L. pneumophila* AA488 or AA502 at 48 h postinfection compared to that in mice inoculated with the same strain of bacteria. While this increase in bacterial growth is not statistically significant, these results suggest that intrapulmonary *H. vermiformis* may potentiate the growth of *L. pneumophila*, at least in part, by a mechanism which is independent of *L. pneumophila* virulence for *H. vermiformis*. We have previously demonstrated that intrapulmonary *H. vermiformis* alters host immune responses during replicative *L. pneumophila* lung infections, resulting in exaggerated production of cytokines, including gamma interferon and tumor necrosis factor alpha (7). Previous studies by our laboratory and others have demonstrated that mononuclear phagocytic cell activation, mediated in large part by gamma interferon and tumor necrosis factor alpha, is required for resolution of replicative *L. pneumophila* infection in permissive mononuclear phagocytic cells (5, 6, 8, 9, 25, 31). However, exaggerated production of proinflammatory cytokines, including tumor necrosis factor alpha, has been shown to be detrimental to resolution of other inflammatory lung diseases, including adult respiratory distress syndrome (30, 39). The potential relationship between *H. vermiformis*-induced altered host immune responses and enhanced intrapulmonary

growth of *L. pneumophila* in mice coinoculated with the amoebae remains to be thoroughly investigated.

Alternatively, it is possible that growth of *L. pneumophila* strains (which are virulent for amoebae) in intrapulmonary *H. vermiformis* may enhance bacterial virulence for mammalian cells, thereby facilitating growth of the bacteria in vivo. Previous in vitro studies have demonstrated that *L. pneumophila*, following growth within *Acanthamoeba castellanii*, another freshwater protozoan, has enhanced invasiveness for cultured mammalian cells (13). The effect of growth of *L. pneumophila* in intrapulmonary *H. vermiformis* on virulence of the bacteria in vivo remains to be investigated.

In summary, we have isolated mutant strains of *L. pneumophila* which have reduced virulence for *H. vermiformis* but are fully virulent for mammalian cells in vivo. These mutant *L. pneumophila* strains suggest that different bacterial gene products are likely required for *L. pneumophila* infection of mononuclear phagocytic cells and freshwater protozoa. We have previously shown that coinoculation of A/J mice with the amoeba *H. vermiformis* potentiates intrapulmonary growth of *L. pneumophila*, thereby demonstrating that coincident inhalation of protozoa may enhance the severity of replicative *L. pneumophila* lung infection (7). The results of our current study demonstrate that *L. pneumophila* virulence for *H. vermiformis* is required for maximal intrapulmonary growth of the bacteria. These results support the hypothesis that intrapulmonary amoebae may facilitate growth of *L. pneumophila* in the lung, at least in part, by providing a niche for bacterial replication in vivo. However, this mechanism is not mutually exclusive, because *H. vermiformis*-induced changes in host immune responses and/or increased bacterial virulence, resulting from intra-amoebic growth of the bacteria, may also contribute to enhanced intrapulmonary growth of *L. pneumophila.*

While our results support a role of coinhaled protozoa in the pathogenesis of experimentally induced legionellosis, the potential role of inhaled amoebae in the pathogenesis of Legionnaires' disease in humans remains unknown. It is likely that people may inhale *H. vermiformis*, because amoebae can be aerosolized (35) and asymptomatic persons frequently have titers of antibody to amoebal species, including *H. vermiformis*, indicative of human exposure (34). However, it is likely impossible to validate a role of inhaled *H. vermiformis* in the pathogenesis of legionellosis in humans by identification of amoebae in the lung. We have previously demonstrated that *H. vermiformis* is identifiable in the murine lung only during the initial phase of replicative *L. pneumophila* lung infection (i.e., at $<$72 h postinoculation [7]). Diagnostic tests of human patients suspected of having Legionnaires' disease are conducted considerably later in the course of the infection, potentially after inhaled amoebae have been cleared from the lung. Nevertheless, the results of our present studies indicate that further investigation into the potential role of coinhaled protozoa in the pathogenesis of Legionnaires' disease in humans is warranted. It is anticipated that the results of these studies may have significant implications with regard to the design of strategies to prevent, control, and/or treat human legionellosis.

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