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Coinoculation with *Hartmannella vermiformis* Enhances Replicative *Legionella pneumophila* Lung Infection in a Murine Model of Legionnaires' Disease

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The effect of inhaled amoebae on the pathogenesis of Legionnaires' disease was investigated in vivo. A/J mice, which are susceptible to replicative *Legionella pneumophila* infections, were inoculated intratracheally with *L. pneumophila* (10^6 bacteria per mouse) or were coinoculated with *L. pneumophila* (10^6 bacteria per mouse) and *Hartmannella vermiformis* (10^6 amoebae per mouse). The effect of coinoculation with *H. vermiformis* on bacterial clearance, histopathology, cellular recruitment into the lung, and intrapulmonary levels of cytokines including gamma interferon and tumor necrosis factor alpha was subsequently assessed. Coinoculation with *H. vermiformis* significantly enhanced intrapulmonary growth of *L. pneumophila* in A/J mice. Histopathologic and flow cytometric analysis of lung tissue demonstrated that while A/J mice inoculated with *L. pneumophila* alone develop multifocal pneumonitis which resolves with minimal mortality, mice coinoculated with *H. vermiformis* develop diffuse pneumonitis which is associated with diminished intrapulmonary recruitment of lymphocytes and mononuclear phagocytic cells and significant mortality. Furthermore, coinoculation of mice with *H. vermiformis* resulted in a fourfold enhancement in intrapulmonary levels of gamma interferon and tumor necrosis factor alpha compared with mice infected with *L. pneumophila* alone. The effect of *H. vermiformis* on intrapulmonary growth of *L. pneumophila* in a resistant host (i.e., BALB/c mice) was subsequently evaluated. While BALB/c mice do not develop replicative *L. pneumophila* infections following inoculation with *L. pneumophila* alone, there was an eightfold increase in intrapulmonary *L. pneumophila* in BALB/c mice coinoculated with *H. vermiformis*. These studies, demonstrating that intrapulmonary amoebae potentiate replicative *L. pneumophila* lung infection in both a susceptible and a resistant host, have significant implications with regard to the potential role of protozoa in the pathogenesis of pulmonary diseases due to inhaled pathogens and in the design of strategies to prevent and/or control legionellosis.

Legionella pneumophila is a bacterial parasite of many species of freshwater protozoa and an occasional intracellular pathogen of humans (2, 3, 20, 24, 26, 28, 30, 34, 39). Infection of humans usually occurs after *L. pneumophila* from natural aquatic sources contaminates potable and nonpotable water systems. The portal of entry is the lung, via inhalation of aerosols or microaspiration of potable water (8, 12–14). 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 (23, 24, 25, 30, 34).

Since the discovery of the bacterial-protozoal interaction, there has been much speculation and limited research directed toward elucidating the relevance of this natural parasitism to human infection. It is believed that protozoa play a key role in the persistence of *L. pneumophila* in the environment, since growth of the bacterium in the absence of these organisms has not been documented (21, 35, 41). In outbreak investigations, amoebae capable of supporting growth of the outbreak-associated strain of *L. pneumophila* have often been found in implicated water sources (4, 9, 18, 19). It is not known whether their presence is a prerequisite for an outbreak, since contamination of potable water systems with protozoa is exceedingly common (23, 34, 39). It has been suggested that protozoa may serve as a vehicle to maintain viable *L. pneumophila* through the process of water treatment and/or serve as a reservoir for growth of the bacteria within permissive sites in water systems (4). Recent evidence also demonstrates that growth of *L. pneumophila* within an amoebal host may enhance the invasiveness of the bacteria for mammalian cells, thereby suggesting that amoebae have a more direct, rather than simply a supportive effect, on pathogenesis (10). Finally, since airborne amoebae have been detected in water aerosols (33), it has also been proposed that *L. pneumophila*-infected protozoa may be the primary infectious particle for Legionnaires' disease (34); however, there is no experimental evidence to support this theory. The potential effect of inhaled protozoa in the pathogenesis of *L. pneumophila* infections in vivo has not been thoroughly investigated.

In this study, we observed the effect of coinoculation of virulent *L. pneumophila* and a permissive amoebal strain in an animal model of infection. We chose *Hartmannella vermiformis* as the protozoal host, since this species is the predominant amoebae in potable water supplies in the United States (17). In addition, this species has been linked with outbreaks of Legionnaires' disease and has been found in aerosols (4, 9, 18, 33). Anti-*Hartmannella* antibodies are commonly detected in the sera of healthy persons, suggesting that exposure to this or a related organism by ingestion or inhalation is a frequent

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occurrence in humans (31). Lastly, a recent report of *H. vermiformis*-associated meningoencephalitis suggests that this agent can produce active infection under some circumstances (32).

Our animal model of infection uses intratracheal (i.t.) inoculation of A/J mice (6). Unlike mononuclear phagocytic cells (MPCs) from other inbred mouse strains, including BALB/c mice, macrophages from A/J mice support intracellular growth of *L. pneumophila* (42, 43). As a result, A/J mice inoculated i.t. with virulent *L. pneumophila* develop a self-limiting pneumonia with histologic features of Legionnaires' disease (6). In the current study, both A/J and BALB/c mice were used to investigate the effect of coinoculation of amoebae on the course of Legionnaires' disease in vivo. We demonstrate that coinoculation with an outbreak-associated strain of *H. vermiformis* potentiated the replication of *L. pneumophila* in both susceptible (A/J) and resistant (BALB/c) mice.

MATERIALS AND METHODS

Animal care. Female pathogen-free 6- to 8-week-old A/J mice (Jackson Laboratory, Bar Harbor, Maine) and BALB/c mice (Charles River Laboratories, Portage, Mich.) were used in all experiments. The animals were housed in micro-isolator cages in Horsfall units and were cared for by standard guidelines.

Bacterial inoculum. *L. pneumophila* AA100, a redesignation of a primary clinical isolate from the Wadsworth Veterans Administration Hospital (Wadsworth, Calif.), was provided by Paul Edelstein. The bacteria were maintained and passaged three times on buffered charcoal-yeast extract agar (22). For preparation of the inoculum, *L. pneumophila* was quantitated on buffered charcoal-yeast extract agar plates that had been incubated for 48 h and resuspended in phosphate-buffered saline (PBS) at 8×10^7 bacteria per ml as previously described (1, 6).

Amoeba culture. *H. vermiformis* CDC-19 (ATCC 50237) 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 (18). This strain of amoebae was originally isolated from a water sample obtained during an investigation of nosocomial legionellosis (18) and was epidemiologically linked to the epidemic strain of *L. pneumophila*. For preparation of the inoculum, the amoebae were removed from the culture flasks, centrifuged ($300 \times g$, 15 min, 22°C), resuspended in PBS, counted on a hemocytometer, washed once in PBS, and resuspended in PBS at 8×10^7 organisms per ml.

Intratracheal inoculation of A/J mice. A/J mice were inoculated i.t. with *L. pneumophila* and/or with *H. vermiformis*, using previously described method (6, 37). Mice were anesthetized with ketamine (2.5 mg per mouse intraperitoneally) and tethered, and an incision was made through the skin of the ventral neck. The trachea was isolated, and 25 μl of PBS containing either *L. pneumophila* (10^6 bacteria per mouse), *H. vermiformis* (10^3 to 10^6 amoebae per mouse), or a mixed inoculum of *L. pneumophila* and *H. vermiformis* followed by 10 μl of air was injected directly into the trachea, using a 27-gauge needle. Results of previous in vitro studies demonstrate that fewer than 0.1% of *L. pneumophila* attach to *H. vermiformis* during 1 h of coculture, while entry of the bacteria into *H. vermiformis* occurs following 1 to 4 h of coculture (21). Consequently, for coinoculation experiments, bacteria and amoebae were combined immediately prior to i.t. inoculation, to prevent *L. pneumophila* infection of *H. vermiformis* in vitro. The skin incision was subsequently closed by using a sterile wound clip.

Recovery of *L. pneumophila* from infected mice. At specific time points postinoculation (p.i.) (24, 48, and 72 h), the mice were humanely sacrificed and the lungs were removed. Lung tissue was finely minced in 10 ml of water and subsequently homogenized (2 min per sample) in a stomacher (Tekmar, Cincinnati, Ohio) as previously described (5, 6). The tissue homogenates were serially diluted and cultured on buffered charcoal-yeast extract agar containing polymyxin B, cefamandole, and anisomycin (Baxter Healthcare Scientific Products, McGaw Park, Ill.) for 72 h, and CFU were determined (6, 15, 36).

Pathology. The inflammatory response in the lung in A/J mice in response to i.t. inoculation of amoebae and/or bacteria was assessed by light microscopy as previously described (6). Mice were inoculated i.t. with *H. vermiformis* (10^6 amoebae per mouse) or were coinoculated with *H. vermiformis* (10^6 amoebae per mouse) and *L. pneumophila* (10^6 bacteria per mouse). At specific time points p.i. (24, 48, and 72 h), the mice were humanely sacrificed. The lungs were excised, inflated, and fixed in 10% buffered formalin. Lungs were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Inflammatory cell recruitment into the lung. The effect of *H. vermiformis* on recruitment of inflammatory cells into the lung during replicative *L. pneumophila* lung infections was determined by a previously described method (11, 27). Mice were inoculated i.t. with *H. vermiformis* (10^6 amoebae per mouse) or *L. pneumophila* (10^6 bacteria per mouse) or were coinoculated with both agents (10^6 amoebae and 10^6 bacteria per mouse). All mice were subsequently processed and analyzed individually. At specific times p.i., the mice were humanely sacrificed. The lungs were excised, minced, and incubated in RPMI 1640 medium containing 5% fetal calf serum, 1 mg of collagenase A (Boehringer Mannheim, Indianapolis, Ind.) per ml, and DNase (Sigma Chemical Company, St. Louis, Mo.) for 30 min at 37°C. The cells were further disaggregated by drawing the resultant lung homogenate repeatedly through a 10-ml syringe 20 to 30 times prior to pelleting of the cells by centrifugation. Erythrocytes were subsequently lysed by brief exposure to ammonium chloride. The resultant lung homogenate sample was washed once in PBS. To obtain a total cell count, an aliquot of the sample was diluted in 2% paraformaldehyde and counted on a hemocytometer. Another aliquot was stained with modified Wright-Giemsa stain (Baxter Health Care Corporation). Differential counts were performed under oil immersion; cells were counted as polymorphonuclear leukocytes (PMNs), as MPCs, or as lymphocytes on the basis of standard light microscopic criteria.

Collection of BALF for cytokine analysis. A/J mice were inoculated i.t. with *H. vermiformis* (10^6 organisms per mouse) or *L. pneumophila* (10^6 organisms per mouse) or were coinoculated with amoebae and *L. pneumophila* (10^6 of each organism per mouse) as previously described. Twenty-four hours p.i. (i.e., when gamma interferon [IFN-γ] and tumor necrosis factor alpha [TNF-α] activities are maximally enhanced in the lungs and/or plasma of *L. pneumophila*-infected A/J mice [6, 7]), the mice were humanely euthanized and bronchoalveolar lavage fluid (BALF) was collected as previously described (5, 6). Briefly, the mice were tethered as for i.t. inoculation, the trachea was exposed, and a 22-gauge angiocatheter was inserted through the trachea. Then 1.6 ml of PBS in two 0.8-ml aliquots was injected into the lungs and withdrawn. The fluid was subsequently filtered through a 0.22-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.) to remove bacteria cells, and debris. Recovery was typically 1.2 ml of fluid per mouse. BALF samples were stored at −20°C until used in cytokine analysis as described below.

IFN-γ analysis by ELISA. IFN-γ activity was assessed in BALF by a murine-specific enzyme-linked immunosorbent assay (ELISA) (interest-γ; Genzyme Corp., Cambridge, Mass.) performed according to the manufacturer's directions (6). BALF samples were diluted 1:5 in buffer medium prior to assessment. A standard of murine IFN-γ was run with each assay.

TNF-α analysis by the WEHI assay. TNF-α activity was assessed in BALF by a cytotoxicity assay using the WEHI 164 subclone 13 cell line as previously described (16). Briefly, BALF samples were serially diluted directly in 96-well microtiter plates (Costar, Cambridge, Mass.). The WEHI cells were resuspended at 5×10^5 cells per ml in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5 μg of actinomycin D (Calbiochem, La Jolla, Calif.) per ml and added to the samples. A standard of human recombinant TNF-α was run in each assay. Samples were incubated overnight at 37°C, after which 20 μl of thiazolyl blue tetrazolium (5 mg/ml; Sigma) was added to the wells, which were allowed to incubate at 37°C for an additional 4 h. Viable cells (i.e., cells not lysed by TNF-α) metabolize the thiazolyl blue tetrazolium to produce dark blue formazan crystals. The crystals were dissolved with isopropanol-HCl, and the plates were read in a microELISA reader at 550 nm. TNF-α activity was calculated on the basis of the human recombinant TNF-α standard that was run in the same assay.

Statistical analysis. The Mann-Whitney test, the Student *t* test, or the chi-square test was used to compare differences between treatment groups. $P < 0.05$ was considered significant.

RESULTS

Clinical signs. Initial experiments were conducted to determine the effect of i.t. inoculation of *H. vermiformis* on the morbidity and/or mortality in A/J mice. Mice were inoculated i.t. with *H. vermiformis* alone (10^3 to 10^6 organisms per mouse, three mice per dose). During the following 72 h, none of the mice inoculated with amoebae alone displayed clinically apparent signs of disease. In subsequent experiments, mice were coinoculated with *H. vermiformis* (10^3 to 10^6 amoebae per mouse) and *L. pneumophila* (10^6 bacteria per mouse) and were observed for 72 h for morbidity and/or mortality. In contrast to mice inoculated with amoebae alone, mice coinoculated with *L. pneumophila* (10^6 bacteria per mouse) and with $\leq 10^5$ *H. vermiformis* displayed mild nonspecific signs of illness at 48 h p.i. These included a ruffled hair coat, a hunched posture, and labored breathing. As shown in Table 1, sporadic mortality was also observed in these mice at 72 h p.i. Mice coinoculated i.t. with *L. pneumophila* and *H. vermiformis* (10^6 organisms of each per mouse) appeared severely debilitated by 48 h p.i., with marked lethargy and severely labored breathing. Mortality in these mice was 50% by 72 h p.i. (Table 1). In contrast, as we have previously reported (6), mice inoculated i.t. with *L. pneumophila* alone (10^6 bacteria per mouse) exhibit nonspecific

TABLE 1. Survival of *L. pneumophila*-infected A/J mice coinfected with *H. vermiformis* at 72 h p.i.[a]

No./inoculum		No. of mice (inoculated)	Mortality (%)
L. pneumophila	*H. vermiformis*		
10^6	10^6	14	50[b]
10^6	10^5	5	20
10^6	10^4	5	20
10^6	10^3	6	16
10^6	0	14	14

[a] A/J mice were coinoculated i.t. with *H. vermiformis* and *L. pneumophila* as described in Materials and Methods and were observed twice daily for 3 days for clinical signs of illness or mortality. Results represent combined results of two separate experiments, 5 to 14 animals per treatment group.

[b] Significantly more compared with mice inoculated with bacteria alone, $P < 0.05$.

clinical signs of illness for the first 24 to 48 h p.i. These clinical signs resolved by 72 h p.i. with minimal mortality. In all subsequent experiments, A/J mice were inoculated i.t. with 10^6 *L. pneumophila* and/or 10^6 *H. vermiformis.*

Pathology. No significant lesions were evident grossly in lungs of mice inoculated i.t. with *H. vermiformis* alone (10^6 amoebae per mouse) at 24 h p.i. Similarly infected mice sacrificed at 48 and 72 h p.i. had numerous petechia on the lung surface and within the lung parenchyma, representing areas of hemorrhage and inflammation, involving $\leq$25% of the lung lobes. Mice coinoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse) and sacrificed at 24 h p.i. had lung lesions similar to those described above for mice inoculated with amoebae alone at 48 h p.i. However, by 72 h p.i., the lesions became more extensive, with involvement and consolidation of $\geq$75% of all lung lobes. Lung lesions in A/J mice inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse), first evident at 48 to 72 h p.i., have been previously described (6) and are similar to, although much less extensive than those described above for mice coinfected with bacteria and amoebae.

Lungs from mice inoculated with *H. vermiformis* (10^6 amoebae per mouse) or coinoculated with *H. vermiformis* (10^6 amoebae per mouse) and *L. pneumophila* (10^6 bacteria per mouse) were subjected to histological examination. (Lungs from three mice per treatment group were examined histologically; lesions in lung tissue were similar between mice in a given treatment group.) Significant microscopic lesions in mice inoculated with *H. vermiformis* alone were first evident at 24 h p.i. and consisted of patchy areas of pneumonia which occupied no more than 10% of the total lung. The pneumonia was characterized by clusters of neutrophils in the alveoli. Many of the neutrophils were either surrounding or near individual amoebae. In addition, alveolar septae in these areas were thickened. Pulmonary lesions in mice inoculated i.t. with amoebae and sacrificed at 48 h p.i. were similar to those described above in mice at 24 h p.i. except that the affected areas contained a mixed inflammatory cell infiltrate, consisting of both neutrophils and mononuclear phagocytic cells. Also, fewer amoebae were evident within the alveoli at 48 than at 24 h p.i., and intra-alveolar amoebae were encysted or were surrounded by neutrophils and appeared to be necrotic. Pulmonary lesions in mice inoculated with *H. vermiformis* alone and sacrificed at 72 h p.i. were similar to those described in mice at 48 h p.i. and occupied no more than 20% of the lung parenchyma. By 72 h p.i., the alveolar infiltrate was composed primarily of mononuclear rather than polymorphonuclear leukocytes, and the alveolar septae were thicker, few amoebae were present, and remaining organisms were encysted.

Lung lesions in mice coinoculated with *H. vermiformis* and *L. pneumophila* and sacrificed at 24 h p.i. were similar to those found in mice inoculated with *H. vermiformis* alone except that there were more neutrophils in the alveoli of coinoculated mice and the affected foci were somewhat larger (Fig. 1a). At 48 h p.i. the pneumonia in these mice was similar to that described for mice inoculated with amoebae alone, although it was more extensive. Additional lesions in A/J mice coinoculated with *L. pneumophila* and *H. vermiformis* included multifocal hemorrhage, necrosis and disruption of alveolar walls (Fig. 1b), and edema adjacent to blood vessels. By 72 h p.i., surviving mice had extensive pneumonia (i.e., involving 70 to 80% of the lung parenchyma). Furthermore, there was also pronounced thickening of the alveolar septa (Fig. 1c).

Histologic lesions in the lung of A/J mice inoculated with *L. pneumophila* alone (10^6 bacteria per mouse) have been previously described (6). Briefly, in contrast to mice coinoculated with *L. pneumophila* and amoebae, patchy pneumonia was first evident in *L. pneumophila*-infected mice at 48 h p.i. and was characterized by the presence of numerous PMNs (75%) and MPCs (25%) in the alveoli. At 72 h p.i., the inflammatory infiltrate was more chronic, consisting of fewer PMNs and more MPCs within the alveolar space.

Quantitation of inflammatory cell in infected lung tissue. At specific time points following i.t. inoculation of *H. vermiformis* (10^6 amoebae per mouse) and/or *L. pneumophila* (10^6 bacteria per mouse), A/J mice were sacrificed and pulmonary inflammatory cells were quantitated as described in Materials and Methods. As shown in Fig. 2A, i.t. inoculation of mice with *H. vermiformis* alone did not result in significant recruitment of inflammatory cells into the lung. In contrast, there was a significant increase in the total number of pulmonary inflammatory cell within 48 h following i.t. inoculation of mice with *L. pneumophila* or with *L. pneumophila* and *H. vermiformis*. However, while the number of cells recruited into the lung of mice inoculated with bacteria alone continued to increase until 72 h p.i., fewer inflammatory cells were recruited into the lungs of mice coinoculated with *H. vermiformis* and *L. pneumophila* between 48 and 72 h p.i. Consequently, at 72 h p.i., the number of inflammatory cells in the lungs of mice coinoculated with *L. pneumophila* and *H. vermiformis* was significantly less than that in mice inoculated with *L. pneumophila* alone ($P < 0.05$). Further analysis of recruited pulmonary inflammatory cell populations at 72 h p.i. revealed that similar numbers of PMNs were recruited into the lungs of mice inoculated with *L. pneumophila* or coinoculated with *L. pneumophila* and amoebae (Fig. 2B). In contrast, significantly fewer lymphocytes (Fig. 2C) and MPCs (Fig. 2D) were recruited into the lungs of mice coinoculated with *H. vermiformis* than into the lungs of mice inoculated with *L. pneumophila* alone ($P < 0.05$).

Quantitation of *L. pneumophila* in tissues in i.t.-inoculated A/J mice. The effect of *H. vermiformis* on growth of *L. pneumophila* in the lung was evaluated. A/J mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) or with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 bacteria per mouse). At specific time points thereafter, the mice were sacrificed and growth of *L. pneumophila* in the lung was assessed. As shown in Fig. 3, coinoculation of mice with both *L. pneumophila* and *H. vermiformis* resulted in a significant enhancement in growth of *L. pneumophila* in the lung at 24 to 72 h p.i. compared with mice inoculated with bacteria alone. At 48 h following coinoculation of mice with *L. pneumophila* and *H. vermiformis*, $>10^8$ *L. pneumophila* CFU were recovered in lung homogenates. In previous studies, we

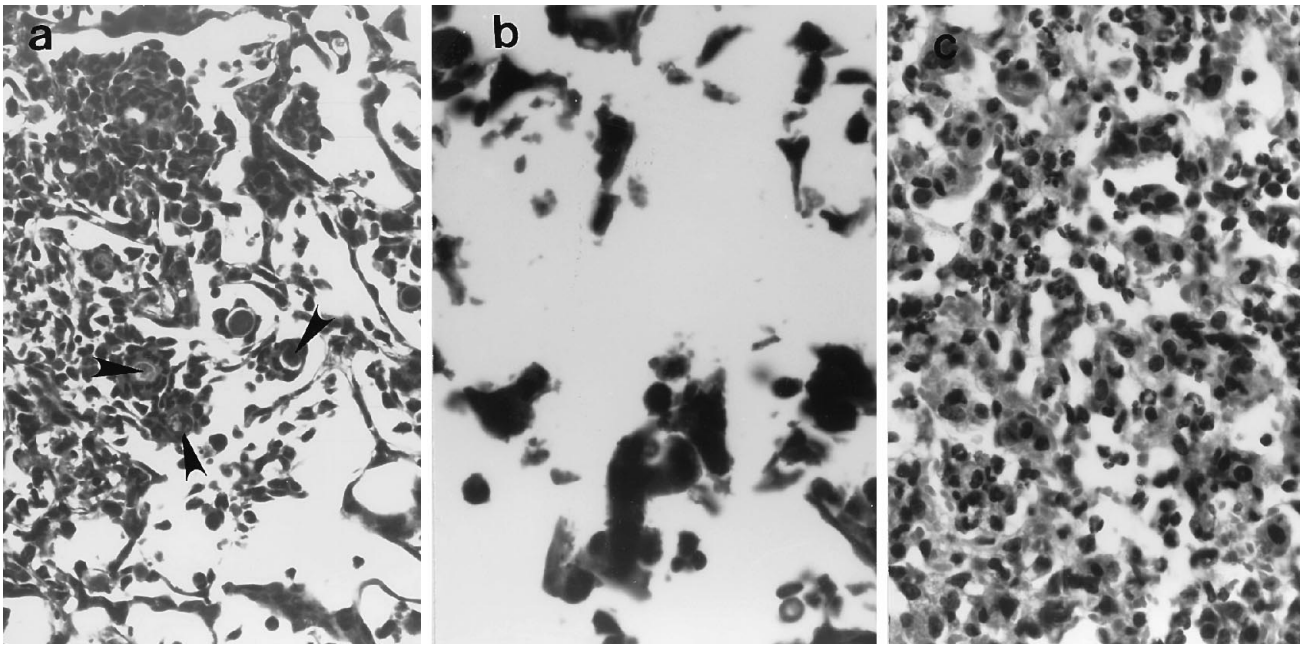

FIG. 1. Lung pathology in A/J mice coinoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse). (a) Mouse lungs 24 h after inoculation with *L. pneumophila* and *H. vermiformis*. Note neutrophils (arrowheads) surrounding and near individual amoebae (arrowheads). (b) Mouse lungs 48 h p.i. Note necrosis and disruption of alveolar walls. (c) Mouse lungs 72 h p.i. Note pronounced thickening of the alveolar septa in lung tissue. Hematoxylin-and-eosin stain; magnification, ×470.

showed that an initial inoculation of $\geq 10^7$ *L. pneumophila* in A/J mice results in ≥66% mortality (6). Thus, the high mortality observed in these mice within 72 h p.i. (Table 1) is most likely reflective of an increased bacterial burden.

Effect of *H. vermiformis* on TNF-α and IFN-γ activities in the lung. A/J mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) or *H. vermiformis* (10^6 amoebae per mouse) or were coinoculated with bacteria and amoebae (10^6 organisms of each). At 24 h p.i., the mice were humanely sacrificed, BALF was collected, and TNF-α and IFN-γ were quantitated in BALF as described in Materials and Methods. As shown in Table 2, both TNF-α and IFN-γ levels were markedly higher in BALF from *L. pneumophila*-infected mice than in that from uninfected A/J mice. Intratracheal inoculation of A/J mice with *H. vermiformis* also resulted in a significant enhancement in TNF-α and IFN-γ activities in BALF when compared with uninfected mice. Furthermore, BALF collected from mice 24 h following coinoculation with both *H. vermiformis* and *L. pneumophila* contained ≥4-fold more TNF-α and IFN-γ than did BALF from mice inoculated with either organism alone (Table 2).

Effect of *H. vermiformis* on growth of *L. pneumophila* in lungs of a resistant host. Results of previous studies have demonstrated that explanted thioglycolate-elicited peritoneal MPCs derived from BALB/c mice are nonpermissive to growth of *L. pneumophila* (42). Because the permissiveness of explanted thioglycolate-elicited peritoneal MPCs to growth of *L. pneumophila* has previously been shown to reflect the susceptibility of the host to replicative *L. pneumophila* infections in vivo, these studies suggest that BALB/c mice are resistant to replicative *L. pneumophila* lung infections. To evaluate the effect of intrapulmonary amoebae on the pathogenesis of *L. pneumophila* lung infection in a resistant host, 6- to 8-week-old female pathogen-free BALB/c mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) or were coinoculated with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse). At specific times p.i. (24, 48, and 72 h), the mice were sacrificed and intrapulmonary growth of *L. pneumophila* was determined by quantification of bacteria in infected lung homogenates. Results of these studies demonstrate that *L. pneumophila* did not replicate in, and was gradually cleared from, the lungs of immunocompetent BALB/c mice inoculated i.t. with the bacteria alone (Fig. 4). In contrast, significantly more *L. pneumophila* organisms were recovered in lung homogenates from BALB/c mice coinoculated with the bacteria and amoebae at 24 to 72 h p.i. than in those of mice infected with *L. pneumophila* alone (Fig. 4; $P < 0.05$). Furthermore, while inoculation of BALB/c mice with *L. pneumophila* alone (total of 18 mice) did not result in any mortality by 72 h p.i., coinoculation of BALB/c mice with *H. vermiformis* and *L. pneumophila* (total of 18 mice) resulted in 30% mortality by 72 h ($P < 0.05$).

DISCUSSION

In the current study, using a murine model of Legionnaires' disease and an axenically maintained strain of *H. vermiformis*, we investigated the effect of coinoculation of amoebae on the pathogenesis of replicative *L. pneumophila* pulmonary infections in vivo. These studies were initiated because previous studies have postulated that *L. pneumophila* pulmonary infections may be acquired by inhalation of *L. pneumophila* in association with amoebae rather than by inhalation of bacteria alone (34). The use of *H. vermiformis* was not arbitrary, as it is the most prevalent species of amoebae in potable water supplies in the United States and has been epidemiologically linked to outbreaks of Legionnaires' disease (18). Mice were inoculated i.t. with both bacteria and *H. vermiformis* in an effort to mimic the route of entry during outbreaks of Legionnaires' disease in humans. Results of our study, demonstrating that intrapulmonary *H. vermiformis* enhances growth of *L. pneumophila* in the lung in vivo, support the hypothesis that coincident

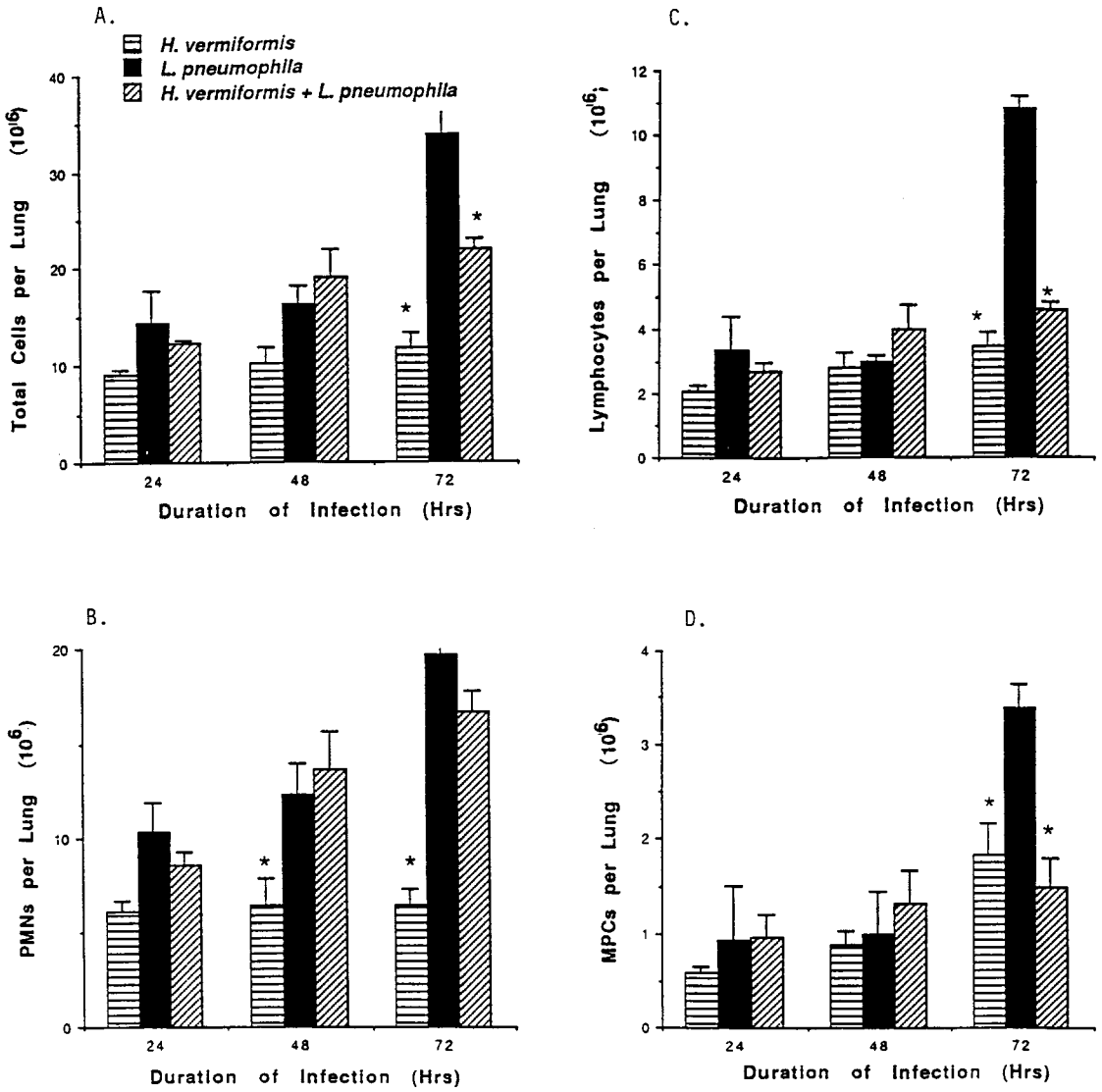

FIG. 2. Effect of *H. vermiformis* on inflammatory cell recruitment into the lungs of A/J mice. A/J mice were inoculated i.t. with *H. vermiformis* (10^6 amoebae per mouse), *L. pneumophila* (10^6 bacteria per mouse) or were coinfected with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse). At specific time points thereafter, the mice were sacrificed and inflammatory cell recruitment into the lung was assessed as described in Materials and Methods. (A) Total inflammatory cells; (B) PMNs; (C) lymphocytes; (D) MPCs. Results represent the mean ± standard error of the mean of three animals per treatment group per time point. *, significantly less compared with mice inoculated with bacteria alone, $P < 0.05$.

inhalation of *H. vermiformis* may enhance the severity of Legionnaires' disease.

These results are in contrast to those of a previous report by Tyndall and Dominique (39), which failed to demonstrate enhanced recovery of *L. pneumophila* from the lungs of weanling ICR mice inoculated intranasally with cocultures of *L. pneumophila* and amoebae (i.e., *Naegleria lovaniensis* or *Acanthamoeba royreba*) compared with mice infected with bacteria alone. Similarly, Vandenesch et al. (40) also failed to demonstrate enhanced mortality in guinea pigs following aerosolization with cocultures of *L. pneumophila* and *Acanthamoeba* spp. compared with animals infected with bacteria alone. The discrepancy between our results and those previously reported may be due to differences between the species of amoebae and/or strain of *L. pneumophila* used, the method of preparation of the mixed inoculum, or the animal models of Legionnaires' disease. As previously noted, *Hartmannella* spp. was chosen in this study because of its relatively common occurrence in potable water supplies and its relationship to human outbreaks of Legionnaires' disease (4, 9, 18).

The mechanism by which intrapulmonary *H. vermiformis* potentiates replication of *L. pneumophila* in the lung in vivo is unclear. There are three possible explanations, any or all of which may apply: (i) *H. vermiformis* may modify the host response to *L. pneumophila* infection, (ii) *H. vermiformis* may be an efficient implanted host cell which enhances *L. pneumophila* replication in the lung, or (iii) association of *L. pneumophila* with *H. vermiformis* may result in enhanced bacterial virulence. We found that coinoculation of A/J mice with *H. vermiformis* modifies the degree and type of pulmonary inflammatory response induced by *L. pneumophila*. Specifically, significantly fewer inflammatory cells were recruited into the lungs of mice coinoculated with *H. vermiformis* at 72 h p.i. than into the lungs of mice inoculated with bacteria alone. Further analysis of

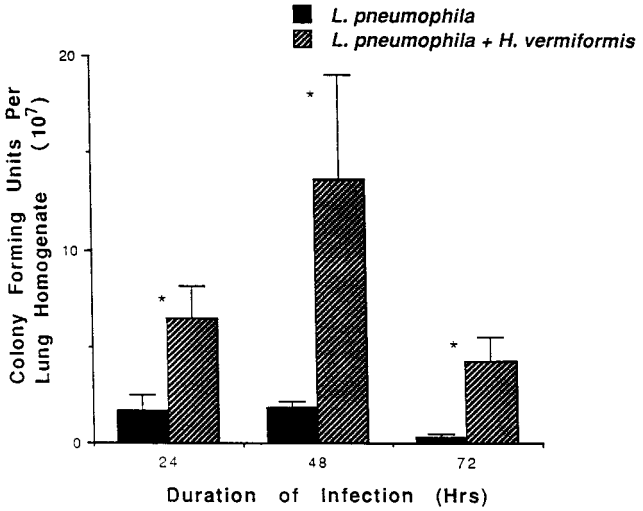

FIG. 3. Effect of *H. vermiformis* on intrapulmonary replication of *L. pneumophila* in A/J mice. A/J mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) or were coinfected with *L. pneumophila* (10^6 bacteria per mouse) and *H. vermiformis* (10^6 amoebae per mouse). At specific time points thereafter, the mice were sacrificed and replication of *L. pneumophila* in the lung was determined by analysis of CFU in lung homogenates as described in Materials and Methods. Results represent the mean ± standard error of the mean of bacteria in the lung of 6 to 11 animals per treatment group per time point. *, significantly enhanced growth compared with mice inoculated i.t. with bacteria alone, $P < 0.05$.

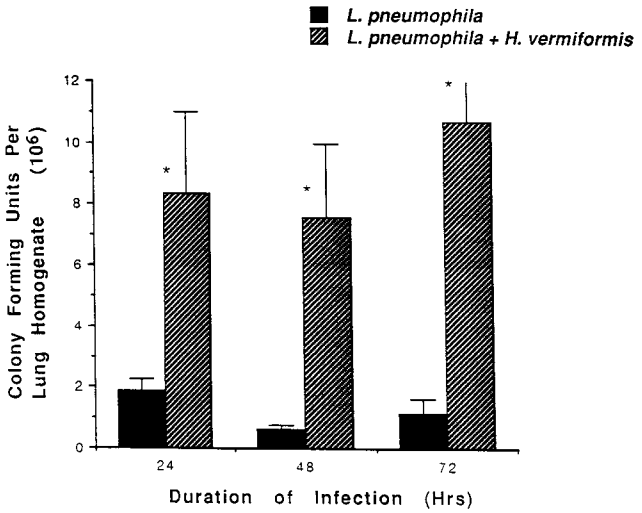

FIG. 4. Effect of *H. vermiformis* on intrapulmonary replication of *L. pneumophila* in BALB/c mice. BALB/c mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) or were coinfected with *L. pneumophila* (10^6 bacteria per mouse) and with *H. vermiformis* (10^6 amoebae per mouse). At specific time points thereafter, the mice were sacrificed and replication of *L. pneumophila* in the lung was determined by analysis of CFU in lung homogenates as described in Materials and Methods. Results represent the mean ± standard error of the mean of bacteria in the lung of five to six animals per treatment group per time point *, significantly enhanced growth compared with mice inoculated i.t. with bacteria alone, $P < 0.05$.

these recruited cell populations demonstrated that this difference in total inflammatory cells was due to the relative lack of lymphocyte and MPC recruitment into the lungs of A/J mice coinoculated with *H. vermiformis*. Previous studies demonstrated that alveolar MPCs play a critical role in both the pathogenesis of, and host defense against, *L. pneumophila* infections (29). Specifically, while unactivated MPCs support growth of the bacteria, activated MPCs facilitate elimination of intracellular *L. pneumophila*. These results suggest that enhanced growth of *L. pneumophila* within the lungs of A/J mice coinoculated with *H. vermiformis* may be mediated, in part, by the lack of recruitment of activated MPCs into the lung.

We have previously shown that cytokines, including TNF-α and IFN-γ, are induced during replicative *L. pneumophila* lung infections in A/J mice inoculated with *L. pneumophila* alone (10^6 bacteria per mouse), with maximal expression of these mediators at 24 h p.i. (6, 7). Furthermore, antibody-mediated neutralization of either endogenous TNF-α and/or IFN-γ inhibits clearance of *L. pneumophila* from the lung, demonstrating that both of these cytokines facilitate resolution of replicative *L. pneumophila* infection (6, 7). Because *H. vermiformis* potentiates growth of *L. pneumophila* in the lung within the first 24 to 48 h p.i. (Fig. 3), subsequent studies were conducted to determine if this effect was due to *H. vermiformis*-mediated inhibition of intrapulmonary TNF-α and/or IFN-γ activity. Results of these studies demonstrate that intrapulmonary levels of TNF-α and IFN-γ were markedly enhanced in A/J mice coinoculated with *L. pneumophila* and *H. vermiformis* (10^6 organisms of each per mouse) compared with mice inoculated with *L. pneumophila* alone. These studies demonstrate that enhanced growth of *L. pneumophila* in the lungs of A/J mice coinoculated with *H. vermiformis* is not due to amoebae-mediated inhibition of induction of intrapulmonary TNF-α and/or IFN-γ in response to the bacteria. Previous studies have demonstrated that high intrapulmonary levels of TNF-α can induce acute lung injury (38). Whether exaggerated local production of TNF-α in mice coinoculated with *H. vermiformis* is in itself detrimental to the host's ability to resolve *L. pneumophila* pulmonary infections has not been investigated.

TABLE 2. Effects of i.t. inoculation of *L. pneumophila* and/or *H. vermiformis* on TNF-α and IFN-γ levels in BALF of A/J mice at 24 h p.i.[a]

Inoculum	(pg/ml of BALF)	
	TNF-α	IFN-γ
None	0	87 ± 14
L. pneumophila	4,357 ± 1,076	498 ± 122
H. vermiformis	3,914 ± 302	795 ± 18
L. pneumophila and *H. vermiformis*	31,118 ± 8,774[b]	3,217 ± 603[b]

[a] A/J mice were inoculated i.t. with *L. pneumophila* (10^6 bacteria per mouse) and/or *H. vermiformis* (10^6 amoebae per mouse). At 24 h p.i., the mice were sacrificed, BALF was collected, and TNF-α and IFN-γ levels were quantitated in BALF as described in Materials and Methods. Results represent the mean ± standard error of the mean of activity in four to five mice per treatment group.

[b] Significant difference in cytokine activity in BALF compared with mice inoculated with *L. pneumophila* alone, $P < 0.05$.

Alternatively, previous studies have demonstrated that *H. vermiformis* provides an environmental niche for growth of *L. pneumophila* (21). Whether inhaled *H. vermiformis* also provides an intracellular niche for *L. pneumophila* replication in the lung in vivo has not been investigated. In the current study, we did not investigated whether inoculated *L. pneumophila* becomes associated with, or is ingested by, inoculated *H. vermiformis* in vivo. In addition, comparative infections with mice inoculated i.t. with *H. vermiformis* carrying preingested, intracellular *L. pneumophila* were not done because of the methodological problems of quantitating the bacteria within the amoebae.

Finally, Cirillo et al. (10) have demonstrated that *L. pneu-*

mophila, grown in *Acanthamoeba castellannii* (another species of amoebae), has enhanced invasiveness for epithelial cells and macrophages in vitro. Whether growth of *L. pneumophila* within amoebae might also enhance the pathogenicity of the bacteria for mammalian cells in vivo remains to be explored.

The potential role of inhaled amoebae in natural human outbreaks of legionellosis is currently unknown. However, amoebae are often present in infectious sources of water, and like *L. pneumophila*, the amoebae may be inhaled in water aerosols. Our study shows that coinoculation of *L. pneumophila* and *H. vermiformis* results in a more profound pneumonia. To determine whether this potentiation actually occurs during human inoculation is highly problematic. It is likely impossible to validate a role of inhaled amoebae in the pathogenesis of legionellosis by active identification of the protozoa in lung samples from patients with Legionnaires' disease. In the mouse, amoebae are identifiable in the lung only during the initial phase of replicative *L. pneumophila* lung infections (i.e., at $\leq$72 h p.i.). Diagnostic tests on human patients suspected of having Legionnaires' disease are conducted considerably later in the course of infection, potentially after any inhaled amoebae would have been cleared from the lung. Nevertheless, results of our studies indicate that further investigation of the potential role of protozoa in pulmonary disease states induced by inhaled pathogens, including *L. pneumophila*, is warranted. With regard to *L. pneumophila* pulmonary infection, these studies have significant implications, both with regard to the pathogenesis of Legionnaires' disease and in the design of strategies to prevent and/or control legionellosis.

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