Title

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Publication Date

2023-12-14

Peer reviewed

LEGIONELLA

Proceedings of the 2nd International Symposium

Editors:

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American Society for Microbiology • Washington, D.C.
1984

STATE OF THE ART LECTURE

Interactions Between *Legionella pneumophila* and Human Mononuclear Phagocytes

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Legionella pneumophila multiplies intracellularly in human mononuclear phagocytes (4; T. W. Nash, D. M. Libby, and M. A. Horwitz, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 93, 1982). This capability is central to the pathogenesis of Legionnaires disease and confronts the human immune defense system with a special challenge. This article summarizes in vitro studies on the interactions between *L. pneumophila* and human mononuclear and polymorphonuclear phagocytes. The article discusses the intracellular replication of *L. pneumophila* in human mononuclear phagocytes, the influence of antibody and complement on *L. pneumophila*-phagocyte interactions, the influence of phagocyte activation on *L. pneumophila*-mononuclear phagocyte interactions, the influence of antibiotics on intracellular *L. pneumophila*, phagocytosis of *L. pneumophila*, the formation of a novel phagosome in mononuclear phagocytes by *L. pneumophila*, and the capacity of *L. pneumophila* to inhibit phagosome-lysosome fusion.

Intracellular multiplication of *L. pneumophila*. *L. pneumophila* multiplies intracellularly in human peripheral blood monocytes (4) (Fig. 1) and in human alveolar macrophages obtained by bronchoalveolar lavage (Nash et al., 22nd ICAAC, abstr. no. 93). *L. pneumophila* does not multiply in the presence of human polymorphonuclear leukocytes, human lymphocytes, or tissue culture medium whether or not the medium is conditioned by human monocytes (4). Inside monocytes, the bacterium multiplies with a doubling time of about 2 h at midlogarithmic phase (4).

L. pneumophila is thus an intracellular parasite. Since the bacterium multiplies extracellularly on complex medium, it is a facultative intracellular parasite. *L. pneumophila* therefore belongs to a special group of bacterial pathogens that can evade host defenses by parasitizing mononuclear phagocytes. This group includes *Mycobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Francisella tularensis, Brucella* spp., and *Salmonella* spp.

Role of humoral immunity. Patients with Legionnaires disease respond to the infection by producing antibody against *L. pneumophila*. In vitro studies have examined three potential functions of such antibody in promoting host defense. The studies have examined (i) whether antibody promotes killing of *L. pneumophila* by complement, (ii) whether antibody promotes phagocytosis and killing of *L. pneumophila* by human phagocytes, and (iii) whether antibody inhibits the multiplication of *L. pneumophila* in monocytes.

Regarding the first potential function of antibody in host defense, virulent egg yolk-grown *L. pneumophila*, Philadelphia 1 strain, is completely resistant to the bactericidal effects of human serum even in the presence of high-titer anti-*L. pneumophila* human or rabbit antibody (5).

Regarding the second potential function of antibody in host defense, antibody in the presence of complement markedly promotes phagocytosis of *L. pneumophila* by human polymorphonuclear leukocytes and human monocytes (5, 6). In the presence of antibody and complement, virtually all the bacteria in an inoculum are ingested and enclosed within membrane-bound vacuoles (5). In contrast, in the presence of antibody alone or complement alone, phagocytosis is very inefficient (5, 6).

Whereas phagocytosis of *L. pneumophila* coated with antibody and complement is very efficient, killing of these bacteria by human phagocytes is not. Human polymorphonuclear leukocytes and monocytes kill only about 0.5 log of an inoculum. In contrast, at the same time and under the same conditions, polymorphonuclear leukocytes very effectively kill an encapsulated serum-resistant strain of *Escherichia coli*, reducing the number of viable bacteria by 2.5 log (5) (Fig. 2). When *E. coli* and *L. pneumophila* are mixed together in the same test tube, polymorphonuclear leukocytes kill the *E. coli* as effectively as when the *E. coli* are present alone, indicating that the killing capacity of polymorphonuclear leukocytes is not reduced in cultures containing *L. pneumophila*.

Thus anti-*L. pneumophila* antibody in the

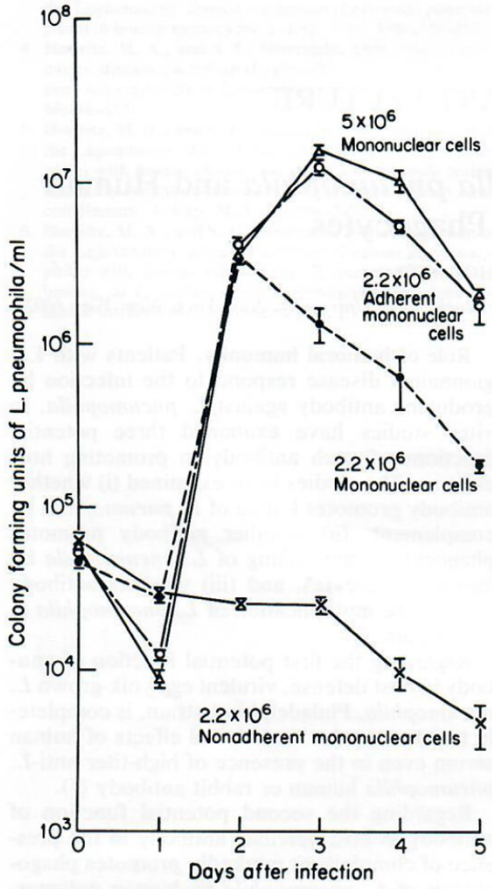

FIG. 1. *L. pneumophila* multiplies in the presence of monocytes (adherent cells) but not lymphocytes (nonadherent cells). *L. pneumophila* (10^5 CFU) was added to petri dishes containing 2.2 ml of RPMI, 15% serum, and either 2.2×10^6 or 5×10^6 mononuclear cells, 2.2×10^6 adherent mononuclear cells selected from 5×10^6 mononuclear cells, or 2.2×10^6 nonadherent mononuclear cells selected from the same mononuclear cell population. The cultures were incubated at 37°C in 5% CO_2–95% air on a gyratory shaker (100 rpm) for 3 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each petri dish were determined daily. Each point represents the average for three replicate petri dishes ± standard error. (Reproduced from reference 4.)

presence of complement markedly promotes phagocytosis of *L. pneumophila* but promotes only a modest degree of killing by human phagocytes.

Regarding the third potential function of antibody in host defense, antibody does not significantly influence the rate of intracellular multiplication of *L. pneumophila* in human monocytes (Fig. 3). In cultures containing antibody and complement, a small proportion (0.25 to 0.5 log) of the bacterial inoculum is killed initially. *L. pneumophila* surviving the initial encounter with monocytes multiply at as rapid a rate as bacteria that enter monocytes in the absence of antibody (6).

Thus, in vitro studies have examined three major ways by which antibody might function to promote host defense. These studies have revealed (i) that specific antibody fails to promote killing of *L. pneumophila* by complement, (ii) that antibody and complement together fail to promote effective killing of *L. pneumophila* by polymorphonuclear leukocytes and monocytes, and (iii) that antibody and complement fail to inhibit the rate of multiplication of *L. pneumophila* in monocytes. These findings suggest that humoral immunity may not be an effective host defense against *L. pneumophila* and that a vaccine that results only in antibody production against *L. pneumophila* may not be efficacious (5, 6).

Role of cell-mediated immunity. Cell-mediated immunity plays an important role in host defense against many intracellular parasites (3). In vitro studies have examined the potential role of cell-mediated immunity in Legionnaires disease by investigating (i) whether activated human mononuclear phagocytes have the capacity to inhibit *L. pneumophila* multiplication and (ii) whether patients with Legionnaires disease develop cell-mediated immunity to *L. pneumophila*.

Regarding the microbistatic capacity of activated mononuclear phagocytes, human monocytes activated by incubation with human lymphocytes in the presence of the mitogen concanavalin A strongly inhibit the intracellular multiplication of *L. pneumophila* (7). Under these conditions, both lymphocytes and concanavalin A are required to activate the monocytes (7).

Human monocytes and human alveolar macrophages activated by incubation with cell-free filtered supernatants of concanavalin A-stimulated mononuclear cell cultures (cytokines) inhibit *L. pneumophila* multiplication (7; Nash et al., 22nd ICAAC, abstr. no. 93). The degree of inhibition is proportional to the concentration of supernatant added (7; Nash et al., 22nd ICAAC, abstr. no. 93) (Fig. 4). With monocytes, the degree of inhibition is proportional to the length of time the monocytes are preincubated with supernatant (48 > 24 > 12 h), and monocytes treated with supernatant daily after infection are more inhibitory than monocytes treated before infection only.

Monocytes activated with antigen-induced mononuclear cell cytokines also inhibit *L. pneumophila* multiplication (see below).

Activated monocytes inhibit *L. pneumophila*

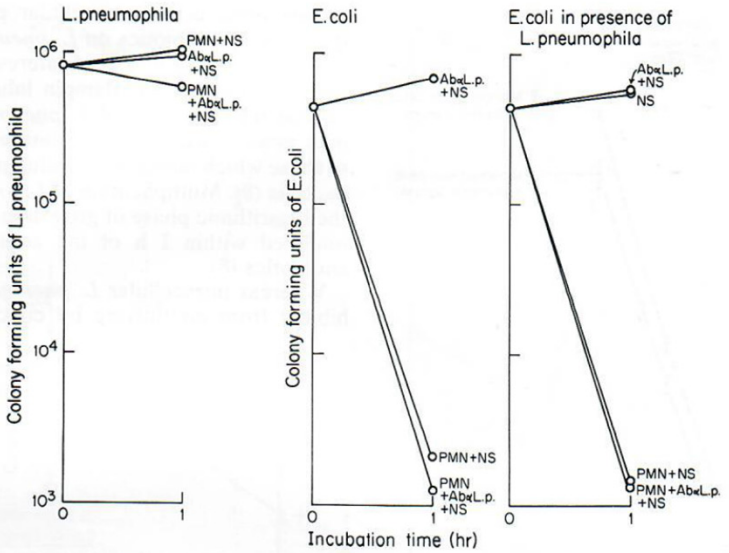

FIG. 2. *L. pneumophila* resists killing by polymorphonuclear leukocytes (PMN) under conditions in which polymorphonuclear leukocytes effectively kill a serum-resistant encapsulated strain of *E. coli*. Polymorphonuclear leukocytes (2.5×10^6) and 5×10^5 CFU each of *L. pneumophila*, *E. coli*, or both were incubated at 37°C for 1 h on a gyratory shaker in 0.9 ml of medium (final volume) containing 10% fresh normal human serum (NS) alone or 10% normal serum plus 8.5 agglutinating units of dialyzed rabbit anti-*L. pneumophila* antiserum (AbαL.p.) per ml. CFU of *E. coli* and *L. pneumophila* were determined initially and at the end of the incubation. (Reproduced from reference 5.)

multiplication in two ways. First, they phagocytize approximately 50% fewer bacteria than nonactivated monocytes, thereby restricting access of the bacteria to the intracellular milieu they require for multiplication (7). Second, they markedly slow the multiplication rate of those bacteria that are internalized; the doubling time is prolonged by greater than threefold (7).

Activated monocytes strongly inhibit *L. pneumophila* multiplication, but they do not kill *L. pneumophila* any better than nonactivated monocytes (7). As with nonactivated monocytes, activated monocytes kill *L. pneumophila* only in the presence of both specific antibody and complement, and even then they kill only a small proportion (<0.25 log) of an inoculum. In fact, activated monocytes consistently kill fewer bacteria than nonactivated monocytes, perhaps because they phagocytize less avidly (7).

Thus, inhibition of *L. pneumophila* multiplication is accomplished by activating the mononuclear phagocytes and not by coating the bacteria with specific antibody and complement. This indicates that cell-mediated immunity could play a major role in host defense against *L. pneumophila*.

Do patients with Legionnaires disease in fact develop cell-mediated immunity to *L. pneumophila*? In vitro studies indicate that they do. Mononuclear cells from patients recovered from Legionnaires disease respond to *L. pneumophila* antigens with both proliferation (lymphoproliferation), as measured by their capacity to incorporate [^{3}H]thymidine, and the production of monocyte-activating cytokines (2). Such cytokines have the capacity to activate normal monocytes such that they inhibit *L. pneumophila* intracellular multiplication, and the degree of inhibition is dose dependent (2). The response of patient mononuclear cells to *L. pneumophila* antigens as measured by both assays (lymphoproliferation and production of monocyte-activating cytokines) is specific in the sense that mononuclear cells from patients recovered from Legionnaires disease respond more strongly to *L. pneumophila* antigens than to *M. leprae* antigens, whereas mononuclear cells from patients with tuberculoid leprosy respond more strongly to *M. leprae* antigens than to *L. pneumophila* antigens (2). Furthermore, mononuclear cells from patients recovered from Legionnaires disease respond much more strongly to *L. pneumophila* antigens in both assays than do mononuclear cells of age- and sex-matched persons with no history or serological evidence of Legionnaires disease (2).

Thus, cell-mediated immunity develops in patients with Legionnaires disease. The capability of activated mononuclear phagocytes to inhibit *L. pneumophila* intracellular multiplication indi-

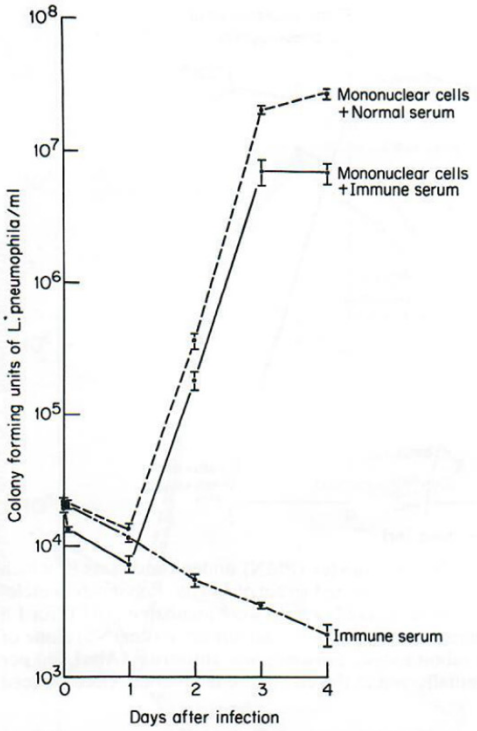

FIG. 3. Specific antibody fails to inhibit the multiplication of *L. pneumophila* in monocytes. *L. pneumophila* (2.5×10^4 CFU) was incubated at 37°C for 10 min in 33% fresh normal human serum or 33% fresh immune human serum. The bacteria were then incubated at 37°C in 5% CO_2–95% air with 5×10^6 mononuclear cells (or RPMI 1640 as control) in medium that contained a final concentration of 10% of the same type of serum to which the bacteria were initially exposed. The cultures were shaken for 1 h and incubated under stationary conditions thereafter for 4 days. CFU were determined at 0, 1, 24, 48, 72, and 96 h after the monocytes were infected. Each point represents the average for five replicate tubes ± standard error. (Reproduced from reference 6.)

cates that cell-mediated immunity plays a major role in host defense in Legionnaires disease.

Influence of antibiotics on intracellular *L. pneumophila*. Erythromycin and rifampin are the drugs of choice for the treatment of Legionnaires disease. Clinical experience and in vivo studies indicate that these drugs are efficacious in the treatment of Legionnaires disease. In vitro studies of *L. pneumophila* multiplying extracellularly on artificial medium show that such bacteria are highly susceptible to these antibiotics (8–10); erythromycin and rifampin inhibit the extracellular multiplication of *L. pneumophila* and kill these bacteria at relatively low concentrations of antibiotic (8–10). However, since *L. pneumophila* is an intracellular pathogen, the influence of antibiotics on *L. pneumophila* multiplying intracellularly is of interest.

Erythromycin and rifampin inhibit the intracellular multiplication of *L. pneumophila* in human monocytes, at concentrations comparable to those which inhibit extracellularly multiplying bacteria (8). Multiplication of *L. pneumophila* in the logarithmic phase of growth in monocytes is inhibited within 1 h of the addition of these antibiotics (8).

Whereas intracellular *L. pneumophila* are inhibited from multiplying by concentrations of

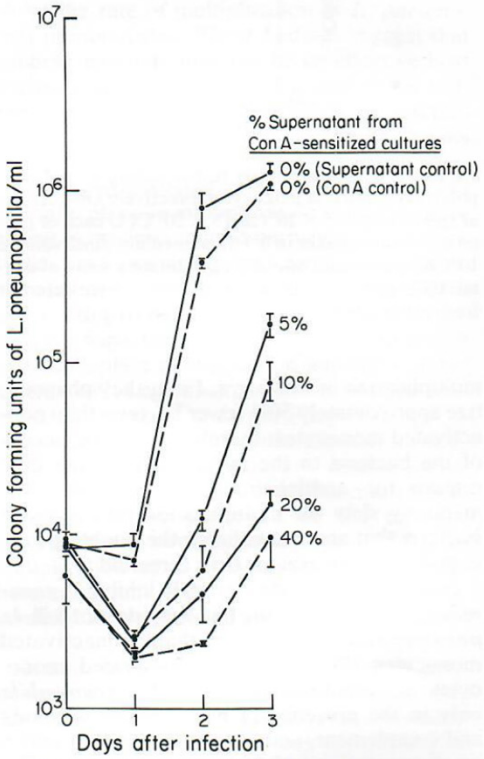

FIG. 4. Monocytes incubated with the supernatant of concanavalin A (ConA)-sensitized mononuclear cell cultures inhibit *L. pneumophila* multiplication. Monocytes in monolayer culture were incubated at 37°C in 5% CO_2–95% air in 2 ml of RPMI medium containing 15% fresh normal human serum and 0 to 40% cell-free supernatant from concanavalin A-sensitized mononuclear cell cultures. Control monolayers were incubated with supernatant from a mononuclear cell culture from which concanavalin A was omitted (Supernatant control) or with 15 μg of concanavalin A per ml (ConA control). After 24 h, *L. pneumophila* (2×10^4 CFU) was added to the cultures, and CFU in each culture were determined daily. Each point represents the average for three replicate petri dishes ± standard error. (Reproduced from reference 7.)

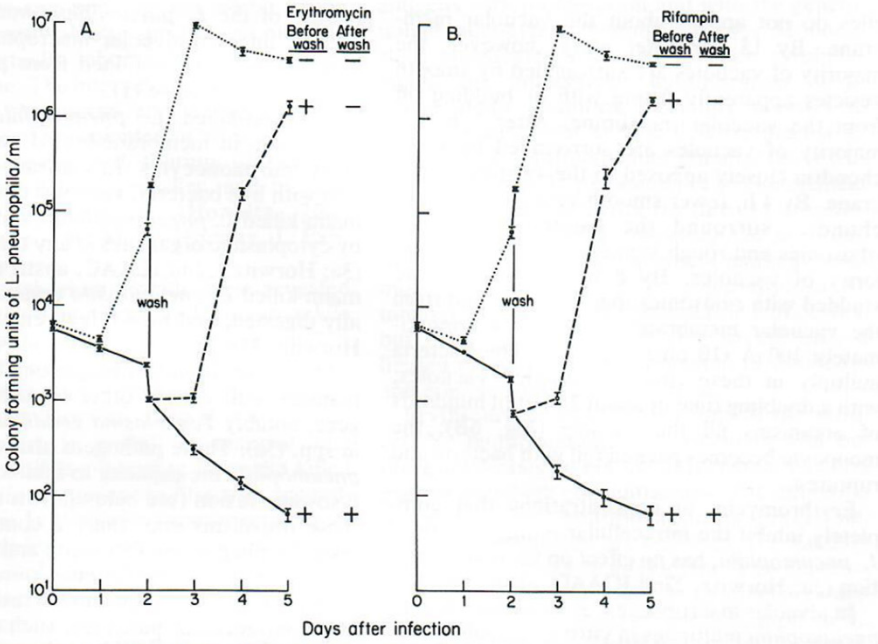

FIG. 5. *L. pneumophila* that have been inhibited from multiplying in monocytes by erythromycin and rifampin multiply after the antibiotics are removed. Mononuclear cells (5×10^6) in 2 ml of RPMI 1640 medium containing 15% fresh human serum were added to plastic tubes, infected with *L. pneumophila*, and incubated for 48 h without antibiotic or with 1.25 μg of erythromycin per ml (A) or 0.01 μg of rifampin per ml (B). After 48 h, the cultures were washed to remove antibiotics. Cultures that initially contained antibiotics were split into two groups; one group was incubated without antibiotic, and the other group was incubated with the same antibiotic as before washing. CFU of *L. pneumophila* in the medium were determined daily. Each point represents the mean for three replicate cultures ± standard error.

erythromycin and rifampin comparable to those that inhibit extracellular *L. pneumophila*, these bacteria exhibit markedly different susceptibilities to killing by these antibiotics. *L. pneumophila* organisms multiplying extracellularly are killed by concentrations of erythromycin and rifampin that are near the minimal inhibitory concentration; the minimal bactericidal concentration is 1 μg/ml for erythromycin and 0.009 μg/ml for rifampin (8). In contrast, *L. pneumophila* organisms multiplying intracellularly are resistant to killing by inhibitory concentrations of these antibiotics or by much higher concentrations equal to or greater than peak serum levels in humans, levels 12 times the minimal bactericidal concentration for erythromycin and 10^4 times the minimal bactericidal concentration for rifampin (8). By electron microscopy, intracellular bacteria that are inhibited from multiplying by antibiotics appear intact within membrane-bound vacuoles in the monocytes (8).

The inhibition of *L. pneumophila* multiplication in monocytes by erythromycin and rifampin is reversible (Fig. 5). When these antibiotics are removed from infected cultures after 2 days, *L. pneumophila* resumes multiplying (8).

These findings indicate that patients with Legionnaires disease under treatment with erythromycin and rifampin require host defenses to eliminate *L. pneumophila*. Viewed in this way, the role of antibiotics in Legionnaires disease may be to buy time for the patient, i.e., inhibit *L. pneumophila* multiplication long enough for the patient to develop an effective immune defense against *L. pneumophila*. These findings also suggest that patients with inadequate host defenses may suffer relapse after cessation of therapy.

Phagocytosis and formation of a novel phagosome. *L. pneumophila* enters phagocytes by a highly unusual process. Long phagocyte pseudopods coil around the bacterium as the bacterium is internalized (unpublished data). After phagocytosis, *L. pneumophila* forms a novel phagosome in mononuclear phagocytes that involves an unusual sequence of cytoplasmic events that take place over 4 to 8 h (3a; M. A. Horwitz, 22nd ICAAC, abstr. no. 94).

Immediately after entry, a vacuolar membrane surrounds the bacterium; cellular organ-

elles do not appear about the vacuolar membrane. By 15 min after entry, however, the majority of vacuoles are surrounded by smooth vesicles apparently fusing with or budding off from the vacuolar membrane. After 1 h, the majority of vacuoles are surrounded by mitochondria closely apposed to the vacuolar membrane. By 4 h, fewer smooth vesicles and mitochondria surround the vacuole, and now ribosomes and rough vesicles surround the majority of vacuoles. By 8 h, all vacuoles are studded with ribosomes that are separated from the vacuolar membrane by a gap of approximately 100 Å (10 nm) (Fig. 6A). The bacteria multiply in these ribosome-studded vacuoles, with a doubling time of about 2 h, until hundreds of organisms fill the vacuole (Fig. 6B); the monocyte becomes packed full with bacteria and ruptures.

Erythromycin, at concentrations that completely inhibit the intracellular multiplication of *L. pneumophila*, has no effect on vacuole formation (3a; Horwitz, 22nd ICAAC, abstr. no. 94).

In alveolar macrophages, as in monocytes, *L. pneumophila* multiplies in vitro in vacuoles studded with ribosomes (T. W. Nash, D. M. Libby, and M. A. Horwitz, unpublished data). This feature of the *L. pneumophila* vacuole is also seen in infected alveolar macrophages in lung biopsy specimens obtained from patients with Legionnaires disease (1).

Formalin-killed *L. pneumophila* organisms also reside in membrane-bound vacuoles after entry into monocytes. In contrast to the situation with live bacteria, vacuoles containing Formalin-killed *L. pneumophila* are not surrounded by cytoplasmic organelles at any time after entry (3a; Horwitz, 22nd ICAAC, abstr. no. 94). Formalin-killed *L. pneumophila* organisms are rapidly digested, and by 4 h few remain intact (3a; Horwitz, 22nd ICAAC, abstr. no. 94).

The *L. pneumophila* phagosome shares some features with certain other intracellular pathogens, notably *Toxoplasma gondii* and *Chlamydia* spp. (3a). These pathogens also share with *L. pneumophila* the capacity to inhibit phagosome-lysosome fusion (see below). This suggests that these organisms may share a common mechanism for phagosome formation and inhibition of phagosome-lysosome fusion.

Inhibition of phagosome-lysosome fusion. Some intracellular parasites, such as *T. gondii*, *M. tuberculosis*, and *Chlamydia psittaci*, inhibit fusion of phagosomes with lysosomes. Others,

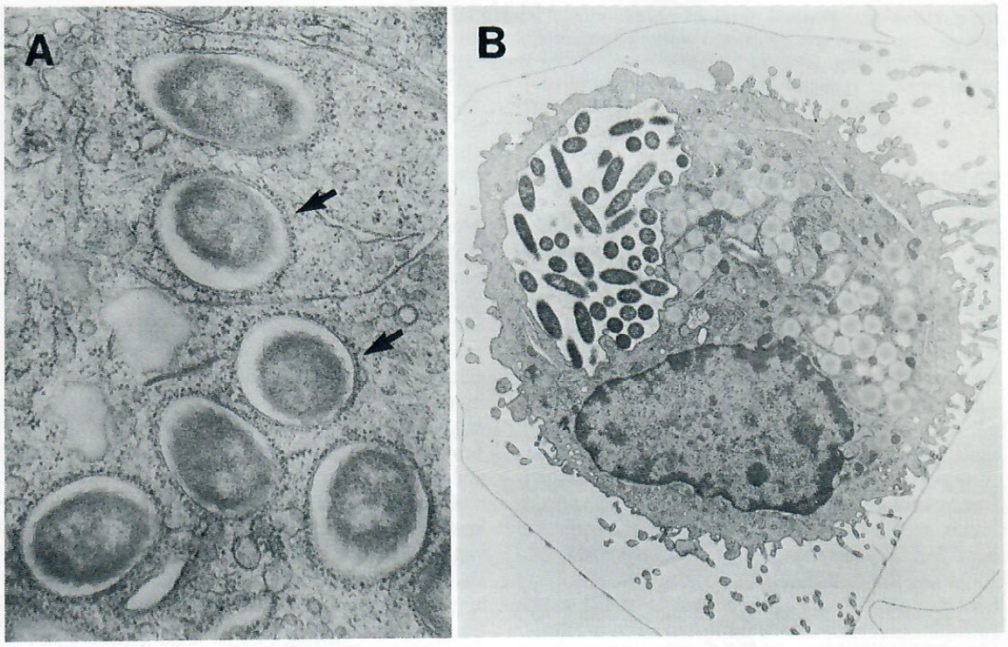

FIG. 6. Electron micrographs of human monocytes infected with *L. pneumophila*. (A) Six membrane-bound vacuoles, each containing a single *L. pneumophila* bacterium, are located in this portion of the monocyte cytoplasm. The vacuoles are studded with monocyte ribosomes (arrows) (×27,000). (B) Later in the course of infection, this monocyte contains a single large membrane-bound vacuole enclosing many *L. pneumophila* cells. Several bacteria appear to be in the process of dividing by binary fission. Although not apparent at this magnification, this vacuole is seen studded with ribosomes at higher magnification (×4,500).

such as *Leishmania* spp., do not inhibit fusion and are apparently able to survive and multiply within the normally inhospitable milieu of the phagolysosome. The interactions between the *L. pneumophila* phagosome and monocyte lysosomes have been investigated in vitro by prelabeling the lysosomes with thorium dioxide, an electron-opaque colloidal marker, and by acid phosphatase cytochemistry (Horwitz, 22nd ICAAC, abstr. no. 94; M. A. Horwitz, J. Exp. Med., in press).

Studies with thorium dioxide have revealed that phagosomes containing live *L. pneumophila* do not fuse with secondary lysosomes at 1 h after entry into monocytes, or at 4 or 8 h after entry, by which time the ribosome-studded vacuole has formed (Horwitz, 22nd ICAAC, abstr. no. 94; Horwitz, in press). In contrast, the majority of vacuoles containing Formalin-killed *L. pneumophila* organisms do fuse with secondary lysosomes. In the same experiments, vacuoles containing live *E. coli* and live *Streptococcus pneumoniae* also fuse with secondary lysosomes (Horwitz, in press).

Erythromycin, a potent inhibitor of bacterial protein synthesis, has no influence on fusion at concentrations which completely inhibit intracellular multiplication of *L. pneumophila*. However, coating the bacteria with antibody and complement or activating the monocytes promotes a modest degree of fusion of live *L. pneumophila* (Horwitz, in press).

Acid phosphatase cytochemistry has revealed that live *L. pneumophila* bacteria also do not fuse with primary lysosomes. In contrast to phagosomes containing live bacteria, the majority of phagosomes containing Formalin-killed *L. pneumophila* do fuse with lysosomes by acid phosphatase cytochemistry (Horwitz, in press).

Thus *L. pneumophila* inhibits phagosome-lysosome fusion. This capacity may be important to its survival in mononuclear phagocytes.

Conclusions. Humans are probably an incidental host for *L. pneumophila*, which likely acquired its capacity to multiply intracellularly in simpler beings such as amoebae or other protozoa. Like *L. pneumophila*, these unicellular organisms are ubiquitous in aquatic environments. *L. pneumophila* may utilize similar mechanisms to survive intracellularly in such disparate cells as amoebae and mononuclear phagocytes. These mechanisms remain to be elucidated, but the capacity of *L. pneumophila* to inhibit phagosome-lysosome fusion may be important.

Cell-mediated immunity appears to play a major role in host defense against *L. pneumophila*. Patients with Legionnaires disease develop cell-mediated immunity to *L. pneumophila*: their mononuclear cells respond to *L. pneumophila* antigens with proliferation and with the generation of monocyte-activating cytokines. Mononuclear phagocytes activated by such cytokines inhibit the intracellular multiplication of *L. pneumophila*. Humoral immunity appears to play a modest role in host defense against *L. pneumophila*. Antibody does not alter the resistance of virulent *L. pneumophila* to complement lysis, nor does it influence the rate of intracellular multiplication of *L. pneumophila* in monocytes. Antibody and complement markedly promote phagocytosis of *L. pneumophila* by monocytes and polymorphonuclear leukocytes, but these phagocytes are able to kill only a limited proportion of an inoculum of antibody- and complement-coated bacteria.

Erythromycin and rifampin inhibit the intracellular multiplication of *L. pneumophila*, but these antibiotics do not kill intracellular bacteria even at very high concentrations. The inhibition of intracellular multiplication is reversible. When the antibiotics are removed, the bacteria resume multiplying in monocytes. This suggests that the role of antibiotics in Legionnaires disease may be to suppress multiplication of *L. pneumophila* long enough for the host to develop an effective immune defense against the bacterium.

L. pneumophila is phagocytized by an unusual process and then forms a novel phagosome in mononuclear phagocytes that is studded with ribosomes. Formation of this phagosome entails a remarkable sequence of cytoplasmic events that involves smooth vesicles, mitochondria, and ribosomes. How *L. pneumophila* orchestrates this sequence of cytoplasmic events and the role of the ribosome-studded vacuole in intracellular survival remain to be determined. Phagosomes of certain other intracellular pathogens have features in common with the *L. pneumophila* phagosome, and these pathogens also share with *L. pneumophila* the capacity to inhibit phagosome-lysosome fusion. This suggests that a common mechanism may underlie the capacity of these organisms to form a specialized phagosome and their capacity to inhibit phagosome-lysosome fusion.

I am supported by the John A. Hartford Foundation, The American Cancer Society, and National Institutes of Health grants AI 17254 and CA 30198.

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