Treatment of Alveolar Macrophages with Cytochalasin D Inhibits Uptake and Subsequent Growth of *Legionella pneumophila*

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*Legionella pneumophila* multiplied rapidly in guinea pig and rat alveolar macrophages but failed to grow when phagocytic activity was inhibited by pretreatment with 0.5 or 1.0 μg of cytochalasin D per ml. Attachment was not inhibited by cytochalasin D. No extracellular multiplication occurred when *L. pneumophila* were in close proximity to viable functional macrophages or even when the bacteria were attached to plasma membranes of the macrophages. Nonopsonized *L. pneumophila* were avidly phagocytized by alveolar macrophages. When bacteria were centrifuged onto a cell pellet, more than 85% of the phagocytes contained one or more bacteria within 15 min. In contrast, under the same conditions only approximately 15% of the macrophages contained nonopsonized *Escherichia coli* or *Staphylococcus aureus*. Phagocytosis of *L. pneumophila* by untreated guinea pig macrophages occurred by extension of pseudopodia around the bacteria in a classical manner. The failure of the bacteria to actively penetrate the phagocyte suggests that their intracellular survival must not depend on avoidance of a phagosome but rather on an inhibition of or resistance to subsequent microbicidal functions of the macrophage.

*Legionella pneumophila* is a ubiquitous gram-negative bacterium which is an important cause of endemic and epidemic pneumonia (24, 38). As a facultative intracellular parasite, the bacterium has a close relationship to macrophages in vivo.

The mechanism by which *L. pneumophila* enters a cell has not been determined. Phagocytosis by macrophages may be enhanced by the addition of antibody and complement (18, 19), although they are not required.

Within a few hours after ingestion, the bacterium is located within a ribosome-studded phagosome (10, 20). The significance of this unusual phagosome has yet to be determined, although Horwitz has suggested (16) that it may be important for the intracellular survival of the bacterium. This unusual intracellular location led Katz and Hashemi to suggest that the bacterium enters a cell directly instead of through mechanisms that are controlled by the phagocyte (20). We tested this hypothesis by determining the ability of virulent *L. pneumophila* to enter macrophages in which phagocytosis had been inhibited by cytochalasin D. The cytochalasins have been extensively used for similar purposes (1, 3, 12, 13) and apparently have little effect on the attachment of relatively large particles, such as bacteria (6, 23, 25, 30, 33, 34).

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**MATERIALS AND METHODS**

**Bacteria.** Virulent *L. pneumophila* serogroup 1, Burlington 1 strain, was initially recovered from a patient with pneumonia. Spleen suspensions (10% in H2O) from infected guinea pigs were used as a source of *L. pneumophila*. *Escherichia coli* (O9:K29:H−) was a gift from Marcus Horwitz, Rockefeller University. *Staphylococcus aureus* ATCC 29213 was obtained from the American Type Culture Collection (Rockville, Md.). All bacteria were grown on buffered charcoal-yeast extract agar (31). *L. pneumophila* was passaged on agar no more than three times before use in an assay (37).

**Animals.** Specific-pathogen-free male Hartley strain guinea pigs weighing between 250 and 350 g and male Lewis rats weighing between 150 and 250 g were obtained from Charles River Breeding Laboratories, Inc., Kingston, N.Y., and Montreal, Canada. Animals were housed in filter top cages and were fed sterile water and food ad libitum.

**Macrophages.** Animals were anesthetized by intramuscular injection of Innovar-Vet (0.1 ml/100 g) (Pittman-Moore, Inc., Washington Crossing, N.J.) and were exsanguinated by cardiac puncture. The tracheas were exposed and cannulated, and the lungs were lavaged in situ a total of eight times with 7-ml boluses of phosphate-buffered saline (0.01 M PBS, pH 7.2). Cells were pelleted at 300 × g for 15 min and resuspended to approximately 5 × 10⁶ cells per ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing fetal calf serum (10%) and 5.8 μg of cefazolin per ml. The cell suspension was dispensed at 2 ml per well into plastic petri dishes (35 mm; Costar, Cambridge, Mass.). After 2 to 3 h of incubation at 37°C in 5% CO₂–95% air, the wells were washed once with Earle balanced salt solution (GIBCO). RPMI 1640 containing fetal calf serum (10%) without antibiotics was then added to the wells (2 ml per well). After an additional 18 to 24 h of incubation, the wells were washed once with Earle balanced salt solution, and fresh antibiotic-free medium was added. At this time, the monolayers were at least 99% macrophages, as assessed by Wright stain, and greater than 98% viable by Nigrosin exclusion (27).

**Cytochalasin D treatment.** Guinea pig and rat alveolar macrophages were treated with cytochalasin D (Sigma Chemical Co., St. Louis, Mo.) at various concentrations (Fig. 1) for 1 h before infection with virulent *L. pneumophila* at a ratio of two bacteria per macrophage. After 1 h, free bacteria were removed by washing with tissue culture medium, and fresh medium containing the original concentration of cytochalasin D was added to the wells. We found that the bacteria did not stick to the plastic tissue culture dish and could easily be removed by washing the macrophage monolayers two to three times with medium. The total
number of bacteria in the tissue culture wells was determined by quantitative plate count after the initial 1-h infection period and after an additional 48-h incubation at 37°C in 5% CO₂-air.

**Phagocytosis.** Macrophages (10⁶/ml) were added to siliconized conical centrifuge tubes (0.5 ml per tube). One set was treated with cytochalasin D (1 μg/ml). Both treated and nontreated macrophages were incubated for 1 h at 37°C. Bacteria at a concentration of 10⁶ CFU/ml were incubated with or without cytochalasin D (1 μg/ml) for 10 min at 37°C. Macrophages and bacteria were cooled to 4°C, and 0.5 ml of the bacterial suspension was added to the macrophages (approximately 100 bacteria per macrophage). The mixture was centrifuged at 220 × g for 10 min at 4°C followed by 10 min at 850 × g. The tubes were then warmed to 37°C for various times. Phagocytosis was stopped by gently adding 5 ml of cold Karnovsky fixative. After overnight fixation at 4°C, the tip of the centrifuge tube was removed, which allowed the cell pellet to be recovered intact. The pellet was fixed in osmium tetroxide and prepared for transmission electron microscopy. A Zeiss EM10 microscope was used to view and to photograph the macrophages. The minimum percent phagocytosis was estimated by counting the number of bacteria in 200 consecutive macrophages in one section.

**Growth in parabiotic tubes.** Parabiotic tubes (Bellco Glass Inc., Vineland, N.J.) were used to determine the ability of *L. pneumophila* to grow in close proximity to functional macrophages without physical contact. Macrophages (10⁵/ml; 8 ml total) and bacteria (1.5 × 10⁵ or 1.5 × 10⁶/ml; 8 ml total) were separated by a filter membrane (pore size, 0.22 μm). At 24-h intervals, 0.5 ml of the bacterial suspension was removed for quantitative plate counts. Controls consisted of macrophages (10⁵/ml; 4 ml total) and bacteria (1.8 × 10⁶/ml; 4 ml total) on the same side of the tube. Membrane permeability was tested by using crystal violet (molecular weight, 407 daltons) and blue dextran 200 (molecular weight, 2 × 10⁶ daltons; Pharmacia, Inc., Piscataway, N.J.).

**RESULTS**

**In vitro growth.** The effect of cytochalasin D on the in vitro growth of *L. pneumophila* in alveolar macrophage cultures was examined (Fig. 1). The growth of the bacteria was greatly reduced in both guinea pig and rat macrophage tissue cultures containing 0.5 μg or 1.0 μg of cytochalasin D per ml. A concentration of 0.5 μg of cytochalasin D per ml reduced the phagocytosis of serum-opsonized *S. aureus* by 73% (data not shown). The ability of cytochalasin D (1.0 μg/ml) to inhibit the phagocytosis of nonopsonized *L. pneumophila* was even more dramatic. No intracellular *L. pneumophila* were seen in these macrophages after a 15-min infection period (see Fig. 3F). When examined by scanning electron microscopy, *L. pneumophila* cells were attached to the

![FIG. 1. Growth of *L. pneumophila* Burlington 1 in guinea pig (A) and rat (B) alveolar macrophage cultures. Symbols: ○, no cytochalasin D; △, 0.25 μg of cytochalasin D per ml; ●, 0.5 μg of cytochalasin D per ml. Results represent the average of three determinations. Vertical lines depict one standard deviation.](http://iai.asm.org/)

![FIG. 2. Electron micrographs of guinea pig alveolar macrophages infected for 24 h with *L. pneumophila*. The medium contains 1.0 μg of cytochalasin D per ml (A) or no cytochalasin D (B). Bars, 1 μm.](http://iai.asm.org/)
plasma membrane of the treated macrophages. In transmission electron micrographs there were no intracellular bacteria in treated macrophages infected for 24 h with virulent L. pneumophila. In contrast, many bacteria were found in untreated phagocytes (Fig. 2). The viability of the macrophages over 48 h was not affected by treatment with 1.0 μg of cytochalasin per ml. Cytochalasin D at 1.0 μg/ml also had no effect on the viability or motility of L. pneumophila or on the growth rate of the bacteria in charcoal-treated, supplemented yeast extract broth (8). Dimethyl sulfoxide (0.1%), which was used to dissolve the cytochalasin D, did not alter the growth rate of the bacteria in guinea pig alveolar macrophage cultures.

Phagocytosis. Several L. pneumophila were closely associated with the plasma membranes of untreated macrophages warmed to 37°C for 30 s (Fig. 3A). Phagocytosis was not observed, and these macrophages could not be differentiated from those treated with cytochalasin D (Fig. 3E). Clear differences in the extent of phagocytosis by treated and nontreated macrophages were seen after 1.5 min at 37°C. Untreated macrophages were beginning to engulf L. pneumophila by extension of pseudopods (Fig. 3B). No phagocytosis by treated macrophages was observed (data not shown). Bacteria were engulfed after 5 min by untreated (Fig. 3C) but not by treated macrophages. By 15 min, approximately 90% of the untreated macrophages contained one or more bacteria and 35% contained more than five (Fig. 3D). In contrast, no bacteria were seen in macrophages treated with cytochalasin D (Fig. 3F).

Nonopsonized E. coli and S. aureus were ingested by extension of the plasma membrane around the bacterium, a process which was consistent with conventional phagocytosis (Fig. 4A and C) and which was inhibited by cytochalasin D (Fig. 4B and D). Neither of these species of
bacteria was phagocytized as avidly as was nonopsonized _L. pneumophila_. Only 15% of the macrophages contained _E. coli_ or _S. aureus_ after 15 min.

**Growth in parabiotic chambers.** No growth occurred when bacteria and macrophages were separated by a membrane (Fig. 5). The membrane was freely permeable to crystal violet. Blue dextran 200 also passed through the membrane, although not as well.

**DISCUSSION**

_L. pneumophila_ is considered a facultative intracellular pathogen because it will grow on artificial media (26) as well as in cell cultures (18, 21, 22). However, the evidence for exclusive intracellular growth is indirect. The presence of a large number of _L. pneumophila_ in some tissue culture cells seen in electron photomicrographs (21, 22) suggests that the bacteria grow intracellularly. In addition, Horwitz and Silverstein reported that _L. pneumophila_ would multiply only in the presence of intact macrophages in tissue cultures and would not grow in macrophage-conditioned media (18). Our results provide further substantiation that no extracellular growth occurs in cell cultures. We found that _L. pneumophila_ could not grow in macrophage cultures in which phagocytic activity was inhibited by cytochalasin D. Attachment was not inhibited by cytochalasin D, because similar numbers of bacteria were associated with treated and nontreated macrophages after 1 h. If macrophages release any essential growth factors into the medium, those factors should be available to the attached bacteria. It may be argued, however, that the cytoskeletal alterations in macrophages treated with cytochalasin D may interfere with the release of any nutrients. We tested this possibility by incubating _L. pneumophila_ in parabiotic chambers. The absence of growth or significant reduction of bacteria in these cultures over a period of 4 days is identical to the results obtained with disrupted macrophages and with macrophage-conditioned media (18; personal observation). These results suggest that _L. pneumophila_ will not grow in close association with macrophages. Apparently an intracellular location is essential for growth in macrophage tissue cultures. The fact that most of the viable _L. pneumophila_ in the lungs of infected guinea pigs are cell-associated within a few hours after infection (7) suggests that intracellular growth is also important in vivo.

We found little evidence that guinea pig or rat alveolar macrophages engulfed virulent _L. pneumophila_ Burlington 1 by coiling phagocytosis as described by Horwitz (17). Rarely, a bacterium was encompassed by more than one layer of pseudopodia (Fig. 3C, arrow). The bacteria were phagocytized predominately by classical processes (Fig. 3). There were several important differences, however, between the methods we used and those used by Horwitz. These differences, which include the procedure used to obtain the bacteria, the isolate of _L. pneumophila_, and the source of macrophages (17), may explain the variations between our results. Whatever the manner by which _L. pneumophila_ is engulfed, our evidence suggested that an intact, functional macrophage cytoskeleton was required. The bacteria do not actively penetrate a macrophage and do not passively enter a cell through hydrophobic interactions between the membranes of the bacterium and macrophage (35). The marked propensity for _L. pneumophila_ to enter a macrophage com-
FIG. 5. Growth of L. pneumophila in parabolic chambers. Symbols: ▲, 8 × 10^8 macrophages and 1.5 × 10^3 bacteria separated by a semipermeable membrane (pore size, 0.22 μm); ⦿, 8 × 10^5 macrophages and 1.5 × 10^5 bacteria separated by a membrane; ●, 1.5 × 10^5 bacteria incubated in tissue culture medium; ■, 8 × 10^5 macrophages and 1.5 × 10^5 bacteria incubated together. Results represent the average of two determinations.

pared to that of nonopsonized E. coli and S. aureus may indicate that the bacteria induce their uptake in a manner similar to that observed in Chlamydia spp., Rickettsia prowazeki, and Rickettsia tsutsugamushi (4, 5, 36). The initial location within apparently normal phagosomes (Fig. 3) suggested that the bacteria were able to inhibit or circumvent the microbial killing mechanisms that are triggered during phagocytosis. In fact, recent evidence indicates that L. pneumophila is capable of preventing phagosome-lysosome fusion (15) and may have the potential to interfere with the oxidative metabolism of macrophages (9). Both of these are important killing mechanisms of phagocytes (2, 11, 14, 28, 29, 32). L. pneumophila appears, therefore, to be well adapted for intracellular survival; the enhanced capability for triggering phagocytosis by macrophages is an important first step in assuring itself an intracellular location.

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LITERATURE CITED


