Intracellular Multiplication of *Legionella pneumophila* in Cultured Human Embryonic Lung Fibroblasts

MAE C. WONG,¹* EDWIN P. EWING, JR.,² CAREY S. CALLAWAY,² AND
WILLIAM L. PEACOCK, JR.¹

Bacteriology¹ and Pathology² Divisions, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333

Cell cultures of normal human embryonic lung fibroblasts were inoculated with two strains of *Legionella pneumophila*, the etiological agent of Legionnaires disease. Large numbers of intact organisms, including many dividing forms, were seen within fibroblasts by light and electron microscopy. Intracellular multiplication of organisms was demonstrated by the progressive increase in bacterial colony counts from plated extracts of infected fibroblast cultures in which extracellular multiplication had been eliminated by bactericidal concentrations of antibiotics. It is evident that *L. pneumophila* can thrive in an intracellular environment, and this property may be significant in the pathogenesis of Legionnaires disease.

Of the many species of bacteria recognized as human pathogens, only a few, such as *Mycobacterium tuberculosis* (12), *Brucella abortus*, and *Salmonella typhi* (9), are known to survive and multiply intracellularly. Dividing forms of *Legionella pneumophila*, the etiological agent of Legionnaires disease, have been demonstrated within human (1, 2, 8), cynomolgus monkey (11), and guinea pig (4) macrophages by electron microscopy, but it has not been clear whether this finding represented intracellular multiplication of the bacteria or phagocytosis of organisms already in the process of division. To determine if *L. pneumophila* can grow intracellularly, we inoculated cultures of human lung cells not specialized for phagocytosis and measured bacterial growth occurring exclusively within host cells.

MATERIALS AND METHODS

Bacterial strains. Two strains of *L. pneumophila* were used. Strain Philadelphia 1 (P1), grown in embryonated eggs, was obtained from K. H. Wong of the Center for Disease Control, Atlanta, Ga. Strain Knoxville 1 (K1), which had been passaged on agar media for over 2 years, was obtained from William B. Cherry (Center for Disease Control).

Tissue culture. Normal human embryonic lung fibroblasts (MRC-5) (10), passages 26 to 38, were provided by the tissue culture laboratory of the Center for Disease Control. The growth medium consisted of Eagle minimum essential medium (EMEM) in Earles balanced salt solution supplemented with 10% inactivated fetal calf serum, pH 7. Except where specified, no antibiotics were included in the growth medium.

Inoculations and maintenance of cultures. A 1-ml amount of P1 in sterile phosphate-buffered saline (PBS) (pH 7) containing approximately 1×10^5 colony-forming units (CFU) as determined by plating on charcoal-yeast extract (CYE) agar was inoculated onto

a monolayer consisting of about 2×10^7 MRC-5 cells in a 75-cm² tissue culture flask with 15 ml of the growth medium.

Strain K1 grown on CYE agar for 48 to 72 h in a candle extinction jar at 37°C was harvested in sterile PBS. A 1-ml amount containing 1×10^7 CFU was similarly inoculated onto a monolayer of MRC-5 cells. Cultures were incubated at 37°C and checked daily with an inverted phase-contrast microscope at a magnification of $\times 400$. For maintenance of bacterial infection in tissue culture, portions of 1 to 3 ml of infected cultures showing a cytopathic effect of 3+ to 4+ were transferred to fresh MRC-5 monolayers. Samples from infected cultures were periodically stained with fluorescein-conjugated antibodies to *L. pneumophila* for direct immunofluorescent examination (5). Samples were also inoculated onto CYE agar and Trypticase soy agar plates for viability and purity check (14).

Light microscopy. Infected and noninfected fibroblasts grown on cover slips were fixed for 1 h in 10% neutral buffered Formalin and examined with Gimenez staining (7) for presumptive demonstration of intracellular *L. pneumophila* (P. W. Greer, F. W. Chandler, and M. D. Hicklin, Am. J. Clin. Pathol., in press).

Electron microscopy. Cultured fibroblasts were fixed in situ with 2% (vol/vol) glutaraldehyde in 0.1 M *s*-collidine buffer for 1 h and then rinsed with 0.1 M *s*-collidine buffer. They were then scraped from the flask and centrifuged into a pellet at $350 \times g$. After postfixation for 45 min in 1% OsO₄ in *s*-collidine buffer (13), the pellets were stained in a saturated solution of uranyl acetate in 70% ethanol for 1 h, dehydrated through graded concentrations of ethanol, and embedded in Epon 812. Sections were cut on a Reichert OMV 2 ultramicrotome, mounted on bare copper grids, and examined with a Phillips 200 electron microscope.

Determination of intracellular multiplication. Bacteria from infected fibroblasts were plated on CYE agar. After incubation for 48 h at 37°C, bacteria were suspended in EMEM growth medium at a concentration of approximately 10^5 to 10^6 organisms per ml.

Each monolayer of lung fibroblasts grown in a Leighton tube (16 by 100 cm) was inoculated with 1 ml of the bacterial suspension. The infected cultures and uninoculated controls were incubated at 37°C for 13 to 16 h. The growth medium was removed, and the monolayers were washed three times with Hanks balanced salt solution. To each washed monolayer was added 3 ml of EMEM growth medium containing a combination of 100 U of penicillin G sodium (The Upjohn Co., Kalamazoo, Mich.) and 45 µg of streptomycin sulfate (Eli Lilly & Co., Indianapolis, Ind.) per ml to kill the extracellular organisms. Incubation was continued at 37°C. At various intervals, the infected cultures were assayed in duplicate for intracellular bacterial multiplication. After the antibiotic-containing growth medium was removed, the monolayers were washed once with PBS, scraped, and quantitatively transferred in PBS to separate centrifuge tubes. The cell suspensions were centrifuged at $3,000 \times g$ for 30 min. The cells were washed once with PBS, resuspended in 1 ml of PBS, and disrupted with a Ten Broeck tissue grinder to release intracellular organisms. Aliquots of 0.1 ml of serial 10-fold dilutions of the tissue extract were plated in duplicate on CYE agar for bacterial counts.

The number of bacteria associated with each infected cell culture immediately before antibiotics were added was determined. After three washings with PBS, infected monolayers were randomly selected for recovery of organisms with a Ten Broeck tissue grinder and plated on CYE agar as described above.

To determine the bactericidal effects of the antibiotic mixture on extracellular bacteria, aliquots of 1 ml of serial 10-fold dilutions of a bacterial suspension containing 3.5×10^6 to 6.5×10^6 organisms per ml were pipetted into sterile centrifuge tubes. EMEM growth medium, with the antibiotics, was added to each tube

to a final volume of 3 ml containing 100 U of penicillin G sodium and 45 µg of streptomycin sulfate per ml as in the tissue culture. The tubes were incubated at 37°C. At intervals, bacterial counts in the incubation mixtures were determined. Duplicate tubes were centrifuged at $4,500 \times g$ for 1 h. The pelleted bacterial organisms were washed once with PBS, resuspended in 0.1 ml of PBS, and plated on CYE agar.

RESULTS

Infection and maintenance of cultures. Observation of strain P1-infected cultures by phase-contrast microscopy at day 1 postinoculation revealed granulation and disorganization of the monolayer. At day 2 or 3 postinoculation, the entire cell sheet appeared very granulated and disorganized, with large numbers of fibroblasts in various degrees of distension (Fig. 1). Necrosis of the monolayer and a steady increase in the number of extracellular organisms were observed after day 3 or 4, and the pH of the medium shifted to the acidic side. When samples from infected cultures were plated onto CYE agar and Trypticase soy agar plates, bacterial colonies typical of *L. pneumophila* (6) appeared on the former, but no growth was observed on the latter; this indicated that any viable bacteria present were likely to be *L. pneumophila* and not contaminants (14). Direct immunofluorescent examination of the organisms from infected tissue culture and from the CYE agar plates showed 4+ fluorescent staining of organisms in both cases, confirming their identity as *L. pneumophila*.

FIG. 1. (A) Intracellular presence of *L. pneumophila* strain P1 in human embryonic lung fibroblasts. $\times 400$. (B) Normal human embryonic lung fibroblasts. $\times 400$.

Strain K1-infected cultures showed effects similar to those seen in P1-infected cultures, but there was an initial delay of at least 10 days before these changes appeared. However, subsequent transfers of 2 to 3 ml of these infected cultures to fresh fibroblast monolayers were followed by the characteristic changes within 2 or 3 days, as observed with strain P1. Both of these tissue culture-grown strains retained their ability to produce similar changes in the fibroblasts even after 10 passages on CYE agar.

Light microscopy. With the Gimenez stain, bacteria were visible within the cytoplasm of many fibroblasts on days 2 and 3 postinoculation, sometimes in such great numbers that the cell appeared as a distended, thin-walled sac full of organisms. Many intracellular bacteria showed the transverse central pinching characteristic of binary fission, and they were often aligned in closely packed columns parallel to the long axis of the host cell. Occasional chains and elongated forms were also observed.

Electron microscopy. The intracellular presence of intact bacteria with the previously described ultrastructure of *L. pneumophila* (3, 15) was confirmed by electron microscopy (Fig. 2). In lightly infected and better-preserved cells, the organisms appeared singly or in small groups within membrane-bound vacuoles. In more heavily infected cells, vacuoles containing organisms tended to become confluent, and vacuolar membranes were often indistinct or absent. Bacteria in various stages of binary fission were observed both within vacuoles and apparently free in the cytoplasm. Most infected cells retained their characteristic long, narrow configuration, but rounded cells reduced to essentially a plasma membrane packed with organisms were also observed. In the latter situation, the bacteria were markedly vacuolated.

Intracellular multiplication. After bactericidal concentrations of antibiotics were added, the number of bacteria in P1-infected fibroblast cultures increased by 100 times in 4.5 h, as determined by bacterial plate counts. The increase in bacterial counts corresponded to the increase in numbers of bacteria-laden rounded fibroblast cells. The counts then leveled off and declined as the pH of the growth medium shifted to the acid side; these changes were accompanied by lysis of cells, as seen by phase-contrast microscopy. A similar curve was obtained with strain K1, by using a schedule slightly different from that for strain P1 (Fig. 3).

The antibiotics in the growth medium were highly effective in killing extracellular *L. pneumophila* organisms. The total number of bacteria associated with each monolayer of fibroblasts in the culture medium before antibiotics were

added was approximately 10^4 to 10^5 , as determined by plating of extracts of the infected fibroblasts. Antibiotic treatment of bacterial suspensions of strains P1 (6.5×10^5 CFU per ml) and K1 (3.5×10^5 CFU per ml) in EMEM medium for 3 h resulted in complete killing of the bacteria (Table 1). When the respective bacterial concentrations were increased by 10 times, only 21 and 2 colonies were recovered from the suspensions.

DISCUSSION

The entry of *L. pneumophila* into human lung fibroblasts followed by intracellular multiplication was clearly demonstrated in this study. Numerous intact *L. pneumophila* bacteria, their identity confirmed by the direct fluorescent-antibody technique, were observed within lung cells by light and electron microscopy. The entry of bacteria into cells was presumed to occur by phagocytosis, since organisms often appeared within membrane-lined vacuoles which were interpreted as phagosomes by electron microscopy; actual entry was not observed. Phagosome membranes were usually indistinct or absent in cells containing large numbers of organisms, suggesting possible destruction of the membranes by the bacteria. Dividing bacteria were observed both within phagosomes and apparently free in the cytoplasm.

Multiplication of *L. pneumophila* within human lung fibroblasts was suggested by the frequency of dividing forms and the frequent arrangement of organisms in long parallel columns within the host cell cytoplasm. Intracellular multiplication was confirmed by the progressive increase in bacterial colony counts from plated extracts of infected fibroblast cultures in which extracellular multiplication had been eliminated by bactericidal concentrations of antibiotics. Complete killing of extracellular bacteria by the antibiotics was demonstrated with both strains. The actual magnitude of intracellular multiplication may have been larger than the bacterial counts reflected, since the antibiotics would have killed any bacteria released into the culture medium from lysed fibroblasts. In addition, a damaged plasma membrane of an infected fibroblast could have permitted the entry of antibiotics in concentrations high enough to kill intracellular bacteria. Infected monolayers treated with antibiotics were degraded much more slowly than those without antibiotics, suggesting interruption of a cell lysis-reinfection cycle or interference with intracellular growth.

Virulence of *L. pneumophila* may be intimately related to its ability to enter and grow within host cells. The intracellular growth could be significant in terms of host cell destruction

FIG. 2. Electron micrographs showing intracellular *L. pneumophila* at various stages of infection. (A) Dividing bacterium within phagosome of intact fibroblast ($\times 16,900$). (B) Coalescence of phagosomes containing bacteria ($\times 8,450$). (C) Numerous bacteria, including dividing forms, within host cell ($\times 3,970$). (D) Dead fibroblast essentially filled with prominently vacuolated bacteria ($\times 3,970$).

and interference with the host immune response. Although *L. pneumophila* has been observed chiefly in intraalveolar macrophages and neutrophils in fully developed human Legionnaires disease, the early host-pathogen interactions at the cellular level are unknown. We have shown that this organism can enter a type of human lung cell not specialized for phagocytosis, thrive intracellularly, and exert a cytopathic effect. The marked pulmonary pathology associated with fatal infections by this organism may be related

to initial destruction of lung cells by a similar mechanism. Subsequent bacterial multiplication within inflammatory cells may account for the inflammatory cell necrosis often prominent in the histopathology of Legionnaires disease, and release of lysosomal enzymes from lysed cells may intensify the pulmonary damage. Intracellular growth could thus account for the observed failure of the phagocyte component of the host defenses to successfully deal with infection by *L. pneumophila*.

FIG. 3. Intracellular growth of *L. pneumophila* strains P1 and K1 in cultured human embryonic lung fibroblasts. After fibroblast monolayers were incubated with *L. pneumophila* bacteria for 13 to 16 h and washed, a mixture of penicillin and streptomycin (PS) was added to the growth medium to kill extracellular bacteria. Intracellular growth of the bacteria was assayed by extraction of tissue cells and plating for colony counts at various intervals.

TABLE 1. Effect of a mixture of 100 U of penicillin G and 45 μ g of streptomycin (PS) per ml on *L. pneumophila*

<i>L. pneumophila</i> strain	Initial count ^a (no PS)	CFU after 3-h PS	CFU after 24- h PS
Philadelphia 1	6.5×10^7	190	5
	6.5×10^6	21	0
	6.5×10^5	0	0
	6.5×10^4	0	0
Knoxville 1	3.5×10^7	55	2
	3.5×10^6	2	0
	3.5×10^5	0	0
	3.5×10^4	0	0

^a Colony-forming units per milliliter as determined by plating on CYE agar.

ACKNOWLEDGMENTS

We thank Patricia Greer for assisting us with the Gimenez staining and Roger M. McKinney for his valuable advice and comments.

LITERATURE CITED

- Blackmon, J. A., M. D. Hicklin, F. W. Chandler. 1978. Legionnaires' disease: pathological and historical aspects of a "new" disease. *Arch. Pathol. Lab. Med.* **102**: 337-343.
- Chandler, F. W., J. A. Blackmon, M. D. Hicklin, R. M. Cole, and C. S. Callaway. 1979. Ultrastructure of the agent of Legionnaires' disease in the human lung. *Am. J. Clin. Pathol.* **71**:43-50.
- Chandler, F. W., R. M. Cole, M. D. Hicklin, J. A. Blackmon, and C. S. Callaway. 1979. Ultrastructure of the Legionnaires' disease bacterium. A study using transmission electron microscopy. *Ann. Intern. Med.* **90**:642-647.
- Chandler, F. W., J. E. McDade, M. D. Hicklin, J. A. Blackmon, B. M. Thomason, and E. P. Ewing, Jr. 1979. Pathologic findings in guinea pigs inoculated intraperitoneally with the Legionnaires' disease bacterium. *Ann. Intern. Med.* **90**:671-675.
- Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver. 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. *J. Clin. Microbiol.* **8**:329-338.
- Feeley, J. C., G. W. Gorman, and R. J. Gibson. 1979. Primary isolation media and methods. In Gilda L. Jones and G. Ann Hebert (ed.), "Legionnaires": the disease, the bacterium and methodology. U.S. Department of Health, Education, and Welfare Publication no. (CDC) 79-8375:78-84.
- Gimenez, D. F. 1964. Staining rickettsiae in yolk-sac culture. *Stain Technol.* **39**:135-140.
- Glavin, F. L., W. C. Winn, Jr., and J. E. Craighead. 1979. Ultrastructure of lung in Legionnaires' disease. *Ann. Intern. Med.* **90**:555-558.
- Holland, J. J., and M. J. Pickett. 1956. Intracellular behavior of *Brucella* variants in chick embryo cells in tissue culture. *Proc. Soc. Exp. Biol. Med.* **93**:476-479.
- Jacobs, J. P., C. M. Jones, and J. P. Baille. 1970. Characteristics of a human diploid cell designated MRC-5. *Nature (London)* **227**:168-170.
- Kishimoto, R. A., M. D. Castello, J. D. White, F. G. Shirey, V. G. McGann, E. W. Larson, and K. W. Hedlund. 1979. In vitro interaction between normal cynomolgus monkey alveolar macrophages and Legionnaires disease bacteria. *Infect. Immun.* **25**:761-763.
- Lurie, M. B. 1942. Studies on the mechanism of immunity in tuberculosis. The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals. *J. Exp. Med.* **75**:247-267.
- Lynn, J. A., J. H. Martin, and G. J. Race. 1966. Recent improvements of histological techniques for the combined light and electron microscopic examination of surgical specimens. *Am. J. Clin. Pathol.* **45**:704-713.
- McDade, J. E., C. C. Shepard, D. W. Fraser, et al. 1977. Legionnaires' disease: isolation of bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* **297**:1197-1203.
- Rogers, F. G., A. D. Macrae, and M. J. Lewis. 1978. Electron microscopy of the organism of Legionnaires' disease. *Nature (London)* **272**:825-826.