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Marcus A. Horwitz, Samuel C. Silverstein

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Research Article

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MARCUS A. HORWITZ and SAMUEL C. SILVERSTEIN,
Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

ABSTRACT We have studied the interaction between virulent egg yolk-grown *Legionella pneumophila* Philadelphia 1 and human blood monocytes in vitro. The leukocytes were cultured in antibiotic-free tissue culture medium supplemented with 15% autologous human serum.

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Several lines of evidence indicate that *L. pneumophila* grows within monocytes. (*a*) In the absence of leukocytes, *L. pneumophila* did not grow in tissue culture medium with or without serum even if the medium was conditioned by monocytes. (*b*) *L. pneumophila* did not grow in sonicated mononuclear cells. Lysis of these cells at various times during logarithmic growth of *L. pneumophila* was followed by cessation of bacterial multiplication. Growth resumed when intact mononuclear cells were added back to the culture. (3) In parabiotic chambers separated by 0.1-μm Nuclepore filters, *L. pneumophila* multiplied only when placed on the same side of the filter as mononuclear cells.

These findings indicate that *L. pneumophila* falls into a select category of bacterial pathogens that evade host defenses by parasitizing monocytes. It remains to be determined whether cell-mediated immunity plays a dominant role in host defense against *L. pneumophila* as it does against other intracellular pathogens.

INTRODUCTION

Legionnaires' disease entered our lexicon when it struck 221 people, mostly American Legionnaires, and claimed 34 lives in Philadelphia in July 1976 (1, 2). Since then, we have learned that the disease is newly recognized but not new (3, 4), and that it occurs worldwide in epidemic and endemic forms (4–8). It is apparently a major cause of pneumonia in the United States (9, 10) and responsible for a substantial proportion of fatal nosocomial pneumonia (11, 12). It affects all age groups, but particularly people over 30 yr, and men more often than women (8, 13). It has a predilection for people who smoke or abuse alcohol, travellers, construction workers, and immunocompromised patients (6, 10, 14–16), and it is probably spread by the airborne route (10). The case-fatality rate has been in the 15–20% range (10, 17).

The causative agent of Legionnaires' disease, *Legionella pneumophila* is an aerobic gram-negative bacterium with fastidious growth requirements. It was originally isolated in guinea pigs and embryonated hens' eggs by a procedure designed for the isolation of rickettsia (2) but it can now be grown on synthetic culture media (18).

Although much has been learned about the epidemiology and clinical features of Legionnaires' disease and the properties of the causative agent, the

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immunobiology of the disease and the roles of cellular and humoral immunity remain poorly understood. Central to an understanding of these immunological issues is the categorization of the Legionnaires' bacillus as an intracellular or extracellular parasite.

Certain features of Legionnaires' disease suggested to us that *L. pneumophila* might be a facultative intracellular pathogen. First, in lung specimens from patients with Legionnaires' disease, clusters of bacteria have been observed in alveolar macrophages (19, 20). Similar findings have been reported in the lungs of experimentally infected guinea pigs (21). From these observations, however, it cannot be determined whether the bacteria multiplied intracellularly or were phagocytosed after extracellular multiplication. Secondly, *L. pneumophila* was originally isolated by procedures designed for the isolation of rickettsia, which are obligate intracellular parasites. Growth on synthetic medium is fastidious.

We have examined the interaction of virulent egg yolk-grown *L. pneumophila* Philadelphia 1 and human blood monocytes in vitro under antibiotic-free conditions. We used egg yolk-grown organisms because of the evidence that agar-adapted bacteria lose their virulence for chicken embryos and guinea pigs (22). In this paper, we shall present our evidence that *L. pneumophila* multiplies intracellularly in human blood monocytes and thus should be categorized as a facultative intracellular parasite.

METHODS

Reagents. Potassium phosphate dibasic ($K_2HPO_4 \cdot 3H_2O$), potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic anhydrous (Na_2HPO_4), and sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), all analytical reagents (Mallinckdrodt, Inc., St. Louis, Mo.); L-cysteine hydrochloride $\cdot H_2O$, glycerol, and sodium chloride, all reagent grade (Fisher Scientific Co., Pittsburgh, Pa.); Bacto-agar and yeast extract (Difco Laboratories, Detroit, Mich.); activated charcoal acid washed with phosphoric and sulfuric acids (Sigma Chemical Co., St. Louis, Mo.); ferric pyrophosphate soluble (Center for Disease Control, Atlanta, Ga.).

Media. RPMI 1640 with L-glutamine (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.); egg yolk buffer (EYB)[1] consisted of 12 parts 0.15 M KH_2PO_4, 38 parts 0.15 M Na_2HPO_4, 50 parts 0.85% NaCl, pH 7.2 as described (23); 100 ml of EYB with 1% bovine serum albumin contained 0.3 ml of a 35% solution of bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.). No antibiotics were used in any medium in any of the experiments.

Serum. Venous blood was clotted in siliconized glass test tubes, stored for 1 h at room temperature and an additional 1 h at 4°C, and the normal serum separated and stored at −70°C until use as described (24). Autologous serum was used in all experiments. The serum was negative for anti-*L. pneumophila* antibody by the indirect immunofluorescence test (25).

Culture of L. pneumophila in embryonated hens' eggs. *L. pneumophila* from a single batch of eggs were used in all the experiments in this study. 6-d-old embryonated hens' eggs from chickens fed an antibiotic-free diet were injected with 0.4 ml of a 10^{-3} dilution in EYB of a 0.2% homogenate of spleen from a guinea pig that had died after intraperitoneal inoculation with *L. pneumophila* Philadelphia 1; the 0.2% splenic homogenate was obtained from the Center for Disease Control. Infected yolk sacs from embryos dying 4–8 d after infection were harvested as described (26), homogenized in 10 ml of EYB, flash-frozen in an alcohol/dry ice bath, and stored in 1-ml aliquots at −70°C. Cultures from each egg were tested for viability by culture on complete charcoal-yeast extract agar, and for the presence of contaminants by culture on CYE agar without cysteine, tryptic soy broth agar, and 5% sheep blood tryptic soy broth agars, none of which support the growth of *L. pneumophila*; all cultures were positive only on complete charcoal-yeast extract agar. Cultures from each egg were also treated for *L. pneumophila* by the direct fluorescent antibody assay (27) and by staining by the Gimenez method (26). After quick-thawing, these chicken embryo cultures contained $5–10 \times 10^6$ colony-forming units (CFU)/ml on modified charcoal-yeast extract agar.

Partial purification of chicken embryo cultures of L. pneumophila by differential centrifugation. 1-ml aliquots of chicken embryo cultures were quick-thawed, diluted in 10 ml EYB, and centrifuged at 45 *g* for 10 min at 4°C; the large debris was pelleted but *L. pneumophila* and egg yolk lipid remained in the supernate under these conditions. *L. pneumophila* were pelleted by centrifugation of the supernate at 1,800 *g* for 20 min at 4°C; most of the lipid remained with the supernate. The bacteria were washed twice more in RPMI by centrifugation at 1,800 *g* for 20 min at 4°C. These partially purified *L. pneumophila* were used in all experiments.

Modified charcoal-yeast extract agar (CYE). CYE agar in 100 × 15 mm bacteriologic petri dishes was prepared as described (18) except that a buffer consisting of 5.24 g/liter $K_2HPO_4 \cdot 3H_2O$ and 1.0 g/liter KH_2PO_4 was added to maintain pH at 6.9.

Determination of L. pneumophila CFU. Test samples were serially diluted in EYB with 1% bovine serum albumin and 100 μl of appropriate dilutions spread onto 100 × 15 mm CYE agar plates with a glass spreader. The plates were incubated in candle jars at 37°C for 96 h and colonies counted manually.

Human blood mononuclear cells. Heparinized blood was diluted 1:1 in 0.9% saline and the mononuclear cell fraction obtained by centrifugation at 800 *g* for 30 min at 24°C over a Ficoll-sodium diatrizoate solution (Ficoll-Paque, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as described (28). The layer containing the mononuclear cell fraction was removed, diluted 1:1 in RPMI and the mononuclear cells collected by centrifugation at 400 *g* for 10 min at 4°C. To remove most of the residual platelets, the mononuclear cells were washed two or three times in RPMI by centrifugation at 115 *g* for 10 min at 4°C. The cells were resuspended in RPMI, counted in a hemocytometer (Clay Adams Div., Becton, Dickinson & Co., Parsippany, N. J.) and adjusted to the required concentration. The cells were >99% viable by trypan blue exclusion. A sample of the cell suspension was cytocentrifuged and stained; the cell population contained ~1.5% polymorphonuclear leukocytes, 40% monocytes, and 58.5% lymphocytes.

Selection of adherent and nonadherent subpopulations of mononuclear cells. Mononuclear cells (5×10^6) in RPMI and 15% serum were incubated in 35-mm Falcon plastic petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) for 1 h at 37°C in 5% CO_2-95% air. The

[1] *Abbreviations used in this paper*: CFU, colony-forming units; CYE, modified charcoal yeast extract agar; EYB, egg yolk buffer.

adherent subpopulation (2.2×10^6 cells) consisted of that fraction of the mononuclear cells that adhered to the petri dish during this incubation and remained adherent after the supernate was removed and the petri dish was vigorously washed in RPMI (37°C). The nonadherent subpopulation (2.2×10^6 cells) consisted of that fraction of the same mononuclear cell population that did not adhere to petri dishes after three successive 1-h incubations (including the one used to select the adherent fraction) at 37°C in 5% CO_2-95% air. The number of adherent cells on each petri dish was determined by counting the number of cells within a calibrated area using an inverted phase contrast microscope. Cell counts from different sections of each petri dish were averaged.

Infection of mononuclear cells in suspension. In experiments in which monocytes were plated after infection (Fig. 1B, 2), *L. pneumophila* ($\sim 4 \times 10^7$ CFU) were mixed with 2×10^7 mononuclear cells (containing $\sim 8 \times 10^6$ monocytes for a CFU:monocyte ratio of 5:1) in 2 ml RPMI and 15% serum in 17 × 100 mm plastic tubes (2057, Falcon Labware); the tubes were gased with 5% CO_2-95% air mixture, sealed with Parafilm (American Can Co., Greenwich, Conn.), and shaken at 37°C for 30 min on a gyratory shaker at 200 rpm. 100 μl of this suspension was plated on 13 mm Diam glass coverslips for 1 h at 37°C in 5% CO_2-95% air; during this incubation, the monocytes adhered to and spread out on the coverslips. The coverslips (two or three per 35-mm petri dish) were then washed with RPMI (22°C) to remove nonadherent bacteria and leukocytes and the infected monolayers were incubated in 2 ml RPMI and 15% serum at 37°C in 5% CO_2-95% air.

In experiments in which monocytes were maintained in 17 × 100-mm plastic tubes after infection, various numbers of *L. pneumophila* and mononuclear cells at the concentrations indicated were incubated together for 1 h under the same conditions as above. Afterward, the Parafilm was removed and the loosely capped tubes were held at 37°C in 5% CO_2-95% air under stationary conditions.

Culture of L. pneumophila and mononuclear cells in parabiotic chambers. Six Karush-type parabiotic chambers (Bellco Glass, Inc., Vineland, N. J.) with 0.05- or 0.1-μm Nuclepore filters separating the two compartments of each chamber (Nuclepore Corp., Pleasanton, Calif.) were mounted on Bellco 3-set chamber holders. The junction of each chamber was sealed with a water-insoluble silicone lubricant (high vacuum grease, Dow Corning Corp., Midland, Mich.) and the opening to each compartment of each chamber was capped with sterile Swinnex 13-mm millipore filter holders (Millipore Corp., Bedford, Mass.) packed with cotton. The entire apparatus was sterilized by autoclaving, after which the junction connecting the two compartments of each chamber was wrapped with Parafilm as an additional precaution against leakage. 1.5×10^6 mononuclear cells in RPMI were placed in one compartment of each of the six chambers; 3×10^4 *L. pneumophila* in RPMI were placed in the compartment on the same side of the filter as the mononuclear cells in three chambers and in the compartment on the opposite side of the filter from the mononuclear cells in the other three chambers. Both compartments of each chamber were brought to a final vol of 1.2 ml and a final serum concentration of 15% with RPMI and serum.

To promote rapid passage of molecules from the compartment on one side of the filter to the compartment on the other side of the filter, the mounted chambers were attached to a Nutator (Clay-Adams Div.) and rotated continuously for the duration of the experiment. The entire apparatus was placed in a 37°C incubator with a 5% CO_2-95% air atmosphere. CFU in both compartments of each chamber were determined daily; these culture determinations showed that *L. pneumophila* did not cross the filter from one side of the chamber to the other. At the end of each experiment, patency of the six filters to small molecules and fluid was confirmed by placing colored dye in the compartment on one side of each chamber and observing its rapid appearance in the compartment on the other side.

Sonication of mononuclear cells and L. pneumophila. Leukocytes and/or *L. pneumophila* in tubes (held in an ice-water bath) were sonicated with a micro tip, and infected monocyte monolayers in petri dishes were sonicated with a 2.54 cm Diam high gain disrupter horn, attached to a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Sonication was performed under sterile conditions for 60 s continuously with the output control of the sonicator set at the 4 position. This amount of sonic energy lysed the leukocytes completely (as determined by phase-contrast microscopy) but did not reduce *L. pneumophila* CFU.

Direct fluorescent antibody staining. Infected monocytes in monolayers on coverslips were fixed in Diff-Quik fixative (Harleco, American Hospital Supply Corp., Gibbstown, N. J.), overlaid with 5–10 μl of fluorescein isothiocyanate-conjugated rabbit anti-*L. pneumophila* Knoxville 1 (group 1) antiserum, globulin fraction (provided by the Center for Disease Control), placed in a covered chamber, incubated for 20 min at 37°C, washed and incubated for 10 min at 22°C in phosphate-buffered saline (pH 7.6) (23), washed in water, air dried, mounted in buffered glycerol (23), and examined with a Zeiss fluorescent photomicroscope III (Carl Zeiss, Inc., New York).

Electron microscopy studies. Monocyte monolayer cultures were prepared by incubating 7×10^6 mononuclear cells for 1 h in 2 ml RPMI and 15% serum in 35-mm plastic petri dishes at 37°C in 5% CO_2-95% air, washing away nonadherent leukocytes, and incubating the adherent cells for an additional 24 h in RPMI and 15% serum. The cultures were washed with RPMI to remove nonadherent cells, and the adherent cells were infected by incubating them for 1 h at 37°C with 7×10^6 CFU *L. pneumophila* in 2 ml RPMI and 15% serum without shaking. The CFU:monocyte ratio was about 5:1 at this time. The monolayers were then washed with RPMI to remove noncell-associated bacteria, and incubated at 37°C in 2 ml RPMI and 15% serum. At daily intervals, the infected monolayers were fixed for electron microscopy with a solution consisting of 1% OsO_4 (two parts) and 2.5% glutaraldehyde (one part) in 0.1 M cacodylate buffer, pH 7.4, stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer, pH 6.3, dehydrated with ethanol, released from the surface of the petri dishes with propylene oxide, and embedded in Epon (Shell Chemical Co., Houston, Tex.) as described (29). The sections were stained with lead citrate (30) and uranyl acetate and examined with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.).

Safety precautions. All handling of infectious material, such as that involved in inoculating and harvesting embryonated hens' eggs, infecting and sonicating leukocytes, transferring and diluting suspensions of organisms, and plating organisms on agar was done in a biological safety cabinet.

RESULTS

L. pneumophila multiplies in the presence of monocytes. We incubated *L. pneumophila* with monocytes in monolayer culture and washed away noncell-adherent bacteria, as described in Fig. 1. We then followed the course of the interaction between bacteria

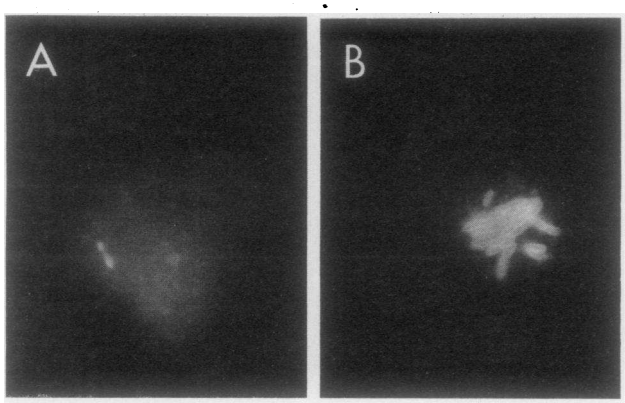

FIGURE 1 Course of *L. pneumophila* infection of monocytes as visualized by fluorescence microscopy. *L. pneumophila* were incubated with 24-h explanted monocytes on glass coverslips for 1 h at 37°C in 5% CO_2-95% air at a 5:1 ratio of CFU:monocytes and nonadherent bacteria were washed away. The monocytes were fixed, incubated with fluorescein-conjugated rabbit anti-*L. pneumophila* antibody, and examined by fluorescence microscopy immediately after the infection (A) and 4 d later at the peak of the infection (B). ×1200.

and monocytes by fluorescence microscopy and, in parallel cultures, by disrupting and assaying the cultures for CFU of *L. pneumophila*.

Few (<5%) of the monocytes examined immediately after infection had bound or ingested *L. pneumophila*, and these monocytes usually had only one cell-associated bacterium (Fig. 1A). Over the next few days, CFU of *L. pneumophila* increased 4–5 logs, and by fluorescence microscopy, the number of monocytes containing *L. pneumophila* increased in parallel with bacterial growth. At the peak of infection, monocytes appeared packed full with organisms (Fig. 1B). Maximum growth of *L. pneumophila* coincided with destruction of the monocytes.

To infect a larger proportion of monocytes and thereby increase the synchrony of infection, we incubated *L. pneumophila* with mononuclear cells in suspension on a gyratory shaker. We then plated the infected monocytes on coverslips and washed away nonadherent bacteria and leukocytes. By fluorescence microscopy, nearly all the monocytes had cell-associated bacteria; on the average, they had 10 *L. pneumophila*/monocyte. In monocyte cultures infected in this way, CFU of *L. pneumophila* increased 3 log within 24–48 h (Fig. 2). The maximal log phase doubling time of *L. pneumophila* in monocytes infected in suspension was 114 min (Fig. 2), a rate of growth that is twice as fast as that reported in specialized media used to grow this fastidious organism (31, 32).

Control experiments showed that in the absence of cells, *L. pneumophila* did not multiply in RPMI alone, in RPMI containing 15% fresh human serum, or in RPMI conditioned for 4 d by viable human

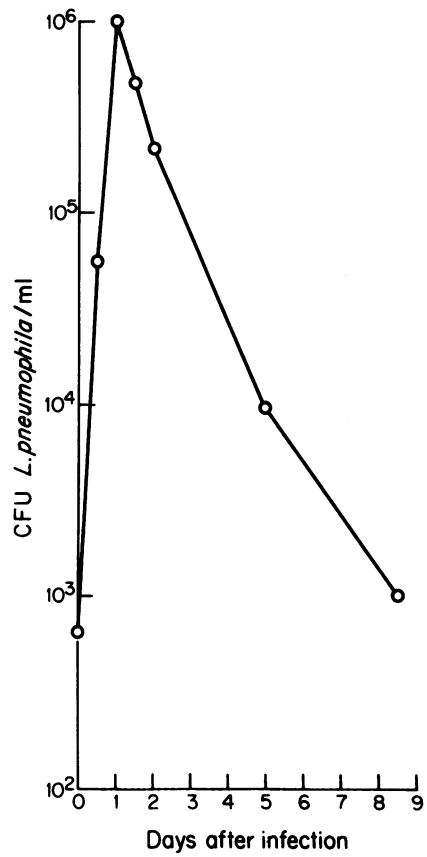

FIGURE 2 Multiplication of *L. pneumophila* in association with monocytes. *L. pneumophila* were incubated with mononuclear cells in suspension at a 5:1 ratio of CFU:monocytes at 37°C in 5% CO_2-95% air for 30 min on a gyratory shaker. The monocytes were then plated on glass coverslips, and non-adherent bacteria and leukocytes washed away. The monocyte monolayers were incubated in 2 ml RPMI and 15% serum and CFU in the medium determined at the indicated times. Zero time is immediately after the nonadherent bacteria and leukocytes were washed away.

monocytes. Fresh serum was present during *L. pneumophila* infection of monocytes and, subsequently, when *L. pneumophila* multiplied in these cultures; these data indicate that *L. pneumophila* are resistant to human serum. Other experiments, not reported here, confirmed that egg yolk-grown *L. pneumophila* are not killed by human serum even in the presence of high titer anti-*L. pneumophila* antiserum.

L. pneumophila multiplies in the presence of monocytes but not lymphocytes. The above experiments show that *L. pneumophila* multiplies in the presence of monocytes. To find out if the bacteria also multiply in the presence of lymphocytes, we compared the growth of *L. pneumophila* in the presence of the total mononuclear cell population with growth in the presence of monocytes and lymphocytes.

We added *L. pneumophila* (10^5 CFU) to petri dishes

containing (*a*) 2.2 or 5 × 10^6 mononuclear cells (~40% monocytes and 60% lymphocytes); (*b*) 2.2 × 10^6 monocytes selected by adherence from 5 × 10^6 mononuclear cells; and (*c*) 2.2 × 10^6 lymphocytes (nonadherent mononuclear cells) selected from the same mononuclear cell population as (*b*). We incubated the cells at 37°C in 5% CO_2-95% air on a gyratory shaker at 100 rpm for 3 h and under stationary conditions thereafter. We sampled the medium of each petri dish daily to determine the CFU of *L. pneumophila* in each petri dish.

L. pneumophila multiplied only in cell populations containing adherent cells (Fig. 3). It grew comparably in the total mononuclear cell population and the adherent (monocyte enriched) fraction of that population, but did not grow in the nonadherent (lymphocyte enriched) fraction. These findings indicate that *L. pneumophila* grows in monocytes but not lymphocytes.

L. pneumophila growth is proportional to the number of monocytes in the culture. The results of the experiment described in Fig. 3 suggested that the yield of *L. pneumophila* was proportional to the number of mononuclear cells in the culture. To investigate this further, we varied the number of mononuclear cells and the number of *L. pneumophila* independently of each other. Peak growth of *L. pneumophila* was independent of the number of bacteria used to initiate the infection (Fig. 4A) but was proportional to the concentration of mononuclear cells in the culture (Fig. 4B). Since monocytes, but not lymphocytes, support the growth of *L. pneumophila* (Fig. 3), these findings show that growth of this bacterium is proportional to the number of monocytes in the culture.

In experiments where *L. pneumophila* were maintained with infected mononuclear cells after infection (Figs. 4–6) there was an apparent 1-d lag in bacterial

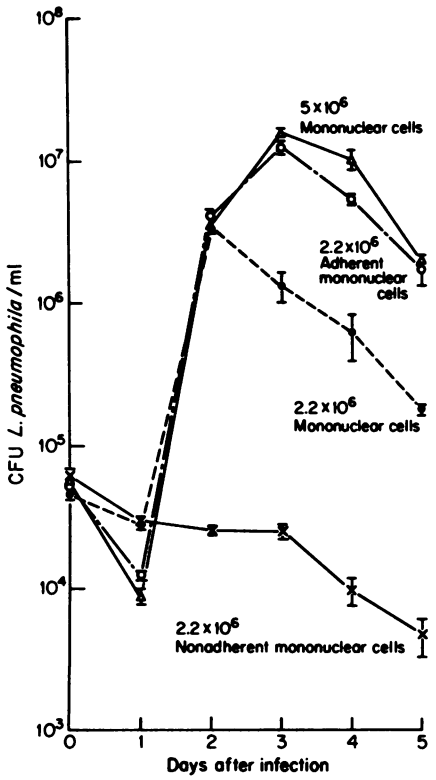

FIGURE 3 *L. pneumophila* multiplies in the presence of monocytes (adherent cells) but not lymphocytes (nonadherent cells). *L. pneumophila* (10^5 CFU) were added to petri dishes containing 2.2 ml RPMI, 15% serum, and (*a*) 2.2 or 5 × 10^6 mononuclear cells; (*b*) 2.2 × 10^6 adherent mononuclear cells selected from 5 × 10^6 mononuclear cells; (*c*) 2.2 × 10^6 nonadherent mononuclear cells selected from the same mononuclear cell population as in (*b*). Adherent and nonadherent mononuclear cells were selected as described in Methods. 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 ±SE.

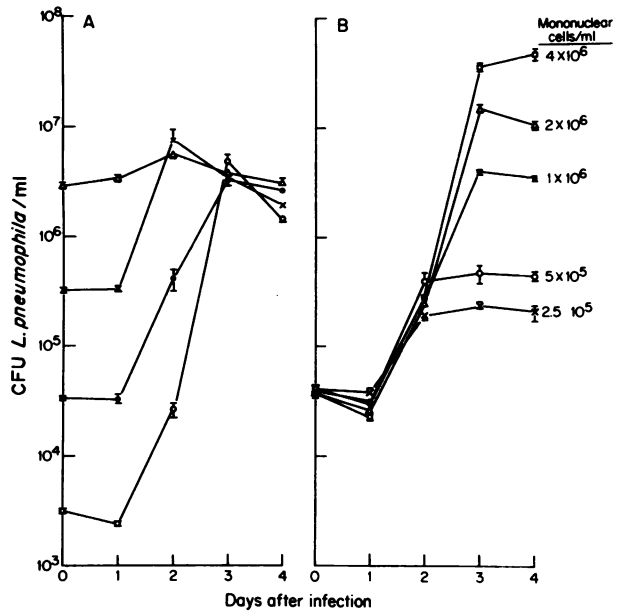

FIGURE 4 *L. pneumophila* growth is proportional to the number of monocytes in the culture. (A) *L. pneumophila* at concentrations ranging from 3 × 10^3 to 3 × 10^6 CFU/ml, as indicated, were incubated in tubes with a fixed number of mononuclear cells (10^6/ml) in 2 ml RPMI and 15% serum. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 1 h and under stationary conditions thereafter. CFU in each culture were determined daily. Each point represents the average for three replicate tubes ±SE. (B) *L. pneumophila* at a concentration of 3 × 10^4 CFU/ml were incubated in tubes with monocytes at concentrations ranging from 2.5 × 10^5 to 4 × 10^6 mononuclear cells/ml, as indicated, in 2 ml RPMI and 15% serum. The cultures were incubated under the same conditions as in A. CFU were determined daily. Each point represents the average for three replicate tubes ±SE.

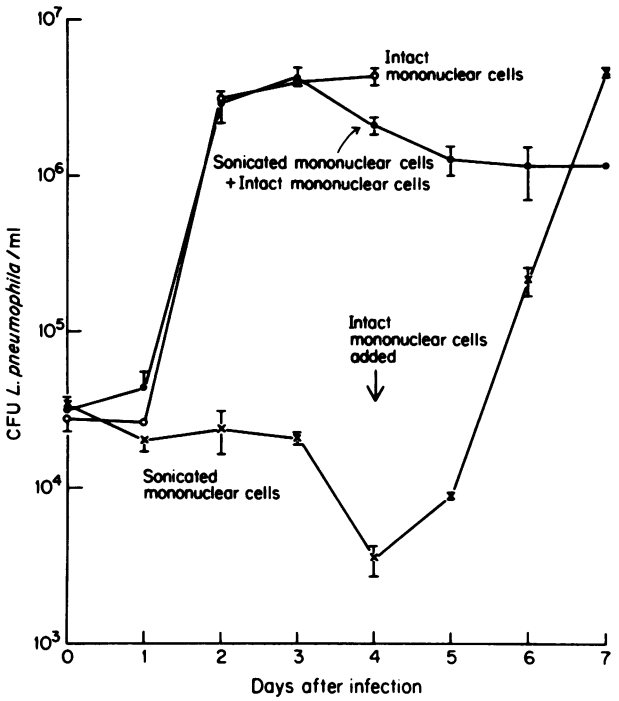

FIGURE 5 *L. pneumophila* multiplies only in the presence of intact leukocytes. *L. pneumophila* (~6 × 10^4 CFU) were added to tubes containing 2 ml RPMI, 15% serum, and (*a*) 3 × 10^6 intact mononuclear cells; (*b*) 3 × 10^6 sonicated mononuclear cells; or (*c*) 3 × 10^6 sonicated and 3 × 10^6 intact mononuclear cells. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 1 h and thereafter under stationary conditions until 4 d after the start of the experiment. At that time, 3 × 10^6 intact mononuclear cells were added to tubes that initially contained *L. pneumophila* and sonicated mononuclear cells. The cultures were shaken again for 1 h and then incubated again at 37°C under stationary conditions. CFU were determined daily. Each point represents the average for three replicate tubes ±SE.

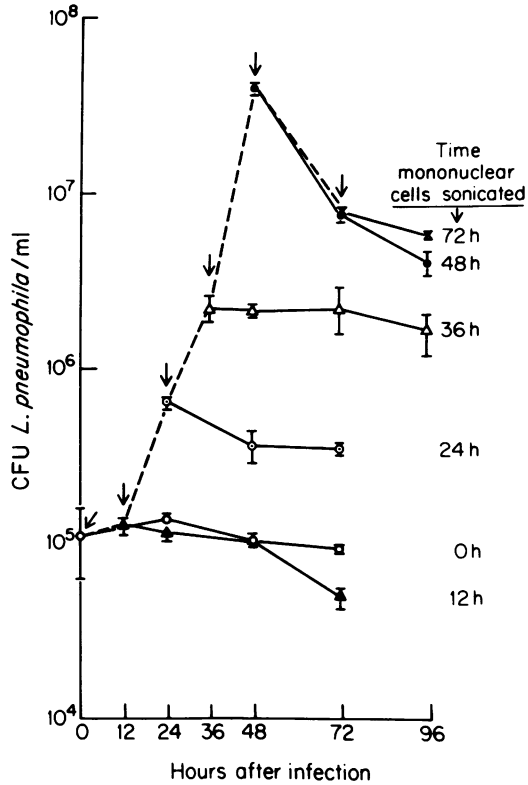

FIGURE 6 *L. pneumophila* requires intact monocytes for multiplication at every phase of its growth. *L. pneumophila* (~2 × 10^5 CFU) were added to tubes containing 3 × 10^6 intact mononuclear cells in 2 ml RPMI and 15% serum. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 1 h and under stationary conditions thereafter. Every 12 or 24 h, three replicate cultures were sonicated (arrow) and CFU determined thereafter. Each point represents the average for three replicate tubes ±SE.

multiplication; this lag was not seen when similarly infected monocytes were plated and bacteria that were not adherent to monocytes were washed away, as in Fig. 2. The probable reason for the lag was that, early after infection, a large number of nonmonocyte-adherent (and nonreplicating) bacteria were in the culture. These masked the contribution to total CFU of the relatively small number of *L. pneumophila* that had infected monocytes but had not yet multiplied to a high level.

L. pneumophila multiplies only in the presence of intact monocytes. To determine whether *L. pneumophila* requires intact viable monocytes for growth, we incubated the bacteria with intact mononuclear cells and/or an equivalent number of sonically disrupted mononuclear cells. *L. pneumophila* multiplied only in cultures containing intact mononuclear cells (Fig. 5). When we added intact mononuclear cells to cultures containing sonically disrupted mononuclear cells 4 d after the start of the experiment, the remaining live *L. pneumophila* then multiplied.

To see if *L. pneumophila* multiplies extracellularly after an initial exposure to monocytes or after entering the logarithmic phase of growth in monocytes, we infected replicate cultures of mononuclear cells and sonically disrupted them at various intervals thereafter. (A control experiment showed that the intensity of sonication used to disrupt the leukocytes did not inhibit or slow the growth of *L. pneumophila* in monocytes [data not shown].) The dashed line in Fig. 6 connects points representing CFU in each set of cultures immediately after sonication. It thus depicts the growth curve of *L. pneumophila* in intact leukocytes. The solid lines in Fig. 6 represent the CFU in each set of cultures after sonication.

Regardless of when in the growth phase we sonicated the leukocytes, growth of *L. pneumophila* ceased and

did not resume thereafter. Intact leukocytes were thus required for multiplication of *L. pneumophila* at every phase of its growth.

L. pneumophila multiplies only when in direct contact with monocytes. Using parabiotic chambers, we tested the possibility that *L. pneumophila* might require not intact monocytes themselves but some short-lived product produced by intact monocytes; such a labile product would presumably be deficient in medium conditioned by monocytes since *L. pneumophila* does not grow in such conditioned medium. We separated the two compartments of each chamber with a filter that contained pores large enough to allow ready passage of molecules but too small to allow passage of bacteria (0.3–0.9 μm Diam) or mononuclear cells from one compartment to the other. We placed mononuclear cells in one compartment of each of the six chambers and *L. pneumophila* in the compartment on the same side of the filter as the mononuclear cells in three chambers and in the compartment on the opposite side of the filter from the mononuclear cells in the other three chambers.

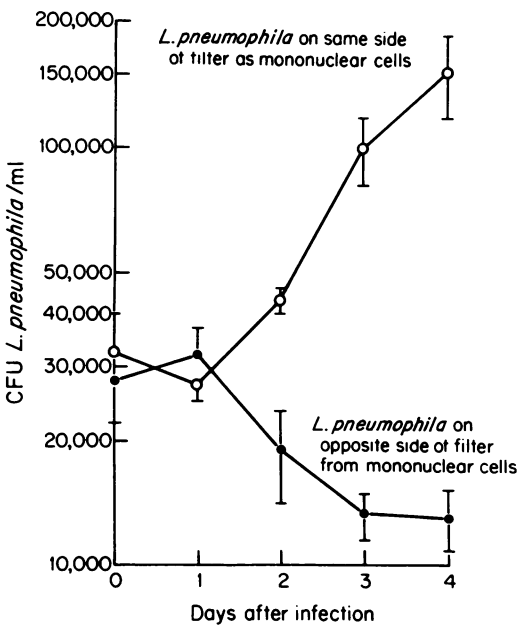

FIGURE 7 *L. pneumophila* multiplies only when in direct contact with monocytes. The two compartments of each of six parabiotic chambers were separated by 0.05-μm Nuclepore filters, as described in Methods. Mononuclear cells (1.5×10^6) were placed in one compartment of each of the six chambers. *L. pneumophila* (3×10^4) were placed in the same compartment as the mononuclear cells in three chambers and in the compartment on the opposite side of the filter from the mononuclear cells in the other three chambers. Both compartments of each parabiotic chamber contained a total volume of 1.2 ml RPMI and 15% serum. The chambers were rotated continuously at 37°C in 5% CO_2-95% air. CFU were determined daily. Each point represents the average for three chambers ±SE.

L. pneumophila multiplied only when placed on the same side of the filter as the mononuclear cells (Fig. 7), i.e., *L. pneumophila* multiplied only when in direct contact with intact mononuclear cells.

L. pneumophila multiplies within membrane-bound cytoplasmic vacuoles studded with structures resembling host cell ribosomes. We examined monocytes infected in a monolayer culture by electron microscopy. Immediately after infection, we observed no organisms in random ultrathin sections of adherent monocytes, but after 2 or 3 d, we observed monocytes that were full of bacteria (Fig. 8A). The bacteria were found inside the monocytes and not on the surface.

All intracellular organisms were in membrane-bound cytoplasmic vacuoles. In some monocytes, most of the vacuoles contained one or two bacteria (Fig. 8B). In other monocytes, a single large vacuole contained numerous *L. pneumophila* (as many as 100 in one section). The membranes of vacuoles containing one bacterium were sometimes closely apposed to the surface of the bacteria. The plasma and outer membranes of these bacteria stained less intensely than the vacuolar membrane, and could be distinguished from it (Fig. 8B). Some bacteria appeared fixed in the process of dividing.

Strikingly, the cytoplasmic sides of the membrane-bound vacuoles surrounding the *L. pneumophila* were studded with structures resembling monocyte ribosomes (Fig. 8B). These structures were a feature of vacuoles containing single or multiple numbers of organisms. Occasionally, the vacuolar membrane surrounding the *L. pneumophila* exhibited an outpocketing that was lined with ribosomelike structures.

DISCUSSION

Our experiments show that *L. pneumophila* multiplies intracellularly in human monocytes. Moreover, under tissue culture conditions, this bacterium multiplies only intracellularly. *L. pneumophila* can multiply extracellularly on complex media. Thus this bacterium is a facultative intracellular parasite. As such, it falls into a select category of bacterial pathogens that can evade host defenses by parasitizing monocytes. Other members of this category are *Listeria monocytogenes* and the mycobacteria including *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

Two groups of investigators, studying other cell types, have recently reported findings consistent with ours. Daisy et al. (33) have reported that agar-adapted *L. pneumophila* grow in the presence of MRC-5, HeLa, Hep 2, and McCoy cells in tissue culture medium and that *L. pneumophila* can be recognized within the

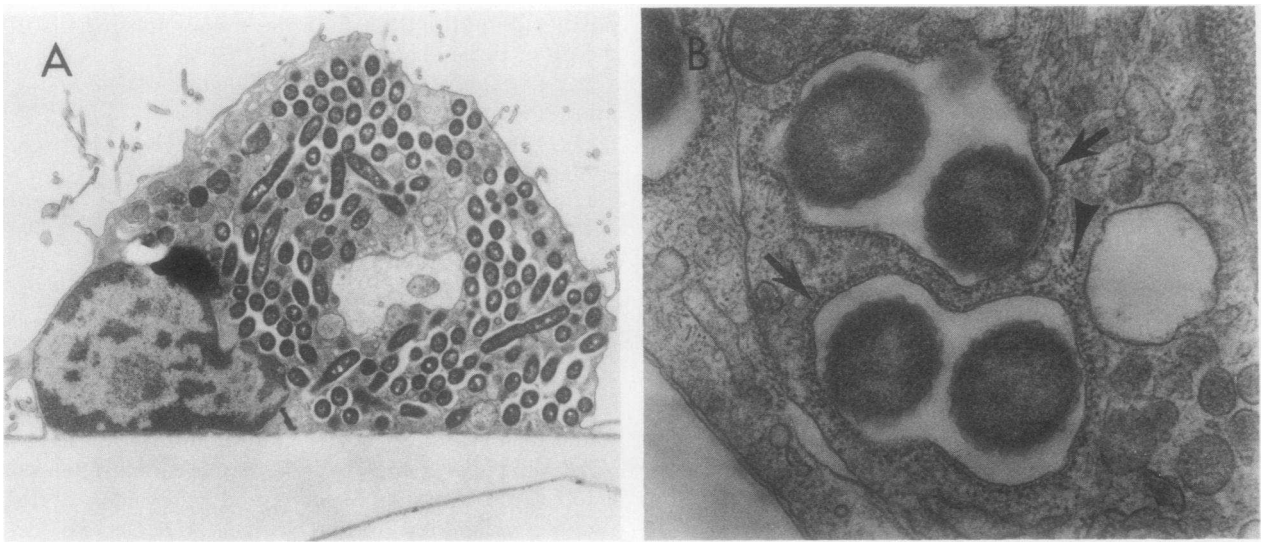

FIGURE 8 Electron micrographs of monocytes infected with *L. pneumophila*. Monocytes in a monolayer culture in plastic petri dishes were infected with *L. pneumophila* as in Fig. 1A, incubated in RPMI and 15% serum at 37°C for 2 d, fixed in glutaraldehyde and osmium, and processed for electron microscopy. (A) Monocyte heavily infected with *L. pneumophila*. ×5,400. (B) *L. pneumophila* in vacuoles studded with ribosomelike structures. Each vacuole contains two bacilli. The ribosomelike structures (arrows) appear to be separated from the cytoplasmic face of the vacuolar membrane by a gap of ~100 Å. Numerous 10-nm filaments in longitudinal and cross-section (arrowhead) are visible in the monocyte cytoplasm. Note that their diameter is smaller than that of the ribosomelike structures. ×32,400.

cytoplasm of infected MRC-5 cells by transmission electron microscopy. Kishimoto et al. (34) have reported that cynolmolgus monkey alveolar macrophages infected in vitro with agar-adapted *L. pneumophila* contain few organisms at 3 h but are packed full with organisms at 24 h after infection; their electron micrographs of heavily infected monkey alveolar macrophages bear a striking resemblance to our micrographs of heavily infected human monocytes.

We have no direct information regarding the mechanism of entry of *L. pneumophila* into the monocyte's cytoplasm. It seems likely that the bacteria are phagocytosed. After gaining entry, *L. pneumophila* resists monocyte microbicidal systems by as yet unknown mechanisms. Other intracellular parasites resist these systems by a variety of strategies. *M. tuberculosis* (35, 36) and *Toxoplasma gondii* (37) among others (38) interfere with host phagosomelysosome fusion. *Mycobacterium lepraemurium* (39), *M. tuberculosis* (40), *Leishmania mexicana* (41), and *Leishmania donovani* (42) are able to thrive within the normally inhospitable milieu of the phagolysosome. Whether phagosomes containing *L. pneumophila* fuse with monocyte lysosomes is also unknown.

L. pneumophila that have multiplied within human monocytes are found in membrane-bound cytoplasmic vacuoles. A remarkable feature of these vacuoles is that they are studded with structures resembling host cell ribosomes. This morphologic feature has also been noted on electron microscopic examination of leukocytes in human lung tissue specimens from patients with Legionnaires' disease (43). *L. pneumophila* is evidently unique among bacterial pathogens in promoting the formation of these vacuoles. The origin of these vacuoles and their role in *L. pneumophila* growth remain to be elucidated.

The roles of humoral and cellular immunity in host defense against *L. pneumophila* also remain to be determined. We have found that egg yolk-grown *L. pneumophila* are resistant to killing by complement even in the presence of anti-*L. pneumophila* antibody and are inefficiently killed by human polymorphonuclear leukocytes in the presence of anti-*L. pneumophila* antibody and serum. These results, viewed in conjunction with the increased incidence of *L. pneumophila* infections in immunosuppressed patients (14–16), suggest that cell-mediated immunity may play an important role in host defense against *L. pneumophila* as it does against other intracellular parasites.

Lymphokines produced by sensitized lymphocytes have been shown to confer on animal macrophages or human monocytes enhanced ability to resist or kill *M. tuberculosis* (44, 45), *L. monocytogenes* (46–49), *Leishmania enrietti* (50), *T. gondii* (51–56), and *Trypanosoma cruzi* (57, 58). Lymphokine-activated

macrophages may play a similar role in eradicating *L. pneumophila* in vivo.

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