CHARACTERIZATION OF AVIRULENT MUTANT *LEGIONELLA PNEUMOPHILA* THAT SURVIVE BUT DO NOT MULTIPLY WITHIN HUMAN MONOCYTES

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Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular bacterial pathogen that multiplies intracellularly in human monocytes and alveolar macrophages (1, 2). In addition to humans, the organism is highly virulent for guinea pigs and lethal to this animal by both the intraperitoneal and aerosol routes of inoculation (3–5).

Virulence of *L. pneumophila* is likely dependent upon several characteristics of this organism. First, *L. pneumophila* is resistant to the bactericidal effects of serum (6). Second, the bacterium is highly resistant to killing by monocytes, polymorphonuclear leukocytes, and alveolar macrophages (2, 6, 7). Third, the organism multiplies within mononuclear phagocytes including monocytes and alveolar macrophages (1, 2). This last characteristic of *L. pneumophila* is dependent upon its capacity to enter mononuclear phagocytes, which it does by a process termed coiling phagocytosis (8), and may be dependent upon its capacity to form a distinctive ribosome-lined replicative phagosome (9), to inhibit phagosome-lysosome fusion (10), and to inhibit phagosome acidification (11).

Mutants, particular avirulent mutants, can be powerful tools for analyzing virulence determinants. When passaged on suboptimal artificial media, *L. pneumophila* spontaneously converts to a mutant form avirulent for guinea pigs (12). Neither the functional nor genetic basis for this avirulent phenotype has been characterized. Theoretically, avirulent mutants would fall into two general phenotypic classes herein designated types I and II. Type I mutants would be those unable to survive extracellularly, e.g., complement-sensitive mutants. Type II mutants would be those unable to replicate in host mononuclear phagocytes. The latter class might include three functional subclasses of mutants. One subclass (type IIA) would be those unable to enter mononuclear phagocytes and thus gain entry into the intracellular milieu the organism requires to replicate. A second subclass of mutants (type IIB) would be those able to enter mononuclear phagocytes but unable to survive intracellularly, e.g., a mutant that has lost its capacity to resist an antimicrobial substance produced by the phagocyte. A third subclass of mutants (type IIC) would be those able to enter and survive within mononuclear phagocytes but unable to multiply intracellularly.

M. A. Horwitz is a Gordon McDonald Scholar at the University of California, Los Angeles and a recipient of a Faculty Research Award from the American Cancer Society.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/87/11/1310/19 $2.00
Volume 166 November 1987 1310–1328

The objective of the present study was to characterize a spontaneously occurring mutant of *L. pneumophila*. This study will show that this mutant is a type IIC mutant as defined above, i.e., the mutant is able to enter and survive within human monocytes, but it is unable to multiply intracellularly. In addition, this study will show that this mutant enters monocytes in the usual way, i.e., coiling phagocytosis, but that the mutant does not form the distinctive replicative phagosome characteristic of the wild-type nor inhibit phagosome-lysosome fusion. The mutant survives intracellularly in a phagolysosome.

Materials and Methods

Media. Egg yolk buffer (EYB),[1] with or without 1% BSA, PBS, and RPMI 1640 medium were prepared or obtained as described previously (1). Antibiotics were not added to media in any of the experiments.

Agar. Buffered charcoal yeast extract (CYE) agar was prepared as described (1). Mueller-Hinton agar supplemented with 1% hemoglobin and 2% Iso Vitalex was purchased from Baltimore Biological Laboratories, Cockeysville, MD.

Serum. Normal (nonimmune) human sera were obtained from adult donors not known to have had Legionnaires' disease as described (1). All sera had indirect fluorescent antibody (IFA) anti-*L. pneumophila* titers of <1:64. Immune human serum with an IFA anti-*L. pneumophila* titer of 1:65,536 or 1:512 were obtained from adult donors who had recently recovered from Legionnaires' disease.

Bacteria. Wild-type *L. pneumophila*, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, as described (1). The bacteria were passed one time only on CYE agar and stored at −70°C.

Mutant bacteria were selected by passaging wild-type *L. pneumophila* on supplemented Mueller-Hinton agar. The bacteria were batch passaged at 4-d intervals nine times. Then 44 colonies were selected and passaged individually an additional three times. The 44 mutants were then passaged on CYE agar, harvested, and stored at −70°C. All 44 mutants were identified as *L. pneumophila* by the direct fluorescent antibody assay using fluorescein-conjugated anti-*L. pneumophila* antiserum. All 44 mutants were tested for the presence of contaminants by culture on CYE agar without cysteine, tryptic soy broth agar, and 5% sheep blood tryptic soy broth agar, none of which support growth of *L. pneumophila*; none of the cultures grew on these media.

Escherichia coli K12, strain MC4100, is a complement-sensitive rough unencapsulated strain without an O antigen on its LPS (13). *E. coli* serotype 09:K29$^-$:H$^-$ (*E. coli* K$^-$), an unencapsulated mutant derived from strain Bi 161-42, is a smooth bacterium with an O antigen on its LPS (14).

Human Blood Mononuclear Cells. Blood mononuclear cells were obtained from normal adult donors not known to have previously had Legionnaires' disease and with an IFA anti-*L. pneumophila* titer of <1:64 as described (1).

Bacterial Intracellular Multiplication Assay. Freshly explanted monocytes were purified and cultured as a monolayer in 16-mm diameter flat-bottomed wells in 0.5 ml of RPMI 1640 medium containing 20% fresh normal human serum as described (13). Then, wild-type or mutant *L. pneumophila* were added to the cultures. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker (100 rpm) for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily after infection as described (1).

Serum Bactericidal Activity. Wild-type *L. pneumophila*, mutant *L. pneumophila*, and *E. coli* K12 (a serum-sensitive control) were suspended in PBS containing 0, 10, 25, or 50% fresh normal human serum and 0 or 20% heat-inactivated human immune serum with an

[1] *Abbreviations used in this paper:* AYE broth, albumin yeast extract broth; CYE agar, buffered charcoal yeast extract agar; EYB, egg yolk buffer; EYB-BSA, egg yolk buffer containing 1% BSA; IFA, indirect fluorescent antibody assay; YE, yeast extract broth.

anti-*L. pneumophila* serogroup 1 IFA titer of 1:65,536 or 1:512. The bacteria were incubated in plastic test tubes in a total volume of 0.5 ml at 37°C for 0, 1, or 24 h. At the end of the incubation, the contents of each tube were serially diluted in EYB-BSA, and assayed for CFU of *L. pneumophila* on CYE agar or CFU of *E. coli* on blood agar as described (6).

Bacterial Binding Assay. Wild-type or mutant *L. pneumophila* (5×10^7 bacterial particles/ml) were incubated in plastic test tubes with mononuclear cells (2×10^7/ml) in RPMI and 20% heat-inactivated normal serum at 37°C in 5% CO_2-95% air on a gyratory shaker at 200 rpm for 30 min. After the incubation, 100-μl aliquots of the suspension were placed on 13-mm diameter glass cover slips and the preparation was incubated for 1.5 h at 37°C in 5% CO_2–95% air to allow the monocytes to adhere. The coverslips were washed three times in RPMI, and the cells were fixed and stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum as described (1), and examined by fluorescence microscopy. 200 consecutive monocytes were examined and the percentage of monocytes with cell-associated bacteria and the mean number of bacteria/monocyte were determined.

Bacterial Intracellular Survival Assay. Intracellular survival was examined by two assays. In the first assay, wild-type or mutant bacteria (10^2–10^3 CFU/ml) were mixed in a conical centrifuge tube with mononuclear cells (10^6/ml) at 4°C in RPMI containing 10% heat-inactivated serum in a total volume of 0.5 ml. The cell preparation was centrifuged at 4°C, first at 220 *g* for 10 min and then at 850 *g* for 20 min, so that the bacteria and leukocytes were pelleted together in the cone of the centrifuge tube. The centrifuge tubes were placed in a 37°C water bath and the bacteria and leukocytes were incubated together in the cone of the tubes for 0 or 2 h. Under these conditions, bacteria are rapidly internalized (8); this was confirmed for mutant *L. pneumophila* by electron microscopy (see below). The cells were then resuspended and sonicated for 10 s continuously with a microtip attached to a sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) under sterile conditions with the output control of the sonicator set at the three position; this amount of sonic energy lysed the mononuclear cells completely but did not reduce bacterial CFU. CFU in replicate cultures were then determined.

In the second assay, wild-type *L. pneumophila* (10^6 CFU/ml), Mutant 25C (10^6 CFU/ml), or Mutant 25D (10^7 CFU/ml) were added to monocytes that had been cultured 1 d in monolayer culture in RPMI containing 20% fresh normal serum. The cultures were incubated at 37°C for 30 min on a gyratory shaker at 100 rpm and washed four times with RPMI to remove extracellular bacteria. The infected monocytes were resuspended in RPMI containing 20% serum, incubated for 0, 4, or 24 h and sonicated for 5 s with a 0.5-inch diameter disruptor horn with the output control of the sonicator set at the 2.5 position; under these conditions of sonication, CFU are not reduced. CFU in triplicate cultures were determined.

Phagocytosis Assay. Phagocytosis of wild-type and mutant *L. pneumophila* was studied by electron microscopy as described (8). Briefly, monocytes and bacteria were mixed and pelleted together at 4°C in the cone of a centrifuge tube, rapidly warmed to 37°C for a few minutes to allow phagocytosis to proceed, and fixed for electron microscopy.

Phagosome Formation Assay. The interaction of phagosomes of wild-type and mutant *L. pneumophila* with monocyte organelles was studied as described (9) or by a modification that simplified embedding and allowed the experiments to be conducted with much fewer monocytes that were cultured on plastic cover slips. Briefly, monocytes were cultured for 1 d as a monolayer in 35-mm diameter plastic petri dishes or at the center of 14-mm diameter toluene-resistant plastic cover slips (Wako Chemical Co., Dallas, TX) placed in 16-mm diameter tissue culture wells (14). The monocytes were infected with wild-type or mutant *L. pneumophila*, washed vigorously to remove non–monocyte-associated bacteria, incubated for 1, 2, 6, or 24 h, and fixed for electron microscopy. Monocytes in plastic petri dishes were released from the surface of the dishes with propylene oxide and embedded as described (9). Monocytes on plastic cover slips were embedded while on the cover slip, and the cover slips were peeled away from the basal surface of the monocytes before sectioning. The sections were stained with lead citrate and uranyl acetate as

described (9) and examined with a JEOL JEM-100 CX electron microscope (JEOL, Ltd., Tokyo, Japan). In each experiment, at least 20 phagosomes containing wild-type or mutant *L. pneumophila* were studied at each time point. Phagosomes were examined for the presence of smooth vesicles, mitochondria, and ribosomes closely apposed to the external phagosomal membrane.

Phagosome-Lysosome Fusion Assay. Fusion of phagosomes containing wild-type or mutant *L. pneumophila* with monocyte lysosomes prelabeled with the electron-opaque marker thorium dioxide was studied by electron microscopy as described (10). Briefly, monocytes in monolayer culture (in petri dishes or on plastic cover slips as in the phagosome formation assay) were incubated at 37°C overnight with thorium dioxide, washed vigorously to remove unbound thorium dioxide, and incubated an additional 3 h to allow monocytes to internalize thorium dioxide absorbed on the cell surface and to concentrate the marker in lysosomes. The monocytes were then infected with live or formalin-killed, wild-type, or mutant *L. pneumophila*, washed to remove non–monocyte-associated bacteria, incubated 1, 6, or 24 h, and fixed and processed for electron microscopy. In each experiment, at least 20 phagosomes containing wild-type or mutant *L. pneumophila* were studied at each time point. Phagosomes were examined for the presence of thorium dioxide, indicating that fusion had taken place with lysosomes.

Protein Profile of Whole Bacteria. Wild-type and mutant *L. pneumophila* were suspended in 1 ml EYB to an OD of 0.8 at 540 nm, as measured in a Coleman 44 model spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), twice pelleted by centrifugation, and washed in 50 mm Tris-HCl buffer, pH 7.4, resuspended in 200 μl of SDS-PAGE sample buffer containing 125 mM Tris-HCl, 20% glycerol (Fisher Scientific Co., Pittsburgh, PA), 4% SDS (BDH Chemicals Ltd., Poole, England), 4 mM EDTA disodium salt (Sigma Chemical Co., St. Louis, MO), 10% 2-ME (Sigma Chemical Co.), and 0.01% bromophenol blue (Sigma Chemical Co.), pH 6.8, and heated at 100°C for 5 min. Proteins in the sample buffer were analyzed by SDS-PAGE (reagents from BDH Chemicals Ltd.) performed according to Laemmli (17) as modified by Ames (18). The separating gel contained 12.5% acrylamide and 0.33% *N,N'*-methylene bis-acrylamide. 50 μl of each preparation were loaded onto the gel. Molecular weight standards were obtained from Sigma Chemical Co. After electrophoresis, the gels were fixed in 10% acetic acid, 25% isopropanol, and stained for proteins with 0.2% Coomassie Brilliant Blue R-250 (Sigma Chemical Co.) in fixing solution.

Secretory Proteins. Secretory proteins of wild-type and mutant *L. pneumophila* were studied by radiolabeling the proteins with [^{35}S]-L-cysteine, subjecting the proteins to SDS-PAGE, and revealing the radiolabeled polypeptides autoradiographically. Bacteria were grown in yeast extract (YE) broth, equivalent to AYE broth without albumin, at 37°C to an OD of 0.5–1.0, pelleted by centrifugation, resuspended in L-cysteine–deficient YE broth (containing 0.001% L-cysteine), and grown to an OD of 0.6. Then 0.1 mCi [^{35}S]-cysteine (New England Nuclear, Boston, MA) was added per milliliter of bacterial suspension and the bacteria were incubated in a test tube for 20 min at 37°C at 120 rpm. The bacteria were pelleted by centrifugation at 15,600 *g* for 10 min at 4°C in an Eppendorf centrifuge, and the supernatant was filtered through 0.2-μm filters (Millipore, Bedford, MA) to remove any residual membranes. Previous experiments had demonstrated that this procedure removes all residual bacterial membranes and that ultracentrifugation of the preparation is unnecessary. Proteins were precipitated by adding 200 μl of 60% TCA (Fisher Scientific Co.) per milliliter of supernatant fluid, vortexing immediately, and incubating at 4°C overnight. The protein precipitate in each milliliter of fluid was collected by centrifugation at 15,600 *g* for 20 min at 4°C, resuspended in 1 ml cold 50 mM sodium acetate in ethanol, collected by centrifugation, resuspended in 1 ml acetone (4°C), collected by centrifugation, dried in a dessicator for 20 min, resuspended in 200 μl sample buffer, heated to 100°C until completely dissolved, and heated an additional 5 min at 100°C. The proteins were diluted 16- or 32-fold in sample buffer and 40-μl aliquots loaded onto 14% polyacrylamide-urea gels, prepared as described above. Radiolabeled polypeptides were revealed by autoradiography of the dried gel using x-ray film (Kodak X-Omat XAR-5 film; Eastman Kodak Co., Rochester, NY). As a control,

[^{35}S]-L-cysteine was added to L-cysteine–deficient YE broth from which bacteria were removed, and the broth was processed in parallel with the supernatants of broth inoculated with bacteria. Such broth controls were always negative for radiolabeled polypeptides.

Lipopolysaccharide Assay. Wild-type and mutant LPS was isolated, subjected to SDS-PAGE (14% acrylamide, 4 M urea), and the gel was stained with silver nitrate as described (13) with the kind assistance of Dr. Joel Gabay (The Rockefeller University, New York).

Results

All Mutant Clones of L. pneumophila Are Avirulent for Human Monocytes. 44 mutant clones were selected and assayed for their capacity to multiply in human monocytes in monolayer culture. None of the 44 mutant clones multiplied at all in experiments in which wild-type *L. pneumophila* multiplied 2.5–4.5 logs. An experiment representative of five experiments that compared growth of Mutant 25 with that of wild-type is shown in Fig. 1; growth of the other mutants resembled that of Mutant 25.

Paralleling these results, all monocyte monolayers infected with the 44 clones of mutant *L. pneumophila* were completely intact 5 d after infection whereas monocyte monolayers infected with wild-type *L. pneumophila* were destroyed by 3 d after infection.

Thus, in contrast to wild-type *L. pneumophila*, the mutant *L. pneumophila* do not multiply in monocytes nor produce a cytopathic effect on monocyte monolayers.

Colony Morphology and Ultrastructure of Mutant L. pneumophila. Mutant *L. pneumophila* formed both diffuse and compact colonies on CYE agar similar in morphology to colonies formed by wild-type *L. pneumophila* (Fig. 2); as with the wild-type bacteria, the formation of these colony types paralleled the production of certain bacterial proteins (Horwitz, M.A., unpublished data). Colony type of mutants had no bearing on intracellular multiplication or any other interaction with monocytes studied.

Ultrastructurally, mutant *L. pneumophila* resembled wild-type *L. pneumophila*. Like the wild-type, the mutants were bacillary in shape, had readily identifiable cytoplasmic and outer membranes, frequently contained electron-lucent cytoplasmic vacuoles, and divided by a pinching nonseptate process.

Mutants Are Resistant to the Bactericidal Effects of Serum. Wild-type *L. pneumophila* are resistant to the bactericidal effect of human serum in the presence or absence of high-titer human anti-*L. pneumophila* antiserum (6). Mutant *L. pneumophila* were examined for their sensitivity to complement. In the presence or absence of anti-*L. pneumophila* antibody, CFU of Mutants 25 and 38 and of wild-type *L. pneumophila* were not significantly reduced upon incubation for 1 h 0% serum; in contrast, CFU of a complement-sensitive strain of *E. coli* were reduced by 2.5 logs in 10% serum and >6 logs in 50% serum (Table I, Exp. A). Both variants of Mutant 25, those forming colonies of compact morphology (Mutant 25C) and those forming colonies of diffuse morphology (Mutant 25D), were resistant to the bactericidal effects of serum in the presence or absence of anti-*L. pneumophila* antibody (Table I, Exp. B). These mutants were also resistant to the bactericidal effects of serum after 24 h of incubation (Table I, Exp. C).

Thus, like the wild-type, both Mutants 25 and 38 are resistant to complement in the presence or absence of anti-*L. pneumophila* antibody. Therefore, the

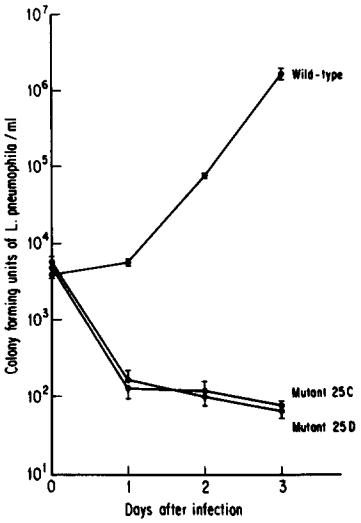


FIGURE 1. Mutant *L. pneumophila* do not multiply in the presence of monocytes. Wild-type *L. pneumophila*, Mutant 25C, or Mutant 25D were added to human monocytes in monolayer culture, and the cultures were incubated at 37°C in 5% CO_2–95% air for 72 h. CFU/ml were determined daily. Each data point represents the mean of triplicate culture wells ± SE.

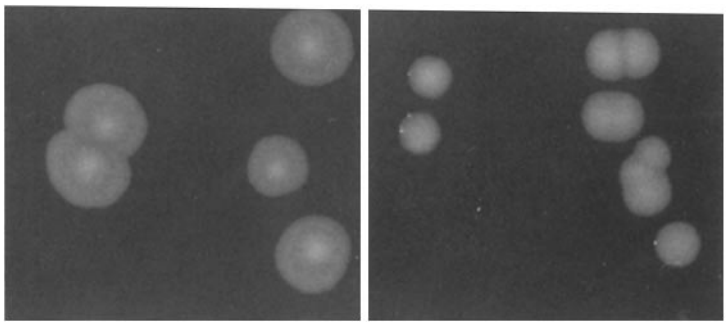


FIGURE 2. Colony morphology of mutant *L. pneumophila*. Mutant *L. pneumophila* form colonies of diffuse (*left*) and compact (*right*) type on CYE agar similar in morphology to those formed by wild-type *L. pneumophila*. × 2.

mutants, at least under tissue culture conditions, were not type I mutants as defined above, i.e., mutants unable to survive extracellularly. This indicated that the avirulent phenotype of the mutants reflected a defect in their capacity to enter monocytes (type IIA), survive intracellularly in monocytes (type IIB), or multiply intracellularly in monocytes (type IIC). Subsequent experiments examined these possibilities.

Mutants Bind to Monocytes and Are Phagocytized by Coiling Phagocytosis. The capacity of mutant *L. pneumophila* to bind to monocytes was examined to determine if avirulence for monocytes was a result of a failure of mutants to enter monocytes and gain access to the intracellular milieu they require to multiply under tissue culture conditions. All 44 mutant *L. pneumophila* clones bound readily to monocytes. The percentage of monocytes that bound or ingested mutant or wild-type *L. pneumophila* was comparable and independent of colony morphology. The number of bacteria bound per monocyte differed somewhat among the different bacteria tested (Table II). More wild-type *L. pneumophila* that form colonies typical in morphology to those of *L. pneumophila*

TABLE I

Viability of Mutant L. pneumophila in the Presence of Human Serum and Anti–L. pneumophila Antibody

Exp.	Bacteria	Initial CFU/ml	CFU/ml after 1 h incubation in 0, 10, or 50% serum		
			0%	10%	50%
A	*L. pneumophila*, mutant 25	$6.8 \pm 0.04 \times 10^4$	$5.8 \pm 0.2 \times 10^4$	$7.6 \pm 0.2 \times 10^4$	$7.0 \pm 0.1 \times 10^4$
	L. pneumophila, mutant 38	$4.8 \pm 0.01 \times 10^4$	$4.3 \pm 0.01 \times 10^4$	$5.3 \pm 0.1 \times 10^4$	—
	L. pneumophila, wild-type	$6.4 \pm 0.2 \times 10^4$	$6.6 \pm 0.9 \times 10^4$	$8.0 \pm 0.3 \times 10^4$	$5.2 \pm 1.0 \times 10^4$
	E. coli	$2.8 \pm 0.1 \times 10^7$	$8.0 \pm 0.3 \times 10^7$	$9.8 \pm 6.9 \times 10^4$	$1.5 \pm 1.1 \times 10^1$
			CFU/ml after 1 h incubation with anti-*L. pneumophila* antibody and 0, 10, or 50% serum		
			0%	10%	50%
	L. pneumophila, mutant 25	$6.8 \pm 0.04 \times 10^4$	$7.6 \pm 0.1 \times 10^4$	$6.9 \pm 0.1 \times 10^4$	$6.8 \pm 0.3 \times 10^4$
	L. pneumophila, mutant 38	$4.8 \pm 0.01 \times 10^4$	$4.7 \pm 0.02 \times 10^4$	$3.3 \pm 0.03 \times 10^4$	$2.0 \pm 0.07 \times 10^4$
	L. pneumophila, wild-type	$6.4 \pm 0.2 \times 10^4$	$7.4 \pm 0.5 \times 10^4$	$4.2 \pm 0.04 \times 10^4$	$2.2 \pm 0.5 \times 10^4$
	E. coli	$2.8 \pm 0.1 \times 10^7$	$1.2 \pm 0.1 \times 10^8$	$5.3 \pm 3.6 \times 10^4$	$7.8 \pm 1.6 \times 10^0$
			CFU/ml after 1 h incubation in 0 or 25% serum		
			0%	25%	
B	*L. pneumophila*, mutant 25C	$1.9 \pm 0.02 \times 10^4$	$2.0 \pm 0.08 \times 10^4$	$2.0 \pm 0.4 \times 10^4$	
	L. pneumophila, mutant 25D	$8.0 \pm 0.04 \times 10^4$	$9.8 \pm 0.8 \times 10^4$	$10.3 \pm 0.7 \times 10^4$	
	L. pneumophila, wild-type	$3.4 \pm 0.4 \times 10^4$	$3.8 \pm 0.4 \times 10^4$	$4.3 \pm 0.8 \times 10^4$	
	E. coli	$4.8 \pm 0.2 \times 10^6$	$6.9 \pm 0.5 \times 10^6$	$1.0 \pm 0.8 \times 10^1$	
			CFU/ml after 1 h incubation with anti-*L. pneumophila* antibody and 0 or 25% serum		
			0%	25%	
	L. pneumophila, mutant 25C	$1.9 \pm 0.02 \times 10^4$	$2.8 \pm 0.05 \times 10^4$	$2.0 \pm 0.5 \times 10^4$	
	L. pneumophila, mutant 25D	$8.0 \pm 0.04 \times 10^4$	$8.9 \pm 1.3 \times 10^4$	$6.3 \pm 0.3 \times 10^4$	
	L. pneumophila, wild-type	$3.4 \pm 0.4 \times 10^4$	$5.8 \pm 0.4 \times 10^4$	$3.0 \pm 0.5 \times 10^4$	
	E. coli	$4.8 \pm 0.2 \times 10^6$	$1.7 \pm 0.07 \times 10^7$	$6.7 \pm 0.3 \times 10^0$	
			CFU/ml after 1 h incubation with 0 or 25% serum		
			0%	25%	
C	*L. pneumophila*, mutant 25C	$2.1 \pm 0.01 \times 10^4$	$2.9 \pm 0.05 \times 10^4$	$1.8 \pm 0.1 \times 10^4$	
	L. pneumophila, mutant 25D	$3.8 \pm 0.4 \times 10^4$	$1.8 \pm 0.2 \times 10^4$	$3.3 \pm 0.07 \times 10^4$	
	L. pneumophila, wild-type	$2.3 \pm 0.06 \times 10^3$	$1.7 \pm 0.4 \times 10^3$	$1.9 \pm 0.8 \times 10^3$	
	E. coli	$5.6 \pm 0.2 \times 10^6$	$5.2 \pm 0.2 \times 10^6$	$1.7 \pm 1.1 \times 10^2$	
			CFU/ml after 24 h incubation with 0 or 25% serum		
			0%	25%	
	L. pneumophila, mutant 25C	$2.1 \pm 0.01 \times 10^4$	$1.3 \pm 0.06 \times 10^4$	$1.5 \pm 0.1 \times 10^4$	
	L. pneumophila, mutant 25D	$3.8 \pm 0.4 \times 10^4$	$1.7 \pm 0.1 \times 10^4$	$4.9 \pm 0.01 \times 10^3$	
	L. pneumophila, wild-type	$2.3 \pm 0.06 \times 10^3$	$1.3 \pm 0.3 \times 10^3$	$5.0 \pm 0.8 \times 10^2$	
	E. coli	$5.6 \pm 0.2 \times 10^6$	$4.4 \pm 0.2 \times 10^6$	$2.8 \pm 2.3 \times 10^2$	

isolated from embryonated hens' eggs or guinea pigs (and thus referred to as "standard" wild-type *L. pneumophila*) bound per monocyte than wild-type *L. pneumophila* of the diffuse or compact colony type. Comparable numbers of wild-type *L. pneumophila* of compact or diffuse colony type bound per monocyte;

TABLE II

Capacity of Monocytes to Bind or Ingest Mutant and Wild-type L. pneumophila

L. pneumophila type	Percentage of monocytes binding or ingesting *L. pneumophila*	Average number of *L. pneumophila*/monocyte with ≥1 associated bacteria	Index of monocyte–*L. pneumophila* association*
Wild-type, standard	75	5.0	375
Wild-type, compact	66	3.2	211
Wild-type, diffuse	74	3.4	252
Mutant 25, compact	70	3.4	238
Mutant 25, diffuse	60	2.4	144

Wild-type or mutant *L. pneumophila* with the colonial morphology indicated were incubated with monocytes as described in the text. The cells were fixed and stained with fluorescein-conjugated anti-*L. pneumophila* antibody, examined by fluorescence microscopy, and 200 consecutive monocytes in each preparation were examined for associated bacteria.

* The index of monocyte–*L. pneumophila* association is the percentage of monocytes with bound or ingested *L. pneumophila* multiplied by the average number of *L. pneumophila* per monocyte with ≥1 associated bacteria.

however, fewer Mutant 25 *L. pneumophila* of diffuse colony type bound per monocyte than Mutant 25 *L. pneumophila* of compact colony type in two independent experiments.

Electron microscopy revealed that Mutants 25 and 38 of compact or diffuse colony type, and wild-type *L. pneumophila* of standard, compact, or diffuse colony type were readily ingested by monocytes. All these types of bacteria were ingested by "coiling phagocytosis," as described previously for wild-type (standard) *L. pneumophila* (8). In coiling phagocytosis, long monocyte pseudopods coil around the bacteria as they are internalized.

Thus, the mutant *L. pneumophila* are all phagocytized efficiently by monocytes. The mutants are not type IIA mutants, i.e., mutants avirulent because of an inability to gain entry into monocytes.

Mutant L. pneumophila Survive Intracellularly in Monocytes but Do Not Replicate. Mutants that gain entry into monocytes may be avirulent either because of an inability to survive intracellularly or because of an inability to replicate. The capacity of mutant *L. pneumophila* to survive intracellularly was examined under two conditions: mutants were incubated with pelleted monocytes in the cone of a centrifuge tube or mutants were incubated with monocytes in monolayer culture (Tables III and IV). Neither mutant nor wild-type bacteria were killed by the monocytes under either of the two conditions of incubation. During the 2 h of incubation in pelleted monocytes and during the first 4 h of incubation in monocytes in monolayer culture, CFU of mutant and wild-type bacteria changed relatively little and if anything slightly increased (Tables III and IV). Between 4 and 24 h of incubation in monocytes in monolayer culture, CFU of mutant *L. pneumophila* remained essentially unchanged, whereas CFU of wild-type *L. pneumophila* increased 1.8 log (Table IV), reflecting intracellular multiplication of wild-type *L. pneumophila*.

TABLE III

Viability of L. pneumophila Mutants Mixed and Incubated with Pelleted Monocytes

Mutant	CFU of *L. pneumophila*/ml		Δlog CFU 0–2 h*
	0 h	2 h	
Wild-type	$3.8 \pm 0.1 \times 10^2$	$6.8 \pm 0.5 \times 10^2$	+ 0.25
Mutant 25	$4.2 \pm 0.6 \times 10^2$	$7.3 \pm 0.9 \times 10^2$	+ 0.25
Mutant 38	$2.1 \pm 0.1 \times 10^3$	$2.2 \pm 0.1 \times 10^3$	+ 0.02

Monocytes were incubated with wild-type or mutant *L. pneumophila* in the cone of a centrifuge tube for 0 or 2 h, lysed by sonication to release intracellular bacteria, and CFU were determined. Data are the mean ± SE for duplicate cultures.

* Δlog CFU = (log CFU at end of incubation period, 2 h) − (log CFU at start of incubation period, 0 h).

TABLE IV

Viability of L. pneumophila Mutants Bound to and Incubated with Adherent Monocytes

Mutant	CFU of *L. pneumophila*/ml			Δlog CFU*		
	0 h	4 h	24 h	0–4 h	4–24 h	0–24 h
Wild-type	$1.2 \pm 0.5 \times 10^2$	$1.5 \pm 0.3 \times 10^2$	$8.4 \pm 0.4 \times 10^3$	+ 0.1	+ 1.8	+ 1.9
Mutant 25C	$4.5 \pm 0.2 \times 10^2$	$1.5 \pm 0.2 \times 10^3$	$1.4 \pm 0.1 \times 10^3$	+ 0.5	− 0.03	+ 0.5
Mutant 25D	$4.5 \pm 0.3 \times 10^3$	$1.1 \pm 0.1 \times 10^4$	$4.7 \pm 0.2 \times 10^3$	+ 0.4	− 0.4	+ 0.02

Monocytes in monolayer culture were infected with wild-type or mutant *L. pneumophila*, washed to remove extracellular bacteria, incubated for 0, 4, or 24 h, lysed by sonication to release intracellular bacteria, and CFU were determined. Data are mean ± SE for triplicate cultures.

* Δlog CFU = (log CFU at end of incubation period; 4 or 24 h, as indicated) − (log CFU at start of incubation period; 0 or 4 h, as indicated).

Thus, mutant bacteria are not killed by monocytes and their avirulence results from an inability to multiply intracellularly. The mutants are therefore type IIC mutants as defined above.

Mutant L. pneumophila Do Not Form the Ribosome-lined Replicative Phagosome Characteristic of Wild-type L. pneumophila. The defect in the capacity of mutant *L. pneumophila* to multiply intracellularly was examined further by investigating the capacity of the mutants to form the distinctive replicative phagosome characteristic of wild-type *L. pneumophila* (9). After phagocytosis, wild-type *L. pneumophila* form a ribosome-lined phagosome by a process that involves the sequential interaction of the phagosome with monocyte smooth vesicles, mitochondria, and ribosomes and requires 4–8 h (9). The bacterium then multiplies within the ribosome-lined vacuole.

Unlike phagosomes of wild-type *L. pneumophila*, phagosomes of mutant *L. pneumophila* were not frequently surrounded by smooth vesicles and mitochondria early (1 or 2 h) after ingestion or by ribosomes later after ingestion (Table V; Fig. 3). At 1 or 2 h after ingestion, 85–96% of phagosomes of wild-type *L. pneumophila* were surrounded by smooth vesicles, compared with 0–4% of phagosomes of Mutants 25C, 25D, or 38C (Table V, Exps. A–C). Similarly, 40–50% of phagosomes of wild-type *L. pneumophila* were surrounded by mitochondria, compared with 0–5% of phagosomes of the mutant *L. pneumophila* (Table

TABLE V

Phagosome Formation by Mutant and Wild-type L. pneumophila

Exp.	*L. pneumophila* type	Incubation period	Phagosomes surrounded by:		
			Smooth vesicles	Mitochondria	Ribosomes
		h	%	%	%
A	Wild-type	1	90	50	0
	Mutant-25D	1	0	3	0
	Mutant 38C	1	0	5	0
B	Wild-type	2	85	40	0
	Mutant 25D	2	4	4	0
	Mutant 38C	2	0	0	0
	Wild-type	6	15	0	82
	Mutant 25D	6	4	4	0
	Mutant 38C	6	0	0	0
C	Wild-type	1	96	50	5
	Mutant 25C	1	0	0	0
	Mutant 25D	1	0	0	0
	Wild-type	6	10	0	95
	Mutant 25C	6	0	0	0
	Mutant 25D	6	0	0	0
	Mutant 25C	24	0	8	0
D	Wild-type	6	13	13	94
	Mutant 25	6	0	0	0
	Mutant 25	24	0	0	0

Monocytes were infected with wild-type or mutant *L. pneumophila*, washed to remove non–monocyte-associated bacteria, incubated for 1, 2, 6, or 24 h, and fixed and processed for electron microscopy. In each experiment, the percentage of 20 consecutive phagosomes surrounded by monocyte smooth vesicles, mitochondria, or ribosomes was enumerated for each bacterium studied at each time point.

V, Exps. A–C). At 6 h after ingestion, 82–95% of phagosomes of wild-type *L. pneumophila* were surrounded by ribosomes, compared with 0% of phagosomes of mutant *L. pneumophila* (Table V, Exps. B–D). At 24 h after ingestion, phagosomes of Mutant 25C were still not surrounded by ribosomes (Table V, Exps. C and D). Phagosomes of wild-type *L. pneumophila* could not be examined at 24 h after ingestion because monocyte monolayers containing these bacteria were destroyed by this time as a result of intracellular multiplication of these bacteria.

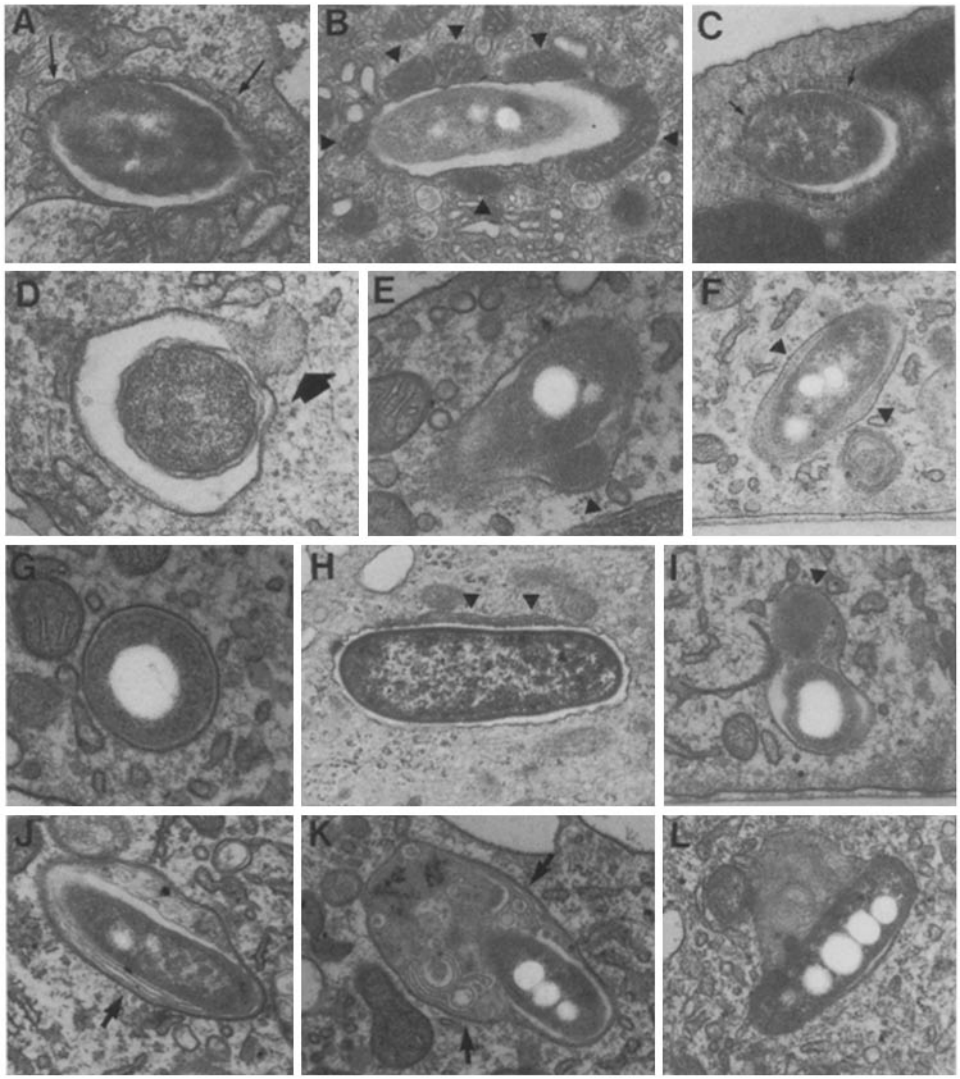
Within 2 h of infection, phagosomes of mutant bacteria contained what appeared to be lysosomal contents (Fig. 3, *D–F*), consistent with the finding that these phagosomes fuse with lysosomes (see below). At 6 and 24 h after infection, phagosomes of mutant bacteria frequently contained large amounts of membranous material (Fig. 3, *J–L*).

There was no electron microscopic evidence of multiplication of mutant *L. pneumophila*. At 24 h after ingestion, most phagosomes contained only a single bacterium (94% of phagosomes examined in Exps. C and D).

Thus, in contrast to phagosomes of wild-type *L. pneumophila*, phagosomes of mutant bacteria do not interact with monocyte smooth vesicles, mitochondria, or ribosomes. In this respect, mutant bacteria resemble formalin-killed wild-type

bacteria. In contrast to formalin-killed *L. pneumophila*, which are rapidly digested in monocytes so that by 4 h few bacteria remain intact, mutant *L. pneumophila* are not digested in monocytes and appear fully intact at least up to 24 h after ingestion (Fig. 3).

Mutant L. pneumophila Do Not Inhibit Phagosome-Lysosome Fusion As Do Wild-type L. pneumophila. Wild-type *L. pneumophila* inhibit fusion between the phagosome and monocyte lysosomes (10). The capacity of mutant *L. pneumophila* to do so was examined by preloading monocyte lysosomes with thorium dioxide, an electron-opaque marker, infecting the monocytes with mutant or wild-type *L.*



pneumophila, and determining by electron microscopy whether phagosomes had fused with lysosomes as evidenced by the presence of thorium dioxide within them. Whereas only 9–10% of phagosomes containing live wild-type *L. pneumophila* were fused at 1 h after ingestion, 57–95% of phagosomes containing mutant *L. pneumophila* were fused (Table VI, Exps. A and B; Fig. 4,*A*, *C*, and *D*). Similarly, at 6 h after ingestion, no phagosomes containing live wild-type *L. pneumophila* were fused, whereas 96% of phagosomes containing live Mutant 25 were fused (Table VI, Exp. C; Fig. 4,*B*, *E*, and *F*). At 24 h after ingestion, only phagosomes containing mutant *L. pneumophila* could be examined because monocyte monolayers containing wild-type *L. pneumophila* were destroyed by this time. The majority of phagosomes containing live Mutant 25 were fused at this time as at earlier time points (Table VI, Exp. C; Fig. 4,*G* and *H*). At all time points, most phagosomes containing formalin-killed *L. pneumophila*, whether the bacteria were wild-type or mutant, were fused (Table VI, Exps. A and C).

Thus, in parallel with their loss of capacity to form a distinctive ribosome-lined phagosome, mutants have lost the capacity to inhibit phagosome-lysosome fusion. The mutants remain intact and viable (as evidenced above by their capacity to survive intracellularly) within a phagolysosome.

Biochemical Characterization of Mutants. Mutant *L. pneumophila* were partially

FIGURE 3. Morphology of phagosomes containing wild-type and mutant *L. pneumophila*. Monocytes in monolayer culture were infected with wild-type *L. pneumophila* (*A–C*) or Mutant 25 (*D–L*), washed to remove non–monocyte-associated bacteria, incubated for 10 min to 24 h, and fixed for electron microscopy. Studies conducted with Mutant 38 yielded similar results to those conducted with Mutant 25.

(*A–C*) Phagosomes containing wild-type *L. pneumophila* interact sequentially with smooth vesicles (*A*), mitochondria (*B*), and ribosomes (*C*), a process culminating in the formation of a ribosome-lined replicative vacuole (*C*). (*A*) Phagosome containing a wild-type bacterium at 1 h after infection. The phagosome is surrounded by smooth vesicles (*arrows*) that appear to be fusing with and/or budding off from the phagosome membrane (× 26,000). (*B*) Phagosome containing a wild-type bacterium 1 h after infection at later stage of phagosome development. The phagosome is surrounded by smooth vesicles and by six mitochondria (*arrowheads*) closely apposed to the phagosomal membrane. As is frequently the case, the bacterium contains an electron lucent fat vacuole (× 20,500). (*C*) Phagosome containing a wild-type bacterium at 6 h after infection. The phagosome is surrounded by monocyte ribosomes (*arrows*) located at a distance of ~100 Å from the phagosomal membrane. As is frequently the case at this point after infection, the phagosome is located in an invagination of the monocyte nuclear envelope (× 28,500).

(*D–L*) Phagosomes containing mutant *L. pneumophila* do not interact with monocyte smooth vesicles, mitochondria, or ribosomes. These phagosomes appear to fuse with lysosomes (see Fig. 4), and at later stages of infection, they contain large amounts of membranous material. The mutant bacteria remain intact, but do not replicate in the phagosome. (*D*) Phagosome containing a mutant bacterium at 10 min after infection. The phagosome appears to be fusing with a monocyte lysosome (*arrow*) (× 52,000). (*E* and *F*) Phagosomes containing mutant bacteria at 2 h after infection. They typically contain what appears to be lysosomal contents (*arrowheads*) sandwiched between the bacterium and inner phagosomal membrane. (*E*) × 39,500. (*F*) × 26,000. (*G–K*) Phagosomes containing mutant bacteria at 6 h after infection. A few phagosomes, as in *G*, appear devoid of nonbacterial contents, but most contain what appear to be lysosomal contents (*arrowheads*) (*H* and *I*). Many phagosomes contain abundant membranous material (*arrows*) by this point after infection (*J* and *K*). (*G*) × 52,000. (*H*) × 15,000. (*I–K*) × 26,000. (*L*) Phagosome containing mutant bacterium at 24 h after infection. Phagosomes at this point are typically large and many contain membranous and other material (× 20,500).

TABLE VI
Phagosome-Lysosome Fusion by Mutant and Wild-type L. pneumophila

Exp.	*L. pneumophila* type	Incubation period	Mean number bacteria/phagosome	Phagosomes fused	Bacteria in fused phagosomes
		h		%	%
A	Wild-type, live	1	1.05	10	14
	Wild-type, dead	1	1.14	100	100
	Mutant 25D, live	1	1.32	90	93
	Mutant 25D, dead	1	1.44	100	100
	Mutant 38C, live	1	1.25	95	96
	Mutant 38C, dead	1	1.96	96	98
B	Wild-type, live	1	1.18	9	8
	Mutant 25C, live	1	1.12	71	63
	Mutant 25D, live	1	1.05	57	59
C	Wild-type, live	6	1.00	0	0
	Mutant 25, live	6	1.14	96	96
	Mutant 25, dead	6	1.22	100	100
	Mutant 25, live	24	1.00	63	63
	Mutant 25, dead	24	1.33	100	100

Monocytes containing lysosomes that had been prelabeled with thorium dioxide were infected with live or formalin-killed, wild-type or mutant *L. pneumophila*, incubated for 1, 6, or 24 h, and processed for electron microscopy. In each experiment, the percentage of ≥20 consecutive phagosomes that were fused was determined for each bacterium studied at each time point.

characterized biochemically in an attempt to identify a specific molecular difference between mutant and wild-type *L. pneumophila* that might relate to the difference in virulence between them. The total proteins, secretory proteins, and LPS of Mutants 25 and 38 were compared with that of wild-type *L. pneumophila*. The total proteins were studied by SDS-PAGE of whole bacteria. The secretory proteins were studied by radiolabeling the bacteria with [^{35}S]cysteine during broth culture, collecting and precipitating the supernatant proteins, and examining them autoradiographically. The LPSs were studied by isolating the molecules, electrophoresing them, and silver staining the gels.

No consistent differences between wild-type and mutant *L. pneumophila* were found in these studies (Figs. 5–7).

Discussion

This paper demonstrates that *L. pneumophila*, upon passage on suboptimal medium, converts to a mutant form that is avirulent for human monocytes. The basis for the avirulence is an inability to multiply intracellularly. In association with this defect, the mutant has lost the the capacity to form the distinctive ribosome-lined replicative phagosome characteristic of wild-type *L. pneumophila*, and it has lost the capacity to inhibit phagosome-lysosome fusion. Earlier interactions of mutant *L. pneumophila* with monocytes appear normal. The mutants

bind to and are ingested by monocytes, and they enter monocytes by coiling phagocytosis.

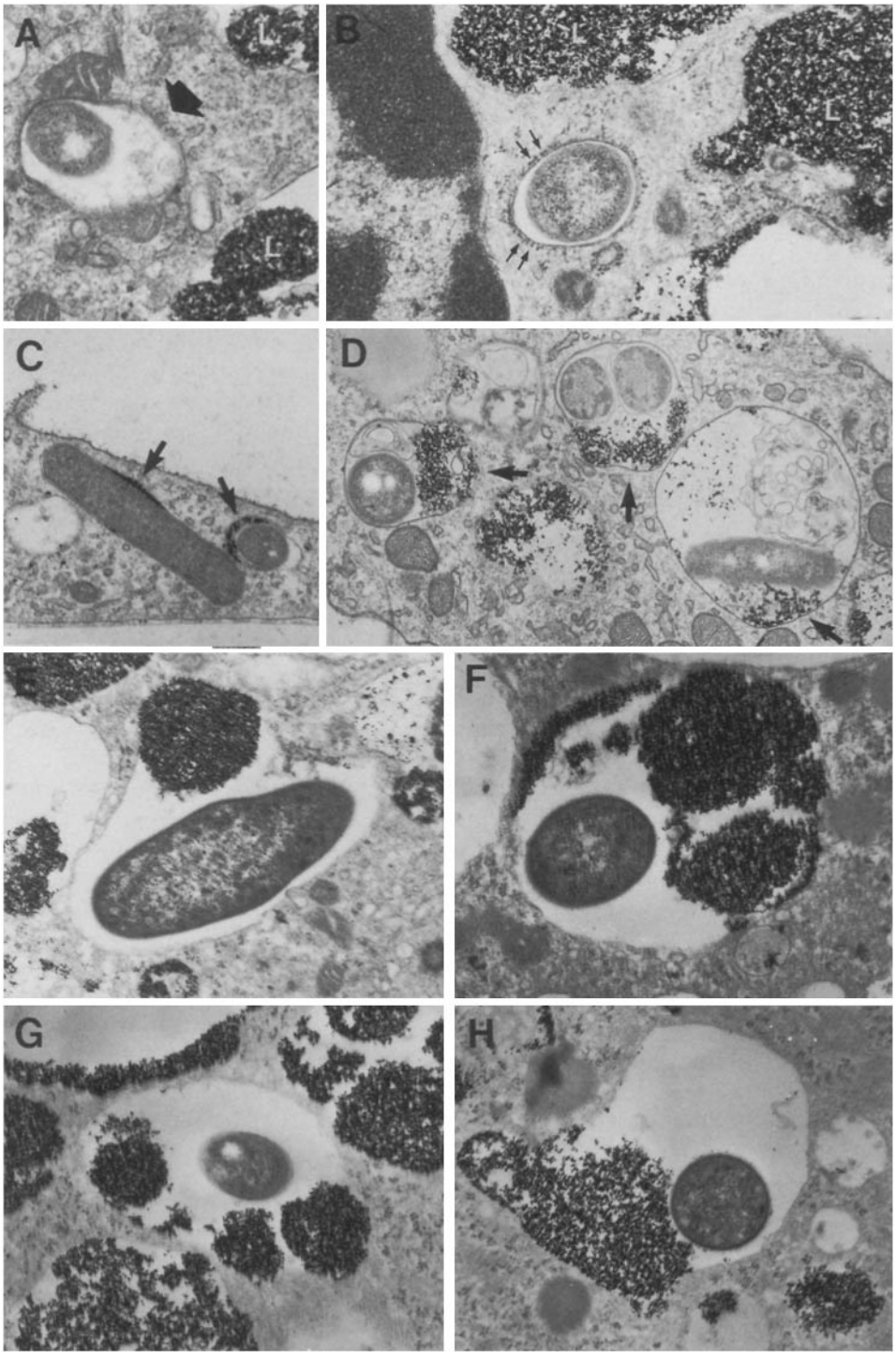
In all other respects examined, the mutant resembles the wild-type. The two types of bacteria have similar colony morphology; both form compact and diffuse colonies on CYE agar. The two types have similar ultrastructure; both are typically appearing Gram-negative bacilli with a cytoplasmic and outer membrane, both have electron-lucent fat vacuoles, and both divide by a pinching nonseptate process. The two types are complement resistant in the presence or absence of specific antibody. Biochemically, the two types of bacteria have similar structural and secretory protein profiles by SDS-PAGE, and similar LPS profiles.

Although mutant *L. pneumophila* do not multiply intracellularly, they are capable of surviving within a phagolysosome in human monocytes. This typically low pH intracellular compartment is rich in hydrolytic enzymes, and is sometimes considered an inhospitable milieu for microorganisms. However, some intracellular parasites are specially adapted for life in the phagolysosome. For example *Leishmania donovani* carries out a variety of physiologic activities optimally in the low pH range (4.0–5.5) characteristic of the phagolysosome (19); this organism is able to survive and multiply within the phagolysosome (20, 21). The ability of mutant *L. pneumophila* to survive but not to multiply in the phagolysosome may reflect suboptimal adaptation to intraphagolysosomal conditions. For example, *L. pneumophila* multiplication in artificial media is dependent upon a relatively high pH (optimally 6.9) (22), and in monocytes, wild-type *L. pneumophila* maintains a high pH in the distinctive phagosome in which it replicates (11). If the pH of the phagolysosome in which mutant *L. pneumophila* reside is characteristically low, mutant *L. pneumophila* would probably not be able to multiply within it for this reason alone.

Previous studies (23, 24) have shown that avirulent *L. pneumophila* (Philadelphia 2 strain) obtained by passage of wild-type bacteria on suboptimal artificial media convert back to virulence upon passage through guinea pigs, embryonated hens' eggs, or cultured human embryonic lung fibroblasts. In these studies, the passaged bacteria were not reported as cloned from a single colony so it is not known whether the conversion to virulence resulted from back mutation or the selection of residual wild-type organisms in the passaged inocula.

The avirulent mutant characterized in this paper has tremendous potential for enhancing our understanding of the intracellular biology of *L. pneumophila* and perhaps of other intracellular parasites, such as *Toxoplasma gondii* and *Chlamydia psittaci*, that follow similar pathways through the mononuclear phagocyte (9, 10). Current studies are aimed at identifying the genetic defect in the avirulent mutant, and subsequently the precise molecular basis for avirulence. Such studies may shed light on mechanisms underlying phagosome formation and inhibition of phagosome-lysosome fusion.

This mutant (type IIC), which survives but does not multiply intracellularly, is one of several avirulent phenotypes that might serve to enhance our understanding of *L. pneumophila*–monocyte interaction. Mutants that are unable to enter monocytes (type IIA), or mutants able to enter but not able to survive intracellularly (type IIB), would also be extremely useful for this purpose. Future studies will seek to obtain such mutants.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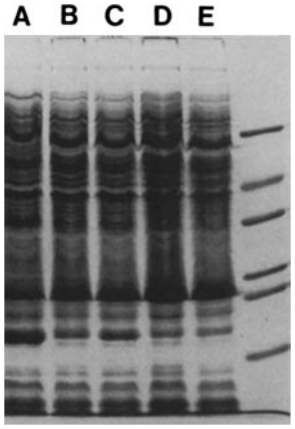

FIGURE 5. SDS-PAGE analysis of wild-type and mutant *L. pneumophila*. Bacterial proteins were analyzed by SDS-PAGE (12.5% acrylamide) and stained with Coomassie Blue. Molecular weight standards were trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3 phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine albumin (66,000). Wild-type or mutant bacteria that form colonies of diffuse morphology have less of the 28-kD major outer membrane protein and more of a 22-kD protein than bacteria that form colonies of compact morphology. No consistent differences were found between wild-type and mutant bacteria of similar colony morphology. (*A*) Mutant 25, diffuse; (*B*) Mutant 25, compact; (*C*) wild-type, diffuse; (*D*) wild-type, compact; (*E*) wild-type, standard.

The avirulent mutant also has great potential for enhancing our understanding of immunity to *L. pneumophila*. The mutant is nonlethal to guinea pigs, and studies in this animal of its capacity to induce protective immunity after immunization may provide new strategies for developing safe and effective vaccines against *L. pneumophila* and other intracellular parasites.

Summary

Legionella pneumophila, the causative agent of Legionnaires' disease, is a Gram-negative bacterium and a facultative intracellular parasite that multiplies in human monocytes and alveolar macrophages. In this paper, mutants of *L. pneumophila* avirulent for human monocytes were obtained and extensively characterized. The mutants were obtained by serial passage of wild-type *L.*

FIGURE 4. Interaction of phagosomes containing wild-type and mutant *L. pneumophila* with lysosomes. Monocytes in monolayer culture were preincubated with thorium dioxide, an electron-opaque marker that is concentrated in monocyte lysosomes. The monocytes were washed to remove uningested thorium dioxide, infected with wild-type (*A* and *B*) or Mutant 25 (*C–H*) *L. pneumophila*, incubated for 1, 6, or 24 h, and processed for electron microscopy as described in the text. Studies conducted with Mutant 38 yielded results similar to those conducted with Mutant 25.

(*A* and *B*) Phagosomes containing wild-type *L. pneumophila* do not fuse with monocyte lysosomes. (*A*) Phagosome (*arrow*) containing a wild-type *L. pneumophila* at 1 h after infection. The phagosome is surrounded by smooth vesicles and mitochondria as is typical at this point after infection. The phagosome is in the vicinity of monocyte lysosomes (*L*), but it has not fused with them as evidenced by the absence of electron-opaque material (thorium dioxide) within it (× 30,000). (*B*) Phagosome containing a wild-type bacterium at 6 h after infection. The phagosome is lined by ribosomes (*arrows*), as is typical at this point after infection. The phagosome has not fused with monocyte lysosomes (× 33,000).

(*C–H*) Phagosomes containing mutant *L. pneumophila* fuse with monocyte lysosomes. (*C* and *D*) Phagosomes containing mutant *L. pneumophila* at 1 h after infection. The phagosomes contain thorium dioxide (*arrows*), indicating that they have fused with monocyte lysosomes. (*C* and *D*) × 18,000. (*E–H*) Phagosomes containing mutant *L. pneumophila* at 6 h (*E* and *F*) and 24 h (*G* and *H*) after infection. The phagosomes have fused with lysosomes and they contain large amounts of thorium dioxide label. The bacteria remain intact within the phagolysosome. (*E* and *H*) × 17,000. (*F* and *G*) × 26,500.

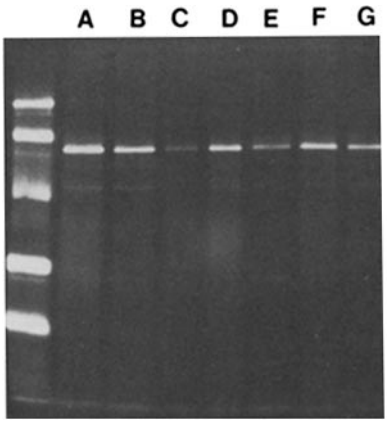

FIGURE 6. Secretory proteins of wild-type and mutant *L. pneumophila*. Secretory proteins were radiolabeled, subjected to SDS-PAGE, and revealed autoradiographically. Molecular weight standards were methylated ^{14}C-labeled bovine serum albumin (69,000 mol wt), ovalbumin (46,000), carbonic anhydrase (30,000), lactoglobulin A (18,367), cytochrome *c* (12,300). Both wild-type and mutant bacteria released the 40-kD major secretory protein. (*A* and *F*) Mutant 25, diffuse; (*B* and *G*) Mutant 25, compact; (*C*) wild-type, standard; (*D*) wild-type, compact; (*E*) wild-type, diffuse.

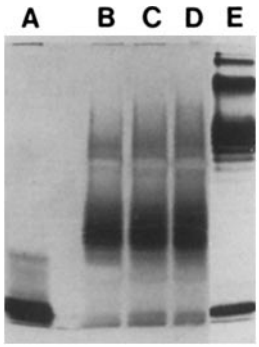

FIGURE 7. LPS of wild-type and mutant *L. pneumophila*. LPS was isolated from wild-type and mutant *L. pneumophila*, and from *E. coli* K12 (rough) and *E. coli* K$^-$ (smooth), subjected to SDS-PAGE, and the gel stained with silver nitrate. 5 μg of each preparation were loaded on the gel. The size distribution of LPS molecules of the three strains of *L. pneumophila* was markedly different from that of LPS molecules from the two strains of *E. coli*. The average size of LPS molecules of *L. pneumophila* (*B–D*) was greater than that of the rough *E. coli* (*A*) but less than that of smooth *E. coli* (*E*). The size distribution and average size of LPS molecules from wild-type *L. pneumophila* (*B*) were similar to those of LPS molecules of mutant *L. pneumophila* (*C* and *D*). (*A*) *E. coli* K12; (*B*) wild-type; (*C*) Mutant 25; (*D*) Mutant 38; (*E*) *E. coli* K$^-$.

pneumophila on suboptimal artificial medium. None of 44 such mutant clones were capable of multiplying in monocytes or exerting a cytopathic effect on monocyte monolayers. Under the same conditions, wild-type *L. pneumophila* multiplied 2.5–4.5 logs, and destroyed the monocyte monolayers.

The basis for the avirulent phenotype was an inability of the mutants to multiply intracellularly. Both mutant and wild-type bacteria bound to and were ingested by monocytes, and both entered by coiling phagocytosis. Thereafter, their intracellular destinies diverged. The wild-type formed a distinctive ribosome-lined replicative phagosome, inhibited phagosome-lysosome fusion, and multiplied intracellularly. The mutant did not form the distinctive phagosome nor inhibit phagosome-lysosome fusion. The mutant survived intracellularly but did not replicate in the phagolysosome.

In all other respects studied, the mutant and wild-type bacteria were similar. They had similar ultrastructure and colony morphology; both formed colonies of compact and diffuse type. They had similar structural and secretory protein profiles and LPS profile by PAGE. Both the mutant and wild-type bacteria were completely resistant to human complement in the presence or absence of high titer anti-*L. pneumophila* antibody.

The mutant *L. pneumophila* have tremendous potential for enhancing our understanding of the intracellular biology of *L. pneumophila* and other parasites

that follow a similar pathway through the mononuclear phagocyte. Such mutants also show promise for enhancing our understanding of immunity to *L. pneumophila*, and they may serve as prototypes in the development of safe and effective vaccines against intracellular pathogens.

I am grateful to Ms. Diane Chodkowski and Ms. Barbara Jane Dillon for expert technical assistance and to Ms. Judy Adams and Ms. Birgitta Sjostrand for preparing the electron microscopy specimens.

Received for publication 12 June 1987 and in revised form 22 July 1987.

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