THE LEGIONNAIRES' DISEASE BACTERIUM (*LEGIONELLA PNEUMOPHILA*) INHIBITS PHAGOSOME-LYSOSOME FUSION IN HUMAN MONOCYTES*

By MARCUS A. HORWITZ‡

From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

Legionella pneumophila multiplies in a novel intracellular vacuole in mononuclear phagocytes (1). In this paper, the interaction between this *L. pneumophila*–containing vacuole and host cell lysosomes is examined.

Some intracellular pathogens, such as *Toxoplasma gondii* (2), *Mycobacterium tuberculosis* (3, 4), *Mycobacterium microti* (5), *Chlamydia psittaci* (6), and *Encephalitozoon cuniculi* (7, 8) have been reported to inhibit fusion of phagosomes with lysosomes. Others, such as *Leishmania* (9–13) and *Mycobacterium lepraemurium* (5), have been reported not to inhibit fusion, and they are apparently able to survive within the normally inhospitable milieu of the phagolysosome. Still other intracellular pathogens such as *Trypanosoma cruzi* have been shown to avoid the unfavorable environment of the phagolysosome altogether by escaping into the host cell cytoplasm (14, 15). The host cells in these studies have almost invariably been mouse peritoneal macrophages or mouse cell lines.

This paper demonstrates that (*a*) *L. pneumophila* inhibits phagosome-lysosome fusion in human monocytes. The *L. pneumophila*–containing vacuole does not fuse with either primary or secondary lysosomes; (*b*) this inhibition is partially overcome by coating the bacteria with antibody or activating the monocytes; and (*c*) the inhibition is not influenced by inhibiting bacterial protein synthesis with erythromycin.

Materials and Methods

Media, Agar, and Human Blood Mononuclear Cells. Media, agar, and human blood mononuclear cells were obtained or prepared as described (1). No antibiotics were added to medium except where specifically indicated.

Reagents. Thorium dioxide (thorotrast) was produced by Fellows Testagar, Detroit, MI, but is no longer commercially available. Erythromycin base was prepared and stored as described (1). Cytidine 5′-monophosphate, sodium salt, was obtained from Sigma Chemical Co., St. Louis, MO. Lead nitrate was obtained from Fisher Scientific Co., Fair Lawn, NJ.

Sera. Sera were obtained and stored as described (1). Normal (nonimmune) human serum (type AB) with an indirect fluorescent antibody anti–*L. pneumophila* titer of <1:64 was obtained from an adult donor not known to have had Legionnaires' disease. Immune

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human serum with an indirect fluorescent antibody anti-*L. pneumophila* titer of 1:4,096 was obtained from an adult donor who had recently recovered from Legionnaires' disease.

Complement was inactivated by heating the serum at 56°C for 30 min just before use.

Bacteria. L. pneumophila, Philadelphia 1 strain, was cultured and prepared and, where indicated, formalin-killed, as described (1).

Escherichia coli, serotype 09:K29:H^-, strain Bi161-42, an encapsulated serum-resistant strain, was cultured and prepared for experiments as described (16), and resuspended in RPMI 1640 medium to 10^{10} CFU/ml.

Streptococcus pneumoniae, strain R6 (17), was obtained from Dr. Alexander Tomasz, The Rockefeller University. The bacteria were grown to mid-logarithmic phase in C medium and yeast extract generously prepared by Ms. Helene Fischer as described (18), washed by centrifugation at 12,000 *g*, and resuspended in RPMI 1640 medium to 10^{10} bacterial particles/ml.

Assay for Fusion of L. pneumophila-containing Vacuoles with Secondary Lysosomes Using Thorium Dioxide Labeling. Mononuclear cells (12×10^6) were incubated in 35-mm plastic petri dishes in 2 ml RPMI 1640 medium containing 10% fresh normal human serum for 1.5 h at 37°C in 5% CO_2-95% air to allow monocytes to adhere. The dishes were then vigorously washed to remove the nonadherent lymphocyte-enriched fraction of the mononuclear cell population. The monocyte monolayers were then incubated for 20 h at 37°C in 5% CO_2–95% air in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and either 12.5 or 6.25 μl undiluted thorium dioxide/ml. After this incubation, the monocyte monolayers were washed three times with RPMI 1640 medium to remove unbound thorium dioxide and incubated at 37°C for an additional 3 h to allow the monocytes to ingest thorium dioxide adsorbed on the cell surface and to concentrate internalized thorium dioxide in secondary lysosomes (4, 5). The cultures were then incubated with 10^{10} live or formalin-killed *L. pneumophila*/ml that had been pretreated with a source of complement alone (fresh normal human serum), 10^{10} live *L. pneumophila*/ml that had been pretreated with a source of antibody alone (heat-inactivated immune human serum), or 7.5×10^8 live *L. pneumophila*/ml that had been pretreated with a source of both anti-*L. pneumophila* antibody and complement (fresh immune human serum). Other cultures were incubated with 10^{10} live unencapsulated *S. pneumoniae*/ml pretreated with a source of complement (fresh normal human serum) or 10^9 live encapsulated *E. coli*/ml pretreated with a source of both complement and anti-*E. coli* antibody (fresh normal human serum); the normal human serum used contains natural antibody to this strain of *E. coli* (16). Pretreatment consisted of incubating the bacteria in 50% of the serum preparation indicated for 10 min at 24°C, immediately before adding the bacteria to the monocyte cultures. The final concentration of serum in all cultures was 25%. The monocyte cultures were incubated with the bacteria for 30 min at 37°C in 5% CO_2–95% air on a gyratory shaker at 100 rpm. At the end of the incubation, the monolayers were washed three times with RPMI 1640 medium (37°C) to remove non-monocyte-associated bacteria and incubated for an additional 1, 4, or 8 h in RPMI 1640 medium containing 10% of the same serum preparation in which they had been cultured before washing. The monolayers were then fixed and processed for electron microscopy as described (1).

In studies on the effect of erythromycin on fusion of *L. pneumophila*-containing vacuoles with secondary lysosomes, monocytes were preincubated with erythromycin (1.25 μg/ml) for 3 h before infection, infected with *L. pneumophila* in the presence of erythromycin (1.25 μg/ml), washed, incubated for 4 h additional in the presence of this concentration of erythromycin, and fixed for electron microscopy, as above. Control monocytes were treated in the same way but without erythromycin present.

In studies on the effect of monocyte activation on fusion, monocytes were incubated with 40% Concanavalin A–induced mononuclear cell supernatant, supernatant control, or buffer control for 20 h at the same time that they were incubated with thorium dioxide. The cultures were then washed, incubated 3 h, infected, and again incubated for 1 h in the presence or absence of these supernatant preparations as indicated, and fixed for electron microscopy as above. Concanavalin A–induced mononuclear cell supernatant was prepared by incubating 6×10^6 mononuclear cells at 37°C in 5% CO_2–95% air in 35-mm plastic petri dishes in 2 ml RPMI 1640 medium containing 25% fresh normal

human serum and 15 μg/ml Concanavalin A for 48 h. At the end of the incubation, the cultures were transferred to conical tubes and the cells were sedimented by centrifugation at 200 *g* for 10 min at 4°C. The supernatant was removed, filtered through 0.2-μm Millipore filters (Millipore Corp., Bedford, MA) and stored at −70°C. The supernatant control was prepared in the same way at the same time except Concanavalin A was added at the end of the 48-h incubation period rather than at the beginning.

Assay for Fusion of L. pneumophila-containing Vacuoles with Lysosomes Using Acid Phosphatase Cytochemistry. Monocyte monolayers were prepared in plastic petri dishes exactly as in the above assay and then incubated 20 h at 37°C in 5% CO_2–95% air in 2 ml RPMI 1640 medium containing 15% fresh normal human serum. The cultures were then incubated with live or formalin-killed *L. pneumophila* (10^{10} bacteria/ml) for 40 min on a gyratory shaker at 100 rpm. At the end of the incubation, the monolayers were washed three times with RPMI 1640 medium (37°C) to remove non-monocyte-associated bacteria and incubated at 37°C in 5% CO_2–95% air for 1 h additional in RPMI 1640 medium containing 10% fresh normal human serum. The monocyte monolayers were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 0°C for 30 min, incubated in 0.88 M sucrose at 0°C for 15 min, washed in 0.9% NaCl (0°C), incubated with prewarmed and filtered acid phosphatase substrate (37°C) for 40 min, washed in 5% sucrose in 0.1 M cacodylate buffer (pH 7.4, 24°C), and processed for electron microscopy as described (1). The acid phosphatase substrate was prepared fresh and consisted of 25 mg cytidine 5′-monophosphate (sodium salt); 12 ml double distilled water; 10 ml 0.05 M acetate buffer (pH 5.0); and 3 ml 1% lead nitrate, added in that order. Control substrate was prepared the same way but without cytidine 5′-monophosphate. This procedure is an amalgam of previously published procedures of Novikoff et al., Edelson and Cohn, and Muller et al. (19–21).

Results

L. pneumophila Inhibits Fusion of Phagosomes with Secondary Lysosomes. Fusion of *L. pneumophila*–containing intracellular vacuoles with monocyte secondary lysosomes was examined by prelabeling the lysosomes with thorium dioxide, an electron-opaque colloidal marker (3). The best results were obtained with 6.25 μl thorium dioxide/ml; 12.5 μl/ml was satisfactory but 25 μl/ml resulted in monocytes engorged with label. After the monocyte lysosomes were labeled, the monocytes were infected, incubated, and fixed as described in Materials and Methods. The monocytes were then examined by electron microscopy to determine how many *L. pneumophila*—containing vacuoles had fused with lysosomes containing thorium dioxide.

In normal monocytes infected and incubated with live *L. pneumophila* for 1 h, few *L. pneumophila*–containing vacuoles were fused with thorium dioxide–labeled lysosomes (Table I, lines *a*, *b*, and *j*; Fig. 1, *A–C*). Of a total of 88 vacuoles examined with the 2 concentrations of thorium dioxide presented in Table I, only 3 (3%) were positive for thorium dioxide. Of the 95 bacteria in these vacuoles, only 4 (3%) were in vacuoles positive for thorium dioxide.

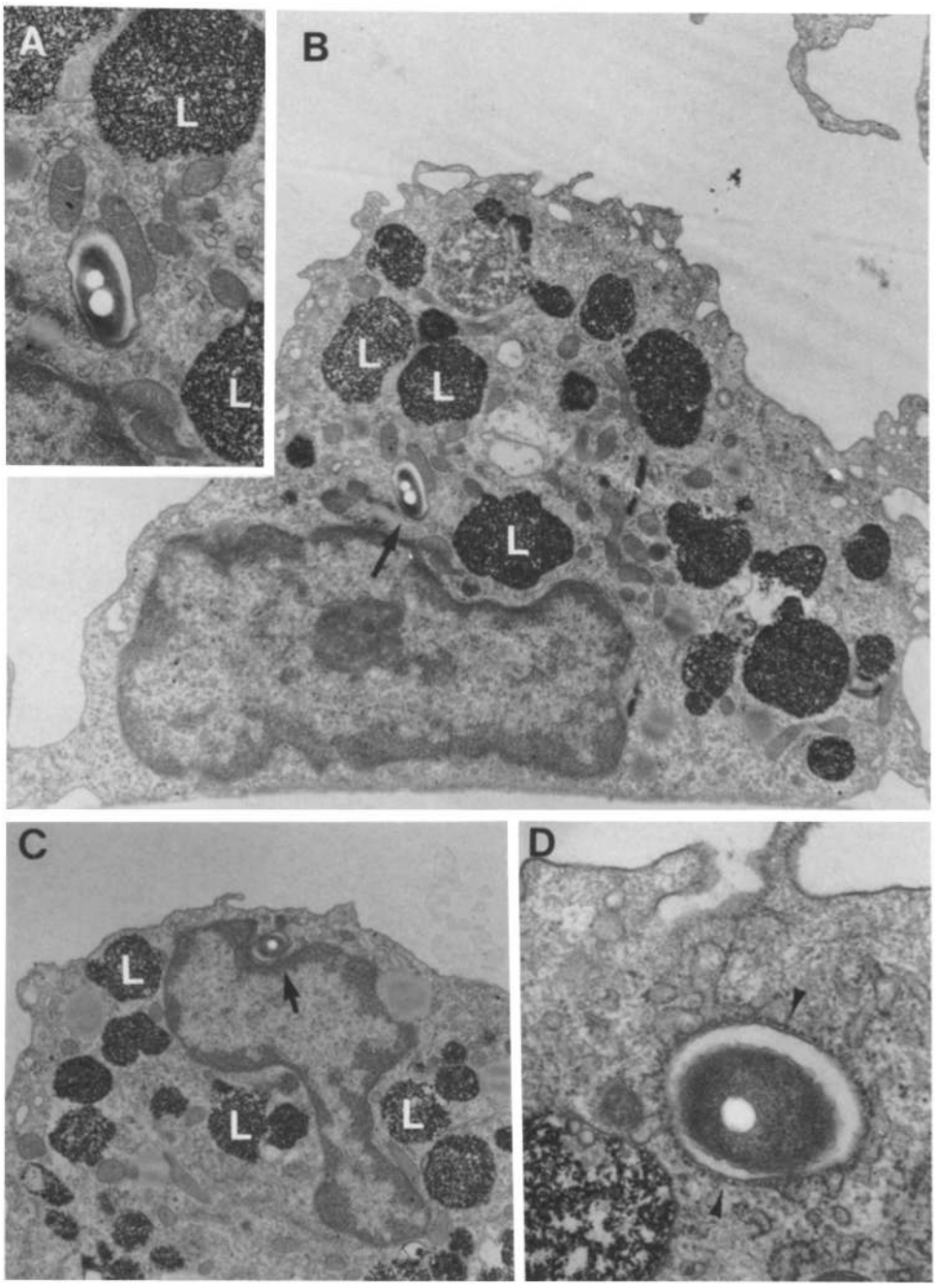
In contrast, in normal monocytes incubated with formalin-killed *L. pneumophila*, a majority of the *L. pneumophila*–containing vacuoles were fused with thorium dioxide-labeled lysosomes (Table I, lines *g* and *h*; Fig. 2, *A* and *B*). Of a total of 159 vacuoles, 120 (75%) were positive for thorium dioxide. Of the 311 bacteria in these vacuoles, 264 (85%) were in vacuoles positive for thorium dioxide. Vacuoles containing formalin-killed *L. pneumophila* frequently contained more than 1 bacterium (1–12), whereas vacuoles containing live *L. pneumophila* only occasionally had more than 1 bacterium and then only 2 bacteria.

TABLE I

L. pneumophila Phagosomes Do Not Fuse with Secondary Lysosomes

Experiment		Bacterial particle	Type of serum	Incubation period	Thorium dioxide concentration	No. monocytes examined	No. vacuoles containing bacteria	Vacuoles positive thorium dioxide		No. bacteria in vacuole	Bacteria in positive vacuoles	
								No.	%		No.	%
				h	*μl/ml*							
I.	a.	*L. pneumophila*, live	Normal	1	6.25	211	35	1	3%	36	1	3%
	b.	"	Normal	1	12.5	162	26	0	0%	30	0	0%
	c.	"	Normal	4	12.5	131	28	0	0%	31	0	0%
	d.	"	Normal	8	12.5	62	29	0	0%	38	0	0%
	e.	"	Immune	1	6.25	80	50	10	20%	51	11	22%
	f.	"	Immune	1	12.5	94	29	5	17%	29	5	17%
	g.	*L. pneumophila*, formalin-killed	Normal	1	6.25	26	116	97	83%	165	146	88%
	h.	"	Normal	1	12.5	17	43	23	53%	146	118	81%
	i.	*S. pneumoniae*, live	Normal	1	12.5	7	21	17	67%	32	25	78%
II.	j.	*L. pneumophila*, live	Normal	1	6.25	286	27	2	7%	29	3	10%
	k.	"	HI-immune	1	6.25	178	45	17	38%	57	25	44%
	l.	"	Immune	1	6.25	80	109	31	28%	120	40	33%
	m.	"	Normal	4	6.25	46	25	1	4%	26	1	4%
	n.	" (+Erythromycin)	Normal	4	6.25	66	34	3	9%	25	3	9%
	o.	*E. coli*, live	Normal	1	6.25	29	25	23	92%	18	26	93%

Freshly explanted monocytes in monolayer culture were incubated for 20 h at 37°C in 5% CO_2–95% air in 35-mm petri dishes in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and 6.25 or 12.5 μl undiluted thorium dioxide/ml of tissue culture medium. After the incubation, the monocyte monolayers were washed to remove unbound thorium dioxide and incubated an additional 3 h to allow the monocytes to ingest thorium dioxide adsorbed on the cell surface and to concentrate internalized thorium dioxide in secondary lysosomes. The cultures were then incubated on a gyratory shaker for 30 min with 10^{10} live or formalin-killed *L. pneumophila*/ml pretreated with a source of complement alone (fresh normal human serum), 10^{10} live *L. pneumophila*/ml pretreated with a source of anti–*L. pneumophila* antibody alone (heat-inactivated immune human serum), 7.5×10^8 live *L. pneumophila*/ml pretreated with a source of both anti–*L. pneumophila* antibody and complement (fresh immune human serum), 10^{10} live unencapsulated *S. pneumoniae*/ml pretreated with a source of complement (fresh normal human serum), or 10^9 live encapsulated *E. coli*/ml pretreated with a source of both anti–*E. coli* antibody and complement (fresh normal human serum). After 30 min, the monolayers were washed to remove non-monocyte–associated bacteria and incubated for an additional 1–8 h as indicated. Some cultures were preincubated, infected with *L. pneumophila*, and then incubated again in the presence of erythromycin (1.25 μg/ml), as indicated. The monolayers were then fixed and processed for electron microscopy.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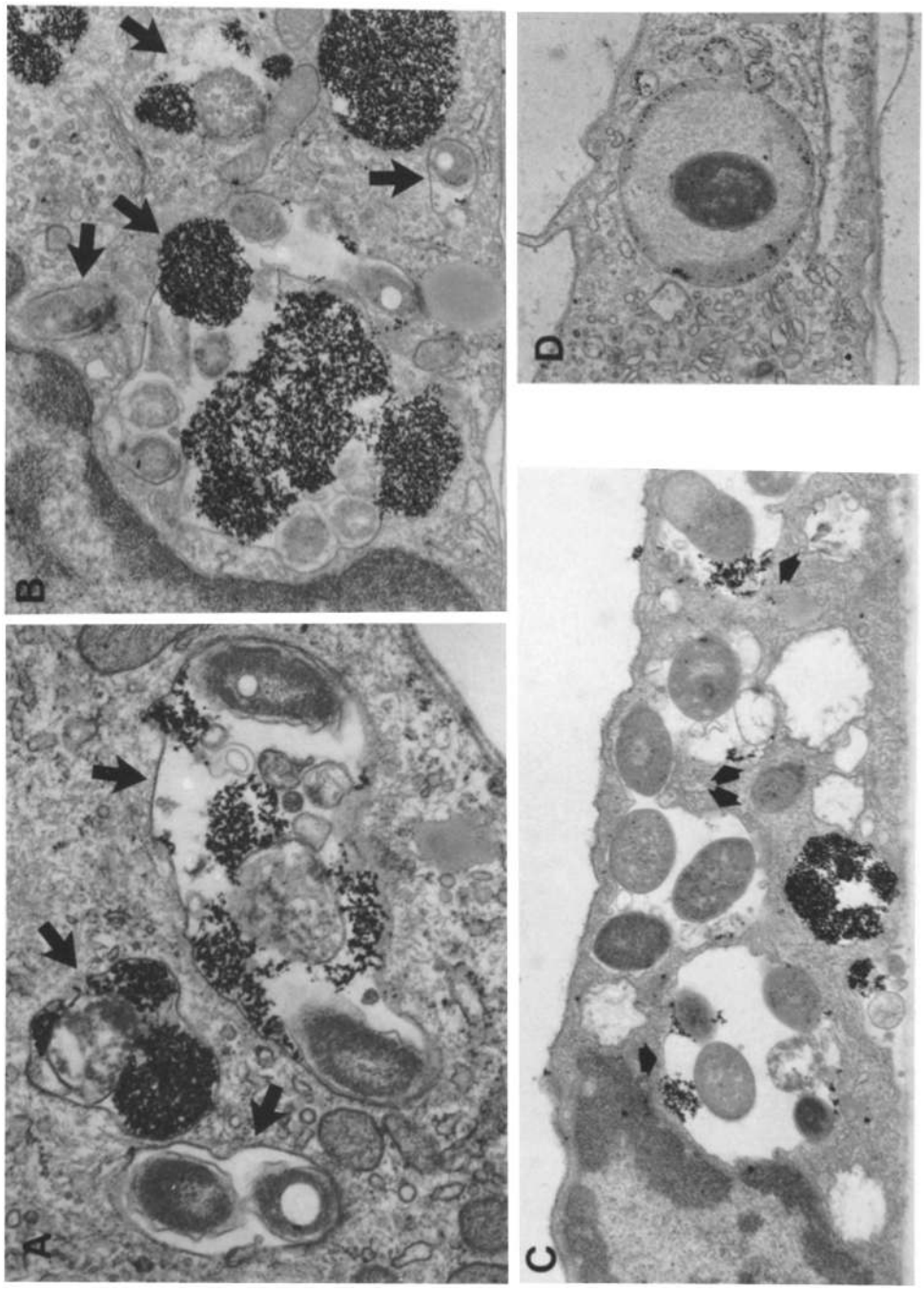
In addition to formalin-killed *L. pneumophila*, live *S. pneumoniae* and live *E. coli* were used as controls (Table I; Fig. 2, *C* and *D*). As with vacuoles containing formalin-killed *L. pneumophila*, the majority of vacuoles containing *S. pneumoniae* (67%) or *E. coli* (92%) were positive for thorium dioxide; the positive vacuoles contained 78% of all *S. pneumoniae* and 93% of all *E. coli* in vacuoles.

Fusion was also examined later after entry of *L. pneumophila* into monocytes at a time when the ribosome-lined vacuole was forming or formed; (Table I, lines *c*, *d*, and *m*; Fig. 1 *D*). At both 4 and 8 h after entry, few *L. pneumophila*–containing vacuoles were fused. Of a total of 53 vacuoles containing 57 bacteria at 4 h, only 1 vacuole containing 1 bacterium was positive for thorium dioxide. Of 29 vacuoles containing 38 *L. pneumophila* examined at 8 h, none were positive for thorium dioxide. The majority of these vacuoles, 71% at 4 h and 90% at 8 h, were studded with ribosomes.

Influence of Antibody and Complement on Fusion of L. pneumophila Phagosomes with Secondary Lysosomes. In studies discussed thus far, monocytes were infected with *L. pneumophila* in the presence of a source of complement alone. However, antibody markedly affects both phagocytosis and killing of *L. pneumophila* by monocytes (22). In the presence of complement alone, phagocytosis is relatively inefficient and monocytes do not kill or kill an insignificant proportion of an inoculum of *L. pneumophila* (22). In contrast, in the presence of both antibody and complement, phagocytosis of *L. pneumophila* is very efficient and monocytes kill approximately half of an inoculum of *L. pneumophila*; the surviving bacteria multiply in the monocytes (22). Therefore, the influence of both antibody and complement on fusion of *L. pneumophila* phagosomes with secondary lysosomes was studied. Live *L. pneumophila* were pretreated with fresh immune human serum containing high-titer anti–*L. pneumophila* antibody and complement and thorium dioxide–treated monocytes were infected with these bacteria in the presence of both antibody and complement.

Antibody significantly promoted fusion (Fig. 3). In Experiment I, in monocytes pretreated with either 6.25 or 12.5 μl/ml thorium dioxide and then infected with *L. pneumophila* in the presence of both antibody and complement, 15 (19%) of 79 *L. pneumophila*–containing vacuoles were positive for thorium dioxide

FIGURE 1. *L. pneumophila* phagosomes do not fuse with monocyte secondary lysosomes. Freshly explanted monocytes in monolayer culture were preincubated with thorium dioxide and then incubated with live *L. pneumophila* in the presence of fresh normal human serum for 30 min, as described in Materials and Methods. Afterwards, the monocyte monolayers were washed to remove non-monocyte associated bacteria, incubated for an additional 1, 4, or 8 h, and processed for electron microscopy. (*A–C*) At 1 h, *L. pneumophila* phagosomes (arrows) are found in the vicinity of thorotrast-labeled secondary lysosomes (*L*) in some monocytes (*A* and *B*) and apparently at a distance from prelabeled lysosomes in other monocytes (*C*). The *L. pneumophila* phagosome in *B* is shown at higher magnification in *A*. The phagosome is surrounded by smooth vesicles and by a mitochondrion closely apposed to the vacuolar membrane; these features are characteristic of the *L. pneumophila* phagosome at this point after infection (1). There is no thorium dioxide within the phagosome, indicating that the phagosome has not fused with thorium dioxide–labeled secondary lysosomes. *A*, × 16,700; *B*, × 8,300; *C*, × 8,400. (*D*) At 4 h, the *L. pneumophila* phagosome is studded with ribosomes (arrow heads), a characteristic of the *L. pneumophila* phagosome at this point after infection (1). As at 1 h, phagosomes at 4 h and 8 h typically are not fused with secondary lysosomes. × 24,600.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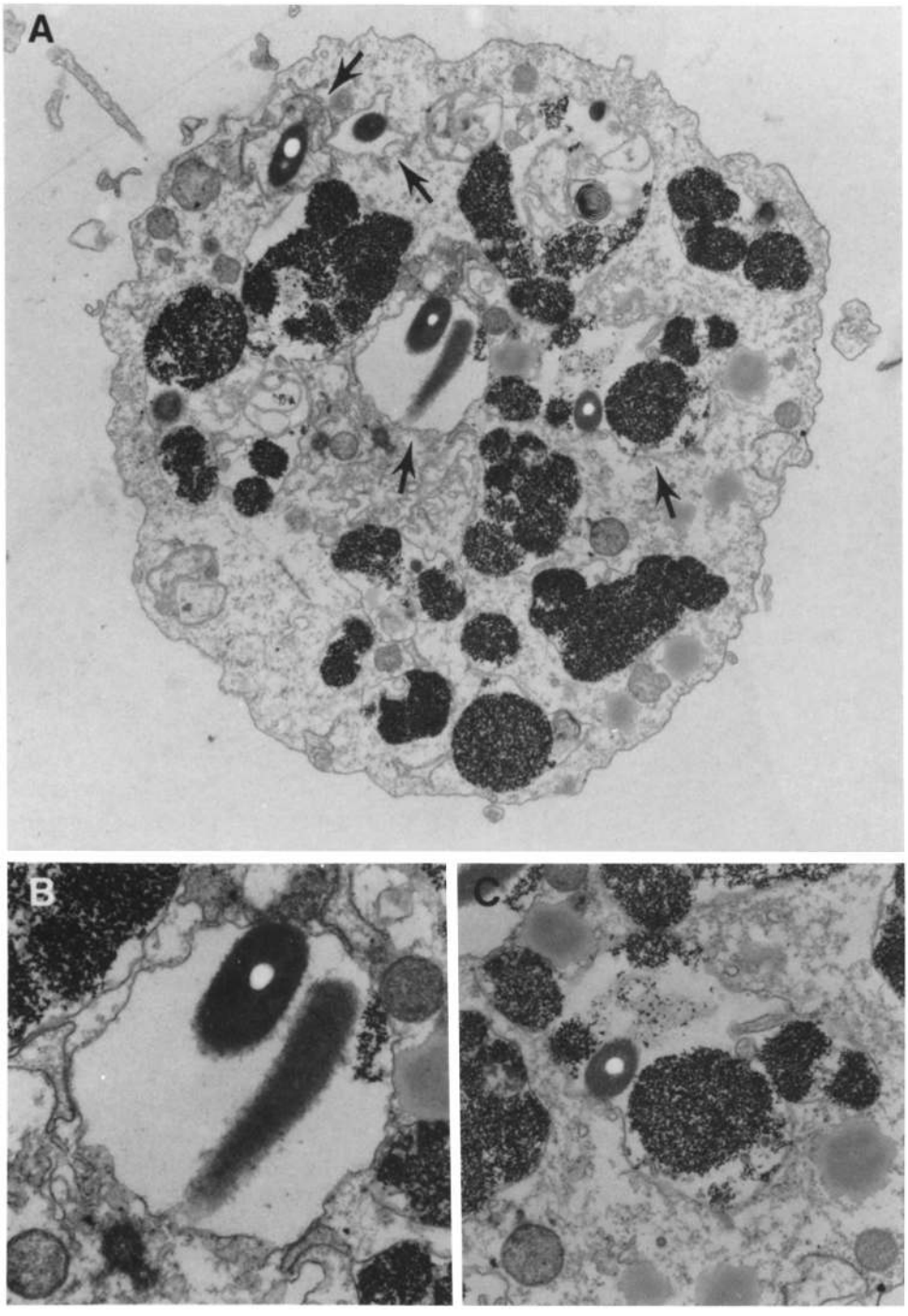
(Table I, lines *e* and *f*); the positive vacuoles contained 20% of all bacteria. This was a significant increase over the percentage of positive vacuoles (2% i.e., 1 in 61 vacuoles) in monocytes infected with *L. pneumophila* and incubated for 1 h in the presence of complement alone (Table I, lines *a* and *b*). In Experiment II, 28% of vacuoles containing 33% of the bacteria were positive in the presence of both antibody and complement, whereas only 7% of vacuoles were positive in the presence of complement alone (Table I, lines *j* and *l*). Of vacuoles that were fused with lysosomes in the presence of antibody and complement, about half resembled vacuoles containing formalin-killed *L. pneumophila* (1) in that no cytoplasmic organelles surrounded the vacuoles (Fig. 3, *B* and *C*). The other half resembled vacuoles containing live *L. pneumophila* (1) in that smooth vesicles and/or mitochondria were clustered about the vacuoles.

In the presence of antibody alone, as in the presence of complement alone, monocytes do not phagocytose *L. pneumophila* as efficiently as in the presence of both antibody and complement, and they do not kill a significant proportion of a bacterial inoculum (20). The influence of antibody alone was examined by pretreating live *L. pneumophila* with heat-inactivated immune human serum, a source of antibody alone, and then infecting monocytes with these bacteria.

Again, antibody significantly promoted fusion. In Experiment II (Table I, line *k*), with antibody alone, 38% of the vacuoles contained thorium dioxide, a proportion comparable to and somewhat greater than that found in the presence of both antibody and complement in the same experiment. Similarly, in Experiment III (Table II), antibody alone (line *k*), or antibody and complement (line *l*) resulted in increased proportions of fused vacuoles (21% and 32%, respectively) in comparison to control levels (10% and 14%; lines *f* and *h*).

Thus, although phagocytosis of *L. pneumophila* is less efficient in the presence of antibody alone than in the presence of both antibody and complement, comparable proportions of those bacteria that are ingested end up in vacuoles fused with secondary lysosomes.

FIGURE 2. Phagosomes containing formalin-killed *L. pneumophila*, live encapsulated *E. coli*, and live *S. pneumoniae* fuse with monocyte secondary lysosomes. Monocytes were cultured and preincubated with thorium dioxide as in Fig. 1. The monocytes were then incubated with formalin-killed *L. pneumophila*, live unencapsulated *S. pneumoniae*, or live encapsulated *E. coli* in the presence of fresh normal human serum for 30 min as described in Materials and Methods. Afterwards, the monocytes were washed to remove non-monocyte–associated bacteria, incubated for an additional 1 h, and processed for electron microscopy. (*A*) Three phagosomes (arrows) containing 1, 2, and 3 formalin-killed *L. pneumophila* are present in this region of monocyte cytoplasm. The phagosomes with 1 and 3 bacteria have fused with secondary lysosomes as evident by the presence of electron-opaque contrast material (thorium dioxide) within them. × 27,300. (*B*) Four phagosomes (arrows) containing formalin-killed *L. pneumophila* are present in this region of the monocyte cytoplasm; three phagosomes contain 1 bacterium each and one large phagosome contains 11 bacteria in this thin section. The large phagosome and two of the three small ones have fused with secondary lysosomes; only a little thorium dioxide is evident in the small phagosome at the base of the monocyte. The uppermost small phagosome, located near the nucleus, does not contain thorium dioxide. × 15,800. (*C*) Three large phagosomes (arrows) containing *S. pneumoniae* are present in this region of the monocyte. All three phagosomes have fused with secondary lysosomes. × 14,600. (*D*) This phagosome contains a single encapsulated *E. coli* and has fused with secondary lysosomes. × 14,600.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Influence of Erythromycin on Fusion of L. pneumophila Phagosomes with Secondary Lysosomes. Erythromycin (1.25 μg/ml), a potent inhibitor of bacterial protein synthesis, inhibits the intracellular multiplication of *L. pneumophila* within 1 h of addition to monocytes containing bacteria in the midlogarithmic phase of growth (23). The antibiotic-inhibited *L. pneumophila* are not killed by the monocytes. Upon removal of the antibiotic, these bacteria resume multiplying (23). Erythromycin has no apparent effect on the formation of the *L. pneumophila* vacuole (1). The influence of erythromycin on fusion of *L. pneumophila* phagosomes with secondary lysosomes was examined by infecting monocytes with *L. pneumophila* and incubating the cultures for 4 h in the presence or absence of erythromycin, as described in Materials and Methods.

Erythromycin had little if any effect on phagosome-lysosome fusion (Table I, lines *m* and *n*). In the presence of erythromycin, 9% of vacuoles were positive for thorium dioxide. In the absence of erythromycin, 4% of vacuoles were positive.

Influence of Monocyte Activation on Fusion of L. pneumophila Phagosomes with Secondary Lysosomes. Activated monocytes inhibit the intracellular multiplication of *L. pneumophila* (24, 25). Therefore, the influence of monocyte activation on fusion was studied by activating monocytes with Concanavalin A–induced mononuclear cell supernatants before infecting them with *L. pneumophila*.

Activation of monocytes promoted fusion of phagosomes with secondary lysosomes to a small degree (Table II; Fig. 4). In Experiment I, 16% and 14% of vacuoles in supernatant-treated monocytes incubated with 6.25 or 12.5 μl/ml thorium dioxide, respectively, were fused (lines *a* and *c*) compared with 3% and 0% of vacuoles in untreated monocytes (lines *b* and *d*). In Experiment III, 28% of vacuoles in supernatant-treated monocytes were fused (line *e*) compared with 14% of vacuoles in monocytes treated with supernatant control (line *f*). Similar results were obtained when monocytes were incubated with these supernatant preparations for 20 h, washed, and incubated without the supernatant preparations for 3 h before and during the infection (lines *g* and *h*). Interestingly, of vacuoles in activated monocytes that were fused with lysosomes, nearly all (>90%) resembled vacuoles containing live *L. pneumophila* at 1 h after infection (1) in that smooth vesicles and/or mitochondria were clustered about the vacuoles (Fig. 4*A–C*).

L. pneumophila Phagosomes Do Not Fuse with Primary or Secondary Lysosomes.

FIGURE 3. Fusion of *L. pneumophila* phagosomes with monocyte secondary lysosomes is promoted by coating the bacteria with antibody and complement. Monocytes were cultured, preincubated with thorium dioxide, infected, washed, incubated for 1 h, and processed for electron microscopy as in Fig. 1 except that the monocytes were incubated with live *L. pneumophila* pretreated with human anti-*L. pneumophila* antibody and complement. (*A*) At least four phagosomes containing antibody- and complement-coated *L. pneumophila* are present in this cross section of the monocyte (arrows); three phagosomes contain 1 bacterium each and one phagosome contains 2 bacteria. The two lower phagosomes have fused with secondary lysosomes. × 11,100. (*B* and *C*) Two phagosomes in *A* that have fused with secondary lysosomes are shown at higher magnification. The phagosomes are not surrounded by either smooth vesicles or mitochondria closely apposed to the phagosome membrane. *B*, × 25,000; *C*, × 16,700.

TABLE II

Influence of Monocyte Activation on Fusion of L. pneumophila Phagosomes with Secondary Lysosomes

Experiment	Bacterial particle	Type of serum	Treatment of monocytes	Incubation period	Thorium dioxide concentration	No. monocytes examined	No. vacuoles containing bacteria	Vacuoles positive thorium dioxide		No. bacteria in vacuoles	Bacteria in positive vacuoles	
								No.	%		No.	%
				h	*μl/ml*							
I.	a. *L. pneumophila*, live	Normal	Supernatant	1	6.25	202	71	10	14	74	10	14
	b. *L. pneumophila*, live	Normal	Buffer*	1	6.25	211	35	1	3	36	1	3
	c. *L. pneumophila*, live	Normal	Supernatant	1	12.5	117	32	5	16	33	6	18
	d. *L. pneumophila*, live	Normal	Buffer*	1	12.5	162	26	0	0	30	0	0
III.	e. *L. pneumophila*, live	Normal	Supernatant	1	6.25	566	104	29	28	107	31	29
	f. *L. pneumophila*, live	Normal	Sup. control	1	6.25	319	51	7	14	51	7	14
	g. *L. pneumophila*, live	Normal	Supernatant‡	1	6.25	293	48	14	29	54	17	31
	h. *L. pneumophila*, live	Normal	Sup. control‡	1	6.25	364	52	5	10	53	5	9
	i. *L. pneumophila*, formalin-killed	Normal	Supernatant	1	6.25	26	55	55	100	149	149	100
	j. *L. pneumophila*, formalin-killed	Normal	Sup. control	1	6.25	31	54	54	100	144	144	100
	k. *L. pneumophila*, live	HI-Immune	Buffer	1	6.25	145	34	7	21	35	7	20
	l. *L. pneumophila*, live	Immune	Buffer	1	6.25	154	25	8	32	28	9	32

Freshly explanted monocytes were prepared as described in the legend to Table I except that in addition to thorium dioxide, cultures were incubated with Con A supernatant, Con A supernatant control (Sup. Control), or buffer control as indicated. Monocytes were incubated with live or formalin-killed *L. pneumophila* at the same concentrations as in Table I in the presence of fresh normal human serum. For purposes of comparison, monocytes were also incubated with live *L. pneumophila* pretreated with a source of antibody alone (heat-inactivated immune serum) or a source of both antibody and complement (fresh immune serum) as in Table I (Experiment III, lines *k* and *l*). The monolayers were then fixed and processed for electron microscopy.

* These data transcribed from Table I.

‡ Cultures incubated for 20 h with Con A supernatant or supernatant control, washed, and incubated without these supernatant preparations for 3 h before and during infection.

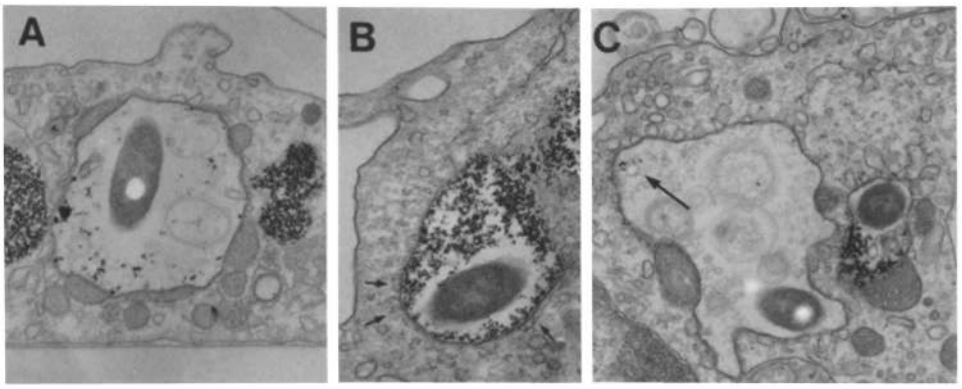


FIGURE 4. Activating monocytes promotes fusion of a small proportion of phagosomes with monocyte secondary lysosomes. Monocytes were cultured as in Figs. 1–3 except that in addition to thorium dioxide they were incubated for 20 h with Concanavalin A–induced mononuclear cell supernatants to activate them. The monocytes were then infected, washed, incubated for 1 h, and processed for electron microscopy as in Fig. 1. (*A*) The phagosome has fused with thorium dioxide–labeled secondary lysosomes. The phagosome is surrounded by smooth vesicles and 5 mitochondria closely apposed to the phagosome membrane. × 14,300. (*B*) The phagosome has fused with secondary lysosomes. Smooth vesicles line segments of the phagosome membrane (arrows). × 23,300. (*C*) Two phagosomes are present in this region of monocyte cytoplasm. Both have fused with secondary lysosomes; the larger phagosome contains only a little thorium dioxide (arrow). Both phagosomes are lined by smooth vesicles and at least 1 mitochondrium. × 17,900.

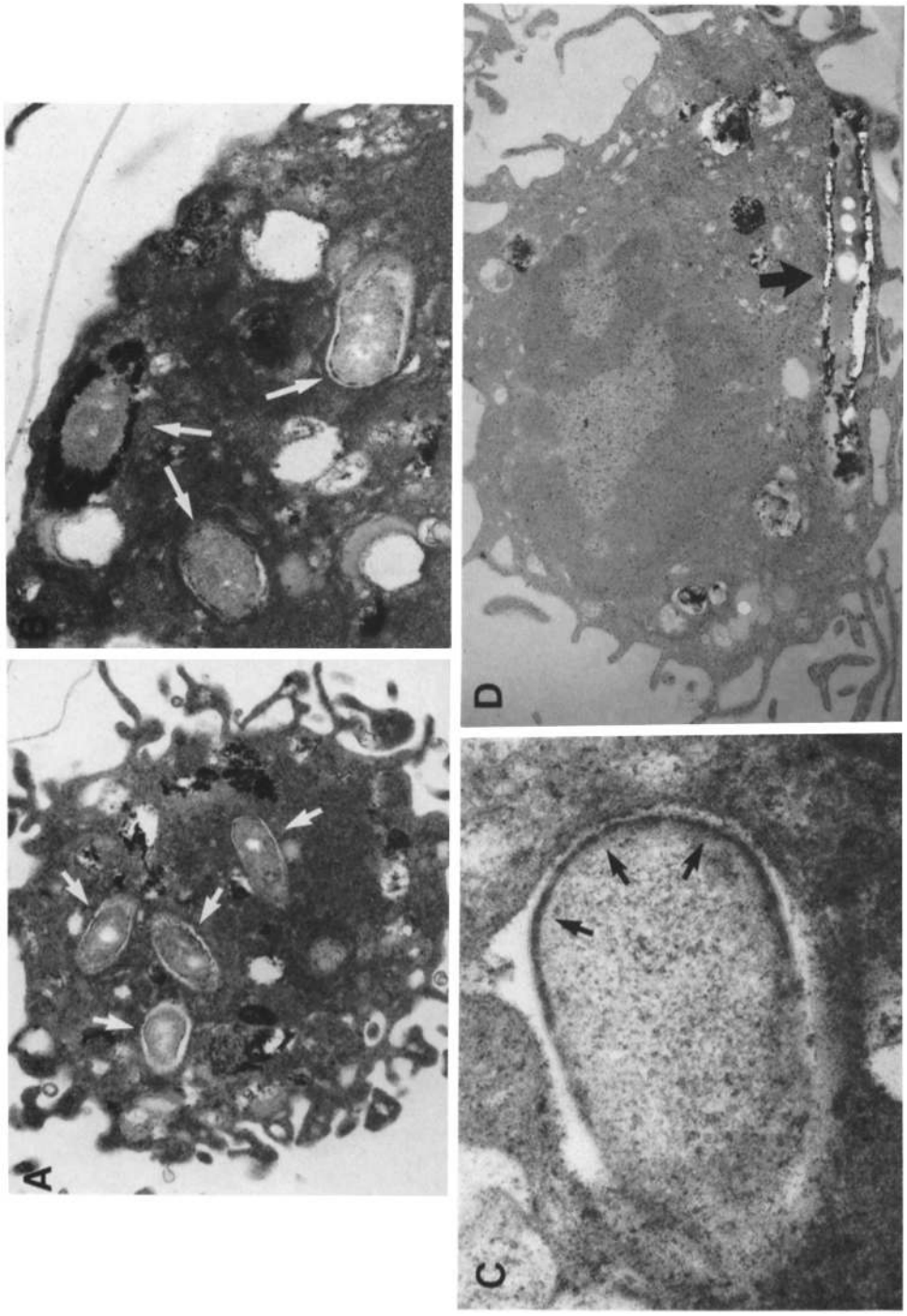
TABLE III

Study of Fusion between L. pneumophila Phagosomes and Monocyte Lysosomes by Acid Phosphatase Cytochemistry

Bacterial particle	CMP	No. monocytes examined	No. vacuoles containing bacteria	Vacuoles positive for acid phosphatase		No. bacteria in vacuoles	Bacteria in positive vacuoles	
				No.	%		No.	%
a. *L. pneumophila*, live	+	78	78	8	10%	79	8	10%
b. *L. pneumophila*, live	−	21	28	0	0%	28	0	0%
c. *L. pneumophila*, formalin-killed	+	62	76	64	84%	271	243	90%
d. *L. pneumophila*, formalin-killed	−	20	51	0	0%	148	0	0%

Freshly explanted monocytes in monolayer culture were incubated for 20 h at 37°C in 5% CO_2–95% air in 35-mm petri dishes in 2 ml RPMI 1640 medium containing 15% fresh normal human serum. The cultures were then incubated on a gyratory shaker for 40 min with 10^{10} live or formalin-killed *L. pneumophila*, washed to remove non-monocyte–associated bacteria, and incubated for an additional 1 h. The monolayers were then gently fixed with glutaraldehyde and incubated with complete acid phosphatase substrate containing cytidine 5′-monophosphate (CMP+), or incomplete acid phosphatase substrate not containing cytidine 5′-monophosphate (CMP−), and lead nitrate as the capture reagent. The monolayers were then processed for electron microscopy.

Although phagosomes containing live *L. pneumophila* do not fuse with secondary lysosomes, these experiments do not exclude fusion with primary lysosomes. This was investigated next by staining the vacuole for acid phosphatase, an enzyme found in monocyte lysosomes. Monocytes were infected with *L. pneumophila*,


A
B
C
D

gently fixed, and stained for acid phosphatase, using cytidine 5′-monophosphate as substrate and lead nitrate as the capture reagent, as described in Materials and Methods. The *L. pneumophila*–containing vacuoles were then examined for the presence of acid phosphatase, as indicated by the presence of electron-opaque lead phosphate reaction product.

In monocytes infected and incubated with live *L. pneumophila* for 1 h, few *L. pneumophila*–containing vacuoles had fused with lysosomes, i.e. few phagosomes contained lead phosphate reaction product in the space between the bacteria and phagosome membrane (Table III; Fig. 5, *A–C*). Of 78 vacuoles examined, only 10% were positive for acid phosphatase (line *a*). In contrast, in monocytes incubated with formalin-killed *L. pneumophila*, 84% of vacuoles were fused (Table III, line *c*; Fig. 5*D*). Control monocytes in the absence of cytidine 5′-monophosphate substrate were uniformly negative (lines *b* and *d*).

Interestingly, 79% of live *L. pneumophila* in vacuoles negative for acid phosphatase in the space betweeen the bacterial surface and the vacuolar membrane had a thin layer of lead phosphate reaction product between the inner and outer bacterial membranes (Fig. 5, *A–C*). This thin layer was absent in control preparations in which the cytidine 5′-monophosphate substrate was omitted. The reaction product indicates the presence of a bacterial acid phosphatase, presumably located in the periplasmic space.

Thus, phagosomes containing live *L. pneumophila* do not fuse with either primary or secondary lysosomes.

FIGURE 5. Interactions between *L. pneumophila* phagosomes and lysosomes as studied by acid phosphatase cytochemistry. Monocytes in monolayer culture were incubated with live or formalin-killed *L. pneumophila* for 40 min, washed to remove non-monocyte–associated bacteria, and incubated for an additional 1 h. The monolayers were then gently fixed with glutaraldehyde, stained for acid phosphatase using cytidine 5′-monophosphate as substrate and lead nitrate as capture reagent, and processed for electron microscopy, as described in Materials and Methods. (*A*) Four *L. pneumophila* phagosomes (arrows) are present in this cross section of a monocyte infected with live *L. pneumophila*. None of the phagosomes has fused with lysosomes as evidenced by the absence of electron-opaque lead phosphate reaction product in the space between the bacteria and the phagosome membrane. Lead phosphate reaction product is present internal to the bacteria; this is seen at higher magnification in *B* and *C*. × 13,700. (*B*) Three *L. pneumophila* phagosomes (arrows) are present in this region of the cytoplasm of a monocyte infected with live *L. pneumophila*. The two lower phagosomes have not fused with lysosomes; as in *A*, lead phosphate reaction product is located internal to the bacteria but not in the space between the bacteria and phagosome membrane. In contrast, the upper phagosome has fused with lysosomes as evidenced by the presence of electron opaque lead phosphate reaction product in the space between the bacteria and the phagosome membrane. In monocytes infected with live *L. pneumophila*, few phagosomes were found fused with lysosomes. × 23,700. (*C*) This is a high magnification of a *L. pneumophila* phagosome in a monocyte infected with live *L. pneumophila*. The phagosome has not fused as evidenced by the absence of lead phosphate reaction product in the space between the bacterium and the phagosome membrane. However, reaction product is present internal to the bacterium between the inner and outer bacterial membranes (arrows). This indicates the presence of a bacterial acid phosphatase, presumably located in the periplasmic space. × 91,000. (*D*) One *L. pneumophila* phagosome (arrow) is present in this cross-section of a monocyte incubated with formalin-killed *L. pneumophila*. As is typical of phagosomes containing formalin-killed *L. pneumophila*, this phagosome has fused with lysosomes. × 13,700.

Discussion

This study demonstrates that *L. pneumophila* belongs to a select group of intracellular parasites whose phagosomes do not fuse with lysosomes. These parasites include the human pathogens *M. tuberculosis* (3 and 4), *T. gondii* (2), and *C. psittaci* (6). In the case of these other human pathogens, mouse peritoneal macrophages or mouse cell lines were used in studies of phagosome-lysosome fusion. In this study, human monocytes were used and *L. pneumophila* phagosomes were found not to fuse with either primary or secondary lysosomes of human monocytes.

In addition to a capacity to inhibit phagosome-lysosome fusion, phagosomes containing *C. psittaci* and *T. gondii* share with *L. pneumophila* certain unusual morphologic features. The phagosomes are surrounded by smooth vesicles, mitochondria, or ribosomes closely apposed to the phagosomal membrane (1). This suggests that a common mechanism may underlie the cytoplasmic reaction about the phagosomes and the capacity to inhibit phagosome-lysosome fusion.

Formalin-killed *L. pneumophila* do not share with live *L. pneumophila* the capacity to inhibit fusion. This suggests that a surface structure is not responsible for the inhibition of fusion by live bacteria unless such a structure is critically altered by formalin treatment. However, formalin treatment does not eliminate *L. pneumophila*'s capacity to induce proliferation and production of monocyte activating cytokines by lymphocytes from patients recovered from Legionnaires' disease (25). Also, formalin-treated *L. pneumophila* are recognized about as well as heat-killed *L. pneumophila* by anti-*L. pneumophila* antibody in immune human serum (26). As with formalin-killed *L. pneumophila*, glutaraldehyde-fixed *T. gondii* (2) and heat-killed *C. psittaci* (6) fail to inhibit phagosome-lysosome fusion.

Erythromycin, a potent inhibitor of bacterial protein synthesis, at concentrations that completely inhibit intracellular multiplication of *L. pneumophila*, has no effect on phagosome-lysosome fusion. This suggests that ongoing bacterial protein synthesis is not required for inhibition of fusion. Similarly, chloramphenicol, a potent inhibitor of chlamydial protein synthesis was found to have no apparent effect on *C. psittaci* inhibition of phagosome-lysosome fusion (6).

Coating live *L. pneumophila* with antibody, with or without complement, promotes fusion with lysosomes of a proportion of the bacterial phagosomes. Antibody has also been reported to promote fusion with lysosomes of phagosomes containing *M. tuberculosis* (27), *T. gondii* (28), *C. psittaci* (6), and *E. cuniculi* (8). The fact that antibody and complement also promote monocyte killing of a proportion (~50%) of an inoculum of *L. pneumophila*, taken together with the fact that formalin-killed *L. pneumophila* are found in fused phagosomes, suggests that the antibody and complement-coated bacteria that are killed are the ones found in fused phagosomes. If so, then the study does not indicate whether fusion precedes or follows bacterial death.

Activation of the monocytes also promoted fusion of a small proportion of *L. pneumophila* phagosomes. In vivo activation of mouse macrophages has also been reported to promote fusion of lysosomes with yeast-containing phagosomes (29). The *L. pneumophila* phagosomes that had fused were virtually always surrounded by smooth vesicles and/or mitochondria, a hallmark of phagosomes containing live bacteria 1 h after entry into monocytes (1). This suggests that the bacteria

were still alive or alive for a substantial proportion of their first hour in the monocyte. What relationship phagosome-lysosome fusion has to the capacity of activated monocytes to inhibit *L. pneumophila* intracellular multiplication is not clear from this study. It is possible that bacteria in fused phagosomes are killed or inhibited from multiplying. However, only a small proportion of phagosomes in activated monocytes are fused at 1 h after infection. Thus, it is not clear that phagosome-lysosome fusion in activated monocytes is sufficiently extensive to account for the strong inhibition of *L. pneumophila* multiplication.

The mechanism by which intracellular parasites inhibit phagosome-lysosome fusion is unknown. Moreover, although this phenomenon is commonly referred to as inhibition of phagosome-lysosome fusion, it is not known whether all such parasites possess an inhibitory factor per se or lack a fusion-promoting factor. In the case of *M. tuberculosis*, two fusion inhibitory factors have been suggested. First, sulfatides of *M. tuberculosis* (anionic trehalose glycolipids), which share a polyanionic character with substances that inhibit phagosome-lysosome fusion (30), were suspected of playing an antifusion role (31). These sulfatides, when taken up into secondary lysosomes of mouse macrophages or when attached to target yeast particles before ingestion by mouse macrophages, were found to inhibit fusion of secondary lysosomes with yeast-containing phagosomes(31). Second, ammonia production by *M. tuberculosis* was suspected of playing an antifusion role when filtrates from cultures of tubercule bacilli, which contain high concentrations of ammonia, were found to inhibit fusion of mouse macrophage secondary lysosomes with yeast-containing phagosomes (32). Ammonia was found to mimic the antifusion effect of the culture filtrates and the ammonia content of the filtrates appeared sufficient to account for their antifusion effect (32). Whether *L. pneumophila* or other intracellular parasites possess antifusion substances or have the capacity to release large amounts of ammonia or other amines that may exert an antifusion effect remains to be determined.

The high correlation between monocyte killing of *L. pneumophila* and phagosome-lysosome fusion—both increased in the presence of antibody and complement—suggests the possibility that inhibition of fusion may be an important mechanism by which *L. pneumophila* resists host cell microbicidal agents. Further evidence for this comes from studies of the susceptibility of *L. pneumophila* to oxygen metabolites, in particular hydrogen peroxide, generated by phagocytes. In the presence of myeloperoxidase and a halide, *L. pneumophila* is susceptible to killing by concentrations of hydrogen peroxide to which it is otherwise resistant (33, 34). Similarly, in the presence of lactoperoxidase and a halide, the concentration of hydrogen peroxide that kills 50% of an inoculum of *L. pneumophila* is reduced by 100-fold (M. A. Horwitz, unpublished data). Therefore, one way by which inhibition of phagosome-lysosome fusion may enhance *L. pneumophila* survival in monocytes and perhaps polymorphonuclear leukocytes is by blocking access of lysosomal or granule myeloperoxidase to the *L. pneumophila* phagosome, thereby protecting the bacterium from otherwise lethal concentrations of hydrogen peroxide generated by the phagocyte.

Summary

The interactions between the *L. pneumophila* phagosome and monocyte lysosomes were investigated by prelabeling the lysosomes with thorium dioxide, an

electron-opaque colloidal marker, and by acid phosphatase cytochemistry.

Phagosomes containing live *L. pneumophila* did not fuse with secondary lysosomes at 1 h after entry into monocytes or at 4 or 8 h after entry by which time the ribosome-lined *L. pneumophila* replicative vacuole had formed. In contrast, the majority of phagosomes containing formalin-killed *L. pneumophila*, live *Streptococcus pneumoniae*, and live *Escherichia coli* had fused with secondary lysosomes by 1 h after entry into monocytes.

Erythromycin, a potent inhibitor of bacterial protein synthesis, at a concentration that completely inhibits *L. pneumophila* intracellular multiplication, had no influence on fusion of *L. pneumophila* phagosomes with secondary lysosomes. However, coating live *L. pneumophila* with antibody or with antibody and complement partially overcame the inhibition of fusion. Also activating the monocytes promoted fusion of a small proportion of phagosomes containing live *L. pneumophila* with secondary lysosomes.

Acid phosphatase cytochemistry revealed that phagosomes containing live *L. pneumophila* did not fuse with either primary or secondary lysosomes. In contrast to phagosomes containing live bacteria, the majority of phagosomes containing formalin-killed *L. pneumophila* were fused with lysosomes by acid phosphatase cytochemistry.

The capacity of *L. pneumophila* to inhibit phagosome-lysosome fusion may be a critical mechanism by which the bacterium resists monocyte microbicidal effects.

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