

FORMATION OF A NOVEL PHAGOSOME BY THE
LEGIONNAIRES' DISEASE BACTERIUM (*LEGIONELLA*
PNEUMOPHILA) IN HUMAN MONOCYTES*

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Legionella pneumophila, the agent of Legionnaires' disease, is a facultative intracellular gram-negative bacterial pathogen (1). The bacterium multiplies intracellularly in human blood monocytes (1) and human alveolar macrophages (2), and under tissue culture conditions, multiplication is exclusively intracellular.

L. pneumophila enters monocytes by an unusual process—a monocyte pseudopod coils around the bacterium as the organism is ingested.¹ Thereafter, the bacterium resides in a membrane-bound cytoplasmic vacuole. Immediately after phagocytosis, cellular organelles do not appear about the *L. pneumophila* vacuole.¹ Later on, however, the bacteria appear to be multiplying within a highly unusual vacuole. A striking feature of this vacuole is that the cytoplasmic side is studded with host cell ribosomes (1). This morphologic feature has also been observed on electron microscopic examination of leukocytes in human lung tissue specimens from patients with Legionnaires' disease (3).

In this paper, the formation of the unusual vacuole in which *L. pneumophila* multiplies within monocytes is examined. This study will show that the formation of the vacuole occurs during the first 4–8 h after phagocytosis and that it involves a complex sequence of cytoplasmic events.

Materials and Methods

Media. Egg yolk buffer and RPMI 1640 medium were prepared or obtained as described previously (1). No antibiotics were added to medium in any of the experiments except where specifically indicated.

Reagents. Formaldehyde solution, 37% wt/wt was obtained from Fisher Scientific Company, Fair Lawn, NJ. Erythromycin base suitable for antimicrobial susceptibility testing (Abbott Laboratories, North Chicago, IL), 980 µg activity/mg, was dissolved in methanol, diluted in water to 1,000 µg activity/ml and stored in small aliquots in vials at –70°C. On the day of the experiments, the frozen preparation was thawed and diluted to the desired concentration in RPMI 1640 medium.

Agar. Modified charcoal yeast extract agar was prepared in 100-mm × 15-mm bacteriologic petri dishes as described (1).

* Supported by grant AI 17254 and Biomedical Research Support grant S07 RR07065 from the National Institutes of Health.

[‡] Dr. Horwitz was the recipient of an American Cancer Society Junior Faculty Research Award and a John A. and George L. Hartford Foundation Fellowship.

¹ Horwitz, M. A. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism. Manuscript in preparation.

Serum. Venous blood was obtained and clotted, and the serum was separated and stored at -70°C until used as described (4). Normal (nonimmune) human serum (type AB) with an indirect fluorescent antibody anti-*L. pneumophila* titer (5) of $<1:64$ was obtained from an adult donor not known to have ever had Legionnaires' disease.

Human Blood Mononuclear Cells. Mononuclear cells used in experiments with *L. pneumophila* were obtained from the blood of a normal adult donor not known to have had Legionnaires' disease and with an indirect fluorescent antibody anti-*L. pneumophila* titer of $<1:64$. The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution as previously described (1); the cells were $>99\%$ viable by trypan blue exclusion. Examination of a stained cytocentrifuged sample revealed that the mononuclear cell fraction contained $\sim 40\%$ monocytes, 58.5% lymphocytes, and 1.5% polymorphonuclear leukocytes.

Bacteria. *L. pneumophila*, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, and stored at -70°C , as described (1). Bacteria used in studies were obtained by culturing egg yolk-grown *L. pneumophila* one time only on modified charcoal yeast extract agar for 80 h. The bacteria were then harvested into egg yolk buffer, washed by centrifugation at $12,000\text{ g}$ for 10 min at 4°C , counted in a Petroff-hausser chamber (Arthur H. Thomas Co., Philadelphia, PA), and resuspended in RPMI 1640 medium at 10^{10} bacterial particles/ml. These bacteria were $75\text{--}100\%$ viable as determined by measuring the number of colony-forming units in the bacterial suspension (1). Formalin-killed *L. pneumophila* were prepared from the same batch of once-passed agar-grown bacteria by incubating these bacteria with 2% formalin for 30 min at 4°C in shaking suspension, washing the bacteria four times by centrifugation at $12,000\text{ g}$ for 10 min, and resuspending the bacteria in RPMI 1640 medium to 10^{10} bacterial particles/ml. The formalin treatment killed 100% of the bacteria as measured by assaying colony-forming units of the treated suspension.

Infection of Monocytes. Mononuclear cells ($6 \times 10^6/\text{ml}$) were incubated in 35-mm petri dishes in 2 ml RPMI 1640 medium containing 10% fresh normal human serum for 1.5 h at 37°C in $5\% \text{CO}_2\text{--}95\%$ 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 overnight in 2 ml RPMI 1640 medium containing 15% fresh normal human serum. The next day the culture medium was removed and the monocyte monolayer was incubated with 10^{10} live or formalin-killed *L. pneumophila* in 2 ml RPMI 1640 medium containing 15% fresh normal human serum for 15 min at 37°C in $5\% \text{CO}_2\text{--}95\%$ air on a gyratory shaker at 100 rpm. At the end of the incubation, the monolayers were washed four times with RPMI 1640 medium to remove non-monocyte-associated bacteria. The monolayers were then incubated under stationary conditions in 2 ml RPMI 1640 medium containing 15% fresh normal human serum for 0–8 h, until fixed for electron microscopy.

To examine the effect of erythromycin, monocytes were prepared as above except they were incubated with erythromycin ($1.25\text{ }\mu\text{g}/\text{ml}$) for 1 h before and during infection with *L. pneumophila*. After infection, the monocytes were washed quickly and immediately incubated again in the same concentration of erythromycin. In one experiment, erythromycin was first added to monocytes immediately after infection.

Electron Microscopy. The infected monocyte monolayers were fixed for electron microscopy with a solution consisting of $1\% \text{OsO}_4$ (two parts) and 2.5% glutaraldehyde (one part) in 0.1 M cacodylate buffer, pH 7.4, stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer, pH 6.3, dehydrated with ethanol, released from the surface of the petri dishes with propylene oxide, and embedded in Epon (Shell Chemical Co., Houston, TX) as described (6). The sections were stained with lead citrate (7) and uranyl acetate and examined with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) or a JEOL JEM-100CX electron microscope (JEOL, Ltd., Tokyo, Japan).

Results

Early Events in the Formation of the L. pneumophila Phagosome. The early events in formation of the *L. pneumophila* intracellular vacuole in monocytes were studied by incubating monocytes in monolayer culture with a high concentration

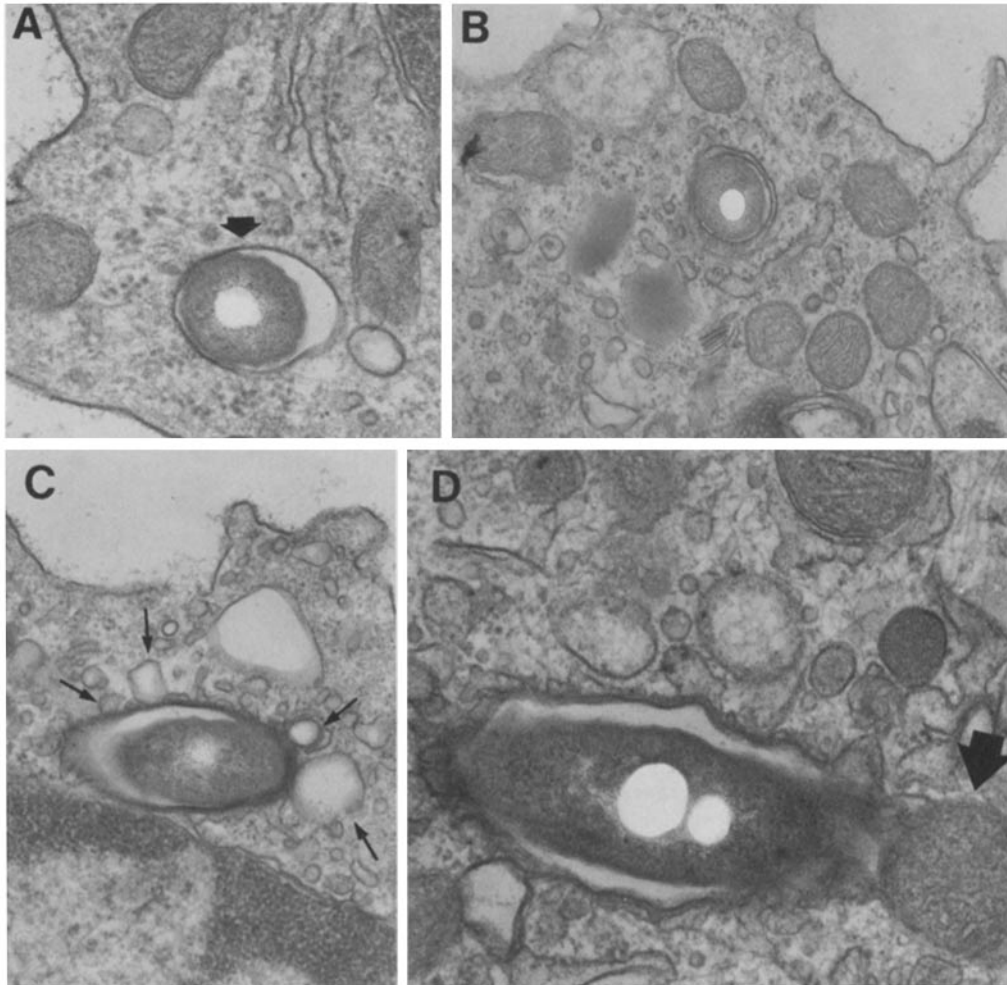
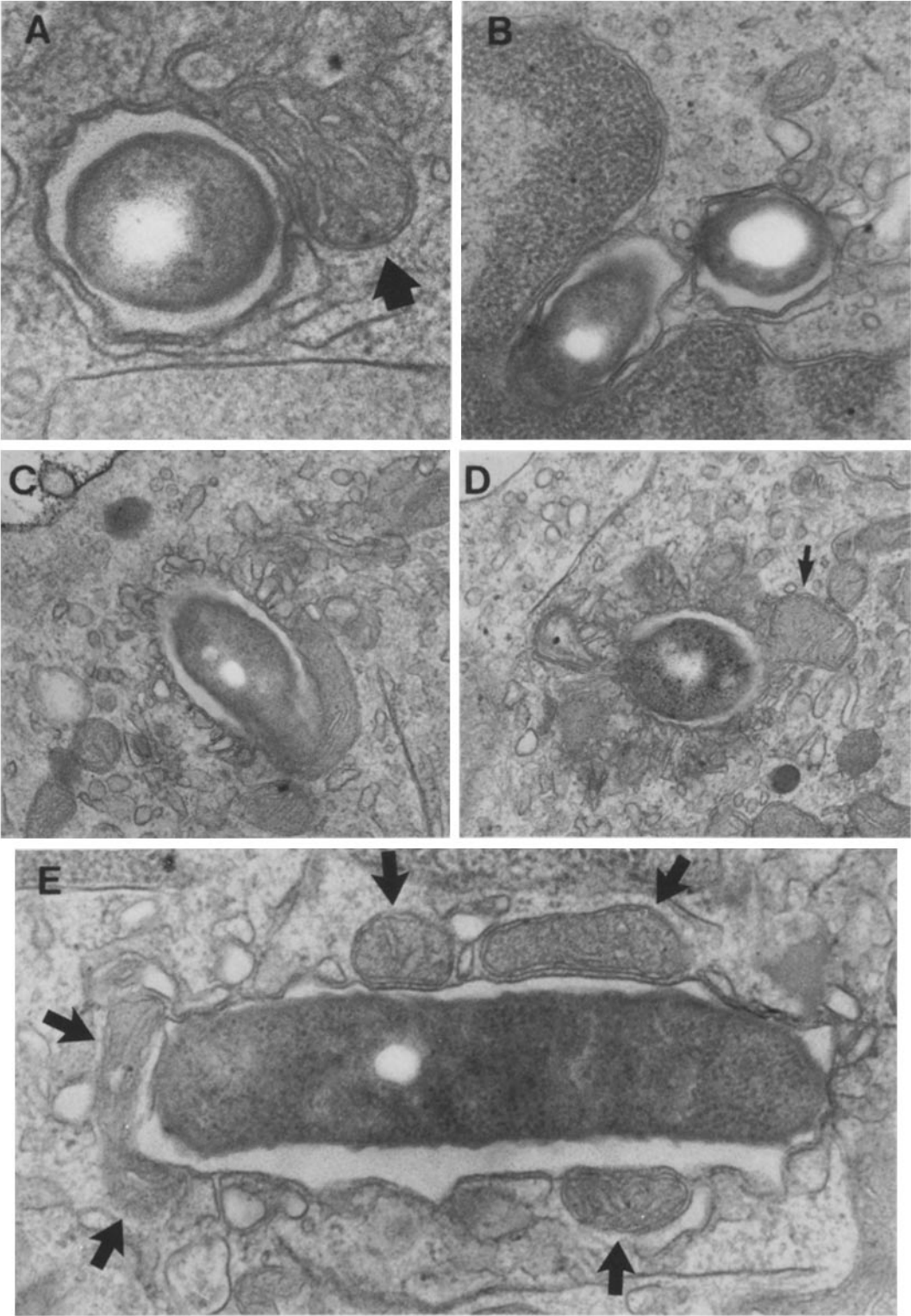


FIGURE 1. Morphology of *L. pneumophila*-containing vacuoles in monocytes 15 min after infection. Monocyte monolayers were cultured overnight at 37°C in 5% CO₂-95% air in RPMI 1640 medium containing 15% fresh normal human serum, incubated with 5×10^9 live *L. pneumophila*/ml on a gyratory shaker for 15 min, and fixed for electron microscopy. All intracellular bacteria were found in membrane-bound cytoplasmic vacuoles. (A) The vacuole (arrow) containing *L. pneumophila* has no cellular organelles about it. As is frequently the case, a lucent vacuole is present within the bacterium. $\times 52,000$. (B) Mitochondria are in the vicinity of the *L. pneumophila* vacuole. $\times 27,000$. (C) The *L. pneumophila* vacuole is surrounded by smooth vesicles (arrows) apparently fusing with or budding off from the vacuolar membrane. $\times 32,400$. (D) The *L. pneumophila* vacuole is surrounded by smooth vesicles and by a mitochondrion (arrow) closely apposed to the vacuolar membrane. $\times 48,600$.

of live *L. pneumophila* (bacteria/monocyte ratio $\sim 5,000:1$) for 15 min, washing the monocytes quickly, and immediately fixing them for electron microscopy. The high bacteria/monocyte ratio was required to obtain an adequate number of monocyte-associated bacteria in random ultrathin sections of adherent monocytes.

All intracellular bacteria were found in membrane-bound cytoplasmic vacuoles



and none was found free in the cytoplasm. Nearly all (>95%) vacuoles contained a single bacterium; and the remainder contained two bacteria. This would remain the case until 8 h after infection.

Three types of *L. pneumophila*-containing vacuoles were observed at 15 min. A minority (<5%) showed no apparent reaction about the vacuolar membrane (Fig. 1, A) and thus resembled vacuoles observed within 3 min of phagocytosis. >95% of vacuoles were surrounded by smooth vesicles apparently fusing with or budding off from the vacuolar membrane (Fig. 1, B–D). Finally, 30% of vacuoles were surrounded by mitochondria closely apposed to the vacuolar membrane (see Fig. 1, D). Usually, smooth vesicles were also present about these vacuoles.

Frequently, when mitochondria were not found adjacent to the vacuolar membrane, they were found in the immediate vicinity of the vacuole (Fig. 1, B). Such mitochondria may have been destined for the *L. pneumophila* phagosome since later after infection, at 1 h, an even higher proportion of vacuoles were surrounded by mitochondria than at 15 min.

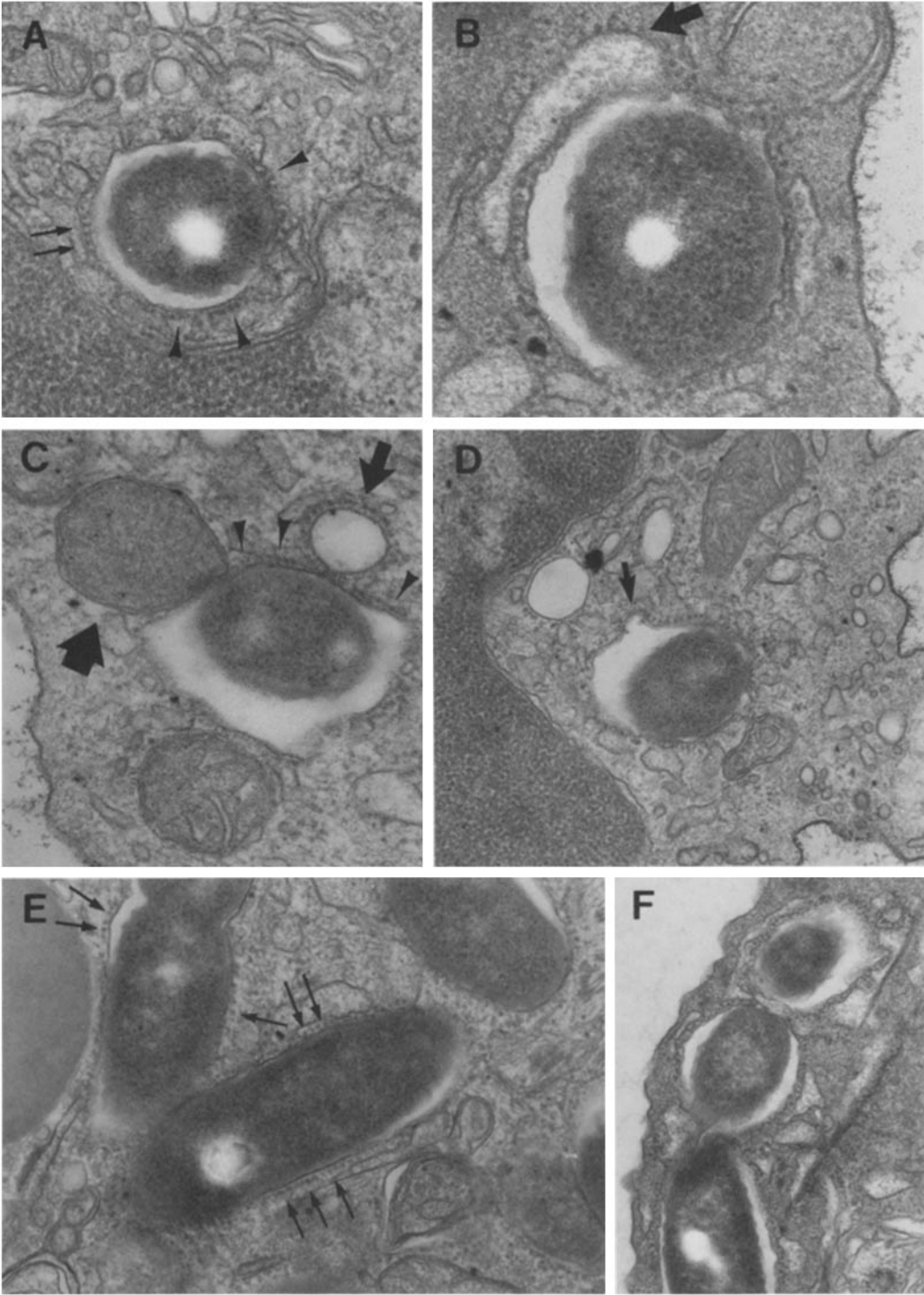
Vacuoles were found at both the base and apex of the adherent monocytes. Only 10% remained near the plasma membrane; 55% were located midway between the plasma membrane and nucleus, and ~35% were located near the nucleus.

Evolution of the L. pneumophila Phagosome. Later events in vacuole formation were studied by examining *L. pneumophila* phagosomes in monocytes 1 h, 4 h, or 8 h after onset of infection.

At 1 h after infection, all vacuoles had cytoplasmic organelles surrounding the vacuolar membrane. Virtually all vacuoles were still surrounded by smooth vesicles, but now the majority of vacuoles (65%) were also surrounded by at least 1 mitochondrion closely apposed to the vacuolar membrane (Fig. 2, A, C–E). One-third of these vacuoles were surrounded by more than 1 mitochondrion and some were surrounded by as many as 5 mitochondria (Fig. 2, E). Sometimes, large clusters of smooth vesicles with or without mitochondria were observed at 1 h (Fig. 2, D).

At 1 h as at 15 min, vacuoles were found at both the base and apex of the adherent monocyte. Vacuoles were rarely (<5%) located near the plasma membrane; about half were located midway between the plasma membrane and nucleus and half were located near the nucleus. Interestingly, of those near the nucleus at 1 h, 45% were found within an invagination of the nuclear envelope (Fig. 2, B).

FIGURE 2. Morphology of *L. pneumophila*-containing vacuoles in monocytes 1h after infection. Monocytes in monolayer culture were infected with live *L. pneumophila* as in Fig. 1. After 15 min incubation with the bacteria, the monocyte monolayers were washed to remove non-monocyte-associated bacteria, incubated for an additional 45 min, and fixed for electron microscopy. (A) *L. pneumophila* vacuole with a single mitochondrion (arrow) closely apposed to the vacuolar membrane. A few smooth vesicles also surround the vacuole. $\times 78,000$. (B) Two *L. pneumophila* vacuoles, one of which is located within an invagination of the nuclear envelope. $\times 41,700$. (C) *L. pneumophila* vacuole surrounded by smooth vesicles and a mitochondrion. $\times 33,000$. (D) *L. pneumophila* vacuole surrounded by a very large number of smooth vesicles and at least one mitochondrion (arrow). $\times 32,400$. (E) *L. pneumophila* vacuole surrounded by 5 mitochondria (arrows) and a few smooth vesicles. $\times 54,000$.



At 4 h after infection, fewer smooth vesicles (60% of vacuoles) and mitochondria (35% of vacuoles) were present about the vacuoles (Fig. 3). At this time, ribosomes were first seen lining the vacuole (Fig. 3, A–D) and 90% of vacuoles had associated ribosomes. The ribosomes were separated from the phagosome membrane by a gap of ~ 100 Å. Adjacent ribosomes were frequently attached to a common membrane which was sometimes an isolated segment of membrane and at other times the membrane of a rough vesicle (Fig. 3, A and B). These membrane-bound ribosomes were evidently part of or derived from rough endoplasmic reticulum. Sometimes, phagosomes were found apparently fusing with a smaller ribosome-studded vacuole whose membrane resembled the phagosome membrane in that the ribosomes were separated from it by a gap of ~ 100 Å (Fig. 3, C). Possibly, the smaller ribosome-studded vacuole was in fact part of the same phagosome, but appeared separate as a result of sectioning the phagosome through an invaginated region of the phagosome membrane, or was part of another phagosome extending from a different plane of the cell. At 4 h, the location of phagosomes within the monocyte relative to the plasma membrane and nucleus was about the same as at 1 h after infection. Also as at 1 h, vacuoles were found within invaginations of the nuclear membrane.

By 8 h, nearly all *L. pneumophila*-containing vacuoles (>95%) were lined with ribosomes (Fig. 3, E and F). For the first time, vacuoles frequently contained more than one bacterium. Under the conditions of this experiment, it was not possible to tell whether this occurred as a result of bacterial multiplication or because of fusion of *L. pneumophila*-containing vacuoles with each other. In experiments not included here, *L. pneumophila* have been found to begin multiplying in monocytes, as measured by colony-forming units, 4–10 h after infection.

Interaction between Formalin-killed L. pneumophila and Monocytes. Monocytes were also incubated with formalin-killed *L. pneumophila*. All intracellular formalin-killed *L. pneumophila* were found within membrane-bound cytoplasmic vacuoles (Fig. 4). In contrast to the situation with live bacteria, vacuoles containing formalin-killed *L. pneumophila* frequently had more than 1 organism at 15 min, 1 hr, and 4 h (Fig. 4, A, D, and E), and occasionally, vacuoles contained >10

FIGURE 3. Morphology of *L. pneumophila*-containing vacuoles in monocytes 4 h and 8 h after infection. Monocytes in monolayer culture were infected with *L. pneumophila* for 15 min and washed to remove non-monocyte-associated bacteria as in Fig. 2. The monocytes were then incubated for 4 h (A–D) or 8 h (E and F) and fixed for electron microscopy as described in Materials and Methods. (A) The *L. pneumophila* vacuole is located within an invagination of the monocyte nucleus. The vacuolar membrane is partially lined by ribosomes (arrows and arrow heads), some of which are attached to a common membrane on the side opposite the phagosome (arrows). $\times 52,000$. (B) The *L. pneumophila* vacuole is surrounded by ribosomes and a large rough vesicle, i.e. a vesicle to which ribosomes are attached (arrow). $\times 75,000$. (C) The *L. pneumophila* vacuole is partially surrounded by ribosomes (arrow heads) and has a mitochondrion closely apposed to the vacuolar membrane (large arrow). A ribosome-studded vacuole (small arrow) appears in the process of fusion with the phagosome membrane; the ribosomes around this vacuole, like those around the phagosome, are separated from the vacuolar membrane by a gap of ~ 100 Å. $\times 50,000$. (D) The *L. pneumophila* vacuole is partially lined by ribosomes. At one point, the vacuolar membrane is evaginated as if fusion has just occurred with a smaller ribosome-studded vacuole (arrow). $\times 33,000$. (E) *L. pneumophila* vacuoles at 8 h are all surrounded by ribosomes (arrows). $\times 41,700$. (F) The three *L. pneumophila* vacuoles are fully surrounded by ribosomes. The lower two vacuoles and perhaps all three vacuoles may be fusing with each other. $\times 27,000$.

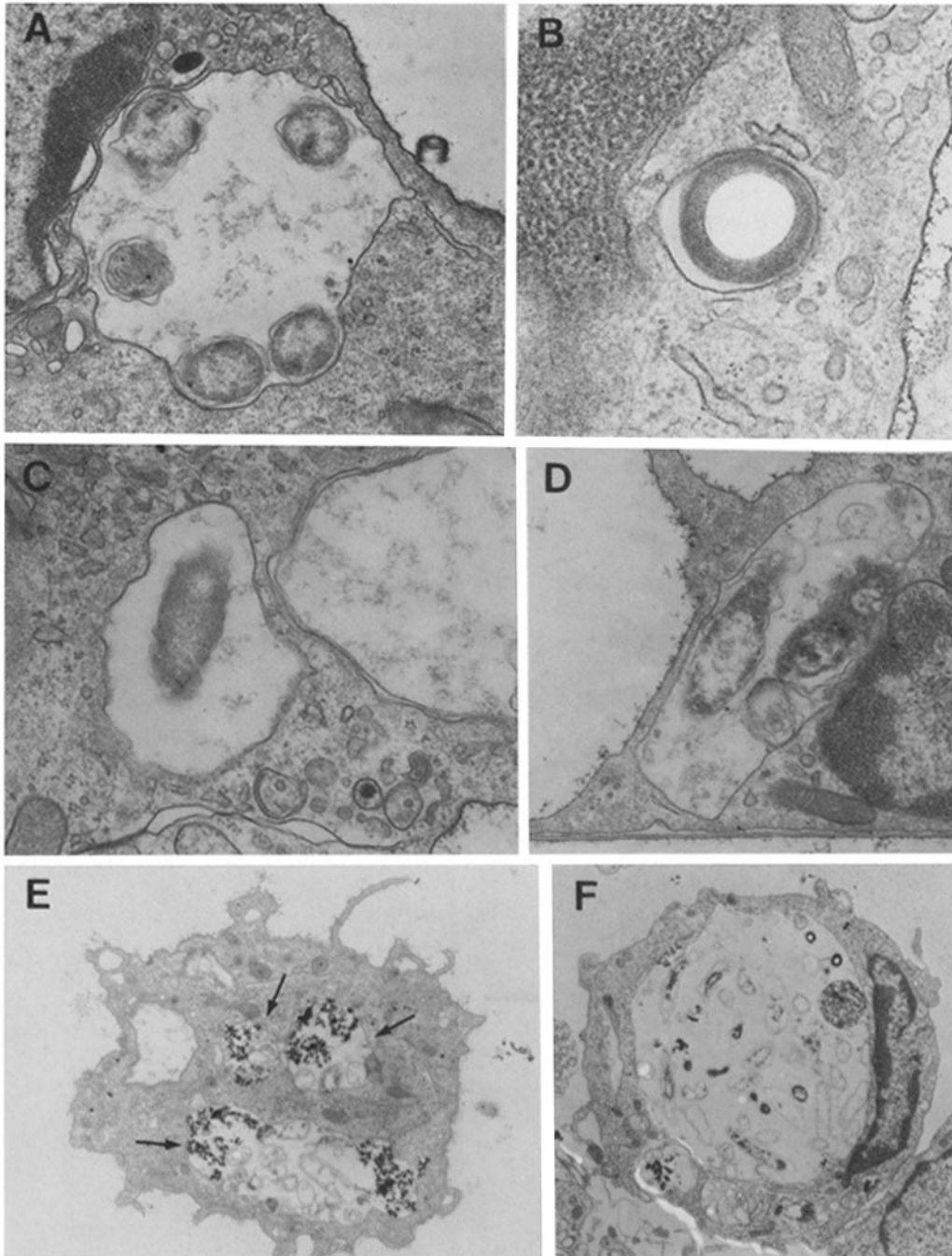


FIGURE 4. Morphology of monocyte vacuoles containing formalin-killed *L. pneumophila*. Monocytes in monolayer culture were incubated with 5×10^9 formalin-killed *L. pneumophila*/ml at the same time that they were incubated with live bacteria and under the same conditions. After 15 min, the monocyte monolayers were washed to remove non-monocyte-associated bacteria. They were then either fixed immediately (A), or after a total incubation time of 1 h

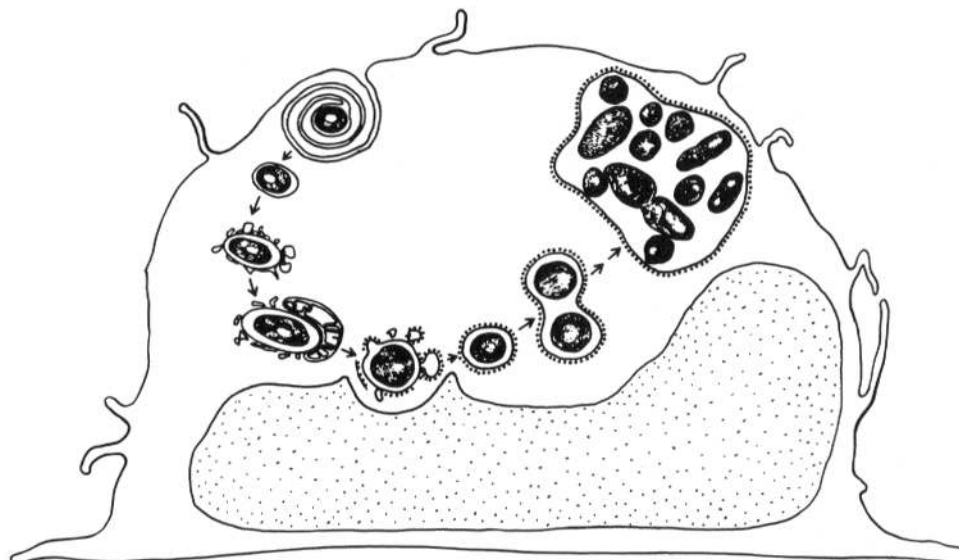


FIGURE 5. Diagram of the sequence of cytoplasmic events involved in formation of the *L. pneumophila* phagosome. (a) *L. pneumophila* is phagocytized by an unusual process in which a monocyte pseudopod is coiled around the bacterium as the bacterium is internalized. (b) Immediately after phagocytosis, no cytoplasmic organelles are found surrounding the *L. pneumophila*-containing vacuole. (c) By 15 min after infection, the majority of *L. pneumophila* vacuoles are surrounded by smooth vesicles, apparently fusing with or budding off from the vacuolar membrane. (d) By 1 h after infection, the majority of vacuoles are surrounded by at least one mitochondrion, closely apposed to the vacuolar membrane. Smooth vesicles are usually also present about the vacuole. (e) By 4 h after infection, fewer smooth vesicles and mitochondria surround the vacuole, but now ribosomes and rough vesicles line the vacuole. (f) By 8 h after infection, the *L. pneumophila* vacuole is studded with ribosomes. (g) The bacteria multiply within the ribosome-lined vacuole with a doubling time of about 2 h. (h) The bacteria multiply until hundreds of organisms fill the vacuole. The monocyte becomes packed full with bacteria and ruptures.

organisms.

No cellular organelles were found surrounding vacuoles containing formalin-killed *L. pneumophila* at 15 min, 1 h, 4 h, or 8 h (Fig. 4). At no time were smooth vesicles, mitochondria, or ribosomes found gathered about these vacuoles.

Vacuoles containing formalin-killed *L. pneumophila* were often larger than

(B-D), 4 h (E), or 8 h (F). As with live *L. pneumophila*, all intracellular formalin-killed bacteria were found in membrane-bound vacuoles. However, in contrast to the situation with live bacteria, cytoplasmic organelles were not found surrounding vacuoles containing formalin-killed *L. pneumophila*. (A) At 15 min, this section of the vacuole contains five formalin-killed *L. pneumophila*. The volume of the vacuole is large relative to the volume of the bacteria within it. $\times 20,000$. (B) At 1 h, the vacuole contains a single intact formalin-killed bacterium. The volume of the vacuole is only slightly greater than that of the bacterium contained within it. $\times 50,000$. (C) At 1 h, this vacuole contains one bacterium, but as in A, the volume of the vacuole is large relative to the volume of the bacterium within it. $\times 27,000$. (D) At 1 h, this vacuole contains at least three formalin-killed *L. pneumophila*; the bacteria appear in various stages of degeneration. $\times 18,400$. (E) at 4 h, this section of a monocyte contains four or five large vacuoles, three of which (arrows) contain formalin-killed *L. pneumophila* at advanced stages of degeneration. Bacterial membrane ghosts are evident in these vacuoles. $\times 7,500$. (F) at 8 h, this monocyte contains several small vacuoles and one enormous vacuole. The vacuoles are filled with degenerated formalin-killed bacteria and bacterial membrane ghosts. $\times 6,100$.

those containing live *L. pneumophila*, (Fig. 4, A and C). The increased volume may reflect fusion of lysosomes with these phagosomes since phagosomes containing formalin-killed but not live *L. pneumophila* fuse with lysosomes (8).

At 1 h, many of the ingested formalin-killed bacteria appeared degenerated (Fig. 4, D). Such bacteria were most frequently observed in vacuoles containing several organisms. At 4 h, only large vacuoles containing many bacteria were found and most of the bacteria were in an advanced stage of degeneration. Frequently only membrane ghosts were evident (Fig. 4, E). At 8 h, few intact bacteria remained. Sometimes, vacuoles containing bacterial remains were huge, apparently occupying more than half the cell volume (Fig. 4, F).

Influence of Erythromycin on Vacuole Formation. Erythromycin (1.25 $\mu\text{g/ml}$) inhibits the intracellular multiplication of logarithmic phase *L. pneumophila* within 1 h of addition to monocyte cultures, but does not kill the bacteria.² Upon removal of the antibiotic, these bacteria resume multiplying. The influence of erythromycin on vacuole formation was studied next.

Erythromycin had no apparent effect on vacuole formation whether added before or after infection. By 4–8 h, *L. pneumophila* in erythromycin-treated monocyte cultures were in ribosome-lined vacuoles.

Discussion

This study demonstrates that the formation of the *L. pneumophila* phagosome entails an unusual sequence of cytoplasmic events that take place during the first 8 h after phagocytosis of this bacterium. This sequence of events is shown diagrammatically in Fig. 5. During the first hour after entry by phagocytosis, smooth vesicles fuse with or bud off from the membrane of the *L. pneumophila*-containing vacuole, and mitochondria cluster about the vacuole in close apposition to the vacuolar membrane. By 4 h, rough vesicles and ribosomes line up about the vacuolar membrane. These ribosomes are separated from the vacuolar membrane by a gap of ~ 100 Å. By 8 h, nearly all *L. pneumophila*-containing vacuoles are studded with ribosomes. Previous studies have shown that at about this time the bacteria begin multiplying with a doubling time of ~ 2 h (1). The bacteria remain within the ribosome-lined vacuole until hundreds of organisms fill the vacuole and the monocyte ruptures (1).

The mechanism by which *L. pneumophila* orchestrates this complex series of cytoplasmic events is unknown. The fact that live, but not formalin-killed, bacteria induce the formation of the specialized vacuole suggests that a secretory or metabolic product of live bacteria is responsible, but does not exclude the possibility that a bacterial surface antigen altered by formalin treatment is involved. Erythromycin, an antibiotic that blocks bacterial protein synthesis, had no influence on vacuole formation when administered to monocytes at a concentration that completely inhibits intracellular multiplication of *L. pneumophila*. Erythromycin also does not influence the capacity of *L. pneumophila* to inhibit phagosome-lysosome fusion (8). This indicates that ongoing bacterial protein synthesis is not required for either vacuole formation or inhibition of phagosome-lysosome fusion.

² Horwitz, M. A. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. Submitted to *J. Exp. Med.*

The origin of the smooth vesicles that appear to fuse with and/or bud off from the phagosome membrane is not clear from this study. They may be derived from smooth endoplasmic reticulum or be of endocytic origin. Possibly, the membranes of these vesicles replace or markedly alter the phagosome membrane that was originally derived from the monocyte plasma membrane during phagocytosis. Isolation and biochemical analysis of the phagosome membrane may clarify its origin.

The origin of the ribosomes surrounding the vacuole, but separated from it by a gap of ~ 100 Å, is also not clear from this study. Those ribosomes that are membrane-bound, i.e. attached to isolated segments of membrane or part of rough vesicles surrounding the phagosome, are evidently derived from rough endoplasmic reticulum. Ribosomes that are not membrane-bound also may be derived from endoplasmic reticulum but have become separated from it. Alternatively, they may have been free ribosomes. Whatever their origin, the ribosomes do not bear the same relationship to the phagosome membrane that they bear to endoplasmic reticulum, since they are separated from the phagosome membrane by a large gap. Further studies are underway in this laboratory to clarify the origin and role of these ribosomes.

The sequence of events involved in formation of the *L. pneumophila*-containing vacuole has not been described for other organisms. However, in other studies, host cell vacuoles containing certain obligate intracellular parasites, notably *Toxoplasma gondii* and *Chlamydia* species, have been described that share at least some of the features of the *L. pneumophila*-containing vacuole (9–11). Lawn et al. (9) found that when BHK-21 cells were infected with the chlamydial agents of trachoma and inclusion conjunctivitis (*Chlamydia oculogenitalis*; *Chlamydia trachomatis*), vacuoles containing the organisms were immediately surrounded by cytoplasmic smooth vesicles. Friis (10) found that L-cell cytoplasmic vacuoles containing *Chlamydia psittaci*, the agent of psittacosis, were surrounded by mitochondria closely apposed to the vacuolar membrane 20 h after entry by phagocytosis. Jones and Hirsch (11) observed that host cell vacuoles containing *T. gondii*, the agent of toxoplasmosis, were also surrounded by mitochondria and that “strips of (rough) endoplasmic reticulum” were closely apposed to the vacuolar membrane; they observed this in vacuoles containing living but not dead *T. gondii* in mouse peritoneal macrophages, L929 fibroblasts, and HeLa cells. It is noteworthy that these intracellular parasites are among the few known to share with *L. pneumophila* the capacity to inhibit phagosome-lysosome fusion (8–10). It is interesting to speculate that these disparate microorganisms may share a common mechanism for vacuole formation and inhibition of phagosome-lysosome fusion.

Summary

Previous studies have shown that *L. pneumophila* multiplies intracellularly in human monocytes and alveolar macrophages within a membrane-bound cytoplasmic vacuole studded with ribosomes. In this paper, the formation of this novel vacuole is examined.

After entry into monocytes, *L. pneumophila* resides in a membrane-bound vacuole. During the first hour after entry, vacuoles containing *L. pneumophila*

are found surrounded by smooth vesicles fusing with or budding off from the vacuolar membrane and by mitochondria closely apposed to the vacuolar membrane. By 4 h, vacuoles are found less frequently surrounded by these cytoplasmic organelles, but now ribosomes and rough vesicles are found gathered about the vacuole. By 8 h, the ribosome-lined vacuole has formed. Erythromycin, at concentrations that completely inhibit the intracellular multiplication of *L. pneumophila*, has no effect on vacuole formation.

Formalin-killed *L. pneumophila* also reside in a membrane-bound vacuole after entry into monocytes. In contrast to the situation with live *L. pneumophila*, cytoplasmic organelles are not found surrounding vacuoles containing formalin-killed *L. pneumophila* at any time after entry. Formalin-killed bacteria are rapidly digested, and by 4 h, few remain intact.

The *L. pneumophila*-containing vacuole has certain features in common with other intracellular organisms that inhibit phagosome-lysosome fusion; these organisms may share a common mechanism for vacuole formation and inhibition of phagosome-lysosome fusion.

I am grateful to Ms. Diane Chodkowski for excellent technical assistance, to Ms. Judy Adams for preparing the electron microscopy specimens and printing the photographs in this paper, and to Dr. Zanvil Cohn for reviewing the manuscript.

Received for publication 11 April 1983 and in revised form 7 June 1983.

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