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Growth of *Chlamydia psittaci* in Macrophages

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Survival and growth of L-cell-cultivated *Chlamydia psittaci* occurred in mouse macrophages in vitro. Two major factors governing the intracellular fate of chlamydiae in macrophages are: (i) the multiplicity of infection (MOI), i.e., the elementary body (EB)-to-macrophage ratio, and (ii) the state of the EB. At a low MOI (1:1), survival and growth of live, untreated chlamydiae were optimal. The chlamydiae were internalized in macrophages within 30 to 40 min. EB proceeded to differentiate into reticulate bodies, which underwent multiplication and further matured into infectious EB in the professional phagocytic cells. In contrast, at a high MOI (100:1), survival of untreated chlamydiae was greatly reduced as a result of immediate damage to the macrophages. EB that were pretreated with heat (56°C for 10 to 30 min) or coated with homologous antibody were rapidly destroyed in macrophage phagolysosomes. Fusion of ferritin-labeled lysosomes with heat-treated or opsonized EB-laden phagosomes occurred in 2 to 4 h, resulting in transfer of the ferritin marker into phagolysosomes.

Chlamydiae are obligate intracellular parasites. Under appropriate conditions, these pathogenic bacteria survive and grow in both nonprofessional (epithelial) (12, 17) and professional (macrophage) phagocytic cells (11) in vivo and in vitro. At the end of their obligate intracellular development, the chlamydiae are released into the extracellular environment where they enter and multiply in neighboring epithelial cells. To perpetuate this cycle the parasites must also escape the scavenging and destructive capacities of the professional phagocytic cells, especially the macrophages. In 1944, Rake and Jones (13) demonstrated that intravenous or intraperitoneal inoculation of mice with chlamydiae resulted in localization of the agents in macrophages of the reticuloendothelial system.

Other investigators have reported the ability of chlamydiae to grow and survive in harvested mouse peritoneal macrophages in vitro (2, 7-9, 11, 16); however, the data are conflicting, and the conditions required for parasite survival appear quite varied.

This paper results from a study of the interaction of chlamydiae with mouse peritoneal macrophages and defines the initial parameters, as monitored by transmission electron microscopy (TEM), necessary for survival and multiplication of this pathogen in one of the first-line host defense cells.

MATERIALS AND METHODS

Propagation of chlamydiae. The Cal 10 meningopneumonitis strain of *Chlamydia psittaci* was grown in 929 L-cell suspension cultures according to the method of Tamura and Higashi (15). Elementary bodies (EB) were harvested and purified as previously reported (15), except that trypsin treatment was not used.

Titration of chlamydiae. For titration, the purified EB preparation was examined by direct particle count in the electron microscope via the method of Sharp (14), and infectivity was determined by a modification of the inclusion-forming unit titer described by Kuo and Grayson (10). Appropriate dilutions of EB were added to L-cell monolayers, 1.5×10^5 to 2×10^5 cells/chamber, in tissue culture chamber/slides (Lab-Tek Products). The Lab-Tek chambers were attached with rubber bands to Omni carriers and centrifuged at $282 \times g$ (1,500 rpm) for 15 min in a GLC centrifuge (Sorvall) at 25°C. May-Grünwald-Giemsa-stained inclusions were counted after 30 h of incubation at 37°C in an atmosphere of 5% CO_2. The infectivity titer of purified preparations of the Cal 10 strain of *C. psittaci* averaged about 50%, or 1 inclusion-forming unit per two particles.

Harvest of mouse macrophages. These studies used both nonstimulated and non-specifically stimulated peritoneal macrophages obtained from 8- to 10-week-old, random bred Swiss Webster female mice. For non-specifically stimulated macrophages, 5 days before harvest, the mice were injected intraperitoneally with 3 ml of sterile thioglycolate medium.

The method for harvesting macrophages was that described by Cohn and Benson (3). Wright-stained smears prepared after cytocentrifugation revealed an average of 90 to 95% macrophages in the thioglycolate-stimulated cell population and an average of 30 to 40% macrophages in the unstimulated peritoneal cell population. A fraction of the cell suspension was diluted with trypan blue, and viable counts were determined in a hemocytometer. The suspension was then ad-

justed to 5×10^6 macrophages/ml in tissue culture medium 199 containing, in solution, 15% heat-inactivated fetal calf serum, sodium bicarbonate and streptomycin (0.02%), and kanamycin (0.01%) (TC199). A 1-ml portion was added to plastic petri dishes (10 by 35 mm), followed by 2 ml of fresh TC199. During overnight incubation in an atmosphere of 5% CO_2, the macrophages adhered to the bottom of the petri dish, forming a near-confluent monolayer. The other cell types in the supernatant were removed by washing the monolayer twice with warm phosphate-buffered saline (PBS) followed by addition of fresh medium.

Uptake of chlamydiae by macrophages. Eighteen hours after inoculation of L-cells with EB, [^{3}H]uridine (1 mCi/liter) was added to infected L-cells. The radiolabeled EB were harvested at 48 h and purified as described. One-milliliter suspensions of ^{3}H-labeled EB (multiplicity of infection [MOI], 100:1) were added to the macrophage monolayers. The petri dishes were centrifuged for 15 min at $282 \times g$ (1,500 rpm) in a GLC centrifuge and then incubated at 37°C in 5% CO_2. The supernatants, washes, and macrophage pellets were hydrolyzed at intervals with 1 N NaOH for 18 h at 37°C, neutralized, precipitated with cold 10% trichloroacetic acid, and processed for scintillation spectrophotometry. The percent uptake represented the ^{3}H-labeled precipitable counts in the monolayer pellet at 20, 40, and 60 min as compared with the precipitable counts in the zero-time sample. A summary of five experiments, each sample performed in triplicate, revealed 92.7% uptake at 40 min compared with 89.1 and 90.5% uptake at 20 and 60 min, respectively. To distinguish between actual internalization and external attachment of EB, the monolayers, after normal washing, were treated for 5 min at 25°C with 1.0 ml of 1 mM ethylenediaminetetraacetic acid–0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1). This resulted in detachment of only 3% at 40 min versus 2% by the normal PBS wash. Maximum internalization had occurred by 40 min.

The routine procedure thus established for infection of macrophages involved the addition of 1.0 ml of purified EB at the appropriate MOI to 24-h macrophage monolayers in petri dishes. The petri dishes were centrifuged as described and then incubated at 37°C in 5% CO_2 for 30 min, this period being designated as the uptake period. The infected monolayers were washed three times in warm PBS, replenished with fresh, warm TC199 medium, and returned to the incubator. At timed intervals, the supernatants were discarded, the monolayer was washed and harvested with a rubber policeman, and fractions of this cell pellet were processed for TEM.

TEM procedure. Control and infected macrophage monolayers were washed thoroughly and then prefixed in 2.5% glutaraldehyde prepared in 0.01 M sodium cacodylate buffer for 60 min at 25°C. After thorough washing in 0.135 M sodium cacodylate buffer, primary fixation of the monolayers was accomplished by 1% osmium tetroxide (OsO_4) in Ringer buffer, pH 7.3, for 90 min at 25°C. The monolayers were then harvested with a rubber policeman, enrobed in agar, and postfixed with 0.5% aqueous uranyl acetate for 60 min in the dark. The samples were dehydrated in acetone and embedded in Vestopal W. Thin sections were cut on a Reichert ultramicrotome and poststained with 5% alcoholic uranyl acetate for 15 min, followed by lead citrate for 4 min. All specimens were examined with an AEI electron microscope, accelerated at 60 kV, with a 30-μm objective aperture.

The technique for ferritin labeling of the macrophage lysosomes was the procedure used by Armstrong and Hart (1). Twice-crystallized horse spleen ferritin (Nutritional Biochemicals, cadmium free) was purified as previously described (18).

Antibody and heat treatment. The rabbit antiserum, a gift from G. P. Manire, had been prepared by repeated intravenous injection of New Zealand white rabbits with purified EB of the Cal 10 strain of *C. psittaci*. The titer, after ammonium sulfate precipitation, was 1:1,256. Equal volumes of antisera and EB in PBS were mixed and allowed to react at 37°C for 1 h, followed by standing at 4°C overnight. The EB suspension was then washed once in PBS and finally suspended again in TC199 medium for inoculation.

For heat treatment, the EB suspension in PBS was placed in a prewarmed test tube and placed in a 56°C water bath for 3, 5, and 10 min with constant shaking. The EB was then diluted in TC199 medium to the appropriate MOI and inoculated onto macrophage monolayers.

RESULTS

Effect of MOI on survival of *C. psittaci* in macrophages. Stimulated-macrophage monolayers, equilibrated after overnight incubation, were inoculated with a range of chlamydial dilutions, with MOIs from 100:1 to 1:100. After 48, 72, and 96 h of incubation at 37°C in an atmosphere of 5% CO_2, the supernatant from harvested, sonically treated macrophages was titrated in L-cell suspension cultures. Since the optimal time for release of the most EB from macrophages was 96 h (11), all subsequent samples were harvested at 96 h and then titrated in the L-cells (Fig. 1). An MOI of 1:1 or less appeared optimal for growth and development of live EB in macrophages. At higher MOIs, growth of chlamydiae was much reduced, probably as a

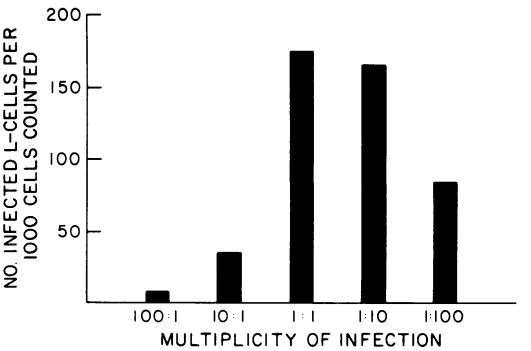


FIG. 1. *Effect of MOI on survival of purified, live chlamydial EB in thioglycolate-stimulated mouse peritoneal macrophages.*

result of early cytotoxic damage to macrophages.

Intracellular fate of chlamydiae in macrophages cultured in vitro. The sequence of events in uptake, intracellular location, and fate of live EB of *C. psittaci* in mouse peritoneal macrophages was followed by TEM (Fig. 2 through 6). There was no apparent difference between thioglycolate-stimulated and nonstimulated macrophages regarding the sequence of events to be described. Thus, the electron photomicrographs presented are ultrathin sections of thioglycolate-stimulated macrophages.

The chlamydiae were phagocytized rapidly and internalized in the macrophage phagosome. Figure 2a illustrates contact of chlamydia with the macrophage surface 30 min after inoculation, followed by engulfment of chlamydia (Fig. 2b). Figure 3 represents localization of apparently live chlamydiae in macrophage phagosomes 2 h after inoculation and showed little change at 6 h. In contrast, heat-treated and opsonized EB at MOIs of both 1:1 and 100:1, as well as untreated EB at an MOI of 100:1, were clearly undergoing degradation by 6 h (Fig. 4). The apparent morphological damage included condensation and margination of cytoplasmic constituents and loss of rigidity of the EB cell envelope.

The evidence provided by the ferritin-labeling experiments strongly suggests that the damage to intracellular, treated EB is the direct result of fusion of host cell lysosomes with chlamydiae-laden phagosomes. Figure 5 illustrates a portion of an uninfected macrophage preincubated with 0.5 mg of purified-horse spleen ferritin per ml, in which the concentration of ferritin marker in the lysosomes is clearly evident. Figure 6 represents the sequence of events resulting in phagosome-lysosome fusion in macrophages infected with heat- or antibody-treated EB. In Fig. 6A, three steps in this sequence may be seen: arrow 1 illustrates apparently healthy chlamydiae in tightly enclosed phagosomes; arrow 2 indicates a juxtaposition of ferritin-labeled lysosomes to chlamydiae-laden phagosomes; arrow 3 illustrates fusion of ferritin-labeled lysosomes with chlamydiae-laden phagosomes with transfer of the ferritin marker into the phagolysosome. Figure 6b, c, and d reveal destruction of chlamydiae in macrophage phagolysosomes.

Live, untreated chlamydial EB at the optimal MOI (1:1), on the other hand, proceeded to differentiate into reticulate bodies, undergo multiplication, and further mature into infectious EB in professional phagocytic cells. Ferritin-la-

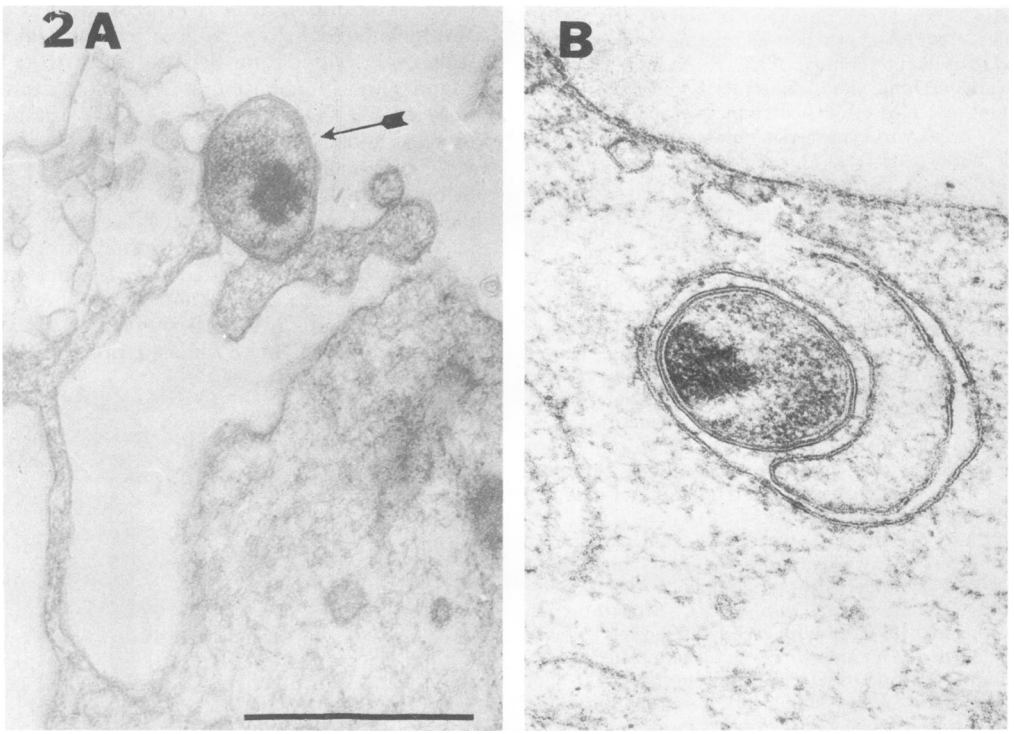
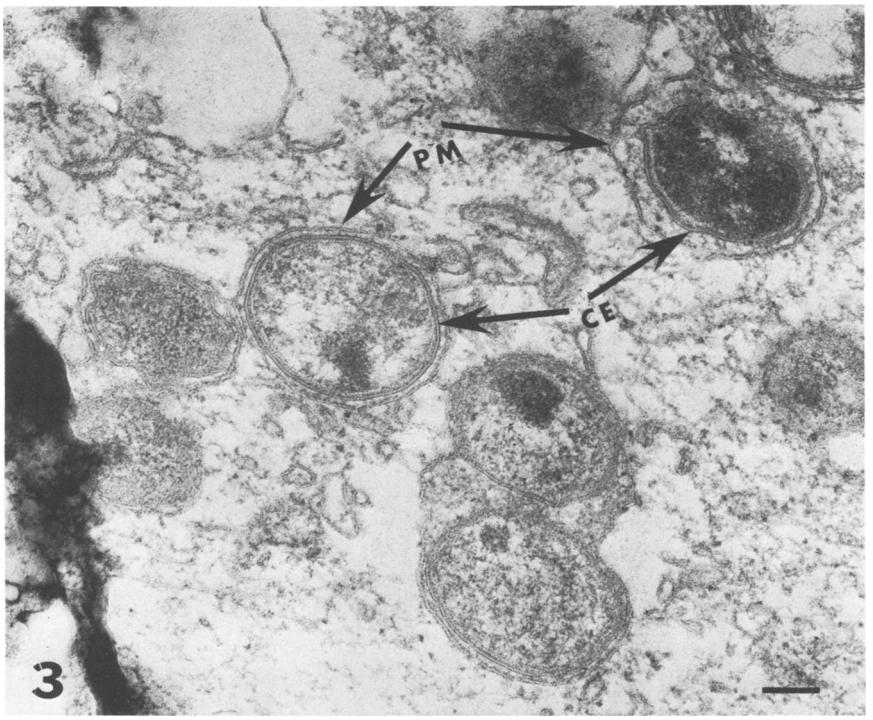


FIG. 2–6. *Representative transmission electron photomicrographs of ultrathin sections cut through control and chlamydiae-infected thioglycolate-stimulated mouse peritoneal macrophages.*

FIG. 2. *(A) Contact of chlamydia with the macrophage surface or pseudopodia 30 min after inoculation. ×60,000; bar, 0.5 μm. (B) Engulfment of chlamydia; ×84,000; bar, 0.1 μm.*



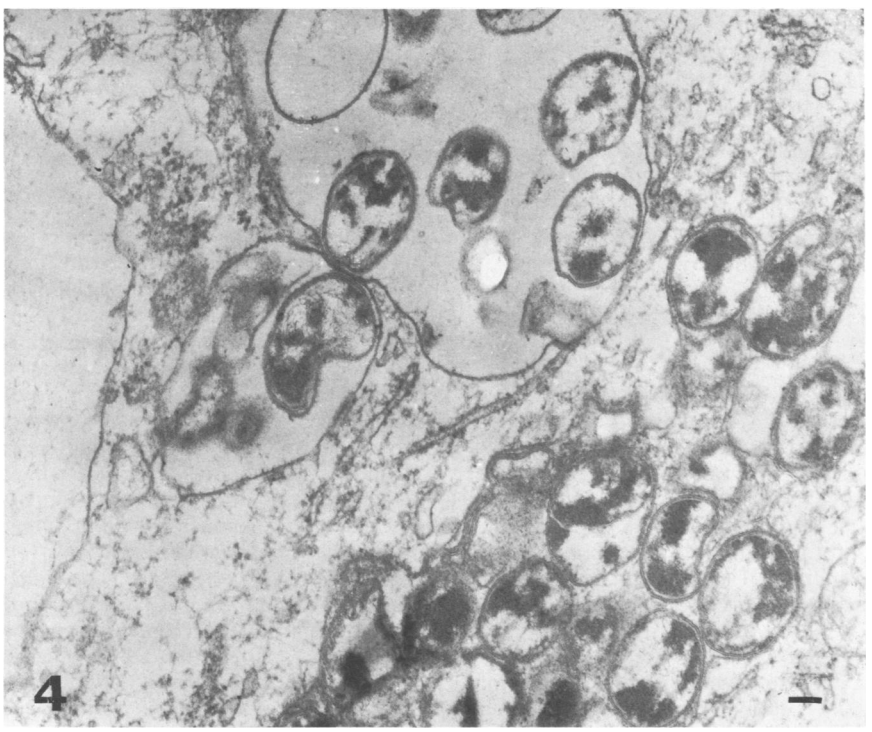


FIG. 3. *Localization of live chlamydiae in macrophage phagosomes 2 h after inoculation. PM, Phagosome membrane; CE, Chlamydiae cell envelope; ×70,000; bar, 0.1 μm.*

FIG. 4. *Intracellular degradation of homologous antibody-coated EB (MOI at 100:1) at 6 h; ×41,000; bar, 0.1 μm.*

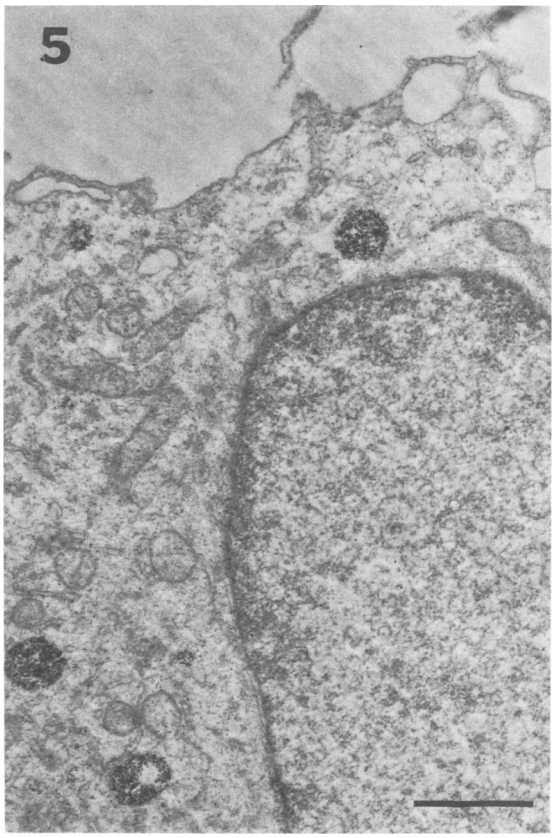


FIG. 5. *Portion of an uninfected macrophage preincubated with 0.5 mg of purified-horse spleen ferritin per ml. The ferritin marker is concentrated in the lysosomes; ×15,000; bar, 1 μm.*

beled lysosomes can be seen adjacent to the chlamydiae-laden phagosome (as in arrow 2, Fig. 6a), but no ferritin was transferred, and phagolysosome formation apparently did not occur.

DISCUSSION

Our results have confirmed that the MOI and the state of the EB are important in regard to the intracellular fate of chlamydiae in macrophages obtained from nonimmune mice.

Regarding the MOI, the lower the MOI, i.e., 1:1, 1:10, or 1:100 versus 100:1, the greater the survival of *C. psittaci* in macrophages. Conversely, a high MOI (100:1) appears to result in early macrophage cytotoxicity (7–9, 16). One hundred *C. psittaci* EB per mouse macrophage result in damage to the macrophage beginning 2 h postinoculation, whereas, with an MOI of 1:1 or less, this cytotoxicity is not apparent. A similar observation was reported for *C. psittaci* interaction with mouse L-cells (12). The effects of high MOI of *C. psittaci* on host cells have been referred to collectively by these authors as "immediate toxicity."

As to the state of the EB, our results show that live EB (MOI, 1:1) will grow and survive in mouse macrophages, whereas, if EB from the same suspension are treated with heat or coated with homologous antibody, they are rapidly destroyed in phagolysosomes. Intracellular survival of chlamydiae in macrophages depends, at least in part, on the absence of fusion of lysosomes with the chlamydiae-laden phagosome. From similar studies, Friis (4) concluded that chlamydiae act to prevent fusion of the lysosomes with the parasite-laden phagosome, as with *Mycobacterium tuberculosis* (1) and *Toxoplasma gondii* (6), rather than being resistant to hydrolytic enzyme destruction in the phagolysosome, as with *Mycobacterium leprae* (5).

The results presented here are based solely on morphological criteria. Though this technique offers many advantages, its limitations are also well known. Distinction between microbes as living or dead is virtually impossible, and quantitative studies are limited. Consequently, TEM should not be adopted as a sole parameter. Biochemical studies, including both radioisotopes

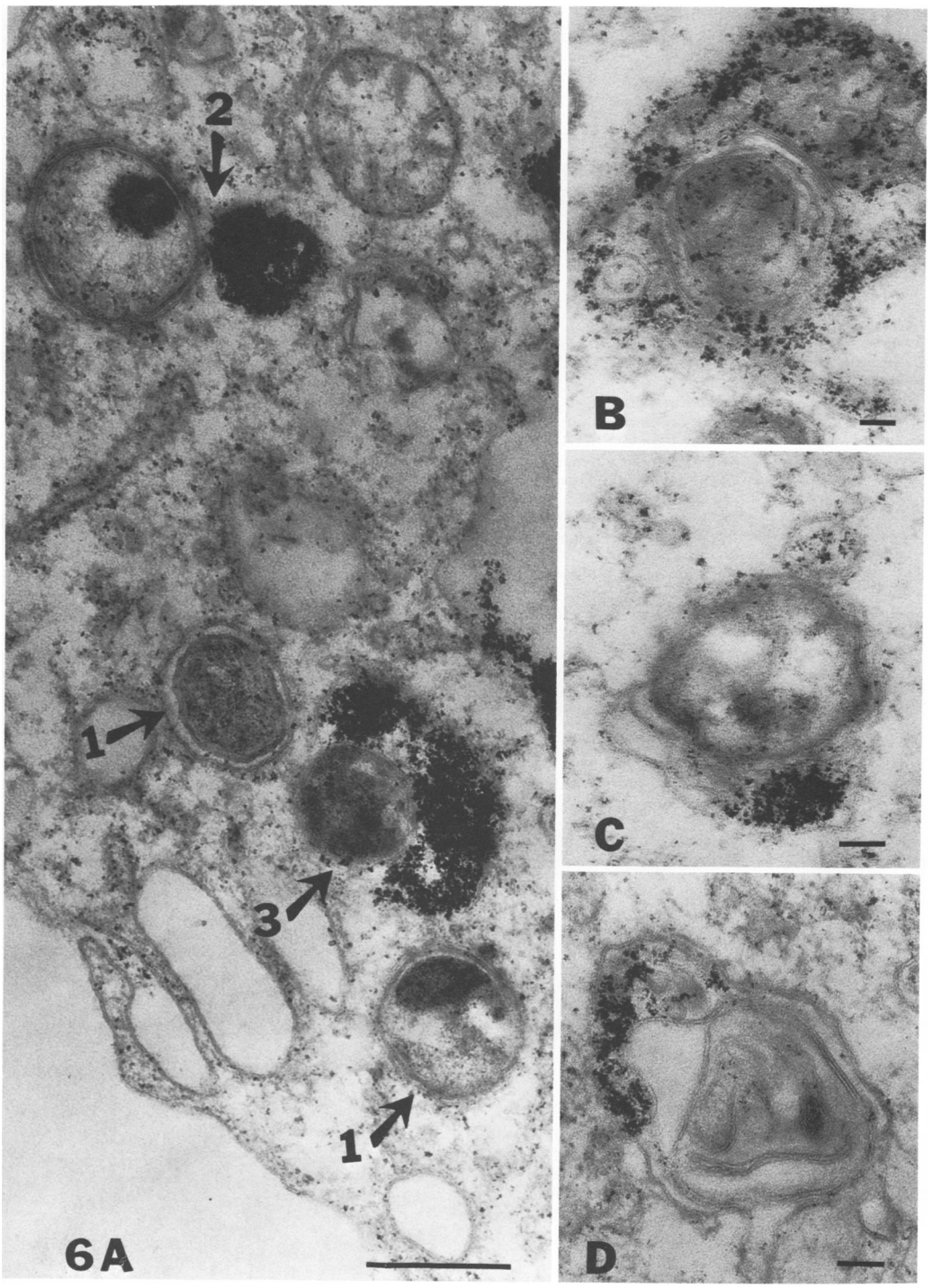


FIG. 6. *Summary photomicrograph illustrating the sequence of events of intracellular fate of chlamydiae in ferritin-labeled macrophages. (A) Arrow 1, Apparently healthy EB in tightly enclosed phagosomes. Arrow 2, Migration of ferritin-labeled lysosomes to chlamydiae-laden phagosomes. Arrow 3, Fusion of ferritin-labeled lysosomes with chlamydiae-laden phagosomes and subsequent transfer of the ferritin marker into the phagolysosome; ×42,250; bar, 0.5 μm. (B), (C), and (D), Destruction of chlamydiae in macrophage phagolysosomes; (B) ×50,000; (C) ×64,500; (D) ×64,200; bars, 0.1 μm.*

and enzyme analyses, have been performed in this laboratory and substantiate these findings (17a).

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