Ultrastructural Study of Mode of Entry of *Chlamydia psittaci* into L-929 Cells

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The entry of *Chlamydia psittaci* into L-929 cells was studied morphologically by transmission electron microscopy and quantitatively by a method that discriminates between attachment and uptake. Upon adsorption of ^{3}H-labeled elementary bodies (EBs) to host cells at 4°C, the EBs bound efficiently to the L-cell surface. Binding reached an equilibrium level of 55% in 3 h. Ultrastructural analysis revealed that EBs were bound preferentially to the tips and sides of microvilli at this temperature. The EBs were also observed in coated pits located at the bases of microvilli and along smooth surfaces of the host cell. No internalization was observed at 4°C. When cells with prebound ^{3}H-labeled EBs were warmed to 37°C, the EBs rapidly became resistant to proteinase K removal (half time = 5 min), indicating ingested chlamydiae. At 37°C, the EBs were internalized within tightly bound vesicles surrounded by an electron-dense coat of fibrillar material. EBs were also present in smooth-surfaced pits and vesicles of the host cell. Using α_2-macroglobulin coupled to colloidal gold (a known marker for receptor-mediated endocytosis), we observed that the entry of EBs into cells via coated pits was identical in appearance to the internalization of α_2-macroglobulin. Also, when the two ligands were mixed together, they could be seen within the same coated pits and were cointernalized within endocytic vesicles of the host cell. These results suggest that *C. psittaci* can enter nonprofessional phagocytic cells by a pathway which is similar to that of receptor-mediated endocytosis of many physiologically important macromolecules, bacterial toxins, and viruses.

Chlamydiae are gram-negative bacteria that live as obligate intracellular parasites within eukaryotic host cells. There are two distinct forms of the organism which are adapted to extracellular survival and intracellular multiplication. The elementary body (EB) is the infectious extracellular form and is involved in the initial attachment and entry of the organism into the host cell. After uptake, the organism converts to the metabolically active reproductive form, or reticulate body, which divides by binary fission to produce new progeny. The entire process occurs within a membrane-bound vesicle inside the host cell and is followed by the release of infectious EB particles (2, 23, 28, 30).

The initial interaction between *Chlamydia psittaci* and host cells involves attachment, ingestion, and escape from phagosome-lysosome fusion. Although crucial to intracellular parasitism, the component(s) or mechanism(s) involved in these early stages of chlamydial pathogenesis is little understood. Once *Chlamydia* spp. attach to the surface of a host cell, it has been assumed that the organisms enter the cell by a phagocytic process involving the participation of eucaryotic microfilaments and the sequential zipperlike engulfment of the bacterium into a tight phagosome (10). However, Byrne and Moulder (4) have shown that chlamydiae are ingested by nonprofessional phagocytic L and HeLa cells at rates far more rapid than those observed for the phagocytosis of *Escherichia coli* and polystyrene latex spheres. This efficient entry process has been termed "parasite-specified phagocytosis" by these investigators to distinguish this ingestion from host-specified phagocytosis by professional phagocytes (i.e., neutrophils and macrophages) and to suggest that specific surface structures on the bacterium play a role in the process. Even EB envelopes alone are efficiently internalized by both professional and nonprofes-

sional phagocytic cells (8). The energy required for the ingestion of chlamydiae comes from the host cell, since the uptake of *C. psittaci* by either L cells or macrophages is interrupted by glycolytic and oxidative inhibitors (10, 17). However, the entry of the organism into these cell types is not inhibited by cytochalasin B in concentrations that prevent phagocytosis of comparable objects (13). This would suggest that the ingestion of *C. psittaci* is a microfilament-independent process and, in this respect, differs from classical phagocytosis.

Upon internalization, *C. psittaci* EBs are surrounded by membrane-bound vesicles which do not fuse with host cell lysosomes (8, 9, 10). The mechanism of escape from phagosome-lysosome fusion is not known but is the function of the EB (3) and involves a surface component of the internalized EB which is already present at the time of entry (9, 10, 32). The escape mechanism does not appear to involve a generalized inhibition of cytoskeletal elements or lysosomal machinery but instead is localized only to EB-containing vesicles (8, 10). Our hypothesis is that the escape from phagosome-lysosome fusion is intimately connected to the attachment and entry of EBs into susceptible host cells. We believe that, once attached, EBs are internalized by an entry process resembling receptor-mediated endocytosis. This process is used by the majority of eucaryotic cells to internalize nutritional and regulatory components, such as low-density lipoprotein and polypeptide hormones, that have become bound to cell surface receptors within electron-dense clathrin-coated pits (12, 22, 24). In most cases, the ligands are delivered to lysosomes, where they are degraded and their byproducts are used for important roles in cellular function. However, other intracellular destinations, not involving lysosomes, have been described (12, 22). Certain viruses (19, 25, 34) and bacterial toxins (20, 21) enter cells by this process and, in doing so, develop effective strategies for
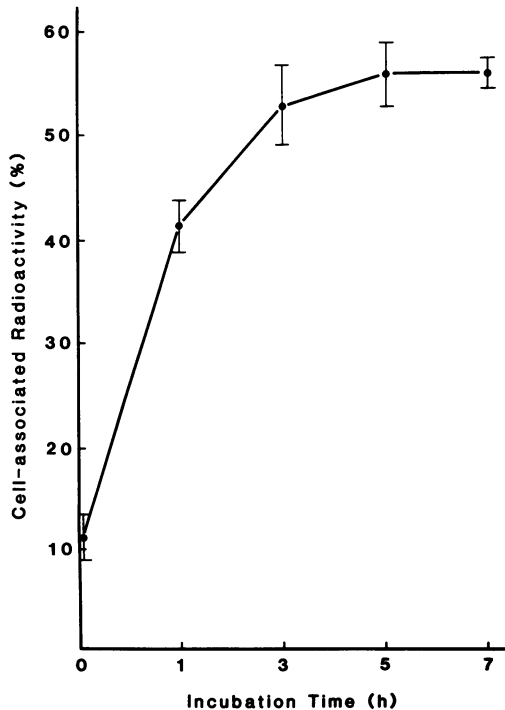
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FIG. 1. Kinetics of binding of ³H-labeled *C. psittaci* to L-929 cells at 4°C. The values shown represent the means ± standard errors for the percent cell-associated radioactivities of three replicate experiments.

controlling their subsequent intracellular fate. Such a process could specifically direct the internalization of EBs into endocytic vesicles that are not destined to fuse with lysosomes and, thereby, secure the survival of this organism within infected cells.

In this paper, we describe the morphological and quantitative examination of the attachment and entry of *C. psittaci* into cultured mouse fibroblasts (L-929 cells). Such studies allow for a critical view of these early stages of infection at the cellular level and should add to our understanding of the pathogenesis of *Chlamydia* spp. and the interrelationships that exist between these bacteria and host cells in vivo.

MATERIALS AND METHODS

Growth, purification, and titration of chlamydiae. The Cal 10 meningopneumonitis strain of *Chlamydia psittaci* was grown in L-929 cell suspension cultures (29). The medium consisted of a 0.01% yeast extract–0.5% lactalbumin hydrolysate–Earle balanced salt solution (YLE) (GIBCO Laboratories, Grand Island, N.Y.) containing calf serum (3.0%), streptomycin (0.02%), and kanamycin (0.01%). The harvest and purification of EBs were done by the method of Wyrick et al. (33). Purified EBs were suspended at 10^{10} particles per ml in a buffer containing 0.02 M $NaH_2PO_4 \cdot H_2O$, 0.02 M Na_2HPO_4, 0.2 M sucrose, and 5 mM glutamine in distilled water (2SPG) and were frozen at −70°C until needed.

The infectivity of the purified EB preparations was determined by a modification of the inclusion-forming unit (IFU) titer technique (17). The percent infectivity was defined as the number of infectious EB particles per total particles (determined spectrophotometrically; Wyrick et al. [33]) mul-

tiplied by 100. Preparations of purified EBs used for these studies routinely averaged 30 to 50% infectivity, or ≤1 IFU per 2 particles.

For the preparation of radiolabeled organisms, chlamydiae were adsorbed to suspension cultures of L cells at 37°C for 2 h with continuous motion. After the adsorption period, [5-³H]uridine (1 mCi/liter) (Research Products International Corp., Mount Prospect, Ill.) was added and the infected cultures were reincubated for 48 h. The growth, harvest, and purification of EBs were performed as described above.

Measurement of attachment and uptake of ³H-labeled *C. psittaci* with L-929 cells. For all experimental procedures, L-929 cells were grown to subconfluent monolayers (approximately 10^6 cells per 25-cm² tissue culture flask) in Hanks buffered Eagle minimum essential medium (MEM) (GIBCO) without antibiotics, supplemented with 4.2 mM sodium bicarbonate–1.6 mM glutamine–10% (vol/vol) heat-inactivated fetal bovine serum (Sterile Systems Inc., Logan, Utah). The cells were washed twice with 1 ml of ice-cold MEM (pH 7.2) and cooled at 4°C for 30 min. Aliquots of frozen chlamydial stock were rapidly thawed at 37°C and sonicated for 20 s in a water bath to disperse clumps before addition of the stock to the L-cell monolayers.

To measure the binding of chlamydiae, radioactive EBs (2000 to 3000 cpm), at a multiplicity of infection of 100 IFU per host cell in 200 μl of ice-cold MEM, were added to each flask, and the flasks were gently shaken every 20 min for various times at 4°C. At this temperature, chlamydiae attach to the host cell surface but are not internalized by the cell (10). After this incubation, unbound chlamydiae were removed and the cell monolayers were washed twice with 0.4 ml of ice-cold phosphate-buffered saline (PBS) (pH 7.2). The removed inoculum and the two PBS washes were combined, placed on ice, and precipitated with an equal volume of cold 10% trichloroacetic acid (TCA). A 0.5-ml amount of PBS was added to each L-cell monolayer, and the cells were harvested with a rubber policeman. The flasks were rinsed with an additional 0.5 ml of PBS. The cells and wash fluid were combined and precipitated with an equal volume of cold 10% TCA.

The TCA precipitates from the inoculum supernatants and the L-cell monolayers were collected onto 2.4-cm-diameter glass microfiber filters (no. 934-AH; Whatman, Inc., Clifton, N.J.), dried, digested with NCS tissue culture solubilizer (Amersham Corp., Arlington Heights, Ill.), and counted in 10 ml of a neutralizing toluene-based scintillation mixture with an LS-8000 scintillation spectrometer (Beckman Instruments, Fullerton, Calif.).

All measurements of the uptake of chlamydiae were taken after the incubation of ³H-labeled EBs (multiplicity of infection of 100 IFU per L cell in 200 μl of MEM per 25-cm² flask) with L cells for 5 h at 4°C and after the subsequent warming of the sample flasks for various periods at 37°C to promote chlamydial entry. At this point, the supernatant medium was collected, and the cell monolayers were washed twice in ice-cold PBS. The combined inoculum medium and washes were analyzed as described above. The cells were either scraped from the flasks and counted directly or incubated with gentle shaking for 45 min at 4°C with 1 mg of proteinase K (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in 1 ml of ice-cold PBS to remove surface-associated chlamydiae. Treatment of the L-cell monolayers with proteinase K detached the cells from the plastic surface of the flasks. The cells were separated from the proteinase K supernatant by centrifugation at 412 × *g* for 10 min. The flasks were washed twice with 0.5 ml of PBS, and the washes
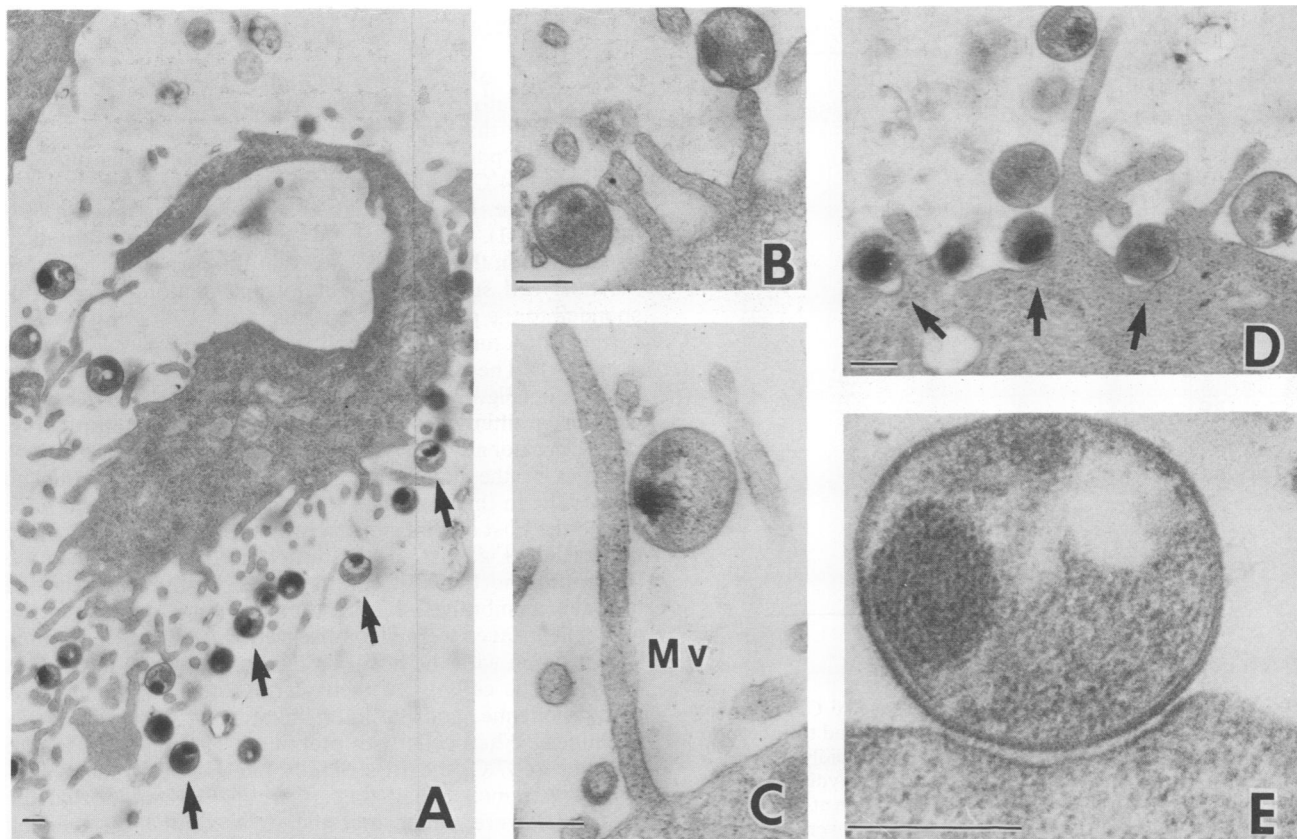
FIG. 2. TEM showing the adherence of *C. psittaci* to L cells after incubation for 5 h at 4°C. (A) Attachment of numerous EBs (arrows) preferentially to cell surface microvilli; (B and C) higher magnifications of EBs bound to the tips and sides of the microvilli; (D) EBs positioned at the bases of these microprocesses (arrows), as well as along the sides of the structures; (E) EB attached to a portion of the L-cell surface which is apparently devoid of a microvillus. Mv, Microvillus. Bar, 0.2 μm.

were combined with the cell suspension before centrifugation. The proteinase K supernatant was collected, and the pelleted cells were resuspended in 1 ml of ice-cold PBS. Both samples were precipitated with an equal volume of 10% TCA. The supernatant was tested for proteinase K-sensitive radioactivity (amount of attached radiolabeled EB not internalized), and the cells were assayed for proteinase K-resistant radioactivity (internalized radiolabeled chlamydiae in the cell pellet) as described above.

The percent attachment of ^{3}H-labeled chlamydiae to L cells was designated by the ratio of the TCA-precipitable counts in the L-cell pellets at various times to the TCA-precipitable counts in the original inoculum, multiplied by 100. The percent internalization of radiolabeled chlamydiae was determined by dividing the proteinase K-resistant fraction by the sum of the proteinase K-sensitive and -resistant radioactivities and multiplying by 100. Each sample for the attachment and uptake assays was performed in duplicate, and the experiments were repeated on at least three separate occasions.

TEM. Subconfluent monolayers of L-929 cells were prepared as described above. Chlamydiae were added to the cell monolayers at a final multiplicity of infection of 300 IFU per host cell in ice-cold MEM, and adsorption was allowed to proceed for 5 h at 4°C with intermittent rocking of the culture flasks. After the adsorption period, the cultures were removed from the cold and either immediately placed on ice

(time zero) or rapidly warmed to 37°C for various times (2, 5, 15, 30, 60, and 90 min) before being processed for transmission electron microscopy (TEM). The inoculum was removed from each flask, and the monolayers were washed twice with PBS lacking Ca^{2+} and Mg^{2+} (pH 7.2) and fixed in situ with 2% glutaraldehyde (electron microscopy grade; Polysciences, Inc., Rydal, Pa.) in 0.1 M Sorensen buffer (pH 7.2) for 1 h at ambient temperature (22°C). The fixed cells were scraped from the flasks, collected by centrifugation at 412 × *g* for 10 min, and agar enrobed with 2% Noble agar in 0.85% saline. The specimens were then washed several times in Sorensen buffer and postfixed for 45 min at room temperature with 1% osmium tetroxide in the same buffer. After dehydration in a graded series of ethanol and two changes of propylene oxide, the samples were infiltrated and embedded in a fresh mixture of Epon-Araldite (Polysciences) and cured at 60°C for 48 h. Silver-to-gold thin sections were prepared with a glass knife on a Reichart ultramicrotome, counterstained with uranyl acetate followed by lead citrate, and examined in a Siemens 101A electron microscope operating at 60 kV.

When examining the binding of gold-labeled α_2-macroglobulin to the host cells, we used a modification of the procedure of Dickson et al. (6). Slightly confluent monolayers of L-929 cells were washed once in serum-free MEM and then incubated for 1 h at 37°C in fresh serum-free medium. The cells were then rinsed three times and maintained in
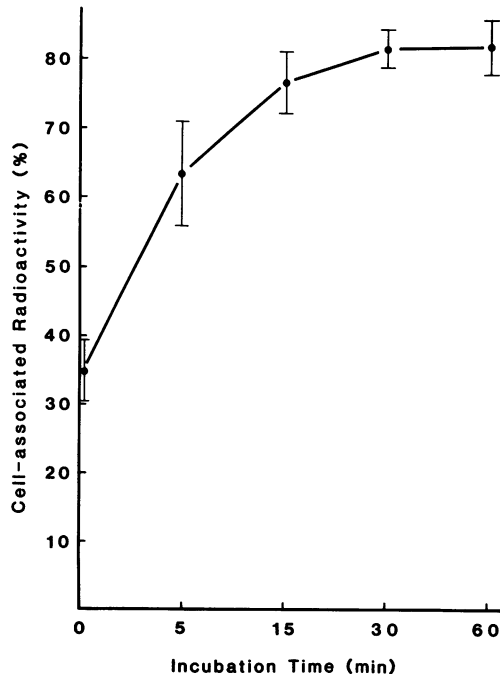
FIG. 3. Synchronous uptake of bound ^{3}H-labeled *C. psittaci* by L-929 cells at 37°C. Chlamydiae were allowed to bind to L cells for 5 h at 4°C, and the L-cell monolayers were then rapidly warmed to 37°C. At various times, surface-associated chlamydiae were removed by proteinase K treatment (1 mg/ml for 45 min at 4°C) and the proteinase K-sensitive and -resistant radioactivities were measured. The values shown represent the means ± standard errors for the percent cell-associated, proteinase K-resistant radioactivities of three replicate experiments.

serum-free medium at 4°C before the addition of the inoculum. The temperature was carefully controlled and monitored at 4°C. The gold-labeled α_2-macroglobulin, at a final concentration of 50 µg/ml in 200 µl of fluid per 25-cm^2 flask, was incubated with the cell monolayers at 4°C for 5 h. The monolayers were either washed three times with ice-cold PBS (pH 7.4) (containing 1.5 mM CaCl$_2$) and fixed with 2% glutaraldehyde in the same buffer without Ca^{2+}, or were rapidly warmed to 37°C for various times (0, 2, 5, 15, and 30 min) to allow for uptake of the α_2-macroglobulin before being washed and fixed. In experiments involving the binding of a mixture of chlamydiae and gold-labeled α_2-macroglobulin to host cells, the components were combined in a final volume of 200 µl to receive chlamydiae at a final multiplicity of infection of 300 IFU per host cell and α_2-macroglobulin at a final concentration of 50 µg/ml. The remaining procedure was as stated above. All TEM experiments were done in duplicate and repeated on two separate occasions.

The procedure for the adsorption of colloidal gold to α_2-macroglobulin was as previously described (6, 11). The colloidal gold suspension (Polysciences) contained 2 × 10^{13} gold particles per ml, ranging in size from 10 to 20 nm, and was in a citrate buffer (pH 5.5). Lyophilized α^2-macroglobulin (Sigma Chemical Co., St. Louis, Mo.) was solubilized in 0.025 M Tris hydrochloride (pH 8.0) containing 0.1 M NaCl and then was extensively dialyzed against distilled, deionized water at pH 6.0. The gold-adsorbed α_2-macroglobulin was stored at 4°C until used and did not flocculate after the addition of a 1% solution of NaCl.

RESULTS

The entry of *C. psittaci* into L-929 cells was studied morphologically by TEM and also quantitatively by a radioactive assay that discriminates between binding and internalization. Upon the adsorption of ^{3}H-labeled chlamydiae to host cells at 4°C, the EBs bound rapidly and efficiently to the L-cell surface. Binding reached an equilibrium level of 55% in 3 h (Fig. 1). Ultrastructural analysis of cells exposed to chlamydiae in the cold revealed that the organisms bound to the host cell surface in a patchy distribution. This corresponded to the preferential binding of the EBs to preexisting surface structures of the cell which are termed microvilli (Fig. 2A). The EBs bound to the tips and sides of the microvilli (Fig. 2B and C), and some appeared to be in position to enter the cell at the bases of these structures (Fig. 2D). Occasionally, EBs were seen attached along smooth surfaces of the host cell plasma membrane which lacked microvilli. In this instance, the association appears to have forced the host cell membrane inward so as to form a cup or depression (Fig. 2E). In the initial interaction between the bacterium and the host cell, a gap generally separated the two cell membranes. More intimate contacts between the two cell surfaces were also observed, with the EBs forming a tight union with the host cell membrane of the microvilli.

Infected L cells were then warmed to 37°C for various periods of time, and the internalization of chlamydiae was examined. When cells with prebound ^{3}H-labeled EBs were warmed to 37°C, the EBs rapidly became resistant to proteinase K removal (half time = 5 min), indicating that the organisms were either ingested or remained on the cell surface but no longer were in a position to be removed by the proteinase K (Fig. 3). The amount of intracellular, cell-associated radioactivity leveled off within a 30-min incubation period at 37°C. A lack of increased radioactivity in the supernatant fluids of these samples during incubation at 37°C indicated that dissociation of the chlamydiae from host cell monolayers before exposure to proteinase K did not occur. Of the ^{3}H-labeled EBs that were bound to the L cells after 5 h at 4°C, only 65% of the associated chlamydiae were sensitive to proteinase K removal before being warmed to 37°C. Decreasing the adsorption time did not increase the removal of bound EBs by proteinase K treatment (data not shown). These results would indicate that possibly 35% of the EBs entered the host cells at 4°C. However, morphological observations showed that a population of EBs still remained bound to the host cell surface at 4°C after treatment with proteinase K. Rarely was a chlamydial particle observed to be internalized at this temperature.

Visualization of the internalization process at 37°C revealed that the infectious EBs appeared to travel down the microvilli to their bases, where they entered the host cell via specialized invaginated pits of the plasma membrane (Fig. 4A, B, and C). There did not appear to be a sequential engulfment of the organism by pseudopods, as is characteristically seen in phagocytosis. Also, there were no obvious microfilaments present at the site of internalization, as are frequently seen under areas of classical phagocytosis. Note the presence of a ghostlike structure, perhaps an EB envelope, which is in position to be internalized by the host cell (Fig. 4D). A direct correlation between the quantitative and the TEM results for uptake was observed, since the number of bound but not internalized chlamydiae seen on the cell surface decreased significantly during the 15-min incubation at 37°C.

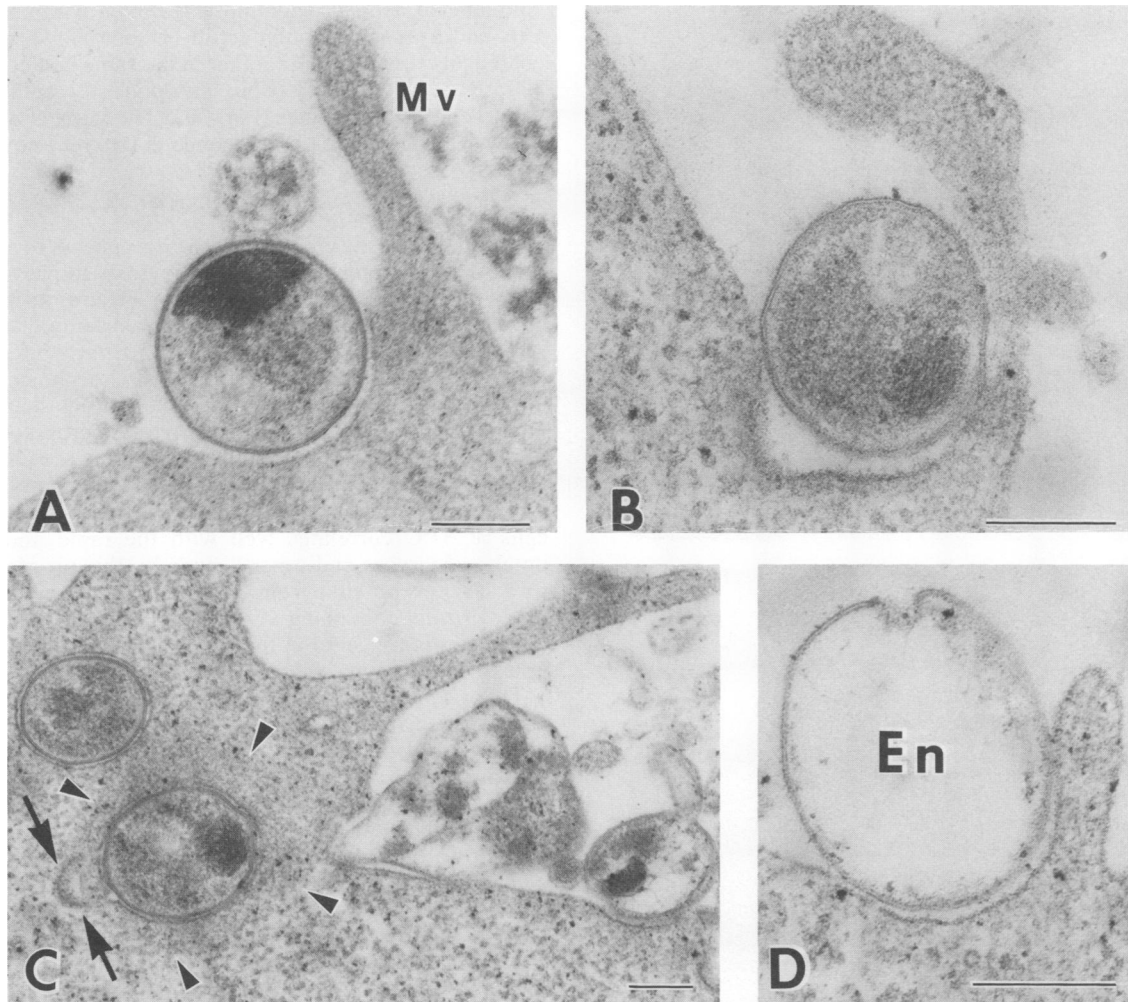Often, the cytoplasmic side of the host cell membrane

FIG. 4. TEM depicting the internalization of *C. psittaci* by L cells after 2 to 5 min at 37°C. (A and B) Entry of EBs into L cells, apparently initiated specifically at the bases of the microvilli; (C) invaginating host cell membrane forming a vesicle that envelopes the elementary body, with a dark, fuzzy coat apparent at the leading portion (arrows) of the entering vesicle, which is immediately surrounded by an electron-dense fibrillar material (arrowheads); (D) EB envelope (En) in position to be internalized. Mv, Microvillus. Bar, 0.2 μm.

beneath the entering EB was more electron-dense than was the rest of the cell surface, suggesting a clathrin-coated pit (Fig. 5A). The coated pits are invaginations of the plasma membrane, with a characteristic dark, fuzzy, bristle coat on the cytoplasmic surface. Many of these pits are localized at or near the bases of the microvilli. Only one EB could be seen in a single coated pit, and chlamydial particles were often located at the edges of the pits (Fig. 5B). EB envelopes were observed to enter the host cells at these coated pit regions (Fig. 5C). EBs were also present in smooth-surfaced invaginations frequently localized at the bases of microvilli.

The EBs were internalized within tightly bound vesicles surrounded by an electron-dense coat of fibrillar material (Fig. 6A). This fibrillar material surrounded the EB-laden vesicles for at least 30 to 60 min. However, its composition and function are not yet known. Although a number of EBs simultaneously invaded a single cell, only one chlamydial particle was present in each coated vesicle and it remained membrane bound (Fig. 6B). Note in Fig. 6C that an EB was internalized within a vesicle that remained connected to the cell surface by a long neck and was encompassed by the same electron-dense fibrillar material. This is similar to what

has been observed for certain macromolecules which enter cells by receptor-mediated endocytosis (22). Release of the coat from an EB-containing vesicle results in the formation of an endosome which may consist of a vesicle plus a tubule structure (Fig. 7). Although morphologically similar to the compartment of uncoupling of receptor and ligand (CURL) described for receptor-mediated endocytosis (22), we have no evidence that the tubular membrane extending from the EB-containing vesicle is involved in recycling receptors back to the cell surface.

To determine that chlamydiae were indeed internalized by host cells through coated pits, we compared the entry of EBs into cells with that of a molecule known to enter cells via receptor-mediated endocytosis and associated clathrin-coated pits. α_2-Macroglobulin was coupled to colloidal gold and added to uninfected L cells to monitor its entry. The gold-labeled α_2-macroglobulin was observed in coated pits at the bases of microvilli, along smooth surfaces of the cell, and in coated vesicles (Fig. 8A and B). The coated pits and vesicles were morphologically similar to those associated with the chlamydial particles, except that EB-containing vesicles appeared to possess a larger, more diffuse coat. If
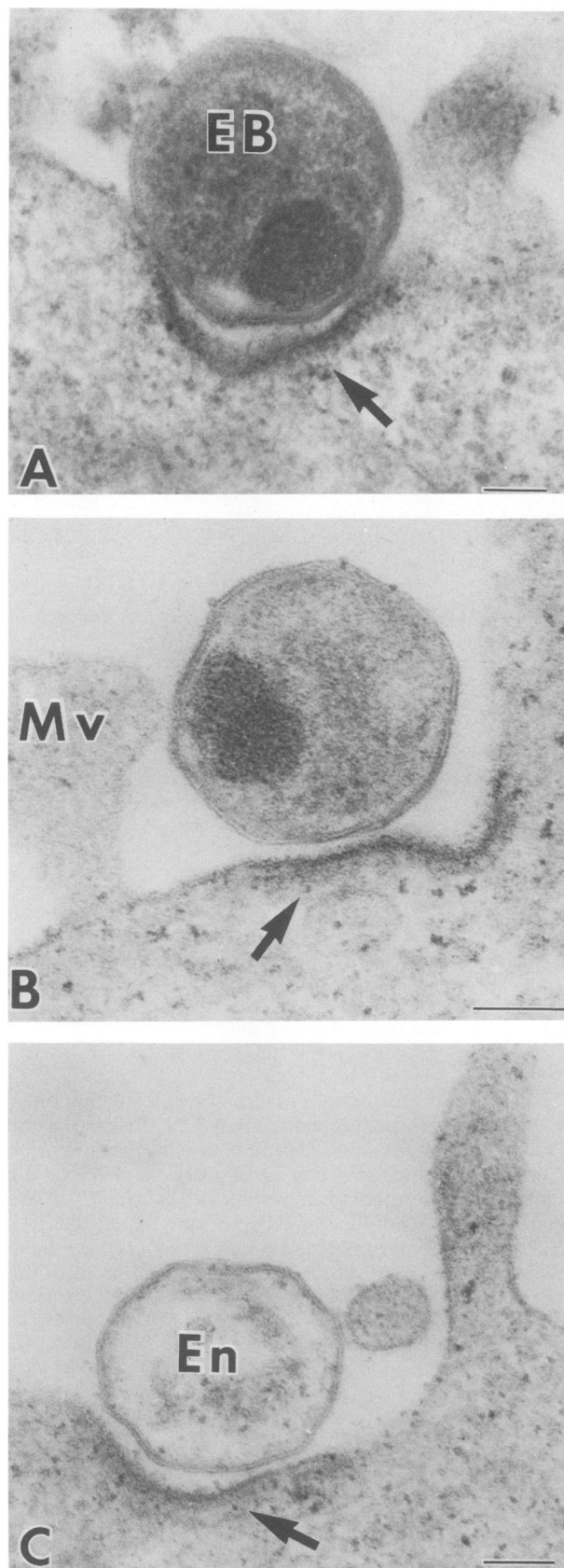
gold-labeled α_2-macroglobulin was mixed with chlamydiae, EBs and α_2-macroglobulin could be seen alone in separate coated pits (Fig. 8C) or together in the same coated pits (Fig. 8D), from which they were subsequently cointernalized within endocytic vesicles (Fig. 8E). The intracellular fate of the vesicles harboring both ligands is not yet known.

DISCUSSION

Like a number of viruses, *C. psittaci* appears to exploit the process of receptor-mediated endocytosis to provide for its entry into eucaryotic cells and to ensure its continued development within the cell. The ultrastructural views presented by TEM observations of *C. psittaci*-infected host cells have provided a unique perspective of the cellular and subcellular aspects of the initial interactions between this parasite and its host. Our electron photomicrographs appear to represent the morphological features that are characteristic of the mechanism of receptor-mediated endocytosis. Also, the speed with which *C. psittaci* is internalized by host cells at 37°C correlates well with the rapid and efficient uptake of viruses and other ligands by this process. Therefore, it seems possible from our observations that the escape of *C. psittaci*-containing vesicles from fusion with host cell lysosomes is triggered at the early stages of attachment and uptake and involves a specialized mode of entry.

That *C. psittaci* attaches preferentially to the microvilli of the L-cell surface may be an important feature of the initial interaction of this organism with the host cell. Considering that the membrane regions at the bases of the microvilli are areas of active transport of extracellular materials into the cell, it would be advantageous for an obligate intracellular parasite to bind to a portion of the plasma membrane that might assist in rapid and efficient entry into the host cell. Clearly, many of the viruses which enter cells by receptor-mediated endocytosis attach specifically to cell surface microvilli (14, 19, 34). The microprocesses may initially be used to bridge the electrostatic repulsion between electronegative membranes of the parasite and host, possibly allowing for subsequent receptor-ligand interactions. This seems to be true for Semliki Forest virus, which binds preferentially to microvilli of host cells (14) and has been shown to have a high affinity for the major histocompatibility antigens on both mouse and human cell surfaces (15). This does not exclude the possibility that receptors which are recognized by a given parasite are, in fact, concentrated on these microvilli, with nonspecific forces playing a lesser role in the initial interaction between membranes of the organism and host.

The involvement of cell surface receptors in chlamydial adhesion to a host cell is possible, since our ultrastructural studies revealed that coated pits, coated vesicles, and endosomes may play a role in *C. psittaci* entry. Consequently, the interaction between chlamydiae and host cells should theoretically be a receptor-ligand binding. Although a specific ligand or receptor has not yet been isolated for *Chlamydia* spp., investigators have demonstrated the need for specific glycoproteins on the surfaces of both the bacte-

FIG. 5. Association of *C. psittaci* with electron-dense, coated invaginations of the L-cell plasma membrane after 2 to 5 min at 37°C. (A) EB within a coated pit (arrow); (B) EB located at the edge of a coated pit, with the membrane beneath the EB (arrow) as electron dense as the pit itself; (C) EB envelope (En) within a coated pit at the base of a microvillus (arrow). Mv, microvillus. Bar, 0.1 μm.
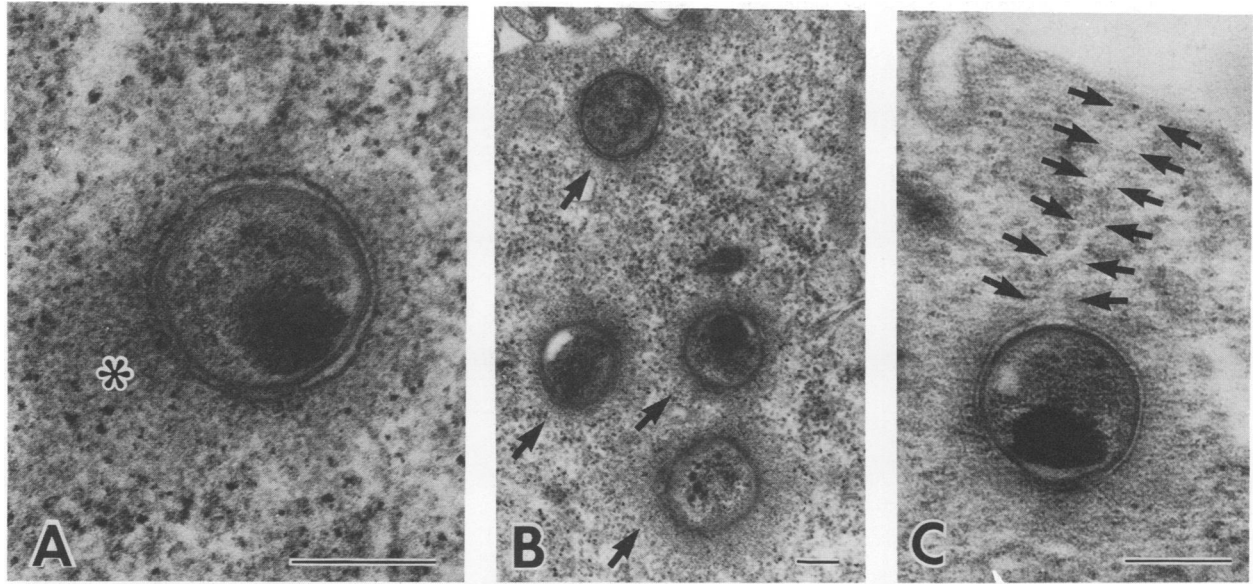
FIG. 6. TEM showing *C. psittaci* EBs internalized within tightly bound vesicles surrounded by an electron-dense coat of fibrillar material after 2 to 5 min at 37°C. (A) High magnification of the EB-containing vesicle and the surrounding material (*); (B) single host cell simultaneously infected with several vesicle-bound chlamydial particles, with only one EB per vesicle, which is encompassed by a fibrillar coat; (C) EB contained within a vesicle which remains associated with the host cell surface by an extended neck (arrows). The fibrillar coat completely surrounds the vesicle except for the opened portion of the vesicle membrane making up the base of the neck. Bar, 0.2 μm.

rium and host cell to bind chlamydiae to these cells (for a review, see references 23 and 30). However, chlamydial attachment may also be influenced by nonspecific factors. The work of Kuo et al. (18) has suggested that the trachoma biovar of *Chlamydia trachomatis* possesses a greater electrostatic barrier to adhesion than does the corresponding lymphogranuloma venereum biovar. Söderlund and Kihlström (26) have found the two biovars to differ in their propensity to hydrophobic interaction and the trachoma biovar to be more negatively charged than the lymphogranu-

loma venereum biovar. To overcome this barrier, certain manipulations have been routinely used to alter the susceptibility of host cells to infection with the trachoma biovar (5, 18). Since *Chlamydia* spp. lack a smooth lipopolysaccharide and possess no surface appendages which might bridge electronegative barriers between bacterium and host, these manipulations appear to increase the chances of the organism to contact and adhere to a host cell. Such techniques are normally not required for the adsorption of *C. psittaci* to cells. The avid binding of *C. psittaci* to L cells at 4°C would indicate that the molecular nature of the membrane of this organism is such that it can readily associate with host cells under the appropriate conditions. By suspending the EB in a small volume of inoculating fluid, we initiated a more than adequate infection by adsorption at this temperature. This enabled us to synchronize the cell infections and to view the various stages of attachment separately from internalization.

The idea that *Chlamydia* spp. can enter cells by receptor-mediated endocytosis has received increasing attention in the last several years. Our findings support the observation of Doughri et al. (7) that a bovine strain of *C. psittaci* associates with electron-dense areas at the bases of the brush borders of intestinal epithelial cells. Also, Söderlund and Kihlström (27) have determined that the internalization and intracellular development of the E serovar of *C. trachomatis* within McCoy cells is disrupted by treatment of these cells with chemical inhibitors of receptor-mediated endocytosis. However, the findings of similar studies by Ward and Murray (31) on the entry of the lymphogranuloma venereum biovar of this organism into HeLa cells are diametrically opposed to those suggesting this endocytic pathway. These contradictory results may be attributed to a number of factors, including the use of different serovars and host cell types, as well as specific disparities in experimental design. It seems unlikely that the biovars of *C. trachomatis* would enter cells by totally dissimilar mechanisms, although this hypothesis has not been experimentally tested. A comparison of our results obtained by TEM with those of Ward
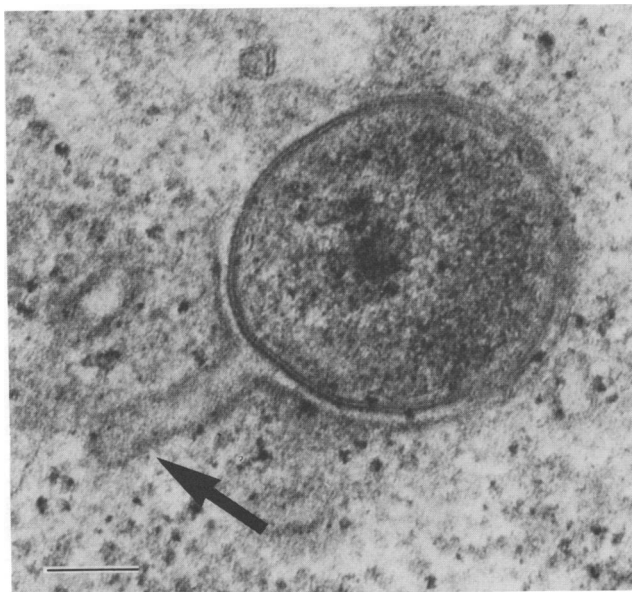

FIG. 7. Chlamydial EB in an endosome after warming at 37°C for 15 to 30 min. Note the absence of coat material and the formation of a tubular membrane structure (arrow) extending from the EB-containing vesicle. Bar, 0.1 μm.
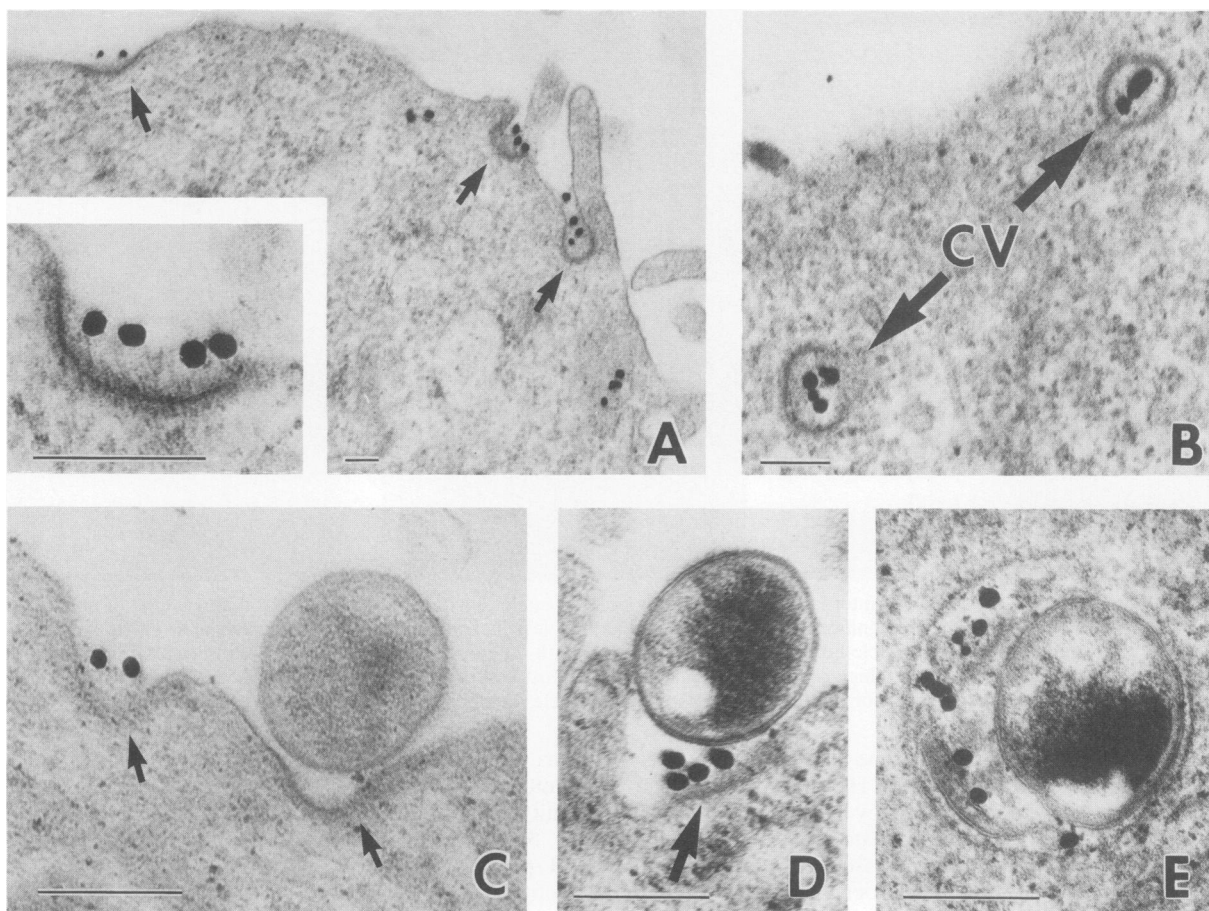
FIG. 8. Similarities in the association of gold-labeled α_2-macroglobulin and *C. psittaci* organisms with L cells as revealed by TEM. (A [including insert]) Gold-labeled α_2-macroglobulin within coated pits (arrows) after incubation for 5 h at 4°C; (B) coated vesicles (CV) of the host cell containing gold-labeled α_2-macroglobulin (arrows) after 2 min at 37°C; (C) association of an EB and gold-labeled α_2-macroglobulin with separate invaginations of the host cell plasma membrane (arrows) after 5 h at 4°C; (D) EB and gold-labeled α_2-macroglobulin entering an L cell at a coated region of the membrane at the base of a microvillus after incubation at 37°C for 2 min; (E) cointernalization of an EB and gold-labeled α_2-macroglobulin within an endocytic vesicle after 15 min at 37°C. Bar, 0.2 μm.

and Murray (31) suggests that the entry process for *C. psittaci* may differ from that of *C. trachomatis*. Though the developmental cycles for both organisms are presumed to be the same, Ward (30) has suggested that *C. trachomatis* enters eucaryotic cells by phagocytosis and that chlamydia-laden vesicles fuse with one another early in the cycle to produce a large inclusion. In contrast, *C. psittaci* appears to enter cells by a mechanism similar to receptor-mediated endocytosis and forms small multiple inclusions within the host cell. Is there a difference in the intracellular fate of these two bacterial species that may be linked to the entry process? Such observations warrant further investigation.

A major argument against the internalization of chlamydiae by coated pits is that an EB (200 to 300 nm) should be too large to fit in an ordinary coated pit (100 to 150 nm). However, the size of such coated pits may simply reflect the molecules and the techniques used to visualize them. Heuser (16) has observed coated pits ranging in size from <10 to >300 nm on the surface of fibroblasts. Also, the work of Aggeler and Werb (1) suggests that only part of the membrane has to be coated for internalization to proceed. Our further observation of *C. psittaci* in uncoated invaginations of the host cell membrane may reflect the given plane of section examined and the procedure used to observe them or

it may truly depict the entry of the organism into specialized uncoated pits. Therefore, it is possible that the chlamydiae are internalized by a specific mechanism involving attachment to and entry at the bases of microvilli regardless of any coating of the membrane pits beneath the entering EBs. Although we were able to observe chlamydiae regularly within coated pits, we have not quantitated this process. However, we do feel confident that this is not a chance association. Again, viruses which enter cells through receptor-mediated endocytosis can be found to also associate with uncoated pits on the cell surface (19). Also, we were able to observe α_2-macroglobulin associated with pits seemingly devoid of coat material. Definitive proof of coated pit involvement requires the identification of the protein clathrin surrounding the chlamydiae-containing pits and vesicles. Using immunoelectron microscopy and gold-labeled anti-clathrin antibody, we will attempt to localize the cytoskeletal components involved in the entry process and to verify that EBs are truly associated with coated pits and coated vesicles.

In conclusion, our electron microscope data suggest a receptor-mediated endocytosislike pathway by which *C. psittaci* is internalized into nonprofessional phagocytes. Parasitism of this exquisitely elegant entry process may offer a

novel explanation for how this organism escapes phagosome-lysosome fusion. We do realize, however, that further studies are necessary, since our interpretations and conclusions were based mainly on morphological observations with a single strain of *C. psittaci* and one host cell type.

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