INFECTION AND IMMUNITY, Dec. 1996, p. 5366–5372 Vol. 64, No. 12
0019-9567/96/$04.00+0

Vesicular Interactions of the *Chlamydia trachomatis* Inclusion Are Determined by Chlamydial Early Protein Synthesis Rather Than Route of Entry

MARCI A. SCIDMORE,[1] DANIEL D. ROCKEY,[1] ELIZABETH R. FISCHER,[2] ROBERT A. HEINZEN,[1] AND TED HACKSTADT[1]*

Host-Parasite Interactions Section, Laboratory of Intracellular Parasites,[1] and Microscopy Branch,[2] National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840

Received 14 June 1996/Returned for modification 15 August 1996/Accepted 29 August 1996

Chlamydiae replicate intracellularly within a vacuole that has recently been characterized as intersecting an exocytic pathway. One of the initial events during chlamydial infection is the expression of a chlamydial early gene product(s) that effectively isolates the inclusion from the endocytic-lysosomal pathway and makes it fusogenic with sphingomyelin-containing exocytic vesicles. Associated with this change in vesicular interaction is the delivery of the vacuole to the peri-Golgi region of the host cell. Inhibition of chlamydial early transcription or translation causes *Chlamydia trachomatis*-containing vesicles to remain dispersed throughout the cytoplasm, where they eventually fuse with lysosomes. Chlamydiae that have been internalized by Fc-mediated endocytosis also avoid lysosomal digestion by a mechanism that requires chlamydial protein synthesis. These results suggest that the vesicular interactions of the chlamydial inclusion are defined by parasite-directed modification of the endocytic vesicle rather than by the route of internalization.

Intracellular parasites can occupy a variety of distinct niches in eukaryotic host cells, including the cytoplasm, lysosomal vacuoles, and nonlysosomal vesicles (11, 28). Several significant obligate and facultative intracellular pathogens of humans, including *Mycobacterium*, *Salmonella*, *Legionella*, *Toxoplasma*, and *Chlamydia* spp., replicate within vacuoles that do not fuse with lysosomes. The nonlysosomal vesicles inhabited by these intracellular parasites exhibit a diverse assortment of endosomal or lysosomal markers, suggesting that maturation of endocytic vesicles to lysosomes may be blocked at discrete steps in the pathway (34). The mechanisms employed by intracellular parasites to prevent lysosomal fusion are for the most part unknown. It is likely that the molecular mechanisms differ depending upon the parasite. A paradigm for the cellular trafficking of intracellular parasites involves *Toxoplasma gondii*, an intracellular parasite that occupies a vacuole that does not fuse with lysosomes or endocytic vesicles. Trafficking of *T. gondii* to the appropriate intracellular compartment and successful replication are determined by the receptor mediating entry of the parasite. When opsonized *T. gondii* is taken up via Fc-mediated endocytosis, lysosomal fusion occurs and the parasites are killed. Although the blocking of lysosomal fusion requires entry of viable *T. gondii*, the properties of the vesicle do not change if the parasite is killed after entry (22). The implication is that the properties of the vacuole are determined at the time of formation and that active modification of the vacuole by the parasite to prevent lysosomal fusion is not required.

Chlamydia trachomatis is the etiologic agent of trachoma and the most common cause of sexually transmitted disease in the United States (32). Chlamydiae replicate within an intracellular vacuole, termed an inclusion, that does not fuse with lysosomes (9, 12, 18, 23). The inclusion displays minimal interaction with the endocytic-lysosomal pathway, as evidenced by the lack of delivery of fluid phase markers and the absence of early or late endosomal markers (18, 33). Instead, the chlamydial inclusion is fusogenic with sphingomyelin-containing exocytic vesicles in transit from the Golgi apparatus to the plasma membrane (16, 17). Endocytosed *C. trachomatis* is rapidly transported to the peri-Golgi region of the cell (6, 16, 20, 25, 26) and gains entry to a specialized compartment that begins to accept exported sphingomyelin. The acquisition of sphingomyelin is initiated before differentiation of elementary bodies (EBs) to reticulate bodies is morphologically apparent (16). How chlamydiae establish themselves in an intracellular site that intersects an exocytic pathway is unknown. Unlike results obtained with *T. gondii* (22), experiments utilizing antibody-coated *C. trachomatis* EBs have demonstrated that chlamydial entry into HeLa 229 cells expressing FcγIII receptor may be enhanced by Fc-mediated endocytosis (35). Thus, EBs that have entered via a pathway otherwise destined to fuse with lysosomes develop and replicate normally.

We demonstrate here that expression of early chlamydial gene products is required for both the physical transport of EBs to the peri-Golgi region of the host cell and establishing fusion competence with sphingomyelin-containing exocytic vesicles. Modification of the vacuole to interact with this exocytic pathway is not dependent upon the route of entry, suggesting that chlamydiae secrete an early gene product that determines the cellular interactions of the inclusion.

MATERIALS AND METHODS

Cells and cell culture. Monolayer cultures of HeLa 229 epithelial cells (CCL 2.1; American Type Culture Collection) were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 10 μg of gentamicin per ml (MEM-10) or in RPMI 1640 supplemented with 10% FBS plus 10 μg of gentamicin per ml. Chinese hamster ovary (CHO) K1 and CHO B1 cells (22), generously provided by I. Mellman, were propagated in MEM containing 600 μg of G418 per ml. Cells were grown at 37°C on 12-mm-diameter glass coverslips (no. 1 thickness) in 24-well plates in an atmosphere of 5% CO_2 in humidified air. *C. trachomatis* LGV-434, serotype L2, was grown in HeLa 229 cells, and infections were carried out as described previously (5, 17).

Antibodies. Anti-*C. trachomatis* monoclonal antibodies (MAbs) L2I-10 (anti-major outer membrane protein [anti-MOMP]); immunoglobulin G3 [IgG3]) and EVI-H1 (anti-lipopolysaccharide; IgG2a) were prepared as described previously (4). The mouse anti-human FcγRIII MAb (3G8) was obtained from Medarex (Annandale, N.J.), the rat anti-mouse FcγRII MAb (2.4G2) was obtained from

* Corresponding author. Phone: (406) 363-9308. Fax: (406) 363-9204. Electronic mail address: Ted_Hackstadt@NIH.GOV.

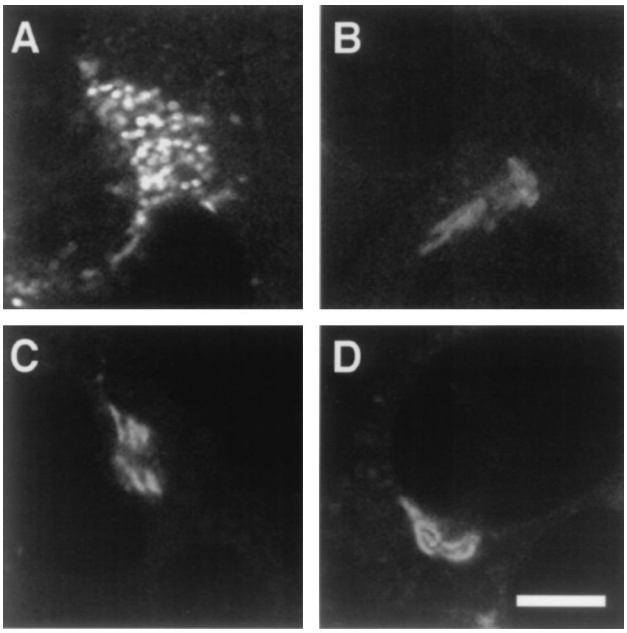

FIG. 1. Inhibition of chlamydial transcription or translation prevents fluorescent sphingomyelin acquisition. Monolayer cultures of HeLa 229 cells were infected with *C. trachomatis* LGV-434, serotype L2, at a multiplicity of infection of about 50 infectious EBs per cell and incubated in MEM-10 containing 0.004% ethanol as a control (A), 200 μg of chloramphenicol per ml (B), 50 μg of tetracycline per ml (C), or 150 μg of rifampin per ml (D). All subsequent incubations contained inhibitor at these concentrations. After 3 h of incubation at 37°C, the cultures were labeled with 5 μM C_6-NBD-ceramide as described previously (16, 29). Following 10 min of incubation at 37°C, the cultures were rinsed and incubated for 1 h in MEM plus 0.34% DFBSA to back-exchange excess probe from the plasma membrane prior to mounting for confocal microscopy. The bright spheres visible in panel A represent *C. trachomatis* EBs. Staining of only the Golgi apparatus is observed in panels B to D. Bar, 10 μm.

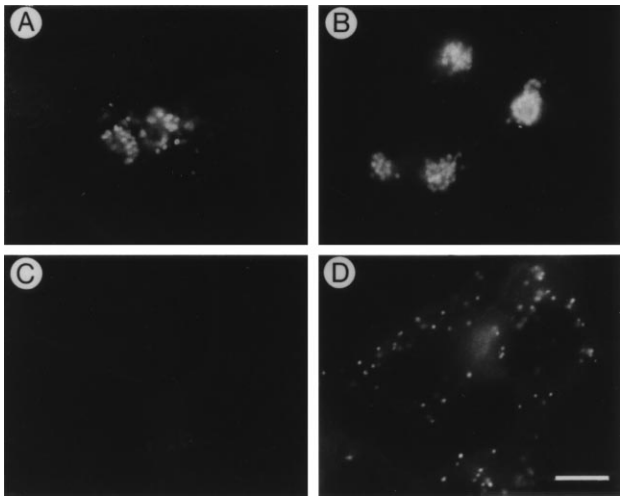

FIG. 2. Inhibition of chlamydial early protein synthesis blocks intracellular transport of endocytosed chlamydiae to the peri-Golgi region of the host cell. Cells were infected with *C. trachomatis* L2 at a high multiplicity of infection as described in the legend to Fig. 1 and incubated for 8 h in the absence (A and B) or presence (C and D) of 200 μg of chloramphenicol per ml. (A and C) Cultures were labeled with C_6-NBD-ceramide as described in the legend to Fig. 1 except that the back-exchange interval was increased to 2 h to reduce background staining of the Golgi apparatus. (B and D) A parallel set of coverslips was fixed with methanol and stained by indirect immunofluorescence with polyclonal anti-*C. trachomatis* L2 EB antiserum. It is important to note that the EBs in the presence of chloramphenicol neither aggregate at the peri-Golgi region nor incorporate fluorescent sphingolipid. Both panels demonstrating C_6-NBD-ceramide labeling (A and C) were taken at the same exposure and printed under identical conditions. Bar, 10 μm.

Pharmingen (San Diego, Calif.), and the mouse anti-*C. trachomatis* MOMP MAb (MAb 8331) was obtained from Chemicon (Temecula, Calif.). The MAb H4A3 to the lysosomal glycoprotein LAMP-1 was obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Fluorescent secondary antibodies were purchased from Zymed Laboratories, Inc. (San Francisco, Calif.).

Opsonization of chlamydiae. MAbs and EBs were diluted in 0.25 M sucrose–10 mM phosphate–5 mM glutamic acid medium (SPG) (pH 7.2), mixed 1:1, and incubated for 60 min at 37°C. Control EBs were mixed with SPG alone and also incubated for 60 min at 37°C. Two hundred microliters of the EB suspension was adsorbed to HeLa cells for 30 min at 37°C, washed once with Hank's balanced salt solution (HBSS), and incubated in RPMI 1640 supplemented with 10% FBS and 10 μg of gentamicin per ml for 36 h at 37°C prior to methanol fixation, staining, and quantitation of productive infections.

C_6-NBD-ceramide labeling. 6-{*N*-[7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl}sphingosine (C_6-NBD-ceramide) was complexed with 0.034% defatted bovine serum albumin (DFBSA) in Dulbecco's MEM as described previously (29). Labeling of *C. trachomatis*-infected monolayers was also performed as described previously (17). For all experiments involving C_6-NBD-ceramide trafficking in the presence of antibiotics, monolayers were infected with *C. trachomatis* for 30 min at 4°C and rinsed with HBSS, and the medium was replaced with prewarmed MEM-10 with or without the desired antibiotic. The antibiotics used and their concentrations were as follows: chloramphenicol, 200 μg/ml; tetracycline, 50 μg/ml; rifampin, 150 μg/ml. Following incubation at 37°C for the desired period, the cultures were labeled with C_6-NBD-ceramide for 10 min in the presence of antibiotic, rinsed with HBSS, and back-exchanged (17) for 1 h in MEM plus 0.34% DFBSA in the presence of the desired antibiotic.

Immunofluorescence. For indirect immunofluorescence, monolayers were fixed at 4°C with 4% paraformaldehyde in 25 mM sodium phosphate–150 mM NaCl (pH 7.4). The cells were permeabilized by 3 min of incubation in 0.1% Triton X-100–0.05% sodium dodecyl sulfate (SDS) and blocked with 10% BSA in 50 mM sodium phosphate–150 mM NaCl (pH 7.4; PBS) plus 0.1% Triton X-100–0.05% SDS for 30 min at room temperature. Antibodies were diluted in 10% BSA in PBS plus 0.1% Triton X-100–0.05% SDS, incubated for 1 h at room temperature, rinsed twice with PBS, and incubated with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin.

Microscopy. Micrographs were taken with a Nikon FXA photomicroscope with 60× Planapochromat objectives. Photomicrographs were obtained with T-Max ASA 400 film (Eastman Kodak Co., Rochester, N.Y.). An MRC-1000 confocal imaging system equipped with a krypton-argon laser (Bio-Rad Laboratories, Hercules, Calif.) on a Zeiss inverted microscope with a 63× objective was utilized for laser scanning confocal microscopy. Confocal images were processed with Adobe Photoshop 2.5.1 (Adobe Systems, Inc., Mountain View, Calif.).

Electron microscopy. Chloramphenicol- and mock-treated *C. trachomatis*-infected HeLa cells on 13-mm-diameter Thermanox coverslips were fixed at 8 h postinfection with 4% paraformaldehyde–2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 1 h at 4°C. The cultures were postfixed in Karnovsky's 0.5% OsO_4–0.8% $K_3Fe(CN)_6$ followed by 1% tannic acid and stained overnight en bloc in 1% uranyl acetate. Samples were dehydrated in graded ethanol and embedded in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome, stained with 1% uranyl acetate and Reynold's lead citrate, and observed at 80 kV on a Philips CM-10 electron microscope.

RESULTS

***C. trachomatis* early gene expression is a prerequisite for fusion with sphingomyelin-containing exocytic vesicles.** C_6-NBD-ceramide has proven to be a valuable tool to examine the early events in chlamydia-host interactions since *C. trachomatis* makes the transition from an endocytic vesicle to a vacuole that interrupts an exocytic pathway. We examined the effects of inhibitors of bacterial transcription and translation on chlamydial entry into an exocytic pathway as defined by the ability to acquire endogenously synthesized sphingomyelin from C_6-NBD-ceramide (Fig. 1). Inhibitors of chlamydial transcription or translation present during the initial 4 h of infection precluded delivery of sphingolipids to the chlamydial inclusion and, by definition, entry into this exocytic pathway (16). Not only was sphingolipid acquisition prevented by inhibition of chlamydial protein synthesis but the characteristic convergence of internalized EBs at the peri-Golgi region of the cell did not occur (Fig. 2 and 3). Early chlamydial gene expression is thus required for both the trafficking of recently endocytosed EBs

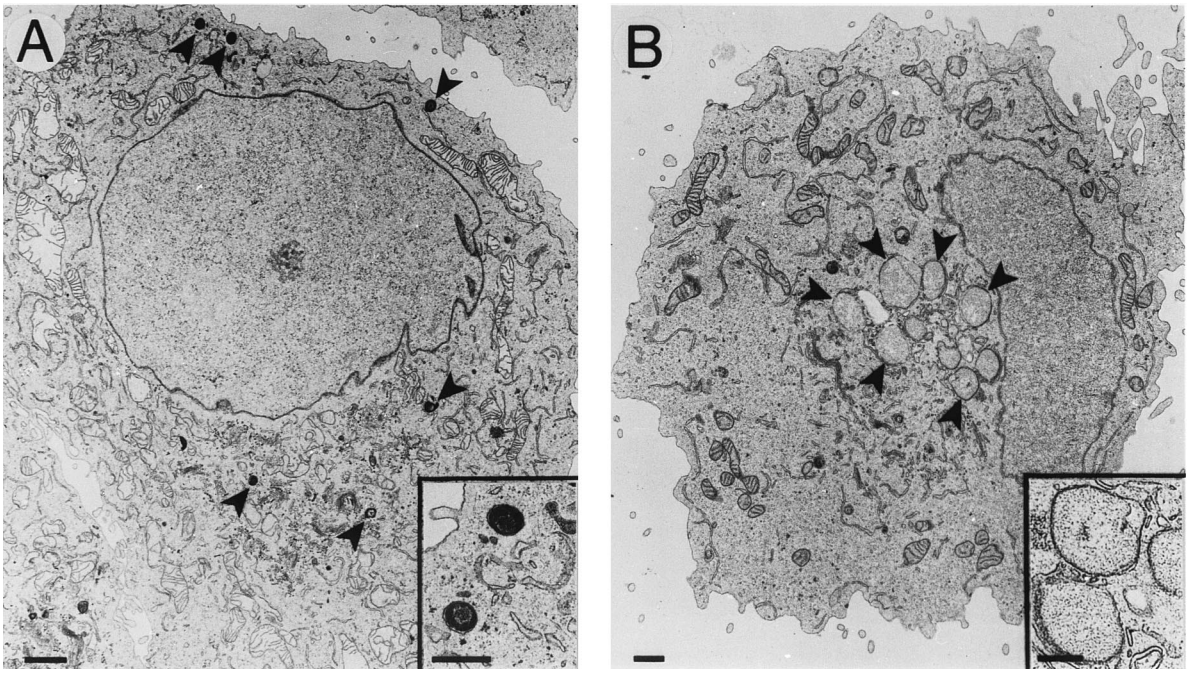

FIG. 3. Electron micrographs demonstrating convergence of chlamydiae in the peri-Golgi region and the interference with this intracellular transport by chloramphenicol. (A) Chloramphenicol treated; (B) mock treated. Cultures were fixed and processed for electron microscopy at 8 h postinfection. It is important to note that the EBs in the presence of chloramphenicol retain the appearance of typical EBs while the untreated controls have increased in size and morphology characteristic of differentiation to reticulate bodies. Bars, 1 μm in large panels and 0.5 μm in inserts.

to the appropriate intracellular location and the functional modification of the vacuoles to initiate fusion competence with sphingolipid-containing exocytic vesicles. The critical events during this early interaction with the host cell occur within the first 2 h following entry (16). If EBs were allowed to attach, enter, and develop within cells for 6 h and then were inhibited with chloramphenicol for an additional 6 h before addition of the fluorescent ceramide analog, the chlamydiae were observed to cluster in the peri-Golgi region and to procure sphingomyelin similar to the way they did in untreated controls (data not shown). Thus, once the unidentified early genes are expressed and the vacuole is modified to establish interaction with the exocytic pathway, the interaction continues after cessation of chlamydial protein synthesis. Extended incubation in the presence of chloramphenicol, however, resulted in the eventual abatement of sphingolipid transport to the inclusion. The chlamydial polypeptides required for this functional interaction are thus unstable or, possibly, are a structural requirement for intercalation of sphingomyelin into the inclusion membrane.

Lysosomal fusion occurs if *C. trachomatis* is prevented from modifying the inclusion. Chlamydial EBs which are endocytosed and inhibited by chloramphenicol are ultimately trafficked to lysosomes. EBs allowed to attach and enter but prevented from transforming the vacuolar properties by incubation in the presence of chloramphenicol eventually died within the host cell with a half-life of approximately 12 h (Fig. 4). Concomitant with the declining numbers of surviving EBs was an increase in the number of EBs that colocalized with antibodies to lysosomal glycoproteins. After 72 h in the presence of chloramphenicol, virtually all of the intracellular EBs colocalized with LAMP-1 (Fig. 5). Incubation for longer periods of time resulted in a loss of fluorescence signal and morphological integrity of the EBs consistent with lysosomal degradation (data not shown). Lysosomal glycoproteins are not

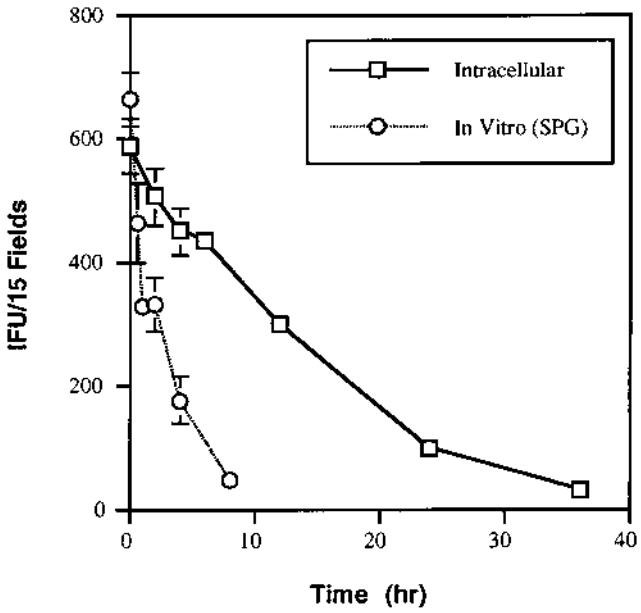

FIG. 4. Survival of endocytosed *C. trachomatis* EBs reversibly inhibited by chloramphenicol. HeLa cells were infected at a low multiplicity of infection and maintained in the presence of chloramphenicol for the indicated times. At the times indicated in the *x*-axis, the medium was removed, the cultures were washed three times with HBSS, and the medium was replaced with fresh medium without chloramphenicol. After incubation to permit chlamydial growth and inclusion formation, the cultures were fixed and stained by indirect immunofluorescence to determine numbers of viable inclusion-forming units (IFU) as described previously (17). The dashed line with open circles indicate survival times of EBs incubated at 37°C in SPG alone.

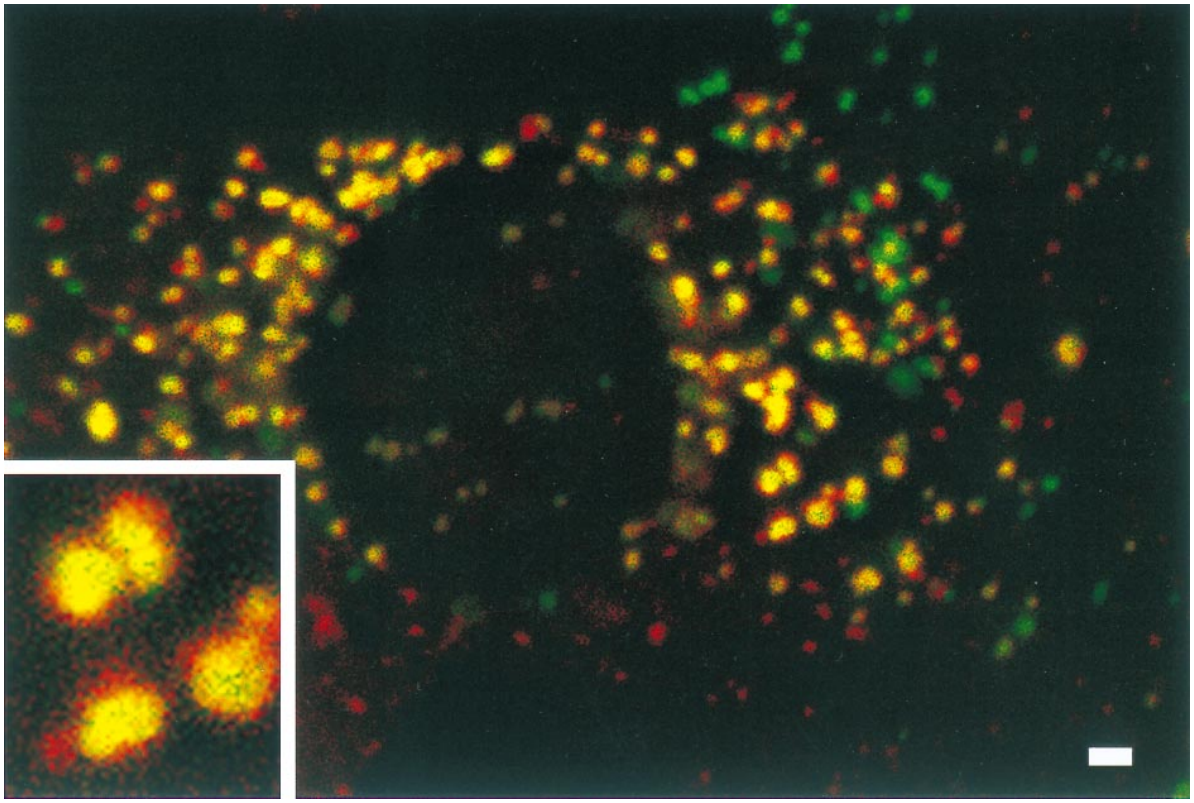

FIG. 5. Inhibition of chlamydial modification of the endocytic vesicle results in eventual lysosomal fusion. The confocal micrograph demonstrates colocalization of LAMP-1 marker with chloramphenicol-treated EBs. HeLa cells were infected as described in the legend to Fig. 1 and incubated in the presence of 200 μg of chloramphenicol per ml for 72 h. The cultures were fixed and doubly stained with polyclonal anti-*C. trachomatis* (green) antibodies and monoclonal anti-LAMP-1 (red). Bar, 10 μm. The insert is a higher magnification of the image, demonstrating the peripheral staining of the LAMP-1 antibody around EBs.

present in the inclusion membrane of viable, replicating chlamydiae (18); thus, unless chlamydiae actively modify the endocytic vesicle, lysosomal fusion occurs and the chlamydiae are eventually degraded.

Avoidance of lysosomal fusion is not dependent upon route of entry. To determine whether the route of chlamydial entry has a role in defining the properties of the vacuole, we examined the fate of EBs internalized via Fc-mediated endocytosis. Fc receptors are normally trafficked to lysosomes (27), and lysosomes fuse with vacuoles containing *T. gondii* internalized by Fc-mediated endocytosis (22). Specific antibodies against surface-exposed chlamydial antigens are nonneutralizing and enhance chlamydial infectivity in HeLa 229 cells, presumably as a result of the expression of FcγRIII by HeLa cells (35). To define the role of Fc-mediated uptake of chlamydiae, EBs were opsonized with the MAb L2I-10 and adsorbed to HeLa cells and productive infections were quantified. As shown in Fig. 6A, opsonization of EBs slightly, but reproducibly, enhanced chlamydial infectivity. Increased infectivity was also observed with several other MAbs (data not shown). To demonstrate that the increased infectivity was due to Fc-mediated uptake, infections were performed in the presence of heparin, which inhibits the normal route of chlamydial entry (38). Although heparin decreased inclusion formation by both opsonized and nonopsonized EBs, infection was enhanced fivefold as compared with that of nonopsonized EBs (Fig. 6A). As shown in Fig. 6B, the uptake of opsonized EBs in the presence of heparin was dependent upon the concentration of antibody. Furthermore, incubation of cells with MAb to FcγRIII prior to infection substantially decreased the uptake of opsonized EBs by approximately 40% percent but had no effect on the uptake of nonopsonized EBs (Fig. 6C).

These observations were confirmed in the CHO cell line stably transfected with an isoform of the mouse FcγRII receptor (FcγRII-B1) which has been used to define the influence of route of entry on survival of *T. gondii* (22). Chlamydial infectivity was enhanced in an isotype-specific manner in CHO cells expressing the FcγRII receptors but not in the parental CHO cell line (Fig. 7A). Specifically, an IgG2b MAb (MAb 8331), an isotype preferentially bound by FcγRII (19), conferred enhancement of chlamydial uptake as compared with opsonization with IgG2a (EVI-H1) (Fig. 7A) or IgG3 (L2I-45) (data not shown). As with the HeLa cell experiments, pretreatment with anti-FcγRII MAb reduced the uptake of opsonized EBs to levels comparable to that of nonopsonized EBs (Fig. 7B). Indirect immunofluorescence demonstrated colocalization of the FcγRII receptor and EBs to confirm that EBs were present in vesicles containing FcγRII receptor (Fig. 8). As shown above for nonopsonized EBs, chlamydiae internalized via Fc-mediated endocytosis were concentrated at a peri-Golgi region and acquired NBD-sphingomyelin. Like nonopsonized EBs, treatment with chloramphenicol prevented targeting and concentration of opsonized EBs at the peri-Golgi region and acquisition of NBD-sphingomyelin (data not shown).

DISCUSSION

The chlamydial inclusion appears not to interact with the endocytic or lysosomal pathway but converges with an exocytic pathway as evidenced by the incorporation of sphingomyelin-

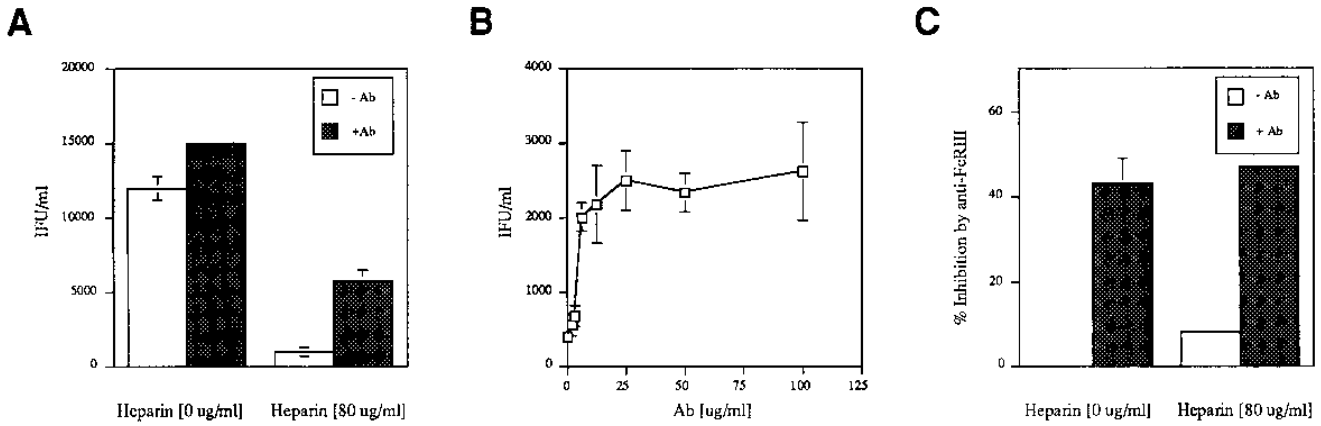

FIG. 6. Opsonized EBs are internalized by the FcγRIII receptor in HeLa 229 cells and produce productive infections. (A) *C. trachomatis* EBs were incubated in the absence or presence of MAb L2I-10 (100 μg/ml) for 60 min at 37°C prior to adsorption to HeLa 229 cells in the presence or absence of heparin at a final concentration of 80 μg/ml. Infection with opsonized EBs resulted in a slight increase in inclusion-forming unit (IFU) formation relative to that of nonopsonized EB infection. This difference in infectivity is enhanced in the presence of heparin. (B) IFU formation by opsonized EBs in the presence of heparin is dependent upon the concentration of antibody (Ab). EBs were opsonized with increasing concentrations of MAb L2I-10 for 60 min at 37°C prior to adsorption to HeLa cells in the presence of 80 μg of heparin per ml. (C) HeLa cells were pretreated with anti-FcγRIII antibody in PBS for 2 h at 4°C prior to infection. Opsonized and nonopsonized EBs were adsorbed in the presence or absence of 80 μg of heparin per ml. Pretreatment of cells with anti-FcγRIII decreased IFU formation of opsonized EBs by approximately 40%. IFUs were determined by indirect immunofluorescence on methanol-fixed cells 36 h postinfection by using a polyclonal anti-*C. trachomatis* serum. Each point represents the average of duplicate determinations, with the standard error of the mean indicated.

containing vesicles in transit to the plasma membrane. This interaction with an exocytic pathway has been hypothesized to constitute a mechanism for evasion of lysosomal fusion as well as to provide a potential source of lipid for both the chlamydiae and the growth of the inclusion membrane (17). The acquisition of fluorescent sphingomyelin, endogenously synthesized from C_6-NBD-ceramide, was used to document a very early transition in the properties of vesicles containing endocytosed chlamydial EBs such that they become fusogenic with exocytic vesicles but not with lysosomes (16, 18). In this study, we addressed the mechanisms involved in the change in vacuolar properties since vesicles containing endocytosed EBs are transformed to intersect an exocytic pathway. Inhibitors of early chlamydial transcription or translation prevent not only the acquisition of ceramide derivatives but the characteristic trafficking of internalized EBs to the peri-Golgi region of the host cell. These results suggest that chlamydiae actively modify the response of the host cell to chlamydial infection by a process that requires de novo chlamydial protein synthesis. The ability of IgG-coated EBs to enter by Fc-mediated endocytosis and replicate normally suggests that the route of entry appears less important in defining the properties of the mature chlamydial inclusion than the expression of chlamydial gene products to modify the vesicle is. This mechanism differs from that of *T. gondii*, in which the route of entry determines the characteristics of the parasitophorous vacuole (22).

Chlamydiae attach to and enter cells so efficiently that the process has been termed "parasite-directed endocytosis" (3).

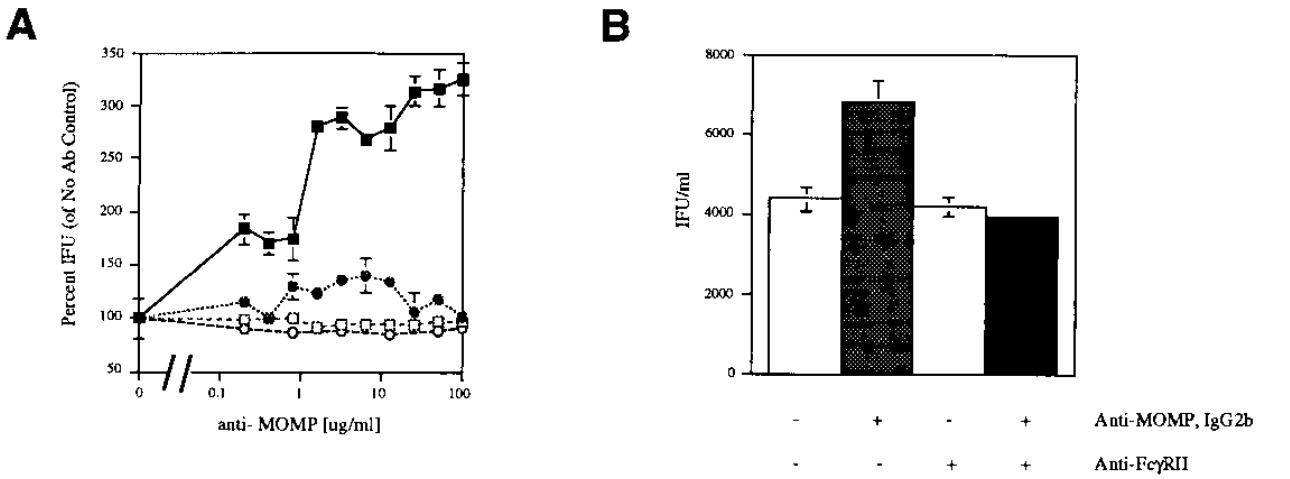

FIG. 7. Opsonized EBs are internalized via the Fc receptor in CHO cells expressing the FcγRII isoform B1. (A) EBs were opsonized with either MAb 8331 (IgG2b) or EVI-H1 (IgG2a) for 60 min at 37°C prior to adsorption to either parental CHO K1 or CHO B1 cell lines. IFU formation in CHO B1 cells increased with increasing antibody (Ab) concentration for EBs opsonized with MAb 8331 but not EVI-H1. Antibody had no effect on chlamydial infectivity in the parental CHO K1 cells. Symbols: circles, CHO K1 cells; squares, CHO B1 cells; open symbols, opsonization with EVI-H1; closed symbols, opsonization with MAb 8331. (B) CHO B1 cells were pretreated with anti-FcγRII antibody for 2 h at 4°C prior to infection with MAb 8331 (50 μg/ml)-opsonized or nonopsonized EBs. Each point represents the average of duplicate determinations, with the standard error of the mean indicated. Open bars indicate nonopsonized EBs; filled bars indicate EBs opsonized with MAb 8331.

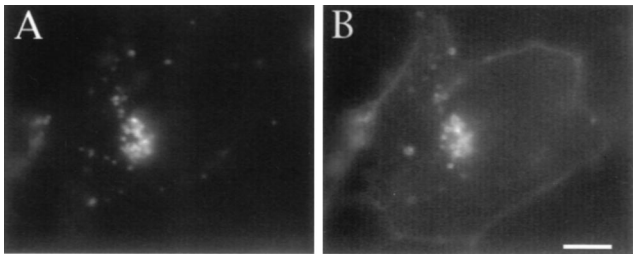

FIG. 8. Immunofluorescent staining of CHO B1 cells infected with opsonized *C. trachomatis* EBs. EBs were opsonized with MAb 8331 (50 μg/ml) for 60 min at 37°C and used to infect CHO B1 cells at a multiplicity of infection of 50. Following 30 min of adsorption at 37°C, the cultures were rinsed with HBSS and incubated in RPMI 1640 supplemented with G418 for 6 h at 37°C. The cultures were then fixed and doubly stained with rabbit anti-*C. trachomatis* antisera (A) or rat anti-FcγRII antibody (B). Bar, 10 μm.

The implication is that chlamydial attachment to the appropriate host cell surface receptor induces chlamydial uptake. The precise mechanisms and pathways involved in chlamydial uptake are not clear, however. It is believed that chlamydiae enter host cells by receptor-mediated endocytosis via vesicles that may (21) or may not (36) be clathrin-coated. EBs allowed to attach and enter even in the presence of chloramphenicol do not fuse with lysosomes for up to 30 h postinfection (12). Purified cell walls from EBs are similarly endocytosed by host cells and not targeted to lysosomes (10). Because EBs and purified cell walls are endocytosed and delivered to a nonlysosomal compartment, it has been thought that the cellular pathway by which EBs are endocytosed directs them to an intracellular compartment not destined to fuse with lysosomes. This mechanism would be analogous to the situation with *T. gondii* in which the route of entry defines the properties of the vacuole. This model has been attractive since EBs display little or no metabolic activity and such a mechanism would obviate the necessity of chlamydial metabolism to determine the intracellular fate of endocytosed EBs. Our results, however, demonstrate that EBs in the presence of chloramphenicol are killed within the host cell with a half-life of survival of approximately 12 h. Furthermore, EBs inhibited by chloramphenicol are not trafficked normally and eventually show lysosomal markers associated with the vesicle, a characteristic not seen on viable inclusions (18). *C. trachomatis* EBs, prevented from modifying the host's response, are eventually degraded by the host cell. In this regard, it appears that EBs taken up naturally by HeLa 229 cells are not rapidly transported to lysosomes even when prevented from modifying the vacuolar environment by inhibition of protein synthesis. Although we have not done a detailed kinetic analysis of the acquisition of lysosomal markers in vesicles containing chloramphenicol-treated EBs, it does appear that the process is relatively slow, with up to 2 to 3 days of incubation required before a large proportion of vesicles containing EBs display lysosomal markers. This process may be accelerated in professional phagocytes, as shown by the fusion of vesicles containing opsonized EBs with ferritin-labeled lysosomes in mouse peritoneal macrophages (37).

A currently favored model of chlamydial attachment and entry involves heparan sulfate-like glycosaminoglycans as a critical ligand mediating chlamydial attachment (38). It is not known whether heparan sulfate-like glycosaminoglycans potentially involved in the attachment process are endocytosed with EBs, where they might be postulated to inhibit lysosomal fusion in a manner similar to that of sulfatides, which have been proposed to prevent lysosomal fusion with *Mycobacterium tuberculosis*-containing vesicles (2, 13). Whether polyanions have a physiological role in prevention of lysosomal fusion with any intracellular parasite is equivocal (8, 14, 15).

It is apparent that chlamydiae actively modify the host cell's response to EB-containing vesicles to prevent lysosomal fusion and to create a specialized environment favorable to chlamydial replication. Chlamydiae initiate protein synthesis relatively soon after endocytosis (24, 30). Among the functions mediated by chlamydial early polypeptide synthesis are the directed transport of endocytosed EBs to the peri-Golgi region of the host cell and establishment of the interaction with an exocytic pathway. A likely means of controlling the intracellular movement of the early chlamydial inclusion as well as altering its fusogenic properties with exocytic vesicles would be the modification of the chlamydial inclusion membrane by the insertion of a parasite-specific protein(s). To date, only a very few parasite-specified proteins are known to be inserted into the vacuolar membrane (1, 7, 31). The function of these polypeptides is unknown. Understanding how intracellular parasites alter vesicular interactions with the host should improve our understanding of intracellular pathogenesis as well as suggest novel mechanisms of intervening in parasite development.

ACKNOWLEDGMENTS

We thank J. Sager for excellent technical assistance, G. Hettrick and B. Evans for graphic arts, and H. Caldwell, W. Cieplak, and J. Bannantine for critical review of the manuscript.

The MAb H4A3 to LAMP-1, developed by S. H. Mane, L. Marzella, D. F. Bainton, V. K. Holt, Y. Cha, J. E. K. Hildreth, and J. T. August, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Md., and the Department of Biological Sciences, University of Iowa, Iowa City, under contract N01-HD-2-3144 from the National Institute of Child Health and Human Development.

REFERENCES

1. **Achbarou, A., O. Mercereau-Puijalon, A. Sadak, B. Fortier, M. A. Leriche, D. Camus, and J. F. Dubremetz.** 1991. Differential targeting of dense granule proteins in the parasitophorous vacuole of *Toxoplasma gondii*. Parasitology **103:**321–329.
2. **Armstrong, J. A., and P. D'Arcy Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134:**713–740.
3. **Byrne, G. I., and J. W. Moulder.** 1978. Parasite-specified phagocytosis of *Chlamydia psittaci* and *Chlamydia trachomatis* by L and HeLa cells. Infect. Immun. **19:**598–606.
4. **Caldwell, H. D., and P. J. Hitchcock.** 1984. Monoclonal antibody against a genus-specific antigen of *Chlamydia* species: location of the epitope on chlamydial lipopolysaccharide. Infect. Immun. **44:**306–314.
5. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31:**1161–1176.
6. **Campbell, S., S. J. Richmond, and P. S. Yates.** 1989. The effect of *Chlamydia trachomatis* infection on the host cell cytoskeleton and membrane compartments. J. Gen. Microbiol. **135:**2379–2386.
7. **Cesbron-Delauw, M. F., B. Guy, G. Torpier, R. J. Pierce, G. Lenzen, J. Y. Cesbron, H. Charif, P. Lepage, F. Darcy, J. P. Lecocq, and A. Capron.** 1989. Molecular characterization of a 23-kilodalton major antigen secreted by *Toxoplasma gondii*. Proc. Natl. Acad. Sci. USA **86:**7537–7541.
8. **D'Arcy Hart, P., M. R. Young, M. M. Jordan, W. J. Perkins, and M. J. Geisow.** 1983. Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit saltatory lysosomal movements. J. Exp. Med. **158:** 477–492.
9. **Eissenberg, L. G., and P. B. Wyrick.** 1981. Inhibition of phagolysosome fusion is localized to *Chlamydia psittaci*-laden vacuoles. Infect. Immun. **32:** 889–896.
10. **Eissenberg, L. G., P. B. Wyrick, C. H. Davis, and J. W. Rumpp.** 1983. *Chlamydia psittaci* elementary body envelopes: ingestion and inhibition of phagolysosome fusion. Infect. Immun. **40:**741–751.
11. **Falkow, S., R. R. Isberg, and D. A. Portnoy.** 1992. The interaction of bacteria with mammalian cells. Annu. Rev. Cell Biol. **8:**333–363.
12. **Friis, R. R.** 1972. Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110:**706–721.
13. **Goren, M. B., P. D'Arcy Hart, M. R. Young, and J. A. Armstrong.** 1976.

Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **73:**2510–2514.
14. **Goren, M. B., A. E. Vatter, and J. Fiscus.** 1987. Polyanionic agents do not inhibit phagosome-lysosome fusion in cultured macrophages. J. Leukocyte Biol. **41:**122–129.
15. **Goren, M. B., A. E. Vatter, and J. Fiscus.** 1987. Polyanionic agents as inhibitors of phagosome-lysosome fusion in cultured macrophages: evolution of an alternative interpretation. J. Leukocyte Biol. **41:**111–121.
16. **Hackstadt, T., D. D. Rockey, R. A. Heinzen, and M. A. Scidmore.** 1996. *Chlamydia trachomatis* interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. EMBO J. **15:**964–977.
17. **Hackstadt, T., M. A. Scidmore, and D. D. Rockey.** 1995. Lipid metabolism in *Chlamydia trachomatis*-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. Proc. Natl. Acad. Sci. USA **92:**4877–4881.
18. **Heinzen, R. A., M. A. Scidmore, D. D. Rockey, and T. Hackstadt.** 1996. Differential interaction with endocytic and exocytic pathways distinguish the parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. Infect. Immun. **64:**796–809.
19. **Heusser, C. H., C. L. Anderson, and H. M. Grey.** 1977. Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J. Exp. Med. **145:**1316–1327.
20. **Higashi, N.** 1965. Electron microscopic studies on the mode of reproduction of trachoma virus and psittacosis virus in cell cultures. Exp. Mol. Pathol. **4:**24–39.
21. **Hodinka, R. L., C. H. Davis, J. Choong, and P. B. Wyrick.** 1988. Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. Infect. Immun. **56:**1456–1463.
22. **Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman.** 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transformed fibroblasts. Science **249:**641–646.
23. **Lawn, A. M., W. A. Blyth, and J. Taverne.** 1973. Interactions of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy. J. Hyg. **71:**515–532.
24. **Lundemose, A. G., S. Birkelund, P. M. Larsen, S. J. Fey, and G. Christiansen.** 1990. Characterization and identification of early proteins in *Chlamydia trachomatis* serovar L2 by two-dimensional gel electrophoresis. Infect. Immun. **58:**2478–2486.
25. **Majeed, M., and E. Kihlström.** 1991. Mobilization of F-actin and clathrin during redistribution of *Chlamydia trachomatis* to an intracellular site in eucaryotic cells. Infect. Immun. **59:**4465–4472.
26. **McBride, T., and E. Wilde III.** 1990. Intracellular translocation of *Chlamydia trachomatis*, p. 36–39. *In* W. R. Bowie, H. D. Caldwell, R. P. Jones, P.-A. Mardh, G. L. Ridgway, J. Schachter, W. E. Stamm, and M. E. Ward (ed.), Chlamydial infections. Cambridge University Press, Cambridge.
27. **Mellman, I. S., H. Plutner, R. M. Steinman, J. C. Unkeless, and Z. A. Cohn.** 1983. Internalization and degradation of macrophage Fc receptors during receptor-mediated endocytosis. J. Cell Biol. **96:**887–895.
28. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49:**298–337.
29. **Pagano, R. E., and O. C. Martin.** 1988. A series of fluorescent N-acylsphingosines: synthesis, physical properties, and studies in cultured cells. Biochemistry **27:**4439–4445.
30. **Plaunt, M. R., and T. P. Hatch.** 1988. Protein synthesis early in the development cycle of *Chlamydia psittaci*. Infect. Immun. **56:**3021–3025.
31. **Rockey, D. D., R. A. Heinzen, and T. Hackstadt.** 1995. Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized to the inclusion membrane of infected cells. Mol. Microbiol. **15:**617–626.
32. **Schachter, J.** 1988. Overview of human diseases, p. 153–165. *In* A. L. Barron (ed.), Microbiology of chlamydia. CRC Press, Inc., Boca Raton, Fla.
33. **Scidmore, M. A., E. R. Fischer, and T. Hackstadt.** 1996. Sphingolipids and glycoproteins are differentially trafficked to the *Chlamydia trachomatis* inclusion. J. Cell Biol. **134:**363–374.
34. **Small, P. L. C., L. Ramakrishnan, and S. Falkow.** 1994. Remodeling schemes of intracellular pathogens. Science **263:**637–639.
35. **Su, H., G. J. Spangrude, and H. D. Caldwell.** 1991. Expression of Fc γRIII on HeLa 229 cells: possible effect on in vitro neutralization of *Chlamydia trachomatis*. Infect. Immun. **59:**3811–3814.
36. **Ward, M. E., and A. Murray.** 1984. Control mechanisms governing the infectivity of *Chlamydia trachomatis* for HeLa cells: mechanisms of endocytosis. J. Gen. Microbiol. **130:**1765–1780.
37. **Wyrick, P. B., and E. A. Brownridge.** 1978. Growth of *Chlamydia psittaci* in macrophages. Infect. Immun. **19:**1054–1060.
38. **Zhang, J. P., and R. S. Stephens.** 1992. Mechanism of *C. trachomatis* attachment to eukaryotic host cells. Cell **69:**861–869.

Editor: A. O'Brien