Differential Interaction with Endocytic and Exocytic Pathways Distinguish Parasitophorous Vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*

ROBERT A. HEINZEN, MARCI A. SCIDMORE, DANIEL D. ROCKEY, AND TED HACKSTADT*

Host-Parasite Interactions Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories,
National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840-2999

Received 12 September 1995/Returned for modification 7 November 1995/Accepted 30 November 1995

Coxiella burnetii and *Chlamydia trachomatis* are bacterial obligate intracellular parasites that occupy distinct vacuolar niches within eucaryotic host cells. We have employed immunofluorescence, cytochemistry, fluorescent vital stains, and fluid-phase markers in conjunction with electron, confocal, and conventional microscopy to characterize the vacuolar environments of these pathogens. The acidic nature of the *C. burnetii*-containing vacuole was confirmed by its acquisition of the acidotropic base acridine orange (AO). The presence of the vacuolar-type (H^+) ATPase (V-ATPase) within the *Coxiella* vacuolar membrane was demonstrated by indirect immunofluorescence, and growth of *C. burnetii* was inhibited by bafilomycin A_1 (Baf A), a specific inhibitor of the V-ATPase. In contrast, AO did not accumulate in *C. trachomatis* inclusions nor was the V-ATPase found in the inclusion membrane. Moreover, chlamydial growth was not inhibited by Baf A or the lysosomotropic amines methylamine, ammonium chloride, and chloroquine. Vacuoles harboring *C. burnetii* incorporated the fluorescent fluid-phase markers, fluorescein isothiocyanate-dextran (FITC-dex) and Lucifer yellow (LY), indicating trafficking between that vacuole and the endocytic pathway. Neither FITC-dex nor LY was sequestered by chlamydial inclusions. The late endosomal-prelysosomal marker cation-independent mannose 6-phosphate receptor was not detectable in the vacuolar membranes encompassing either parasite. However, the lysosomal enzymes acid phosphatase and cathepsin D and the lysosomal glycoproteins LAMP-1 and LAMP-2 localized to the *C. burnetii* vacuole but not the chlamydial vacuole. Interaction of *C. trachomatis* inclusions with the Golgi-derived vesicles was demonstrated by the transport of sphingomyelin, endogenously synthesized from C_6 -NBD-ceramide, to the chlamydial inclusion and incorporation into the bacterial cell wall. Similar trafficking of C_6 -NBD-ceramide was not evident in *C. burnetii*-infected cells. Collectively, the data indicate that *C. trachomatis* replicates within a nonacidified vacuole that is disconnected from endosome-lysosome trafficking but may receive lipid from exocytic vesicles derived from the *trans*-Golgi network. These observations are in sharp contrast to those for *C. burnetii*, which by all criteria resides in a typical phagolysosome.

Chlamydia trachomatis is the causative agent of several significant human diseases including trachoma, the leading cause of infectious blindness worldwide (38), and is the most common sexually transmitted disease in the United States and developed countries. The rickettsial organism *Coxiella burnetii* is the etiologic agent of human Q fever, a zoonosis that normally presents as an atypical pneumonia (4).

C. trachomatis and *C. burnetii* are obligate intracellular bacterial parasites of eucaryotic cells that carry out a biphasic life cycle within intracellular membrane-bound vacuoles. There are several superficial similarities between the life cycles of these diverse bacteria. Both have resistant cell types adapted for extracellular survival and metabolically active cell types for intracellular multiplication. *Chlamydia* infection is initiated by a small, metabolically inactive cell type called the elementary body (EB). Following endocytosis, an EB differentiates into a larger, pleomorphic, and metabolically active cell type called the reticulate body (RB). The RBs divide by binary fission throughout the remainder of the infection until the cell lyses at 40 to 48 h postinfection. At about 18 h postinfection the developmental cycle becomes asynchronous as some of the RBs begin to differentiate back to EBs which accumulate within the vacuole (termed an inclusion) until cell lysis occurs (33). The developmental cycle of *C. burnetii* is less well characterized. It

is believed, however, that once internalized, metabolically inactive small-cell variants morphologically differentiate to more metabolically active large-cell variants (28). Since the small-cell variant is far more resistant than the large-cell variant to physical and chemical disruption (29), it is presumed that the small-cell variant is the extracellular survival form that initiates natural infections. The environmental signals within the inclusion that regulate development of chlamydiae are unknown. It has been proposed that acidification of the phagocytic vacuole triggers metabolism and growth of *C. burnetii* (18).

The chlamydial inclusion is not believed to fuse with lysosomes. The lysosomal enzyme acid phosphatase does not localize to the chlamydial inclusion (14, 23), and fusion with secondary lysosomes labeled with ferritin does not occur (44). In contrast to chlamydiae, acid phosphatase activity has been localized to vacuoles containing *C. burnetii* (1, 8). Thorium dioxide, as a marker for secondary lysosomes, is also delivered to *C. burnetii*-containing vacuoles (1). The metabolic activity of purified, cell-free *C. burnetii* is maximal at a pH of 4.7 to 4.8, suggesting that the organism is adapted to metabolism and growth under acidic conditions. An *in vivo* requirement for acidic pH is implied by the inhibition of *C. burnetii* development by lysosomotropic amines, agents known to raise the pH of lysosomal vesicles (18). In contrast, the metabolic activity of purified chlamydial RBs is maximal at pH 7.0 to 7.5 (20).

There are many fundamental questions regarding the nature of the parasitophorous vacuoles of obligate and facultative

* Corresponding author. Phone: (406) 363-9308. Fax: (406) 363-9204. Electronic mail address: ted_hackstadt@nih.gov.

intracellular parasites. *C. burnetii* and *C. trachomatis* offer a unique opportunity to compare and contrast the intravacuolar environment of two obligate intracellular bacterial pathogens that interact very differently with the host cell. In this study we have employed a variety of contemporary probes for endocytic and lysosomal pathways in conjunction with conventional and confocal fluorescence microscopy to more precisely define the cellular organelles and vesicles that interact with *Chlamydia* and *Coxiella* parasitophorous vacuoles. Our results support the concept that *C. burnetii* resides in an acidified vacuole with lysosomal characteristics whereas *C. trachomatis* resides in a vacuole apparently disconnected from endosomal-lysosomal trafficking. The chlamydial inclusion appears to be intimately connected with the *trans*-Golgi network such that it intercepts Golgi-derived vesicles containing sphingomyelin en route to the plasma membrane.

MATERIALS AND METHODS

Reagents and chemicals. C_6 -NBD-ceramide, C_6 -NBD-sphingomyelin, Lucifer yellow (LY), and Texas red-dextran (TR-dex) (molecular weight, 70,000) were obtained from Molecular Probes (Junction City, Ore.). Acridine orange (AO), fluorescein isothiocyanate (FITC)-dextran (FITC-dex) (molecular weight, 70,000), and bafilomycin A₁ (Baf A) were obtained from Sigma Chemical Co., St. Louis, Mo. Rhodamine-conjugated goat anti-mouse immunoglobulin G and fluorescein-conjugated goat anti-rabbit immunoglobulin G antiserum were purchased from Pierce, Rockford, Ill. Monoclonal antibody against human cathepsin D was purchased from Triton Labs, Alameda, Calif. Monoclonal antibodies against human LAMP-1 (H4A3) and LAMP-2 (H4B4), lysosomal glycoproteins (lgp), were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Monoclonal antibody 2G11 against bovine cation-independent mannose-6-phosphate receptor (CI-M6PR) was generously provided by Suzanne Pfeffer, Department of Biochemistry, Stanford University, Stanford, Calif. Monoclonal antibody 3.2-F1, which recognizes the 73-kDa subunit of the bovine coated vesicle V-ATPase, was generously provided by Michael Forgac, Department of Physiology, Tufts University School of Medicine, Boston, Mass.

Organisms. *C. trachomatis* LGV-434, serotype L2, was cultivated in HeLa 229 cells as previously described (9). *C. burnetii*, Nine Mile strain, in phase II was propagated in African green monkey kidney (Vero) fibroblasts (CCL 81; American Type Culture Collection) (16). The phase II variant (truncated lipopolysaccharide [LPS]) of *C. burnetii* was employed in these studies because it is more efficiently internalized by host cells than the phase I variant (full-length LPS) (4). When required, organisms were purified by Renografin (E. R. Squibb & Sons, Inc., Princeton, N.J.) density gradient centrifugation (16). Infectivity of *C. trachomatis* EBs was determined by numbers of inclusion-forming units (IFU) as described by Furness et al. (15) except that inclusions were visualized by indirect immunofluorescence employing polyclonal rabbit antisera against formalin-killed *C. trachomatis* L2 EBs and FITC-conjugated goat anti-rabbit immunoglobulin G serum.

Infection of tissue culture cells and use of inhibitors. Twelve-millimeter-diameter coverslips were seeded with 2×10^5 Vero cells and cultivated overnight at 37°C in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah) and 20 µg of gentamicin (Whittaker Bioproducts, Walkersville, Md.) per ml. *C. burnetii* suspended in 3.7% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and *C. trachomatis* suspended in SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM glutamic acid [pH 7.2]) were used to infect monolayers at a multiplicity of infection of 0.1 to 1.0 for 1 h at room temperature. After infection, the cells were washed once with M199 medium (GIBCO) supplemented with 2% FBS. Fresh M199 medium plus 2% FBS was then added, and incubation was continued at 37°C for the indicated times. For certain experiments requiring the use of species-specific antibodies, HeLa 229 or Madin-Darby bovine kidney (MDBK) cells (CCL 22; American Type Culture Collection) were cultivated and infected as described above. When desired, Baf A was added to the culture medium to a final concentration of 0.5 µM to inhibit endosome acidification. The lysosomotropic amines methylamine, ammonium chloride, and chloroquine were employed at final concentrations of 10 mM, 10 mM, and 100 µM, respectively. To inhibit chlamydial protein synthesis, chloramphenicol was added to a final concentration of 34 µg/ml.

Immunofluorescence staining. All fixation and staining procedures were conducted at room temperature. All antibody incubations were carried out for 20 min with three washes of phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaPO₄ [pH 7.2]) containing 0.5% bovine serum albumin following fixation and permeabilization, and between applications of different antisera. Infected cells on coverslips were fixed for 20 min with 4% paraformaldehyde in PBS. Cells were then permeabilized for 4 min with 100% methanol (for lgp and cathepsin D labeling) or 0.1% Triton X-100 in PBS (for CI-M6PR and V-ATPase labeling). For dual fluorescence staining of microorganisms and lgp, infected Vero cells

were first incubated with rabbit antiserum directed against formalin-fixed *C. burnetii* or *C. trachomatis*. The organisms were then stained with a goat anti-rabbit immunoglobulin G fluorescein conjugate. lgp were then stained by incubation with undiluted hybridoma culture supernatant containing monoclonal antibodies to LAMP-1 or LAMP-2, followed by incubation with a goat anti-mouse rhodamine conjugate. Infected MDBK cells were stained for the V-ATPase and infected HeLa 229 cells were stained for CI-M6PR and cathepsin D by incubation with the respective monoclonal antibodies followed by incubation with a goat anti-mouse rhodamine conjugate. Coverslips were then mounted onto glass slides using Vectashield (Vector Laboratories, Inc., Burlingame, Calif.) mounting medium and viewed.

Fluorescence staining of infected cells with AO, FITC-dex, and LY. The acidity of bacterium-containing vacuoles was qualitatively determined by staining infected cells with 5 µg of AO per ml in Hanks balanced salt solution (HBSS) (GIBCO) as described elsewhere (45). The fluid-phase endocytic probes FITC-dex and LY were added to the culture medium at a final concentration of 1 mg/ml. Coverslips with live infected cells were washed four times with HBSS and viewed by fluorescence microscopy.

C_6 -NBD-ceramide labeling. Fluorescent C_6 -NBD-ceramide (N-[7-(4-nitrobenzyl-2-oxa-1,3-diazole)]aminocaproyl sphingosine) (Molecular Probes, Eugene, Ore.) was complexed with 0.034% defatted-bovine serum albumin (DFBSA) in Dulbecco's minimal essential medium (MEM) as described previously (24) to yield complexes approximately 5 µM in both DFBSA and C_6 -NBD-ceramide. Infected Vero cells were incubated with the DFBSA-NBD-ceramide complex at 4°C for 30 min, washed with 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered calcium and magnesium-free Puck's saline, pH 7.4 (HCMF), and incubated for 1 h in MEM plus 0.34% DFBSA to "back exchange" excess probe from the plasma membrane. Cultures on coverslips were rinsed in HCMF prior to mounting for fluorescence microscopy.

To examine incorporation of NBD-lipid by purified organisms, Renografin density gradient-purified *C. burnetii* and *C. trachomatis* were incubated in 5 µM C_6 -NBD-ceramide or C_6 -NBD-sphingomyelin in MEM plus 0.034% DFBSA for 30 min at 37°C. The cells were then pelleted in a microcentrifuge, washed once with HCMF, and dispersed in HCMF for photography. Micrographs were taken at 1-s fixed exposures and developed identically.

To examine trafficking of C_6 -NBD-ceramide and the fluid-phase marker TR-dex in Vero cells coinfecting with *C. burnetii* and *C. trachomatis*, cells were first labeled with TR-dex (0.5 mg/ml) in tissue culture medium for 2 h at 37°C. Cells were washed three times with HBSS and then labeled with C_6 -NBD-ceramide for 10 min at 37°C. The cells were washed three times with HBSS and incubated in back exchange medium containing TR-dex for 1 h at 37°C. Cultures on coverslips were rinsed in HCMF prior to mounting for fluorescence microscopy.

Microscopy. Fluorescent and Nomarski differential interference contrast micrographs were taken on a Nikon FXA photomicroscope using a 60× Planapochromat objective. Photomicrographs were obtained using T-max ASA 400 film (Eastman Kodak Co., Rochester, N.Y.). In some cases, digital images were recorded using a Dage MTI CCD-72 camera, and the images were captured with a DSP-2000 image processor (Dage-MTI, Inc., Michigan City, Ind.). A Bio-Rad 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 and digital images were processed using Adobe Photoshop 2.5.1 (Adobe Systems, Inc., Mountain View, Calif.).

Electron microscopy cytochemistry for acid phosphatase. Confluent Vero cell monolayers in Contour Permax tissue culture dishes (60 by 15 mm; Lab-Tek, Naperville, Ill.) were infected at a multiplicity of infection of 5 to 10 for 2 h at 4°C. The cultures were washed twice with cold HBSS to remove unabsorbed bacteria, fed with prewarmed MEM-10 (GIBCO), and incubated at 37°C for 20 h. The cultures were then washed once with cold HBSS and prefixed for 15 min at 4°C with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.6. Cytochemical localization of the lysosomal enzyme acid phosphatase was done essentially as described previously (5). Briefly, after prefixation, the monolayers were washed twice with cold 0.2 M Tris-maleate, pH 5.0, and incubated for 1 h at 37°C in 40 mM Tris-maleate, pH 5.0, with sodium-beta-glycerophosphate as the substrate and lead nitrate as the capture agent. The culture was then washed twice with 0.2 M Tris-maleate, pH 5.0, and postfixed overnight at 4°C with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.6, prior to embedding and sectioning.

RESULTS

Vacuole acidification, localization of the V-ATPase, and effect of Baf A and lysosomotropic amines on growth. To qualitatively determine the acidity of parasitophorous vacuoles, *C. burnetii* (phase II)- and *C. trachomatis* (L2)-infected Vero cells were vitally stained with AO and viewed by fluorescence microscopy. AO is a basic dye that accumulates within acidic vesicles (43). Intense AO fluorescence was apparent in *C. burnetii*-containing vacuoles, indicating an acidic intravacuolar

FIG. 1. Fluorescence localization of AO in Vero cells infected with *C. burnetii* or *C. trachomatis*. Living cells were stained with 5 μ g of AO per ml for 10 min prior to viewing. Corresponding Nomarski (A) and fluorescent (B) images of Vero cells infected for 48 h with *C. burnetii* showing a parasite-containing vacuole heavily stained with AO, indicating the vesicle is acidic. Corresponding Nomarski (C) and fluorescent (D) images of Vero cells infected for 22 h with *C. trachomatis* showing an inclusion with little accumulation of AO. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

pH. (Fig. 1A and B). The Nomarski image shows the characteristic clumping of intravacuolar phase II *C. burnetii*. The fluorescence intensity was greatly diminished if the cells were pretreated for 2 h with Baf A prior to staining with AO (data not shown). Baf A is a specific inhibitor of the V-ATPase, a proton pump responsible for acidifying coated vesicles, endosomes, lysosomes, and the Golgi apparatus (7, 27, 45). When the drug was added to the culture medium 1 h postinfection, growth of *C. burnetii* was completely inhibited (Fig. 2A and B). The inhibitory effect of Baf A was reversible. When Baf A-containing medium was subsequently removed from cells infected for 48 h and replaced with fresh medium without Baf A, approximately the same number of *C. burnetii*-containing vacuoles developed in 48 to 72 h as in the untreated control (data not shown). This result indicated that the drug is not directly cytotoxic to the rickettsia. These data are consistent with previous *in vitro* and *in vivo* studies (18) demonstrating that *C. burnetii* metabolism is activated only under mildly acidic conditions ($\sim$ pH 4.5 to 5.0). These results also provide a qualitative confirmation of the acidic pH determined for *C. burnetii*-containing vacuoles in a persistently infected macrophage-like cell line (1).

To confirm the presence of V-ATPase in the *Coxiella* vacuolar membrane, indirect immunofluorescence was performed using monoclonal antibody 3.2-F1, which recognizes the 73-kDa cytoplasmic head subunit of the V-ATPase of bovine brain coated vesicles (27). As depicted in Fig. 3A and B, a distinctive rim-like fluorescence was observed coincident with the membrane surrounding internalized *C. burnetii*, indicating the presence of V-ATPases. Prominent juxtanuclear labeling of the Golgi apparatus, as previously described by Márquez-Sterling et al. (27), was also evident. In certain cells, smaller immunoreactive vesicles were juxtaposed to the *Coxiella* vacu-

ole. It was not clear whether these vesicles are actively fusing with the parasite-containing vacuole.

In contrast to vacuoles harboring *C. burnetii*, sequestration of AO by chlamydial inclusions was negligible, suggesting a neutral intravacuolar pH (Fig. 1C and D). The lack of AO uptake coincided with no obvious colocalization of V-ATPases with the inclusion membrane (Fig. 3C and D). Chlamydial growth was not inhibited when infected Vero cells were treated with Baf A 1 h after infection (Fig. 2C and D). At 30 h postinfection the morphology of inclusions was indistinguishable between untreated control and Baf A-treated cultures. Normal chlamydial growth was also observed in infected cells treated with the lysosomotropic amines chloroquine, methylamine, and ammonium chloride (Table 1), compounds also known to raise the pH of acidic vesicles (35) and which inhibit development of *C. burnetii* at the concentrations used here (18). This is in contrast to the results of Söderlund and Kihlström, who used a much higher concentration of methylamine (41).

Differential trafficking of fluid-phase markers to *C. trachomatis*- and *C. burnetii*-containing vacuoles. Vacuoles harboring *C. burnetii* or *C. trachomatis* were tested for ability to fuse with endosomes by examining uptake of the fluid-phase markers LY and FITC-dex. Both fluorescent tracers are pinocytosed and sequestered in early and late endosomes and lysosomes (43). Infected monolayers were loaded with LY and FITC-dex 6 to 12 h prior to viewing. This labeling period is sufficient to load both early and late endosomes in addition to lysosomes. Both LY (molecular weight, 454) (Fig. 4A and B), and FITC-dex (molecular weight, 70,000) (Fig. 5A and B) were delivered to, and retained by, *C. burnetii*-containing vacuoles. In contrast, very little, if any, tracer accumulated in chlamydial inclusions (Fig. 4C and D and Fig. 5C and D), suggesting that these vacuoles are unable to fuse with endosomes or lysosomes.

Localization of late endosomal-prelysosomal and lysosomal markers. Late endosomal and prelysosomal vesicles, and to a lesser extent *trans*-Golgi vesicles, are enriched for CI-M6PR. This integral membrane protein sorts lysosomal enzymes containing terminal mannose 6-phosphate moieties from the *trans*-Golgi network to late endosomal-prelysosomal compartments (22). To ascertain whether *C. burnetii* and *C. trachomatis* parasitophorous vacuoles are enriched for CI-M6PR, infected HeLa cells were stained by indirect immunofluorescence with the anti-bovine CI-M6PR monoclonal antibody 2G11. This monoclonal antibody cross-reacts with human CI-M6PR (25). The receptor was detectable within the Golgi but below the limits of detection in the parasitophorous vacuolar membranes of both *C. burnetii* (Fig. 6A and B) and *C. trachomatis* (Fig. 6C and D).

We next tested for the presence of the soluble lysosomal enzymes acid phosphatase and cathepsin D within *C. burnetii*- or *C. trachomatis*-containing vacuoles. Cathepsin D is trafficked to lysosomes via an M6PR-dependent pathway, while acid phosphatase is not (22). Ultrastructural cytochemistry revealed acid phosphatase activity within the *C. burnetii* vacuole but not within *C. trachomatis* inclusions, which is consistent with previous observations (1, 8, 14, 23) (data not shown). Indirect immunofluorescence was employed to assess the presence of cathepsin D. The enzyme was detected within the *C. burnetii* vacuole (Fig. 7A and B) but not those harboring *C. trachomatis* (Fig. 7C and D).

To identify lysosomal characteristics of the vacuolar membranes encompassing *C. burnetii* and *C. trachomatis*, monoclonal antibodies against two predominant human IgG, LAMP-1 and LAMP-2 (26), were employed in indirect immunofluorescence of infected Vero cells. There was a strong coincidence of

FIG. 2. Effect of Baf A on growth of *C. burnetii* and *C. trachomatis*. Vero cells were infected for 1 h, the monolayer was washed, and new medium was added containing 0.5 μ M Baf A. Monolayers were fixed, and bacteria were stained by indirect immunofluorescence. Samples were viewed by confocal microscopy. (A) Vero cells infected with *C. burnetii* for 53 h in the absence of Baf A show numerous small parasitophorous vacuoles with clumps of replicating bacteria. (B) Vero cells infected with *C. burnetii* for 53 h in the presence of Baf A show total growth inhibition of *C. burnetii*. Fluorescence in panel B represents organisms that are either adherent or internalized and nonreplicating. Vero cells infected with *C. trachomatis* for 30 h in the absence of Baf A (C) show no growth inhibition of chlamydiae when compared with the untreated control (D). Bar, 10 μ m.

FIG. 3. Immunofluorescence localization of the vacuolar-type (H^+)-ATPase (V-ATPase). *C. burnetii*- and *C. trachomatis*-infected MDBK cells were fixed and stained by indirect immunofluorescence with monoclonal antibody 3.2-F1 directed against the 73-kDa subunit of the bovine brain clathrin-coated vesicle V-ATPase. Samples were viewed by confocal microscopy. Corresponding Nomarski (A) and fluorescent (B) images of MDBK cells infected for 70 h with *C. burnetii* showing a distinctive rim-like fluorescence of the parasitophorous vacuolar membrane. Corresponding Nomarski (C) and fluorescent (D) images of MDBK cells infected for 26 h with *C. trachomatis* showing no labeling of the inclusion membrane. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

TABLE 1. Effects of lysosomotropic amines on *C. trachomatis* replication

Treatment ^a	IFU ^b	% of untreated control
Untreated	$7.6 \times 10^4 \pm 2.0 \times 10^3$	100
Chloroquine (100 μ M)	$6.0 \times 10^4 \pm 1.3 \times 10^4$	79 ± 17
Methylamine (10 mM)	$7.8 \times 10^4 \pm 1.2 \times 10^4$	103 ± 16
Ammonium chloride (10 mM)	$8.8 \times 10^4 \pm 8.6 \times 10^3$	117 ± 11

^a Lysosomotropic amines were added to the culture medium 2 h preinfection and maintained throughout the 40-h incubation.

^b IFU were counted from 15 fields. Results are from three experiments and are expressed as means $\pm$ standard errors of the means.

both LAMP-1 (Fig. 8A) and LAMP-2 (Fig. 8B) with the vacuolar membrane surrounding replicating *C. burnetii*. Figure 8 also illustrates the two parasitophorous vacuole morphologies witnessed with phase II *C. burnetii*. The organisms are typically clumped and residing in a spacious vacuole (Fig. 8A) or tightly constrained within the surrounding vacuolar membrane (Fig. 8B). No colocalization of either LAMP-1 (Fig. 8C) or LAMP-2 (Fig. 8D) with the *C. trachomatis* inclusion membrane could be detected. Typical perinuclear labeling with some punctate labeling of the cytoplasm (11) was evident in cells infected with either parasite. To test whether continuous chlamydial protein synthesis is required for inhibition of fusion with lgp-containing vesicles, Vero cells infected for 24 h and subsequently treated for 24 h with chloramphenicol were stained for lgp. Inhibition of chlamydial protein synthesis caused no detectable association of lgp with the chlamydial inclusion membrane (data not shown).

Taken together, the presence of four lysosomal markers

(acid phosphatase, cathepsin D, LAMP-1, and LAMP-2), in addition to the V-ATPase and low pH, strongly suggest that *C. burnetii* is terminally targeted to vacuoles that classify as secondary lysosomes, with quick transit through the late endosomal-prelysosomal CI-M6PR-rich compartment. This is in stark contrast to *C. trachomatis* vacuoles, which were devoid of all endosomal and lysosomal markers tested.

Differential trafficking of Golgi-derived sphingolipids. Fusion of Golgi-derived vesicles is an important mechanism for adding lipid to distal cellular sites such as the plasma membrane (31). We have shown that Golgi-derived sphingolipids are trafficked specifically to the chlamydial inclusion in a time-dependent manner and incorporated into the cell wall of the intracellular chlamydiae (17). To identify any relationship between the Golgi apparatus and *C. burnetii*-containing vacuoles, infected Vero cells were vitally stained with C₆-NBD-ceramide (24). After incubation of the infected cells with NBD-ceramide followed by 60 min of back exchange (24), intense fluorescence was observed with the intracellular chlamydiae (Fig. 9C and D). In contrast, there was no indication of transfer of fluorescent sphingolipid from the *trans*-Golgi to the vacuolar membrane surrounding *C. burnetii* or to the organisms themselves (Fig. 9A and B). To eliminate the possibility that fluorescent sphingolipid is trafficked to the *C. burnetii*-containing vacuole but not incorporated by the bacterium, purified *C. burnetii* and *C. trachomatis* EBs were exposed to either C₆-NBD-sphingomyelin or C₆-NBD-ceramide. Both bacteria readily incorporated C₆-NBD-sphingomyelin and C₆-NBD-ceramide (data not shown) into their cell walls when incubated with these compounds in vitro. Because *C. burnetii* does not incorporate the fluorescent probe in vivo we concluded that ceramide or its derivatives are not directly trafficked to the vacuoles containing *C. burnetii*.

To investigate trafficking of vesicles to the parasitophorous vacuoles of Vero cells coinfecting with *C. burnetii* and *C. trachomatis*, cells were dual labeled with C₆-NBD-ceramide and the fluid-phase marker TR-dex. (Cells were first infected with *C. burnetii* for 53 h, because of its slower growth rate, and then coinfecting with *C. trachomatis* for 19 h). TR-dex was delivered by endosomes only to the vacuole harboring *C. burnetii*, whereas fluorescent sphingolipid was selectively trafficked only to the chlamydial inclusion (Fig. 10A and B). This result indicated that infection by *C. burnetii* does not disrupt Golgi-directed transport of sphingolipid, at least to the chlamydial inclusion, and that *C. trachomatis* infection does not disrupt normal pinocytosis and sequestration of fluid-phase markers by the *C. burnetii*-containing vacuole. On no occasion were the two organisms detected in the same compartment.

DISCUSSION

It is becoming increasingly evident that the vacuoles inhabited by different intracellular parasites are heterogeneous and that parasites can interrupt maturation of the phagosome at different stages of the endosomal-lysosomal pathway (12, 40, 42). The vacuoles occupied by viable *C. burnetii* and *C. trachomatis* appear to represent extreme ends of this spectrum. Every marker studied differentiated *C. burnetii*-containing vacuoles from chlamydial inclusions with the exception of CI-M6PR, which was absent from both. (A summary of the characteristics of *C. burnetii* and *C. trachomatis* parasitophorous vacuoles is presented in Table 2.) Vacuoles harboring *C. burnetii* displayed the characteristics of secondary lysosomes. As such, these vacuoles interacted extensively with vesicles of the endocytic compartment. *C. trachomatis* inclusions, on the other hand, evidenced no apparent interaction with the early or late endo-

FIG. 4. Fluorescence localization of the endocytic tracer LY in infected Vero cells. *C. burnetii*- and *C. trachomatis*-infected Vero cells were incubated for 6 h with LY (1 mg/ml) at 54 and 27 h postinfection, respectively. Corresponding Nomarski (A) and fluorescent (B) images of a *C. burnetii*-containing vacuole filled with LY, indicating fusion with the endosomal compartment. Corresponding Nomarski (C) and fluorescent (D) images of *C. trachomatis* inclusions showing no sequestration of LY. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

FIG. 5. Fluorescence localization of the endocytic tracer FITC-dex in infected Vero cells. *C. burnetii*- and *C. trachomatis*-infected Vero cells were incubated for 12 h with FITC-dex (1 mg/ml) at 24 and 12 h postinfection, respectively. Corresponding Nomarski (A) and fluorescent (B) images of *C. burnetii*-containing vacuoles filled with FITC-dex, indicating fusion with the endosomal compartment. Corresponding Nomarski (C) and fluorescent (D) images of *C. trachomatis* inclusions showing no sequestration of FITC-dex. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

cytic pathway once the inclusion membrane was established. Instead, chlamydiae appeared to interrupt an exocytic pathway between the *trans*-Golgi and the plasma membrane.

The *C. trachomatis* inclusion is characterized by a lack of fusion with endosomes and lysosomes. The avoidance of lysosomal fusion by closely related *Chlamydia psittaci* is not due to a general repression of lysosome function as fusion of lysosomes with phagocytosed zymosan in *C. psittaci*-infected macrophages occurs normally (13). As demonstrated in this study, *C. trachomatis*-infected cells actively pinocytose fluid-phase markers but these markers are not delivered to the inclusion. Normal endocytic function in *C. trachomatis*-infected cells was clearly illustrated by demonstrating that sequestration of fluid-phase TR-dex by *C. burnetii*-containing vacuoles occurred normally in cells coinfecting with *C. trachomatis*. In addition, lysosome distribution, as assessed by the presence of acid phosphatase, cathepsin D, LAMP-1, and LAMP-2, appeared normal in cells infected with *C. trachomatis*. However, these lysosomal markers were absent or below the limits of detection within the chlamydial vacuole.

Confirmation of the neutrality of the chlamydial inclusion was provided by the relatively small amount of AO accumulation in the inclusion as compared with that in *C. burnetii*-containing vacuoles. The observation that the metabolism of purified chlamydial RBs is optimal between pH 7.0 and 7.5 (20) suggests that the chlamydial inclusion must approximate a neutral pH. This is in contrast to *C. burnetii* cell-free organisms, which display optimal metabolic activity at pH 4.7 to 4.8 (18) and, as demonstrated here and elsewhere (1), reside in an acidic vacuole. V-ATPases were not detected immunochemically within the chlamydial inclusion membrane. The 3.2-F1 monoclonal antibody used in this study recognizes the 73-kDa

cytoplasmic head subunit of the V-ATPase. Thus, we are unable to distinguish between rapid, selective dissociation of this subunit and complete absence of the V-ATPase complex. However, either situation would result in a vacuole that is unable to acidify. We conclude that the neutral pH of the vacuole apparently results from the absence of at least a portion of this pump, and not from inhibition of V-ATPase activity or chlamydia-mediated buffering of the inclusion. Consistent with a relatively neutral pH of the chlamydial inclusion was the lack of growth inhibition by lysosomotropic amines that block the development of *C. burnetii*. Similarly, an inhibitor of the V-ATPase, Baf A, when added to the culture medium 1 h postinfection, inhibited replication of *C. burnetii* but did not inhibit replication of *C. trachomatis*. There was also no detrimental effect on chlamydial growth if cells were pretreated with Baf A for 2 h prior to infection (data not shown), suggesting that transient acidification of phagosomes containing newly endocytosed chlamydiae is not required to trigger metabolism and differentiation. Collectively, the results indicated that, at least after the initial internalization event, the chlamydial inclusion is not acidified and is isolated from the endocytic and lysosomal pathways.

In contrast to the chlamydial inclusion, vacuoles containing *C. burnetii* fulfilled various criteria to be characterized phenotypically as mature phagolysosomes. The fluid-phase markers FITC-dex and LY were readily sequestered in vacuoles harboring *C. burnetii*, and the vacuoles retained IgG, cathepsin D, and acid phosphatase, which are markers of lysosomes and late endosomes. In addition, the presence of V-ATPase within the *Coxiella* vacuolar membrane was confirmed immunochemically. Although not detected by immunofluorescence, the vacuole probably transits through the late endosomal, CI-M6PR-

FIG. 6. Immunofluorescence localization of CI-M6PR. HeLa cells were infected for 24 h with *C. burnetii* or *C. trachomatis* and fixed and stained by indirect immunofluorescence with the anti CI-M6PR monoclonal antibody 2G11. Corresponding Nomarski and fluorescent images of *C. burnetii* (A and B)- and *C. trachomatis* (C and D)-containing vacuoles showing an absence of parasitophorous vacuolar membrane staining for CI-M6PR. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

rich compartment en route to a mature phagolysosome. *C. burnetii* flourishes in this harsh environment despite the presence of acid hydrolases and other bactericidal constituents of lysosomes. This intracellular niche is not unique to *C. burnetii*—the protozoan parasite *Leishmania* sp. (2) and the obligate intracellular bacterium *Mycobacterium lepraemurium* (19) also replicate in vacuoles that have been classified as phagolysosomes. Other intracellular parasites that occupy endosomal pathways can block development to a mature phagolysosome. For example, the vacuolar membrane encompassing internalized *Mycobacterium avium* contains LAMP-1, but, surprisingly, no V-ATPase complexes. The authors speculate that the my-

cobacterial vacuole selectively fuses with vesicles carrying lgp but inhibits fusion with those carrying V-ATPases (42). Similarly, *Mycobacterium tuberculosis*-containing phagosomes acquire low levels of lysosomal glycoproteins and cathepsin D but do not fuse with primary lysosomes (12). Based upon these findings, it was proposed that although the *M. tuberculosis* phagosome interacts with endosomes, maturation into a mature phagolysosome does not occur. These interactions are distinct from those of phagosomes containing *Legionella pneumophila*, which do not acquire LAMP-1, LAMP-2, or cathepsin D (12).

C. burnetii and *C. trachomatis* appear to inhabit vacuoles of

FIG. 7. Immunofluorescence localization of the lysosomal enzyme cathepsin D. HeLa cells were infected for 40 h with *C. burnetii* or *C. trachomatis* and fixed and stained by indirect immunofluorescence with a monoclonal antibody directed against human cathepsin D. Corresponding Nomarski (A) and fluorescent (B) images of *C. burnetii*-containing vacuoles staining for cathepsin D. Corresponding Nomarski (C) and fluorescent (D) images of *C. trachomatis* inclusions showing no localization of cathepsin D. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

fundamentally different cellular origins. It is clear, however, that *C. psittaci* modifies the inclusion membrane by the insertion of a parasite-derived polypeptide (37). The function of this protein is as yet unknown. It is presumed that this inclusion membrane-specific protein modulates the interaction of the inclusion with cellular organelles and/or may promote nutrient acquisition from the cytoplasm (30). A possible parallel to the chlamydial inclusion is the parasitophorous vacuole of the protozoan parasite *Toxoplasma gondii*. This vacuole also does not fuse with the endocytic and lysosomal compartments (21). The vacuolar membrane surrounding *T. gondii* contains parasite-derived proteins (6) and has channels that allow the free ex-

change of molecules up to 1,900 Da (39). It remains to be determined if *T. gondii* proteins make up these channels.

There is some evidence that *C. burnetii* may modify its intravacuolar environment. Ingestion of *C. burnetii* by macrophage-like cell lines results in a greatly diminished respiratory burst and little production of superoxide anion (3). This suggests the absence, defective assembly, or inhibition of the NADPH oxidase enzyme complex (10). *C. burnetii* is known to secrete proteins; one of these possesses acid phosphatase activity (3, 36). It is tempting to speculate that one or more of these proteins function in inhibiting the oxidative burst. This would be an obvious pathogenic mechanism for an aerosol-

FIG. 8. Double immunofluorescence localization of LAMP-1 and LAMP-2. Infected cells were fixed and dual stained by indirect immunofluorescence with monoclonal antibodies directed against human LAMP-1 or LAMP-2 (lgp (rhodamine) and polyclonal rabbit antiserum against formalin-fixed *C. burnetii* or *C. trachomatis* (FITC). Samples were viewed by confocal microscopy. Vero cells infected for 49 h with *C. burnetii* and stained for LAMP-1 (A) and LAMP-2 (B) showing intense staining of the parasitophorous vacuolar membrane for both lgp. Vero cells infected for 26 h with *C. trachomatis* and stained for LAMP-1 (C) and LAMP-2 (D). Neither antibody labeled the chlamydial inclusion membrane. Bar, 10 μ m.

FIG. 9. C_6 -NBD-ceramide staining of *C. burnetii*- and *C. trachomatis*-infected Vero cells. Cells were labeled with the fluorescent C_6 -NBD-ceramide at 4°C, warmed to 37°C, and photographed. Corresponding Nomarski (A) and fluorescent (B) images of Vero cells infected for 40 h with *C. burnetii*. Intense Golgi staining is observed with minimal labeling of intracellular bacteria or the parasitophorous vacuolar membrane. Corresponding Nomarski (C) and fluorescent (D) images of Vero cells infected for 40 h with *C. trachomatis*. Note the intense staining of intracellular chlamydiae. Selected parasitophorous vacuoles are indicated with arrows. Bar, 10 μ m.

borne pathogen such as *C. burnetii* that likely encounters alveolar macrophages as a first line of host defense.

Recently, trafficking of Golgi-derived sphingolipids to the chlamydial inclusion has been demonstrated (17). This trafficking is specific to chlamydiae because *C. burnetii* does not directly receive fluorescent lipids after C_6 -NBD-ceramide labeling. A model was proposed in which the chlamydial inclusion is situated such that it intercepts anterograde vesicular traffic from the *trans*-Golgi that is otherwise destined for the plasma membrane (17). Thus, the chlamydial inclusion is disconnected from the endocytic and lysosomal pathways but it appears to be integrated into an exocytic pathway which delivers endogenously synthesized sphingolipids from the Golgi apparatus to the plasma membrane.

C. burnetii-containing vacuoles showed no evidence of incorporation of fluorescent sphingolipid. However, they retained lgp, cathepsin D, acid phosphatase, and the V-ATPase, which are markers of primary lysosomes and late endosomes. These and other vesicles that are derived from the *trans*-Golgi are heterogeneous in both lipid and protein composition (22, 34). The failure to detect fluorescent ceramide or its metabolites in vacuoles containing *C. burnetii* suggests that they do not interact with Golgi-derived vesicles containing newly synthesized

sphingolipids in transit to the plasma membrane or other cellular sites. The possibility remains, however, that *C. burnetii* could obtain host sphingolipids through endocytosis and recycling of plasma membrane lipids. Endocytic vesicles clearly fuse with the parasitophorous vacuole, and it is likely that specific, lysosomally targeted Golgi-derived vesicles would do so as well. Thus, the lipid composition of the *C. burnetii* vacuole likely represents a contribution from several sources.

Although both *C. burnetii* and *C. trachomatis* replicate within membrane-bound vacuoles, these vacuoles differ dramatically in their properties and interactions with other cellular organelles and vesicles. This is perhaps not surprising since intracellular parasites inhabit a variety of intracellular niches within host cells (32). It has become increasingly evident that many invasive microorganisms have the capacity to selectively modify their intravacuolar environment and/or to enter cells via a pathway that results in a parasitophorous vacuole amenable to growth (40). Greater understanding of vesicular trafficking to parasitophorous vacuoles may suggest improved methods to specifically target antimicrobial compounds to microbes residing in unique intracellular niches.

FIG. 10. Selective trafficking of TR-dex and C_6 -NBD-ceramide in Vero cells coinfecting with *C. burnetii* and *C. trachomatis*. Cells infected for 53 h with *C. burnetii* were subsequently coinfecting for 19 h with *C. trachomatis*. Infected cells were then dual labeled with TR-dex and C_6 -NBD-ceramide. Live cells were viewed by fluorescence microscopy. Corresponding Nomarski (A) and fluorescent images (B) showing selective trafficking of the fluid-phase marker TR-dex to the vacuoles containing *C. burnetii* (red) and C_6 -NBD-ceramide to the *C. trachomatis* inclusions (green). *C. burnetii* and *C. trachomatis* parasitophorous vacuoles are designated in panel A with white and black arrows, respectively. Bar, 10 μ m.

TABLE 2. Characteristics of *C. burnetii* and *C. trachomatis* parasitophorous vacuoles

Assay	Microorganism	
	<i>C. burnetii</i>	<i>C. trachomatis</i>
Uptake of vital stains ^a		
AO	+	—
C ₆ -NBD-ceramide	—	+
Uptake of fluid phase markers ^a		
LY	+	—
FITC-dex	+	—
Colocalization of endosomal and lysosomal markers ^b		
Vacuolar (H ⁺) ATPase	+	—
CI-M6PR	—	—
LAMP-1	+	—
LAMP-2	+	—
Acid phosphatase	+	— ^d
Cathepsin D	+	—
Growth inhibition ^c		
Baf A	+	—
Lysosomotropic amines	+	—

^a Uptake was determined by fluorescence microscopy.^b Colocalization was determined by indirect immunofluorescence microscopy (V-ATPase, CI-M6PR, cathepsin D, LAMP-1, and LAMP-2) and transmission electron microscopy histochemistry (acid phosphatase).^c From references 1 and 8 and data not shown.^d From references 14 and 23 and data not shown.^e Growth inhibition was determined by enumeration of parasite-containing vacuoles by indirect immunofluorescence microscopy.

ACKNOWLEDGMENTS

We are grateful to E. Lewis for excellent secretarial assistance, F. Hayes for electron microscopy, P. Small for use of the confocal microscope, G. Hettrick and B. Evans for graphics, and H. Caldwell and W. Cieplak for review of the manuscript. The H4A3 (LAMP-1) and H4B4 (LAMP-2) monoclonal antibodies developed by S. H. Mane, L. Marzella, D. F. Bainton, V. K. Holt, Y. Cha, J. E. K. Hildreth, and J. T. August were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, Md., and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under contract N01-HD-2-3144 from the NICHD.

REFERENCES

- Akporiaye, E. T., J. D. Rowatt, A. A. Aragon, and O. G. Baca. 1983. Lysosomal response of a murine macrophage-like cell line persistently infected with *Coxiella burnetii*. *Infect. Immun.* **40**:1155–1162.
- Antoine, J.-C., E. Prina, C. Jouanne, and P. Bongrand. 1990. Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. *Infect. Immun.* **58**:779–787.
- Baca, O. G., Y. P. Li, and H. Kumar. 1994. Survival of the Q fever agent *Coxiella burnetii* in the phagolysosome. *Trends Microbiol.* **2**:476–480.
- Baca, O. G., and D. Paretsky. 1983. Q fever and *Coxiella burnetii*: a model for host-parasite interactions. *Microbiol. Rev.* **47**:127–149.
- Barka, T., and P. J. Anderson. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem.* **10**:741–753.
- Beckers, C. J. M., J. F. Dubremetz, O. Mercereau-Puijalon, and K. A. Joiner. 1994. The *Toxoplasma gondii* rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm. *J. Cell Biol.* **127**:947–961.
- Bowman, E. J., A. Siebers, and K. Altendorf. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA* **85**:7972–7976.
- Burton, P. R., N. Kordova, and D. Paretsky. 1971. Electron microscopic studies of the rickettsia *Coxiella burnetii*: entry, lysosomal response, and fate of rickettsial DNA in L-cells. *Can. J. Microbiol.* **17**:143–150.
- 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.
- Chanock, S. J., J. E. Benna, R. M. Smith, and B. M. Babior. 1994. The respiratory burst oxidase. *J. Biol. Chem.* **269**:24519–24522.
- Chen, W. J., T. L. Murphy, M. C. Willingham, I. Pastan, and J. T. August. 1985. Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* **101**:85–95.
- Clemens, D. L., and M. A. Horwitz. 1995. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J. Exp. Med.* **181**:257–270.
- Eissenberg, L. G., and P. B. Wyrick. 1981. Inhibition of phagolysosome fusion is localized to *Chlamydia psittaci*-laden vacuoles. *Infect. Immun.* **32**:889–896.
- 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.
- Furness, G., D. M. Graham, and P. Reeve. 1960. The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J. Gen. Microbiol.* **23**:613–619.
- Hackstadt, T., R. Messer, W. Cieplak, and M. G. Peacock. 1992. Evidence for the proteolytic cleavage of the 120-kilodalton outer membrane protein of rickettsiae: identification of an avirulent mutant deficient in processing. *Infect. Immun.* **60**:159–165.
- 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.
- Hackstadt, T., and J. C. Williams. 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. *Proc. Natl. Acad. Sci. USA* **78**:3240–3244.
- Hart, P. D., J. A. Armstrong, C. A. Brown, and P. Draper. 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. *Infect. Immun.* **5**:803–807.
- Hatch, T. P., M. Miceli, and J. A. Silverman. 1985. Synthesis of protein in host-free reticulate bodies of *Chlamydia psittaci* and *Chlamydia trachomatis*. *J. Bacteriol.* **162**:938–942.
- 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-transfected fibroblasts. *Science* **249**:641–646.
- Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**:483–525.
- 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.
- Lipsky, N. G., and R. E. Pagano. 1985. Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucocerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. *J. Cell Biol.* **100**:27–34.
- Lombardi, D., T. Soldati, M. A. Riederer, Y. Goda, M. Zerial, and S. R. Pfeffer. 1993. Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J.* **12**:677–682.
- Mane, S. M., L. Marzella, D. F. Bainton, V. K. Holt, Y. Cha, J. E. K. Hildreth, and J. T. August. 1989. Purification and characterization of human lysosomal membrane glycoproteins. *Arch. Biochem. Biophys.* **268**:360–378.
- Márquez-Sterling, N., I. M. Herman, T. Pesacreta, H. Arai, G. Terres, and M. Forgac. 1991. Immunolocalization of the vacuolar-type (H⁺)-ATPase from clathrin-coated vesicles. *Eur. J. Cell Biol.* **56**:19–33.
- McCaul, T. F. 1991. The developmental cycle of *Coxiella burnetii*, p. 223–258. *In* J. C. Williams and H. A. Thompson (ed.), *Q fever: the biology of Coxiella burnetii*. CRC Press, Inc., Boca Raton, Fla.
- McCaul, T. F., T. Hackstadt, and J. C. Williams. 1981. Ultrastructural and biological aspects of *Coxiella burnetii* under physical disruptions, p. 267–279. *In* W. Burgdorfer and R. L. Anacker (ed.), *Rickettsiae and rickettsial diseases*. Academic Press, Inc., New York.
- McClarty, G. 1994. Chlamydiae and the biochemistry of intracellular parasitism. *Microbiology* **2**:157–164.
- Moreau, P., and C. Cassagne. 1994. Phospholipid trafficking and membrane biogenesis. *Biochim. Biophys. Acta* **1197**:257–290.
- Moulder, J. W. 1985. Comparative biology of intracellular parasitism. *Microbiol. Rev.* **49**:298–337.
- Moulder, J. W. 1991. Interaction of chlamydiae and host cells in vitro. *Microbiol. Rev.* **55**:143–190.
- Pelham, H. R. B., and S. Munro. 1993. Sorting of membrane proteins in the secretory pathway. *Cell* **75**:603–605.
- Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intraphagolysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* **90**:665–669.
- Redd, T., and H. A. Thompson. 1995. Secretion of proteins by *Coxiella burnetii*. *Microbiology* **141**:363–369.
- Rockey, D. D., R. A. Heinzen, and T. Hackstadt. 1995. Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol. Microbiol.* **15**:617–626.
- Schachter, J., and C. R. Dawson. 1990. The epidemiology of trachoma predicts more blindness in the future. *Scand. J. Infect. Dis.* **69**:55–62.

39. Schwab, J. C., C. J. M. Beckers, and K. A. Joiner. 1994. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *Proc. Natl. Acad. Sci. USA* **91**:509–513.
40. Small, P. L. C., L. Ramakrishnan, and S. Falkow. 1994. Remodeling schemes of intracellular pathogens. *Science* **263**:637–639.
41. Söderlund, G., and E. Kihlström. 1983. Effect of methylamine and monodansylcadaverine on the susceptibility of McCoy cells to *Chlamydia trachomatis* infection. *Infect. Immun.* **40**:534–541.
42. Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell. 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton ATPase. *Science* **263**:678–681.
43. Swanson, J. 1989. Fluorescent labeling of endocytic compartments. *Methods Cell Biol.* **29**:137–151.
44. Wyrick, P. B., and E. A. Brownridge. 1978. Growth of *Chlamydia psittaci* in macrophages. *Infect. Immun.* **19**:1054–1060.
45. Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai, and Y. Tashiro. 1991. Bafilomycin A₁, a specific inhibitor of vacuolar-type H⁺-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* **266**:17707–17712.

Editor: A. O'Brien

B