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Vesicles Containing *Chlamydia trachomatis* Serovar L2 Remain above pH 6 within HEC-1B Cells

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***Chlamydia trachomatis* serovar L2 is an obligate intracellular bacterium which is internalized in target epithelial cells by endocytosis and resides within a membrane-bound vesicle. Over the next several hours following entry, individual serovar L2-containing vesicles fuse with one another to form a single membrane-bound vesicle (or inclusion) within which the microcolony develops. The experiments reported here directly examined the pH of vesicles containing chlamydiae. The pH was determined by measuring emission ratios of the fluorescent, pH-sensitive probe SNAFL (5- [and 6-]-carboxyseminaphthofluorescein-1, succinimidyl ester) conjugated to chlamydiae. The pH remained above 6.0 at 2, 4, and 12 h after infection, while the pH of vesicles containing heat-killed organisms fell to 5.3. In the presence of amines, which raise the pH of acidic compartments, *C. trachomatis* inclusion formation was unaffected. Inactivation of Na^+,K^+-ATPases, the ion pumps responsible for maintaining a pH above 6 within early endocytic vesicles, inhibited the growth of *C. trachomatis* within epithelial cells. Preventing vesicular acidification by inhibiting the vacuolar proton ATPase did not affect chlamydial growth. Thus, chlamydiae do not reside within highly acidic vesicles and avoid the pathway leading to lysosomes.**

Intracellular pathogenic bacteria have evolved mechanisms to ensure their survival within eukaryotic cells. Instead of facing destruction by lysosomes, these organisms are able to subvert the endocytic route which leads to lysosomes, block acidification of the vesicle in which they reside, escape from the vacuole, or adapt to a harsh acidic environment in order to thrive within host cells. Direct measurement of the pH within vesicles containing bacteria is essential in revealing the nature of the environment in which these pathogens reside.

The obligate intracellular bacterium *Chlamydia trachomatis* has evolved into a highly successful pathogen causing an estimated 4 million new cases of sexually transmitted diseases per year in the United States (7). The infectious forms of this pathogen, the elementary bodies (EB), attach to microvilli and enter host epithelial cells by receptor-mediated endocytosis at clathrin-coated pits (24, 31, 38, 47) which are then enveloped into clathrin-coated vesicles. Alternatively, investigators have demonstrated EB entry, especially into nonpolarized cells, at sites lacking clathrin (26, 44). Endocytosis without clathrin, however, has been found to direct molecules to the same intracellular compartments as those endocytosed by classic receptor-mediated endocytosis (23, 43). Regardless of the mode of EB entry, these *C. trachomatis*-containing vesicles fuse early after entry to form one large vesicle, the inclusion. By 8 to 12 h postinfection (p.i.), depending on the host cell type, infectious EB convert into the replicative, metabolically active forms, the reticulate bodies (RB). The RB divide by binary fission to fill the inclusion, which expands to take up the majority of the cytoplasmic space of the host cell. Late in the developmental cycle, RB mature back into infectious EB in an asynchronous fashion, and EB are released between 48 and 60 h p.i. to infect neighboring cells. Neither the pH within the inclusion nor the source of the inclusion membrane is known.

Chlamydia psittaci, the agent of psittacosis, is capable of growth within macrophages. Vesicles containing *C. psittaci* have been shown to avoid fusion with lysosomes in macrophages and L cells. This lack of fusion with lysosomes was demonstrated by the absence of acid phosphatase within live *C. psittaci*-laden vacuoles (17, 42), while heat-killed chlamydiae were directed to vacuoles containing this lysosomal enzyme (17). Live, UV-exposed, and isolated EB envelopes of *C. psittaci* have been shown to evade fusion with ferritin-labeled lysosomes (16, 46). The most direct assessment of chlamydial inclusion pH was performed within macrophages infected with *C. psittaci*, by transmission electron microscopy analysis of immunogold staining of a marker [3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine] (DAMP) (4) known to be concentrated in acidic compartments (41). *C. psittaci*-containing vesicles were not labeled, i.e., were DAMP negative, from 5 to 30 min after infection unless the EB were first heat killed.

There has been a tendency to assume that the biological and growth properties of all chlamydial species in all cell types are similar despite differences in disease manifestations and invasiveness. The demonstrated lack of fusion of *C. psittaci*-containing endosomes with lysosomes in macrophages and L cells has been assumed to be true for *C. trachomatis* biovars in target epithelial cells. *C. psittaci* grows in macrophages, whereas there is little to no growth of *C. trachomatis* in macrophages. Individual *C. psittaci* EB-containing endosomes remain separate from one another, resulting in multiple inclusions in infected host cells. In contrast, individual *C. trachomatis* EB-containing endosomes do fuse with one another over several hours to form one large inclusion in infected epithelial cells. Whether or not the fusion of *C. trachomatis* EB-containing endosomes extends to fusion with lysosomes has not been reported. To begin to address early intracellular events, this study used fluorescence to directly measure the pH of the intravesicular environment of *C. trachomatis* serovar L2

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TABLE 1. pH values within vesicles containing various pathogens as determined by fluorescence ratio measurements

Pathogen	pH of bacterium-containing vacuole	Fluorescent probe used to measure pH	Host cell	Reference
Histoplasma capsulatum	6.2–7.0	FITC-labeled *Histoplasma* cells	P388D1 macrophages	15
Toxoplasma gondii	6.8–7.2	FITC-labeled *Toxoplasma* cells	Mouse peritoneal macrophages	36
Legionella pneumophila	6.1 ± 0.4	FITC-labeled *Legionella* cells	Human monocytes	25
Mycobacterium avium	6.3–6.5	FITC-labeled *Mycobacterium* cells	BMD macrophages[a]	40
Leishmania mexicana	≤5.5	FITC-labeled *Leishmania* cells	BMD macrophages	40
Salmonella typhimurium	5.5–4.9	FITC-dextran	BMD macrophages	2
Leishmania amazonensis	4.74–5.26	FITC-dextran	BMD macrophages	6
Coxiella burnetii	5.21 ± 0.07	FITC-dextran	J774 macrophages	1
	4.4–5.08	FITC-dextran	P388D1 macrophages, L929 fibroblasts	27

[a] BMD macrophages, bone marrow-derived macrophages.

within its target host genital epithelial cell over a time course of 2 to 12 h p.i.

Direct measurement of the pH of intracellular vesicles has been successfully performed with pH-sensitive fluorescent probes (39). By measuring a fluorescence ratio, one can avoid the errors that arise from single-wavelength measurements because of variations in dye concentration, photobleaching, differences in optical pathlength, and heterogeneity in illumination and detector sensitivities (14). The pH within vesicles containing intracellular pathogenic organisms has been determined by measuring fluorescence emission ratios of pH-sensitive dyes (Table 1). The majority of this work has been performed within macrophages, and therefore, little is known about the pH of the pathogen-containing vesicles within nonprofessional phagocytic cells such as epithelial cells. Pathogens that reside within an acidic environment (pH < 6.0) must be resistant to the action of lysosomal hydrolases or inhibit the incorporation of these enzymes into the vacuole. These organisms may require an acidic pH to trigger metabolic activity and replication. Pathogens which reside within slightly acidic to neutral compartments (pH > 6.0) may not be exposed to lysosomal contents but may face other host defenses, such as increased nitric oxide production within nonacidic phagosomes of macrophages (9). One can hypothesize that it is this pH difference (pH above or below 6) that determines whether organisms simply follow the endocytic pathway to the lysosomes or follow a nondegradative route within the host cell.

The work presented here examined the pH of vesicles containing *C. trachomatis* serovar L2 within the human endometrial epithelial cell line HEC-1B. Serovar L2 is an invasive strain of *C. trachomatis* belonging to the biovar causing lymphogranuloma venereum. This pathogen initially infects the columnar epithelial cells of the genital mucosae, causing urethritis or cervicitis, before invading underlying tissues and spreading through the lymphatics to the regional lymph nodes of its host. The obligate intracellular pathogen *Coxiella burnetii*, like chlamydiae, resides within a membrane-bound vesicle throughout its developmental cycle and can exist within the host cell for long periods during which cell growth, damage, and bacterial replication are in balance (19, 27). *C. burnetii* requires the acidification of its vesicle for intracellular multiplication (22). Therefore, vesicles containing *C. burnetii* were examined within HEC-1B cells to provide a control for pH measurement within pathogen-containing vesicles in which the pH is known to be acidic (1, 27).

MATERIALS AND METHODS

Cells and medium. The human endometrial epithelial cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.) was used as the host cell during these experiments. Poly-L-lysine-coated glass coverslips (12-mm diameter) were seeded with 5×10^4 HEC-1B cells and incubated for 2 to 3 days, until the cells reached subconfluency (10^5 cells), at 35°C in an atmosphere of 5% CO_2 in Dulbecco's minimal essential medium (DMEM-H) containing Earle's salts, 584 mg of L-glutamine per liter, 4,500 mg of glucose per liter, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10% (vol/vol) fetal calf serum (HyClone).

Bacterial growth and infection of HEC-1B cells. *C. trachomatis* biovar Lymphogranuloma venereum L2/434/Bu (serovar L2) was used in these experiments. Chlamydial stocks were harvested from McCoy cells grown on Cytodex 3 beads (Sigma) as described previously (33). HEC-1B cells were inoculated at 35°C with purified serovar L2 stock diluted in 0.02 M phosphate buffer containing 0.2 M sucrose and 0.73 mg of glutamine per ml (2SPG). The titer of the chlamydial inoculum was determined in McCoy cells (34) to yield a 90 to 100% infection of the monolayer. To ensure an even infection of the monolayer, a small inoculum volume (50 μl) was used and the tissue culture plates were tapped by hand at 15-min intervals during the 2-h adsorption period. Fresh DMEM-H (500 μl) was then added, and the cells were incubated in an atmosphere of 5% CO_2 at 35°C. The Nine Mile phase II isolate of *C. burnetii*, generously provided by Ted Hackstadt, Rocky Mountain Laboratories, Hamilton, Mont., was propagated within Vero 76 cells (ATCC CRL 1587) in Hanks buffered Eagle minimal essential medium containing 10% (vol/vol) fetal calf serum in 150-cm^2 flasks for 7 days, purified, and inoculated onto HEC-1B cells as described for chlamydiae.

Effect of ion pump inhibition and amines on inclusion development. HEC-1B cells were infected with a dilution of serovar L2, calculated to infect 40% of the host cells, in the presence of *N*-ethylmaleimide, bafilomycin A1, digoxin, ouabain, chloroquine (Sigma), methylamine hydrochloride (ICN), or ammonium chloride (Fisher), at the concentrations listed in Table 2, and incubated for 24 h. After cold (−20°C) methanol fixation for 15 min at room temperature, serovar L2-infected HEC-1B cells were stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies specific for the major outer membrane protein of *C. trachomatis* suspended in an Evan's blue counterstain (Syva stain) (Syva Microtrak, San Jose, Calif.). Drug-exposed infected HEC-1B cells were examined to determine whether mature chlamydial inclusions had formed in comparison with untreated infected control cells by using epifluorescence microscopy at a magnification of ×400 with a Zeiss Axiovert inverted microscope (Carl Zeiss Inc., Thornwood, N.Y.) equipped with a mercury bulb and fluorescein filter set (excitation, 450 to 490 nm; emission, >520 nm; dichroic, 510 nm). The methods used for visualization of size and enumeration of chlamydial inclusions within HEC-1B cells by immunofluorescence have been published previously (34). The examination of inclusion formation in 500 drug-exposed HEC-1B cells, compared with that in an equivalent number of infected control cells, was performed on two separate occasions with triplicate coverslips.

Host cell viability. HEC-1B cell viability was assessed by two methods. In the first method, a mixture of the fluorescent dyes fluorescein-diacetate (0.1 μg/ml; Sigma) and propidium iodide (0.1 μg/ml; Sigma) in phosphate-buffered saline (PBS) was used to stain live cells green and dead cells red. In the second method, the presence of viable mitochondria within HEC-1B cells was determined by staining with the lipophilic, cationic fluorescent dye rhodamine 123 (5 μg/ml; Molecular Probes). This dye detects the presence of a transmembrane potential across the mitochondrial membrane. Cells were incubated for 10 min at 35°C in DMEM-H containing dye, washed three times with PBS, mounted in PBS, and examined by epifluorescence microscopy.

SNAFL conjugation. The pH-sensitive, dual-emission fluorescent probe 5-(and 6-)-carboxyseminaphthofluorescein-1, succinimidyl ester (SNAFL) (Molecular Probes) was used to measure the pH of intracellular vesicles containing bacteria. *C. trachomatis* serovar L2 or *C. burnetii* was incubated with 13 μg of SNAFL in 1 ml of physiological buffer (138 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 5 mM glucose) at pH 7.6 for 30 min at 4°C with rocking. The bacteria were then pelleted at 12,000 × *g* for 15 min and resuspended twice in 2SPG to remove unbound dye. SNAFL-

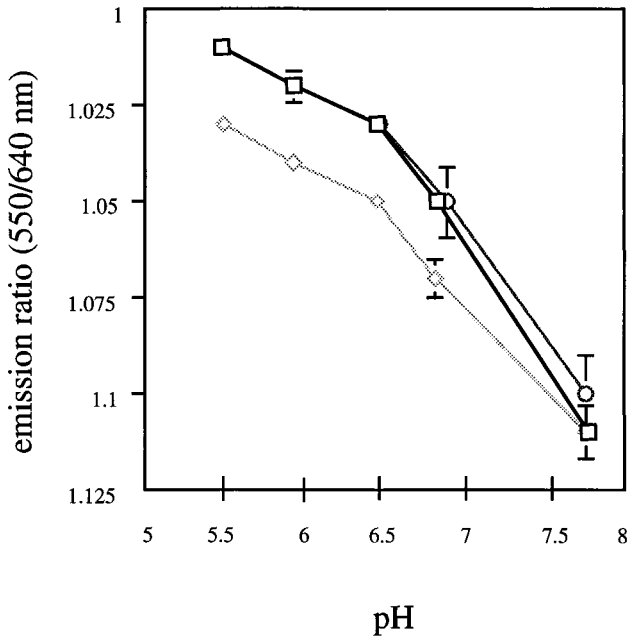

FIG. 1. Intracellular standard curves of emission ratio versus pH for SNAFL conjugated to *C. trachomatis* serovar L2 or *C. burnetii*. The emission ratios plotted are the means calculated from the fluorescent images from 10 microscopic fields of infected cells incubated in a buffer of known pH. The standard curves shown are representatives from three separate experiments, depicted by circles, squares, and diamonds, respectively, with the standard error of the mean (n = 10).

conjugated bacteria were immediately inoculated onto HEC-1B cells as described above and incubated in DMEM-H at 35°C for various times.

Chlamydial viability. To determine the effects of SNAFL conjugation on chlamydial viability, SNAFL-conjugated serovar L2 (SNAFL-L2) was grown within HEC-1B cells for 44 h, harvested, diluted in 2SPG, and inoculated onto untreated coverslips supporting HEC-1B subconfluent monolayers. Each dilution of the chlamydial inoculum was inoculated onto four replicate coverslips. The number (~800 to 1,000) and size of the inclusions as well as the percentage of cells containing chlamydial inclusions 24 h later, compared with results for cells infected with mock-conjugated SNAFL-L2, were determined. Chlamydial inclusions were stained with Syva stain in methanol-fixed HEC-1B cells and examined by epifluorescence microscopy. In addition, HEC-1B cells infected with heat-killed SNAFL-L2, as well as ouabain-exposed HEC-1B cells infected with SNAFL-L2, were examined as stated above for the ability to produce viable progeny.

pH measurement. The pH of SNAFL-containing vesicles within HEC-1B cells was determined by dual-emission fluorescence ratio measurements. Fluorescence microscopy was performed at room temperature to reduce metabolic changes within infected cells during pH determination. SNAFL was excited by using the 514-nm line of a Uniphase argon ion laser operating at 2 mW. Cells were examined with a Zeiss LSM 10 microscope equipped with a dual-emission detection system as well as by standard epifluorescence with a mercury light source. Dual-wavelength emission detection was achieved by using a dichroic mirror (610-nm-long pass), directing the light through two emission filters (550 ± 30 and 640 ± 35 nm) to independent photomultiplier tubes. The emission signals were digitized, displayed on a video monitor as two separate images, and saved as picture files (PIC) for computer analysis. Emission ratios (signal at 550 ± 30 nm/signal at 640 ± 35 nm) were calculated by computer analysis with the aid of the NIH-IMAGE program on a Macintosh 840 AV computer. The average pixel intensities from selected areas of the image at 550 ± 30 nm were divided by those from the complementary areas of the image at 640 ± 35 nm after subtraction of background from a cell-free area. Infected HEC-1B cells were scanned by standard epifluorescence to locate intracellular SNAFL-conjugated bacteria before dual-channel split-screen emission fluorescence images were captured.

Four parameters were examined: (i) untreated infected HEC-1B cells were incubated in physiological buffer at pH 7.3 just before pH analysis; (ii) heat-killed chlamydiae were exposed to 56°C for 5 min after conjugation to SNAFL; (iii) HEC-1B cells were exposed to 1 mM ouabain during the 2-h adsorption period, after which the ouabain was removed; and (iv) HEC-1B cells infected with SNAFL-conjugated bacteria were exposed to 20 mM ammonium chloride for 1 h before pH measurement. Trypan blue (0.2 mg/ml in phosphate buffer, pH 7.6) was added to the cells just before microscopic examination to quench extracellular fluorescence (32). Emission ratios from SNAFL-containing vesicles within cells from 10 to 20 microscopic fields were averaged for each coverslip. Measurement of vesicular pH, at each time point examined, was repeated on three separate occasions. The results of three experiments for each time point examined were averaged and are shown with the standard error of the mean.

pH standard curve. An intracellular standard curve of the emission ratio versus pH was generated at each time point for each experiment by incubating HEC-1B cells, which were previously infected with SNAFL-conjugated bacteria, in buffers of known pH containing ionophores. Infected HEC-1B cells were incubated in pH buffer containing 20 μM nigericin (Molecular Probes), 40 μM valinomycin (Sigma), 140 mM KCl, 1.0 mM $MgCl_2$, and 30 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid (MES) (pH 5 to 6.5) or HEPES (pH 6.5 to 8). The buffer was adjusted to a pH of between 5.5 and 8 with KOH and HCl and added to infected HEC-1B cells 1 h prior to microscopic analysis to equilibrate intra- and extracellular pH. Cells were examined by determining ratios of dual-emission fluorescence as stated above. Emission ratios were determined after subtraction of background from a field containing no cells. For each experiment, the mean of the emission ratios from SNAFL-containing vesicles within cells from 10 microscopic fields was determined for each of the pH buffers from separate coverslips. The mean ratio versus pH was plotted, and the curve was used to calculate the pH of each vesicle examined during that experiment. Figure 1 shows representative standard curves generated from three separate experiments. Only SNAFL fluorescence associated with HEC-1B cells was examined by scanning the infected cells by using differential interference contrast and epifluorescence microscopy with the Zeiss LSM 10 microscope before dual-emission images were captured.

RESULTS

Inclusion development in the presence of ion pump inhibitors. The ion pumps Na^+,K^+-ATPase and the vacuolar proton ATPase (H^+-ATPase) are involved in regulating the pH of endocytic compartments and controlling their acidification (11, 18). The action of the Na^+,K^+-ATPase, internalized with ligands during endocytosis, creates an interior-positive membrane potential in the endosome, which limits acidification by the H^+-ATPase. Thus, the pH of early endosomes does not fall below 6.0 until the Na^+,K^+-ATPase is removed from the vesicle, permitting acidification to lysosomal levels. The activity of the Na^+,K^+-ATPase can be inhibited by ouabain or digoxin, and that of the H^+-ATPase can be inhibited by *N*-ethylmaleimide or bafilomycin A1 (18). The effects of these ion pump inhibitors on the growth of serovar L2 within HEC-1B cells were analyzed.

As controls, monolayers of HEC-1B cells alone were incubated for 24 h in medium containing the individual ion pump inhibitors at the concentrations listed in Table 2. The viability of the epithelial cells was not affected by the inhibitors when assessed by two sensitive vital dye epifluorescence assays. Importantly, exposure of serovar L2 EB to 1 mM ouabain prior to infection of untreated HEC-1B monolayers did not affect inclusion development as determined by comparison in size and number of inclusions within mock-exposed EB-infected epithelial cells.

The effects of the ion pump inhibitors on chlamydial inclusion formation in infected HEC-1B cells were then examined by comparison of inclusion size and number with those within mock-exposed control cells. Inhibition of the vacuolar proton ATPase by *N*-ethylmaleimide or bafilomycin A1, which subsequently prevents endosome acidification, did not affect chlamydial inclusion formation within HEC-1B cells by 24 h p.i. In contrast, specific inhibition of the Na^+,K^+-ATPase by ouabain or digoxin, which allows early activation of the H^+-ATPase and rapid endosome acidification, resulted in an inhibition of *C. trachomatis* serovar L2 inclusion development (Table 2). Exposure of HEC-1B cells to ouabain at low concentrations (0.01 and 0.001 mM) only during the 2-h chlamydial adsorption period, followed by the washout of ouabain, resulted in chlamydial inclusion development by 24 h p.i. (data not shown).

These experiments suggest that the initiation of chlamydial growth and development within host cells depends on the

TABLE 2. Effects of various drugs on *C. trachomatis* serovar L2 inclusion formation within HEC-1B cells[a]

Drug	Concn	Inclusion formation[b]
H^+-ATPase inhibitors		
N-Ethylmaleimide	0.01 mM	Unaffected
Bafilomycin A1	10 nM	Unaffected
	50 nM	Unaffected
Na^+,K^+-ATPase inhibitors		
Digoxin	1.0 μM	Inhibited
	10 μM	Inhibited
Ouabain	0.001 mM	Inhibited
	0.01 mM	Inhibited
	0.1 mM	Inhibited
	1.0 mM	Inhibited
Amines		
Chloroquine	100 μM	Unaffected
	300 μM	Inhibited
Methylamine	10 mM	Unaffected
	20 mM	Unaffected
Ammonium chloride	10 mM	Unaffected
	20 mM	Unaffected

[a] HEC-1B cells infected with serovar L2 were incubated for 24 h in the presence of the drug. The cells were fixed with cold methanol and stained with Syva stain before inclusions were examined by epifluorescence microscopy.
[b] Unaffected, no change in inclusion size or number; inhibited, no chlamydial inclusions were seen (compared with results for mock-exposed infected control cells).

action of the Na^+,K^+-ATPase to maintain a mildly acidic to neutral environment in early chlamydia-containing vesicles. To substantiate these results, the effects of various amines on inclusion development in exposed, infected HEC-1B cells were examined.

Inclusion formation in the presence of amines. The effects of amines on chlamydial growth were determined by the presence or absence of mature chlamydial inclusions in drug-exposed cells compared with the size and prevalence of inclusions within mock-exposed infected control cells 24 h after infection. The amines, chloroquine, methylamine, and ammonium chloride, are weak bases which are able to permeate membranes in their unprotonated forms. They diffuse into acidic compartments, become unable to permeate membranes when they acquire a positive charge, and neutralize acidic vesicles (10). Incubation of control, uninfected HEC-1B cells in chloroquine, methylamine, or ammonium chloride at the concentrations listed in Table 2 did not affect the viability of the epithelial cells as assessed by using two fluorescence assays of cell vitality. Importantly, the added amines, at low concentrations, did not alter serovar L2 inclusion formation in infected host cells (Table 2). However, chloroquine, at 300 μM, completely inhibited the growth of serovar L2 within HEC-1B cells.

Viability of SNAFL-conjugated chlamydiae. The pH-sensitive fluorescent probe SNAFL was selected to measure the pH of *C. trachomatis* serovar L2-containing vesicles in infected genital epithelial cells. To determine if the process of conjugating SNAFL to EB altered chlamydial viability, the ability of SNAFL-L2 to produce infectious EB progeny within HEC-1B cells was compared with that of mock-conjugated SNAFL-L2. There was no difference in the number or size of mature chlamydial inclusions formed at 24 h p.i. by SNAFL-L2 relative to those formed by mock-conjugated SNAFL-L2 after a 44-h incubation and subpassage in HEC-1B cells. In contrast, no inclusions were formed when the epithelial cells were infected with heat-killed SNAFL-L2 or ouabain-exposed SNAFL-L2; less than 1% of the HEC-1B cell monolayer contained mature inclusions upon subpassage (data not shown).

pH of SNAFL-containing vesicles. *C. burnetii* was used as a control organism for the examination of an acidic vesicle containing bacteria. Because 24 h p.i. is the earliest time at which the pH within vesicles containing *C. burnetii* has been measured (27), *C. burnetii* conjugated to SNAFL was incubated with HEC-1B cells for 24 h before pH ratio measurement. The pH of vesicles containing untreated SNAFL-conjugated *C. burnetii* was 5.82, while vesicles containing SNAFL-conjugated *C. burnetii* within cells exposed to 20 mM ammonium chloride had a pH of 6.47 (Table 3). Therefore, the coxiella positive control for an acid environment is confirmed. Further, the increase in pH in the presence of ammonium chloride confirmed that this technique was detecting intravesicular changes in pH.

The pH within vesicles containing SNAFL-conjugated chlamydiae in HEC-1B cells was then examined at 2, 4 and 12 h p.i. (Table 3). The pH of untreated SNAFL-L2-containing vesicles was neutral at 2 h p.i., dropped to 6.26 at 4 h p.i., and was 6.60 at 12 h p.i. HEC-1B cells infected with either heat-killed SNAFL-L2 or ouabain-exposed SNAFL-L2 had a pH approximately 1 unit below that of vesicles containing untreated SNAFL-L2. This result would be expected if the proton ATPase was active within these vesicles. The pH of SNAFL-L2-containing vesicles within HEC-1B cells exposed to 20 mM ammonium chloride for 1 h before examination was raised only at 4 h p.i. At this time, the SNAFL-L2-containing vesicles were slightly acidic, and ammonium chloride collects only within acidic compartments. The slight increase in pH measured at 12 h p.i. may be due to an instability of SNAFL within cells over

TABLE 3. pH values within vesicles containing SNAFL conjugated to *C. trachomatis* serovar L2 or *C. burnetii* within HEC-1B cells[a]

Organism	Time p.i. (h)	pH[b] after the following treatment:			
		None	Heat[c]	Ouabain[d]	NH_4Cl[e]
C. trachomatis serovar L2	2	7.01 ± 0.14	6.22 ± 0.22	6.43 ± 0.19	6.98 ± 0.14
	4	6.26 ± 0.24	5.37 ± 0.24	5.37 ± 0.21	6.76 ± 0.32
	12	6.60 ± 0.14	5.81 ± 0.22	5.91 ± 0.28	6.67 ± 0.27
C. burnetii	24	5.82 ± 0.12			6.47 ± 0.14

[a] HEC-1B cells were infected with serovar L2 or *C. burnetii* conjugated to SNAFL, and the intravesicular pH was determined as described in the text.
[b] Values are the means for three separate experiments ± standard errors of the mean.
[c] SNAFL-L2 EB were exposed to 56°C for 5 min prior to inoculation of HEC-1B cells.
[d] HEC-1B cells were exposed to 1 mM ouabain for 2 h at 35°C during infection with SNAFL-L2.
[e] Infected HEC-1B cells were exposed to 20 mM NH_4Cl for 1 h prior to pH determination.

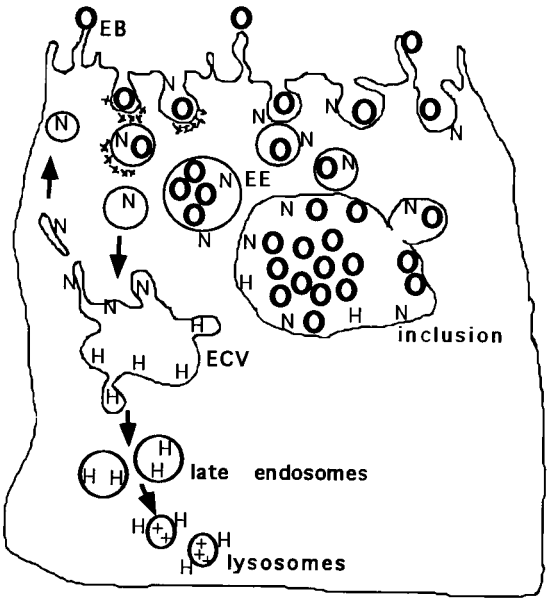

FIG. 2. Model for the formation of a *C. trachomatis* serovar L2 inclusion within an epithelial cell, showing regulation of pH and avoidance of lysosome fusion. EB attach and enter at the surfaces of epithelial cells at clathrin-coated (x) or noncoated sites and are then encased in an early endosomal membrane containing Na^+,K^+-ATPases (N). These early endosomes (EE), containing EB, avoid fusion with the ECV but fuse together to form an inclusion. The pH of the inclusion is maintained above 6 by the action of Na^+,K^+-ATPases, which inhibit acidification by H^+-ATPases (H). The ECV sorts these ion pumps by recycling Na^+,K^+-ATPases to the cell surface and directing H^+-ATPases toward the formation of late endosomes and lysosomes.

time or to a complexing of the probe with proteins during bacterial division, which may alter its fluorescent properties. This may also explain why the pH measured for *C. burnetii*-containing vesicles in our system at 24 h p.i. (pH 5.8) was slightly higher than that previously reported (pH 5.5) (27).

There were a few difficulties encountered in performing direct pH measurements on chlamydia-containing vesicles which may account for the standard error of the mean in our data. Every chlamydial inoculum contains a mixture of live and dead EB. Since the intracellular fates of these two forms are known to be different, vesicles with different pH values were examined. Also, because the infection was not synchronized, EB-containing vesicles which entered the cell at many different times after infection were analyzed. Therefore, the pH values reported are the averages for a population of vesicles with slightly different pH values. Finally, the intravesicular pH is known to vary within the cells of a given population (48, 49). This finding may be due to variations in the number of ion pumps within vesicle membranes that are involved in pH regulation, which is known to vary among cell types (35, 37).

The fluorescence of SNAFL is temperature sensitive (45). The emission intensity of the dye decreases with increasing temperature, and a 10°C increase in temperature raises the pH measurement by approximately 0.16 unit (10). Because the pH measurements were performed at room temperature, it may be that at 35°C the intravesicular pH would be 0.16 unit higher than our measurements. Also, the pK_a of SNAFL is 7.6 at room temperature (Molecular Probes), which makes the optimal pH range of the probe 6.3 to 8.6. Thus, the intracellular standard curve did become more linear in the acidic pH range (Fig. 1). However, this pH-sensitive probe was the only bioreactive fluorescent molecule available to us that, upon conjugation, did not render the EB noninfectious.

DISCunitSSION

The results presented here show that *C. trachomatis* serovar L2 resides within a vesicle which is only slightly acidic and remains above pH 6 for 12 h after infection. The data further suggest that *C. trachomatis* serovar L2 relies on the activity of Na^+,K^+-ATPases and not H^+-ATPases for intracellular survival and replication within HEC-1B cells.

It is not clear why the pH within vesicles containing heat-killed EB required more than 2 h to become acidic. Acidification of phagosomes containing heat-exposed *C. psittaci* EB envelopes within macrophages takes as little as 30 min (16); however, the acidification of bacterium-containing vesicles within epithelial cells may take longer. In this study, by microscopic examination, heat-killed EB were seen to enter HEC-1B cells as efficiently as viable EB. Thus, the delay observed in the acidification of heat-killed EB-containing vesicles may reflect the host cell receptor bound by EB at the time of entry which directs these vesicles away from the degradative pathway leading to lysosomes. If this is true, an active maintenance of pH within the inclusion by chlamydiae may not be required within epithelial cells until after chlamydia-containing vesicles fuse and EB convert into metabolically active RB.

Chloroquine, at 300 μM, inhibited the growth of serovar L2 within HEC-1B cells, while lesser concentrations of this amine did not. It is known that the concentration of chloroquine required to increase the intravesicular pH by 1 unit within 3T3 fibroblasts is 300 μM (13). Chloroquine, at 300 μM, may have collected within chlamydia-containing vesicles until the pH was raised to near 8. From these results, we estimate that serovar L2 survives only within a pH range of above 6.0 and below 8.0. The structural integrity of the EB envelope is known to be compromised at pH 8 (30). A previous investigation demonstrated that methylamine at concentrations of above 40 mM decreased the number of serovar E inclusions within McCoy cells (38). Thus, in infected cells incubated with high concentrations of amine, the level of amine within chlamydia-containing vesicles may become bactericidal over time. Importantly, our study showed that low concentrations of amine, incubated with chlamydia-infected cells for extended periods, did not inhibit inclusion development as would be expected if the organism required an acidic environment in which to grow.

Low concentrations of the Na^+,K^+-ATPase inhibitor, ouabain, present only during the first 2 h of serovar L2 infection in HEC-1B cells did not inhibit inclusion formation. Ouabain is unable to permeate membranes and inhibits the Na^+,K^+-ATPase by binding to the extracellular portion of the alpha subunit of this membrane ion pump at a stoichiometry of 1:1 (3). Thus, chlamydia-containing vesicles formed in the presence of 1 mM ouabain have inactive Na^+,K^+-ATPases and possess excess unbound ouabain which is able to inactivate any Na^+,K^+-ATPases which join this vesicle. The survival of serovar L2 within HEC-1B cells exposed to low concentrations of ouabain (0.001 to 0.01 mM) may be explained by the lack of free ouabain within the EB-containing vesicles. Fusion of EB-containing vesicles with vesicles containing functional Na^+,K^+-ATPases may rescue vesicles exposed to low doses of ouabain by inhibiting acidification by H^+-ATPases, thus maintaining a pH of above 6.0 and permitting chlamydial growth.

Chlamydiae which enter cells by receptor-mediated endocytosis probably follow the endocytic pathway within the host cell. Following this route may depend solely on the receptor bound at the time of entry. Although heat-killed EB are destroyed in lysosomes (17), purified EB envelopes and unitV-exposed EB remain undamaged within intracellular vesicles (16). These data support the role of chlamydial surface com-

ponents in directing intracellular fate. The observed drop in pH from 7.01 at 2 h p.i. to 6.26 at 4 h p.i. may be required for the recycling of the receptor(s) to the cell surface. The pH drop may also play a role in catalyzing the conversion of metabolically inactive EB to metabolically active RB. A drop to pH 6.0 at 4 h p.i. has also been reported for vesicles containing *Salmonella typhimurium* in infected MDCK cells (20).

The data from these experiments combined with information on vesicle transport within the endocytic pathway have led to the formation of a model for chlamydial survival within epithelial cells (Fig. 2). Bafilomycin A1 inhibits the acidification of early endosomes, which leads to a lack of formation of the endosomal carrier vesicle (ECV) (12). Since early endosomes cannot fuse directly with late endosomes (5, 8, 21), the ECV is thought to be necessary for the transfer from early to late endosomes. Because bafilomycin A1 does not affect serovar L2 inclusion development within HEC-1B cells, this serovar probably does not reside within vesicles which fuse with the ECV, leading to the formation of late endosomes and lysosomes (Fig. 2). Instead, *C. trachomatis* serovar L2 may reside within vesicles originating from the host cell plasma membrane which retain the characteristics of early endosomes. If so, these serovar L2-containing vesicles are guaranteed to avoid fusion with lysosomes because early endosomes are unable to fuse with lysosomes (28) while late endosomes readily fuse with lysosomes (29). This model hypothesizes that chlamydiae remain within vesicles which retain the characteristics of early endosomes and do not mature to lysosomes, possibly by avoiding fusion with H^+-ATPase-containing vesicles as reported for *Mycobacterium avium* (40). However, if chlamydia-containing vesicles retain Na^+,K^+-ATPases, any H^+-ATPases incorporated into this vesicle will remain inactive until the Na^+,K^+-ATPases are removed or inactivated.

The conjugation of pH-sensitive fluorescent probes to pathogenic organisms provides a useful tool to monitor the pH of intracellular compartments in which these pathogens reside within specific target host cells. In order to determine how the pH is maintained within the chlamydial inclusion, the components of the inclusion membrane which regulate pH must be identified. Retention of Na^+,K^+-ATPases within the parasite-containing vesicular membrane is a novel method for the inhibition of vesicle acidification by intracellular parasites and warrants further investigation. Research aimed to determine if Na^+,K^+-ATPases and H^+-ATPases are present within the inclusion membrane is in progress.

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