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Phagolysosomes of *Coxiella burnetii*-Infected Cell Lines Maintain an Acidic pH during Persistent Infection

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***Coxiella burnetii*, the agent of Q fever, is an obligate intracellular bacterium that multiplies within vacuoles of phagolysosomal origin. Persistently infected cell lines were maintained in continuous culture for months. We studied the pH of the phagolysosomes by using two murine cell lines during early propagation of the bacteria and after establishment of persistent infection. Three strains of *C. burnetii* were studied because of the purported propensity of each strain to cause acute or chronic disease and to be resistant or susceptible to antibiotics. The pHs were calculated from fluorescence experiments with fluoresceinated dextran as a lysosomal probe. Phagolysosomal vacuoles maintained an acidic pH during a 36-day infection. Minimal variation of the pH occurred over the duration of the experiment with strains that caused either acute or chronic disease. Phagolysosomal pH remained stable for as long as 153 days with the Nine Mile phase II isolate. Thus, neither the course of *C. burnetti* infection nor the diversity of antibiotic susceptibility of the strains is related to variations in the phagolysosomal pH.**

Intracellular organisms have evolved several stratagems to resist the microbicidal mechanisms of the host cells, particularly to the lethal consequences of phagosome-lysosome fusion (10). Some organisms escape from the phagosomes, whereas others prevent the fusion of these organelles with lysosomes. *Coxiella burnetii*, the agent of Q fever, resists the attack of the lysosomal environment and has adapted to the acidic environment of the phagolysosome (6, 14). In vitro infected-cell models have provided essential information on the intracellular behavior of this pathogen (1, 4, 5). This bacterium can establish persistent infection in cell culture, in which it not only survives but also multiplies in vacuoles of phagolysosomal origin. Using J774 murine macrophage-like cells infected with the Nine Mile phase II strain of *C. burnetii*, Akporiaye et al. reported a phagolysosomal pH of 5.2 (1). Hackstadt and Williams demonstrated that such acidity enhances the metabolism and growth of the bacteria (6). Moreover, alkalinization of the phagolysosomes by basic lysosomotropic agents such as chloroquine, methylamine, and ammonium chloride inhibits parasite multiplication (6, 13). Under these conditions the bactericidal activity of antibiotic compounds appears to be enhanced (13).

Q fever may be an acute or chronic illness. Since pH conditions within phagolysosomes influence the multiplication of *C. burnetii*, the purpose of the present study was to determine the vacuolar pHs of infected cells during early propagation of bacteria and after establishment of persistent infection. We also compared the vacuolar pHs after infection with strains causing acute and chronic disease (15, 16). Measurements of pH were calculated from fluorescence experiments with fluoresceinated dextran as a lysosomal probe. This technique was first described by Ohkuma and Poole (12). The study shows that *C. burnetii*-containing vacuoles maintain an acidic pH during early and persistent infection in two different infected cell lines. Only small variations in pH were observed during the course of the experiments. Also, the strain did not influence the phagolysosomal pH.

MATERIALS AND METHODS

***C. burnetii* propagation.** The Nine Mile phase II isolate of *C. burnetii* (ATCC VR-615) was obtained from O. Baca (University of New Mexico, Albuquerque), whereas the Priscilla and Q212 isolates were obtained from T. Hackstadt (Rocky Mountain Laboratory, Hamilton, Mont.). Strain ATCC VR-615 is the reference strain for acute disease, and the other two strains are associated with chronic disease. All strains were propagated in two murine cell lines: the P388D1 macrophage-like cell line and the L929 fibroblast cell line. Infected cells were grown in 25-cm^2 culture flasks in Eagle minimal essential medium with 10% fetal calf serum and 2 mM glutamine at 37°C in an atmosphere of 5% carbon dioxide. The cells were passaged three times a week. Cell cultures were examined by using a Gimenez stain daily for 2 weeks and then at the time of measurement of phagolysosomal pH for determination of the percentage of infected cells. Cell viability was evaluated on the same samples by using the trypan blue dye exclusion test.

Phagolysosomal pH measurements. Measurements of phagolysosomal pH were calculated from fluorescence experiments as described by Antoine et al. for *Leishmania amazonensis* (3). *C. burnetii*-infected cells in 25-cm^2 culture flasks were harvested and allowed to attach for 15 min to 14-mm-diameter coverslips (1×10^5 to 5×10^5 cells per coverslip) in 12-well plates (Corning no. 25815). Then 2 mg of fluoresceinated dextran (fluorescein isothiocyanate-labeled dextran, FD40; Sigma) per ml was added to the culture medium. After 24 h of incubation, cell monolayers were washed three times with Dulbecco phosphate-buffered saline (pH 7.4) and examined for fluorescence at room temperature with ×400 magnification. Fluorescence intensity measurements were conducted on an Olympus IMT2 inverted microscope system equipped with interchangeable blue (wavelength, 455 to 490 nm) or purple (wavelength, 370 to 430 nm) band-pass excitation filters (2). Video images of fluorescence

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were obtained with a high-sensitivity video camera (model 1036; Lhesa, Cergy Pontoise, France) mounted on the microscope. The camera output was connected to a digitizer (Imaging Technology, Woburn, Mass.) mounted on an IBM-compatible Zenith 148 computer. Digitized fields were analyzed immediately. This equipment allowed measurement of the fluorescence intensity of only the lysosomal compartment of each individual cell with automatic subtraction of the extracellular background. The total fluorescence intensities after blue or purple excitations were recorded, and the blue/purple fluorescence ratio was calculated. Each phagolysosomal pH value corresponded to fluorescence ratios of at least 15 cells. Fluorescence ratio measurements on uninfected P388D1 cells served as controls.

A calibration curve for deriving pH values from fluorescence intensity ratios was constructed with uninfected cells. These were loaded with fluoresceinated dextran, fixed with 4% formaldehyde for 15 min, and equilibrated with acetate or phosphate buffers containing 20 μM monensin at pHs that ranged from 4.5 to 7. Fluorescence intensities were recorded as described above.

Statistical analysis. Statistical evaluation of the differences in phagolysosomal pH among the various infected cell models and over the experimentation time was performed by using the Student *t* test at the 95% confidence limit.

RESULTS

Course of *C. burnetii* infection. The propagation of *C. burnetii* in cell culture was evaluated by determining the percentage of infected cells. An infection rate of 100% was observed 7 days after inoculation of the Q212 isolate and 5 days later with the Priscilla and Nine Mile isolates. Subsequently, at least 95% of P388D1 or L929 cells remained persistently infected. Infected cells showed single or multiple vacuoles of different sizes (Fig. 1A).

Some cells contained distended vacuoles that occupied more then two thirds of the cell volume. With the Nine Mile and Priscilla isolates, less than 15% of cells showed such vacuoles, but over 50% of such cells were noted with the Q212 strain.

Intracellular localization of fluoresceinated dextran. Examination by fluorescence microscopy of either P388D1 or L929 cells loaded with fluoresceinated dextran showed that they accumulated this probe essentially in round cytoplasmic granules whose pattern was typical of lysosomes. Fluoresceinated dextran concentrated within *C. burnetii*-containing vacuoles of infected cells, at which time the lysosomes had almost completely disappeared (Fig. 1B). These morphological findings, and the stain control showing that at least 95% of cells were infected, indicate that during the fluorescence experiments we were truly measuring the fluorescence intensity of phagolysosomes of *C. burnetii*-infected cells.

Phagolysosomal pH measurements. Figure 2 shows the calibration curve that allowed determination of pH values from fluorescence intensity ratios. The means ± standard errors of lysosomal pHs of uninfected P388D1 and L929 cells were 4.59 ± 0.16 and 4.40 ± 0.23, respectively. Figure 3D presents the variations in lysosomal pH of uninfected P388D1 cells throughout the experiment. The phagolysosomal pHs of P388D1 and L929 cells infected with the Nine Mile, Priscilla, or Q212 isolate were determined twice a week for 36 days (Fig. 3A, B, and C). The fluorescence experiments were continued for up to 153 days with P388D1 cells infected with the Nine Mile isolate (Fig. 4). The means ± standard deviations of vacuolar pHs ranged from 4.54 ± 0.15 to 4.89 ± 0.17, 4.5 ± 0.15 to 4.88 ± 0.38, and 4.47 ± 0.14 to 5.08 ± 0.2, respectively, with the Nine Mile, Q212, and Priscilla isolates cultured in P388D1 cells. They ranged from 4.42 ± 0.16 to 5.07 ± 0.23, 4.43 ± 0.18 to 4.8 ± 0.37, and 4.47 ± 0.16 to 5.06 ± 0.16, respectively, with the same isolates in L929 cells. These experiments confirmed the acidity of the intracellular environment of *C. burnetii*. Moreover, the phagolysosomal pH remained acidic throughout the experiment with both acute or chronic strains. In P388D1 cells infected with the Nine Mile strain, acidity within *C. burnetii*-containing vacuoles was noted for as long as 5 months. Variations in phagolysosomal pH were noted with each infected cell culture model. The highest variations in pH over the experimentation time were 0.35 ($P < 0.01$) and 0.38 ($P < 0.01$) pH unit with the Nine Mile strain cultured in P388D1 or L929 cells, respectively. They were 0.61 ($P < 0.01$) and 0.65 ($P < 0.01$) pH unit, respectively, with the Q212 strain in P388D1 and L929 cells and 0.43 ($P < 0.01$) and 0.79 ($P < 0.01$) pH unit, respectively, with the Priscilla strain in P388D1 and L929 cells. Variations in vacuolar pH among the three *C. burnetii* strains were up to 0.67 pH unit ($P <$

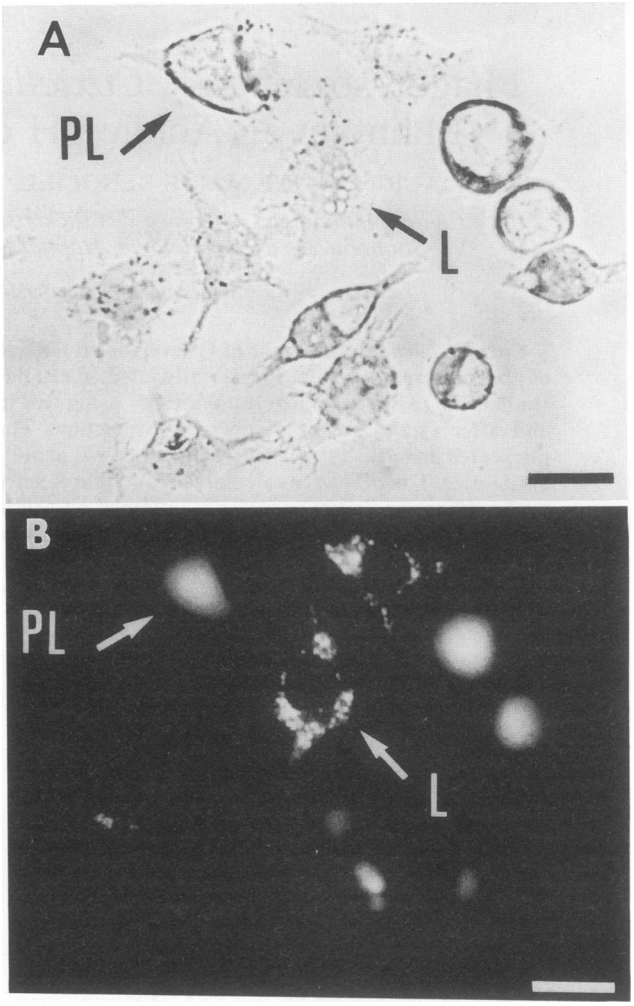

FIG. 1. P388D1 cells infected with the Q212 isolate of *C. burnetii*. (A) Photomicroscopic study of the infected cells. (B) Fluorometric analysis of the same cells. Magnification, ×400 for both panels. Bars, 30 μm.

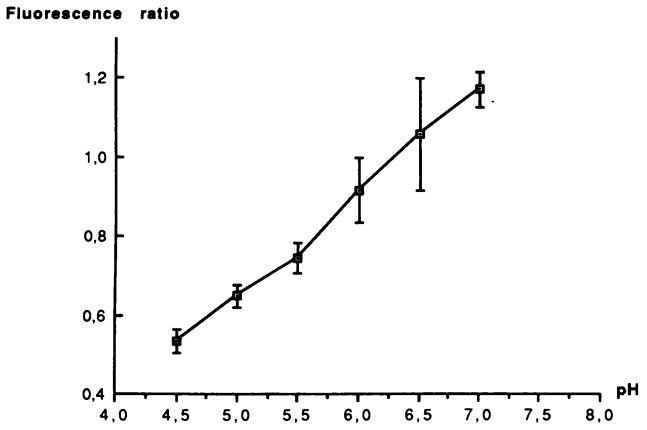

FIG. 2. Calibration curve for pH. The calibration curve for deriving lysosomal pH from fluorescence intensity ratios was constructed by using P388D1 macrophage-like cells loaded with fluoresceinated dextran and then fixed with formaldehyde and equilibrated with phosphate or acetate buffer. Each point of the graph represents the means ± standard deviation of the fluorescence ratios of at least 15 cells.

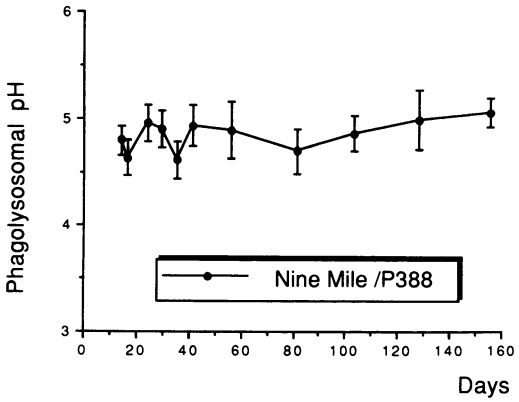

FIG. 4. Phagolysosomal pH of P388D1 cells infected with the Nine Mile isolate of *C. burnetii*, as determined by the fluorescence probe technique, throughout a 153-day infection.

0.01). Although these variations were significant when analyzed by the Student *t* test, they could not be related to the use of different *C. burnetii* isolates or to the duration of infection. They may be related to experimental conditions, since we noted variations in lysosomal pH of uninfected P388D1 cells up to 0.4 pH unit ($P < 0.01$). Finally, even the cells with distended vacuoles full of bacteria displayed the same acidic pH. Some of them displayed a vacuolar pH of 7, but the trypan blue dye exclusion test revealed that such cells were dead. Thus, the phagolysosomal pH remained acidic until *C. burnetii* multiplication led to cell lysis.

DISCUSSION

C. burnetii can establish persistent infection of several cultured cell lines, including L929 mouse fibroblast cells and P388D1 murine macrophage-like cells (1, 4, 5). These infected cell populations can be maintained in continuous culture for months. The cells continue to maintain normal cell cycle progression and division capacity.

Using the murine J774 macrophage-like cell line persistently infected with the Nine Mile isolate of *C. burnetii*, Akporiaye et al. (1) defined the properties of the environ-

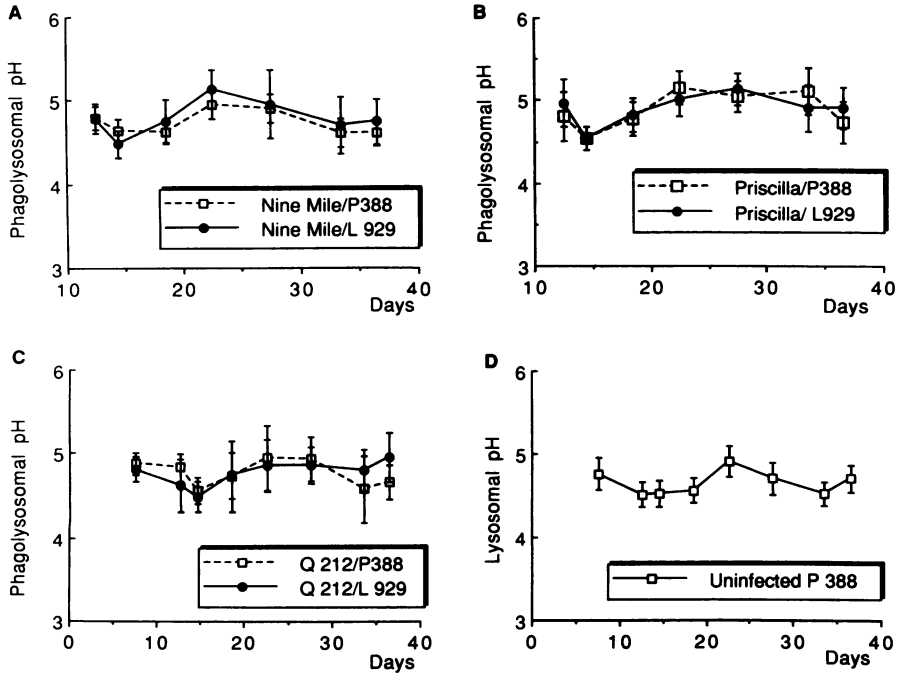

FIG. 3. Phagolysosomal pHs of P388D1 and L929 cells infected with *C. burnetii* as determined by the fluorescence probe technique. Three different isolates of *C. burnetii* were used: (A) the Nine Mile isolate, (B) the Priscilla isolate, and (C) the Q212 isolate. Panel D shows the lysosomal pHs of the uninfected P388D1 cell controls.

ment in which the bacteria survived. They reported acid phosphatase activity and an acidic pH of 5.21 and concluded that *C. burnetii* was located in vacuoles of phagolysosomal origin. Hackstadt and Williams (6) provided an explanation for the survival of this bacterium in such hostile medium. Metabolism and growth of the bacteria are activated at acidic pH, as shown by the enhanced incorporation of several metabolites under such conditions (7, 8, 19). Our results confirm the acidity of the vacuoles in which *C. burnetii* grows and extend the results of Akporiaye et al. (1). We have demonstrated that acidic pH is maintained in the vacuolar growth chamber during early propagation of the bacteria and after maintenance of the persistent infection for as long as 153 days postinoculation with the Nine Mile isolate. Moreover, we did not find any significant differences in the pHs of vacuoles containing the Nine Mile acute disease strain and those of vacuoles containing the Priscilla and Q212 chronic disease strains throughout 36 days of growth. Thus, although these isolates lead in vivo to different pathologic states and progression of disease, the fate of infected cells in vitro is similar.

Although Yeaman and Baca (17) did not find any difference in the penetration of antibiotics into *C. burnetii* incubated at acidic or alkaline pH, the pH conditions within phagolysosomes actually can alter the susceptibility of *C. burnetii* to some antibiotics (13). To be active against this intracellular organism, drugs must enter mammalian host cells, reach the pararasitophorous vacuoles, and be active at the acidic pH. Only a few antibiotics, including rifampin and cyclines, which are currently used in chronic Q fever treatment (9), display such properties. Yeaman et al. (18) reported some variability in antibiotic susceptibility of *C. burnetii* during persistent infection; antibiotics were more effective in the early stage of propagation of bacteria than after establishment of persistent infection. Moreover chronic disease organisms such as the Priscilla and Q212 isolates were less susceptible to antibiotics than the acute disease Nine Mile isolate. We conclude that such differences cannot be explained by variations of phagolysosomal pH.

Other obligate intracellular bacteria capable of persistently infecting cell populations maintained in vitro include *Chlamydia psittaci*, *Rickettsia rickettsii*, and *Ehrlichia sennetsu* (10). None of them survive in phagolysosomes. Recently Antoine et al. (3), using the fluorescence pH quantification technique, demonstrated that amastigote forms of *L. amazonensis*, which is an obligate intracellular parasite, are located in acidic vacuoles of phagolysosomal origin. Thus, *L. amazonensis* shares with *C. burnetii* the ability to survive and multiply in harsh phagolysosomal environmental conditions without alkalinizing these cell compartments (6, 11). How these organisms can resist the attack of microbicidal activities is unknown, but activation of the metabolism at acidic pH indicates an effective adaptation of these pathogens to the microbicidal environment of the phagolysosome (6).

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