Developmental Cycle of Coxiella burnetii: Structure and Morphogenesis of Vegetative and Sporogenic Differentiations

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Coxiella burnetii is a gram-variable obligate intracellular bacterium which carries out its development cycle in the phagolysosome of eucaryotic cells. Ultrastructural analysis of C. burnetii, in situ and after Renograin purification, by transmission electron microscopy of lead-stained thin sections has revealed extreme pleomorphism as demonstrated by two morphological cell types, a large cell variant (LCV) and a small cell variant (SCV). Potassium permanganate staining of purified rickettsiae revealed a number of differences in the internal structures of the cell variants. (i) The outer membrane of the SCV and LCV were comparable; however, the underlying dense layer of the SCV was much wider and more prominent than that of the LCV. The periplasmic space of the SCV was not readily visualized, whereas the periplasmic space of the LCV was apparent and resembled that of other gram-negative bacteria. (ii) Complex internal membranous intrusions which appeared to originate from the cytoplasmic membrane were observed in the SCV. The LCV did not harbor an extensive membranous system. (iii) Some LCVs contained a dense body in the periplasmic space. This endogenous structure appeared to arise in one pole of the LCV as an electron-dense “cap” formation with the progressive development of a dense body approximately 130 to 170 nm in diameter which was eventually surrounded by a coat of at least four layers. Our observations suggest that the morphogenesis of C. burnetii is comparable, although not identical, to cellular differentiation of endospore formation. A developmental cycle consisting of vegetative and sporogenic differentiation is proposed.

Coxiella burnetii, the etiological agent of Q fever, differs from other members of the family Rickettsiaceae in its high degree of resistance to physical and chemical agents (4), unique phase variation (55), variable Gram stain reaction (21), and DNA base composition (53, 64). C. burnetii has been shown to be highly pleomorphic during multiplication within phagolysosomes of host cells (7). The pleomorphic nature of C. burnetii was first described by Davis and Cox (15), who observed by light microscopy that minute coccolid and granular forms, as well as bacillary forms, of C. burnetii were present in cells of infected guinea pigs and in tissue cultures. The size of C. burnetii was so variable that some forms passed through filters. Ultrastructural analysis of C. burnetii, studied in chicken embryonic yolk sacs (YS) (1), tissue culture cell lines (51), infected animal tissues (2, 26), and infected peritoneal macrophages (31, 32) revealed distinct cell variants with marked morphological differences.

Cell variants of C. burnetii could be separated into two distinct zones in CsCl, sucrose, or isopycnic Renograin gradients (9, 61). Although the relationship between the cell variants of C. burnetii has not been fully elucidated, Wiebe et al. (61) speculated that they represented two stages in a complex developmental cell cycle similar to the one of Chlamydia psittaci, another order of obligate intracellular parasites. An alternative mechanism for the generation of cell variants was suggested by Wiebe et al. (61) to include partial degradation of the small cell variants (SCVs) by lysosomal enzymes of host cell origin, thereby rendering them large cell variants (LCVs). Thus, the SCV was considered a “normal” nondegenerated cell apparently unaffected by enzymatic processes. Physical stresses associated with storage of infected embryonic YS and purification of samples were also thought to play contributing factors in generating LCVs (58).

In contrast to the observations implicating transverse binary fission (61) as the mode of replication of C. burnetii, a quite different intracellular behavior was proposed by Kordova et al. (33-36, 51). The multiplication process of C.
burnetii was thought to be similar to that of viral replication during the early phase of the infection cycle. Evidence for this interpretation was the demonstration of infectious, round particles of C. burnetii, which passed through 40-nm collodion membranes (33). Such ultrafilters were capable of causing infection in YS tissue culture cells (36). During this infection cycle, fine granules appeared, and as the infection proceeded, the vacuolated cytoplasm was filled with antigenic material of differing size and form. Subsequently, C. burnetii particles appeared in the cytoplasm as masses of recognizable rickettsiae. Most authors (46, 59, 63) have, however, expressed some doubts as to the validity of these observations, ascertaining that transverse binary fission is the customary and only mode of replication.

The purpose of this study is to resolve the apparent discrepancy in our understanding of the relationship between the distinct cell variants and the developmental cycle of C. burnetii. Ultrastructural analysis, using several conditions of preparation, was therefore carried out on C. burnetii in situ in YS tissues and on viable Renografin-purified cells. The results indicate that C. burnetii has certain features comparable to those of bacteria which undergo differentiation leading to endospor formation.

MATERIALS AND METHODS

Bacteria. C. burnetii strains employed in this study were in various passage levels in guinea pigs, chicken embryo fibroblast tissue culture, and chicken embryo YS. The Ohio strain (5EP/2GP/2EP) was in phase I without detectable phase II antigens, whereas the Nine Mile strains were in phase I (907GP/1TC/1EP, clone 7) and phase II (908EP/1TC/4EP, clone 4), hereafter designated CBOI, CB9MI, and CB9MII, respectively.

Preparation of rickettsiae. C. burnetii were propagated in specific pathogen-free type IV, antibiotic-free, fertile hen egg YS (H & N Hatchery, Redmond, Wash.). Stock cultures of C. burnetii were maintained as 50% (wt/vol) grams of YS per milliliter of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) YS suspensions at -70°C. Stock cultures were prepared by injecting C. burnetii into the YS of chicken embryos on the 5th day postincubation. Incubation was carried out at 35°C in a humidity-controlled Jamesway incubator. On the 7th day postinfection, the YS from live embryos were harvested, and a 50% YS suspension in brain heart infusion was prepared by blending (Waring blender, model F.C. 114) for 30 s at a powerstat setting of 100. Stock cultures were shell-frozen as 2-ml aliquots in sealed glass ampoules. Plaque-forming units inoculated into each YS were 1.1 x 10^8 for CBOI, 1.5 x 10^8 for CB9MI, and 5.6 x 10^5 for CB9MII. Mean survival time of the infected embryos was 7.4 ± 0.2 days at 35°C. Cultures destined for the separation of C. burnetii from host material were frozen in batches of 25 to 35 YS and stored at -70°C, or they were used as fresh YS without freezing.

Harvest and purification of C. burnetii. The rickettsiae from the infected YS were purified by isopycnic Renografin gradients as outlined in a previous study (62). Some rickettsiae, however, were purified through gradients containing 0.25 M sucrose, which was also present in buffer components (phosphate-buffered saline-sucrose, pH 7.35) used for resuspending the pellets throughout purification.

Light microscopy. The Renografin-purified rickettsiae (CB9MI) were inactivated with 1% formaldehyde for 24 h at room temperature. Formaldehyde was removed by washing the organisms three times with sterile deionized distilled water at 12,100 x g for 30 min. The pellet was resuspended in sterile deionized distilled water and applied to the glass slides with a loop to avoid overlap of the bacterial cells in the sample. All samples were air dried, heat fixed, and cooled before staining. The following staining procedures were employed. (i) Gram staining was carried out as described by Gimenez (21), employing mordants of either aqueous or alcoholic solutions. (ii) The acid-fast staining was carried out by the Kinyoun carbol-fuchsin method (17). (iii) The spore-staining procedure was a modification of the methods of Dorner (17) and Wirtz-Conklin (47). Samples were examined under oil immersion field with a Zeiss standard 10 light microscope.

Electron microscopy. Rickettsiae in YS and from purified rickettsial suspensions were fixed overnight at 4°C in a primary fixative containing 2.5% glutaraldehyde (Polysciences, Warrington, Pa.), 2.0% formaldehyde prepared from paraformaldehyde (Baker, Phillipsburg, N.J.), and 2.5 mM CaCl₂ in 66 mM cacodylate buffer (pH 6.8). After a brief rinse with buffer, the specimens were postfixed in 1% osmium tetroxide buffered with 66 mM cacodylate buffer for 1 h at 4°C. The suspensions in microsample tubes (1.5 ml) were centrifuged (Beckman Microfuge B) after each stage of both fixation and rinsing. The pellets were preembedded in 2% Difco Noble agar (23), and the blocks were dehydrated through serial dilutions of methanol. The cells were stained for 1 h at room temperature with 0.5% uranyl acetate in 30% methanol during dehydration.

The nucleoids of C. burnetii were preserved by a procedure which was described by Ryter and Kellenberger (52). The Ryter-Kellenberger fixative (30) containing 1% osmium tetroxide was also employed as an alternative primary fixative. In this instance, the purified sample was fixed by adding 10 volumes of Ryter-Kellenberger fixative (pH 6.1) for 1 h at 4°C. Fixation was followed by three 15-min rinses in Ryter-Kellenberger fixative Veronal-acetate buffer (pH 6.1). After each stage of fixation and rinsing, the solution was centrifuged at 9,750 x g for 30 min at 4°C. The pellet was resuspended and stained with 0.5% uranyl acetate in Ryter-Kellenberger fixative Veronal buffer (pH 6.1) for 2 h at room temperature. After one rinse in buffer, the samples were embedded in 2% Difco Noble agar (23), cut into 1-mm³ blocks, and dehydrated through serial dilutions of methanol. All blocks were embedded in Spurr epoxy resin (54). Ultrathin sections were cut on a Reichert OMEU microtome, collected on uncoated Pelco 300 grids, stained with either lead citrate
(50) or potassium permanganate (37, 56, 65), and examined in a Hitachi EM-HU-11E-1 electron microscope operated at 75 kV.

Samples purified with phosphate-buffered saline-sucrose as the diluent (62) were fixed with primary fixative containing the following components: 3% glutaraldehyde and 2.5 mM CaCl₂ in 66 mM cacodylate buffer (pH 6.8). Postfixation was carried out with 1% osmium tetroxide buffered with 66 mM cacodylate buffer (pH 6.8). The osmolarity of this buffer and the buffer (66 mM cacodylate, pH 6.8) used for washing after fixation was adjusted from 137 mOsmol/kg to approximately 640 mOsmol/kg with the addition of sucrose (0.37 M). The osmolarity of solutions was determined by the freezing point depression method with an osmometer (model 3W, Advanced Instrument, Needham Heights, Mass.). Ryter-Kellenberger fixative was used as an alternative primary fixative (30).

Biochemical assay: dipicolinic acid. The colorimetric assay for dipicolinic acid in bacterial spores was carried out by the method of Janssen et al. (28), employing a dipicolinic acid (Sigma Chemical Co., St. Louis, Mo.) standard curve as a positive control.

RESULTS

Staining of C. burnetii. The following observations were made on C90MI purified by Renografin gradients (62).

(i) Gram-staining. A variable Gram stain reaction was obtained with ethyl-alcohol iodine solution as a mordant, whereas aqueous iodine gave a gram-negative reaction. Such findings agree with those of Gimenez (21).

(ii) Acid-fast stain. Approximately 10% of the organisms were stained red or were "acid fast," suggesting that C. burnetii cells exhibit some of the staining properties of tubercle bacilli.

(iii) Spore stains. With the Wirtz-Conklin method, faint-green sphehores and rods were barely distinguishable in a field of red-stained rods. Clear visualization of the green-staining bodies, characteristic of spores, was limited due to the resolution of the light microscope. With the Dorner method, most cells were stained red, characteristic of spores, whereas bacterial cells appeared colorless against a dark grey background.

Pleomorphism of C. burnetii before and after Renografin purification: a comparison of lead-stained thin sections. Morphological features of C. burnetii growing in YS of an infected embryo is shown in Fig. 1A. At least two morphological cell types, designated as LCVs and SCVs, of C. burnetii were observed. Some cells appeared to be undergoing cellular deterioration, as evidenced by loose outer cell membranes and fibrillar nuclear regions (Fig. 1A, arrows), whereas other cells were more electron dense (Fig. 1A).

After the long, arduous Renografin purification procedure (62), similar morphological cell variants were observed in the final, highly concentrated preparation (Fig. 1B). These cells resembled the starting YS material; however, cell damage was apparent, as shown by the loose outer membrane and apparent outer membrane bleb formation (Fig. 1B, arrows). Morphological features of the SCVs appeared to be unaffected during the purification procedures (40). Indeed, in an earlier study, we showed that the LCVs were sensitive to decreasing osmotic conditions, whereas the SCVs were not affected by extremes of osmotic pressures and sonic disruption (40). The SCVs were compact and rod-shaped with a very dense central region of condensed nucleoid filaments (Fig. 1B). This nucleoid region was surrounded by an electron-dense and granular material, presumably ribonucleoprotein (Fig. 1C). The outer membrane of the SCV was barely resolvable, yet a dense layer beneath the outer membrane, possibly a peptidoglycan, was very prominent (Fig. 1C). Staining with lead, however, was too intense to allow reasonable visualization of the cytoplasmic membrane (Fig. 1A, B, and C).

The LCVs, which resembled gram-negative bacteria, were larger and more pleomorphic than the SCVs (Fig. 1). A slightly swollen, and perhaps damaged, LCV is depicted in Fig. 1C, showing a loose outer membrane and cytoplasmic membrane. The nucleoid region, recognized by dense filaments radiating from the central region into the cytoplasm, appeared to be more dispersed in the LCV than in the SCV. Also, the outer membrane of the LCV was more clearly separated from the cytoplasmic membrane by the periplasmic space, whereas the characteristic dense layer of the SCV underlying the outer membrane was not observed. The ribonucleoprotein material was displaced to the periphery of the cell, leaving a transparent zone between the nucleoid mass and the granular cytoplasm.

Comparison of Renografin-purified LCVs and SCVs stained with potassium permanganate. Morphological features of C. burnetii growing in the phagolysosome of an infected YS cell is depicted in Fig. 2A. Extreme cellular pleomorphism was observed in this thin section, demonstrating the heterogeneity of cell types. In general, these potassium permanganate-stained cells show similar morphological features as those described in lead-stained thin sections at low magnification (compare Fig. 1A and 2A). Importantly, some cells (Fig. 2A) appeared to be undergoing cellular deterioration, as evidenced by loose cell membranes and increased periplasmic space; however, some LCVs were undergoing cell division with septate formation (Fig. 2, arrows).
FIG. 1. Electron micrographs of thin sections of C. burnetii cells observed in situ in YS and after Renografin purification. C. burnetii cells were fixed with primary fixative and stained with lead. (A) Demonstration of the pleomorphic nature of C. burnetii in situ in YS before Renografin purification. Both the LCV (L) and the SCV (S) are clearly depicted. The LCV appears to be undergoing deterioration since, in some cells, the outer membrane is distended, thereby increasing the periplasmic space (arrows). Bar, 0.6 μm. (B) Renografin-purified C. burnetii cells, showing both the LCVs and the SCVs. The LCVs appear to be damaged by the purification procedure. The outer membrane of the LCVs appears to form blebs (arrows). The morphology of the SCVs appears to be unaffected by purification. Bar, 0.6 μm. (C) Resolution of LCV ultrastucture, employing primary fixative and lead staining. The cytoplasmic membrane (CM) and outer membrane (OM) are separated by a periplasmic space (PS). DL, Dense layer. The nucleoplasm, with dispersed nucleoid filaments and ribonucleoprotein, is clearly shown. Bar, 0.2 μm.

Morphological differences between the cell variants were more striking (Fig. 2B) at higher magnifications when potassium permanganate staining was carried out on Renografin-purified C. burnetii. Sections of the LCVs and SCVs showed a well-defined outer membrane and a recognizable cytoplasmic membrane. However, the SCVs are markedly different from the LCVs. Notably, a dense material filled the periplasmic space between the outer and cytoplasmic membranes (Fig. 2B, C, and D), and multilayered trilaminar membranes were also observed within the cytoplasm of the SCVs (Fig. 2C and D, arrows). These membranes consisted of distinct electron-lucent layers separated by electron-dense layers. In both transverse (Fig. 2C) and longitudinal (Fig. 2D) sections, the membranes appeared mostly on one hemisphere of the cell. The outer membrane was 4.5 nm thick, whereas the internal membranes were 3.5 nm thick. The complex intracellular membranes appeared to be continuous with the cytoplasmic membrane, and each set of two electron-lucent layers separated by an electron-dense layer seemed to be infoldings of a unit membrane. These membranes were so closely compacted that the contiguous layers, in some cells, were unresolvable. The LCVs, in contrast to the SCVs, did not appear to have internal membranes. Instead, the LCV cell wall consisted simply of a trilaminar cytoplasmic membrane separated from a threelayered outer membrane by a periplasmic space. The peptidoglycan layer was not readily visible. This is in sharp contrast to the electron-dense material found in the periplasmic space of the SCV.

Sporogenic differentiation. Although electron microscopic studies cannot simulate time-lapse sequence of cellular differentiation, we have arranged selected photomicrographs of a purported differentiation progression (Fig. 3). The interior of the cell was composed of a very
dense nucleoplasm. As the purported differentiation progressed, fibrils radiated from the nucleoplasm, and the dense granules or ribonucleoproteins formed a tight package surrounding the nucleoplasm (Fig. 3A). Thus, the cell appeared to differentiate into a sporogenic phase with the following features. (i) In some cells, an unusual morphological feature was observed. Initial observations suggested that an endogenous structure could be shown in one pole of the LCV. Furthermore, cells purified with phosphate-buffered saline as diluent showed fewer of these ultrastructural features than did those cells purified with phosphate-buffered saline-sucrose as diluent. Therefore, cells were purified in phosphate-buffered saline-sucrose as diluent, and potassium permanganate-stained thin sections were examined under high magnification. Indeed, an endogenous structure was observed developing in one pole of the LCV (Fig. 3A). The structure appeared as a “cap” with electron-dense material surrounded by a single trilaminar membrane (Fig. 3A). The membrane resembled the cytoplasmic membrane, but the infolding of the membrane surrounding the cap did not appear to be continuous with the cytoplasmic membrane. The space between the cap and the cytoplasmic membrane seemed to share the area with the periplasmic space (Fig. 3A and B). (ii) The LCV appeared to undergo a stage in vege-
Fig. 3.
tative differentiation which corresponded to endospore formation during a process of unequal cell division (Fig. 3B). The cap structure appeared to be initiated in an intermediate stage of SCV to LCV differentiation, thus proceeding to an endogenous structure characteristic of sporogenic differentiation. (iii) As the progression continued, an endospore was clearly depicted in one pole of the LCV (Fig. 3C). The coat of the dense body or endospore was approximately 26.4 ± 2.5 nm in thickness and consisted of three electron-lucent layers separated by two electron-dense areas (Fig. 3C). (iv) Separation of the dense body from the main cytoplasmic component of the cell is depicted in Fig. 3D. The LCV containing the endospore appeared to be deteriorating, suggesting that the endospore may be liberated upon lysis of the LCV.

The purification procedure probably contributed to the distended appearance of the LCVs containing endospores. Since we observed these marked increases in the periplasmic space of some cells (compare Fig. 2A and 3), it is conceivable that some cellular damage was promoted by the purification procedure. However, the LCVs showed extreme pleomorphism in situ during intraphagolysosomal growth (Fig. 2A). It is worth noting that the LCVs in situ appeared to deteriorate, shedding cytoplasmic components as well as the cell envelope material.

Nuclear region of the endospore. Since the endospore could be visualized in those cells which had been fixed with potassium permanganate, it was of interest to demonstrate the purported nuclear regions. We therefore used the Ryter-Kellenberger procedure (52) in an attempt to demonstrate subcellular nucleic acid bounded by membranes (Fig. 4A). This method showed at least one electron-dense body surrounded by an electron-lucent zone within several LCVs (Fig. 4A and B, arrows). Since the Ryter-Kellenberger procedure had been shown to stabilize the nuclear material of bacterial cells, the extreme density of the subcellular structures may reflect an aggregation of nuclear material enclosed in membranes within the boundary of the LCVs. The subcellular orientation of the endospore in purified C. burnetii cells was demonstrated by the Ryter-Kellenberger (Fig. 4B) and potassium permanganate (Fig. 4C) methods. Similar subcellular structures were observed when the potassium permanganate procedure was carried out on C. burnetii, phase I or II, in situ in chicken embryo tissue culture cells (Fig. 4D).

Mode of propagation of C. burnetii. There are at least two modes of propagation of C. burnetii. Binary transverse fission was observed to occur in both the SCVs and LCVs (Fig. 5). Transverse septate formation appeared to occur in both cell variants during cell division (Fig. 5A and B, arrows); however, some LCVs appeared to undergo cell division without septate formation (Fig. 5C). Some LCVs were observed to undergo concurrent binary transverse fission with unequal cell division characterized by endospore formation (Fig. 3C).

Biochemical assay for dipicolinic acid. The assay for dipicolinic acid, employing 50 mg (dry weight) of Renografin-purified rickettsial suspension of CBOI per ml, was negative (28).

DISCUSSION

The observations outlined in this study were made on C. burnetii cells before and after Renografin purification. Although extreme pleomorphism was noted in the purified cells, similar morphological properties of C. burnetii were observed in the phagolysosome of infected eukaryotic cells. Our results can be explained on the basis of differences of morphology of two cell variants of C. burnetii. The LCVs appeared similar to the gram-negative bacteria in possessing outer and cytoplasmic membranes separated by a periplasmic space. The SCVs, on the other hand, had an extremely electron-dense area between the cytoplasmic and outer membranes and a well-defined membranous system, possibly continuous with the cytoplasmic membrane. At the ultrastructural level, the combination of

Fig. 3. Purported differentiation progression of endospore formation by C. burnetii. Selected photomicrographs from Renografin-purified C. burnetii cells were arranged in a proposed differentiation progression. Cells were fixed with primary fixative and stained with potassium permanganate. (A) Initial stages of endospore formation within the polar end of the large cell variant. Note the periplasmic orientation of the cap formation (arrow). PS, Periplasmic space. Bar, 0.2 μm. (B) Intermediate stage of endospore formation showing the polar and periplasmic orientation of endospore development during a process of unequal cell division. The periplasmic space is greatly distended, probably as a result of purification. Bar, 0.2 μm. (C) Complete formation of the endospore (E) in an LCV concurrently undergoing unequal cell division. Note the nuclear regions of the dividing cell and the separation of the spore from cytoplasmic contents by the membranes of the endospore. Bar, 0.2 μm. (D) Apparent degeneration of an LCV containing an endospore (E). Bar, 0.2 μm. The endospore may be released from the LCV upon complete lysis of the cell.
Fig. 4. Comparison of electron micrographs of thin sections of C. burnetii cells fixed and stained by the Ryter-Kellenberger and potassium permanganate methods. (A) Nucleoid region of the endospore (E) is clearly visible in electron micrographs of thin sections of C. burnetii fixed with Ryter-Kellenberger fixative and stained with lead. Note two LCVs with endospores (E) (arrows). Bar, 0.4 μm. (B, C, and D) Higher magnification illustrating the polar location and the density of the endospore (E), reflecting the limiting membranes of the endospore and aggregation of nucleic acid. Bar, 0.2 μm. (B) Ryter-Kellenberger procedure, (C) potassium permanganate procedure, and (D) potassium permanganate procedure carried out in situ in chicken embryo tissue culture cells infected with C. burnetii, phase I and II. In (D), the cell was in phase II and the surface was coated with antibody (manuscript in preparation).

Macromolecules and peptidoglycan may contribute to an electron-dense area in the cell walls of the SCVs. The peptidoglycan was not as obvious in the LCVs. Such vast differences in morphology between LCVs and SCVs provide some possible clues in solving the puzzling phenomena of both the variable Gram stain reaction of C. burnetii and the
relationship between the two cell variants. *C. burnetii* is generally regarded as gram negative (29), but under certain conditions of staining, it can be shown to be gram positive, as demonstrated in this and a previous study (21). Biochemically, muramic acid (35) and diaminopimelic acid (41) have been detected in the cells of *C. burnetii*, although the concentration of diaminopimelic acid was lower than that reported for gram-negative bacteria (41). Lipopolysaccharide has been isolated from *C. burnetii* and shares properties with that of gram-negative bacteria (5, 6). The structure of the cell wall, the composition and possession of the muramic and diaminopimelic acid components, and the presence of lipopolysaccharide suggest that the cell walls of *C. burnetii* resemble those of gram-negative bacteria. Different views, however, have been expressed by other investigators. Nermut et al. (43) observed that the peptidoglycan layer could only be removed from the outer layer by prolonged treatment with hot trichloroacetic acid. Burton et al. (8) reported a thin, moderately electron-dense intermediate layer associated with the inner surface of the outer membrane. This intermediate layer was unaffected by lysozyme and EDTA treatment, which indicated that this structure might not be comparable to that of the peptidoglycan of gram-negative bacteria. We showed that the SCVs possess
a complex internal cytoplasmic membranous system that appears to consist of several trilaminar membranes. These membranes may explain the findings of Burton et al. (8), who described three instead of two peripheral membranes in some *C. burnetii*. They could not determine whether this third or intermediate membrane was equivalent to the cytoplasmic or the outer membrane. In our studies, cells were observed with three peripheral membranes that may represent the intermediate stage between the SCV and LCV during a process consistent with vegetative differentiation (20). Postassium permanganate staining in the SCVs revealed a very dense central nucleoid region, showing a fibrillar network resembling ribonucleoprotein filaments.

In contrast to the SCVs, complex membranous systems were not observed in the LCVs. Structurally, the LCVs was bounded by both the outer and cytoplasmic membranes, which were separated by a periplasmic space. Peptidoglycan was not clearly observed; however, preliminary chemical analysis of the cell wall shows that the LCVs possess quantitatively less peptidoglycan than do the SCVs (K. Amano and J. C. Williams, unpublished data). As reported by others (9, 31, 42, 61), the central nucleoid filaments in most LCVs were more dispersed, and the ribonucleoprotein particles were less concentrated and situated more peripherally than in the SCVs. More important, some LCVs possessed an endogenous structure that was more clearly observed after staining with potassium permanganate. Thus, *C. burnetii* appears to have an additional mode of multiplication in conjunction with transverse binary fission. This additional mode results from unequal cell division and is unique among any obligately intracellular parasite, but it is similar to the bacterial endospore differentiation of gram-positive bacteria (20). The electron-dense body, about 130 to 170 nm in diameter, was surrounded by a well-defined coat of at least four layers. The endospore appeared to develop endogenously in the periplasmic space of one pole of LCVs during unequal cell division (Fig. 3D). The formation of endospores was also observed in both phase I and phase II cells within the phagolysosome of infected tissue culture cells (Fig. 4D).

Endospores (14) are formed under various experimental conditions by members of many genera of bacteria (20), such as *Bacillus* and *Clostridium*, which are widely distributed, especially in soil. An important aspect of the bacterial endospore is the degree of both heat resistance and survival in nature. *C. burnetii* is also unique in that it is more resistant to physical and chemical agents than any other pathogenic rickettsia (45). The heat resistance of *C. burnetii* is considerable. In milk, for instance, *C. burnetii* is resistant for 30 min at 63°C and even for 15 s at 74°C (4). The resistance to cold is even more remarkable, in that *C. burnetii* held at −20°C remained viable for almost 2 years (4). *C. burnetii* has been known to survive on wool, clay, and sand (27, 60), and its ability to persist outside its host has also been shown by isolation from air and dust (16).

Recently, a study was made to test the effect of physical stress on *C. burnetii* (40). Suspensions of organisms were subjected to osmotic shock in water at 45°C followed by sonication at 4°C, incubation at elevated temperatures at 45°C for 3 h, and finally, centrifugation through a 40 to 70% sucrose density gradient at 4°C. Examination of the cells by transmission electron microscopy revealed that the final fraction contained SCVs which were found to be infectious in chicken embryo-cultured eggs, whereas the LCVs were virtually eliminated in the final preparation (40). The final product, however, was shown to retain metabolic activity although at a slower rate than that of the starting material. These data suggest that the SCV of *C. burnetii* retains its viability during exposure to extreme environmental conditions. Thus, the SCV is a heat-resistant relatively dormant structure which has the ability to survive in an adverse environment(s).

Bacterial endospores are also known to have structural characteristics that distinguish them from the corresponding growing or vegetative organisms. The multilayered membranes and the dense layer in the cell wall discriminate the SCVs from the vegetative counterparts, the LCVs. Bacterial endospore differentiation proceeds through well-defined stages which are morphologically identifiable and well documented for *Bacillus* sp. and *Clostridium* sp. (3, 20, 66, 67). However, the sporulation process for *C. burnetii* requires further study before we can describe each phase of sporogenic differentiation.

Dipicolinic acid, a common chemical constituent of most spores (3), was not detected in *C. burnetii* cells. However, some cells of *C. burnetii* were stained acid fast by the Kinyoun carbol-fuchsin method, which detects acid-fast bacilli. Apparent differences in morphology of both the SCVs and LCVs stress the need to establish the identity of chemical components in the cell walls of each cell variant and to correlate such findings to the Gram variability and the acid-fast properties of *C. burnetii*.

Based on the morphology of *C. burnetii* in
unsynchronized cultures, we hypothesize a speculative developmental cycle which consists of vegetative and sporogenic differentiations (Fig. 6). This putative developmental cycle of *C. burnetii* proposes the morphogenesis of spore formation which should be advantageous for the microbe since it would offer considerable survival value in nature. Although both physiological and biochemical factors involved in the formation and germination of *C. burnetii* endospores have not been determined, previous knowledge of the proliferation and metabolism of *C. burnetii* provides some insight into these phenomena. Differentation of the spore to the vegetative state may require the participation of the host eucaryotic cell since *C. burnetii* is known to reside in the phagolysosomal compartment. This compartment is a hostile environment in which the microbe is exposed to low pH (44), hydrolytic enzymes (48), and other...

![Diagram of the putative developmental cycle of *C. burnetii* within the phagolysosome of eucaryotic cells.](http://jb.asm.org/)

Fig. 6. Schematic of the putative developmental cycle of *C. burnetii* within the phagolysosome of eucaryotic cells. (1) The developmental cycle may be initiated coincident with engulfment of the spore or SCV by a phagocytic cell. Upon entry of the SCV into the phagolysosome, the acid pH of the phagolysosome may activate generalized metabolism of *C. burnetii* (24, 25, 40). (2) The SCV may undergo multiplication by transverse binary fission. (3 and 4) Alternatively, the SCV may differentiate to the vegetative cell variant. Changes initiated by pH, enzymatic systems, and/or nutritional status within the phagolysosome may be the triggering mechanisms that induce vegetative differentiation. At this stage, the multilayered membranes should become less visible, and the dense nucleoid may begin to disperse. (5) Further differentiation of the LCV may proceed to a transverse binary division stage. (6) Alternatively, the division stage may coincide with sporogenic differentiation, resulting in unequal cell division. Changes initiated during the progressive infection of the eucaryotic cell may signal the LCV to undergo sporogenesis. Indeed, the eucaryotic cell becomes filled with the phagolysosome containing large numbers of *C. burnetii* in different stages of development (see Fig. 2A). (7, 8 and 9). Putative sequences during the sporogenic differentiation must include the polar development of the spore ranging from 130 to 170 nm in diameter. (10) Release of the spore from the LCV may occur upon lysis of the LCV. Release of the spore from the LCV may lead to the maturation of the SCV.
microbicidal mechanisms (22). The signals required to induce differentiation must therefore initiate in the phagolysosome of the host cell. The function of the SCV or spore, due to the thick periplasmic wall, is perhaps to survive in the phagolysosome and in an extracellular environment during aerosol dissemination.

Further, proliferation and multiplication of C. burnetii in the phagolysosome may involve the pH activation of protein and DNA and RNA biosynthesis (24, 25). Indeed, Hackstadt and Williams (24) have shown that phagosome-lysosome fusion is required to generate conditions favorable to C. burnetii multiplication. Growth of C. burnetii in the phagolysosome of the host cell may inevitably lead to a depletion of essential nutrients or an increase in the pH within the vacuoles. Thus, an alteration in the nutritional status and pH of the phagolysosome may bring about an induction of endospore formation. Both the primary induction of synthesis for sporogenesis and initiation of cellular division by binary fission appear to be independently triggered since dividing cells can undergo sporogenesis (Fig. 3). However, binary transverse fission appears to be a common feature for both cell variants. In Escherichia coli, a large cell that initiates DNA replication will proceed faster to division than cells that initiate at a small size (17). Since the two cell variants of C. burnetii show obvious size differences, the LCV is more likely to undergo binary fission than the SCV.

Interesting parallels in ultrastructure and developmental cycle may be drawn between the genera Chlamydia and Coxiella. However, the following comparison clearly delineates the marked differences which exist between these genera. (i) These intracellular rickettsiae occupy different compartments within the eucaryotic cell; Chlamydia organisms carry out their developmental cycle in the phagosome (18), whereas C. burnetii growth occurs only in the phagolysosome (24). Indeed, the metabolism of exogenous substrates by C. burnetii is activated at acid pH, whereas Chlamydia spp. metabolize substrate at neutral pH (24, 25). (ii) Morphological heterogeneity of both genera has been demonstrated in situ in eucaryotic cells and in purified preparations (1, 7, 8, 10–13, 18, 26, 31–36, 40, 42, 49, 51). Chlamydia spp. and C. burnetii show a range of particle sizes with varying degrees of osmotic stability and infectivity. Only the elementary body of Chlamydia organisms is infectious (57), whereas for C. burnetii, the LCV, SCV, and filter-passing particles are infectious (33, 40, 61). (iii) Ultrastructural evidence suggests that these rickettsiae are quite similar; however, these microorganisms are, in fact, quite different. Both genera exhibit extreme pleomorphism during growth in their respective intracellular compartments (10–13, 33–36, 51). More important, Chlamydia organisms lack a well-defined peptidoglycan (10, 19), whereas Coxiella organisms possess a clearly distinguishable peptidoglycan, as evidenced by chemical analysis of typical bacterial cell walls (29). Indeed, preliminary fractionation of cell walls and chemical analysis suggest to us that the SCVs possess a more complete peptidoglycan than do the LCVs (Amano and Williams, unpublished data). Detailed ultrastructural studies indicate that Chlamydia spp. are propagated within phagocytic vacuoles by means of a biphasic developmental cycle which consists of the transition of infecting elementary bodies to reticulate bodies dividing by binary transverse fission, without apparent septation, followed by nuclear condensation to form the infectious elementary body (10–13, 18, 49). In comparison, some investigators have suggested that the genus Coxiella has a complex developmental cycle (33–36, 61) in which two cell types represent separate stages similar to those of Chlamydia spp. (51). We have illustrated a putative developmental cycle of C. burnetii, showing a progression through both vegetative and sporogenic differentiations characterized by binary transverse division, with septation, and unequal cell division during endospore formation. Thus, there was no evidence for “condensation” of the LCV or reorganization of the nucleoid region with the cell that would represent the differentiation of a reticulate body to an elementary body of Chlamydia sp. Litwin (38) and Litwin et al. (39) have discussed the possible occurrence of endospore formation in Chlamydia spp. and contrasted this aberrant developmental cycle to the formation of bacterial spores. An interesting parallel might be drawn if the question of the developmental cycle of Chlamydia sp. were studied again, employing the techniques used in our study, especially potassium permanganate as a membrane stain. The present data, however, indicate marked differences in the ultrastructures and developmental cycle of C. burnetii and Chlamydia spp.

The discovery of sporogenic differentiation by C. burnetii should facilitate future studies on the pathogenicity of this highly infectious and resistant bacterium. Physiological and biochemical factors involved in the formation and germination of endospores are currently being evaluated.

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