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ELECTRON MICROSCOPY OF *COXIELLA BURNETII* IN TISSUE CULTURE. INDUCTION OF CELL TYPES AS PRODUCTS OF DEVELOPMENTAL CYCLE

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Summary. - An *in vitro* ultrastructural study was carried out on tissue cultures (J774, murine macrophage-like tumour cell line, and BHK-21, baby hamster kidney cell line) persistently infected with *C. burnetii* to investigate whether the events of cellular differentiation could be visualized. At a given stage of the developmental cycle, a proportion of the cells within the affected phagolysosomes clearly underwent cellular differentiation. The cells initially showed asymmetrical septation, the primary stage of cellular differentiation, and ended with the formation of the differentiated product, a precursor to the small cell. The results verified our initial observation that the events occurring during growth in a phagolysosome represent stages of a complex developmental cycle consisting not only of i) vegetative growth by typical transverse binary fission, but also ii) cellular differentiation.

Key words: Coxiella burnetii; tissue culture; developmental cycle; sporogenesis; electron microscopy

Introduction

Coxiella burnetii the etiological agent of Q fever, is an obligate intracellular bacterial pathogen of eukaryotic cells, and an acidophilic microorganism (Hackstadt & Williams, 1981), that multiplies in the phagolysosome of host cells. The events that occur during growth in a phagolysosome are consistent with a typical bacterial growth cycle and cellular differentiation, both of which are characteristics of the *C. burnetii* developmental cycle (McCaul and Williams, 1981; McCaul *et al.*, 1990*a*). Ultrastructural studies of *C. burnetii* have

provided evidence of i) symmetrical (binary) cell division which was identified as constriction of the equatorial region with the concomitant appearance of two nucleoids giving rise to separate daughter cells with equivalent size and fate (Anacker *et al.*, 1964; Burton *et al.*, 1975; McCaul and Williams, 1981; Nermut *et al.*, 1968, 1972), and ii) differentiation process involving asymmetrical septate formation and segregation of cytoplasmic DNA with one daughter cell becoming a functionally differentiated cell and the other a lysed mother cell (McCaul and Williams, 1981; McCaul *et al.*, 1990*a*). The formation of sporelike forms have been identified in i) renografin-purified *C. burnetii* from yolk sac of embryonated eggs (McCaul and Williams, 1981; McCaul *et al.*, 1981; McCaul *et al.*, 1990*a*), ii) sucrose density gradient purified *C. burnetii* cells from infected cell cultures (Aitken *et al.*, 1987; Schaal *et al.*, 1987; Schmeer *et al.*, 1987), and iii) *C. burnetii* cells naturally released from infected culture cells, and are independent of phase variation (McCaul *et al.*, 1990*a*). Recent improvements in the electron microscopic procedures revealed, in *C. burnetii* cells, that the differentiation process involved, as demonstrated in Figure 2, i) a primary septum (S1), ii) layers of membrane-like material that cicrumscribed the developing cell, iii) formation of a dense band (peptidoglycan layer), and iv) a secondary septum (S2) (McCaul and Williams, 1990*a*). Since these observations were recorded from either purified preparations, or cells that were naturally released from infected culture cells, a more detailed study of the events of cellular differentiation require an *in vitro* ultrastructural examination of *C. burnetii* cells within the affected host phagolysosomes. We were able to show using eukaryotic cell cultures (J774, murine macrophage-like tumour cell line, and BHK-21, baby hamster kidney cell line) that, at a given stage of the developmental cycle, a proportion of the *C. burnetii* cells within the affected phagolysosomes clearly underwent cellular differentiation.

Materials and Methods

Bacterial strains. Coxiella burnetii (9 Mile phase I - clone 7, and RSA514 phase I) was purified from infected and antibiotic-free, hen egg yolk sacs by isopycnic Renografin gradient centrifugation (Williams *et al.*, 1981). Purified preparations were propagated further in both baby hamster kidney (BHK-21) fibroblast cultures, and murine macrophage-like tumour (J774) cell line.

Infection of BHK-21 and J774 cells with C. burnetii. The BHK-21 cells were suitable for examining naturally released cells whereas the macrophage cell line was chosen for examining the *C. burnetii* cells within the phagolysosomes. The procedure of infecting the cells with *C. burnetii* and harvesting the released bacteria was carried out according to the protocol outlined by Zuerner and Thompson (1983) with some modifications (McCaul *et al.*, 1990*a*). The BHK-21 cells were grown in RPMI 1640 medium prior to inoculation with *C. burnetii* whereas J774 cells were grown in P-25 medium (pH 7.4) prior to inoculation. After absorption of bacteria onto the cells, the medium for the BHK-21 cells was changed to DMEM containing glutamate (0.584 $g.l^{-1}$), and 10 % (v/v) newborn calf serum. For the J774 cells, the medium was replaced with fresh P-25, (pH 7.4), containing 5 % newborn calf serum. The infected BHK-21, and J774 cells were cultivated and passaged 3 to 6 times (>20 days) before a persistent infection was established.

Coxiella burnetii cells that were naturally released from infected BHK-21 cells were harvested by replacing the medium with DMEM, pH 4.5, containing both glutamate and glucose (0.5 mM each). On the 1st day, the culture medium was decanted into an ultracentrifuge tube and prepared for electron microscopy. The J774 cells containing infected phagolysosomes were also subjected to electron microscopy.

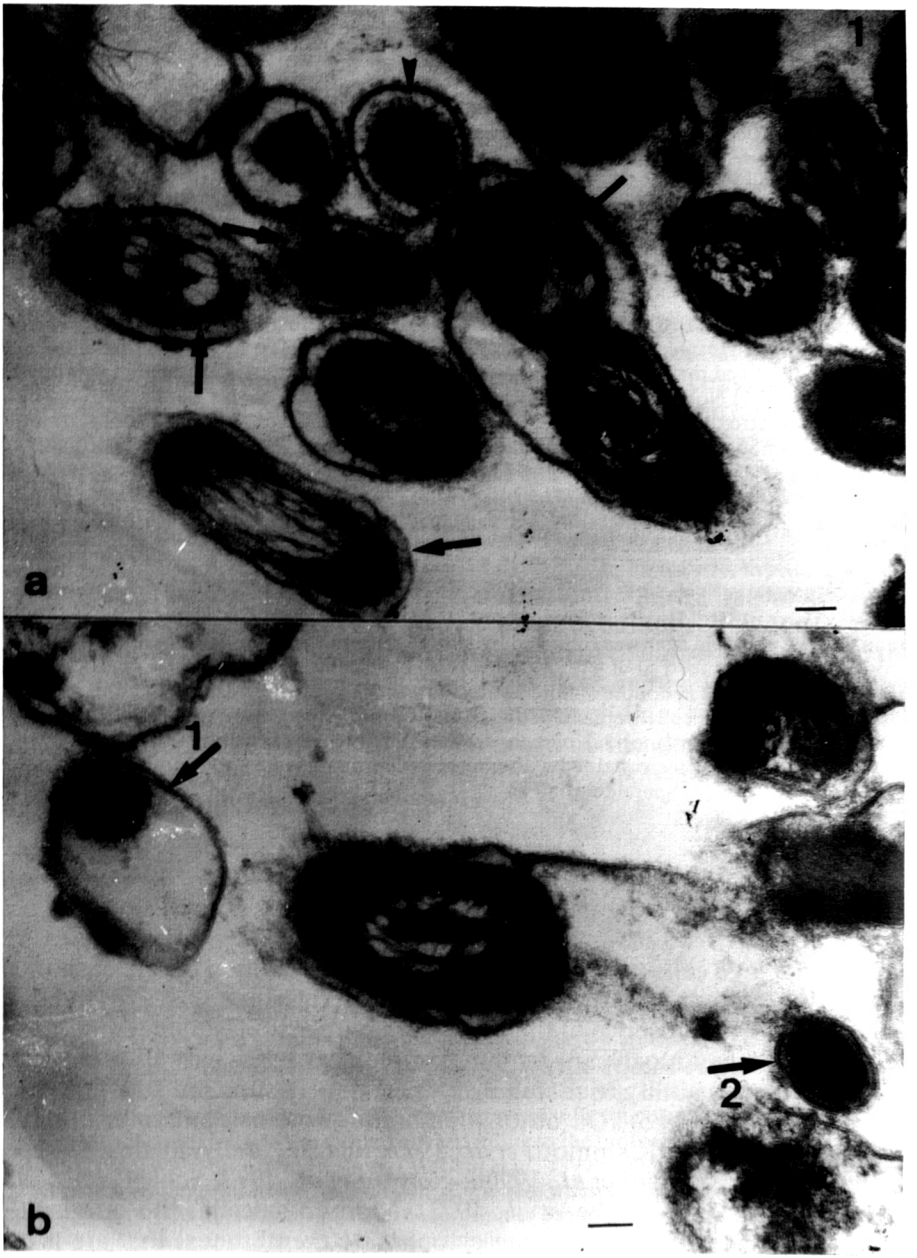
Electron microscopy: Naturally released C. burnetii cells. Culture medium was used as a vehicle for buffering the fixatives (McCaul *et al.,* 1990*a*) to lessen drastic changes in osmolarity, that may cause disruption of the fragile large cells, especially in the extracellular environment. This procedure also has shown to greatly enhance the morphological integrity of the membranous structures in the naturally released *C. burnetii* (McCaul *et al.,* 1990*a*). Glutaraldehyde as fixative (final concentration, 3 % v/v) was added into the centrifuge tube containing the decanted DMEM medium and harvested bacteria. The tube was left at 22 °C for 3 hr and then transferred to an ice-bath and incubated at 4 °C for an additional 1 hr before centrifugation at 53 000 x g at 4 °C. The pellet was suspended in DMEM without serum. The *C. burnetii* cells were centrifuged (17 000 x g) in ultracentrifuge adaptors (Ernest F. Fullam, Inc., Latham, New York, U.S.A.) that allowed the collection of the fixed material onto the bottom of the conical capsules. The pellet was pre-embedded in 2 % (w/v) Noble agar. After a brief rinse in DMEM, the blocks were post-fixed in 1 % (V-V) osmium tetroxide in DMEM for 1 hr at 4 °C. Two washes in 66 mM cacodylate buffer containing 370 mM sucrose were followed by dehydration through serial dilutions of methanol. The blocks were stained for 1 hr at room temperature with 0.25 % (w/v) uranyl acetate in 30 % (v/v) methanol during dehydration. The block were embedded in Spurr epoxy resin. Ultrathin sections were stained with potassium permanganate and examined in a Joel 100CX electron microscope operated at 80 kV.

Intact C. burnetii cells within the phagolysosomes. The cells were very carefully loosened from the flasks either by gently rocking the flasks or scraped with a policeman before fixing overnight at 4 °C in 3 % (v/v) glutaraldehyde in 66 mM cacodylate buffer, 2.5 mM $CaCl_2$ (pH 7.9). Pre-embedding in 2 % (w/v) Noble agar was followed by a brief rinse in the same buffer containing 0.37 M sucrose. Post-fixation for 1 hr at 4 °C with 1 % (v/v) osmium tetroxide in 66 mM cacodylate was followed by two washes in double distilled water. The blocks were dehydrated through serial dilutions of methanol. The cells were stained for 1 hr at room temperature with 0.5 % (w/v) uranyl acetate in 10 % (v/v) methanol during dehydration. The blocks were embedded in Spurr epoxy resin. Ultrathin sections were stained with potassium permanganate and examined in a Joel 100 CX electron microscope operated at 80 kV.

Results and Discussion

Naturally-released cells

Small and large cells of *C. burnetii* are generally identified on the basis of the i) dimension, ii) density of the nucleoid and the periplasmic space, iii) thickness of the cell wall, iv) presence of the multi-laminate cytoplasmic membranes, v) quantity of the peptidoglycan-protein complex, vi) sensitivity to osmotic and pressure lysis, vii) ability to metabolize exogenously supplied substrates, and viii) presence of a 29.5-kDa outer membrane protein (Amano *et al.,* 1984; Canonico *et al.,* 1972; Kishimoto *et al.,* 1977; McCaul and Williams, 1981; Mc Caul *et al.,* 1981; McCaul *et al.,* 1990b; Nermut *et al.,* 1972; Schaal *et al.,* 1987; Wachter *et al.,* 1975; Weibe *et al.,* 1972). Morphologically, the intracellular membranes (or the multi-laminate cytoplasmic membranes) that are prominent in the small cell, are noticeably absent in the large cells (McCaul and Williams, 1981; McCaul *et al.,* 1981; Schaal *et al.,* 1987). However, sensitivity of



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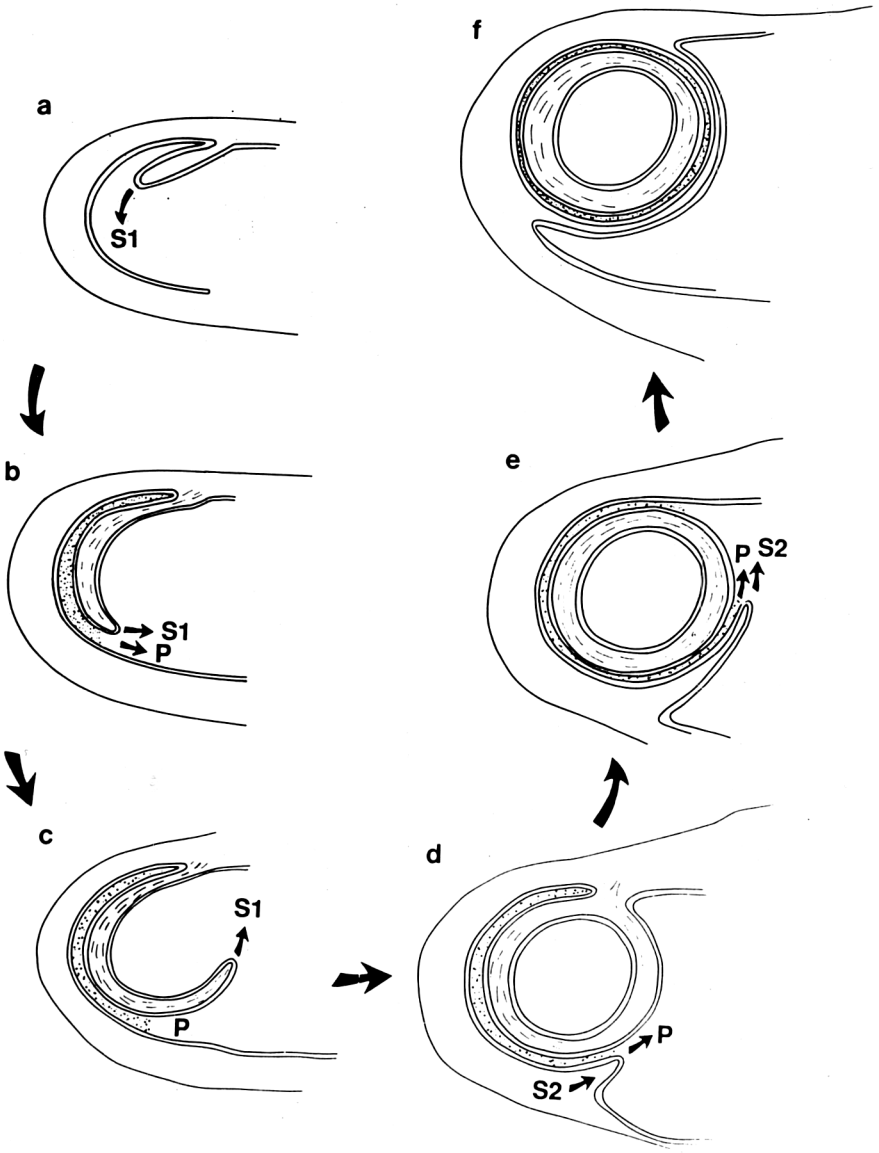

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the large cells to conventional fixatives especially after prolonged purification procedures may cause a dissolution to some degree of the intracellular membranes. An addition of fixatives to the culture medium containing the naturraly-released *C. burnetii* has shown an improvement in the retention of the intracellular membranes, especially in the large cells (Fig. 1a) (McCaul *et al.*, 1990*a*). Such an improvement in the integrity of the membrane structures provides an important clue to the structure and function of the intracellular membranes. The membranes are the important component of the primary septum (McCaul *et al.*, 1990*a*). (Fig. 2) that is formed during the formation of the differentiating cell in the polar region of the mother (large) cell. The membranes therefore play a crucial and important role in forming the cell wall of the differentiating cell (McCaul *et al.*, 1990*a*) (Figs. 1a, 3b), although not all the large cells carry the intracellular membranes. The morphology of the differentiating cell has been recently characterized (McCaul *et al.*, 1990*a*). At the final stage of the development, the membrane-like layers completely circumscribed the dense core (Fig. 3b), which has been shown to contain DNA using monoclonal antibody against single- and double- stranded DNA and colloidal gold (McCaul *et al.*, 1990*a*; McCaul and Williams, 1990). The membrane-like layers are surrounded by an electron-dense peptidoglycan, and a single trilaminar outer membrane (Fig. 3b). These membrane-like layers in terms of position correspond to the intracellular membranes commonly seen in the small cells (Fig. 3a). The differentiated cell was apparently the precursor to the small cell (McCaul *et al.*, 1990*a*). The ultrastrutural similarities between the small cells and the differentiated cell clearly verified the existence of a developmental cycle in *C. burnetii.*

Fig. 1

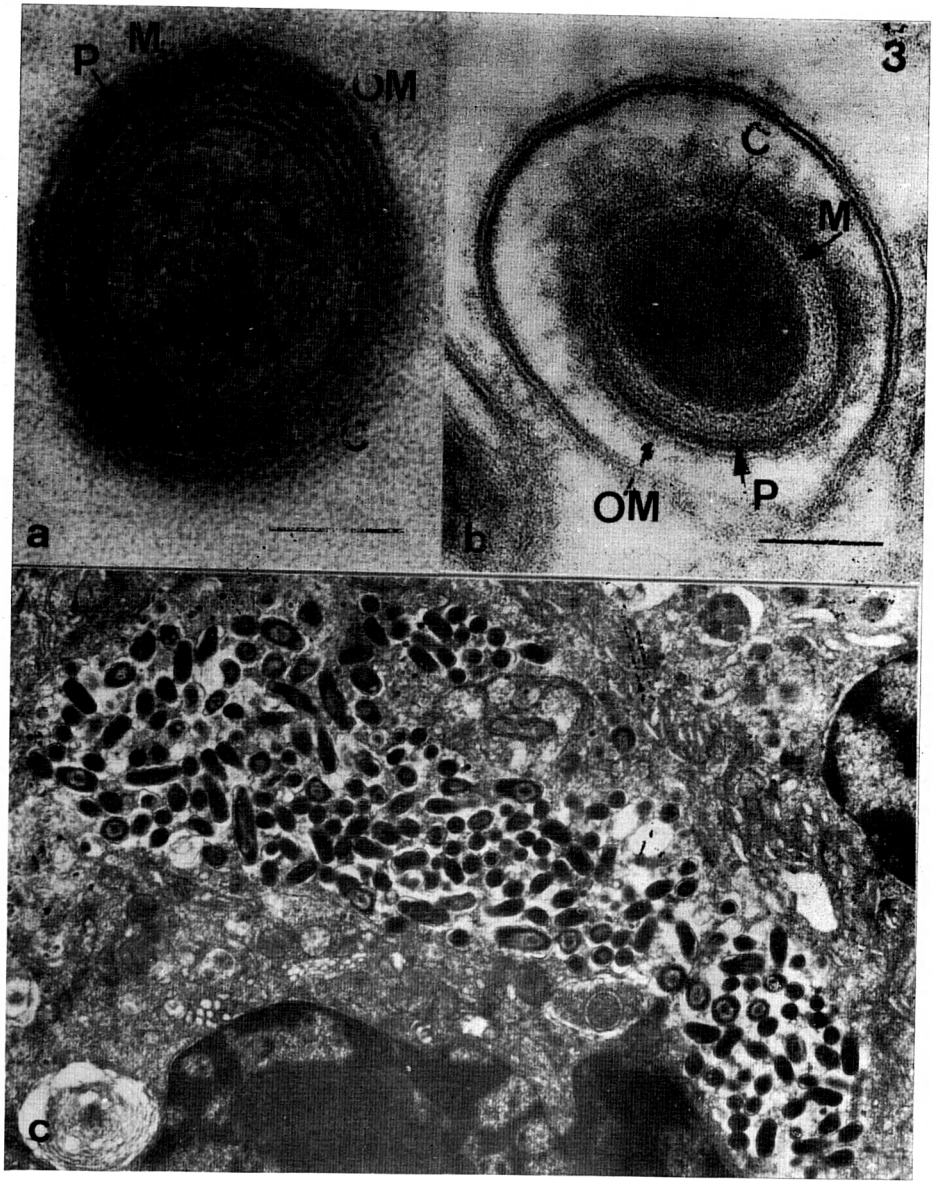
Naturally-released *Coxiela burnetii* cells from infected BHK-21 cells

a) The addition of primary fixative into the culture medium preserves the integrity of the membranous structures of the large cells (arrows). The membranous structures constitute the cell wall of the differentiating cell (arrowhead). B) The differentiated product is retained within the periplasmic space by the mother cell's outer membrane integument (arrow # 1), and is only released on lysis of the mother outer membrane (arrow # 2). Bars=100 nm.

Fig. 2

Diagram of morphological changes occurring during the formation of the small cell in *Coxiella burnetii* as seen in purified preparations and naturally-released cells from infected culture cells

a) The primary asymmetrical septum (S1) appears as an invagination at the polar region of the cell; b) The S1 curves along the cytoplasmic face of the cytoplasmic membrane of the mother cell. Simultaneously the peptidoglycan (p) is synthesized; c) After rouding the pole, the S1 then penetrates into the cytoplasm; d) As S1 rejoins the original initation site, where it fuses directly with the cytoplasmic membrane of the mother cell, a second septum (S2) is formed at the opposite site of the initiation site of the S1; e) The peptidoglycan is synthesized as the S2 continues to migrate towards the initiation sit of the S1; f) The S2 has completed its migration, and the differentiating cell is now separated from the mother cell cytoplasm, although it is still within the mother cell envelope.



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The differntiated cells were generally loosely retained in the periplasmic space by the mother cell's outer membrane integument (Fig. 1b). However, the mother cell undergoes disintegration. During the later stages of cellular differentiation, the loss of morphological integrity of the mother cell has been shown to correlate with the disintegration of the mother cell's nucleoid DNA (McCaul and Williams, 1990). The sole function of the mother cell is therefore to produce the small cell to survive extracellularly, and to guarantee preservation of the DNA for future propagation. On lysis of the mother cell, the differentiated cell was finally released (Fig. 1b).

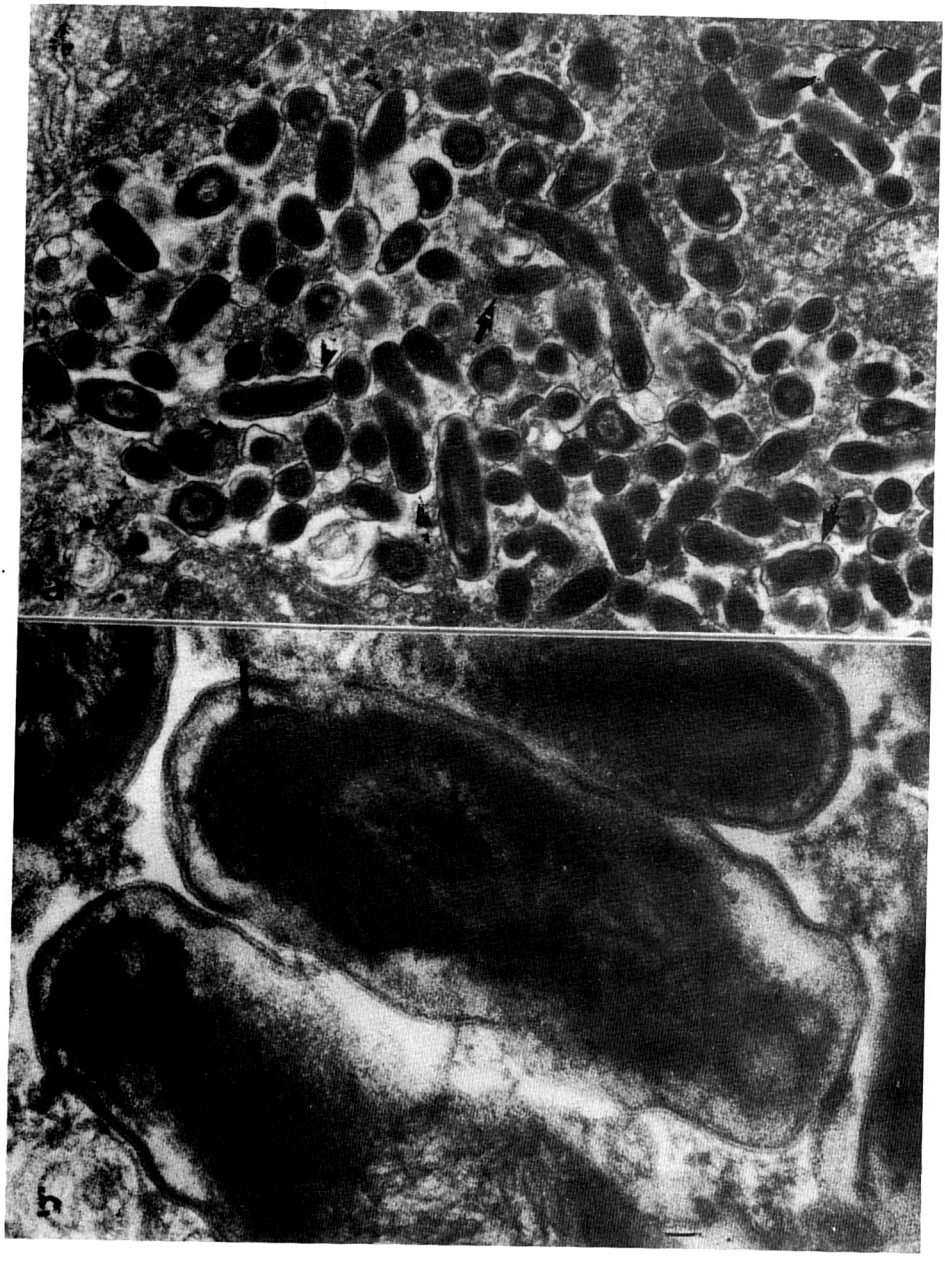
Intraphagolysosomal C. burnetii cells

A proportion (10–20 % in the plane of the thin section) of the *C. burnetii* cells within a phagolysosome may undergo cellular differentiation (Figs. 3c, 4a). The events of cellular differentiation recorded from previous studies using naturally-released cells and purified preparations (Mc Caul *et al.*, 1990*a*) are depicted in Fig. 2. Two structural features, the primary septum (S1), and the seondary septum (S2), are involved in the process of cellular differentiation. The structure of the asymmetrical septum S1 septum is clearly separate and apart from the septum observed in symmetrical transverse binary division (McCaul and William, 1990a). The S1 (Fig. 4b) curves along the cytoplasmic face of the cytoplasmic membrane of the mother cell. This was also seen in the phagolysosome (Fig. 4b). An electron-dense region observed between the outer face of the S1 and the cytoplasmic face of the cytoplasmic membrane of the mother cell in naturally released cells and in purified preparations may represent the peptidoglycan-protein complex of the small cell. Such an electron-dense region could also be seen in cells within the phagolysosome (Fig. 5a). As shown in Fig. 2, the S1 migrated around the pole of the cell and then invaginated and penetrated into the cytoplam. This was also seen *invitro* (Fig. 5a). The completion of the engulfment process by the S1 led to the formation of a spherical structure that was still surrounded by the mother cell cytoplasm (Fig. 5a). A secondary septum is therefore involved in separating the differentiating cell from the mother cell cytoplasm (Fig. 2). The result of separating the differentiated cell from the mother cell cytoplasm is demonstrated in Fig. 5b.

The events occurring during growth of *C. burnetii* in a phagolysosome represent stages of a complex developmental cycle consisting not only of vegetative growth by typical transverse binary fission, but also cellular differentiation.

Fig. 3

A) and B) Comparison in morphology between a) resistant and mature small cell, which was obtained from renografin-purified *C. burnetii* from infected yolk sacs, and b) differentiating cell spore within the mother cell. P=Peptidoglycan; M=Intracellular membranes; C=Core; OM=Outer membrane. C) Phagolysosome of J774 cells showing the proliferation of *C. burnetii* cells. Bars = 50 nm (a,b); 1 μm (c).



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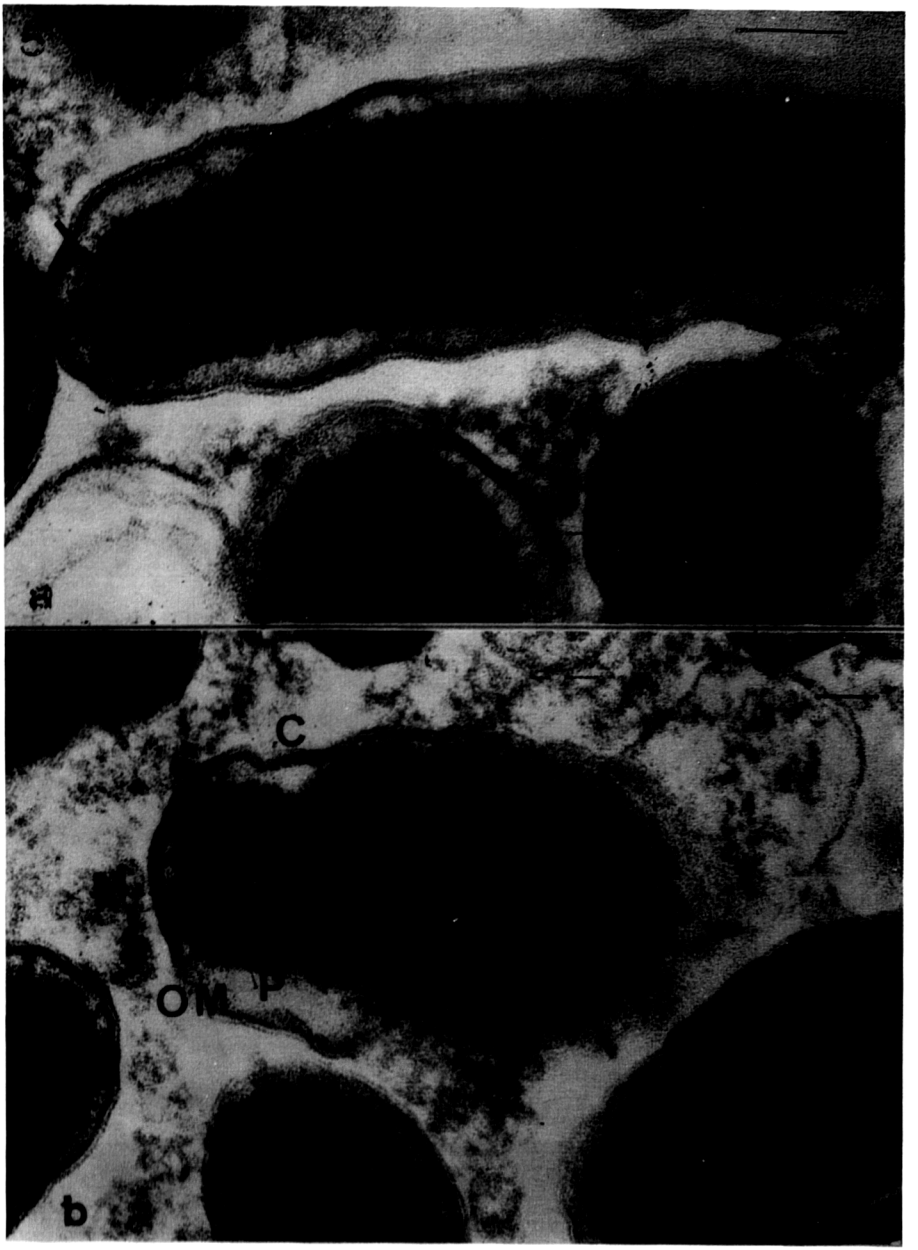

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The next question that needs to be answered is what are the determinative host factors that induce cellular differentiation? At the present time, the physiological and biochemical factors that induce sporogenic cellular differentiation in *C. burnetii* are unknown. The regulatory signals and genetic mechanisms of initiation of cellular differentiation are also unknown. However, an environment poor in nutrients is commonly one of several biological responses of the spore formers that leads to sporulation (Sudo and Dworkin, 1973). Since there was no morphological indication of overt cellular degradation within the cytoplasm of the host cell beyond the affected phagolysosomes, the signals that induce cellular differentiation may have originated specifically within the phagolysosomes themselves. Since phagosome-lysosome fusion is required to generate conditions favourable to *C. burnetii* replication (Hackstadt and Williams, 1981), the continuous growth of the microorganism may inevitably lead to a depletion of essential nutrient or an increase in the pH within the vacuoles. An alteration in the nutritional status and pH of the phagolysosome may therefore bring about an induction of cellular differentiation.

In conclusion, the observations within the affected phagolysosomes clearly verified our initial observation that the developmental cycle of *C. burnetii* consists of cellular differentiation in addition to vegetative growth by typical transverse binary division.

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Fig. 4

A) Higher magnification of Fig. 3c, showing spontaneous initiation of cellular differentiation (arrows) in *Coxiella burnetii* cells within the phagolysosome of an infected J774 cell b) *C. burnetii* cell showing the involvement of primary septum (S1) (arrow) during the initial stage of cellular differentiation. Bars=500 nm (a); 100 nm (b)

Fig. 5

Coxiella burnetii cells in the phagolysosome of an infected J774 cell

a) The primary septum (S1) (arrow) has terminated its migration during differentiation. b) The differentiated cell on completion of cellular differentiation, although still retained within the confinements of the mother cell, harboured structural features of the small cell. C=Core; M= Intracellular membranes; OM=Outer membrane; P=Peptidoglycan. Bars=100 nm.

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