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Three-Dimensional Reconstruction of *Coxiella burnetii*-Infected L929 Cells by High-Voltage Electron Microscopy

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Previous examination of thin sections of L929 cells heavily infected with the Q fever Priscilla isolate by conventional transmission electron microscopy indicated that the rickettsiae resided within multiple vacuoles. The present study using high-voltage electron microscopy and three-dimensional reconstruction revealed that, in heavily infected cells, the rickettsiae, in fact, reside in one multilobed vacuole. As a result of asymmetric cell division, the multilobed vacuole containing the rickettsiae apparently segregates into one daughter cell, while the companion daughter cell emerges parasite free. This likely explains the appearance of naive uninfected cells in long-term-infected (i.e., ca. 2 years) cell populations that had not been supplemented with uninfected L929 host cells.

Coxiella burnetii is an obligate intraphagolysosomal rickettsial pathogen of eucaryotic cells which causes both acute Q fever and chronic Q fever endocarditis in humans (reviewed in reference 1). In vivo, *C. burnetii* infects a variety of cells, including macrophages, hepatocytes, epithelial, connective-tissue cells, and other unspecified strand cells of lymphoid and hematopoietic tissues. Investigations of the causative mechanisms of the chronicity of Q fever have shown, by restriction endonuclease mapping of *C. burnetii* plasmids, a possible correlation between the presence of certain plasmid types in the rickettsiae and the disease entity (4). Studies of the Q fever pathobiology in model eucaryotic cell systems (1) have also shown that persistent infection may last for years in tissue cultures without the need to replenish the culture with naive eucaryotic host cells. Furthermore, the apparent number of vacuoles per rickettsia-containing infected host cell resulting from long-term infection differed from one strain to the other. It was observed (6) that a number of cells heavily infected with the acute-phase Nine Mile isolate (QpH-1) exhibited one large vacuole containing the rickettsiae (Fig. 1A). In contrast, infection with the Priscilla isolate of *C. burnetii* (QpRS), a representative of the chronic endocarditis-type strains, resulted in heavily infected cells exhibiting (by conventional transmission electron microscopy) several vacuoles containing the rickettsiae (Fig. 1B) (7). Because of the long-term infection of the cell cultures with the Nine Mile isolate and the presence of one vacuole in a number of infected cells, Roman et al. (6) concluded that heavily infected host cells are capable of undergoing what they termed asymmetric division. The infected cell undergoes mitosis and cytokinesis, resulting in an infected daughter cell, while the companion daughter cell emerges parasite free. The proposed model of asymmetric division explained, in part, the emergence of naive cells in long-term-infected cell cultures. The infected populations generated uninfected cells susceptible to infection, which explains why it is unnecessary to add uninfected cells to keep the population from dying out. However, this model could not be readily applied to cells heavily infected with the Priscilla isolate because they apparently contained more than one heavily infected vacuole (7). No satisfactory explanation was available to explain long-term infection of host cells with the Priscilla isolate, even though infected populations were observed to fluctuate between 100% and partial infection (7). This implied that there was production of uninfected cells as was observed in Nine Mile isolate-infected cultures.

We have examined the vacuolation of L929-infected fibroblast cells with the Priscilla isolate by high-voltage electron microscopy of serial thick sections and computer-generated three-dimensional reconstructions (5). With this approach, we were able to resolve the issue of the number of vacuoles in the eucaryotic host cell heavily infected with the Priscilla isolate and hence explain, at least in part, why infected cell populations continue to proliferate and remain persistently infected (for years), without requiring the addition of uninfected host cells.

L929 fibroblast cells uninfected and infected with the Priscilla isolate were maintained in static culture for 912 days as described previously (7). Infected and uninfected cells were processed for high-voltage electron microscopy essentially as described before (2). Cultures were fixed in a 2% glutaraldehyde solution for 2 h, washed in buffer, and resuspended in 2% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4). The cells were rinsed in cacodylate buffer, resuspended in 1% osmium tetroxide (in cacodylate buffer) at 25°C for 1 h, and rinsed in cacodylate buffer. To further increase contrast, the cells were suspended in 0.5% uranyl acetate in distilled water at 25°C for 2 h. The cells were then rinsed in distilled water, embedded in 2% agar, dehydrated with a graded series of ethanol, treated with propylene oxide, and embedded in Epon-Araldilt. Trapezoid-shaped sections were prepared with a Reichert Ultracut E microtome (5). One-micrometer sections were collected on Formvar-coated copper slot grids (one section per grid), stained with 2% aqueous uranyl acetate solution at 60°C for 1 h, and further stained with Reynolds lead citrate solution at 25°C for 45 min. The sections were examined at 1 MeV in the high-voltage microscope.

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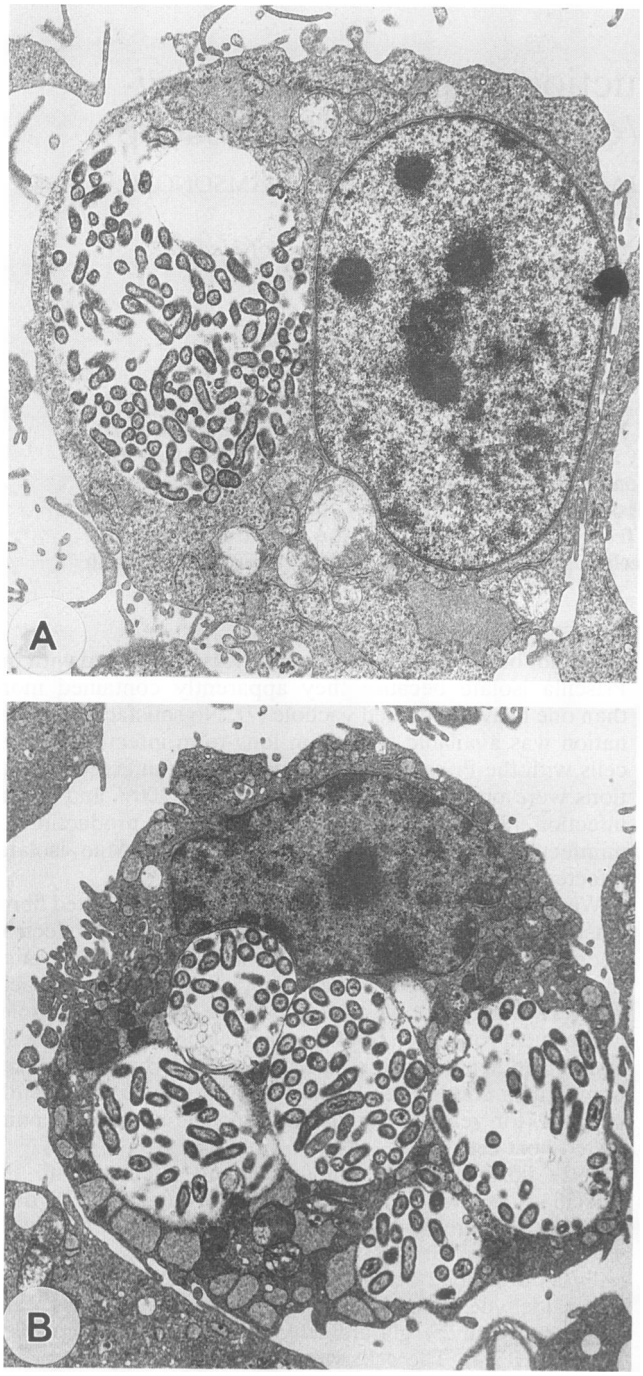

FIG. 1. Conventional transmission electron microscopy of 0.08-μm sections of Nine Mile isolate-infected (A) and Priscilla isolate-infected (B) L929 cells.

Serial sections of 12 cells were examined. Landmarks in the sections (e.g., creases, adjoining cells, and the trapezoid shape of the sections) allowed us to recognize and monitor the various cells for their entire length in adjacent sections. The electron micrograph negatives of infected and naive cells from infected cell cultures and naive cells from noninfected cultures (16 sections from each cell), which encompassed entire cells with average diameters of 16 μm, were further processed for image reconstruction by using STERECON (5). The images on the negatives were digitized by the video camera of the system, stored by the computer, and displayed on the graphics monitor. Each section was displayed on the screen, and color-coded contour lines were then drawn on the cell structures of interest. In this case, the cell membrane (blue), the nuclear membrane (red), the vacuolar membrane (green), and representatives of the rickettsiae (yellow) were traced with the cursor.

Figure 2 represents the high-voltage microscopy and the computer rendition of the three-dimensional reconstruction of the 16 sections. Figure 2A and B are a rendition of the rickettsia-free host cells. In Fig. 2A, the cells were from a noninfected culture. In contrast, in Fig. 2B, the naive cells were from a rickettsia-infected cell culture. The membrane is defined with contour lines, while the nucleus and vacuoles are shaded. The computer rendition showed that the vacuoles of noninfected cells were, for the most part, not interconnected. A few relatively large vacuoles appeared to be interconnected or extending to other sections.

Two sequential thick sections, no. 6 and 7, through a Priscilla isolate-infected cell are represented in Fig. 2C and D, respectively. In section 6, two vacuoles were apparent, while, in section 7, only one vacuole profile was observed. This indicates either that two vacuoles may have merged at this depth of the infected cell or that the depth of one of the vacuoles was less than 1 μm. A computer rendition of the superimposed 16 sections is shown in Fig. 2E. Only the contour lines are depicted, except for the nucleus, which we shaded for contrast enhancement. This representation was the initial indication that the vacuole profiles in the infected cells probably comprised one interconnected vacuole. Figure 2F shows the contour of the cell membrane and shaded contours of the vacuole and nucleus. The presence of only one vacuole indicates that the vacuoles were eventually interconnected to form one large vacuole. A wire frame model of the structure (Fig. 2G) indicates the relative volume of each of the structures. The volumes calculated by the STERECON program for the nucleus in the infected and noninfected cultures were between 19 and 24%, respectively. In contrast, the volume of the vacuoles varied. In the noninfected cells, the volume of the vacuoles, as a percentage of the total cell volume, was 7.8%. In noninfected cells present in the infected cell culture, there was a notable increase in percentage of total volume (13%). However, the largest percentage of total volume of a vacuole was found in the infected cells and was similar for both isolates (22%). Surface rendering of the wire frame model gives an appreciation of the three-dimensional structure of the cell (Fig. 2H). This representation clearly shows that the vacuole is multilobed, and the lobes appear to be interconnected. The presence of multiple vacuoles observed by conventional transmission electron microscopy in cells heavily infected with the Priscilla isolate is apparently a consequence of the angle of sectioning through certain areas of the multilobed vacuole.

In conclusion, these results show that L929 host cells heavily infected with the Priscilla isolate contain the rickettsiae in one giant, multilobed vacuole. This is similar to the findings with the Nine Mile isolate of *C. burnetii* (6), which involves the formation of one heavily infected vacuole in a number of host L929 fibroblasts. In light of these findings, it is likely that the model of asymmetric mitotic division of cells heavily infected with the Nine Mile isolate, which results in an infected and naive daughter cell, proposed by Roman et al. (6), also applies to the Priscilla isolate. The generation of uninfected cells from infected parent cells is apparently the key to the maintenance of persistent infec-

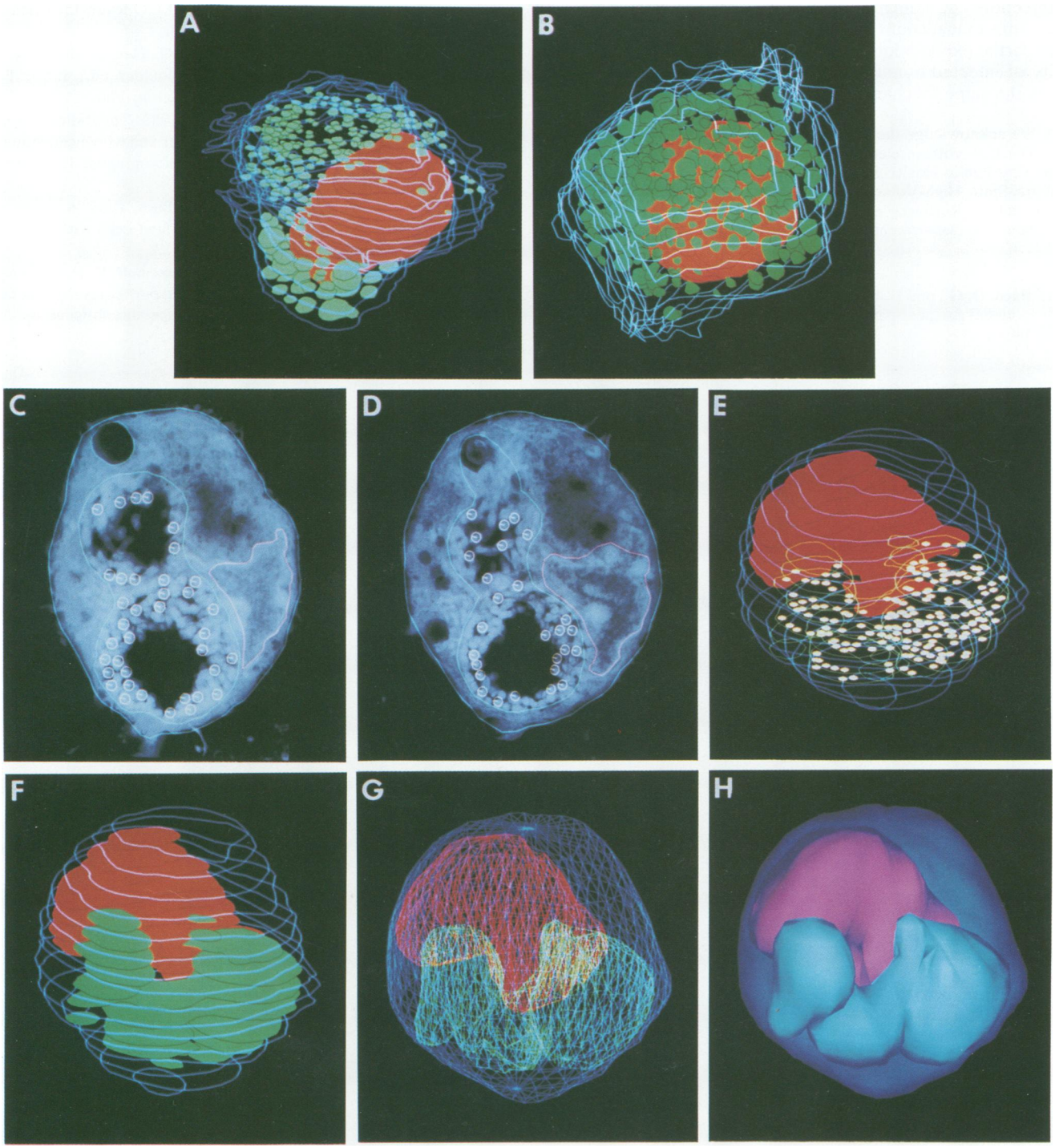

FIG. 2. High-voltage electron microscopy and computer rendition. The blue line represents the outline of the cell membrane, the red line represents the nuclear membrane, the green line represents the vacuole membrane, and the yellow line represents contours of a representative number of rickettsiae. (A and B) Noninfected cells from a rickettsia-free culture (A) and an infected cell culture (B); (C to H) Priscilla isolate-infected cells; (C and D) sections 6 and 7, respectively; (E) contour lines of cell membrane and vacuole (nucleus is color filled and rickettsiae are shown as dots); (F) contour lines of cell membrane (nucleus and vacuole are color filled); (G) wire frame model of the three cell organelles; (H) surface rendering showing that the vacuole is multilobed.

tion. The uninfected cells generated must also be susceptible to infection because, periodically, all of the cells of the population contain rickettsiae (7).

Occasionally, *C. burnetii* causes life-threatening chronic infections in humans. The biological and immunological reasons that account for persistence are not well understood. A partial explanation for this persistence may be the capacity of infected nonmacrophage cells to divide in vivo (1, 3) via the same mechanism described in this communication.

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