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Surface Projections of *Chlamydia psittaci* Elementary Bodies as Revealed by Freeze-Deep-Etching

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The morphology of the surface projections of *Chlamydia psittaci* elementary bodies in the intracytoplasmic inclusion was the same as that of the projections on the purified elementary bodies. Each projection emerged from the center of a flower structure, which was composed of nine leaves arranged radially.

Electron microscopic observations of *Chlamydia psittaci* (meningopneumonitis strain) demonstrated the presence of unique projections on a limited surface area of the elementary bodies (EB) of the organisms (4, 6–8). However, all studies cited were carried out with purified EB, and the question arises as to whether the projections are natural structures or artifacts newly formed during preparation. To avoid this vagueness, a replica of the true outer surface of the EB in an intracytoplasmic inclusion was made by a freeze-deep-etching technique and then examined in detail by electron microscopy.

L-cells in suspension infected at 1 to 2 inclusion-forming units per cell were harvested at 40 h post-inoculation. The cells were washed twice with phosphate-buffered saline, spun down at 300 × *g* for 5 min into a pellet, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) (1) for 60 min in ice. The pellet was divided into small pieces during fixation. After several washings with the buffer solution, the pellet was mounted on a specimen support disk and frozen in liquid Freon 12 at liquid nitrogen temperature. After transfer into liquid nitrogen, the frozen sample was set in a Balzers BAF301 freeze-replica apparatus. No glycerol was used to facilitate the sublimation of ice from the specimen surface after fracturing. The specimen temperature was increased to −95°C and held for 20 or 30 min for deep-etching. To compare the surface morphology of the EB in situ and isolated, the EB were purified by the method of Tamura and Higashi (9), collected into a pellet with centrifugation at 10,000 × *g* for 30 min, and then treated by the same procedure as that for the infected L-cells. The replicas were cleaned in commercial bleaching solution containing sodium hypochlorite, rinsed in distilled water, and then collected on a copper grid without supporting film. The replicas were examined with a Hitachi H-500 or HU-12A electron microscope at 75 kV. For three-dimensional observations of the EB surface, stereo pairs of micrographs were taken at a tilting angle of ±8° and examined stereographically.

Figure 1 illustrates a part of an inclusion in the host cytoplasm. Although the structures of the host cytoplasm were damaged by the formation of ice crystals, one can see many round, convex bodies, ranging from 0.33 to 0.46 μm in diameter. These were regarded as the EB on the basis of their dimensions. Some concavities contain the button structures (B structures), indicating the inside surface of the EB cell walls (3), but many other EB having fractured faces show their true outer surface exposed by the deep-etching. It is to be noted that the EB marked with arrows possess many surface projections which are arrayed hexagonally at a center-to-center spacing of about 50 nm and that the projections are located only on the true outer surfaces. At higher magnification, it was noted that each projection emerged from the center of a flower structure, about 30 nm in diameter, which appeared to be a radial arrangement of several leaves (Fig. 1, inset). An identical morphology was more clearly seen on the purified EB surface when the area containing the projections was viewed stereographically. As seen on the EB in situ, each projection came out from the center of the flower which was located at the base of the projection and was somewhat raised from the EB surface (Fig. 2). To determine the number of flower leaves, the rotation technique (2) was applied to several flowers. Nine leaves were clearly distinguished by rotating nine times, indicating that the flower had nine leaves in a nonagonal arrangement (Fig. 2, inset).

From these results, it is concluded that the projections are not artifacts formed during the preparation, but intrinsic structures, and each projection comes out from the center of the flower, composed of nine leaves. I reported that the rosettes in purified, negative-stained EB cell walls were arrayed in a hexagonal arrangement and that each rosette was a radial arrangement of nine subunits (3). Observations of negatively

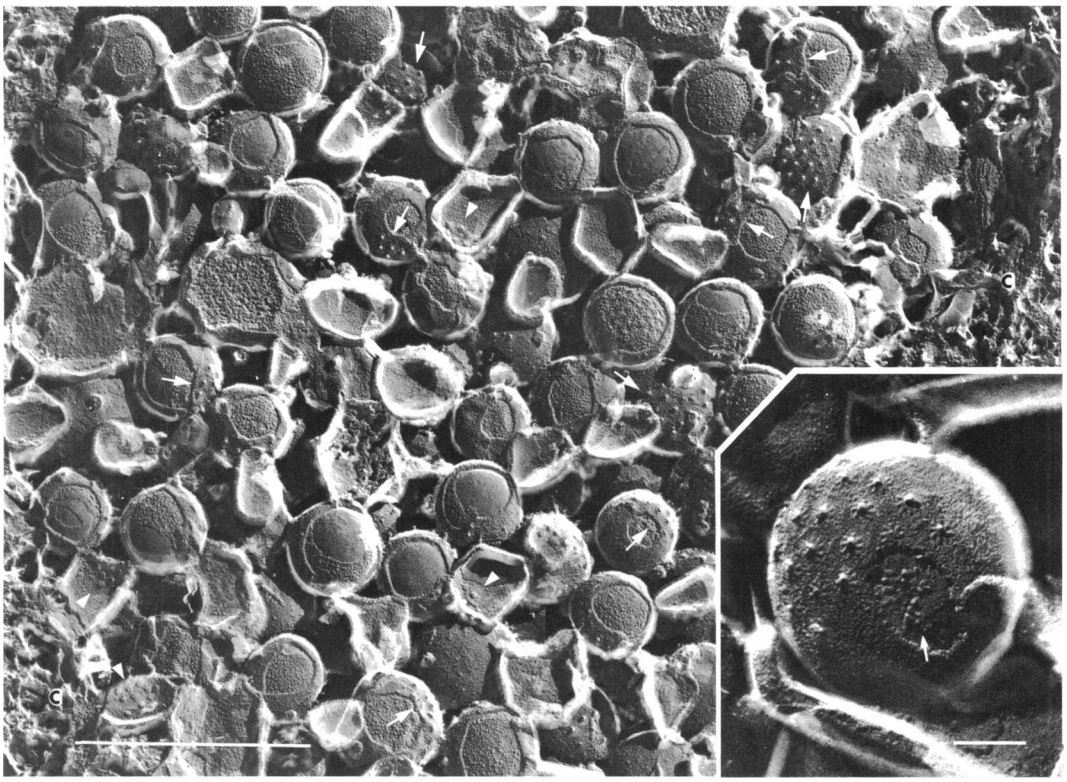

FIG. 1. Part of an inclusion in the host cytoplasm. The true outer surfaces of many EB, as exposed by deep-etching, are seen. EB surfaces having the projections are shown by arrows. No projection is seen on the fractured convex faces. Arrowheads indicate the inside surfaces of EB cell walls containing the B structures. c, Host cytoplasm. Bar, 1 μm. Inset shows the in situ EB surface containing the projections. The arrow points to a fractured area. Each projection comes from the center of a flower structure, which is composed of several leaves. Bar, 100 nm.

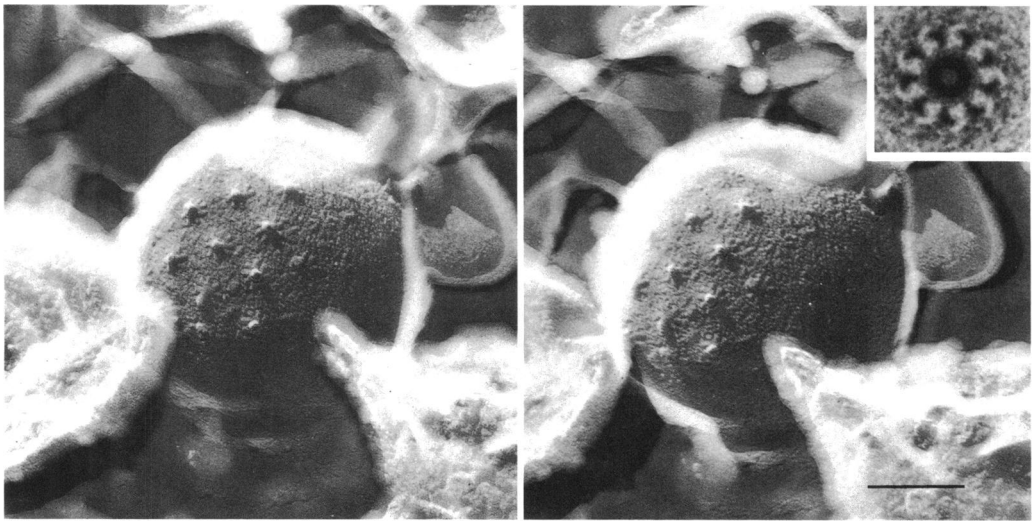

FIG. 2. Stereo pair of purified EB surfaces containing the projections. Stereographic viewing shows more clearly the projections at the center of the flowers. Each flower is raised somewhat from the EB surface. Inset shows an image obtained by rotating nine times, indicating that a flower is composed of nine leaves in a nonagonal arrangement. Bar, 100 nm.

stained cell envelopes (cell wall-cytoplasmic membrane complexes) demonstrated that the rosette was a hole from which one end of each projection protruded beyond the cell wall (6a). Therefore, the flower at the base of each projection shown in the present experiment may be the rosette. Based on observations with complementary freeze-replica and thin-sectioning techniques, Louis et al. (1) presented a diagram of a transversal section of the EB envelope in which the canals were crossing the central granules of craters, which corresponded to the B structures reported by me (3). However, they thought the projections to be structures formed only at a certain physiological stage of the organisms or the results of external conditions. These possibilities seem to be completely excluded by the facts that the EB in the in situ and purified preparations, both fixed with the same fixative as that used by Louis et al., possessed projections of identical morphology, and that the reticulate bodies of the organisms also possessed the projections (5, 6a). Using ruthenium red to enhance the contrast of purified EB in thin sections, I reported that the cytoplasmic membrane sites where one end of each projection was tightly connected were bound to the DNA molecule of the organism through a component sensitive to trypsin (6). I also reported the projections to be cylindrical structures (6, 7). Based on the results obtained from a series of morphological investigations, including the present experiment, it is suggested that the projections may play an important role during multiplication, although the function of the projections is not known.

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