KAWASAKI MED. J. VOL. 8 : NO. 3-4, 149-157 (1982)

Morphology of *Chlamydia psittaci* Elementary Bodies as Revealed by Electron Microscopy

—Dedicated to the memory of the late Professor N. Higashi—

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Accepted for Publication on October 27, 1982

Chlamydiae are obligate intracellular bacteria, which are well known as the causative agents of trachomatis, inclusion conjunctivitis, lymphogranuloma venereum and psittacosis. In the growth cycle of the organisms, there are two morphologically different cell types, infectious elementary bodies (EBs), about 0.3 μm in diameter, and noninfectious reticulate bodies (RBs), 0.5–1 μm in diameter. EBs penetrate a susceptible cell and are converted into RBs, which multiply by binary fission, and then mature to form EBs[1]. This unique growth cycle occurs within intracytoplasmic inclusion bodies limited with a membrane which is derived from the host plasma membrane as EBs are phagocytized. A series of electron microscopic studies of the meningopneumonitis strain of *Chlamydia psittaci* revealed unique morphology of the EBs. Based on the studies, the results are summarized and a schematic diagram of the EB morphology is proposed in this short review.

Surface projections and related intracytoplasmic structure as revealed by thin sectioning

When thin sections of EBs and L cells infected were prepared by the ordinary method for thin sectioning, neither the surface projection nor the cytoplasmic membrane was observed in the EBs. This might be due to their inadequate opacity under the electron microscope. To enhance the opacity of the projections, an additional treatment with tannic acid[2] or ruthenium red[3] was needed during preparation. When the EBs were treated with tannic acid, the projections cylindrical in morphology were clearly seen (Fig. 1). Although tannic acid treatment was highly effective in enhancing the opacity of the projections, other structures in the EBs were also stained strongly. In such preparation, the morphological relationship between the projections and other intracellular structures could not be clearly seen. Therefore, instead of tannic acid, ruthenium red was used[3]. As shown in Fig. 2, the projections were clearly observed due to the fine deposit of the drug. The projections measured approximately 10 nm in diameter and 20 nm in the maximum length, and distributed hexagonally with an approximate center-to-center spacing of 46 nm.

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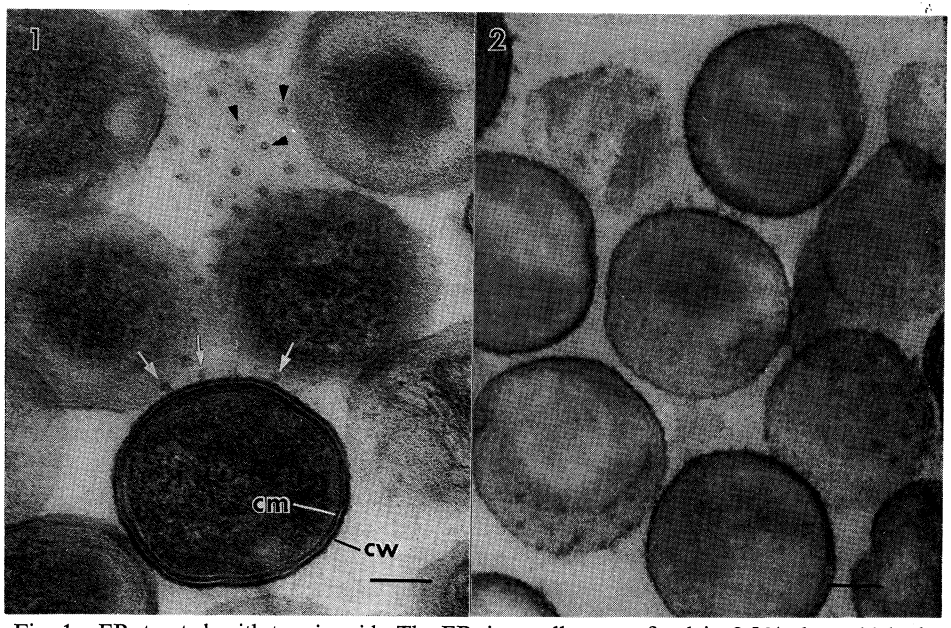

Fig. 1. EBs treated with tannic acid. The EBs in a pellet were fixed in 2.5% glutaraldehyde in0.05M phosphate buffer solution (pH 7.2), treated with 4% tannic acid in distilled water and then fixed with 1% OsO_4 in the phosphate buffer solution. Many projections are seen (arrows). Arrowheads indicate the projections cut crossly. Intracytoplasmic structures, such as nucleus and ribosomes, are not well distinguished, while the cytoplasmic membrane is clearly seen. cm : cytoplasmic membrane, cw : cell wall. Bar indicates 100 nm.

Fig. 2. EBs treated with ruthenium red. The EBs in a pellet were prefixed in a mixed solution of equal volume of 3.6% glutaraldehyde, 0.2M cacodylate buffer and 1.5 mg/ml ruthenium red solutions, washed briefly with 0.15M cacodylate buffer solution and then postfixed in a similar solution with the prefixative, except for the addition of 5% OsO_4 solution instead of the glutaraldehyde solution. Due to the fine deposit of ruthenium red, many projections are seen without additional staining in section. Bar indicates 100 nm.

When the EBs in a pellet were treated with ruthenium red, washed well and then stained doubly in sections, it was revealed that the projections with reduced opacity were located on the surface opposite the nucleus which was normally located in an eccentric cytoplasmic region (Fig. 3). The morphological relationship between the cell envelope and surface projections was clearly seen in the cell envelopes which were prepared by the method of Tamura et al.[4] and then treated with tannic acid[5] or ruthenium red[3]. One end of each projection was fitted into a holding of the cytoplasmic membrane while the other end of the projection protruded beyond the cell wall (Fig. 4). To prepare the cell envelopes, the purified EBs were shaken with glass beads, centrifuged in a sucrose density column, followed by the successive treatment with enzymes, such as DNase, RNase and trypsin[4]. During the preparation, especially shaking, the

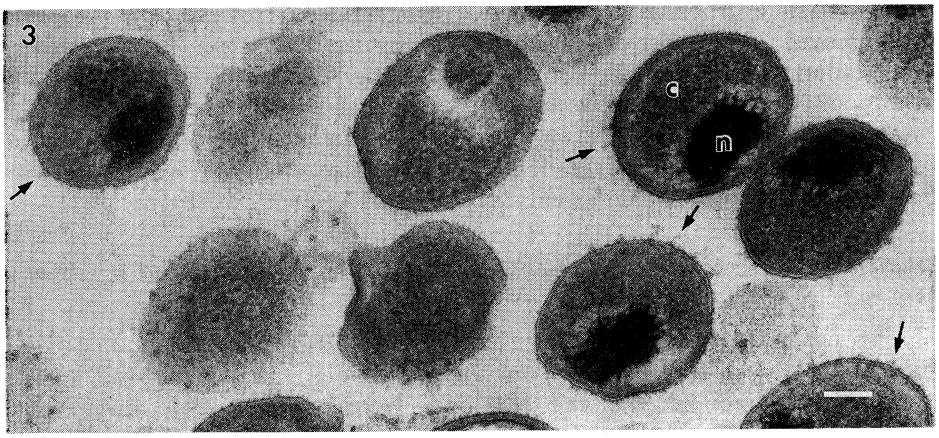

Fig. 3. EBs treated with tannic acid by the method identical with that for the EBs shown in Fig. 2, except for overnight washing and staining with uranyl acetate and lead citrate solutions in section. The cytoplasm and nuclei are well delineated as well as projections (arrows), which are located on the surface at the far side from the nucleus. n : nucleus, c : cytoplasm. Bar indicates 100 nm.

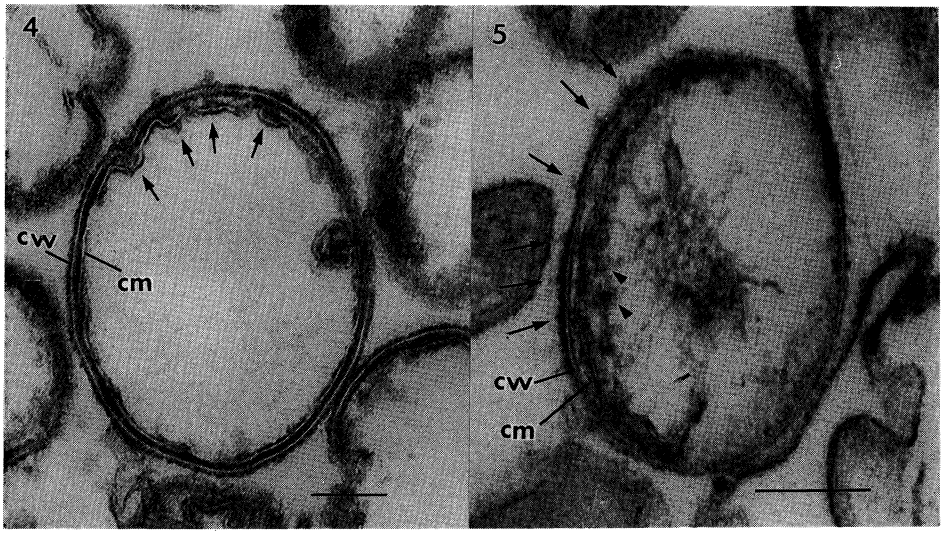

Fig. 4. EB envelope treated with tannic acid. EB envelopes in a pellet were treated by the method identical with that for the EBs shown in Fig. 1. Fitting of one end of each projection into a holding of the cytoplasmic membrane is clearly seen (arrows). Similar result was obtained when the envelopes were treated with ruthenium red.

Fig. 5. EB frozen-thawed, treated with RNase and then fixed by the method identical with that for the EBs shown in Figs. 2 and 3. DNA fibers protruding from the nucleus bind to the "membrane knobs" (arrowheads), which coincide with the sites of the projections (arrows). cm : cytoplasmic membrane, cw : cell wall. Bars indicate 100 nm.

intracytoplasmic structure was completely disintegrated. However, the cytoplasm was removed with mild desruption of EBs by the freeze-thawing followed by the digestion with RNase, one could see DNA fibers bound to the site where the projections were connected with the cytoplasmic membrane (Fig. 5). The connection between DNA fibers and the cytoplasmic membrane was disappeared by the trypsin treatment, suggesting that the DNA molecule was not bound directly, but through a component sensitive to trypsin[3].

Relationship between surface projections and cell envelope as revealed by negative staining

No advantage was provided if the intact EBs were stained negatively. To examine the relationship between the projections and the cell wall and/or cytoplasmic membrane, the cell envelopes were prepared and then stained negatively with phosphotungstic acid at 0.25 or 0.5 % of the final concentration[7]. Fig. 6 shows an EB envelope in which some projections and holes are seen. The projections measured about 5 nm in width and 45 nm in the maximum length. Careful examinations of the EB envelopes stained negatively showed that the projections were readily split off from the cell wall together with the cytoplasmic membrane during preparation, and that each projection was connected with the cytoplasmic membrane at one end and the other end

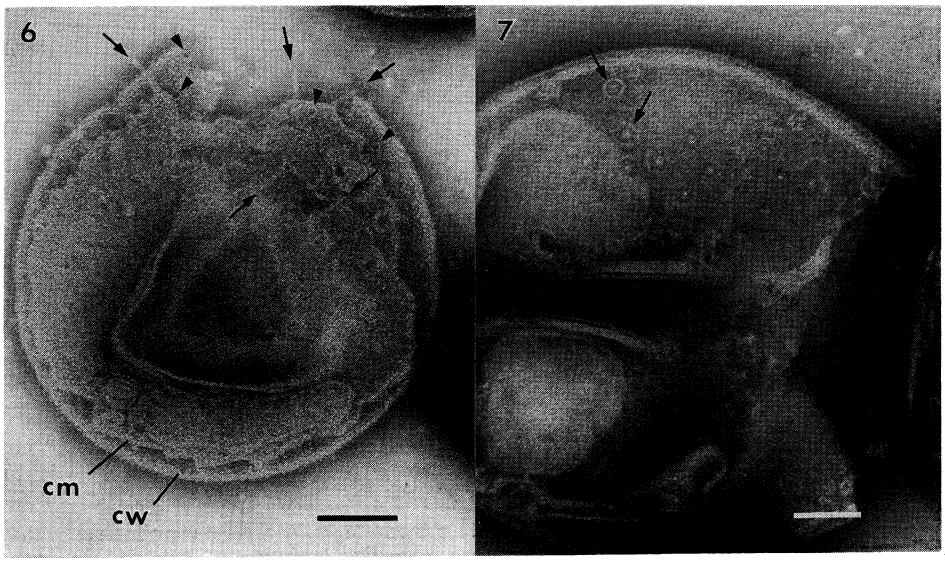

Fig. 6. EB envelope stained negatively. Due to the penetration of the stain, the cell wall (cw) and cytoplasmic membrane (cm) are clearly distinguished. Arrows and arrowheads indicate the projections and holes or rosettes, respectively. Bar indicates 100 nm.

Fig. 7. EB envelope prepared from a fresh envelope fraction. Many projections viewed on end appear to be white spots, which are located at the center of holdings of the cytoplasmic membrane (arrows). Bar indicates 100 nm.

of the projection protruded through the hole in the cell wall. The holes were identical with the "rosettes" reported previously[6]. The observations of the RB envelopes demonstrated that the RB envelopes also possessed the projections identical with those of EB envelopes in morphology[7]. The examinations by means of the rotation technique indicated that each hole or rosette, 19–20 nm in diameter, was composed of a radial arrangement of nine subunits[6]. Fig. 7 shows an envelope which was obtained from a fresh preparation. The projections viewed on end appeared to be white spots, about 5 nm in diameter. Around some projections, one could see white rings, about 38 nm in the outer diameter. From the location and the dimension of these rings, it is very likely that the rings are identical with the cytoplasmic membrane holdings observed in the sections.

Surface projections as revealed by freeze-replica technique

In previous experiments, the specimen for the freeze-replica technique was commonly impregnated with glycerol to prevent the ice crystal formation[5,6]. However, this treatment did not facilitate the sublimation of ice from the frozen specimen after fracture. Consequently, the true outer surface of the specimen was hard to be widely exposed. To examine the morphology of EB true outer surfaces, no glycerol was used[8]. In such preparations, one could see the true surfaces on which the projections were arrayed hexagonally at a center-to-center spacing of about 50 nm. It was noted that each projection from the center of a "flower" structure, about 30 nm in diameter. The rotation technique applied to several flowers demonstrated nine subunits in a radial arrangement[8]. We reported previously that the rosettes in purified, negative-stained cell walls were arrayed in a hexagonal arrangement and that each rosette was composed of radial arrangement of nine subunits[6]. Therefore, it might be concluded that the flowers were identical with the rosettes seen in the cell walls. We also reported that the rosettes were identical with the B structures which were encountered only on the inside surface of the cell walls in the replicas prepared by the freeze-fracture technique[5,6]. From these facts, it was concluded that the B structure, the rosette and the flower were the same structure with each other observed by the diffierent techniques.

The observations mentioned above were carried out with purified EBs and isolated cell envelopes. Therefore, the question arised as to whether the projections were natural structures or artifacts newly formed during preparation. Actually, Louis et al[9]. reported the projections to be structures formed only at a certain physiological stage of the organisms or the results of external conditions. To exclude these possibilities, the projections in an identical morphology with that in the purified EBs should be demonstrated in the *in situ* EBs in the host cell. Fig. 8 shows the EBs in the host cell which was prepared at 40 hr postinoculation without used of glycerol. While the structures of the host cytoplasm were damaged by the formation of ice crystals, EBs were observed as convex round bodies ranging from 0.33 to 0.46 μm in diameter.

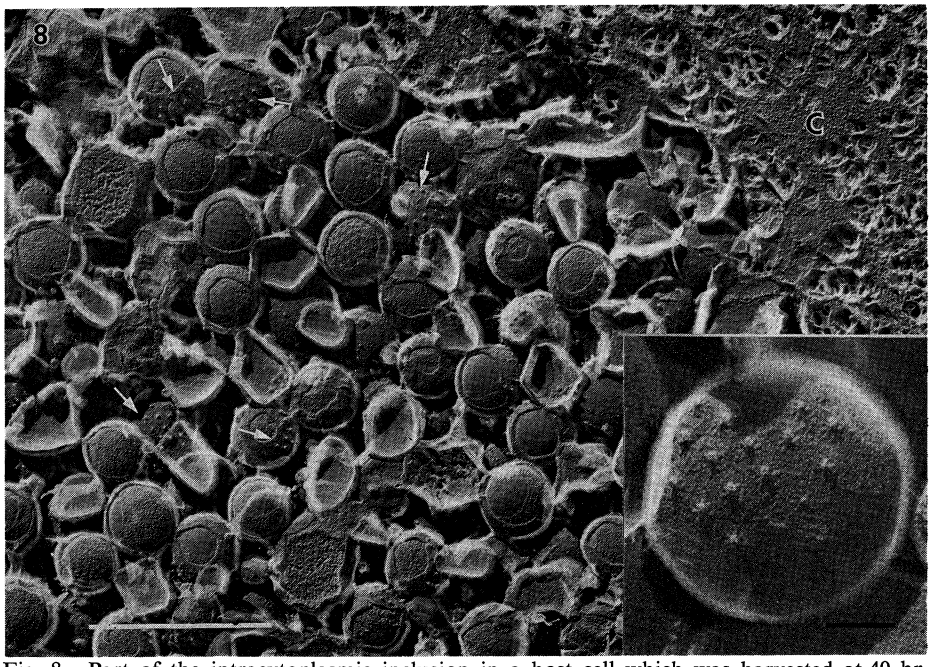

Fig. 8. Part of the intracytoplasmic inclusion in a host cell which was harvested at 40 hr postinoculation and then prepared into a replica by means of the freeze-deep-etching. One can see many EBs showing the true outer surface on which the projections are arrayed (arrows). c : host cytoplasm. Bar indicates 1 μm. Inset shows an *in situ* EB at higher magnification. It is noted that each projection emerged form the center of a flower, which is composed of several leaves arrayed radially. Bar indicates 100 nm.

Some of them possessed the surface projections grouping together in a limited area. The fine structure of each projection was identical with that on the purified EBs. It is, therefore, concluded that the projections are not artifacts formed during preparation, but intrinsic structures of the EBs. The statistical analysis demonstrated that each EB possessed 18 projections in a hexagonal arrangement within a limited surface area[7,10].

Discussion and Summary

Based on the recent studies mentioned above, together with the results obtained in the other previous studies, the EB morphology may be summarized in a diagram in Fig. 9.

The cell walls in sections appeared to be a triple layer, about 8 nm in thickness, which composition was similar to that of so called the "unit membrane"[4]. The outer leaflet of the cell wall was composed of fine particles, about 5 nm in diameter in a negatively stained preparation, and the inner leaflet was observed as a hexagonally arrayed subunit layer (hexagonal layer in Fig. 9) with a 167 Å in diameter in the negative stained preparations[5,11].

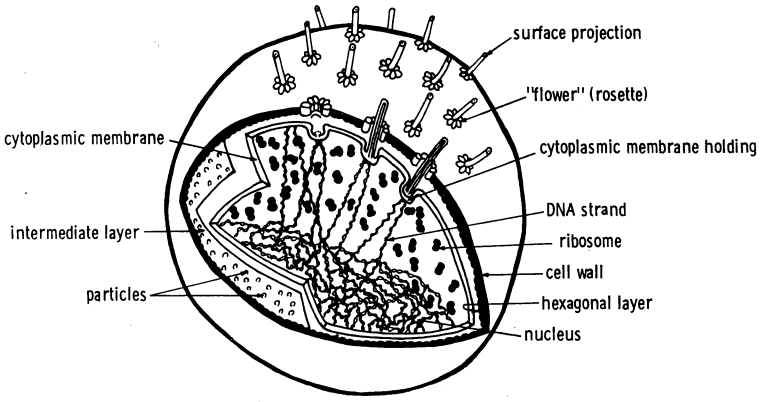

Fig. 9. Diagrammatic representation of the morphology of the EB delineated based on the results obtained from our studies cited.

The previous studies revealed that the hexagonal layer was a proper structure of the EB cell walls and that penicillin strongly inhibited the formation of this layer during the maturation process from the RB to EB[12], while the RB cell walls were synthesized in the presence of the antibiotic, but lacked the hexagonal layer[12,13]. The hexagonal layer seemed to be responsible for the ridigity of the EB cell wall to the mechanical treatment[13,14].

The morphology and biochemistry of the cytoplasmic membrane and the intermediate layer still remain to be studied, because no technique for their isolation was established. In the replicas prepared by the freeze-fracturing, EBs purified, as well as EBs *in situ,* occasionally appeared to be multiple layered faces. The cleavage of such multi-layered EB was never explained if one considered simply the EB envelope as to be composed of two membranes, the cell wall and the cytoplasmic membrane. Careful examinations suggested the presence of an intermediate layer between the cell wall and the cytoplasmic membrane[5,10,11]. Based on this idea, the outside surface of the intermediate layer, which was always appeared as the convex face, was convered with a number of fine paritcles with an approximate diameter of 10 nm, while the inside surface of the layer, and the inside and the outside faces of the cytoplasmic membrane lacked the particle.

From the results obtained by the freeze-fracturing, we reported the presence of other particles, 16 nm in diameter, among the 10 nm-particles. The 16 nm-particles were arrayed hexagonally, indicating that they were complementary with the B structures on the inside surface of the cell wall[5,10,11]. However, Louis et al.[9] demonstrated clearly the 16 nm-particles as fine holes. It was very likely that our 16 nm-particles were resulted from inadequate resolution of the replicas in which the fine holes at the center of the 16 nm-particles were filled up with the metal for shadowing. Therefore, the 16 nm-particles at the bases of the projections delineated in the previous diagram[5] were

modified.

The number of the surface projection of each EB was determined by the statistical analysis of the results obtained by the high resolution scanning electron microscopy, in which the purified EBs were heavily coated with gold-palladium alloy[7,10]. In such preparations, the projections appeared to be hemispheres on a limited surface area, and consequently, the fine structure of the projections was obscure. This problem was settled by the freeze-deep-etching without use of glycerol[8].

We reported that polymixin B sulfate formed many protrusions on the EB cell walls[15]. The protrusions were readily dissolved with sodium dodecyl sulfate, resulting the formation of many pores in the cell walls. Based on these facts, we established a method by which DNA was mildly extracted from the intact EBs in a sucrose density column, and demonstrated that the DNA molecule of the EB was double stranded and displayed 345.6 μm of modal length which corresponded to an average molecular weight of about 660×10^6 daltons[16,17]. This value corresponded positively with that of intact molecules of *Chlamydia trachomatis* reported by Sarov and Becker[18]. They also reported that a molecule in full length was circular. Therefore, it is likely that DNA is bound to the cytoplasmic membrane holdings at some points of the molecule, and that some regions of the DNA molecule are exposed directly to the EB cytoplasm, which contains a number of ribosomes ressemble to those of the eubacteria in size and chemical composition[19,20].

Recent studies on the isolated inclusion bodies demonstrated that the RBs also possessed the surface projections which morphology was identical with that of the EBs[7]. In the isolated inclusions, the RBs close to the inclusion membrane were closely connected to the inside surface of the inclusion membrane by means of the projections, which appeared to pierce the inclusion membrane[21,22]. These facts suggested that the RBs were connected directly with the host cytoplasm through the canales of the projections and that the RBs interchanged information with the host cell through the projections. To clarify the function of the projections, further studies are in progress.

Acknowledgment

These studies were supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture (No. 357149) and a Project Research Grant, Kawasaki Medical School (No. 54-012).

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