Isolation and Electron Microscopic Observations of Intracytoplasmic Inclusions Containing *Chlamydia psittaci*

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Intracytoplasmic inclusions containing *Chlamydia psittaci* were isolated by a newly established method. Infected L-cells at 20 h after infection were suspended in 0.25 M sucrose-tris(hydroxymethyl)aminomethane buffer containing ethylene-diaminetetraacetic acid, homogenized in a Dounce tissue grinder, and filtered through a 2,000-mesh screen. Isolated inclusions were stabilized in 5% bovine serum albumin in 10 mM tris(hydroxymethyl)aminomethane buffer. Electron microscopic observations revealed the presence of surface projections on the vegetative, reticulate bodies and a direct connection between the reticulate bodies and the inclusion membrane by means of projections.

Cell envelopes isolated from the infectious, elementary bodies (EB) and the developmental, reticulate bodies (RB) of *Chlamydia psittaci* have been previously studied by electron microscopy. These observations revealed surface projections with roughly hexagonal arrangements on a limited surface area on EB (8–10). When isolated cell walls were stained negatively, the sites of these projections coincided with the rosettes and B structures seen on the concave surface exposed by cleavage of frozen EB (7, 8). No projections were found on RB surfaces. Examination by the freeze- replica technique demonstrated some groups of fine particles on the smooth convex surface of the intracytoplasmic inclusions which contained only RB in samples harvested at 18 h after infection (7). The arrangement of the fine particles was roughly hexagonal and was similar to that of the projections on EB, suggesting the presence of the projections on the RB surface and structural interrelation between RB and the inclusion membrane by means of the projections. The projections were, however, never seen in inclusions in situ in thin sections. Experiments were then designed to examine inclusions isolated in native form, since the projections on EB apparently possess very low electron opacity and are hard to stain specifically.

The isolation of intact inclusions and a description of their morphology, together with observations on the interrelation between RB and inclusion membrane, are reported in this paper.

**MATERIALS AND METHODS**

Chlamydial strain and its propagation. The meningопneumонitis strain of *Chlamydia psittaci* and L-cells in suspension culture were used throughout this experiment. The propagation of the organisms was carried out by the method of Tamura and Higashi (16).

**Isolation of inclusions.** L-cells inoculated with 5 to 10 inclusion-forming units per cell were cultivated in a Spinner culture bottle at 37°C for 20 h and collected with low-speed centrifugation (350 × g). After washing twice in the phosphate-buffered saline of Dulbecco and Vogt (4), but lacking Ca and Mg, the cells, approximately 10^9 in number, were suspended in 10 ml of 0.25 M sucrose-ET buffer solution (1 mM EDTA in 10 mM Tris buffer, pH 7.4) and kept in ice for 60 min. The cell suspension was then transferred to a Dounce tissue grinder SS, 15 ml in size (Kontes Glass Co., Vineland, N.J.) and homogenized gently for 20 strokes. The homogenate was taken in a 10-ml syringe and filtered through a stainless-steel, 2,000-mesh screen (Twilled Dutch Weave, purchased from Taiyo Wire Cloth Co. Ltd., Osaka, Japan) set in a stainless-steel Millipore membrane filter holder 2.5 cm in diameter. The filtrate was then spun down at 1,500 × g for 5 min in a Kubota K-80 table type centrifuge. The pellet obtained was washed twice with 0.25 M sucrose in 10 mM Tris buffer. This procedure was monitored with a phase-contrast light microscope.

**Electron microscopy.** For thin-sectioning technique, the samples in the pellets were fixed in 2.5% glutaraldehyde in phosphate-buffered saline lacking Ca and Mg or in Kavrovsky formaldehyde-glutaraldehyde mixture (M. J. Kavrovsky, J. Cell Biol. 27: 137A, 1965) for 60 min at room temperature. The fixed samples were treated with tannic acid as previously reported to enhance the electron opacity of the specimens, especially the fine surface projections in thin sections (9). After several washings in distilled water, the samples were fixed again in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in an ethanol series, and embedded in the low-viscosity embedding medium described by Spurr (14). Thin sections were cut with a glass knife on a Porter-Blum microtome MT2-B and doubly stained with 2% uranyl acetate in 70% ethanol and lead citrate solution (13).

For freeze- replica preparations, the samples of infected cells and isolated inclusions, in pellets prefixed
in glutaraldehyde or Karnovsky fixative and impregnated with 10% glycerol, were frozen in Freon 22 at liquid nitrogen temperature and then transferred into liquid nitrogen. The replica was prepared in a Balzers BAF301 freeze-replica apparatus without sublimation of ice. The replica membrane was treated with commercial bleaching solution containing sodium hypochlorite, rinsed several times in distilled water, and then collected on a copper grid.

All specimens were examined with a Hitachi H-500 electron microscope at an accelerating voltage of 75 kV.

RESULTS

Figure 1 presents phase-contrast light micrographs showing the process of inclusion isolation. Figure 1a represents the cells kept in 0.25 M sucrose-ET buffer for 60 min. The cells appear somewhat swollen, and the inclusions can

![Fig. 1. Phase-contrast micrographs of the process of inclusion isolation. The process is shown alphabetically (a to f). (a) L-cells kept in 0.25 M sucrose-ET buffer for 60 min in ice. Inclusions are clearly seen as the bright bodies (arrows). (b) Cells after a five-stroke homogenization in a Dounce tissue grinder. Some inclusions are released, but the majority of the cells retain their shape. (c) Fraction after 20 strokes of homogenization. The cells are disintegrated and make large aggregates. Many inclusions and organelles are released. (d) Filtrate of the 23-stroke homogenate through the screen. The large aggregates are removed. Many inclusions and much small cell debris are obtained. (e) Suspension of pellet in 0.25 M sucrose-Tris buffer, obtained by centrifugation at 1,500 × g for 5 min. Small debris still remains. (f) Suspension in 0.25 M sucrose-Tris buffer after two washings by centrifugation at 1,500 × g for 5 min. Many inclusions in high purity are obtained. All micrographs are shown at the same magnification. Bar, 10 μm.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
be clearly seen as the bright bodies in the central region of cytoplasm (arrows). After a five-stroke homogenization, some inclusions were released, but the majority of the cells retained their shape and the inclusions were still contained in each cell (Fig. 1b). When the homogenization was continued up to 20 strokes, the cells were almost completely disintegrated (c). Many inclusions and cell organelles were mixed in amid the large cell debris, which tended to make large aggregates. These large aggregates were efficiently removed by the filtration through the screen, although many small organelles and much membranous debris remained in this fraction (d). The suspension of the pellet obtained from the fraction after centrifugation contained mainly inclusions (e). The purity of this fraction did not, however, seem sufficient for morphological examination, especially for the freeze-replica technique, since small particulate contaminants were still present. After two washings in 0.25 M sucrose-Tris buffer, the small contaminants were mostly removed (f). When the inclusions were collected into a pellet, cut into thin sections, and followed by electron microscopic examination, they showed severe damage in inclusion membrane and RB (Fig. 2). The inclusion membrane was broken at many places and lacked continuity. RB appeared to be remarkably shrunk and lacked their native morphology, although the fraction was rich in inclusions.

To determine the conditions under which the inclusion membrane and RB can retain their native morphology, the isolated inclusions in a suspension were divided equivalently into seven parts, then spun down at 1,500 x g for 5 min, simultaneously. Each pellet was suspended, in 0.25 M sucrose, 0.5, 1, 2.5, and 5% bovine serum, and 5 and 10% fetal calf serum, respectively. All solutions tested were prepared with 10 mM Tris buffer. The suspensions were incubated at room temperature. The turbidity of the supernatants of the 1,500 x g, 5-min centrifugation was assayed at 0, 30, 60, and 120 min after incubation, with the expectation that the turbidity of the supernatant would indicate the degree of RB liberation due to the disintegration of the inclusion membrane in the suspension. The results (Fig. 3) indicated that bovine serum albumin was capable, in proportion to its concentration in the suspension, of stabilizing the inclusion membrane, and that 5% bovine serum albumin effectively maintained the integrity of inclusions. It is noteworthy that the turbidity in 0.25% sucrose showed rapid increase, indicating that the inclusion membrane was unstable in this solution, which has been widely used for the isolation of organelles from various types of cells and tissues. The inclusions in 5% bovine serum were examined in thin sections by electron microscopy.

As shown in Fig. 4, each inclusion was round in shape, and the RB that were surrounded by the inclusion membrane appeared to retain their natural shapes. At higher magnification, it appeared that some RB were closely connected to the inside surface of the inclusion membrane by means of fine projections, cylindrical in shape and 10 to 13 nm in diameter, which appeared to pierce the inclusion membrane (Fig. 5a). Figure 5b illustrates the projections sectioned tangentially, showing RB adhering closely to the inner surface of inclusion membrane with the projections.

Figures 6 and 7 illustrate the freeze-replica images of the inclusions in situ. In Fig. 6, showing a convex surface of partially fractured inclusion membrane, some particles are seen on the top of the RB in the inclusion (arrows). Large convex and concave surfaces in Fig. 7 show two different faces exposed by the cleavage of inclusion membrane of neighboring inclusions. No RB profile is seen in each inclusion, but both surfaces show clear ruggedness along RB outlines. On the con-
vex surface (conv), several groups of particles, which seem to be the same particles as those shown in Fig. 6, are seen (arrows). These particles are also the same as those I reported previously (7). On the concave surface (conc), fine particle-like depressions can be seen (arrows), which correspond to the pattern of the particles on the convex surface. These results indicate that the particles on the convex surface and the depressions on the concave surface are identical to the structures seen in the inclusion membrane. The convex surface, which may correspond to the "E face" (2) of the host plasma membrane, completely lacks membrane particles, whereas many membrane particles 8 to 10 nm in diameter are seen on the concave surface, which may be the "P face" (2) of the host plasma membrane. The freeze- replica of the isolated inclusions also showed a convex or concave surface, on which several of these structures, as shown in Fig. 6 and 7, were seen in a regularly arrayed pattern. Figure 8 illustrates the convex surface of an isolated inclusion on which the structures in groups are clearly seen. Each structure is surrounded by six structures lying 40 to 50 nm apart. From these results, it appears very likely that the fine particles on the convex surface of the inclusion membrane are RB surface projections, which pierce the inclusion membrane.

It was noticed that many mitochondria were associated with the outer surface of inclusions, with minimum spacing of 5 nm (Fig. 9). However, the relation between the location of mitochondria and the projections is not clear.

**DISCUSSION**

The key points for the isolation of inclusions in their native form seem to be as follows: (i) use of 0.25 M sucrose-ET buffer, in which the plasma membrane swells and becomes unstable in gentle homogenization; (ii) use of the Dounce tissue grinder, with which swollen plasma membrane is broken; (iii) use of the 2,000-mesh screen, with which the larger aggregates were readily removed from the homogenate. The actual pore size of the screen is not known. When a normal L-cell suspension (10^6/10 ml) was filtered in the same way, only about 2 ml of the suspension, with 10^9 to 10^10 cells per ml, was obtained and the majority of L cells remained, showing that the pores in the screen were stopped up with the cells. The pore size of the screen seems, therefore, to be a little bit less than 10 μm in diameter. Direct measurement of the pore size by electron microscopy failed because of the Twilled Dutch Weave.

Many attempts to isolate the inclusions from the homogenate by various techniques such as differential, sucrose-cushioning, and density gradient centrifugations failed.

The inclusion membrane is very unstable even in 0.25 M sucrose solution, although this solution has been widely used for the isolation of various organelles of animal cells. Addition of bovine serum albumin effectively stabilized the integrity of inclusions. The purity of the inclusions in the final fraction was, at least as seen by phase- contrast microscopy, very high. However, the fraction still contained some cell debris. Additional treatment may be necessary for use of the inclusion-rich fraction in further experiments.

We previously reported that the projections on EB, treated with tannic acid and thinly sectioned, are cylindrical in structure (9), and that one edge of each projection is connected with the cytoplasmic membrane while the other edge projects beyond the cell wall through fine holes, which are seen as rosettes in negatively stained cell wall preparations and as B structures on the inner surface of the EB cell wall by either freeze-fracture or freeze-etching technique (8). The B structures are identical to the craters of EB and RB recently reported by Louis et al. (6), but the
Fig. 4. Thin-sectioned inclusions after stabilization in 5% bovine serum albumin. Most inclusions show the natural forms in which a number of RB are contained. Mitochondria associated with the surface of inclusions are visible (arrows). Bar, 1 μm.

Fig. 5. Part of a thin-sectioned inclusion at higher magnification. (a) Inclusion membrane is pierced by the projections of RB (arrow). (b) Projections cut tangentially are clearly seen (arrow). Projections in both figures show the cylindrical structure. im, Inclusion membrane; m, mitochondria. Bars, 100 nm.
location of the crater is, according to their explanation, on the fractured face of the cytoplasmic membrane of the organism. This may be due to incorrect application of technique. With their idea, it may be difficult to make clear the fact that the B structures are located on a surface possessing the hexagonally arrayed subunits which compose the inner surface of the EB cell wall and are visualized only after optimum etching (7).

The results obtained in the present experiment strongly suggest that RB also have surface projections and that RB are connected directly with the host cytoplasm through the canals of the projections. In this manner, RB within an inclusion may regularly connect with the host...
cytoplasm during their multiplication and interchange information with the host cell.

The relation between loci of mitochondria and those of the projections was not clear. Furthermore, it was not clear whether mitochondria touched the inclusion membrane. Considering the obligate parasitism of Chlamydia organisms, the significance of the attachment of mitochondria remains an important problem.

From many studies of the plasma membrane of many types of cells by means of freeze-replica technique, it may be generalized that both P and E faces, according to the nomenclature by Branton et al. (2), are covered by the membrane particles, but the E face is studded with fewer membrane particles than the P face (1, 3, 12, 17). The morphology of the inclusion membrane revealed by the freeze-replica technique apparently differs from that of the plasma membrane. The inclusion membrane is derived from the plasma membrane of the host cell as EB is phagocytized (5). Therefore, the convex surface of the inclusion membrane corresponds to the E face, and the P face is exposed as the concave surface. No membrane particles are encountered on the convex surface, whereas many membrane particles are seen on the concave surface. The inclusion membrane seems to be modified in its nature during the multiplication of these organisms. Biochemical study by Stokes also indicates this modification by Chlamydia-directed glycosylation (15). The modified nature of these membranes is also suggested by the instability of the inclusions in 0.25 M sucrose solution.

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LITERATURE CITED


2. Branton, D., N. B. Gilula, M. J. Karnovsky, H. Moor,


