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Brief Note

Morphology of *Chlamydia* Organisms as Revealed by Freeze-substitution Fixation Technique

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The freeze-substitution fixation technique consists of the combination of rapid freezing and osmium fixation. The samples rapidly frozen by contact on a metal plate at liquid nitrogen temperature are immersed and kept in osmium tetroxide dissolved in absolute acetone at dry-ice temperature for several days, then embedded in the plastic material for thin sectioning.[1] The rapid freezing and the omission of dehydration with a series of ethanol or acetone may preserve the fine structure in the biological specimens.[1,2] Cell morphology of *Chlamydia psittaci* meningopneumonitis strain was studied by this technique.

The infected L cells at 40 hr postinoculation were washed in PBS(-), suspended in 0.75% agar in PBS(-) at 45°C and then solidified on a glass plate cooled on ice. The agar plate, about 2 mm in thickness, was cut into 2×2 mm^2 pieces and divided into two groups. The piece in the first group was put on the top of the plunger, and then frozen rapidly by contact on the clean surface of a gold plate at liquid nitrogen temperature in an EIKO RF-2 rapid freezing device. The frozen specimens were quickly transferred into 4% osmium tetroxide in acetone cooled at -79°C and kept for 3 days and then embedded in Epon after gradual increase the specimen temperature to room temperature for 2 days. The sections perpendicular to the agar surface were cut on a Porter-Blum microtome and stained doubly with uranyl acetate[3] and lead citrate[4] solutions. The agar piece of the second group was fixed in 1% osmium tetroxide in 0.1M cacodylate buffer solution (pH 7.3) for 2 hr in ice, dehydrated in an ethanol series and then embedded in Epon. The sections were treated identically with those of the first group. All sections were examined with a Hitachi H-500 transmission electron microscope at 75 kV.

In the sections of the first specimen group, the freezing damage was little or free in the zone up to 10 to 15 μm depth from the agar surface and the chlamydia cells contained in L cells in this zone well retained their morphology. Both cell types, elementary bodies (EBs) and reticulate bodies (RBs) of this organism were round in shape and possessed dense opacity (Fig. 1). A clear gap between cytoplasmic membrane and cell wall was seen in each EB, but this gap was not so much distinct in the intermediate forms (IFs) which were characterized by the location of the dense nucleus at the cell center. At higher magnification, the nucleus locating at the eccentric region and cytoplasm were

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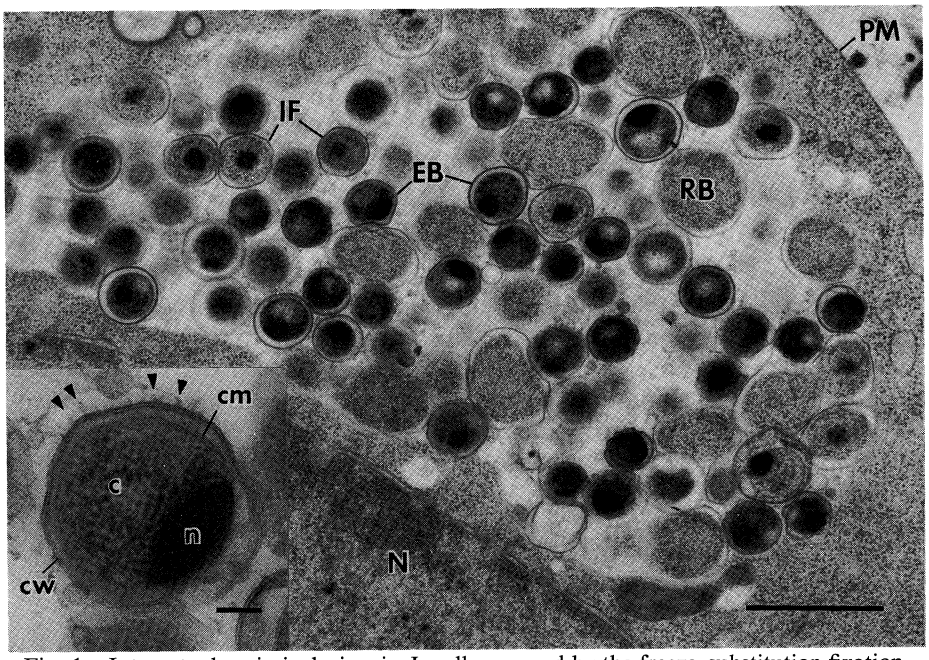

Fig. 1. Intracytoplasmic inclusion in L cell prepared by the freeze-substitution fixation technique. EB : elementary body, IF : intermediate form, N : host nucleus, PM : host plasma membrane. Bar indicates 1 μm. Inset shows an EB at higher magnification. Arrowheads indicate the surface projections. c : cytoplasm, cm : cytoplasmic membrane, cw : cell wall, n : nucleus: Bar indicates 100 nm.

clearly distinguished in each EB. It was noted that the cytoplasmic membrane was clearly recognized as a triple layer, 80Å in thickness, and that some EBs possessed the surface projections which were located on the far side surface from the nucleus (Fig. 1, inset).[5] Fig. 2 shows the organisms in the second specimen group. The gap between the cell wall and the cytoplasm was more remarkable and irregular than that in the cells shown in Fig. 1. Both structures in the EB, the cytoplasmic membrane and the surface projections, were obscure in the EBs prepared without freezing (Fig. 2, inset). Morphology of the RBs and IFs in this group was similar to that of them in the first group, while cell shape in the second group was somewhat irregular.

The advantages in using the freeze-substitution fixation technique are summarized as follows : (i) The cytoplasmic membrane was well preserved and seen as a triple layer, while the usual thin sectioning technique did not retain its morphology. When the cell envelopes (cell wall-cytoplasmic membrane complexes) were examined by the usual sectioning technique, the cytoplasmic membrane was clearly seen.[6] The reason why cytoplasmic membrane was not observed in the specimens of the second group was not known. (ii) No surface projection was seen in the EBs prepared by the usual fixation procedure. This

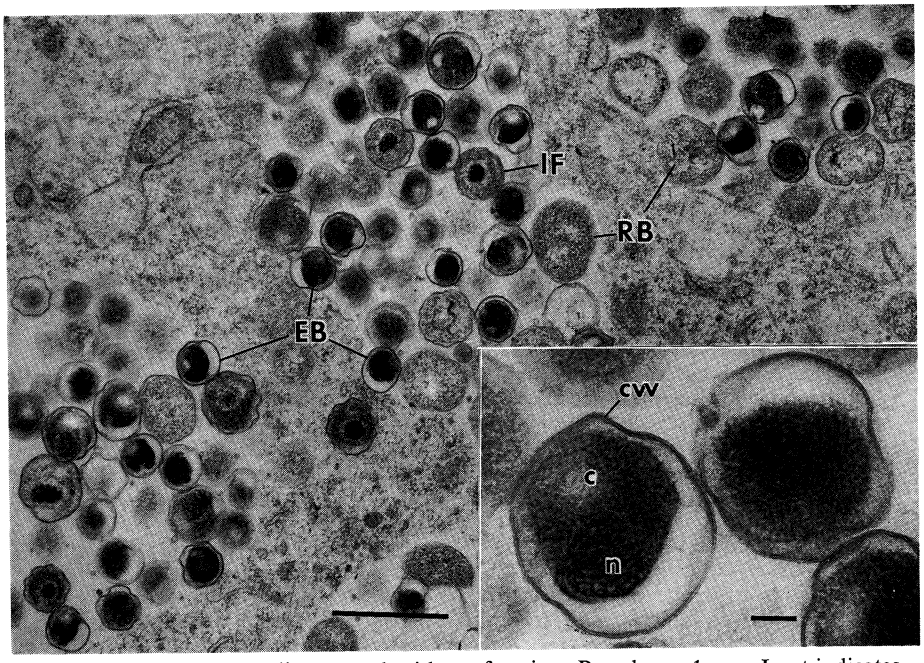

Fig. 2. Inclusions in L cell prepared without freezing. Bar shows 1 μm. Inset indicates EBs, in which cytoplasmic membrane and surface projection are obscure. Bar indicates 100 nm.

might be due to their inadequate opacity in sections.[7,8] To observe the projections in thin sections, the additional treatment with the chemicals, such as tannic acid[7] or ruthenium red,[5] to enhance the specimen opacity was needed. When EBs were examined by the freeze-substitution fixation technique, the surface projections were readily observed, suggesting that this technique can retain the stainability of the specimen.

The gap between the cell wall and the cytoplasmic membrane seemed to be the plasmolitic artifact occurred during preparation, because this gap was never encountered in the EBs examined by the freeze-replica technique.[9] Therefore, further technical modification, presumably at the step of rapid freezing is required.

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