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Electron Microscopic Observations on the Fine Structure of Cell Walls of *Chlamydia psittaci*

AKIRA MATSUMOTO[1] AND G. P. MANIRE

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

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L-cell cultures were infected with elementary bodies (EB) of meningopneumonitis organisms. Cell walls were prepared from reticulate bodies (RB), which are the intracellular developmental forms into which EB are converted, and from EB at appropriate times after infection. When fragmented EB cell walls were shadowcast with platinum palladium alloy, about one-half of the fragments were seen to be composed of hexagonally arrayed structures on the inner side of the cell wall. When EB cell walls were negatively stained with phosphotungstic acid, they all showed this fine structural array. These macromolecular units were estimated to be about 18 nm in diameter. RB cell walls, harvested at various times after infection, were similarly stained; about 20% of RB walls at 15 hr after infection showed traces of these regular structures, but only 2% of them had the structures at 24 hr. When RB cell walls prepared from penicillin-containing culture were examined, they were observed to be similar to RB without penicillin. When EB cell walls were treated with formamide at 160 C, and then centrifuged in a 10 to 40% potassium tartrate density gradient, hexagonal particles about 20 nm in diameter were obtained as a middle band in the gradient column. These particles were not obtained from RB cell walls harvested from cultures with or without penicillin. It is concluded that the particles are macromolecular subunits located on the inner side of the EB cell walls, that the subunits probably provide the structural rigidity found in the EB, and that their synthesis is inhibited by penicillin.

In the developmental cycle of the intracellular parasite *Chlamydia*, there are two different types of particles: one is the rigid infectious elementary body (EB) and the other is the fragile developmental reticulate body (RB). Within 6 to 8 hr after infection of L cells, the EB are converted into the larger fragile RB which undergo binary fission. About 20 hr after infection the RB in turn begin to mature into EB. Recent studies on purified EB and RB showed that these two types of particles differ in a number of respects, including morphology (size being an obvious difference), chemical composition, resistance to sonic treatment (8), and to some enzymes (9). It seems that these differences can be accounted for in part by differences in the cell walls of the two types of bodies. Manire and Tamura (4) studied the chemical composition of highly purified cell walls of the EB of meningopneumonitis organisms, and found a considerable amount of phospholipid and an amino acid composition similar to that of gram-negative bacterial cell walls. In purified RB cell walls, on the other hand, the phospholipid content was less than that in EB, and no sulfur-containing amino acids were detected (9).

In experiments on the effect of penicillin on the multiplication of the meningopneumonitis organisms, Tamura and Manire (10) found that the maturation of RB to EB was completely prevented by penicillin. However, there was no inhibition of RB multiplication, and the RB cell walls obtained from cultures grown in the presence of penicillin were similar to normal cell walls in their fragility and in their chemical composition. In more recent electron microscopic studies on the effect of penicillin on the multiplication of meningopneumonitis organisms, Matsumoto and Manire (6) showed that the drug did inhibit maturation. This suggests that some new components may be synthesized during normal maturation and that this synthesis is inhibited by penicillin.

Based on an electron microscopic study of purified EB cell walls, Manire (3) reported that fragments of the cell walls were composed of a

[1] Present address: Institute for Virus Research, Kyoto University, Kyoto, Japan.

granular outer layer and an inner layer composed of hexagonally arrayed structures estimated to be 10 to 20 nm in periodicity. The experiments reported here extend the study of the fine structure of cell walls at different stages of development, with and without penicillin, and they describe the isolation of these components from cell walls of meningopneumonitis organisms.

MATERIALS AND METHODS

Organisms and cells. The meningopneumonitis strain of *C. psittaci* was routinely cultured in suspended L cells as previously described (10). Large amounts of EB were prepared in a 4-liter Spinner cell culture agitated at 50 cycles/min on a magnetic stirrer in a 37 C incubator, or in a 14-liter table-top fermentor.

RB were propagated by the methods of Tamura et al. (11), with 4-liter Spinner culture bottles used under the conditions mentioned above. When desired, sodium penicillin was added to the culture medium at a final concentration of 200 units/ml; the other growth conditions remained the same.

Preparation of EB cell walls. Purified EB from the infected cell culture harvested at 48 hr after infection were obtained by the methods of Tamura and Higashi (8). By using purified EB as starting material, cell walls were isolated as previously described (4).

Preparation of RB cell walls. RB cultures, with and without penicillin, were purified by the method of Tamura et al. (11), and both types of cell walls were obtained by the method reported previously (9).

After purification of cell walls, each preparation was examined under an electron microscope to determine its suitability for use.

Electron microscopy. Samples of fresh preparations were spread on copper grids covered with collodion membrane and were shadowcast with platinum palladium alloy at a 10° angle against an emitter basket in approximately a 10^{-6}-mm Hg vacuum. Identical samples were stained negatively with phosphotungstic acid (PTA) solution at *p*H 7.2. The specimens prepared were examined either with an Akashi TR-50E1 microscope with a 50-μn objective aperture, or with an AE1 electron microscope, accelerated at 60 kv, with a 30-μm objective aperture.

RESULTS

Fine structure of EB and RB cell walls. When fragmented EB cell walls, obtained by sonic treatment (10 kc/sec) with glass beads for 3 min, were examined as shadowcast specimens, approximately 50% were seen to be composed of regularly arrayed structures, and the remaining fragments showed no regular structure. One partially disintegrated cell wall had the regular patterns only on the inside surface (Fig. 1). The other walls in the top portion do not show any regular structure, because only the outer surface is exposed. A fragment at the bottom shows clearly the regular structural array. It is, therefore, easily recognized that this fragment is oriented with its inner surface upward. When purified EB cell walls were stained negatively with PTA, all walls demonstrated this regular structure. Fragments of EB cell walls seen at high magnification (Fig. 2) showed the structure to be a regular array of macromolecular units. Geometrical analysis revealed that this regular array consisted of an arrangement of subunits which were hexagonally packed with about an 18-nm periodicity as measured from center to center. On the other hand, RB cell walls prepared from the culture harvested at 18 hr after infection did not show any regular structure. To demonstrate this difference between the walls of the two growth stages, RB cell walls and EB cell walls were prepared separately and then mixed together for examination in the same field. The two EB cell walls in Fig. 3 exhibit the regular array, whereas the one RB cell wall exhibited a granular, irregular pattern of small particles.

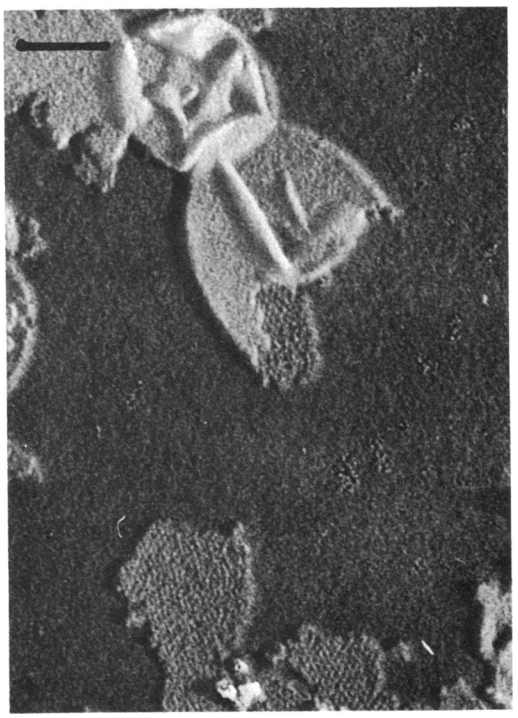

FIG. 1. *EB cell walls shadowcast with platinum palladium alloy. Markers in all figures indicate 250 nm.*

During examination of many RB cell walls, some were seen with small areas showing the regular structures. Figure 4 shows such an RB cell wall harvested at 16 hr after infection. To determine whether this area represented the beginning of EB cell wall maturation or simply remained from the preceding infecting stage, RB

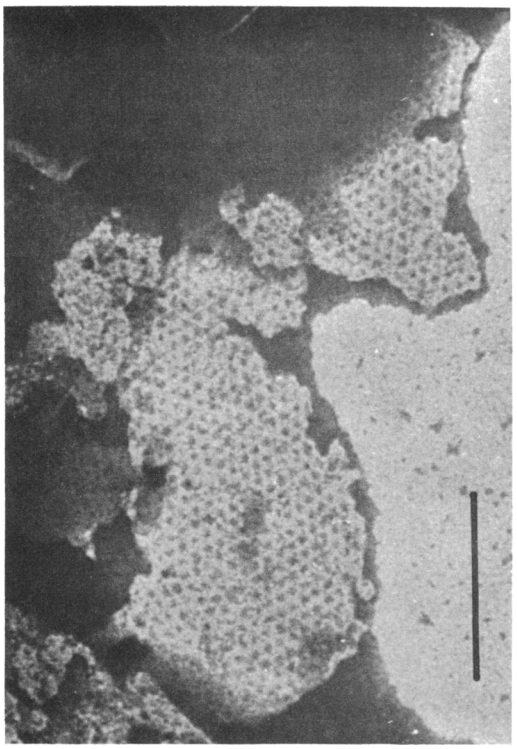

FIG. 2. *Fragment of an EB cell wall stained with phosphotungstic acid.*

cell walls were prepared from a single infected cell culture at 15, 18, 21, and 24 hr after infection and were examined (Table 1). The number of cell walls with and without these structures was counted, and the proportion of walls with the regular array structure was calculated. RB cell walls harvested at 15 hr showed the regular structures to be present in more than 20% of the walls. However, at 24 hr after infection, only 2% of the RB cell walls had such structures. The results indicate that these structures were not newly synthesized but were carried over from the infecting EB and were then diluted out in the multiplying RB.

Isolation and morphology of subunits from EB cell walls. Many workers have described the isolation of peptidoglycan components from bacterial cell walls by the use of hot formamide (1, 2, 7). A similar technique for the chemical disintegration of the EB cell wall was attempted.

Purified EB cell walls (1 to 5 mg, dry weight) were suspended in 1 to 2 ml of formamide (Fisher Scientific Co., Fair Lawn, N.J.) and heated for 10 min in a 160 C oil bath. Within the first 5 min, the suspension became very clear and its color changed to light brown. After heating, the fraction was diluted four times with 0.033 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4) containing 0.01 M $MgCl_2$, and then centrifuged at 25,000 × *g* for 60 min. The supernatant

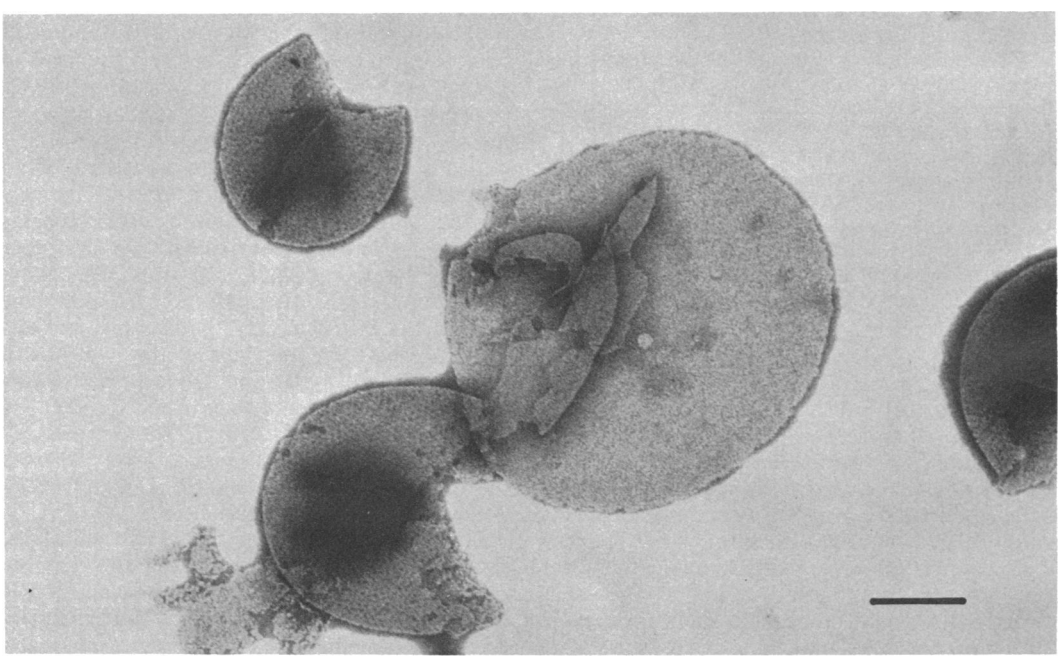

FIG. 3. *Two EB and one RB cell walls stained with phosphotungstic acid. The regularly arrayed structures are evident in the EB cell walls.*

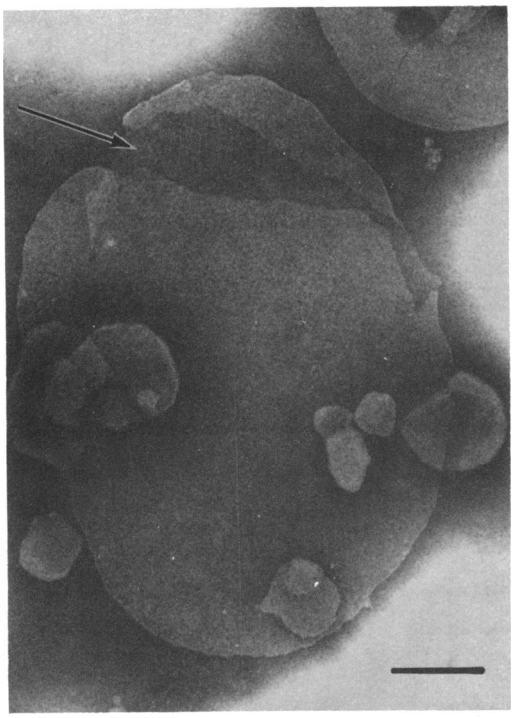

FIG. 4. *RB cell wall harvested at 16 hr after infection. Arrow indicates a small area showing the regular structural array.*

TABLE 1. *Observation of the hexagonally arrayed subunits in RB cell walls prepared at various times after infection*

Time of RB harvest (hr)	No. of walls with subunit/total no. counted	Percentage with subunit
15	29/124	23.4
17	7/158	4.4
21	8/169	4.7
24	6/284	2.1

fluid was centrifuged again at 100,000 × *g* for 120 min with an SW39 rotor in a Spinco L-type centrifuge. The pellets were suspended in 2 ml of Tris buffer, and this suspension was overlayed on a 10 to 40% potassium tartrate density gradient column in cellulose nitrate tubes [0.5 by 2 inches (1.27 by 5 cm)] and centrifuged at 70,000 × *g* for 120 min in an SW39 rotor. One clear band was collected from about the middle of the gradient column and examined under an electron microscope with PTA staining. Figure 5 shows fine particles embedded in PTA. Some of them appear to be doughnut-shaped structures measuring about 20 nm in mean diameter. Careful observation of each particle revealed that they have some polygonal outlines. To determine the exact shape of the particle, the rotation technique (5) was applied to several particles. The results (Fig. 6) show a clear hexagonal profile by three and six

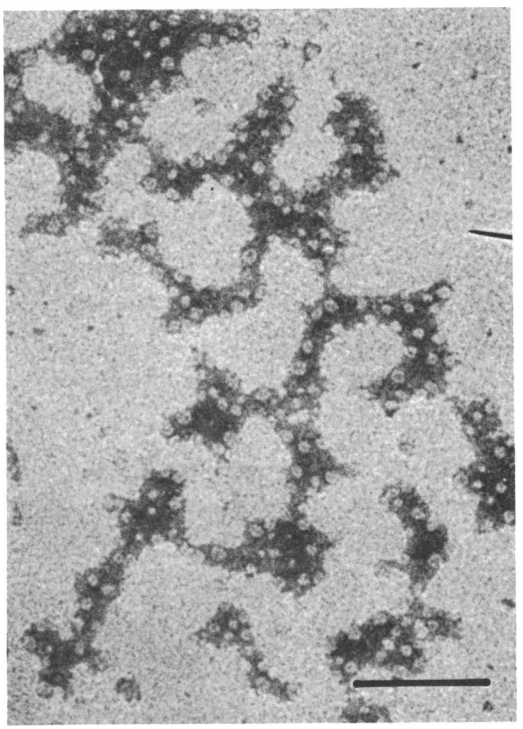

FIG. 5. *Subunit particles isolated from EB cell walls.*

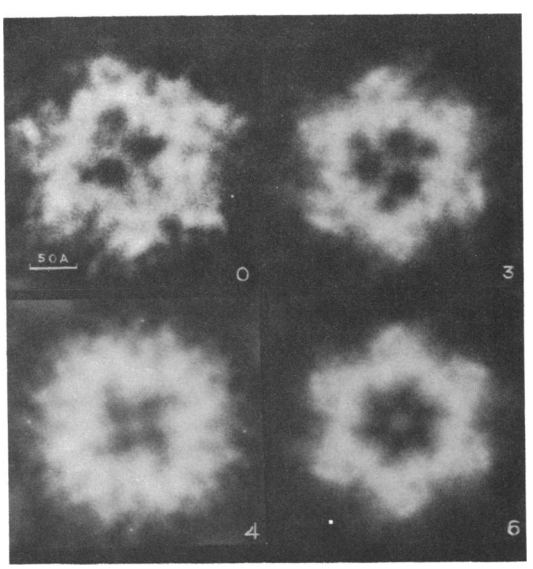

FIG. 6. *Application of the rotation technique to a subunit particle. The particle has been rotated 0, 3, 4, and 6 times.*

rotations; that is, one particle has six elements in hexagonal arrangement. On the basis of particle dimension and fine structure, it appears that these particles are the subunits seen in negative stains of the EB cell walls.

To determine whether any such structures could be found in RB, the following experiment was performed. Cell walls purified from EB, normal RB at 18 hr, and RB from 48-hr cultures with penicillin were treated simultaneously with formamide at 160 C for 10 min. They were then put on potassium tartrate density gradient columns and centrifuged at 70,000 × *g* for 120 min in an SW39 rotor. After hot formamide treatment, both RB cell wall preparations exhibited single bands at identical positions in the gradient (Fig. 7). Electron microscopic examination revealed that the structures contained in both these bands were very fine elongated particles, 5 to 6 nm in diameter (Fig. 7C). The EB cell walls showed two bands in the gradient: the upper band (Fig. 7A) occupied a position identical to that of the RB bands, but it was much sharper in appearance, and electron microscopic examination showed that it contained thin, fibrous structures. The lower gradient band of the EB cell walls (Fig. 7B) contained the hexagonal particles described above.

DISCUSSION

In these experiments, further evidence has been obtained that there are profound differences in the cell wall of *Chlamydia* during the various stages of the developmental cycle. The geometrically arrayed subunit was seen by negative staining to be present in all elementary body cell walls, but was not present in cell walls of reticulate bodies grown without penicillin for 18 hr after infection or with penicillin for 48 hr after infection. These subunits were isolated from

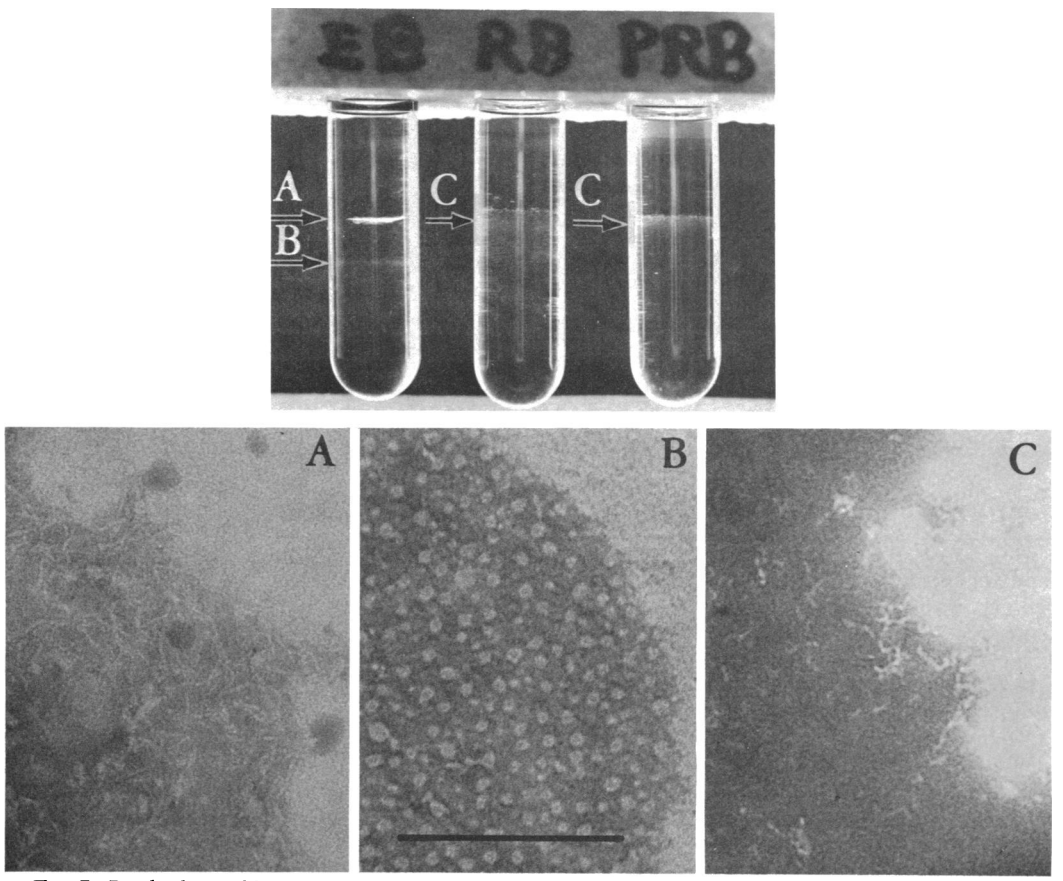

FIG. 7. *Bands obtained by potassium tartrate gradient centrifugation. Arrows indicate the positions of the bands. Left, EB cell walls; middle, RB cell walls from culture without penicillin; right, RB cell walls from penicillin-containing culture. Electron micrographs A, B, and C correspond to the above labeled bands.*

intact cell walls and partially purified by hot formamide extraction and were seen to have a hexagonal outline.

These experiments indicate that these subunits are on the inside part of the cell wall, and they are synthesized during the maturation process. When seen occasionally on the developmental RB, they are apparently left over from the parent EB and there is a rapid dilution of these subunit fragments as binary fission occurs. Very few older RB (24 hr after infection) show any subunits whereas all EB cell walls show a uniform array. The exact time during maturation when synthesis of the subunits occurs is not known.

Matsumoto and Manire (6) reported that the addition of penicillin to cultures of L cells infected with meningopneumonitis organisms completely inhibited the binary fission of RB and the maturation of RB to form EB, but it does not inhibit the growth of RB. In these experiments we have demonstrated that penicillin also completely inhibits the formation of the subunit component of the cell wall.

Although we have not yet determined the chemical composition or the immunological characteristic of the subunits, we have concluded that these structures are newly synthesized during maturation of RB to form EB, that their synthesis is inhibited by penicillin, that they are probably the structural units which provide strength to the EB cell wall which is lacking in the RB cell wall, and that the conversion of the rigid and relatively impermeable EB cell wall to the permeable and fragile RB cell wall is due to an outgrowth of the external wall layer in the absence of subunit synthesis rather than to the removal of an external cell wall layer.

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