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Electron Microscope Observations on the Effects of Polymixin B Sulfate on Cell Walls of *Chlamydia psittaci*

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The effects of polymixin B sulfate on cell walls of mature elementary body (EB) and of immature developmental reticulate body (RB) of *Chlamydia psittaci* were investigated. When purified EB were treated with polymixin (10^4 units per ml or more) at 37 C for 60 min, about 70% of EB was found to be covered with a number of projections. Further incubation did not increase the percentage affected. The infectivity after treatment as assayed by the inclusion counting technique was reduced by 70% of the original titer. These results suggest that EB with the projections are no longer infective. The projections had obscure outlines and were 20 to 40 nm in diameter when seen in thin sections. In the negatively stained preparations, the projections were composed of aggregations of fine particles 4 to 5 nm in diameter. Treatment with sodium dodecyl sulfate at the same concentration used for cell wall isolation removed the projections completely, and the cell walls were converted to rather ragged forms apparently composed of outside and inside layers. When RB cell walls prepared from infected cells at 18 hr after infection were treated with polymixin at the same concentration, the projections having the same morphology with those seen on treated EB cell walls were observed only on the inside surface of cell wall.

Two different types of particles are found in the developmental cycle of *Chlamydia;* one is the rigid infectious elementary body (EB), and the other is the fragile developmental reticulate body (RB) (1, 2). In the previous report on the fine structure of cell walls, Matsumoto and Manire showed a distinct structural difference between the two forms (8). The cell walls of EB had hexagonally arrayed subunit structure, 18 nm in diameter, on the inside surface, whereas RB cell walls did not have this structure. Although RB cell walls were synthesized continuously in the presence of penicillin, the formation of EB cell walls was completely inhibited. Consequently, the formation of cell walls with the hexagonally arrayed structure is inhibited, and the rigidity of the cell walls remains as it is in RB cell walls, suggesting that the regularly arrayed units are responsible for the rigidity of EB cell walls (7, 15). Chemical investigations by Manire and Tamura demonstrated that the phospholipid content in EB cell walls is much greater than that of RB cell walls, that EB cell walls contain all common natural amino acids, and that the composition of amino acids is similar to that of the cell walls of gram-negative bacteria, such as *Escherichia coli* (6). However, no sulfur-containing amino acids, i.e., methionine and half cystine, were contained in RB cell walls (14).

The bactericidal effects of the polymixin group of antibiotics against gram-negative bacilli, including *Pseudomonas* organisms, apparently result from the action of the drug on cell walls with destruction of osmotic regulation (3, 10). Electron microscope studies on the effects of polymixin B on *E. coli* and *P. aeruginosa* revealed the formation of numerous projections on the cell wall surface and the leakage of cell content through the projections (4).

The experiments reported here suggest a similar mechanism for the action of polymixin B sulfate on *Chlamydia* and a remarkable structural difference between EB and RB cell walls.

MATERIALS AND METHODS

Organisms and cells. The meningopneumonitis strain of *Chlamydia psittaci* was routinely cultured in suspended L cells as previously reported (12).

Preparation of EB and EB cell walls. Purified EB from infected cells at 48 hr after infection were prepared by the method previously reported (12).

The cell walls were isolated by using purified EB as starting material by the method reported previously (6, 17). Samples (5 ml) of purified EB suspension were homogenized with 5 g of glass beads (0.1 mm in diameter) in a Cell-Mill shaker (Edmund Bühler, Tübingen, West Germany) at 70 cycles per sec for 5 min. After sucrose density gradient centrifugations (5 to 45%, w/v) in an SW25 rotor at 6,000 rev/min for 30 min, the cell walls in a middle broad band in the column were collected and treated with a mixture of deoxyribonuclease and ribonuclease (10 μg/ml, each) for 120 min. This was followed with trypsin treatment (0.1 mg/ml) for 60 min at 37 C and finally with 0.5% of sodium dodecyl sulfate (SDS) solution for 60 min at 37 C.

Preparation of RB cell walls. RB cell walls were prepared by the method previously reported (14, 17). L cell cultures infected at high multiplicity of infection (more than 100 MOI) were harvested at 18 hr after infection. RB were purified by the method reported previously (16). The RB fraction obtained was treated with a mixture of deoxyribonuclease and ribonuclease (10 μg/ml, each) and then with trypsin (0.1 mg/ml) for 60 min at 37 C. The fraction was finally treated with 0.25% SDS solution for 60 min at 36 C.

All preparations, i.e., EB, EB cell walls, and RB cell walls, were examined for purity by electron microscopy before further experimental use.

Treatment with polymixin B sulfate. Polymixin B sulfate (purchased from Nutritional Biochemical Corp., Cleveland, Ohio) in 0.2 M tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 7.4 was mixed with a suspension containing 2×10^7 whole EB, EB cell walls, or RB cell walls per ml, and the mixture was incubated at 37 C for various intervals with gentle agitation.

Electron microscopy. Whole EB, EB cell walls, or RB cell walls, washed and suspended in Tris buffer, were spread on a copper grid using a smooth agar surface (11). One drop of the sample suspension was spread on a smooth-surfaced agar plate (1 cm^2, 0.5 cm in thickness) and dried at room temperature (25 C). The surface was covered with 1% collodion, and then the excess of collodion was removed with a filter paper. The collodion film was removed from the agar surface by sinking it gradually into redistilled water, and then the film floating on the water surface was transferred to copper grids. To observe the specimen surface and to count the number of EB under an electron microscope, some specimens prepared were shadow-cast with platinum-palladium alloy twice with the second shadow-cast at a 90° angle from the first. The grazing angle of the metal beam to the specimen was 10°.

Identical samples were negatively stained by adding 1% phosphotungstic acid (PTA) solution at *p*H 7.4 directly onto the dried samples on the grids, which were then dried immediately. For negative staining of RB cell walls treated with polymixin alone, the sample suspended in distilled water was mixed with the same volume of 2% PTA solution and then put on the grids covered with a glow-discharged carbon film. After removal of the excess of sample suspension, the grids were dried at room temperature.

For thin sectioning, the samples were doubly fixed with 1% glutaraldehyde at *p*H 7.4 with phosphate buffer solution and 1% OsO_4 in Veronal acetate buffer solution at *p*H 7.4, dehydrated in acetone, and then embedded in Vestopal W. The thin sections cut on an LKB ultratome were doubly stained with saturated uranyl acetate solution (18) and lead hydroxide solution (9) to enhance the specimen contrast, and then examined with a JEM 6C electron microscope with 30-μm objective aperture, at 80 kv.

RESULTS

Biological analysis of the effects of polymixin. Figure 1 shows a group of EB treated with polymixin B. Although some EB were affected and were covered with a number of projections, some appeared to be quite normal. To determine the effect of concentration of polymixin, 10^7 particles of EB per ml were treated with 10, 10^2, 10^3, 10^4, and 10^5 units of the drug per ml at 37 C for 60 min, washed, and then examined (Fig. 2). At a concentration of 10^4 and 10^5 units per ml, about 70% of EB were affected. To determine the effect of time of incubation, EB incubated in 10^4 units of the drug per ml were examined after 0, 5, 10, 15, 20, 30, 40, 60, and 90 min of treatment (Fig. 3). There was an increase in the number of EB affected up to 60 min, but no further increase occurred as about 30% of the EB remained unaffected. The effect of polymixin treatment on infectivity was then studied. Monolayers of L cells on cover slips in Leighton tubes, containing 1.2 to 1.5×10^5 cells per tube, were infected with EB which had been treated with the drug (10^4 units per ml) for 60 min, washed, and then diluted to 10^{-3} or 10^{-4} with fresh culture medium before infection. At 8 hr after infection, the cells were harvested, stained by the Giemsa staining procedure, and examined to determine the number of inclusions seen by light microscopy. The inclusions could be distinguished readily as globular clusters of a number of RB stained deep purple in color. The results are summarized in Table 1. About 25 to 30% of original infectivity remained after treatment; that is, about 70% of EB are inactivated with polymixin. These results, obtained from both electron microscopy and biological experiments, suggest that EB with the projections have no infectivity.

Morphological changes of EB and EB cell walls with polymixin. Thin-section profiles of treated EB are shown in Fig. 4. The inserted figure is from an untreated, normal

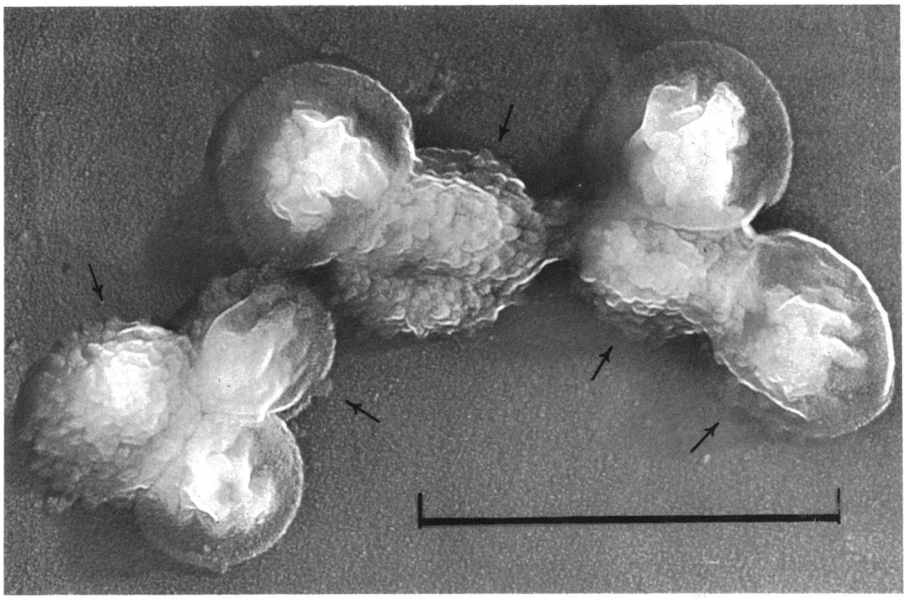

FIG. 1. *Shadowed EB after treatment with 10^4 units of polymixin B sulfate per ml for 60 min at 37 C. EB with projections are pointed by arrows. Bar indicates 1 μm.*

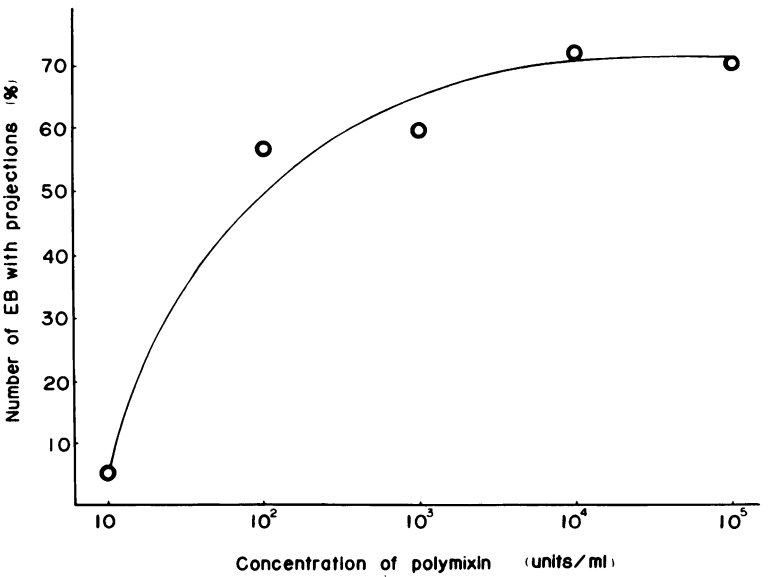

FIG. 2. *Relative number of EB with projections formed with the various concentration of polymixin.*

EB. Definite changes can be seen not only on the cell wall, but also in the cytoplasm. Projections on the outside surface of the cell walls have obscure outlines and particulate, sometimes membranous appearances. The diameter of the projections ranged from 20 to 40 nm. However, the details of the connection between the cell wall surface and each projection are not clear. The clear definition of the nucleoid and cytoplasm in the normal EB (Fig. 4, insert) is completely missing in the treated EB. Some large masses with high density and irregular lamellar structures were frequently observed.

Purified EB cell walls were also affected by polymixin (Fig. 5, insert). In this case, it could be seen that such projections occurred on both inner and outer aspects of the cell wall, with the greater number on the outer surface.

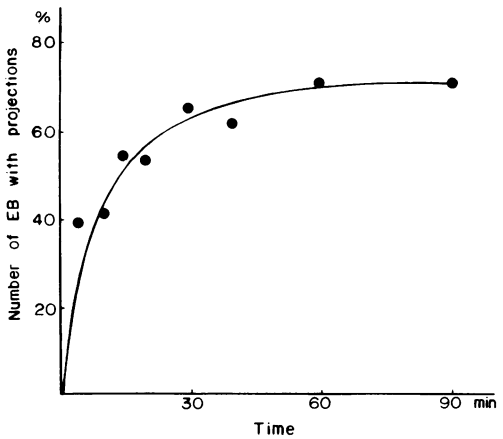

FIG. 3. *Relative number of EB with the projections formed with 10^4 units of polymixin per ml during various intervals of incubation at 37 C.*

In routine preparation, EB cell walls were treated with SDS to eliminate cytoplasmic membranes and the other cellular components, and the resulting cell walls were resistant to SDS (6, 17). However, when the cell walls treated with polymixin were incubated in SDS of the same concentration (0.5%) used in the isolation procedure, the projections completely disappeared (Fig. 5). In most cases, such treated cell walls appeared normal with the cell wall appearing as a triple-layered membrane approximately 8 nm in thickness, the outside layer 2 to 2.5 nm and the inside layer 2.5 to 3 nm in width. In some cell walls sectioned obliquely, however, several pores are clearly seen (Fig. 5, arrows). It is most likely that these pores are located at the loci of projections removed with SDS treatment. When the cell walls were stained negatively, these effects were seen more clearly (Fig. 6). This figure shows an untreated cell wall (Fig. 6a), a polymixin-treated cell wall with projections (Fig. 6b), and an SDS-treated cell wall showing the disarray of the cell wall component. A comparison of the treated and the normal cell walls demonstrated that the normal EB cell wall contained numerous fine particles 4 to 5 nm in diameter and that the sequential treatments with polymixin and SDS caused remarkable disintegration of the arrangement of the particles. The projections were also composed of massive aggregation of the fine particles.

Morphological changes of RB cell walls with polymixin. In RB cell wall preparation stained negatively, outside and inside surfaces appeared to be composed of numerous fine particles, which were similar to those seen in the outside surface of EB cell wall (Fig. 7a). When these cell walls were treated with the drug (10^4 units per ml), many projections occurred (Fig. 7). Their morphology was very similar to the projections seen in treated EB cell wall. All projections were seen only within the cell wall outlines. This indicates that the projections in treated RB cell walls were not formed on the outside surface but only on the inside surface.

After sequential treatments with the drug and SDS, the projections completely disappeared and the cell walls changed into much more ragged forms than the remnants of EB cell walls after the same treatments (Fig. 7b).

DISCUSSION

EB cell wall in thin-sectioned preparation was seen as a trilaminar membrane by electron microscopy, i.e., outside and inside dense layers binding a less dense intermediate layer (17). In negatively stained preparation, the outside layer of EB cell wall appeared to be composed of very fine particles 4 to 5 nm in diameter (Fig. 6a), whereas the inside layer was composed of a hexagonally arrayed subunit structure (5, 8). When EB was exposed to polymixin, many projections were formed on its surface, and intracellular structure was greatly disturbed (Fig. 4). This was apparently the cell wall alteration resulting from the effect of polymixin, since isolated cell wall was also affected in a similar manner by the polymixin treatment. These results indicate that EB response to the drug is similar to that observed in the gram-negative bacterial cell walls (4). However, the drug concentration (10^4 units per ml; this corresponded to about 0.5 mg or more of the

TABLE 1. *Number of inclusions in cell cultures infected with polymixin B sulfate[a] -treated EB*

Expt	No. of inclusions/cell (%)[b]		Relative survival rate (%)
	Control	Treated	
I	12.70 (131/1031)	3.24 (36/1109)	25.51
II	2.99 (30/1002)	0.88 (10/1124)	29.43

[a] 10^4 units/ml at 37 C for 60 min.

[b] Numbers in parentheses: each numerator indicates the number of inclusions observed in the cells shown as the denominator. The proportional relationship between number of inclusions and infectivity was proved when less than 50% of cells in culture were infected (13). Therefore, the percentages shown in this table demonstrate actual decrease of infectivity.

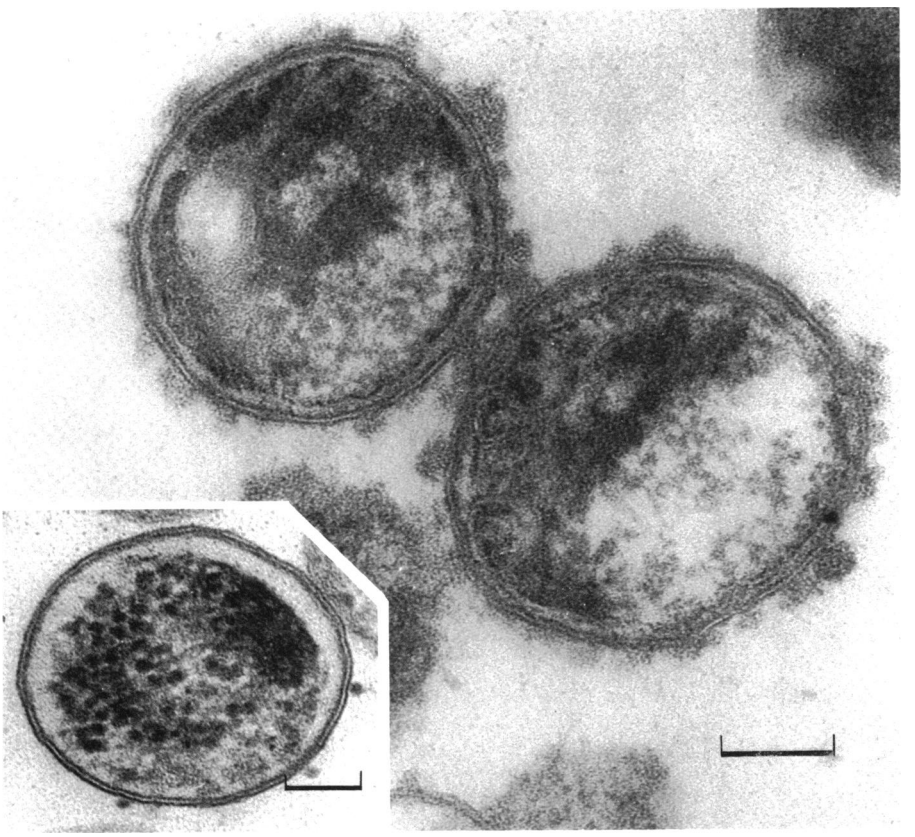

FIG. 4. *Thin-sectioned EB after treatment with 10^4 units of polymixin per ml for 60 min at 37 C. Insert, Normal EB. Bars indicate 0.1 μm.*

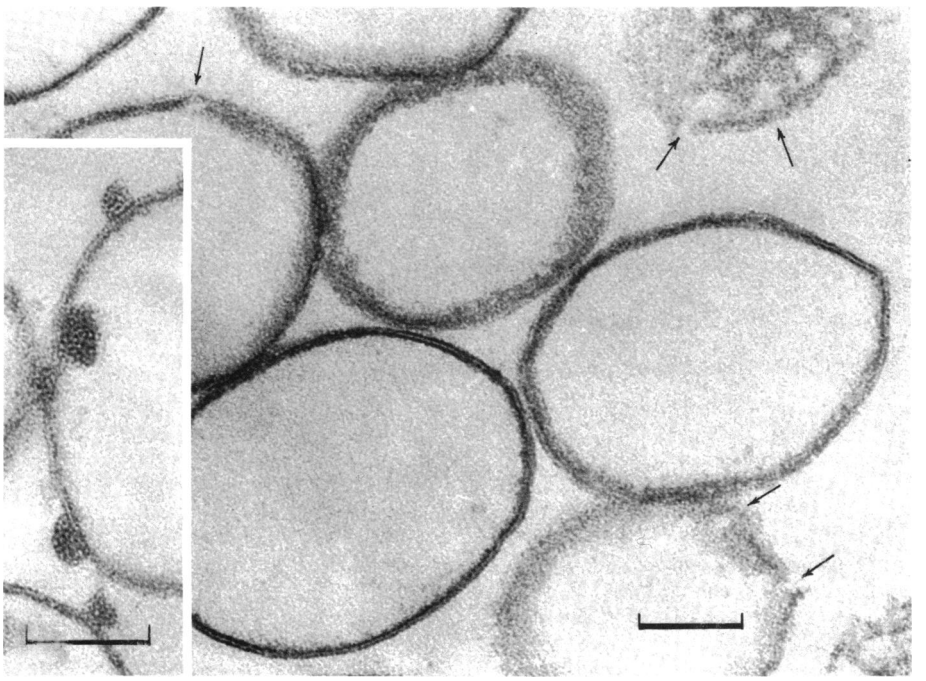

FIG. 5. *Thin-sectioned EB cell walls after treatments with polymixin and SDS. Arrows indicate the pores. Insert, Cell wall treated with polymixin alone. Bars indicate 0.1 μm.*

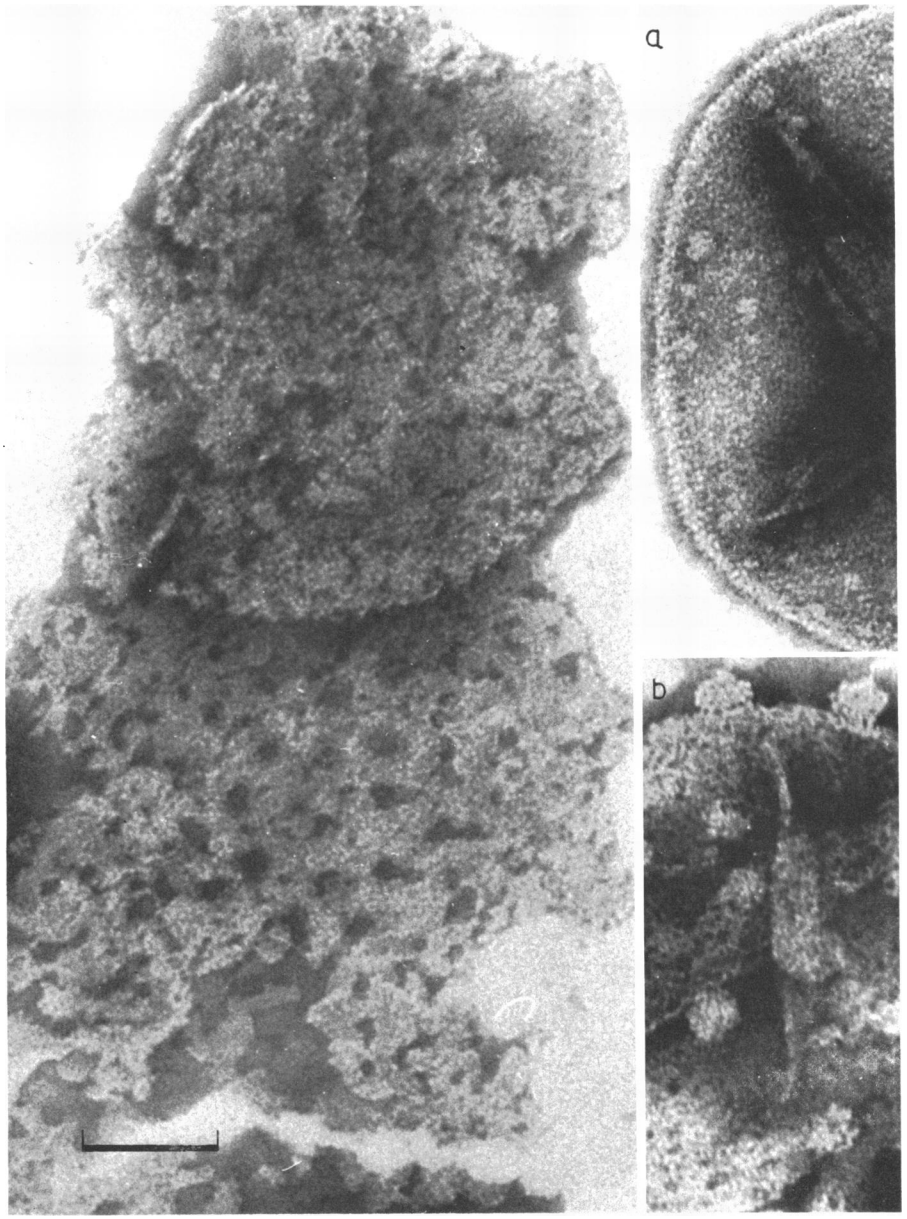

FIG. 6. *Negatively stained EB cell walls after treatments with polymixin and SDS. a, Part of untreated, normal cell wall surface; b, part of cell wall treated with polymixin alone. All figures are at the same magnification. Bar indicates 0.1 μm.*

drug per ml) which caused the maximal reduction of infectivity and the greatest number of projections on EB was almost 20 times greater than that for *E. coli* B cell wall (4).

The projections formed with polymixin were completely dissolved with SDS, and the cell walls were converted to rather rugged forms in negatively stained preparations (Fig. 6). In obliquely sectioned cell walls, many pores appeared, but no remarkable morphological changes were seen in the cross-sectioned cell wall (Fig. 5). From this evidence, it has been suggested that the drug affects some localized sites on, but not the entire, surface layer, and that the pores indicate the loci of these sensitive sites against polymixin. The characteristic con-

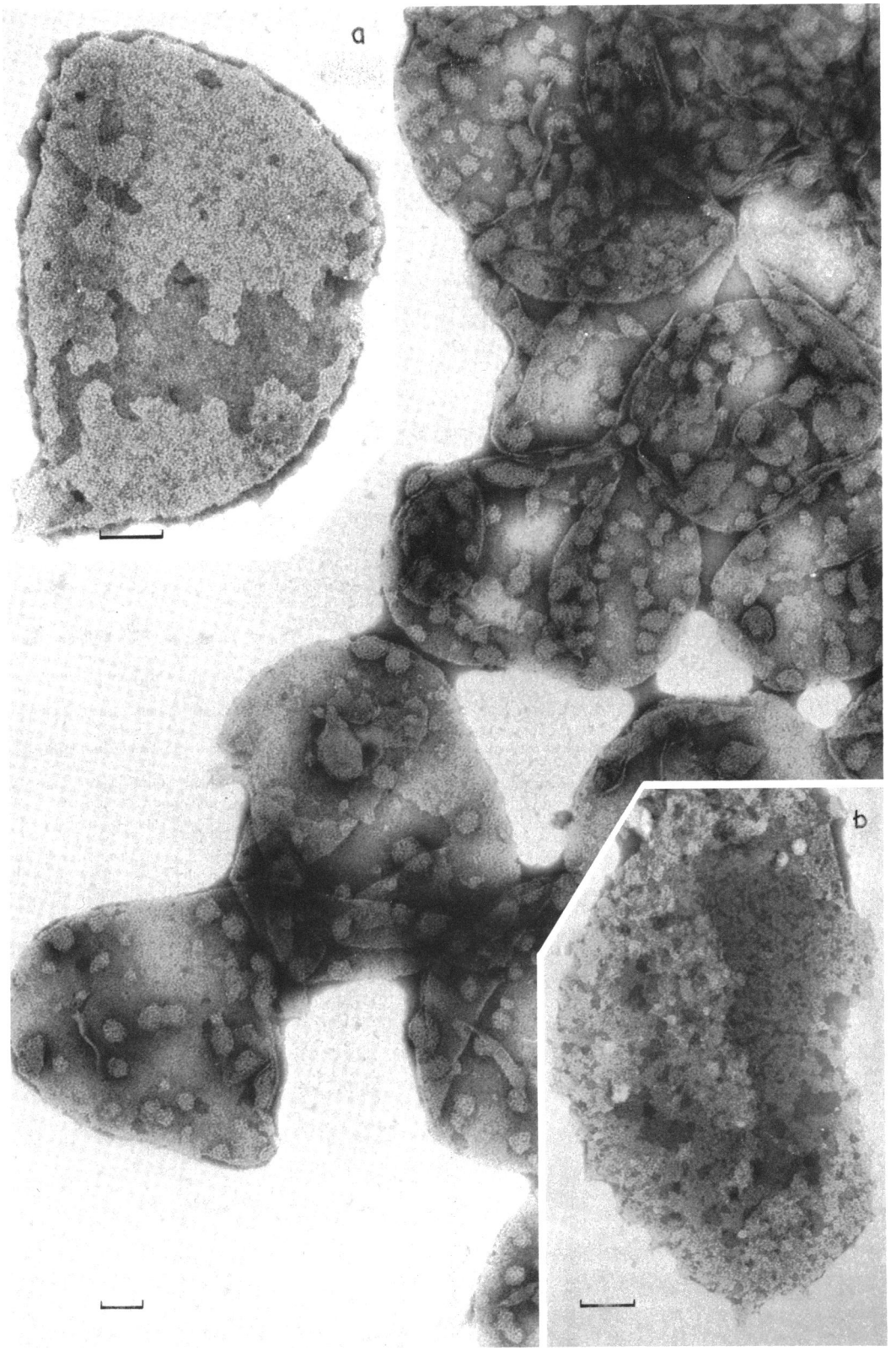

FIG. 7. *Negatively stained RB cell walls after treatment with 10^4 units of polymixin per ml. a, Normal RB cell wall, dark area shows inside surface of cell wall, less dense area is outside surface; b, RB cell wall treated with polymixin and SDS. Bar indicates 0.1 μm.*

version from SDS-resistant cell wall to SDS-soluble projection suggests that the macromolecular structures of the specific sites are changed to SDS-sensitive configuration by polymixin.

In RB cell wall, the projections occurred only on the inside surface (Fig. 7). This was a striking contrast to EB cell walls in which the projections were formed mostly on the outside and rarely on the inside surfaces (Fig. 5). Both outside and inside layers of RB cell wall were composed of fine particles (Fig. 7a) similar to those in the outside surface of EB cell wall. The specific sites attacked by polymixin could not be distinguished morphologically.

As described above, the infectivity of treated EB was reduced to about 30% of the original titer after polymixin treatment. This ratio coincides with that of EB without projections among treated EB. No explanation of EB resistance to polymixin is known.

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LITERATURE CITED

1. Higashi, N. 1964. The mode of reproduction of the psittacosis-lymphogranuloma-trachoma viruses. Int. Rev. Exp. Pathol. **3**:35-64.
2. Higashi, N. 1965. Electron microscopic studies on the mode of reproduction of trachoma virus and psittacosis virus in cell cultures. Exp. Mol. Pathol. **4**:24-39.
3. Kay, L. L., and G. B. Chapman. 1963. Cytological aspects of antimicrobial antibiosis. III. Cytological distinguishable stages in antibiotic action of colistin sulfate on Escherichia coli. J. Bacteriol. **86**:536-544.
4. Koike, M., K. Iida, and T. Matsuo. 1969. Electron microscopic studies on mode of action of polymixin. J. Bacteriol. **97**:448-452.
5. Manire, G. P. 1966. Structure of purified cell walls of dense forms of meningopneumonitis organisms. J. Bacteriol. **91**:409-413.
6. Manire, G. P., and A. Tamura. 1967. Preparation and chemical composition of the cell walls of mature infectious dense forms of meningopneumonitis organisms. J. Bacteriol. **94**:1178-1183.
7. Matsumoto, A., and G. P. Manire. 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. **101**:278-285.
8. Matsumoto, A., and G. P. Manire. 1970. Electron microscopic observations on the fine structure of cells walls of *Chlamydia psittaci*. J. Bacteriol. **104**:1332-1337.
9. Millonig, G. 1961. A modified procedure for lead staining of thin sections. J. Biophys. Biochem. Cytol. **11**:736-739.
10. Newton, B. A. 1956. The properties and mode of action of the polymixins. Bacteriol. Rev. **20**:14-27.
11. Sharp, D. G. 1958. Sedimentation counting of particles via electron microscopy, p. 542-548. *In* W. Bargman, G. Mollenstedt, H. Niehrs, D. Peters, E. Ruska, and C. Wolpers (ed.), 4th Int. Conf. Electron Microsc. Band II. Biologisch-Medizischer Teil.
12. Tamura, A., and N. Higashi. 1963. Purification and chemical composition of meningopneumonitis virus. Virology **20**:596-604.
13. Tamura, A., M. Iwanaga, and N. Higashi. 1961. Biochemical studies of meningopneumonitis virus multiplication in L cell suspension culture. (Japanese) Virus **11**:386-393.
14. Tamura, A., and G. P. Manire. 1967. Preparation and chemical composition of the cell membranes of developmental reticulate forms of meningopneumonitis organisms. J. Bacteriol. **94**:1184-1188.
15. Tamura, A., and G. P. Manire. 1968. Effect of penicillin on the multiplication of meningopneumonitis organisms (*Chlamydia psittaci*). J. Bacteriol. **96**:875-880.
16. Tamura, A., A. Matsumoto, and N. Higashi. 1967. Purification and chemical composition of reticulate bodies of the meningopneumonitis organisms. J. Bacteriol. **93**:2003-2008.
17. Tamura, A., A. Matsumoto, G. P. Manire, and N. Higashi. 1971. Electron microscopic observations on the structure of the envelopes of mature elementary bodies and developmental reticulate forms of *Chlamydia psittaci*. J. Bacteriol. **105**:355-360.
18. Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. **4**:475-478.