Ultrastructural Study of Chitosan Effects on Klebsiella and Staphylococci

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Antibacterial effect of chitosan on the morphofunctional organization of clinical strains of *Klebsiella pneumoniae* and *Staphylococcus aureus* was studied by transmission electron microscopy. Chitosan promoted aggregation of bacterial cells and disorganization of bacterial cell wall and cytoplasmic membrane, which leads to the release of bacterial contents into the environment. These structural changes result in bacterial death.

Key Words: *Klebsiella pneumoniae; Staphylococcus aureus; chitosan; ultrastructure*

Chitosan and its derivatives are actively used in medicine for the treatment of pyoinflammatory diseases [7]. Clinical microbiological studies of the effect of chitosan on bacteria demonstrated its pronounced antibacterial effect. However, the mechanisms of this effect remain poorly studied.

In order to clear out the mechanisms of the effect of chitosan on bacterial cells, we studied the ultrastructural organization of clinical strains of *Klebsiella pneumoniae* and *Staphylococcus aureus* (gram-negative and gram-positive microorganisms) under the effect of chitosans with medium-viscous molecular weight (Mv) 6 and 27 kDa and 85% deacetylation degree.

MATERIALS AND METHODS

High-molecular-weight chitosan from Paralithodes camtschatica shell (Bioprogress Firm) was used. Low-molecular-weight chitosans were obtained by hydrolysis of commercial chitosan by means of *Streptomyces kurssanovii* chitinolytic complex [5]. The chitosan Mv was estimated using previously offered equation and coefficients [4], chitosan deacetylation degree was evaluated as described previously [8].

Klebsiella pneumoniae and *Staphylococcus aureus* were isolated from patients by inoculation of wound discharge into blood agar. To this end, 24-h cultures were placed into 0.1 M acetate buffer (pH 6.5) and 1% chitosan with Mv of 6 and 27 kDa and 85% deacetylation was added. The cultures were then inoculated in three-sugar agar containing glucose, lactose, and sucrose (HiMedia). After 24 h the cultures were fixed by the method of Ito [6], dehydrated in ascending alcohols, and embedded in LR White (PLANO W. Plannet GmbH) [9].

Ultrathin sections (15 nm) were made on an LKB-3 ultramicrotome, stained by Reynolds' method [10], and analyzed under a GEM-100B electron microscope. Preparations for ultrastructural study of chitosan submicroscopic organization were made *in vitro* by precipitation of chitosan with 0.1 M NaOH and subsequent embedding in LR White without intermediate dehydration in alcohols.

RESULTS

After chitosan was added into *Klebsiella pneumoniae* and *Staphylococcus aureus* cultures, only few colonies grew in nutrient medium, while control inoculation of the same cultures, not treated with chitosan, produced

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complete growth on dishes. This seems to indicate a bacteriostatic effect of chitosan; viable cells capable of growth in nutrient media are retained in chitosan-treated culture, but the number of these cells markedly decreased.

Preliminary study of chitosan ultrastructural organization was carried out for detecting it in experimental samples during interaction with bacterial cells. Chitosan structure presented as an amorphous heterogeneous bulk of medium electron density with more dense small incorporations (Fig. 1, *a*).

Comparative ultrastructural analysis of *Klebsiella pneumoniae* first passage cultures (control and incubated with chitosan) showed differences in the fine structure of the bacteria. Cells in control preparations had a pronounced reticular capsule (40-60 nm). The thickness of the cell wall (CW) was 30-35 nm. The contour of the outer layer of CW was clearly seen along the entire cell perimeter and was characterized by high electron density. The inner CW layer was also well discernible on all sections. Ribosomes and polysomes were clearly seen in dense cytoplasm of control cells; nucleotide zones were seen in some areas of the cytoplasm (Fig. 2, *a*).

The structure of the capsule did not change after exposure of Klebsiella to chitosans with different Mv, but thinned in some cells, the cytoplasm density and nucleotide structure remained unchanged.

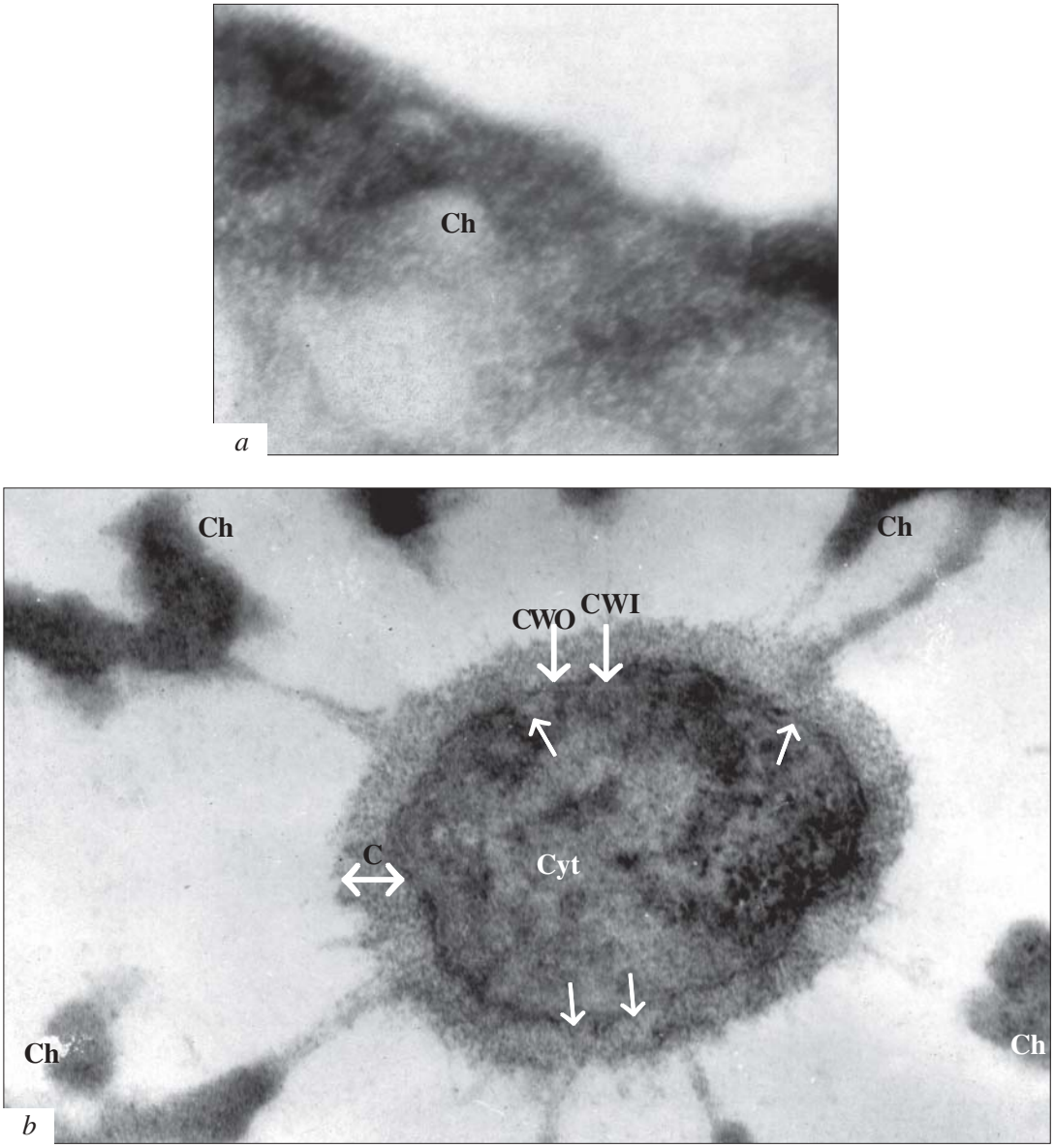

Fig. 1. *Klebsiella pneumoniae* ultrastructure under the effect of chitosan. *a*) chitosan structure on ultrathin sections, ×105,000; *b*) *Klebsiella pneumoniae* in close contact with chitosan, ×100,000. Ch: chitosan. Here and in Figs. 2, 3: CWI: cell wall inner layer; CWO: cell wall outer layer; Cyt: cytoplasm; C: capsule. Arrows show sites of cell wall destruction.

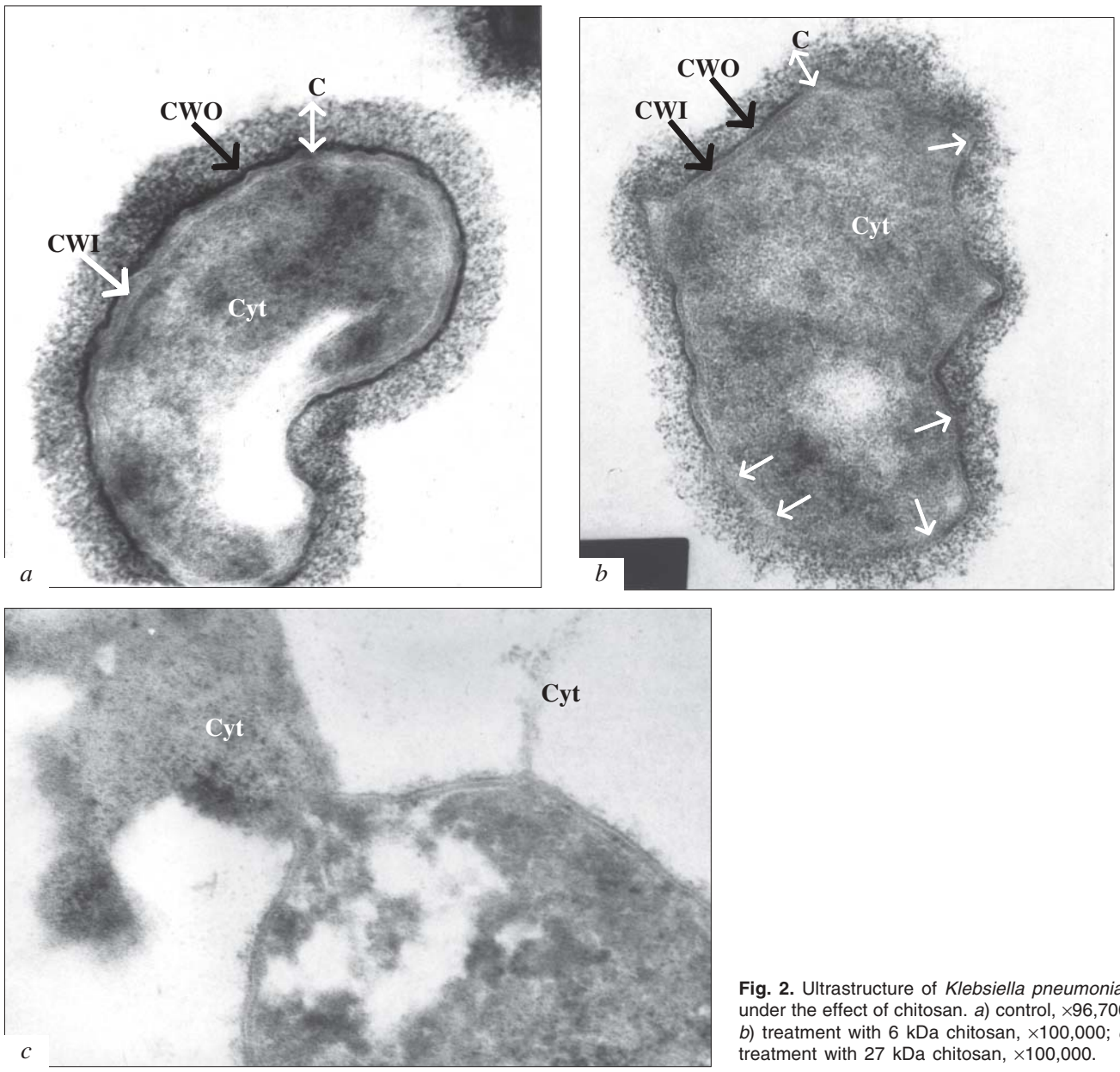

Fig. 2. Ultrastructure of *Klebsiella pneumoniae* under the effect of chitosan. *a*) control, ×96,700; *b*) treatment with 6 kDa chitosan, ×100,000; *c*) treatment with 27 kDa chitosan, ×100,000.

Chitosan was largely washed from electron microscopic preparations during their dehydration, but its presence could be detected (radial cords connected to CW surface, Fig. 1, *b*). Some cells were in close contact with each other and bulks of substance identical to chitosan were situated between them. This fact indicates that chitosan binds to the bacterial cell capsular structures. This type of interaction can lead to cell "adhesion" into colonies, which is essential for the cultural characteristics of the population [3].

The main morphological sign of chitosan effect on Klebsiella is fragmentation of CW outer layer, loosening of the inner layer (Fig. 1, *b*, 2, *b*), and release of cytoplasmic matrix from the cell in these sites (Fig. 2, *c*). Injuries in the CW structure were more distinct in cultures treated with 27 kDa chitosan than in those treated with 6 kDa chitosan.

Staphylococcal cells in control preparations had thick CW (20-30 nm) consisting of two layers: homogeneous outer layer of medium electron density and inner layer of high electron density, which grew together with the outer layer of the cytoplasmic membrane (CPM), forming the so-called membrane-wall complex (Fig. 3, *a*).

Structural changes in staphylococci under the effect of chitosan with Mv 6 kDa are linked with the membrane-wall complex; the structure of the inner electron-dense CW layer and the adjacent CPM is impaired. No changes were detected in other structural components of the cells (Fig. 3, *b*).

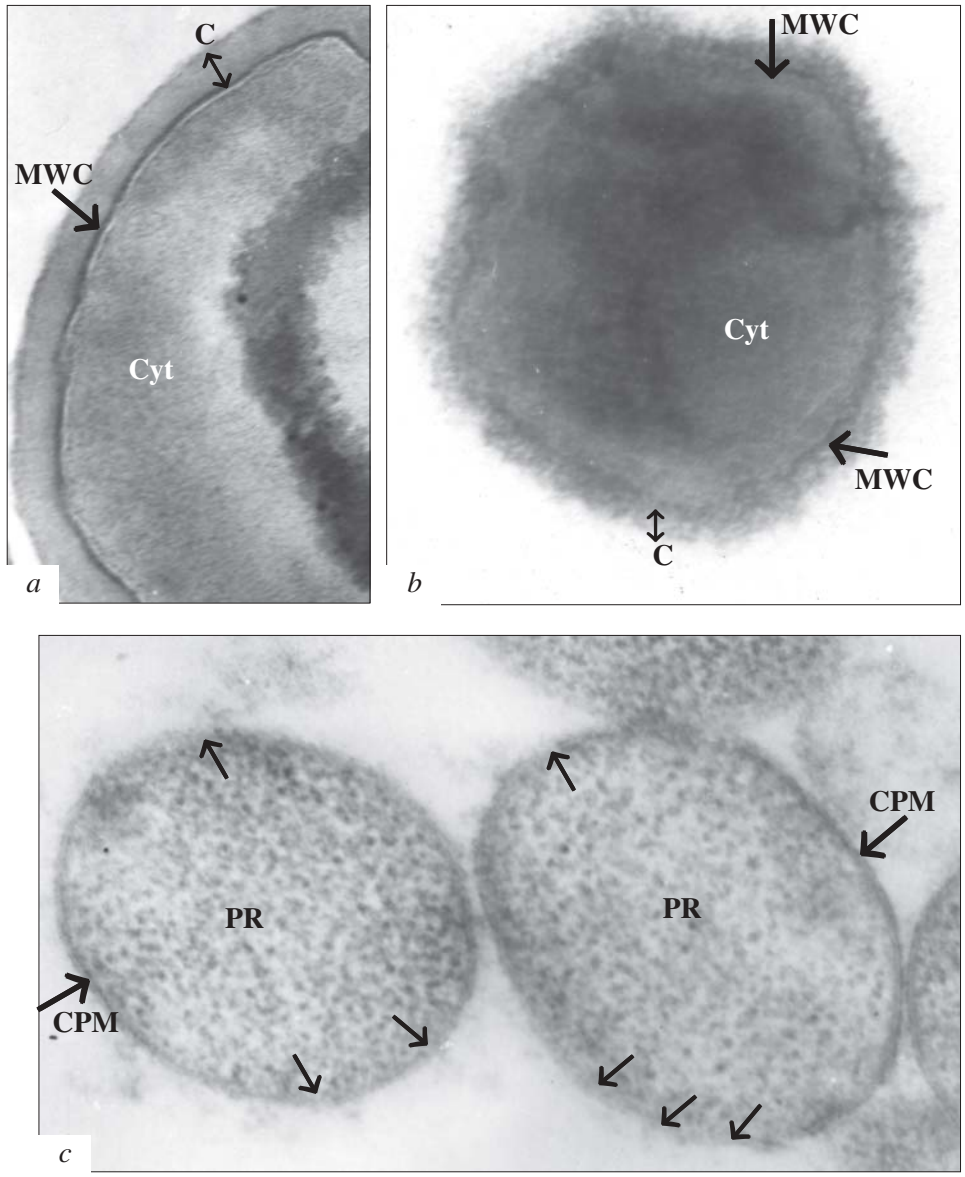

Fig. 3. Ultrastructure of *Staphylococcus aureus* under the effect of chitosan. *a*) cell fragment (control), ×136,500; *b*) treatment with 6 kDa chitosan, ×126,900; *c*) treatment with 27 kDa chitosan, ×98,400. MWC: membrane-wall complex; PR: protoplasts; CPM: cytoplasmic membrane.

Treatment of staphylococci with 27 kDa chitosan resulted in the appearance of protoplasts (bacteria completely lacking CW), in which CPM outer layer was fragmented (Fig. 3, *c*).

Hence, the effect of chitosan on the submicroscopic organization of bacteria of different systematic groups is the same in principle and consists in impairment of surface cell structures. Aggregation of bacterial cell under the effect of chitosan observed in our study is associated with impairment of the architectonics of bacterial colonies and modification of hierarchic interrelationships inside the colony. The final result of these disorders can be bacterial death in an isolated colony.

Cell wall damage in the studied bacteria (representatives of gram-negative and gram-positive microflora) can lead to impairment of the cell rigidity and mitotic processes, determining the death of individual cells and population in general.

Chitosan effect on bacterial forms with defective CW characterized by higher resistance to antibiotics and other antibacterial agents deserves special interest. These forms of bacteria appear in infectious allergic diseases, after antibiotic therapy, maintaining the chronic course of an infectious process [1].

Structural changes in the cells of antibiotic-resistant bacterial strains are more pronounced under the effect of antibiotics in bacteriostatic doses than under

the effect of chitosan. This does not diminish the significance of chitosan as an antibacterial agent, as bacteria do not develop chitosan resistance. This is particularly important in the treatment of infections caused by bacterial strains with multiple antibiotic resistance in wound and burn infections, when the "adhesive" effect of chitosan is maximally pronounced.

Disorders in the bacterial surface structures lead to modification of their metabolism. For example, sugars are the main energy sources for the majority of bacteria. Competitive replacement of sugar receptors with chitosan was hypothesized [2].

The detected disorders in the submicroscopic organization of CW and CPM can indicate destruction of receptors or be a morphological manifestation of competitive replacement of sugar receptors with chitosan. Our data on chitosan effect on the submicroscopic organization of gram-positive and gram-negative bacteria most likely indicate its bacteriostatic effect.

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