Effect of Bactericidal Substance from *Staphylococcus aureus* on Group A Streptococci

II. Structural Alterations

C. CARLYLE CLAWSON AND ADNAN S. DAJANI
Department of Pediatrics, University of Minnesota College of Medical Sciences, Minneapolis, Minnesota 55455

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Ultrastructural alterations brought about by treatment of a β-hemolytic streptococcus with a bactericidal substance from *Staphylococcus aureus* are described and illustrated. The substance causes an early condensation of nucleoid deoxyribonucleic acid (DNA) and a partial loss of ribosomes. These changes are followed by a dissolution of the cell contents resulting in bacterial “ghosts” composed of empty cell wall and capsule. These morphological findings correlate with known biochemical effects of the bactericidal substance on ribonucleic acid degradation and cessation of DNA and protein synthesis.

*Staphylococcus aureus* phage type 71 produces a bactericidal substance that is lethal for various gram-negative rods but has no effect on gram-negative rods (2a, 4). The group A β-hemolytic streptococci are uniformly sensitive to the action of the bactericidal substance and within this group M type 49 is the most sensitive (4). The addition of this proteinaceous bactericidal substance to susceptible streptococci produces immediate cessation of protein and deoxyribonucleic acid (DNA) synthesis and active degradation of preexisting and newly formed ribonucleic acid (RNA; 3).

Previous investigations (3) indicated that despite rapid killing of susceptible cells no lysis occurred, as evidenced by the stable optical density of the cell suspensions. Light microscopic examination failed to reveal significant alterations in the killed bacteria; therefore, fine structural changes were sought by electron microscopy. This report presents the ultrastructural features produced by the bactericidal substance on group A β-hemolytic streptococci. These structural alterations are correlated with the biochemical changes that are reported in the preceding communication (3).

MATERIALS AND METHODS

Bactericidal substance. The bactericidal substance was prepared from the supernatant fluid of broth cultures of *S. aureus* (strain C55) by methods presented in detail previously (4). A precipitate was obtained from the supernatant fluid with 85% saturation of ammonium sulfate. This precipitate was then redissolved in phosphate buffer and fractionated by ammonium sulfate in increments of 10% saturation. The fractions obtained between 60 and 80% were separated by gel filtration on a Sephadex G-100 column. The purified material was assayed by determining the highest dilution that would kill type 49 streptococci when grown on a blood-agar plate (4). Bactericidal substance active at a dilution of 1:32 was employed in these experiments.

Streptococcal cultures. M type 49 group A β-hemolytic streptococcus was studied as the prototype of susceptible streptococci. The bacteria were grown in Todd-Hewitt broth for 18 hr at 37 C. A 2 ml amount of the bactericidal substance in phosphate buffer (pH 7.2) was added to 0.5 ml of the cell culture. Controls consisted of phosphate buffer added to the bacterial cultures. Samples of the streptococcal cultures with bactericidal substance were taken for electron microscopy immediately after mixing and after 5, 15, 30 and 60 min of incubation at 37 C. Controls were sampled at mixing and at 60 min. Smears for Gram staining and light microscopic examination were also taken at each of these times.

Electron microscopy. The samples of streptococcal cultures were first mixed with an equal volume of 0.1% glutaraldehyde in balanced salt solution with sucrose (335 millimoles, pH 7.4; 9) and incubated for 15 min at 37 C as a gentle first fixation. The tubes were then transferred to a centrifuge at room temperature and spun at 1,300 × g for 10 min. The supernatant fluid was poured off and the pellet was resuspended in 1% glutaraldehyde in balanced salt solution without sucrose (415 millimoles). After 15 min at 37 C, the cell suspension was spun again to a pellet at 1,300 × g. This pellet was gently broken into 0.25- to 0.5-mm pieces and transferred to 1% osmic acid and 0.5%
RESULTS

Normal streptococcus at 0 and 60 min. The fine structure of the normal type 49 streptococcus illustrated in Fig. 1 to 3 is in agreement with previously published observations of group A streptococci (1, 2, 8). The bulk of the cytoplasm was filled with densely staining ribosomes in a lighter, amorphous matrix. The nucleoid lacked any distinct margin or limiting membrane and appeared as a region of fine filamentous material (DNA) lying on a clear background in the center of the cell (5). In the normal cells, these strands or filaments were quite fine and relatively lightly staining (Fig. 1).

In many sections, a region of special cytoplasm could be seen which consistently lay immediately adjacent to the cytoplasmic membrane (Fig. 2). This region was homogeneous, darkly staining, and devoid of ribosomes and was most often seen at the plane of cell division. This amorphous cytoplasmic region was 160 to 200 nm in diameter.

The cell periphery of the streptococcal cells consisted of several distinct layers. The innermost layer, the cytoplasmic membrane, appeared as a single dense line by the methods used in these experiments and was approximately 7.0 nm in thickness. Lying exterior to the plasma membrane and separated from it by a thin, electron-transparent zone were the cell wall, capsule, and protein coat (Fig. 3). This type 49 strain is a non-nucoid strain with a relatively thin capsule. The outermost layer of the cell periphery was a "fuzzy" layer which probably represented the protein coat which contains the M and other surface antigens (8). These "fuzzy" coats were not present on all cells and did not follow the cell wall where the latter invaginates at the site of cell division.

Streptococcus incubated with bactericidal substance. Treated bacteria were sampled at 0, 5, 15, 30, and 60 min after the addition of the bactericidal substance. Determinations of optical density of identical cultures and examination by Gram staining at each of these times revealed no cell lysis and only minimal morphological changes. These changes consisted of the presence of occasional large, irregularly shaped cells in the later samples.

Electron microscopy revealed visible interval change even in the zero-time sample. These effects of the bactericidal substance were not completely uniform within each time of sampling. By examining a large number of bacteria from each sample, the extremes of change for each time were noted and a pattern of alteration was defined. This pattern is illustrated in sequence in Fig. 4 to 9. Details of this pattern are seen in Fig. 10 to 18. The earliest changes noted in the 0- and 5-min cultures were in the nucleoid and the ribosomes. The nucleoid became more prominent with enlargement of the clear zone and dense aggregation of the DNA masses. The pattern of ribosomal distribution became altered in these early cultures. Regions of cytoplasm appeared which were devoid of identifiable ribosomes (Fig. 6, 13).

The mesosomes, which were usually tubular structures in the earlier treated cultures (0 and 5 min), lost this configuration and took a laminated, myelin-like form (Fig. 10 to 12). These probably underwent further degradation, because in later cultures all forms of identifiable mesosomes were rare, especially in the effete cells.

A prominent feature of the cultures at 15 min and later were cells which displayed abortive attempts at division (Fig. 7, 14 to 16). These were in the form of erratic membrane formation at the plane of division. These membranes were single or double and appeared to lack orientation in their formation. This gave the effect of wandering about the cytoplasm in apparent random fashion without accomplishing union with the opposite side or division. Some bacteria demonstrated the result of several incomplete divisions resulting in a large, misshapen cell (Fig. 16).

The final stage of cell death in these cultures was a process of dissolution of the entire cytoplasmic contents (Fig. 17). This resulted in bacterial "ghosts" of intact cell wall, capsule, and protein coat which lacked any organized internal structure (Fig. 18). All that remained in the interior were scattered remnants of the cytoplasmic membrane and a few dense precipitates. The one most stable region of the cytoplasm which resisted the dissolution process the longest appeared to be the 200-nm homogeneous mass described above in the normal cultures.
**FIG. 1. Normal type 49 β-hemolytic streptococcus.** A central nucleoid (arrow) is composed of fine filamentous material in a lightly staining matrix. The cytoplasm contains numerous darkly staining ribosomes. The cell is surrounded by a single densely staining layer and an outer very lightly staining layer. × 91,000.

**FIG. 2.** Region of a normal dividing streptococcal cell showing two amorphous regions of cytoplasm (arrows) which are free from ribosomes. These regions often lie at the plane of cell division. × 89,500.

**FIG. 3.** Cell periphery of a normal type 49 streptococcus. At the border of the dark cytoplasm is an indistinct plasma membrane of about 7.0 nm in thickness. Exterior to the plasma membrane is a broader dense layer, cell wall, and beyond that a very lightly staining smooth homogeneous layer which is the capsule. The most exterior layer seen here is a "fuzzy" protein coat. This latter is absent from the cell illustrated in Fig. 1 but present on most of the cells in these cultures. × 192,000.
FIG. 4. Control bacteria typical of both the zero-time and 60-min control cultures. These may be compared with the treated streptococci illustrated in Fig. 5 to 9. $\times$ 27,000.

FIG. 5. Streptococci taken immediately after mixing with the bactericidal substance. The nucleoid region of these cells appears more prominent than in the controls due to expansion of the clear zone and beginning condensation of the filamentous DNA material. $\times$ 32,000.

FIG. 6. Five minutes after addition of bactericidal substance. There has been extension of nucleoid condensation. There is a loss of ribosomes from some regions of cytoplasm (arrow). Mesosomes are prominent in these early treated cultures. $\times$ 28,000.
Fig. 7. Fifteen minutes after treatment with bactericidal substance. The erratic extension of membranes from the cell division plane suggests abortive attempts at cell division. These are seen in greater detail in Fig. 14 to 16. × 34,000.

Fig. 8. Thirty minutes after treatment. Cells undergoing internal clearing and empty bacterial “ghosts” have begun to appear. Other cells still display the effects of the bactericidal substance first seen in the earlier samples. × 30,000.

Fig. 9. Sixty minutes after treatment. At this time the majority of the streptococci are represented by bacterial “ghosts.” × 30,600.
DISCUSSION

Addition of the staphylococcal bactericidal substance to group A streptococci produced a sequence of events which led to the death and dissolution of the living components of the streptococcal cell. The earliest ultrastructural changes were a condensation of nucleoid DNA into dense aggregates and a loss of recognizable ribosomes from scattered areas of cytoplasm. Later abortive attempts at cell division were indicated by erratic development of membrane and the production of large, partially segmented cells. In the final stage, there was a dissolution of the nucleoid, mesosome, and the remainder of the cytoplasmic contents resulting in a bacterial “ghost.”

Specific biochemical sequelae to treatment with bactericidal substance have been documented for the type 49 streptococcus (3). Protein and DNA synthesis are stopped very soon after addition of the substance. This interruption in synthesis is not accompanied by any demonstrable increase in degradation of either protein or DNA. These data may be correlated with the structural changes demonstrated for the early cultures. There is condensation of the nucleoid material into dense DNA aggregates (5) and an expansion of the surrounding light zone. Only in the later cultures is the breakdown of the nucleoid obvious as the interior of the cell begins to be cleared. The general appearance of the cells in the early samples does not suggest any appreciable loss of structural components. This agrees with the noted lack of protein degradation. The cessation of protein production may be entirely secondary to the effect of the bactericidal substance on the bacterial RNA. This is a rapid onset of marked degradation of preexisting and newly formed RNA (3). The observed loss of ribosomes from scattered regions of cytoplasm in the early specimens reflects this RNA degradation.

The mesosomes of the early experimental cultures were usually present as tubules arranged in a well-ordered or paracrystalline array. These subsequently were altered and took on the whorl or myelin-like pattern (Fig. 10 to 12). The tubular appearance is in close agreement with the fine structure of the mesosomes as demonstrated by Pate and Ordal for the Chondrococcus columnaris (6). It should be noted that mesosomes were frequently encountered in all of the early experimental cultures but were not seen in the controls for this study or in several other streptococcal cultures examined by the authors. The role of the mesosomes and the significance of the failure to observe them in our normal cultures are not known.

Biochemical data from cultures identical to those used in this study demonstrated onset of action for the bactericidal substance within 1 min (3). Morphological observations reflected this rapid effect since structural changes were seen even in the zero-time sample. It should be noted, however, that the times indicated for sampling designate the initiation of fixation. The glutaraldehyde does not reach the interior of the cell immediately, and, no doubt, some alteration continued for a short time after fixation was started.

The finding of well-preserved bacterial “ghosts” composed of cell wall, capsule, and protein coat explains the lack of change in optical density and the paucity of light microscopic findings. The maintenance of these exterior cell investments throughout the time of the experiment reflects the stability of these structures and their lack of direct involvement in the action of the bactericidal substance.

Significant variability in structural modification from one cell to another was noted within a single sample. This finding suggests that the bactericidal substance does not act with equal rapidity on each organism in the culture. This may be
explained by a dose effect with some cells receiving less than the optimally effective amounts of bactericidal substance. It may also be true that the individual streptococcus is susceptible to the substance at only certain stages in its sequence of metabolism.

The possible relationship of the bactericidal substance to bacteriocins has been discussed (2a). The mode of action of various bacteriocins was reviewed by Reeves (7). Marked variability in the kinetics of killing and the metabolic site of action have been observed depending on the particular bacteriocin studied. Adsorption of the bacteriocins onto specific receptors on the susceptible cell surface is thought to precede killing. The number of units of various bacteriocins required to kill one bacterium ranges from 1 to 100. Both adsorption and the kinetics of killing are dependent on the concentration of the bacteriocin. These observations on other bacteriocins suggest that careful kinetic studies are important to assess the mode of action of bacteriocins. An answer for the observed structural variabilities may well be found in an investigation of the kinetics of killing of the bactericidal substance. These studies are currently in progress.

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LITERATURE CITED