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Fine Structural Demonstration of Anionic Sites on Streptococcal and Staphylococcal Envelopes by Cationic Dyes

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Abstract: Anionic sites on bacterial envelopes of *Streptococcus β-haemolyticus* and *Staphylococcus aureus* ATCC 6538 P were investigated on materials fixed with Karnovsky fixative containing the cationic dyes ruthenium red (RR) or alcian blue (AB). Electron dense granules and short fibrils were found to be irregularly distributed around the cells on the glycocalyces of both genera. An electron dense stained reaction with RR or AB on the cell membrane and glycocalyx indicates the presence of rich anionic sites on these structures. Electron microscopic observations demonstrate that anionic sites in the glycocalyx are responsible for the adherence phenomenon during the formation of microcolonies. For structural improvement of bacterial envelopes, the RR procedure was more effective than the AB procedure.

Key Words: *Streptococcus β-haemolyticus*, *Staphylococcus aureus* ATCC 6538 P, Glycocalyx, Anionic sites, Ruthenium Red, Alcian Blue

Introduction

Bacterial cells are surrounded by cell envelopes, or cell coats, which include the cell membrane, cell wall and glycocalyx (1,2). The numerous mono-, di- and polysaccharide containing part of the coats is the glycocalyx (3-7). Considerable quantities of negative charges are present in carbohydrate-rich glycocalyx. The carbohydrates in the glycocalyx are especially involved in phenomena recognition. They are responsible for the adhesion of cells to each other and the surfaces in their environment (2, 8-11). Attachment is a prerequisite phenomenon in the colonization and the pathogenesis of bacterial infections. The ultrastructural investigation of bacterial envelopes is important in understanding bacterial adhesion, colonization and infections.

In general, the carbohydrate components of bacterial cell envelopes are not preserved well by routine electron microscopic methods. A significant improvement can be obtained by adding cationic dyes to the fixative (4 - 6, 12-17). In this study, anionic sites were demonstrated using the cationic dyes ruthenium red and alcian blue, on a fine structural level. The aim of the study was to compare the effects of these dyes on the preservation of the glycocalyx on the streptococcal and staphylococcal envelopes.

Materials and Methods

Microorganism Culture: The cultures were obtained from different sources.

Streptococcus β-haemolyticus (synonym of *Streptococcus pyogenes*) was isolated from a sputum culture of a hospitalized patient. Active cultures were grown in Trypticase Soy Broth (15) at 37ºC under static growth condition. *Staphylococcus aureus* ATCC 6538 P was taken from American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852 USA). After a 12 hour growth period both S. aureus ATCC 6538 P and young *S. β-haemolyticus,* and after a 24 hours growth period only the old samples of *S. β-haemolyticus* were centrifuged at 10,000Xg for 15 min, and washed three times with phosphate buffered saline (PBS) at pH 7.2. Cells were handled as pellets until all fixation/wash procedures were completed.

Electron Microscopy: In order to preserve the bacterial glycocalyces and to stain anionic sites, Karnovsky's (16) fixative containing 1 mg of RR or AB per ml was used for two hours at room temperature (6, 14,17). Phosphate buffered 1% weight/volume (w/v) OsO_4 (18) was used as a second fixative. After dehydration, the samples were embedded in EPON 812. Thin unstained or stained sections were examined with a JEOL 100C Electron Microscope at 80kV.

Results

Bacterial glycocalyces occur on the outside of the peptidoglycan layer of gram positive cells. Fine and electron dense granulated material was seen on the glycocalyces of both genera on unstained sections (Figures 1-3). Structural parts of the bacterial envelopes were better observed on stained sections (Figure 4). However, internal structures of the cells could not be identified properly. Short fibrils of glycocalyx were distinguished (Figure 5) around the older cells of *S. β-haemolyticus*. *S. aureus* ATCC 6538 P cells also have a short fibrillar glycocalyx (Figure 6). Several layers within the peptidoglycan cell wall were distinguished. Fibrillar glycocalyx was identified better than the others in the unstained preparation (Figure 3). The internal structure of the cells was not seen.

Observations of an electron-dense reaction with RR on the cell membrane and with the glycocalyx of *S. β-haemolyticus* and *S. aureus* ATCC 6538 P indicate that anionic sites are rich on these structures (Figures 1-3). During the formation of microcolonies, bacterial cells adhere to each other by the interaction of their glycocalyces

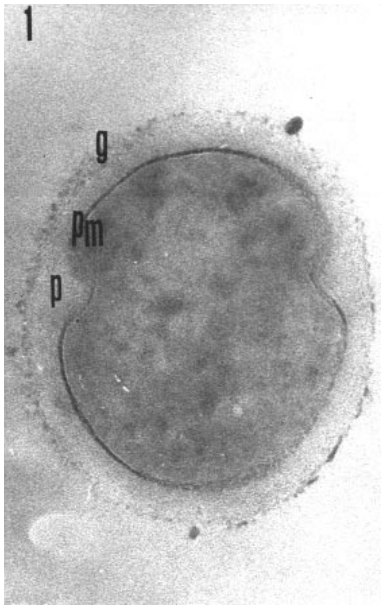

Figure 1. A *Streptococcus β-haemolyticus* cell fixed in RR procedure. Electron-dense reactions are seen on plasma membrane (pm) and glycocalyx (g). (p) indicates a thick peptidoglycan layer. Unstained section, X 100,000.

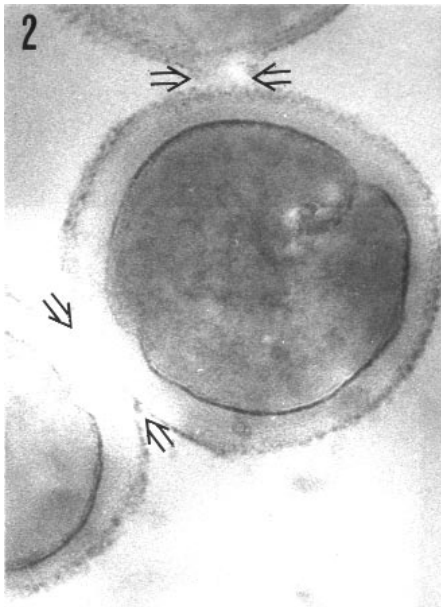

Figure 2. *Streptococcus β-haemolyticus* cells attached (⇒) to each other by their glycocalyces in RR procedure. Unstained section, X 100,000.

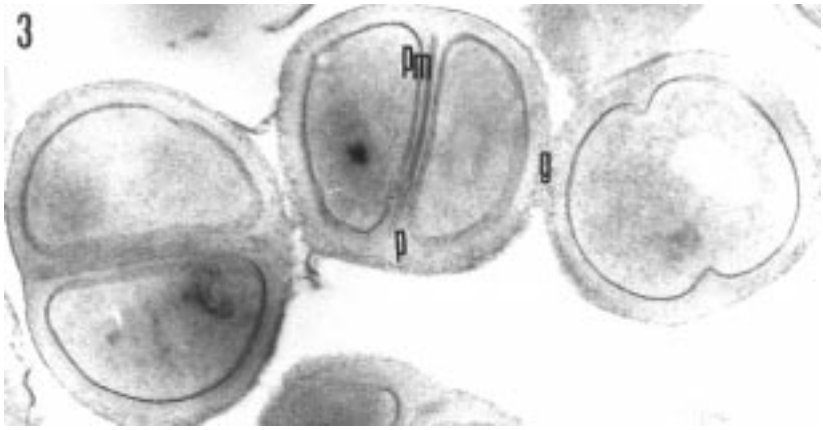

Figure 3 *Staphylococcus aureus* ATCC 6538 P cells fixed in RR procedure. Electron dense reaction on plasma membrane (pm) and glycocalyx (g) are clearly seen on dividing cells. (p) indicates a thick peptidoglycan layer. Unstained section, X 65,000.

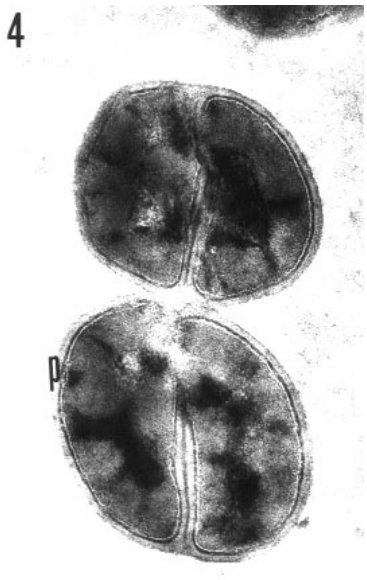

Figure 4. 12-hour-old dividing *Streptococcus β-haemolyticus* cells fixed in RR procedure. The cells are surrounded by a thin peptidoglycan layer (p) and a fine granulated electron-dense glycocalyx. Stained section, X 80,000.

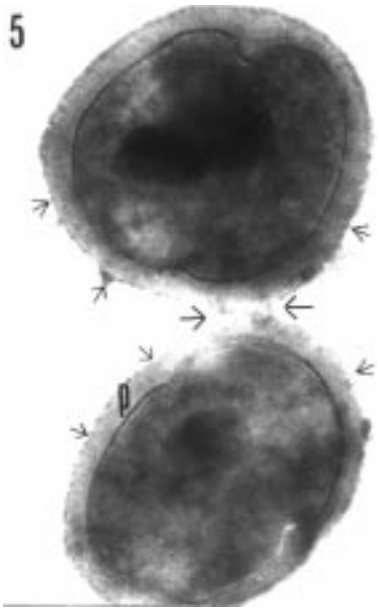

Figure 5. 24-hour-old *Streptococcus β-haemolyticus* cells have a thick peptidoglycan layer (p). Irregularly distributed electron-dense short fibrils (→) are seen on glycocalyx. In addition, cells are attached (➔) to each other by their fibrils. Stained section, X 80,000.

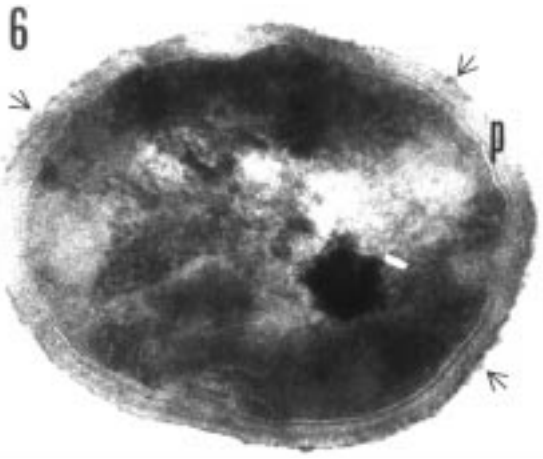

Figure 6. *Staphylococcus aureus* ATCC 6538 P cell fixed in RR procedure. Several layers on thick peptidoglycan (p) coat and short fibrils (→) on glycocalyx are seen. Stained section. X 84,000.

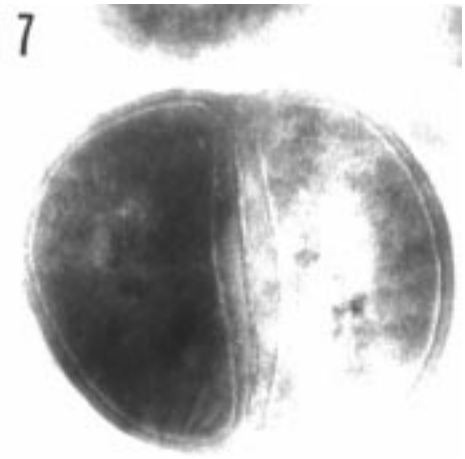

Figure 7. *Staphylococcus aureus* ATCC 6538 P cell fixed in AB procedure. Structural characterization of the bacterial envelope is not distinguished properly. Stained section, X 84,000.

(Figures 2 and 3). It seems that anionic sites (surface charges) on glycocalyces of both genera are responsible for the adherence phenomenon.

Anionic sites on bacterial cell envelopes where AB stain was used were not distinguished clearly (Figure 7), even on the stained section. A few short and very fine irregular fibrils were observed on the glycocalyx of *S. aureus* ATCC 6538 P cells. Fine structural observation of *S. β-haemolyticus* cells was not better than *Staphylococcal* cells. According to our electron microscopic observation, the RR procedure was more effective than the AB procedure for structural improvement of the bacterial envelopes.

Discussion

Differences in appearance of external material appeared to be somewhat species dependent. These included limited fibrous material, thin fibrillar threads and bead-like structures. For example, the glycocalyx material was sparsely fibrillated in *S. aureus* ATCC 25923, was infrequent in *S. hominis* SP 2 and contained bead-like structures in *S. epidermidis* RP 62 (4, 6). Sparse fibrous material around *S. aureus* ATCC 25923 (4, 6) and *S. suis* (19) resemble the appearances observed in this study.

The structural components of bacterial cell envelopes are highly hydrated in nature. During the dehydration stages of electron microscopic preparation methods, collapse and condensation occur (3,20,21). Improvements in preservation at the ultrastructural level can be obtained by adding certain cationic dyes, such as RR or AB, to the fixative (4-6). These dyes contain cationic groups which bind to polyanions within the envelopes of procaryotic and eucaryotic cells and have been used as a marker of anionic sites in many systems (3,12,13). Sharp intracellular material preservation by RR was observed in *Methylomonas* species (4,5). A good intracytoplasmic membrane system in *M. albus* B68 and extremely limited or no glycocalyx material in *S. aureus* ATCC 25923, in *S. hominis* SP 2, and in *S. epidermidis* RP 62, were obtained in glutaraldehyde/OsO_4 fixation (4). In the case of glutaraldehyde/OsO_4 including RR fixative, collapsed or condensed structures appeared on the glycocalyx. When the results of these two dyes were compared, the extended filamentous or fibrous structures are generally similar in AB and RR procedures for methanotrophs (4-6). The most abundant and extended glycocalyces were observed for cells by RR-lysine processing Staphylococci cells (4,6). Consequently, according to our observation, Karnovsky fixative containing RR has a better effect than RR containing glutaraldehyde/OsO_4 fixative in the preservation of structural components. When it is compared with AB results, it seems that the RR procedure has somewhat better effects on structural preservation.

An observation of the relationship between the adherence phenomenon and surface charges in this study shows similar appearances in *Staphylococcus aureus* ATCC 25923, *Staphylococcus hominis* SP 2, *Staphylococcus epidermidis* RP 62 (4) and *Methylomonas albus* BG8, *Methylomonas trichosporium* Ob3b, *Methylocystis paris* OBBP (4, 5) and *Streptococcus suis* (19, 22). Adhesion is the first essential step in the pathogenesis of infections. It is generally known that colonization and the infection capacity of bacterial cells are closely associated. It is clear that anionic sites on the envelopes contribute significantly to the colonizations.

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