Surface ultrastructure of intact and in situ chlorhexidine-treated human buccal cells

A method for scanning electron microscopy

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Air-dried and ethanol-fixed buccal epithelial cell smears from five subjects were observed by scanning electron microscopy. The mucous pellicle was precipitated as a smooth haze covering the cells, and outlines of bacteria were found embedded within it. Rinsing the preparations under running water gradually diminished the mucous pellicle but not the cell-adherent bacteria. A more complete dissolution of the pellicle was accomplished by washing the buccal epithelial cells before smearing. After a chlorhexidine mouthrinse the buccal cells appeared distorted, with only a few adherent bacteria. Three days after the rinsing, the denatured appearance still persisted on many cells, however, simultaneously with the emergence of undenatured epithelial cells with adherent bacteria. The method introduced in this study is useful to investigate the bacteria–mucus–epithelial cell interactions. A possible mode of antibacterial activity of chlorhexidine in vivo may be that it destroys bacterial adhesins. The substantivity of chlorhexidine in the oral cavity may be linked to the turnover rate of the oral epithelial cells. □ *Anti-infective agents; bacterial adhesion; bacterial flora; mouth mucosa; oral hygiene*

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A scanning electron microscopy (SEM) study of high-resolution impressions of the oral epithelial surfaces in vivo showed that the epithelial surfaces of human buccal mucosa are covered by a smooth pellicle, within, or under which, bacteria are embedded (1). The oral mucosal pellicle has been defined as a protein film analogous to acquired tooth enamel pellicle; it is formed by organized and selective adsorption of salivary components of the epithelial cell surface (2, 3). The salivary pellicles have a propensity to modify the process of adherence of bacteria (4). Many studies have shown that oral epithelial cells provide specific receptors for distinct bacterial species to adhere to (5). However, little is known about whether the indigenous oral bacteria actually are tightly attached to the receptors of the epithelial cell proper or to the pellicle covering these cells.

Chlorhexidine mouthrinse formulations are widely established in reducing oral microflora, but the mode of action of chlorhexidine is not entirely understood. Gjermo et al. showed early (6) that many antibacterial agents with power superior to chlorhexidine in vitro did not effect significant reduction of oral microflora in vivo. Chlorhexidine is primarily bound to salivary glycoproteins, the essential ingredients of pellicles that cover mucosal surfaces (7); it was therefore concluded that the crucial feature of chlorhexidine is its substantivity in the oral cavity. The slow release of chlorhexidine from pellicle-covered oral surfaces may be causing leakage and lysis of bacteria, but some studies have suggested that even sublethal amounts of chlorhexidine may impair the adhesivity of bacteria to oral surfaces (8, 9). Audus et al. (10) showed that chlorhexidine prevented the adherence of *Candida albicans* to human buccal epithelial cells. It could be hypothesized that the binding of chlorhexidine either to the mucosal pellicle or to the epithelial cell proper may cause ultrastructure alterations that defer the normal function of bacterial adhesins. Previous reports from our own research projects have shown that chlorhexidine mouthrinses drastically reduce the amount of indigenous bacteria adhering to buccal epithelial cells (11, 12).

This study aimed to develop a simple and inexpensive method for the preservation of the mucous pellicle covering buccal epithelial cells for visualization by means of SEM. I also tried by different methods to gradually dissolve the mucosal pellicles to ascertain whether the cell-adherent bacteria are tightly attached to the epithelial cell proper or loosely associated with the mucus covering them. The surface ultrastructure of buccal epithelial cells was examined before and after the use of a chlorhexidine mouthwash.

Materials and methods

Samples were collected from one male and four female healthy, non-smoking dental health care personnel, with ages ranging from 26 to 38 years, presenting with a good level of oral hygiene and dental health. The samples were collected around 1500 h, allowing about 3½ h to elapse after lunch. During this time no snacks,

coffee, or toothbrushing was allowed. The buccal surface of the oral mucosa was gently wiped with a cotton swab, which was then put into a vial containing about 1 ml of physiologic saline (NatroSteril, Orion, Finland). A drop of this suspension of buccal cells was prepared on a coverglass (13 mm in diameter), which was allowed to air dry at +37 °C. To promote the adhesion of buccal cells, all the coverglasses in this study were treated with 0.1% Poly-L-lysine solution (Cat. no. 8920; Sigma, St Louis, Mo., USA) in accordance with the manufacturer's instructions. Thereafter ethanol solution (95%) was flowed over the coverglass, which was air-dried again. All the coverglasses were kept in a cool (+8 °C), dry place. The samples were coated with gold palladium and observed under SEM (Jeol JSM 6300F, Tokyo, Japan).

Several further tests were conducted to find a method to gradually dissolve the mucosal pellicle covering the buccal epithelial cells, so that the degree of solubility of the mucosal pellicle and the tightness of adherence between bacteria and the epithelial cell could be evaluated. Duplicates and triplicates of prepared coverglasses with air-dried buccal cells were rinsed for 2 and 5 min, respectively, under running tap water, after which they were processed for SEM. Another method for dissolving the pellicle was accomplished by suspending the swabs of buccal cells in variable volumes of distilled water or physiologic saline. It was deemed appropriate to decrease the amount of floating bacteria by first aspirating the washing medium through a Luer-compatible, 5-µm pore size filter disk (Sartorius, Minisart NML SM 17594K, Göttingen, Germany). Thereafter, the epithelial cells that had become arrested in the filter disk were released by counterdirectional injection of a corresponding volume of washing medium. The suspensions were agitated by a Vortex mixer at maximum setting for 20 sec and allowed to stand for from 10 min up to 1 h, after which light centrifugation (1650 *g*, 5 min) was used to harvest the cells at the bottom of the tubes. The supernatants were discharged carefully with a Pasteur pipette, and the pellets of washed epithelial cells were suspended in the remaining approximately 0.3–0.5 ml of washing medium and smeared on a coverglass. Alternatively, another fresh volume of distilled water was added for a second washing and the procedure repeated. The coverglasses were prepared for SEM as described previously.

Ten milliliters of Corsodyl, 2 mg/ml, chlorhexidine gluconate preparation (Smith-Kline-Beecham, Espoo, Finland) was used for rinsing for 1 min. The samples were collected 10 min, 3 h, 24 h, 48 h, and 72 h after rinsing. A washout period of at least 12 days of non-use of chlorhexidine-containing products was carried out before each testing for each participant.

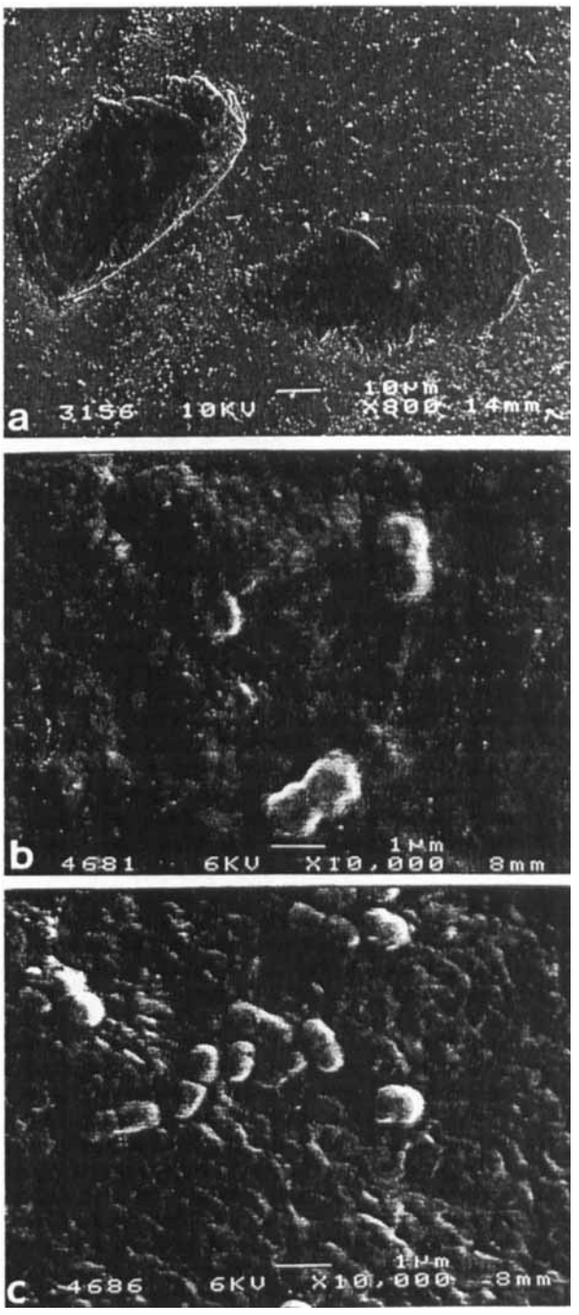

Fig. 1. Air-dried, ethanol-fixed buccal epithelial cells (a). Although the cell surface is meticulously focused, the details of the cell surface cannot be detected owing to the smooth haze of the acquired pellicle (b). Rinsing the fixed specimen for 5 min under running water gradually washed away the mucosal pellicle. The bacteria and the details of the epithelial cell surface became more discernible (c). The magnification and scale are indicated in each picture.

Results

The air-dried unwashed buccal cells appeared to be flat, sometimes folded, with slightly prominent nuclei (Fig. 1a) and to be covered by a smooth haze, through which the particular details of a cell surface typical for epithelial cells could not be distinguished, except for

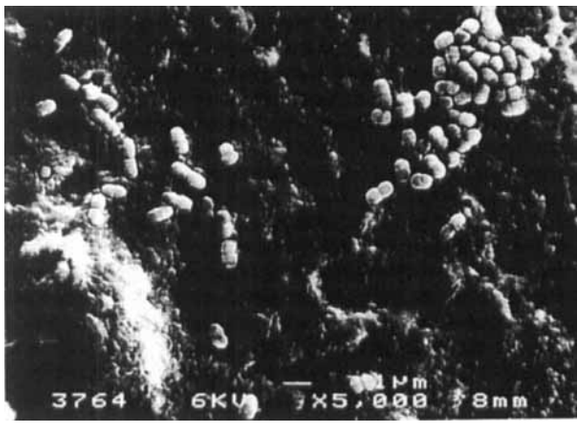

Fig. 2. Improved clarity of the details of buccal cells was obtained after the buccal cells had been washed in two consecutive portions of physiologic saline before the fixation procedure.

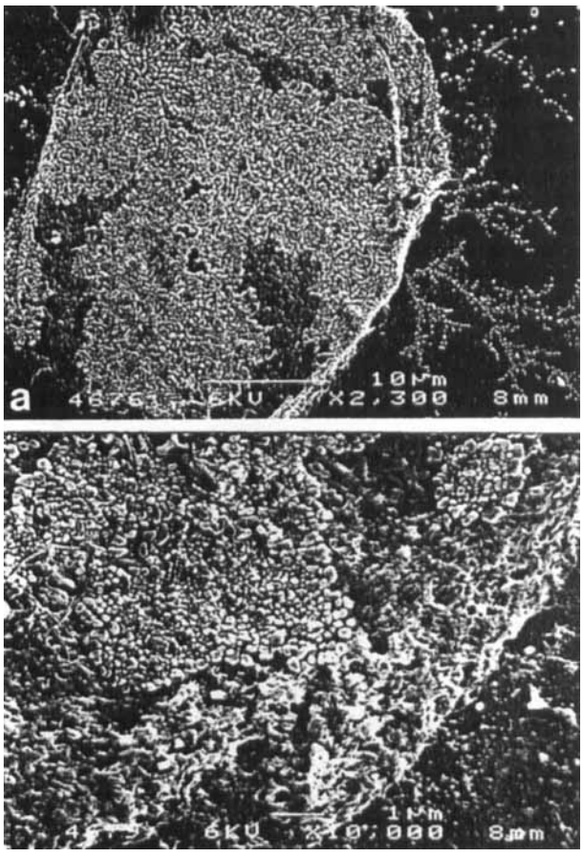

Fig. 3. Surface alterations caused by chlorhexidine rinsing. Initially, 10 min after rinsing, a dense mat of distorted pellicle appeared which was resistant to washing with physiologic saline (a). The distorted pellicle remained present on the epithelial cells 1 day after the rinsing (b).

occasional low convexities, which by their size could be assumed to be the outlines of coccus-shaped bacteria (Fig. 1b). By rinsing these specimens under running tap water, it was possible to gradually diminish the pellicle, sometimes unveiling the normal microplicated ultrastructure of buccal epithelial cells. Different phases of bacterial cell division could often be distinguished (Fig. 1c).

The method with which the suspension of buccal cells was first washed either with distilled water or physiologic saline before smearing and air-drying the samples was found to be more effective to dissolve the pellicle. Although remnants of pellicle remained, the microstructure of oral epithelial cells, reminiscent of collapsed microvilli or microplicae, were distinguishable in most places. The adherent bacteria often appeared to be slightly embedded in cup-like depressions within the epithelial cell surface (Fig. 2).

Mats of globules or dense, rough material, possibly of denaturated pellicle, were found covering the cell surfaces after the chlorhexidine rinse (Fig. 3a). The denatured appearance remained unaltered 24 h after the rinse (Fig. 3b).

Two days after chlorhexidine rinsing it was not unusual to find two adjacent cells, one densely covered with adherent bacteria and the other free of bacteria (Fig. 4a). Striking differences could be seen in the microstructure between these two types of cells. The cell with many adherent bacteria (Fig. 4b) had the appearance of a normal, freshly emerged epithelial cell, whereas the cell with few or no bacteria (Fig. 4c) had the typical surface appearance of a chlorhexidine-treated cell.

Discussion

The air-drying and ethanol fixation method caused the mucosal pellicles to become precipitated, yet the buccal cells still retained their normal structure. Early in the 1970s Cleaton-Jones (13) pointed out that the superficial cells of the oral mucosa were relatively resistant to distortion in the specimen handling that is required for electron microscopy. Apparently, the parakeratinized cells of the buccal mucosa were sufficiently rigid to be adequately preserved irrespective of whether air-drying or critical-point techniques were used. Washing the buccal cells, preferably before the air-drying, could be used to gradually dissolve the mucosal pellicle precipitate. The buccal epithelial cell surface seemed to consist of microstructures reminiscent of collapsed microvilli or microplicae and were in many ways similar to what is known from other studies in which conventional specimen preparation techniques have been used, such as reported for, for example, the oral epithelial cells of the vervet monkey (14).

In contrast, the cell-adherent bacteria seemed to be

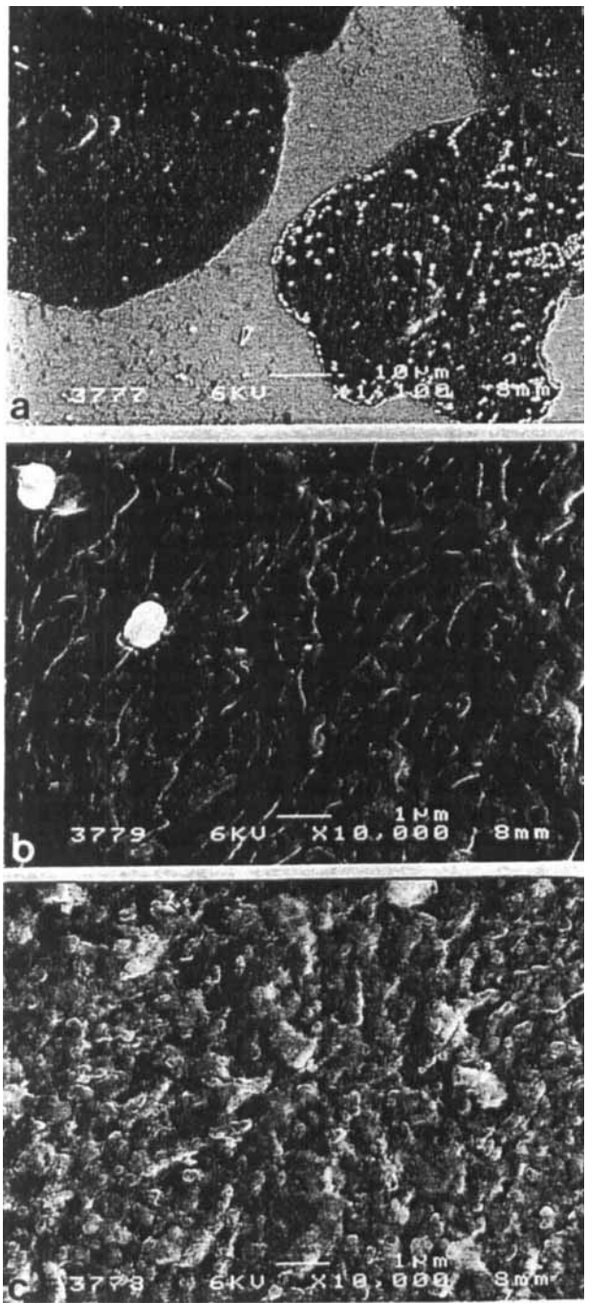

Fig. 4. Two distinct types of buccal cells in a sample taken 2 days after rinsing with chlorhexidine (a). The cell to the right has many adherent bacteria, whereas the cell to the left has only a few. A close-up of the cell to the right in Fig. 4a shows the typical appearance of a normal buccal cell (b). In contrast, the cell to the left in Fig. 4a shows the distorted pellicle typical of chlorhexidine-treated cells (c).

irreversibly bound to the epithelial cell surface. Many adherent bacteria were found in cup-like depressions as if partially engulfed by the cytoplasmic membrane (Fig. 2). Lange & Schroeder (15) suggested early that bacteria may become engulfed inside the cytoplasmic membrane of the epithelial cells of the human gingiva. Bacterial products have been shown to have the ability to alter the ultrastructure of epithelial cells in culture (16), and direct contact of bacteria with the cytoplasmic membrane has been shown to induce cytoskeletal rearrangement in human cells (17, 18). Hoepelman & Tuomanen (19) have pointed out that many of the adhesin molecules on the eukaryotic cell surface may in fact be biologic effector molecules, and the binding of bacteria to these adhesins causes cascades of intracellular processes. Human buccal surfaces are inhabited by a few species of viridans streptococci (20). The bacteria that were seen adhering to the buccal cells most obviously represented the genuine indigenous microflora of buccal mucosa, since they were uniformly coccoid organisms, apparently of a single species, and many of them seemed to be undergoing division.

Many authors have stressed the importance of the acquired pellicle as a modulator of bacterial adherence processes in the oral cavity. The bacterial adherence has been suggested to proceed in two distinct kinetic steps, which include a weak adherence and conformational adaptation of the bacterial receptor molecules in relation to mucosal pellicle, which enables the second step, a firm ligand–receptor bond (21, 22). Håkansson et al. (23) have shown that, whereas *Haemophilus influenzae* strains mainly adhere to respiratory mucus epitopes, the strains of *Streptococcus pneumoniae* rather bind to the cell surface proper. Accordingly, the epithelial cell-associated pellicle may act as a first step in distinguishing which bacteria are allowed to come into contact with the epithelial cell proper. The findings of this study suggest that firmer molecular mechanisms, such as lectins, or stereochemically matching complementary protein structures (5, 24) are responsible for the adherence of indigenous oral streptococci to the epithelial cell surface proper.

It is obvious that chemical handling of the oral mucosa with chlorhexidine devastates the normal ultrastructure of epithelial cells and their receptors for bacteria. The rough material seen after rinsing with chlorhexidine was either an unsoluble, denatured pellicle or a completely altered cell surface. It has been suggested that some of the side effects of chlorhexidine, such as interference with taste sensations, soreness of the oral mucosa, and discoloration of the teeth, could be associated with the denaturation and precipitation of the salivary mucinous proteins (25). Indeed, all the cells in the sample taken after a chlorhexidine rinse seemed to be gravely affected. Practically no bacteria could be found adhering to these denatured cell surfaces, as could be expected from earlier studies (11, 12). Three days later there appeared to be an interesting dichotomy, with two different kinds of cells in the sample. The buccal cells that were free of bacteria appeared to be 'chlorhexidine-affected' (Fig. 4c), whereas the cells with many adherent bacteria appeared

to be freshly emerging cells with the normal surface microstructure of non-chlorhexidine-treated buccal epithelial cells (Fig. 5b). With regard to the fact that the oral mucosa is a major reservoir of the retained chlorhexidine, it may be asked whether the substantivity of chlorhexidine may actually be related to the turnover rate of the buccal epithelium, rather than reversible slow release of the salivary mucin-bound drug.

In conclusion, this study presents an SEM method for studying details of epithelial cells and the mucosal pellicle covering them. Mucosal pellicles appear to be more or less reversibly bound to buccal epithelial cells, whereas the indigenous bacteria seem to be irreversibly and tightly bound to buccal epithelial cell surfaces. The SEM method presented in this study visualizes a possible mode of action of chlorhexidine in vivo and added to our understanding of the pharmacodynamics of chlorhexidine in the oral cavity. The techniques presented in this study can easily be implemented in future studies aiming to clarify drug or treatment effects on host–bacteria interactions.

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