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Bacterial pellicle-like substances and polyphosphate formation by enamel-adherent oral microorganisms*

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Abstract

Association with enamel of strains of streptococci and actinomycetes was studied both visually and electron microscopically. Microorganisms that were noted to form visible plaque on enamel exhibited electron microscopically cell attachment apparently mediated by a bacterially derived "pellicle." The ability to produce this structure may be a critical determinant of initial stages of bacterial attachment to enamel. Electron-lucent "holes" were also noted in cells of all the strains observed. These holes are compatible with the ultrastructural appearance of bacterial polyphosphates.

Introduction

Dental plaque, the predominantly bacterial mass that adheres to tooth surfaces, has been of interest due to its causal relationship with two of the most prevalent diseases in man—caries and periodontal disease. The bacteria that initially colonize teeth are derived from the indigenous microbiota suspended in the oral fluids. Yet, only certain of the many species present in the mouth can be found on the teeth,[1] and those organisms which colonize smooth tooth surfaces can only do so because they are capable of tenacious and essentially irreversible attachment to this hard surface.

After the initial adherence of bacteria to enamel, organisms divide and cohere, and thus bacterial masses, termed plaque, enlarge. Despite the widespread occurrence of bacterial attachment to hard surfaces in many natural environments, remarkably little is known about the mechanisms responsible for these events.[2]

Some models which have been used to observe bacterial attachment to surfaces involve growth of bacteria on plastic[3] or on plastic-embedded hydroxyapatite powder.[4] However, the recent development of a technique for thin sectioning plaque on enamel *in situ*[5] has enabled ultrastructural study of the interactions of plaque-forming bacteria with enamel. We have previously described some of the parameters involved in early plaque formation by *Streptococcus mutans* on enamel *in vitro*.[6]

The purpose of this investigation was to observe electron microscopically the characteristic features of the attachment of other plaque-forming species to enamel, and to compare these features to our previous findings for *S. mutans*.

Methods and materials

Enamel Specimen Preparation

Enamel specimens used as a substratum for bacterial attachment were prepared by cutting blocks (40 mm^2) from the smooth surfaces of extracted human molars which had been stored in distilled water. A hole to accept a 20 gauge nichrome wire was then made through the specimens. The surface enamel was polished with fine pumice and washed with water in an ultrasonic cleaner. The specimens were then sus-

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pended by wire, placed in stoppered test tubes, and autoclaved prior to plaque growth.

Microorganisms, Media, and Growth

The following microorganisms were studied with respect to their attachment to enamel: *Streptococcus sanguis* ATCC 10558, *S. sanguis* UCHC-FG, *S. salivarius* ATCC 13419(rough) *S. salivarius* 13419(smooth), *S. salivarius* UCHC-FL(rough), *S. salivarius* UCHC-FL(smooth), *S. salivarius* TOVE(rough), *S. salivarius* UCHC-EL(smooth), *S. salivarius* UCHC-LL(smooth), *S. salivarius* UCHC-DL(smooth), *S. mutans* 6715-13 WT, *S. mutans* NCTC 10449, *S. faecalis* ATCC 9790, Group D streptococcus, *Actinomyces viscosus* M-100, *A. viscosus* UCHC-R3A, *A. viscosus* TO4, *A. naeslundii* ATCC 12104, *A. naeslundii* I-S-1000.

Stock cultures were maintained by monthly transfer in fluid thioglycollate medium† supplemented with meat extract (20% v/v) and excess $CaCO_3$. Fresh human plaque isolates of streptococci were identified morphologically on mitis salivarius agar and their identities were confirmed biochemically. The "rough" and "smooth" notation for *S. salivarius* characterizes their colonial morphologies on mitis salivarius agar. Prior to experiments, cultures were adapted to growth in the complex medium of Jordan *et al.*[7] supplemented with 50 mg/liter of Na_2CO_3 and 5% sucrose.

For plaque growth on enamel, culture tubes of the complex medium were inoculated with 0.2 ml of 24-hr culture of the adapted strain.[6] Sterile, wire-mounted enamel specimens were then suspended in the culture tubes and incubated microaerophilically at 37°C. The enamel specimens were transferred daily to uninoculated medium, and after 2 days the enamel specimens were observed visually for surface bacterial growth and processed for electron microscopy.

Electron Microscopic Preparation

The specimens to be processed for electron microscopy were removed from the growth medium and fixed with a 2.5% gluteraldehyde in 390 mOsM phosphate buffer (pH 7.4), and postfixed in 1% osmium tetroxide in veronal buffer (pH 7.3).[8] They were then washed in the phosphate buffer and placed in an acidic gel containing 0.1 N HCl and 15% gelatin (BBL) for 3.5 hr to demineralize slightly the enamel surface.[5] After dehydration in acetone and embedment in epoxy medium,[9] the resin was polymerized at 70°C.

The specimens were prepared for sectioning as described previously,[6] so that only the acid-softened enamel and the organic films on the surface of the enamel remained. Thin sections were prepared with a Reichert ultramicrotome using a diamond knife. Silver-gold-colored sections were stained with aqueous uranyl acetate followed by lead citrate[10] and examined at 90 kV with a Zeiss EM 10 electron microscope.

† Difco, Detroit, Michigan.

Results

Adherent bacterial masses on enamel were evident with all *S. mutans, S. sanguis* and *Actinomyces* cultures tested. Only one of the nine *S. salivarius* strains tested failed to form plaque on enamel, whereas both of the enterococcus strains failed to form plaque.

Ultrastructurally, enamel incubated with a non-plaque-forming microorganism, *S. faecalis*, showed neither microorganisms nor films on the enamel surface. The organisms that produced visible deposits on enamel exhibited bacteria surrounded by an extracellular electron-dense matrix (Figs. 1–4). Electron-lucent "holes" (0.02–0.30 μm) were noted in all strains observed, consistent with the presence of polyphosphate granules.[11]

Bacterial attachment to enamel appeared to be mediated by a thin, 0.01–0.10 μm, electron-dense film which covered the enamel surface. This film appeared contiguous with the surfaces of cells in close association with the enamel.

No differences could be noted among the plaque-forming streptococci, either in their morphology or in the matrix between these bacteria and the enamel. The *Actinomyces* strains, however, differed from the cocci in that these organisms were pleomorphic, varying from coccal to bacillary in form (Fig. 4).

Discussion

Many of the current concepts concerning bacterial attachment to teeth have been derived from studies of marine bacterial sorption to glass surfaces. The attachment of these organisms has been reported to involve an initial reversible phase and a time-dependent irreversible phase.

In the first phase the bacteria are not firmly attached to the surface and can be desorbed readily. Later these organisms have been noted to produce polymeric fibrils which may cause an irreversible attachment to the surface.[12] Two stage attachment has also been noted for *in vitro S. mutans* plaque formation. For example, Clark and Gibbons[13] demonstrated initial reversible adsorption of *S. mutans* to enamel. When these organisms were allowed to produce extracellular polymers, the attachment of bacteria to enamel became irreversible, as demonstrated by decreased ability to desorb the cells.

Glucan was suggested as the polymer mediating firm attachment of these organisms to enamel.[13] Glucan production by *S. mutans* has also been implicated as essential for this organism to form large bacterial accumulations.[2, 14–16]

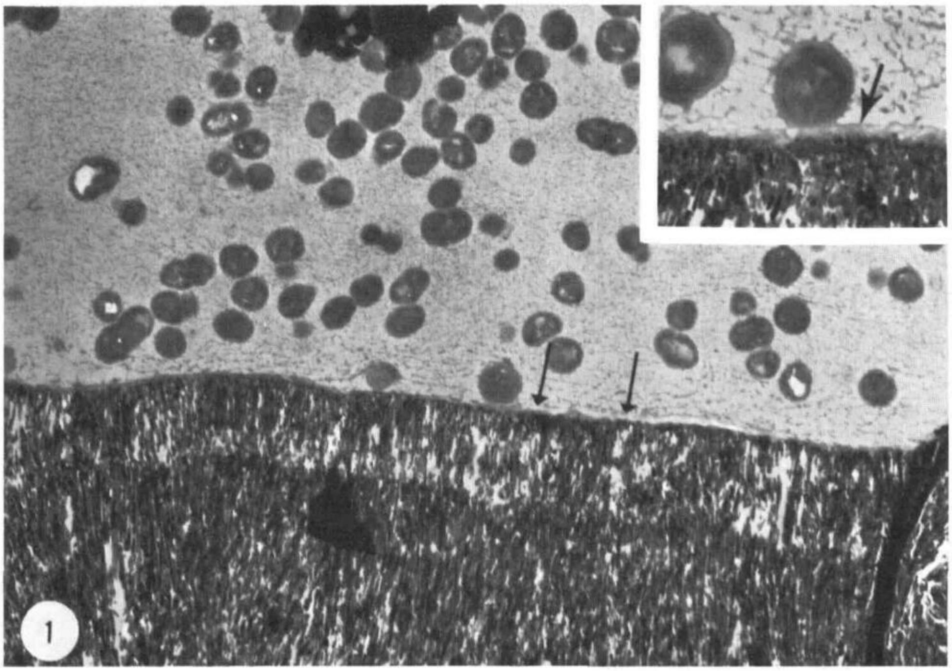

Fig. 1. Transmission electron micrographs of the enamel-bacterial interface after incubation of *Streptococcus sanguis* UCHC-FG with enamel for 2 days in complex medium containing 5% sucrose. Apparent bacterially derived extracellular material (arrows) is present on the enamel surface at low magnification. Note electron-lucent holes in cells. ×9,200. Inset shows one cell approximating the enamel with extracellular material apparently mediating attachment. ×22,500.

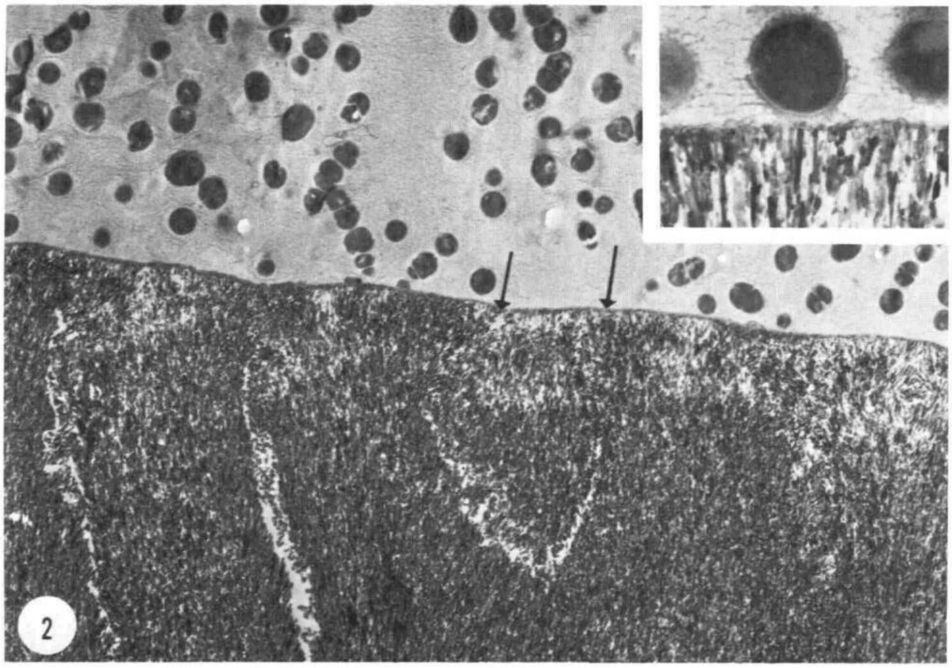

Fig. 2. Electron micrograph of the enamel-bacterial interface after incubation of *S. salivarius* UCHC-FL (rough) with enamel for 2 days in complex medium containing 5% sucrose. Extracellular material on the enamel is evident at low magnification. Also note enamel prism structures and electron-lucent holes in cells. ×5,040. Inset shows a higher magnification of one cell approximating the enamel. ×22,400.

In a previous report, we noted sucrose dependency for plaque formation only by Bratthall serotypes *a, b*, and *d* of *S. mutans*. Serotypes *c* and *e* were noted to form plaque on enamel in glucose-containing as well as in sucrose-containing media, although they adhered less tenaciously under the former condition. Additionally, a bacterially produced "pellicle" on the enamel appeared electron microscopically to mediate attachment of *S. mutans* to the enamel.

Since this extracellular material was formed in glucose-containing cultures as well as in sucrose-containing cultures, it was believed not to be glucan. Chemical analysis confirmed that no glucan was present in the glucose-grown plaques. Furthermore, only bacterial products of adherent cells were believed to have produced this "pellicle" since: (1) polishing of the enamel was noted to remove organic films; (2) enamel was not exposed to saliva to form a "salivary pellicle"; (3) sterile bacterial growth medium incubated with enamel did not precipitate any surface layer on the

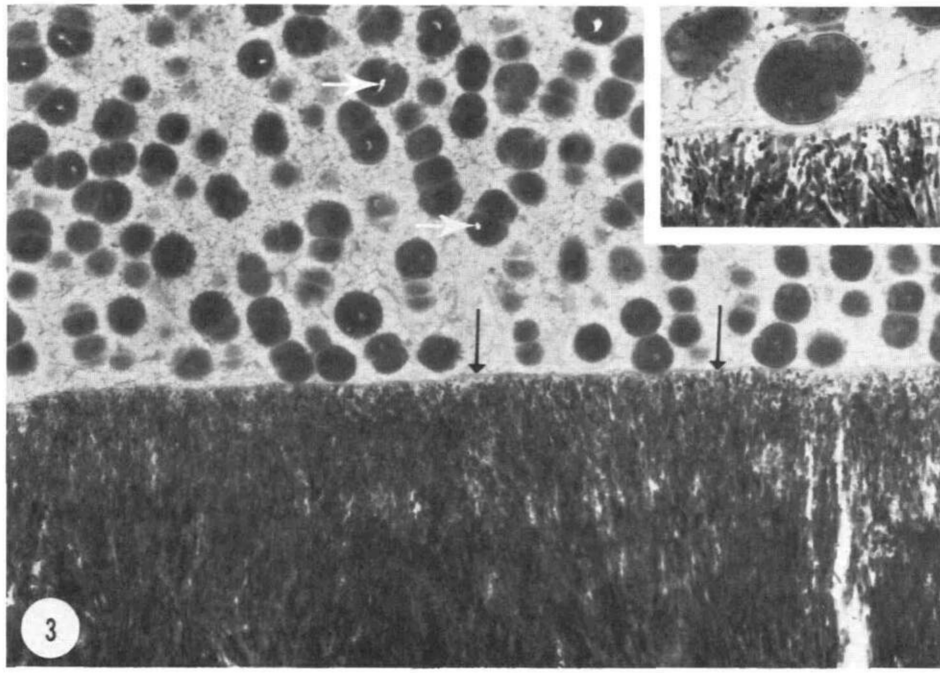

Fig. 3. Electron micrograph of the enamel-bacterial interface after incubation of *S. mutans* NCTC 10449 with enamel for 2 days in complex medium containing 5% sucrose. At low magnification extracellular material can again be observed on the enamel surface and electron-lucent holes are apparent in cells (white arrows). ×9,200. High magnification shows the interaction of one cell with the enamel. ×22,400.

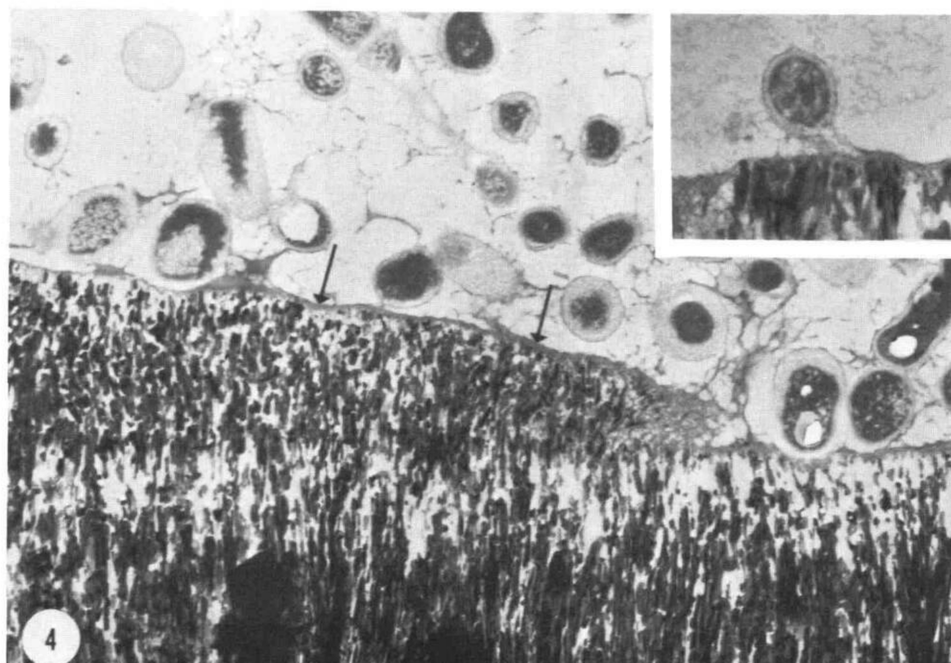

Fig. 4. Electron micrograph of the enamel-bacterial interface after incubation of *Actinomyces viscosus* M-100 with enamel for 2 days in complex medium containing 5% sucrose. A "bacterial pellicle" apparently mediates cell attachment to enamel. A stria of Retzius forming surface perikymata can also be noted as part of the enamel structure. Low magnification, ×19,000. Higher magnification shows one cell approximating the enamel with extracellular material apparently mediating attachment. ×35,000.

enamel; and (4) nonadherent cells failed to form such surface film.[6]

In the present study a similar pellicle-like structure was evident at the enamel-plaque interface for all plaque-forming organisms studied. This bacterially derived, presumptive polymer covered the enamel and apparently mediated bacterial attachment to this surface. Since the production of "bacterial pellicle" was noted to be common to the adherent organisms studied, the ability of bacteria to produce this extracellular polymer may be a critical determinant of initial stages of bacterial attachment to enamel. Further studies are necessary to explore the chemical nature of this structure.

Traditionally, the pellicle found at the enamel-bacterial interface has been thought to be derived from salivary glycoprotein components.[17-19] Such pellicles have been observed in previous studies upon incubation of enamel with saliva for a brief period of time.[6] Since the enamel in this study was not exposed to saliva, salivary pellicle was not found essential for attachment of *S. mutans, S. sanguis, S. salivarius*, or *Actinomyces sp.*

Others have also demonstrated that glycoprotein-rich (hog gastric) mucin is not essential for attachment of *S. mutans* to nichrome surfaces.[20] Salivary films, however, have been shown to enhance adsorption of certain plaque-forming microorganisms to enamel and to inhibit the adsorption of others.[13, 21, 22] Hence, the acquired salivary pellicle may have a role in determining the relative proportion of different bacteria which adsorb to enamel, but it does not appear requisite for the attachment of plaque-forming bacteria to enamel.

Bacterial attachment and subsequent formation of microbial masses on enamel are indeed essential for caries of smooth tooth surfaces.[23] *S. sangius* reportedly adheres to enamel more readily than *S. mutans*, while *S. salivarius* has a weak affinity for enamel.[24] Additionally, relatively large percentages of *A. viscosus* have also been noted in the early dental plaque of some individuals.[1] However, *S. sanguis* has not been shown to be associated with human caries,[25] while *S. mutans* has been shown to have a strong correlation,[25-27] and there is only limited evidence that caries may be induced by *A. viscosus*[28, 29] and *S. salivarius*.[30] Hence, other traits[31] appear to be necessary for caries beside the capability of bacteria to adhere to the teeth.

The electron-lucent "holes" noted incidentally in the bacteria were a common finding in all of the adherent strains. These holes probably represent electron-dense bacterial polyphosphate granules which have been volatilized by the electron beam,[32, 33] which is typical of polyphosphate. Polyphosphates have been identified in a variety of microorganisms,[11] including *S. mutans*.[34] This highly anionic phosphate is believed formed in cells when nutritional conditions are not favorable to growth.[11]

It should not be concluded, however, that the polyphosphates apparent in the adherent cells are related to adhesion because nonadherent cells were not studied ultrastructurally. The presence of polyphosphates in the adherent cells does indicate, however, that these cells are not growing optimally. Other data directly support this conclusion.[35]

Conclusion

Strains of *S. sanguis, S. mutans, Actinomyces*, and *S. salivarius* were noted to attach to enamel *in vitro*. Ultrastructurally, the bacterial attachment to enamel is mediated by an electron-dense film which covered the enamel surface. The formation of this "bacterial pellicle" at the enamel-plaque interface may be necessary for stable bonding of bacteria to this surface.

Electron-lucent "holes" in some of the bacteria were noted in all the strains observed. These holes are believed to represent electron-dense bacterial polyphosphate granules which have been volatilized by the electron beam.

Acknowledgment

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