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, 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.

Some models which have been used to observe bacterial attachment to surfaces involve growth of bacteria on plastic or on plastic-embedded hydroxyapatite powder. However, the recent development of a technique for thin sectioning plaque on enamel in situ 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.

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 mm²) 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-
pended by wire, placed in stoppered test tubes, and autoclaved prior to plaque growth.

Microorganisms, Media, and Growth


Stock cultures were maintained by monthly transfer in fluid thioglycollate medium supplemented with meat extract (20% v/v) and excess CaCO₃. 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. supplemented with 50 mg/liter of Na₂CO₃ 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. 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). 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. After dehydration in acetone and embedment in epoxy medium, the resin was polymerized at 70°C.

The specimens were prepared for sectioning as described previously, 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-lead acetate sections were stained with aqueous uranyl acetate followed by lead citrate and examined at 90 kV with a Zeiss EM 10 electron microscope.

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.

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. Two stage attachment has also been noted for *in vitro S. mutans* plaque formation. For example, Clark and Gibbons 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. Glucan production by *S. mutans* has also been implicated as essential for this organism to form large bacterial accumulations.
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
enamel; and (4) nonadherent cells failed to form such surface film.

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. Such pellicles have been observed in previous studies upon incubation of enamel with saliva for a brief period of time. Since the enamel in this study was not exposed to

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