In Vitro Attachment of Streptococci to the Tooth Surface

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The ability of Streptococcus strains to adhere to the tooth surface in vitro was investigated. Polished enamel slabs, with and without acquired pellicles, were incubated with buffer suspensions of oral streptococci, and attached bacteria were counted under a microscope using incident light. Low numbers of bacteria adhered to uncoated enamel; the presence of an acquired pellicle significantly enhanced the attachment of all strains tested. The adherence of Streptococcus sanguis was significantly greater than that of Streptococcus salivarius, and both of these strains adhered in greater numbers than did Streptococcus mutans. When bacteria were suspended in whole saliva, the adherence of S. salivarius and S. mutans was inhibited, whereas the adherence of S. sanguis was enhanced in some experiments and inhibited in others. The adherence of S. sanguis and S. salivarius was consistently inhibited by parotid fluid; this inhibitory effect persisted after thorough washing and resonication of the bacterial cells. Incubation in oral fluids was associated with the attachment of bacterial clumps to the pellicle, and parallel investigation revealed agglutination of S. sanguis and S. salivarius by whole saliva and, in particular, parotid fluid. The results are discussed in terms of surface microecology, and are related to the development of dental plaque.

Bacterial deposits on teeth (dental plaque) constitute the infective agent in dental caries and periodontal inflammation. Though events underlying dental plaque formation are poorly understood, current theories emphasize the importance of selective interactions of bacteria with the tooth surface, and of interbacterial aggregation mechanisms as well as salivary agglutinating systems (6). Bacteria are preceded on the tooth surface by an acquired pellicle (17) of salivary origin (11). The pellicle is assumed to consist mainly of glycoproteins; studies of experimental acquired pellicles have moreover demonstrated the presence of specific proteins such as immunoglobulins and lysozyme (14). Also, a salivary protein agglutinating bacteria has been reported to possess high affinity for powdered tooth enamel (7) and may be present in the pellicle. Agglutinating factors (7, 8) and specific antibodies (9, 18) are known to act on strains of Streptococcus, the numerically dominant genus in young plaque (2). Adhesion of streptococci to oral and glass surfaces has been studied in vitro (4, 12, 13), and these reports as well as in vivo experiments (21) have shown significant differences in the adhesive characteristics of bacterial species.

In the present experimentation, interactions of oral streptococci with salivary proteins and with the acquired pellicle have been studied in an in vitro system designed for quantitative evaluation of bacterial adherence to the tooth.

MATERIALS AND METHODS

Substrate for bacterial adhesion. Bovine incisor tooth crowns were cut in square slabs, 3 by 3 by 2 mm, and the enamel was polished lightly to a plane, smooth surface. Some slabs were used without further treatment (uncoated); other slabs were coated (in vivo or in vitro) with an acquired pellicle. For growth of pellicle in vivo, 8 to 16 slabs were mounted with sticky wax onto acrylic rods, and 1 to 2 of these rods were kept in the mouth of a subject for 2 h. During this period a pellicle of salivary origin covered the enamel surface (14). Pellicles were grown in vitro on slabs mounted in cellulose acetate boxes and incubated (2 h, 37°C, shaking action) in pooled whole saliva collected as described later. After incubation, all slabs were rinsed with a stream of distilled water to remove loosely attached cells and debris.

Collection of oral fluids. Paraffin-stimulated whole saliva from several subjects was collected in ice-chilled tubes. Portions (10 ml each) of saliva were quick-frozen by immersion in ethanol with dry ice.
For production of in vitro pellicles, samples were thawed immediately before use and applied without further preparative procedures. For use as a vehicle for bacterial suspension, samples were thawed, centrifuged (10,000 x g, 15 min), and filtered through a membrane filter (0.45 μm pore size; Millipore Corp.).

Lemon-candy-stimulated parotid fluid was collected in ice-chilled tubes by means of Curby caps (3). Samples were collected immediately before the experiments, and secretions pooled from three individuals were used unless otherwise indicated in the text.

**Bacteria.** *Streptococcus mutans* strains GS5, GS15 (both courtesy of R. Gilmore), OMZ176E (J. E. Norton) and 6715 (H. J. Sandham), *Streptococcus salivarius* 9GS2 (R. J. Gibbons), and *Streptococcus sanguis* 34 (R. J. Gibbons) and 10556 (H. J. Sandham) were cultured anaerobically for 24 h in Trypticase soy broth (BBL, Cockeysville, Md.). The cells were harvested by centrifugation (10,000 x g, 15 min), washed three times in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), resuspended in PBS to about one-tenth of the original culture volume, and dispersed for 15 s with a sonifier (Branson Instruments, Inc., Stamford, Conn.).

For adherence experiments, this heavy suspension was diluted in PBS, filtered whole saliva, or parotid fluid to an absorbance of 0.5 at 540 nm, corresponding to total counts of 0.9 x 10^9 to 1.2 x 10^9 organisms per ml for all species.

**Incubation for adherence.** Five to 10 enamel slabs, with and without pellicles, were mounted in cellulose acetate boxes. A 10-ml amount of the bacterial suspension to be tested was poured into the boxes that were covered, sealed, and incubated, with shaking action, for 1 h at room temperature. The bacterial suspension was then decanted, and the slabs were washed by shaking with PBS for 10 min.

**Microscope observation and quantitative enumeration of adherent bacteria.** The washed slabs were air dried, immersed for surface fixation in 95% ethanol or methanol for 10 min, rehydrated, and Gram stained without counterstain. The slabs, still mounted in the cellulose acetate boxes, were then washed with water and placed on the microscope stage. Immersion oil was placed directly on the specimens which were observed at ×540 magnification in a Leitz Ortholux microscope equipped with a vertical illuminator for incident light (15). Tungsten light was used unfiltered except for the dichroic mirror TK 400 and the secondary filter K 400 of the illuminator.

A reticle, which consisted of 100 equal squares and was mounted in a focus of the ocular, permitted standardized counts of bacterial cells to be made on each slab. The areas to be counted were randomly chosen, with two restrictions: the avoidance of surface irregularities near the edges and of enamel cracks. Five squares were counted at five locations on each slab surface; 25 squares thus constituted the unit area for counts per slab. In individual experiments, 5 to 20 identically treated slabs were used.

**Bacterial agglutination by oral fluids.** The ability of filtered, pooled whole saliva and pooled parotid fluid to agglutinate *S. sanguis* 34 and *S. salivarius* 9GS2 was monitored (i) by absorbance measurements (K. Pruitt and T. Ericsson, International Association for Dental Research, abstr. no. 587, 1972) and (ii) by microscopy of Gram-stained, standardized droplets. Washed and dispersed cells were suspended in PBS to A_600 ≈ 2, mixed with an equal volume of PBS, whole saliva, or parotid fluid, and incubated at 37 C. The A_600 was read at 10-min intervals over a 1-h period. For microscopy, 10 μlitters were pipetted off each suspension before, and at every 10-min interval of, the incubation period. The samples were air dried, ethanol fixed, Gram stained, and observed for agglutination at ×540 magnification.

**RESULTS**

**Microscope observations of enamel surfaces prior to and after incubation with bacterial suspensions.** Polished slabs without pellicles, stained as described for adherence experiments, revealed a smooth, homogeneous surface with occasional enamel cracks. Bacteria were not seen. The picture was similar when slabs with either in vivo or in vitro pellicles were stained and observed prior to incubation with bacteria; however, clusters of bacteria could sometimes be found along enamel cracks, and 1 to 10 epithelial cells, with some adherent bacteria, were seen scattered over the surface. Incubation of slabs with bacterial suspensions resulted in the adherence of scattered organisms; the density of attached bacteria varied depending on the experimental conditions (e.g., Fig. 1). The usually even distribution of attached bacteria was reflected in the reproducibility of triplicate counts which deviated only 4 to 6% from the mean.

**Influence of the acquired pellicle on bacterial adhesion.** Three test organisms, *S. sanguis* 34, *S. salivarius* 9GS2, and *S. mutans* 6715, suspended in PBS, all adhered weakly to uncoated enamel (Fig. 2). The presence of an acquired pellicle significantly (P < 0.01 by Student's t test) enhanced the adherence of each strain (Fig. 1 and 2). The results illustrated in Fig. 1 and 2 were derived from experiments with pellicles produced by the same individual throughout. When pellicles from four subjects were compared, the attachment of *S. sanguis* 34 and *S. salivarius* 9GS2 to these pellicles indicated small, though not statistically significant, intersubject differences; all pellicles showed enhanced adherence compared to uncoated slabs.
FIG. 1. Adherence of Streptococcus sanguis strain 34 to enamel coated with an acquired pellicle (A) and to uncoated enamel (B). Incident light, crystal violet. ×400.

In vitro pellicles promoted adherence to a similar degree as did in vivo pellicles; the bacterial counts were almost identical and the microscope pictures indistinguishable.

Strain differences in adherence to pellicle. Figure 2 illustrates the marked differences in the ability of buffer-suspended streptococcal strains to adhere to pellicle-coated enamel. The S. mutans strain adhered sparsely and in variable numbers; S. salivarius displayed an intermediate ability for attachment to the coated surface; S. sanguis regularly covered the pellicle densely (Fig. 1) and gave consistently high counts (Fig. 2). The mean estimates for the three strains were significantly different at the 1% level (Student’s t). The same pattern of species differences in adherence was observed when all seven strains were tested on pellicles produced in vitro: S. sanguis 34 and 10556 adhered more readily than did S. salivarius 9GS2, and the four S. mutans strains adhered weakly if at all.

Adherence of bacteria suspended in oral fluids. Marked changes occurred in the microscope picture of bacterial attachment when oral fluids were used as suspension media. The number of single organisms was low; the field was dominated by bacterial clumps of various sizes that adhered to the pellicle. Clumping was most pronounced after incubation in parotid fluid, when large aggregates of bacteria could be found widely scattered over the pellicle. Counting of individual organisms was still possible and was performed when the clumps appeared in the unit area.

When incubated in whole saliva, all four strains of S. mutans became less adherent to pellicle, but the variations due to clumping made the counts of this species unsuitable for statistical treatment; for this and other reasons (see Discussion), S. mutans was not included in subsequent experiments. Consistent and significant (P < 0.01) inhibition of adherence was observed when S. salivarius was suspended in whole saliva, whereas adherence of S. sanguis 34 was decreased in some experiments and enhanced in others (Table 1).

Substitution of the buffer with parotid fluid invariably caused adherence inhibition of both S. salivarius 9GS2 and S. sanguis 34; the decrease in adherence was greater than that encountered with whole saliva, and the effect was similar on both S. sanguis and S. salivarius (Table 1). With S. sanguis 34 and S. salivarius
9GS2 as test organisms, parotid secretions from four subjects were tested separately for inhibition of adherence to pellicles obtained from the corresponding individuals. Reduced attachment was noted in all cases; the subject differences were insignificant. When pooled parotid fluid of controlled pH (7.2) was tested for its effect on adherence of bacteria to in vitro pellicles, the inhibition was similar to that observed for in vivo pellicles. The inhibitory effect persisted with bacterial cells that were preincubated in parotid fluid for 1 h, washed, redispersed, and resuspended in PBS before incubation with in vitro pellicles (Table 1).

**Agglutination of test organisms by oral fluids.** Spectrophotometric measurements of the suspensions of *S. sanguis* 34 and *S. salivarius* 9GS2 incubated with whole saliva and parotid fluid revealed agglutination of the *S. sanguis* strain with parotid fluid only (Fig. 3), whereas *S. salivarius* 9GS2 did not show appreciable changes in optical density with either parotid fluid or whole saliva. Gram-stained smears (Fig. 4) were more sensitive than absorb-
ance readings in detecting agglutination; clumping of both S. salivarius 9GS2 and S. sanguis 34 by parotid fluid was readily observable under the microscope. Also, slight agglutination of both organisms by whole saliva could be noted (Fig. 4). The agglutination by parotid fluid increased during the 1-h incubation period; the largest clumps were found at the termination of the experiment.

DISCUSSION

Different commensal and pathogenic microorganisms in humans attach preferentially to particular kinds of epithelia through presumably specific interactions between the bacterial and mucosal surfaces (5, 16). Morphological (20) and bacteriological (2) characterizations of early dental plaque reveal a predominance of a limited number of microbial species. This suggests that attachment mechanisms of a specific nature also operate on the tooth surface; results of the present study show that such is indeed the case.

A cleaned tooth surface will be rapidly covered by an adsorbed layer of organic constituents from saliva (14, 19). and the bacteria that initiate plaque formation seem to be in contact with this acquired pellicle rather than with the enamel surface itself (17). The importance of this organic film in bacterial attachment was evidenced by the significant increase in adherence to pellicle-coated enamel as compared with uncoated (Fig. 1 and 2). The adherence-promoting effect of the acquired pellicle was observed in all four subjects tested, and the general nature of this phenomenon was further demonstrated in experiments with in vitro pellicles formed in whole saliva pooled from several individuals. It appears that the acquired pellicle does not merely precede bacteria on the tooth surface, but represents a preferred substrate for their attachment and thereby favors plaque formation.

The results of adherence tests for different streptococcal species demonstrated that attachment to the pellicle is a selective process (Fig. 2); the adherence of S. sanguis was significantly greater than that of S. salivarius, and both these strains adhered in greater numbers than did S. mutans. The higher affinity of S. sanguis for the pellicle implies that this organism is of greater importance in plaque initiation than the other streptococci, and this contention is supported by the prevalence of S. sanguis in early plaque (2). The comparatively negligible adherence of S. mutans to the pellicle may be taken to indicate that this organism does not play a major role in the very first stages of tooth colonization. Though it cannot be excluded that local conditions in vivo (e.g., available sucrose) may differ from our model so as to favor the attachment of S. mutans, the presence of S. mutans in plaque may be governed by interactions with preformed plaque rather than by primary adherence to the smooth surface of the tooth.

It is noteworthy that the relative adherences of S. sanguis and S. salivarius to the pellicle were reversed when compared to affinities for human cheek and tongue cells (5). On the one hand, this supports the concept of adherence as an ecological determinant in oral cavity (5); on the other, it demonstrates that the pellicle must differ significantly from the surface coat of oral epithelial cells. By inference, the pellicle is the factor mainly responsible for the ecologic fea-
tures unique to early microbial accumulation on the tooth surface. One may speculate, in this context, that immunoglobulins (10, 14) and salivary agglutinating factors (7) in the acquired pellicle may bind surface components of bacteria in a specific manner. Furthermore, it may be desirable to direct attempts at plaque prevention towards modification of the pellicle, and preliminary experimentation indicates that it is feasible to reduce the adherence-promoting quality of the pellicle by treatment with agents such as chlorhexidine gluconate (unpublished data).

The bacteria, for their part, are subject to modifications of their adherence characteristics by saliva. Whole saliva sometimes inhibited and at other times enhanced the adherence of bacterial cells, but the pattern of attachment of saliva-suspended organisms seen under the microscope always exhibited a larger number of aggregates than that of buffer-suspended controls. One may conclude that the adherence properties of oral bacteria are altered by their salivary environment in vivo. The influence of saliva on adherence seems to be species-specific as indicated by the difference of its effect on S. sanguis and on S. salivarius. However, the salivary influence may vary on a particular bacterial strain depending on the site of attachment. In the present study, S. salivarius 9GS2 adhered to the pellicle in significantly decreased numbers when suspended in whole saliva, whereas its attachment to human cheek cells (5) was unaffected by whole saliva under comparable experimental conditions. It is possible, therefore, that the attachment of S. salivarius to the pellicle and to epithelial cells entails two different mechanisms.

S. sanguis as well as S. salivarius was consistently less adherent when suspended in parotid fluid (Table 1), and the inhibition by parotid fluid was always greater (70 to 95%) than the most pronounced inhibition caused by whole saliva (64%). The decrease in attachment was accompanied by the appearance of large bacterial aggregates on the pellicle, which suggested a relationship between adherence and agglutination. Both test organisms were agglutinated by whole saliva and, more strikingly, by parotid fluid (Fig. 3 and 4); the reaction of S. sanguis 34 with parotid fluid was so extensive that visible aggregates were formed concomitantly with a decrease in absorbance. The simultaneous occurrence of agglutination and adherence inhibition may promote the conclusion that attachment of large aggregates of bacteria to the pellicle is mechanically hampered. However, it is equally possible that adherence effectors on the bacterial surface become inaccessible after interaction with salivary constituents. Salivary IgA which coats salivary bacteria in vivo (1) would seem to be a likely mediator of inhibition (22), but fluorescent protein tracing of organisms incubated with, and agglutinated by, parotid fluid in vitro failed to disclose IgA on the bacterial surfaces (unpublished observation).

The present in vitro study of streptococcal adherence to tooth surfaces demonstrates an interdependence of salivary factors, acquired pellicle, and bacterial cell surfaces. In vivo, the relative importance of various bacteria in plaque initiation may be determined by their tendency, as modified by the salivary environment, to attach to the acquired pellicle.

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LITERATURE CITED


