Site-related streptococcal attachment to buccocervical tooth surfaces

A correlative micromorphologic and microbiologic study

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Bevenius J, Linder L, Hultenby K. Site-related streptococcal attachment to buccocervical tooth surfaces. A correlative micromorphologic and microbiologic study. Acta Odontol Scand 1994;52:294–302. Oslo. ISSN 0001-6357.

Scanning electron (SEM) microscopy of epoxy replicas made from dental impressions has shown that in buccal gingival recession the root surfaces are devoid of cementum, leaving the dentin exposed. In this study replication techniques were applied to correlate the micromorphology of the buccocervical region with early streptococcal attachment. The subjects were 27 healthy young adults. The buccocervical surfaces of all the premolars were meticulously cleaned. The subjects fasted for 2 h before impression-taking. Replicas were made from impressions in hydrophilic A-silicone, and streptococcal attachment was visualized by light microscopy of mitis-salivarius agar replicas incubated anaerobically for 48 h. The surface micromorphology was documented by SEM of corresponding epoxy replicas. Colonization only 2 h after cleaning was very sparse. Sites with healthy or inflamed gingivae had markedly different colonization patterns in the sulcular region. In 4 subjects with a total of 12 sites where gingival recession, undetected clinically, was disclosed by SEM, representative colonies were retrieved and identified microbiologically to species level. Two healthy sites per subject were also sampled. *Streptococcus mutans* and *S. sobrinus* were identified from eight sites with exposed root dentin. *S. oralis* predominated on the enamel surfaces. The method offers a valuable complement to in situ and in vitro microbiologic studies of exposed dentin and a novel technique for sampling clinical isolates of streptococci. □ *Buccocervical region; replica; scanning electron microscopy; streptococcal attachment*

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In dental plaque morphogenesis, pioneer bacterial attachment is considered to have an important influence on the subsequent selective adsorption of other microorganisms (1). Early colonization of the tooth surface, studied mainly by in situ models of bacterial attachment to artificial surfaces (2–5) or to slivers of extracted human or bovine teeth (6, 7), is dominated by streptococci with special affinity for receptors in the salivary pellicle.

Although these adherence characteristics have been intensively investigated in vitro, site-related variations in the pattern of early streptococcal colonization may occur in vivo, owing to variations in the acquired pellicle.

In the gingivocervical region, for example, local conditions may influence pellicle formation. The oral fluids from which pellicular components are derived comprise not only salivary secretions but also crevicular fluid from the gingival sulcus. In health crevicular fluid is regarded as a serum transudate, but in gingivitis it becomes an inflammatory exudate (8). Furthermore, exposure of the root surface by gingival recession offers a potentially different surface for pellicle formation.

Plaque morphogenesis in the dentogingival region has been documented by scanning electron microscopy (SEM) of replicas made from dental impressions (9–11). Although this is a simple, non-invasive method for revealing sites of bacterial proliferation, it is bacteriologically non-specific; that is, it precludes identification of the microflora at the sites of plaque development.

Dental impressions have also been rep-

licated in selective agar culture medium to study *Candida* colonization of denture seating surfaces (12) and to demonstrate *Streptococcus mutans* on occlusal fissures of molar teeth (13, 14).

The present study is one of a series applying replication techniques to study the gingivocervical region of human teeth (15–17). In healthy young adults free from caries and periodontal disease, SEM of replicas has shown a high frequency of clinically undetectable buccal gingival recession, characterized by exposure of root dentin (17). The concomitant presence of healthy sites in the same subjects presented an opportunity for investigating whether early streptococcal attachment differed at sites with exposed dentin.

The streptococci were retrieved in an impression of the tooth surface, cultivated on mitis-salivarius (MS) agar replicas and identified to the species level.

The specific aim of the study was to relate the in vivo pattern of streptococcal adherence to the surface micromorphology of the underlying tooth structure, documented by SEM of corresponding replicas in epoxy resin.

Materials and methods

The subjects, described in an earlier publication (17), comprised 27 volunteers: 14 men and 13 women aged 17–25 years (mean age, 19.4 years). They were non-smokers, in good general health, and were not currently undergoing restorative or orthodontic treatment. Four of the women and 11 of the men were recent matriculants from high schools in suburban Stockholm. The remaining nine women and one man were recent arrivals from Eastern Europe, guest students at Karolinska Institutet. They were informed in writing about the purpose of the study and the procedures involved. The study was approved by the ethical committee of Karolinska Institutet.

On the day of the experiment the subjects were instructed to carry out their usual oral hygiene procedures. Sampling was performed about 90 min after breakfast.

Bacterial sampling

The subjects rinsed their mouths vigorously with water, and the buccal surfaces of all the premolars were carefully cleaned with a slurry of pumice and water in a rotating rubber cup, the flexible lip of the rubber cup gently deflecting the gingiva. The teeth were then vigorously sprayed with water and air. The subjects then fasted for 2 h before impression-taking.

To remove saliva, the experimental surfaces were flushed with a gentle stream of water from the triplex syringe. The area was isolated with an absorbent parotid shield (Dry-tip; Mölnycke, Sweden) and then dried with a gentle stream of air. Automixing hydrophilic polyvinylsiloxane impression material (Panasil Contact, Germany) was expressed through a special tip (Express tip, 3M, St. Paul, Minn., USA), starting at the approximal papilla distal to the second premolar and moving quickly along the gingival margin. To give bulk to the impression, a further layer was expressed over the first. Impressions of maxillary and mandibular teeth on one side of the mouth were taken from the same mix. After a 4-min setting time the impressions were carefully flexed off the teeth with sterile tweezers and transferred to a clean plastic container, which was sealed immediately. A second impression was taken immediately, for replication in epoxy resin for SEM. The procedure was then repeated on the contralateral teeth.

Laboratory procedures

The first impressions were processed within 2 h. A supporting mold of baseplate wax was carefully adapted around the base and sides of each impression, care being taken to avoid contamination of the impression surfaces. MS agar (Difco, Detroit, Mich., USA), prepared the day before and maintained at 50°C, was then carefully pipetted into the impressions.

Once the agar had set, the wax molding was removed with a sterile scalpel. The MS-agar-filled impression was then carefully inverted onto a Petri dish prepared with a thin layer of transparent agar. The im-

pression was gently eased off with a sterile instrument, leaving an agar 'replica' of the dentogingival region, face upwards, on the Petri dish.

Bacterial culture

The replicas were incubated anaerobically for 48 h at 37°C and examined for bacterial colonies at 24 and 48 h. To document the sites of bacterial growth, the replicas were photographed in a stereomicroscope at 16× (Leitz), using a cold light source to avoid thermal damage to the agar.

Pilot investigation

The development and evaluation of procedures for impression-taking and replication for micromorphologic studies of the gingivocervical region by SEM have been reported previously (15). Modifications necessary for replication for microbiologic studies were developed in a pilot investigation, conducted in three healthy volunteers.

The buccocervical surfaces of the right and left maxillary first and second premolar teeth were used in a crossover study. The various steps evaluated were carried out three times, at intervals of 1 week.

The question of establishing base-line cleanliness of the tooth surfaces before impression-taking was addressed: the antimicrobial agent used to reduce surface tension in the earlier SEM method was excluded because this might inhibit recolonization.

The timing of the experiments was standardized so that the potential effect on the oral fluids of a meal followed by oral hygiene procedures (fluoride toothpaste) would be minimized.

Impressions taken without prior professional cleaning—that is, using the subject's own oral hygiene measures as a standard reference—yielded variable streptococcal growth, with marked intraindividual variation from day to day. The cleansing effect of pumicing, depressing the lip of the flexible rubber cup into the sulcus, was assessed by sampling the sites with a sterile toothpick (TePe Röd, Munhygienprodukter AB, Malmö, Sweden) before and after cleaning. Extreme care was taken to avoid contacting the gingiva during sampling. The samples were streaked onto MS agar. On the contralateral teeth, impressions were taken before and immediately after pumicing and replicated in MS agar. Replicas from impressions after pumicing showed only a few isolated colonies in the gingival sulcus region and no colonization on the replicated tooth surfaces. The samples plated from the toothpick showed no streptococcal growth. Pumicing was therefore routinely included to ensure a base-line level of cleanliness in all subjects.

Impressions were taken 30, 60, 90, and 120 min after cleaning, but streptococci were consistently detected on the tooth surfaces of all subjects by the agar replica method at 120 min after cleaning. Consequently, a fasting period of 2 h was adopted.

The use of an absorbent parotid shield to prevent salivary contamination was adopted in preference to the conventional cotton rolls, after several replicas showed cotton fibers with adherent bacterial colonies. It was very difficult to insert cotton rolls into the buccal fold without inadvertently contacting the dentogingival region. No such problem occurred with the parotid shield.

Replicas poured immediately and replicas of the same sites poured up to 2 h after impression-taking showed no quantitative

Table 1. Distribution of gingival recession of the buccocervical region of premolar teeth in four selected subjects

	Tooth site							
Subject	15	14	24	25	45	44	34	35
A		+	+	+			+	+
B		+	+					
C					+	+	+	+
D	+	++	++	+			+	+

Scanning electron microscopy (SEM) of replicas showed that all root surfaces exposed by gingival recession were devoid of cementum, leaving the dentin exposed.

+ = Gingival recession, undetected clinically, but disclosed by SEM of epoxy replica; ++ = clinically detected gingival recession.

differences in bacterial growth. Delays of up to 5 h or storage in transport medium gave inconsistent intraindividual results. All replicas were subsequently prepared no later than 2 h after impression-taking.

Identification of streptococci

The MS agar replicas of four subjects were selected for microbiologic sampling. SEM of the epoxy replicas in SEM had shown that these subjects had sites with clinically undetected buccal recession as well as healthy sites (Table 1).

At a magnification of 20×, single colonies were picked off the MS agar replicas, in accordance with the following criteria: no colony was closer than 0.5 mm to the gingival margin; the colonies had distinctive morphologies and could be retrieved without contamination by neighboring colonies of different morphology; and colonies were representative for colonization of coronal enamel and/or exposed root surfaces. Twelve sites with buccal recession were sampled and, in addition, two healthy sites from each subject. One colony was sampled from the interproximal papilla of a healthy site.

The selected colonies were cultivated on blood agar and on MS agar at 37 °C for 48 h in anaerobic jars (GasPak, BBL Microbiology Systems, Cockeysville, Md., USA) and then transferred from blood–agar plates to 2-ml vials containing glucose-peptone medium with 30% v/v glycerol. The vials were stored at −70 °C.

Identification of the isolates was done mainly with the tests described by Kilian et al. (18). Dextran and levan production was determined with the method of Gibbons & Banghart (19).

Arginine hydrolysis and fermentation of mannitol were determined by means of the API 20E (API system, SA Montalieu-Vercieu, France) and α-fucosidase and β-glucosidase with the APIzym (API system) (20). Esculin and mannitol fermentation were tested with esculin and mannitol agar (21). *S. mutans* was differentiated from *S. sobrinus* by its ability to metabolize *N*-acetyl glucosamine (22).

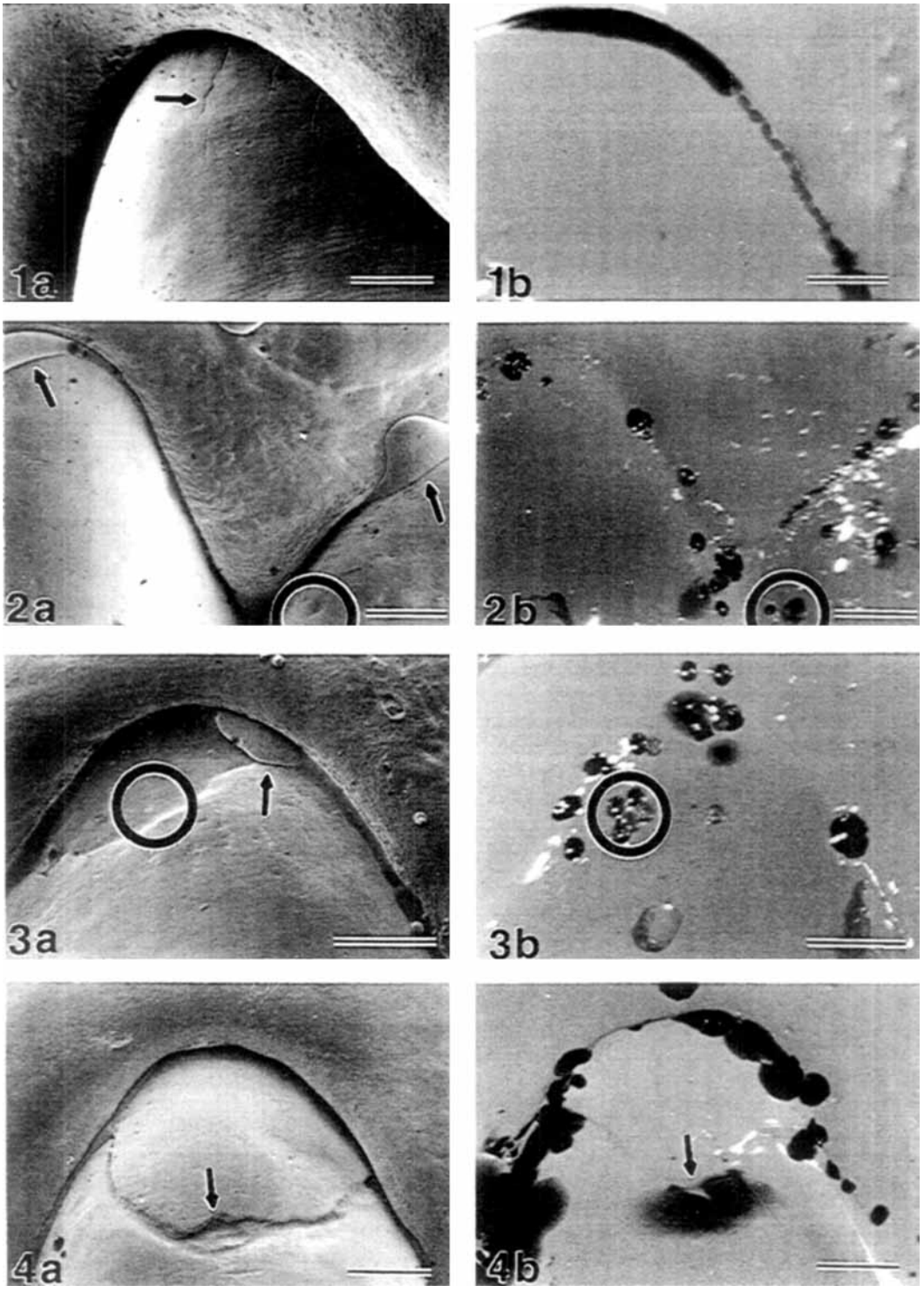
Replication of tooth surfaces for scanning electron microscopy

The second impressions, taken immediately after the scavenger impressions, were poured in Epoxy-die (Ivoclar, Liechtenstein) as described in a previous paper (15), coated with a 20-nm layer of platinum (Polaron, Watford, UK) and examined in the scanning electron microscope at 3 kV (Jeol, JSM 820, Tokyo, Japan). The micromorphology of the dentogingival region was photographed at magnifications from 20× to 280×, with detailed documentation at up to 2000×.

Results

In the total material of 27 subjects, 216 buccocervical surfaces of premolar teeth were replicated in MS agar and epoxy resin. SEM of the epoxy replicas disclosed that in addition to the 32 sites diagnosed clinically as having exposed root surfaces, a further 37 sites had subclinical exposures. All these 69 root surfaces were devoid of cementum, leaving the dentin exposed. Many sites with clinically healthy gingivae showed evidence by SEM of gingival inflammation: seepage of gingival exudate from the sulcus and engorgement of the gingivae, particularly the interproximal papillae, distorting the normal contour (Fig. 2). At healthy sites (Fig. 1a), streptococcal colonization at the entrance to the gingival sulcus was characterized by coalescence of colonies of similar morphology, outlining the coronal limit of the marginal gingiva (Fig. 1b). At sites assessed clinically as healthy but showing evidence of subclinical inflammation on SEM of epoxy replicas (Fig. 2a), colonies on the agar replicas had heterogeneous morphologies, clumping together and spreading onto the replicated tooth surface or scattered along the sulcus entrance (Fig. 2b). Colonies in this region were not selected for identification.

Site-related early streptococcal adhesion to hard tissue surfaces, as evidenced by growth on MS agar replicas, was characterized by sparse populations of organisms belonging to the species *S. oralis, S. mitis* Biovar 2, *S. mutans*, and *S. sobrinus*.


1a
1b
2a
2b
3a
3b
4a
4b

Of the 21 colonies retrieved for subculture, 1 colony was contaminated by staphylococci during laboratory procedures, and 3 did not grow. Seventeen of the clinical isolates were successfully subcultivated: seven were identified as *S. oralis*, three as *S. mitis* Biovar 2, three as *S. mutans*, and two as *S. sobrinus*. One colony was identified as *Veillonella*. The colony retrieved from the healthy interproximal papilla was identified as *S. salivarius*.

As shown in Table 2, the *S. oralis* colonies were retrieved from enamel surfaces, four from intact cervical enamel and three from regions of enamel hypoplasia (Fig. 2b). The mutans streptococci were all from root dentin. The *S. mitis* were retrieved from sites on cervical enamel, near an exposed amelocemental junction and the gingival margin. The surfaces from which the colonies of *S. mutans* and *S. sobrinus* (Fig. 3a,b) were recovered and identified had not been clinically diagnosed as exposed roots but were disclosed by SEM of the epoxy replicas.

Although the fine, fissure-like developmental faults in the coronal enamel of the teeth (Figs. 1a and 3a) were faithfully reproduced in the agar replicas, no evidence of streptococcal attachment was detected in these regions at healthy sites or at sites with exposed roots. However, areas of enamel hypoplasia were preferentially colonized by *S. oralis* (Fig. 2b).

Discussion

To our knowledge, using agar replication techniques to study the dentogingival region has not previously been reported. The concomitant application of SEM replica

Plate I. Epoxy resin replicas at low magnification in scanning electron microscopy (SEM) and the corresponding mitis salivarius (MS) agar replicas photographed in a stereomicroscope after 48 h of anaerobic incubation. Bar = 1 mm.

Fig. 1a. Healthy cervical region, showing the evenly rolled border of the marginal gingiva. Arrows indicate developmental faults, probably enamel tufts, which were a common characteristic of most sites. 1b. Corresponding replica in MS agar. Streptococcal colonization is limited to the sulcul region, and the colonies, all of similar morphology, have coalesced. No colonization has occurred at the sites corresponding to the developmental faults arrowed in Fig. 1a.

Fig. 2a. Low-magnification SEM of epoxy resin replica of maxillary right premolar teeth. These sites were assessed clinically as healthy, but gingival exudate, indicated by the arrows, was present on the tooth surfaces during impression-taking. The interproximal papilla is engorged. The enamel surface of the first premolar had patches of hypoplasia in the form of pits (circled area). 2b. Replica in MS agar of corresponding tooth surfaces. Colonization in the gingival sulcus area is sparse and unevenly distributed, and the colonial morphology is varied. In the area corresponding to the circled area in Fig. 2a, colonization of the hypoplastic pits has occurred. Colonies retrieved from this region were identified as *Streptococcus oralis*.

Fig. 3a. SEM low-magnification view of an epoxy resin replica of a site with subclinical gingival recession and root exposure. The gingiva had been assessed clinically as healthy, but some gingival exudate (arrowed) was present on the tooth surface during impression-taking. At higher magnification the dentin comprising the root surface was characterized by droplets of fluid extruding from the dentinal tubules. 3b. MS agar replica of the same site. The colonies retrieved from the circled area, corresponding to the site in Fig. 3a, were identified as *S. sobrinus*.

Fig. 4a. SEM of epoxy resin replica of a premolar tooth restored with glass ionomer cement because of cervical sensitivity. The arrow indicates a deep defect in the margin of the restoration, which was seen at higher magnification to penetrate to the dentin. 4b. Agar replica of the corresponding site. Streptococcal colonization appears to be restricted mainly to the gingival sulcus and to the deep defect in the margin of the restoration (arrowed).

Table 2. Identification of colonies retrieved from mitis salivarius agar replicas of cervical tooth surfaces in four subjects

Streptococcal strain	Healthy site		Site with recession	
	en	hypopl*	cej*	rt surf*
S. oralis	4	3		
S. mutans				3
S. sobrinus				2
S. mitis (Biovar 2)			3	
Failed to grow				3
Contaminated	1			

en = enamel; hypopl = enamel hypoplasia; cej = amelocemental junction; and rt surf = exposed root surface.

* Not detected clinically, but disclosed by scanning electron microscopy of epoxy replica.

techniques to document the site-related micromorphology has provided information about the in vivo microenvironment in this region, to complement in situ studies.

As with other methods of sampling bacteria in vivo, there are inherent errors (23, 24). A problem seldom addressed in sampling of enamel surfaces but highly relevant in the gingivocervical region is the establishment of base-line cleanliness of the tooth surface. The possibility that some viable bacteria had not been flushed from the gingival sulcus cannot be discounted, and colonies closer than 0.5 mm to the gingival sulcus region on the agar replicas were therefore not selected for identification. The characteristic pattern of colonization for healthy and inflamed sites is, however, in accordance with in vitro observations of the inhibiting effect of inflammatory gingival exudate on streptococcal colonization (8).

The sparse streptococcal adhesion to 2-h pellicle on enamel surfaces in vivo after meticulous pumicing and thorough irrigation is in accordance with earlier SEM studies of 2-h pellicle formation, and analysis of amino acids from such pellicle has shown them to be of salivary, not bacterial, origin (25). In our pilot study replicas from impressions taken less than 90 min after cleaning showed no colonization, and colonization was consistent first after 2 h. In most in situ studies it is extremely difficult to harvest bacterial colonies before 4 h after cleaning, and sucrose rinsing is frequently used in healthy subjects to increase plaque volume, even though the presence of sucrose selectively enhances growth of some streptococcal species. The very sparse colonization of the tooth surfaces in the present study facilitated selection of colonies of distinctive morphology and reduced the risk of contamination when these colonies were recovered for subculturing.

The present study shows that the microenvironment in the gingivocervical region may be modified by clinically indiscernible gingival recession, loss of cementum, and exposure of the underlying dentin. The outward flow of dentinal fluid through the exposed tubules is regarded as a hydrodynamic mechanism to protect the pulp from inward diffusion of molecules from the surface (26–30). However, the potential role of dentinal fluid, a plasma transudate rich in proteins, as a selective substrate for early colonizers of exposed root surfaces warrants further investigation. The specific affinity of certain strains of mutans streptococci for exposed dentin may be important in the pathogenesis of root caries, and a recent in vitro study has shown that the collagen of dentin mediates adhesion of certain strains of *S. mutans* (31).

Comparison of the present results with earlier studies is complicated by the introduction in 1989 of the detailed taxonomic classification of streptococcal species used in the present study (18). The revised classification has been applied in an in situ study of initial streptococcal microflora in caries-active and caries-inactive adolescents (7), which reported that after 4 h the predominant streptococci on enamel were *S. oralis, S. mitis* Biovar 1, and *S. sanguis*. The present study included attachment only 2 h after cleaning, and no *S. sanguis* or *S. mitis* Biovar 1 was recovered, the predominant species being *S. oralis*. The difference in findings may be attributable not only to the difference in intervals before sampling but also to the modifying influence in the present study of local conditions in the gingivocervical region. Similarly, in situ studies of microbial colonization of tooth specimens taken from the amelocemental junction of intact, unerupted teeth have demonstrated attachment to healthy cementum (32) and not to vital dentin as in the present study.

Because the microbiologic data were obtained from highly selected subjects, the results of the present study should be interpreted with caution. A larger study in healthy young adults would provide baseline data as a frame of reference for studies relating micromorphology and early plaque morphogenesis in, for example, root caries risk subjects (33–35).

The following preliminary conclusions can be drawn: 1) streptococcal attachment to 2-h pellicle on the buccocervical surfaces of premolars, although sparse, can be visualized by replication in MS agar; 2) attachment varies qualitatively and quantitatively

in accordance with the condition of the cervical tooth surface—that is, intact cervical enamel protected by the gingiva or exposed cementoenamel junction, root surface denuded of cementum. This condition cannot be determined by clinical examination alone because of the frequency of subclinically exposed root surfaces; and 3) the pattern of streptococcal attachment suggests that in vivo, surface roughness is not the sole determinant of initial bacterial attachment to enamel or dentin.

The streptococcal strains retrieved from the agar replicas and subsequently identified to species level by biochemical and fermentation tests (18–22) have been stored at −70°C, and the characteristics of their adhesion to experimental pellicles are currently being compared with those of corresponding laboratory strains.

Acknowledgements.—The skillful technical assistance of Ms Helena Lönnies and Ms Karin Trollsås is gratefully acknowledged. Technical facilities for scanning electron microscopy were provided at The Clinical Research Center, Huddinge Hospital. The study was supported by grants from The Swedish Dental Association and The Karolinska Institute.

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Received for publication 29 October 1992
Accepted 30 March 1994