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SCANNING ELECTRON MICROSCOPY OF DENTIN CARIES. EXPERIMENTAL *IN VITRO* STUDIES WITH *STREPTOCOCCUS MUTANS*

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Abstract

This study was performed to gain better insight into the mechanisms involved in carious destruction of human dentin by *Streptococcus mutans*. In particular, bacterial colonization of dentin surfaces and bacterial invasion in dentin were studied.

Streptococcus mutans (*S. mutans*), strain NCTC 10449, was grown on sterile dentin blocks in a 10 % CO_2 atmosphere at 37°C. After 72, 120, 144 and 288 h of incubation the specimens were processed for scanning electron microscopic examination. The colonization of the dentinal surface progressed slowly and was nearly complete after 288 h. Invasion of *S. mutans* into the dentinal tubules was found occasionally and was limited to the initial 5 µm of the tubular lumen. The acid metabolites produced by *S. mutans*, caused lesions of the dentinal structures in the close proximity of the bacteria.

From the results of this *in vitro* study it appears that carious destruction of exposed dentinal surfaces by *S. mutans* mainly occurs at the exposed dentin after it has been colonized by bacteria. Destruction of the deeper dentinal layers by bacteria invading the dentinal tubules may play a less important role. However, in the few cases where *S. mutans* invaded the dentinal tubules, rapid destruction of the peritubular dentin sheath occurred. In addition, the possibility remains that acid metabolites produced by *S. mutans* diffuse into the dentinal tubules and cause tissue damage in the deeper parts of the dentin.

KEY WORDS : *Streptococcus mutans*, Dental Caries, Dentin, Bacteriology, Scanning Electron Microscopy, Bacterial Colonization, Bacterial Invasion, Demineralization, Dentinal Tubules.

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Introduction

Streptococcus mutans (*S. mutans*) is considered the most important caries associated oral microorganism (for review : Loesche, 1982, 1987). The cariogenic potential of *S. mutans* has been demonstrated in animal experiments (Fitzgerald and Keyes, 1960; Zinner et al., 1965; Krasse, 1966; Guggenheim et al., 1966; Gibbons et al., 1966; Fitzgerald, 1968; Guggenheim, 1968; Krasse and Carlsson, 1970; Michalek et al., 1977a, 1977b; Hamada et al., 1978). In humans, the relationship between the presence of high levels of *S. mutans* and the development of dental caries was established in cross-sectional epidemiologic studies (Littleton et al., 1970; Ikeda and Sandham, 1971; Englander and Jordan, 1972; Hoerman et al., 1972; Loesche and Syed, 1973; Shklair et al., 1974; Loesche et al., 1975; Street et al., 1976; Duchin and van Houte, 1978) and in longitudinal studies (Krasse et al., 1968; Ikeda et al., 1973; Swenson et al., 1976; Klock and Krasse, 1978; Masuda et al., 1979; Loesche and Straffon, 1979; Köhler et al., 1981).

In experimental animals, *S. mutans* caused fissure caries and smooth surface caries (Gibbons et al., 1966; Krasse and Carlsson, 1970; Michalek et al., 1977a, 1977b; Hamada et al., 1978), as well as root surface caries (Gibbons et al., 1966). In humans, *S. mutans* could be isolated from fissure caries and from smooth surface carious lesions (de Stoppelaar et al., 1969; Edwardsson, 1974; Svanberg and Loesche, 1978; Loesche and Straffon, 1979; Masuda et al., 1979) as well as from root surface carious lesions (Jordan and Hammond, 1972; Sumney and Jordan, 1974; Loesche et al., 1975).

Ultrastructural studies of carious lesions (Takuma and Kurahashi, 1962; Frank et al., 1964; Westbrook et al., 1974; Mjör, 1974; Olgart et al., 1974; Brännström and Nyborg, 1974; Langeland, 1981) showed the presence of microorganisms in the dentinal tubules. However, the mechanisms and the progression rate of this bacterial invasion into the dentinal tubules has not been studied so far.

The aims of the present *in vitro* study were to develop and use an *in vitro* model to study : a) the colonization of dentinal surfaces when exposed to a single episode of exposure to

S. mutans, b) the pattern of invasion of *S. mutans* into the dentinal tubules, and, c) to study the effect of bacterial acid metabolites on the dentinal structures.

Materials and Methods

In vitro model

Under aseptic conditions dentin pieces of approximately 8 mm^3 were removed from the approximal areas of 16 caries-free human premolars. All teeth were obtained from patients seen in the Dept. of Orthodontics in whom the orthodontic treatment included extractions of premolar teeth. Teeth with carious lesions and teeth with white spot enamel lesions were not taken into the study. Dentin pieces were obtained from the coronal interdental portions of the teeth. The isolation of the dentin pieces was done with high speed dental diamond points cooled with a sterile saline spray. The dentin pieces were rapidly frozen in liquid nitrogen and transferred to a liquid nitrogen-cooled copper table (Adriaens, 1987). A freeze-fracture of the dentin piece was induced in a plane perpendicular to the long axis of the dentinal tubules. Using this procedure, 73 dentin blocks with a surface area of approximately 4 mm^2 and a thickness of approximately 1 mm were obtained. After the freeze-fracturing, each dentin block had one surface which was free of contamination or smear layer which would cover the tubular entrances after preparation with diamond points.

Subsequently, the dentin blocks were transferred to individual vials containing 15 ml of thioglycollate broth (29 mg thioglycollate - BBL Microbiology Systems, Cockeysville, MD - in 1 ml distilled water). The vials were placed on a gyrotory shaking apparatus and incubated for 48 h at 37°C in a 10 % CO_2 atmosphere. Fifteen flasks showed bacterial growth, indicating a contamination of the dentin blocks, which may have occurred during the preparatory phases. These dentin blocks were taken out of the experiment.

The remaining 58 dentin blocks were transferred on to blood agar plates (40 mg trypticase soy agar in 1 ml distilled water, supplemented with 5 % human blood). The dentin surface exposed by the freeze-fracturing procedure was directed away from the agar surface. All blocks were gently pressed into the agar, leaving only the freeze-fractured surface exposed to the atmosphere (Fig. 1). One hundred microliters of a suspension of *Streptococcus mutans* (*S. mutans*), strain NCTC 10449 (ATCC 25175), grown for 24 h in thioglycollate broth, was deposited on each dentin block. Four dentin blocks were taken for baseline observations. The remaining plates were incubated in a 10 % CO_2 atmosphere at 37°C for different experimental periods : 72 h (12 blocks), 120 h (13 blocks), 144 h (14 blocks) and 288 h (11 blocks). Four control specimens were sham-infected with sterile thioglycollate broth and incubated for 144 h.

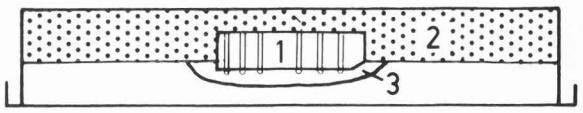


Fig 1 The dentin block (1) is submerged in the agar (2) in such a way that the surface exposed by the freeze-fracture remains exposed to the atmosphere. Before incubation 100 µl of the bacterial suspension containing *S. mutans* (3) is placed on this surface. The specimen is incubated with the exposed dentin surface facing downward.

Scanning electron microscopy

Fixation was performed at the end of the experimental period by flooding the plates with a 2.5 % glutaraldehyde solution in a 0.1 M sodium cacodylate buffer (pH = 7.4). After 12 h the dentin blocks were transferred to individual vials containing a freshly prepared sodium cacodylate buffered 2.5 % glutaraldehyde solution and they were fixed for an additional 2 h. Washing in 0.1 M sodium cacodylate was performed twice, followed by dehydration in a graded ethanol series. Then, the dentin blocks were rapidly frozen in liquid nitrogen and a freeze-fracture along the long-axis of the dentinal tubules was induced. Ethanol was substituted by amylacetate before critical point drying was performed in a Polaron E3000 critical point drying apparatus (Polaron Instruments Inc., Doylestown, PA). The specimens were mounted on stubs, sputter coated with approximately 25 nm gold in a Polaron E5000 diode sputtering apparatus and examined in a JEOL JSM U3 scanning electron microscope (JEOL, Tokyo) at 15 kV.

In randomly chosen areas on the exposed surfaces, the numbers of dentinal tubules associated

Table 1 : Colonization of the dentin surface

incubation period (h)	0	72	120	144	288
specimens (n)	4	12	13	14	11
observed tubules (n)	1429	1068	1447	1131	420
associated tubules[a] (%)	0.6*	22.0*	36.8*	50.4*	92.1*
confidence limit	0.4	2.5	2.5	3.0	2.7

* : statistically significantly different from all other values in row ($p < 0.005$).
[a] : bacteria were present on the peritubular dentin or covered the peritubular dentin and the tubular lumen partially or completely

and not associated with *S. mutans* were counted. Dentinal tubules were considered as associated with *S. mutans* when bacteria were present on the peritubular dentin area or when bacteria covered both the peritubular dentin and the tubular lumen. On the longitudinally fractured surfaces the dentinal tubules without and with bacterial invasion were counted. The dentinal tubules with bacterial invasion were divided into 2 groups. The first group consisted of dentinal tubules with bacterial invasion in the outer 5 µm of the dentinal lumen. In the second group bacteria had invaded deeper than 5 µm into the dentinal tubules. Comparisons between the specimens incubated for different time periods were made and statistical significance was determined using a Chi-square test. The diameter of the decalcification lesions and the corresponding bacteria were measured on photomicrographs (magnification 10,000 times). These data were tested for statistical significance using parametric (Student's t-test) and non-parametric (Wilcoxon rank sum test) techniques.

Results

Colonization of exposed surface

Observations made on the baseline specimens revealed that at the start of the experiment 0.6 % of the dentinal tubules were associated with *S. mutans*. Bacteria were present as single organisms or as small groups. They were well distributed over the surface and, in most cases, were found on the intertubular dentin.

With increasing incubation time the bacterial colonies were growing larger (Figs. 2a and 2b). Confluent microcolonies were found starting from 144 h on (Fig. 2c). With a longer incubation period increasing proportions of intertubular and peritubular dentin, as well as orifices of dentinal tubules became covered by bacteria. Once confluent colonies were established, several superimposed bacterial layers were observed (Figs. 2d and 3a). After 288 h of incubation, 93.1 % of the dentinal tubules were associated with *S. mutans*.

Table 1 shows the percentages of dentinal tubules associated and not associated with *S. mutans* for the different incubation periods. The number of dentinal tubules associated with *S. mutans* increased with time. Statistically significant differences ($p < 0.005$) were found between all incubation periods.

Invasion into dentinal tubules

In the dentin blocks used as baseline specimens, *S. mutans* was never found in the dentinal tubules. From 72 h onwards invasion into the dentinal tubules occurred. However, this invasion remained limited, even after 288 h of incubation (Figs. 3a and 3b). When bacteria were found in the dentinal tubules, they were most frequently located at the tubular entrances. After 288 h of incubation, only 4.6 % of the observed tubules displayed bacterial invasion to a depth greater than the initial 5 µm. For the 288 h specimens the mean depth of invasion in these tubules was 23.3 µm (s.d. = 8.2 µm).

In all dentinal tubules which displayed bacterial invasion, the bacteria were present as a continuous row, starting at the colonized surface and extending to the deepest part of the tubular lumen which contained the bacteria (Figs. 3b and 3c). This observation was made for both the dentinal tubules with bacteria in the initial 5 µm of the tubular lumen and for the tubules containing invading bacteria in the deeper part of the lumen. In those tubules where bacteria had been washed away during specimen preparation, their previous presence was shown by distinct demineralization lesions in the tubular wall (Fig. 3d).

Table 2 gives the percentages of empty tubules, tubules with *S. mutans* in the outer 5 µm of the tubular lumen and tubules with *S. mutans* invading to a depth greater than 5 µm in the dentinal tubules, in function of incubation time. Differences between all experimental periods were statistically significant, except for the differences between 72 h and 120 h and between 144 h and 288 h.

Demineralization of the dentin

In the baseline specimens, bacteria were lying on top of the dentinal surface. The dentin

Table 2 : Invasion of the dentinal tubules

incubation period (h)	0	72	120	144	288
number of specimens	4	12	13	14	11
number of tubules observed	145	240	315	299	138
% empty tubules	100.0	94.6	94.3	82.9	79.0
% invaded tubules (< 5 µm)[a]	0.0	4.2	3.8	15.1	15.9
% invaded tubules (> 5 µm)[b]	0.0	1.2	1.9	2.0	5.1
% invaded tubules	0.0[c]	5.4[d]	5.7[d]	17.1[e]	21.0[e]

[a] : tubules containing invading bacteria in the outer 5 µm of the tubular lumen.
[b] : tubules containing invading bacteria to a depth exceeding the peripheral 5 µm of the tubular lumen.
[c] : statistically significantly different from all other data in row ($p < 0.005$).
[d] : statistically significantly different from values with c and e ($p < 0.005$).
[e] : statistically significantly different from values with c and d ($p < 0.005$).

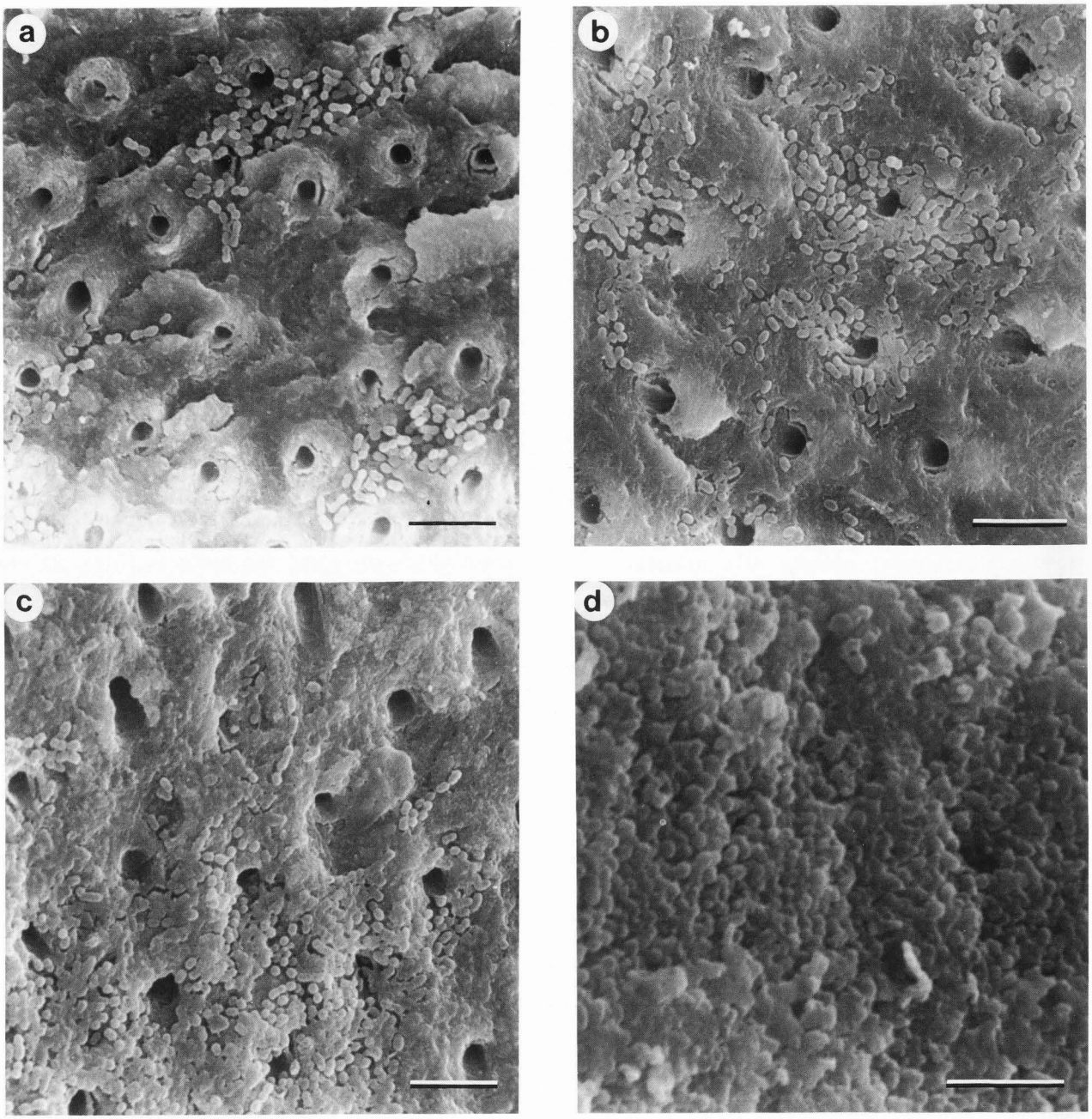
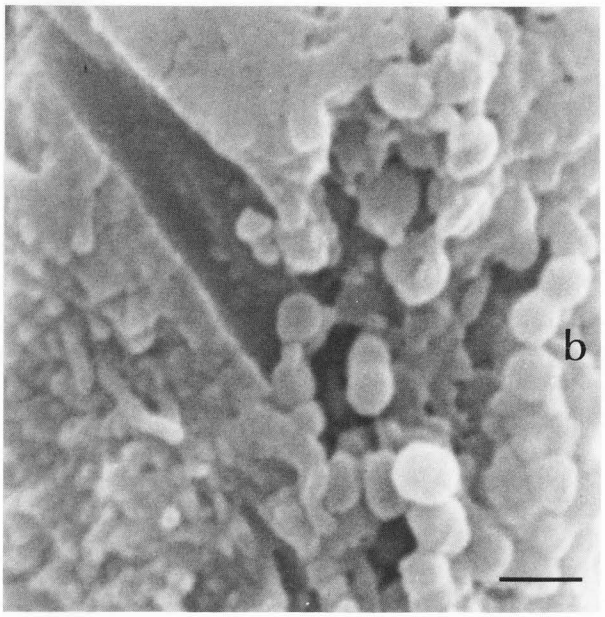


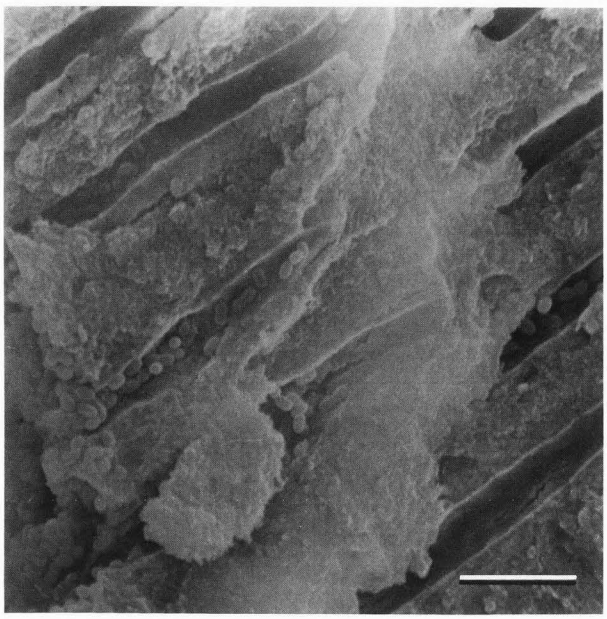
Fig 2 The colonization of the dentinal surface by *S. mutans*, after 72 h (a), after 120 h (b), after 144 h (c) and after 288 h (d). The colonies increased in size due to the cell division of the individual bacteria. As a result of this process the dentinal surface was gradually covered by bacteria. After 144 h (c) the individual colonies became confluent. After 288 h (d) the dentinal surface was completely covered in most areas. Bar = 5 μm.

immediately adjacent to *S. mutans* displayed the normal ultrastructural features. However, for all other experimental periods, in numerous cases bacteria were surrounded by a concave lesion in the dentin. The outline of this lesion was almost congruent with the outline of the corresponding single bacterium or group of bacteria. These lesions were found in intertubular and in peritubular dentin (Figs. 4a to 4d). They were also found in the tubular wall around invading bacteria (Figs. 3c and 3d). Observations on stereopairs of scanning electron micrographs revealed these lesions as being concavities. The exact depth of these lesions, however, could not be assessed, since the deepest part of the concavity was hidden below the bacterium. Structures similar to these lesions were never observed on baseline specimens nor on sham-infected

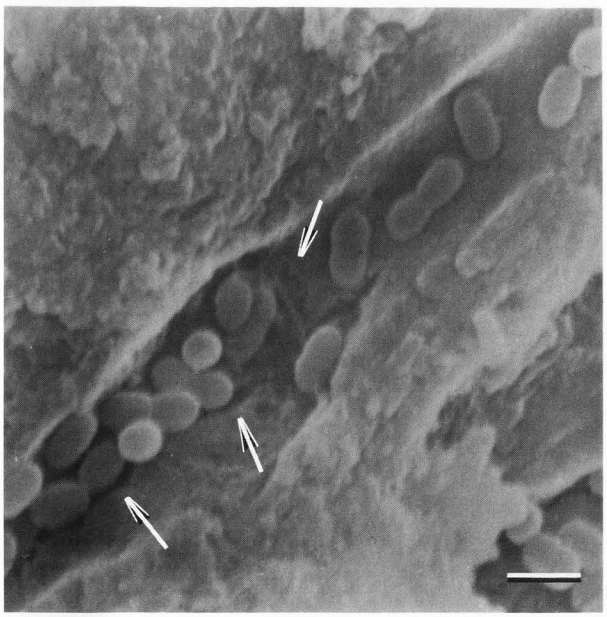
Fig 3 Observations on invasion of *S. mutans* in the dentinal tubules after 288 h of incubation (dentin fractured along the long axis of the dentinal tubules).



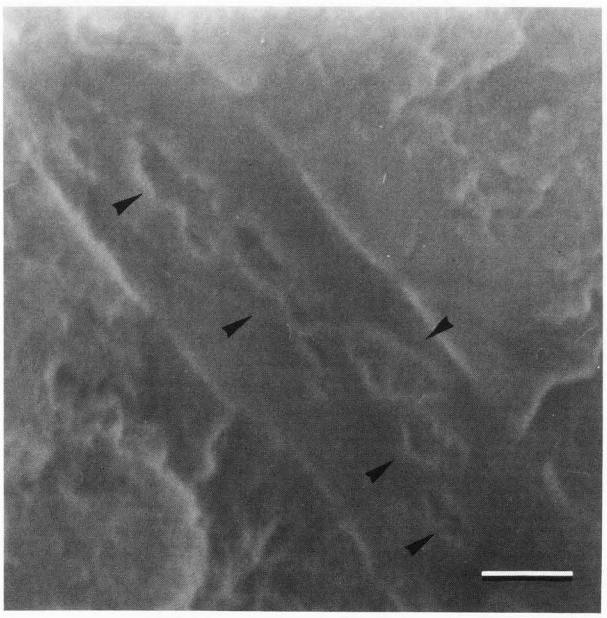
a. Dentinal tubule at the entrance of the tubular lumen. Multiple layers of *S. mutans* (b) cover the dentinal surface. Although the orifice of the dentinal tubules is completely covered by *S. mutans*, limited invasion of bacteria into the dentinal tubule occurred. Bar = 1 µm.



b. The dentinal surface (lower left) is covered with a multilayer of *S. mutans*. Three of the 5 dentinal tubules contain invading bacteria. Bacteria are present in a continuous row starting at the surface and extending to the deepest part of the invasion front. Bar = 5 µm.



c. Dentinal tubules containing invading bacteria. The *S. mutans* caused demineralization lesions in the peritubular dentin wall (arrows). The outline of the lesions follows the outline of the bacterial group. Bar = 1 µm.



d. Demineralization lesions (arrows) in the peritubular dentin lining of a longitudinally fractured dentinal tubule. The *S. mutans* which caused the lesions, were washed away during specimen preparation. Bar = 1 µm.

specimens. For the 288 h specimens quantitative data on the extent of the lesions could not be collected since the dentinal surface was covered by multiple layers of bacteria. In several areas the bacteria produced various amounts of extracellular polysaccharides (for review : Loesche, 1982), coating the cellular wall of the bacteria (Fig. 4b). Since this extracellular material also partially covered the dentinal surface, it was not possible to obtain measurements of dentin lesions in these areas.

Table 3 demonstrates the differences between the diameter of the demineralization lesion and the diameter of the associated bacteria, in function of incubation time. Differences between all experimental periods were statistically significant ($p < 0.01$).

Discussion

The results of this *in vitro* study have demonstrated that for *S. mutans* the colonization of the dentinal surface and the bacterial invasion in the dentinal tubules is determined by a passive translocation. This passive translocation of *S. mutans* on a solid surface is caused by the growth of individual bacterial colonies as a result of cell division by the individual bacteria. Furthermore, it has been shown that, within the limited time of the experiment, *S. mutans* is capable of causing demineralization lesions of the dentin.

In this *in vitro* model the colonization of the dentinal surfaces appeared to be determined by the rate at which the individual microcolonies became larger and thus covered the dentinal surface. Initially, *S. mutans* was most frequently found on intertubular and on peritubular dentin. Only when they covered approximately the entire peritubular dentin, the tubular lumen ultimately got covered by bacteria. The slow colonization rate could be attributed to various factors inherent to this *in vitro* system. In contrast to the *in vivo* situation where a continuous process of adsorption is present by which the oral microorganisms attach to the mineralized surfaces, in the present experiment only a single episode of exposure to the bacterial suspension occurred. Furthermore, in the *in vitro* model, the supply of nutritional factors depended on diffusion of nutrients from the agar through the dentin block, whereas in the *in vivo* situation more favorable nutritional conditions may be present for the development of the plaque bacteria. On the other hand, in the *in vitro* model the growth of *S. mutans* was not hampered, as can be expected to be the case in the oral cavity, by tooth cleaning, chewing, saliva flow and movements of the tongue and cheeks.

In contrast with the findings in the present investigation, an almost complete colonization of an identical surface was found after 144 h when *Capnocytophaga gingivalis* was grown on dentin blocks under identical conditions (Adriaens et al., 1982a). *Capnocytophaga gingivalis*, however, is a motile oral bacterium, possessing the capability to perform a gliding motility (surface translocation), whereas *S. mutans* does not possess any kind of active motility. In this *in vitro* model, *S. mutans* had to rely on passive translocation, i.e., movement on a solid surface as a consequence of growth of the microcolonies, to cover the dentinal surface.

Nutrients diffusing from the agar into the dentinal tubules at the interface between dentin and agar are likely to act as chemoattractants for *S. mutans*. Therefore, it seems reasonable to expect that, once the tubular entrance is covered by bacteria, invasion into the dentinal tubules would follow. However, as was shown from the present observations, the bacterial invasion was occurring in a limited number of dentinal tubules, even after 288 h when the dentinal surface was completely covered by *S. mutans*. Moreover, in those dentinal tubules where bacterial invasion occurred, the invasion was limited to the initial 5 µm portion of the tubular lumen. Furthermore, the invading bacteria were always present in a continuous row in the invaded dentinal tubules. This observation strongly supports the assumption that the bacterial invasion of the dentinal tubules by *S. mutans* is also determined by the rate of growth of the bacterial colonies which in its turn is dependent on the cellular division of the bacteria.

The present observations confirm the findings reported by Mjör (1974). In his *in vivo* study including 45 human premolars, bacterial invasion into the experimentally exposed dentinal tubules was observed during periods up to 101 days. Very few dentinal tubules were found in which bacteria invaded. In addition, when bacteria invaded into the dentinal tubules, bacteria were only present in the outer few micrometers of the tubular lumen. In contrast, Olgart et al. (1974) and Michelich et al. (1980) reported the presence of deeply invading *S. mutans* in the dentinal tubules in their *in vitro* studies. The experimental periods were 7 and 5 days, respectively. The fact that the dentin pieces were treated with acid etching, thereby widening the lumen of the dentinal tubules, might have played an important role in these results.

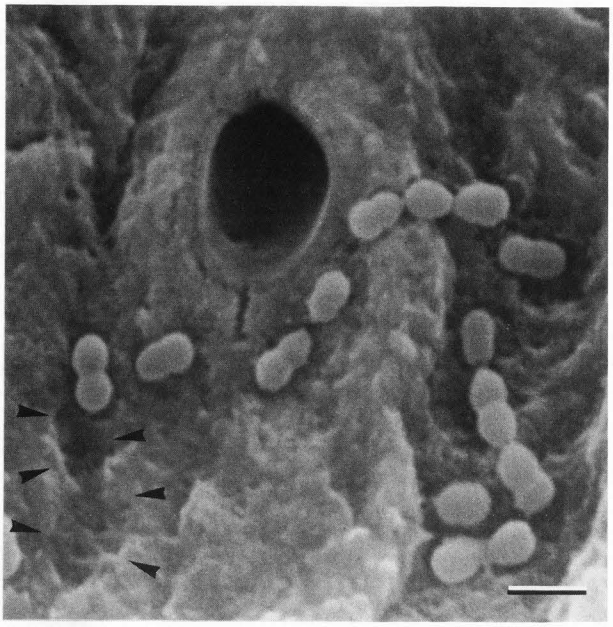
The pattern of invasion into the dentinal tubules observed in this study was different from the pattern observed for *Capnocytophaga gingivalis* (Adriaens et al., 1982a). The latter bacterium invaded in more dentinal tubules in a shorter

Table 3: Demineralization lesions on dentin surface.

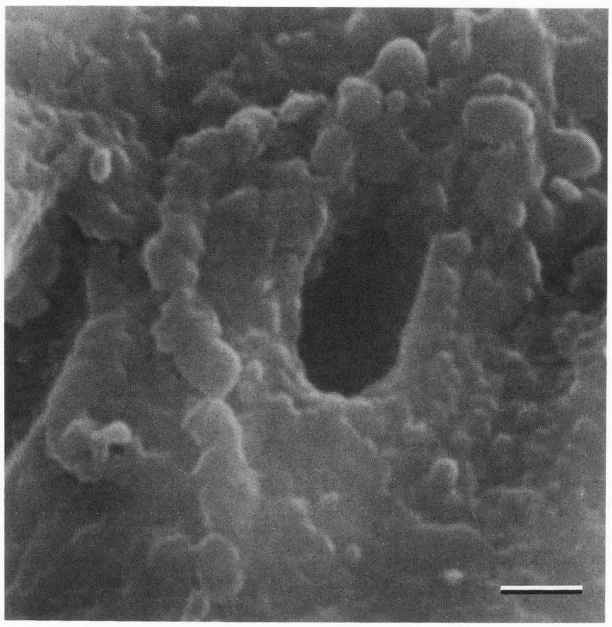
incubation period (h)	0	72	120	144
measured lesions (n)	0	15	22	17
mean diameter[a] (µm)	-	0.132*	0.283*	0.366*
s.d.	-	0.049	0.088	0.078
S.E.M.	-	0.013	0.019	0.019

a: difference between diameter of lesion and diameter of corresponding bacterium.
*: statistically significantly different from other values in row.
s.d.: standard deviation
S.E.M.: standard error of mean

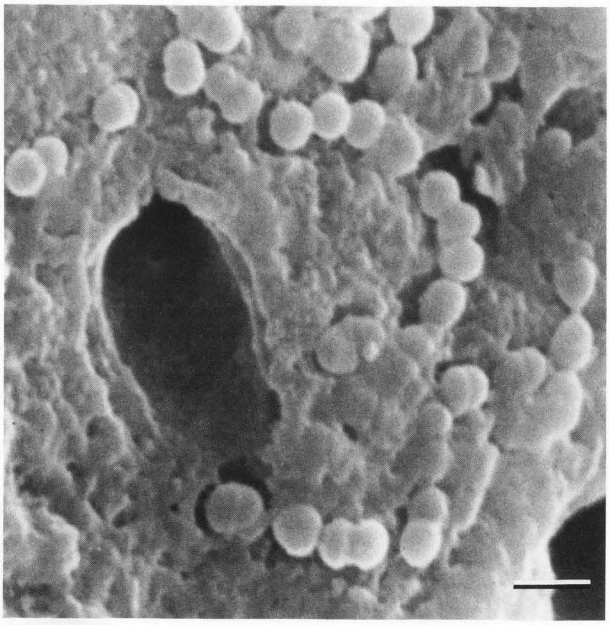
Fig 4 Demineralization lesions of the dentinal surface, induced by *S. mutans*.



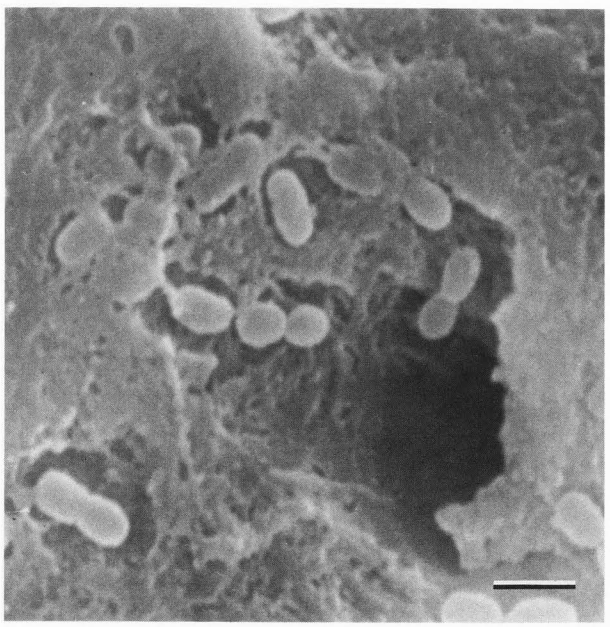
a. After 72 h small concave lesions could be observed surrounding the *S. mutans*. In some areas (arrows) bacteria were dislodged from the demineralization lesions during specimen preparation. Bar = 1 µm.



b. After 72 h some of the bacteria on the dentinal surface were covered by an extracellular material, probably extracellular polysaccharides produced by *S. mutans*. Bar = 1 µm.



c. After 120 h demineralization lesions of the dentin were found in the close proximity of the bacteria. The lesions appear as cavities in which the bacteria are embedded. Bar = 1 µm.



d. After 144 h extensive demineralization resulted in severe lesions of the peritubular and intertubular dentin. In the bottom of the cavities remnants of collagen fibers can be seen. Bar = 1 µm.

time. In addition, invading *Capnocytophaga gingivalis* were found to a mean depth of 55 µm already after 144 h of incubation. Furthermore, in contrast with *S. mutans*, invading *Capnocytophaga* were not present in a continuous row in the lumen of invaded dentinal tubules, thus indicating that this bacterium, due to its gliding motility, actively invaded the dentinal tubules. It seems probable that the invasion pattern of the bacteria currently associated with the development of dentinal caries (Edwardsson, 1974; Loesche, 1982, 1986) will be similar to that observed for *S. mutans* in the present study, since the majority of these bacteria do not possess active motility.

It may be assumed that the concave lesions observed in the dentin immediately adjacent to the bacteria are caused directly by the action of acid metabolites produced by *S. mutans*. These lesions were observed in the intertubular and in the peritubular dentin. They were also observed in the tubular wall of the invaded dentinal tubules. The lesions in the highly mineralized peritubular dentin appeared slightly larger than those in the intertubular dentin, which contains a higher proportion of organic material. In advanced cases of human periodontitis similar demineralization lesions were found at the surface of the alveolar bone when bacteria invading the soft periodontal tissues were in contact with the surface of the alveolar bone (Frank and Voegel, 1978).

The acid metabolites produced by *S. mutans* are known to have a demineralizing action on the mineralized material in dentin (Loesche, 1986). However, they also cause a denaturation of the collagen which can then further be broken down by bacterial enzymes with gelatinase activity. Lactate is the main acid endproduct of *S. mutans*. In addition, other acid endproducts of *S. mutans* (Jordan, 1965; Drucker and Melville, 1968; Robrish and Krichevsky, 1972; Tanzer et al., 1972; van der Hoeven, 1976) may play a role in this process, albeit probably a minor role.

Distinct demineralization lesions could already be detected in the 72 h specimens, indicating that this process has been initiated before that moment. The extent of the lesions further increased with time. This observation indicates that, in this *in vitro* model, the pH drop induced locally by the acid metabolites of single bacteria or small colonies of *S. mutans*, is capable of inducing localized structural changes in the mineralized dentinal structures. It might well be that this phenomenon is not necessarily occurring to the same extent in an *in vivo* situation, as it might be expected that saliva, crevicular fluid and dentinal fluid could act as buffering solutions, thereby reducing the pH drop. However, the presence of extracellular polysaccharides between the plaque bacteria reduces the penetration of these buffering solutions into the plaque mass (Gibbons, 1968).

From this *in vitro* study it may be concluded that, in the absence of a continuous exposure to new adhering microorganisms, the undisturbed colonization of the dentinal surface by *S. mutans* is a slowly progressing process. It is determined primarily by the growth rate of the bacteria and their microcolonies. Since bacterial invasion into the dentinal tubules is limited, it may be expected that the major part of the destruction caused by the acid metabolites produced by *S. mutans*, will occur at the dentinal surface. However, in the cases where dentinal tubules become invaded with acid-producing non-motile bacteria, local demineralization of the dentinal wall will occur, thereby accelerating the carious destruction process.

Acknowledgements

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Discussion with Reviewers

S.J. Jones : How can you be sure that the regions described as demineralized dentin are correctly interpreted ? Did you observe a demineralized collagen fringe at such sites ? Could the same appearance result from differential shrinkage of extracellular polysaccharide, bacteria and dentin upon drying ?

Authors : During the interpretation of the concave lesions we realized that these structures could be artifacts caused by differential shrinkage of bacteria, dentin and extracellular polysaccharides (EPS) during critical point drying. The following arguments and observations led us to the conclusion that these lesions were indeed demineralization lesions. The lesions were observed as concavities in stereopairs. The diameter of the lesion increased with longer incubation periods. This is in agreement with increased amounts of acids produced by *S. mutans* as endproducts of the sucrose metabolism. Sucrose was present in the medium. Similar lesions have been detected in analogous *in vitro* experiments with *Capnocytophaga gingivalis* (Adriaens et al., 1982a) which produce appreciable amounts of lactate, and in experiments with a mixed subgingival flora from a periodontitis lesion (Adriaens et al., 1984). Furthermore, in those specimens where EPS were present, they tended to shrink towards the bacterial wall, thereby masking the bacterial morphology (Fig. 4b).

In the lesions in the intertubular dentin no demineralized collagen fringe could be observed. However, it is unlikely that one would detect the typical collagen network which can be observed at the predentinal surface (Adriaens and De Boever, 1981; Adriaens, 1982). The acid metabolites produced by the bacteria cause denaturation of the collagen which results in the loss of the typical morphology of the collagen network of the intertubular dentin. However, remnants of collagen could be observed in the bottom of demineralization lesions after 144 h of incubation (Fig. 4d). In the peritubular dentin one would not expect to see a collagen fringe when demineralization occurs, because peritubular dentin does not contain collagen (Adriaens, 1982).

J.M. Hardie : The authors quote the earlier work of Edwardsson (1974) on deep dentinal caries which clearly illustrated that bacteria other than *Streptococcus mutans*, especially anaerobic Gram-positive rods of various taxa, are predominant at the advancing front of carious lesions. Similar observations have been made more recently by Japanese workers (Hoshino et al., Japanese J. Oral Biol. 26, 276-279 and 538-542, 1984). It would be interesting to see how such organisms behave in the experimental model used in these experiments, either in pure culture or in association with streptococci.

Authors : We have tested several other oral microorganisms in this experimental model. Pure cultures of *Actinomyces naeslundii*, an organism which has been implicated in the development of root caries, behaved essentially in the same way as *Streptococcus mutans* (Adriaens et al., 1982b). Pure cultures of motile oral bacteria, e.g. *Capnocytophaga gingivalis*, colonized the entire surface in a shorter time and invaded in more dentinal tubules and to a greater depth (Adriaens et al., 1982a). When a mixed flora taken from periodontitis lesions was cultured in this system, non-motile bacteria (cocci and short rods) invaded to a much greater depth than in pure culture experiments (Adriaens et al., 1984). This was due to the piggyback transport of these bacteria by motile microorganisms (*Capnocytophaga* species) present in the flora. Motile bacteria were always present at the invasion front. The anaerobic Gram-positive rods which are present in the advancing front of the *in vivo* carious lesion have not been tested yet. However, one can expect that these bacteria which are non-motile microorganisms, will invade in the dentinal tubules in a way similar to *Streptococcus mutans* and *Actinomyces naeslundii*. On the other hand, the fact that these bacteria are present in the advancing front of the established *in vivo* carious lesion is not so much due to their motility as it is due to their aciduricity and perhaps also to their capability to metabolize the denatured collagen (Loesche, 1982).

A. Linde : The main value of this investigation is that it adds to our understanding of the mechanisms whereby *S. mutans* acts on a denuded dentin surface. The main question is, of course, how representative the results are of the *in vivo* caries situation? The study was performed with a monoculture of *S. mutans* under more or less standard bacteriological growth conditions and with the tissue pieces devoid of their physiological surroundings and possible defence factors. This implies that time and depth values given should be viewed for what they are, results obtained under these specific experimental conditions.

Authors : This statement is correct and applies, of course, to every *in vitro* experimental model. Quantitative values for time and depth given in this study should by no means be extrapolated to the *in vivo* situation. However, it is valid to compare the results of this experiment with the results obtained with other oral bacteria in pure cultures (Adriaens et al., 1982a, 1982b) and with mixed cultures of oral bacteria (Adriaens et al., 1984) in this *in vitro* system. The experiment was designed primarily to study the role of bacterial motility in the colonization of dentin surfaces and invasion of dentinal tubules. In an *in vivo* situation many more variables have to be taken into account.

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