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Diminished Virulence of Glucan Synthesis-Defective Mutants of *Streptococcus mutans*

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The virulence of cell surface-associated, glucan synthesis-defective mutants of *Streptococcus mutans* strain 6715-13 was studied. Representatives from three groups of such mutants were tested for their pathogenicity in conventionalized, specific pathogen-free rats and gnotobiotic rats. The mutants differ from the wild-type strain in that each failed to form plaque on the smooth surfaces of the teeth and to cause smooth surface caries. Although the ability to form cell surface-associated glucans was not a strict requirement for the expression of virulence in the sulci of the teeth, it augmented virulence at such sites. However, the ability to form cell surface-associated glucans and to adhere to the teeth was clearly not the sole determinant of virulence.

Streptococcus mutans is strongly implicated as a prime pathogen in dental caries of rodents (20, 21, 26, 40, 55), non-human primates (3), and man (14, 33, 44). The molar teeth of rats have both sulcal (pit and fissure) and smooth surfaces like those of man (35). Caries is readily induced by *S. mutans* in rats consuming high-sucrose diets (19, 29). Rats thus serve as useful experimental models for the study of dental caries caused by *S. mutans* (20, 26, 29, 51).

Caries of the smooth surfaces of the teeth is well established as essentially sucrose dependent (21, 26, 29, 51). The effects of sucrose metabolism thought to be most germane to the ability of *S. mutans* to colonize, survive, proliferate upon, and ultimately demineralize smooth tooth surfaces are thought to be adhesive glucan synthesis and glucan-induced agglutination (27, 28, 54) and, of course, the production of large amounts of lactic acid (9, 10, 49).

We have isolated and characterized morphologically, chemically, and functionally in vitro a number of *S. mutans* mutants (25), allowing direct test of whether the trait of cell surface-associated glucan synthesis and resultant adhesion are germane to virulence and, if so, at which sites on the teeth. These experiments assess the virulence of three mutants in two types of caries models—conventionalized, specific pathogen-free rats and gnotobiotic rats. Each of the mutants studied is a representative of one of the three groups of mutants described in the preceding paper (25).

MATERIALS AND METHODS

Microorganism and mutants. Streptomycin-resistant *S. mutans* parental strain 6715-13 wild type (WT) and mutants were studied. All strains were maintained in lyophile until ready for the virulence test in experimental animals, at which time they were cultured in fluid thioglycolate medium containing 20% (vol/vol) meat extract (Difco) and excess $CaCO_3$.

The details of mutangensis and mutant selection were reported previously (25). The mutants were shown to be unaltered, as far as we could monitor, in all aspects of their physiology that are hypothesized to play a role in virulence, i.e., rapid acid production from sucrose (9, 10, 49), intracellular polysaccharide synthesis (1), extracellular fructan synthesis (8, 11, 46), and glucan-induced agglutination (27). They all had lost their ability to form tenacious plaque in vitro. Biochemically, they differ from the WT strain only with respect to the diminished amount of cell surface-associated glucan and the increased amount of water-soluble extracellular glucan found in sucrose-containing broth. A detailed description of the altered colonial morphology, in vitro plaque-forming ability, and unaltered glucan-induced agglutination of these mutants was given in the preceding report (25).

Experimental animals, diet, infectious challenges, and recovery of microorganisms. Two types of experimental animal caries models were used. Barrier-maintained, specific pathogen-free Osborne Mendel rats (SPFOM) were obtained from the National Institutes of Health Animal Production Unit. This animal strain was Caesarian-derived, suckled by SPFOM foster mothers, and specifically infected by four microorganisms constituting normal indigenous rodent gut flora: two lactobacilli of unknown species, a strain of *Streptococcus faecalis*, and a strain of *Bacteroides* of unknown species (48). The animals

were maintained until weaning "behind the barrier" in isolation from other animals. At weaning (21 days old), they were conventionalized by housing, generally two animals per cage, in our own animal facility, and caries test diet 2000 (36) containing 56% sucrose was supplied ad libitum, as was demineralized water. One day after weaning and institution of diet 2000, animals were infected with either the WT or mutants 4, 27, or 33, or were not infected. A suspension of a 24-h thioglycolate culture of the respective microorganisms was diluted in thioglycolate medium such that it had an absorbance of 0.54 at 600 nm (1-cm light path). This density is equivalent to 88.6 μg of dry cells/ml. The standard infectious dose was 15 ml of the appropriate cell suspension added to 45 ml of milk contained in a cup. One cup was placed into each cage of animals and water bottles were removed. Uninfected rats received milk only. The animals always consumed the cup contents in a few hours. Deionized water was then resupplied ad libitum.

Recovery of microorganisms was tested 12 days after infectious challenge and at the time of sacrifice, 49 days after infectious challenge. The molar teeth of all animals were swabbed, and the swabs were placed into VMG II transport medium (34, 45) prior to vortexing and serial dilution in 0.05% yeast extract (BBL) in 67 mM phosphate buffer (pH 7.0). Undiluted as well as diluted samples were cultured on Mitis Salivarius (MS) agar (Difco), MS agar containing 200 μg of streptomycin per ml, and meat extract-starch-blood agar (50). Recoveries were recorded as the percentage of total colony-forming units on MS agar which grew on MS agar containing 200 μg of streptomycin per ml. Due to the uncertainties of plate counts of streptococci (51), especially for *S. mutans*, which clumps in the presence of sucrose, and the difficulty of sampling plaque from the teeth of such small animals, recoveries are expressed as negative, low (<25%), moderate (25 to 75%), or high (>75%).

For the gnotobiotic animal caries tests, germfree rats of the National Institutes of Health Sprague-Dawley line were employed. The methods used were those detailed previously (19), with the following slight modifications. Animals were weaned at 19 days of age and housed, generally three per cage, in plastic isolators of the Trexler (52) type. All rats infected with a single microorganism were housed in a single isolator; uninfected rats were housed in separate isolators. Rats received sterile, deionized water and diet 2000 supplemented with vitamins according to the recommendations of Gustafsson (32). The diet was sterilized by gamma irradiation (6 Mrads), maintaining its powdery consistency. Each animal was orally infected at 19 days of age with a swab moistened with an 18-h culture of the WT or one of the mutants. Cultures were grown in Todd-Hewitt broth in sealed ampoules to an absorbance of approximately 0.65, equivalent to about 106 μg of dry cells/ml. The sealed ampoules were brought into the isolators via germicidal locks (52). In addition to the swabbing procedure, 2.0 ml of each culture was added to 100 ml of water supplied to each cage of animals. At 48 h, the unused water was discarded and the animals received sterile deionized water thereafter.

Fecal samples were studied weekly to insure implantation of microorganisms and that extraneous contaminants were absent. At the termination of the experiment, 60 days after infectious challenge, oral swabs were taken to recover infecting microorganisms for comparison of their characteristics with those of the original inoculants and to check for extraneous contamination.

Plaque and caries activity. At sacrifice, 49 and 60 days after infectious challenge in the case of the SPFOM and gnotobiotic rats, respectively, the heads of all animals were fixed in formalin, and the jaws were examined with the plaque in situ. After staining of the plaque with safranin, the appearance of the jaws was recorded photographically.

The jaws were defleshed by dermestid beetles, and the number of carious enamel areas was scored by the method of Keyes (35). The experimental history of jaws was unknown to the scorer. Differences among group caries score means were tested by analysis of variance. Individual contrasts were tested for significance by the method of Tukey when there were equal-sized experimental groups or by the method of Scheffé when there were unequal-sized experimental groups (47).

RESULTS

Specific pathogen-free rats. Rats infected with strain 6715-13 WT had heavy plaque on their teeth (Fig. 1a); those infected with either mutant 4, 27, or 33, or which were uninfected, were virtually plaque free (Fig. 1b). The caries activity of the WT-infected animals was high (Fig. 2a). By contrast, that of the mutant-infected animals was reduced with respect to the smooth surfaces of the teeth (Fig. 2b). Mean smooth surface caries scores for the mutant-infected animals were greatly reduced ($P < 0.01$) from those of the WT-infected animals but were not different from those of the uninfected animals (Fig. 3). Thus, there is a dramatic loss of virulence of three different glucan synthesis-defective mutants on the smooth surfaces of the teeth, the areas at which adhesion would appear to be an important ecological determinant.

Sulcal caries scores were not significantly different in mutant-challenged compared with WT-challenged animals. Nonetheless, mutant 27, as well as the WT, significantly ($P < 0.05$) augmented the amount of sulcal caries activity ascribable to the indigenous flora of the non-*S. mutans*-infected control rats. Thus, adhesion appears to be a crucial determinant of virulence on the smooth surfaces. However, it is clearly not the sole determinant of virulence on the sulcal tooth surfaces of these rats.

The cultures recovered from all SPFOM animals were predominantly streptococcal since the number of colony-forming units on MS agar was essentially the same as on meat extract-

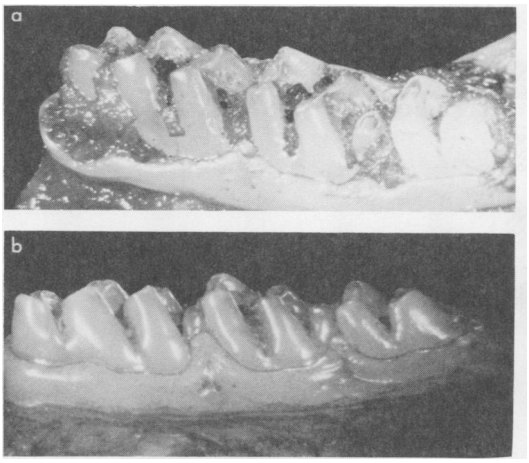

FIG. 1. *Comparison of mandibular teeth from S. mutans 6715-13 WT-infected (a) and mutant 33-infected (b) SPFOM rats with plaque in situ. Lingual view shows safranin-stained heavy plaque on surfaces of teeth of WT-infected animal (a) but essential absence of plaque from surfaces of teeth of mutant-infected animal (b). Specimen (a) is typical of those from WT-infected animals; specimen (b) is typical of those from animals infected by any of the mutants or uninfected.* ×8.

starch-blood agar. Such predominantly streptococcal plaque recoveries are usual in this and other animal models (42, 51). In no case was a streptomycin-sensitive *S. mutans* detected in any animal, and thus no caries activity could be ascribed to an extraneous *S. mutans* infectant. Molar swabs of WT-infected animals uniformly gave moderate to high recoveries of *S. mutans* 6715-13 WT (range, 40 to 90% colony-forming units on MS). Mutant-infected animals uniformly gave negative to low recoveries of the mutants (range, 0 to 5% colony-forming units on MS). The colonial morphologies of the recovered streptomycin-resistant cocci were identical to those used to infect the animals in each instance.

Gnotobiotic animals. The recovery patterns of microorganisms from the SPFOM animals may have reflected either or both of the following. First, the loss of adhesion to the teeth by the mutants may have precluded their establishment on the smooth surfaces of the teeth and, consequently, may have resulted in the absence of decay on these surfaces. Second, it was conceivable that some other characteristic of the mutants diminished their ability to compete with the mixed flora of these rats. To clarify this point, we employed a caries test model which eliminates the possibility of competing flora—the gnotobiotic rat.

As in the case of the SPFOM rats, gnotobiotic rats infected with strain 6715-13 WT had heavy plaque on the smooth surfaces of their teeth, mutant-infected gnotobiotes were essentially free of smooth surface plaque, and uninfected gnotobiotes were totally plaque free (Fig. 4a, b).

The WT-infected animals had extensive carious involvement of both smooth and sulcal surfaces of the teeth (Fig. 5a, 6). The mutant-infected animals (Fig. 5b, 6) had no smooth surface caries ($P < 0.001$). Uninfected gnotobiotes developed no caries. Thus, it was clear that the ability to adhere and to form plaque augments sulcal caries scores in the gnotobiotic animal and is a strict requirement for virulence on the smooth surfaces of the teeth in an animal with no competing flora.

No extraneous contaminants were cultured from either fecal samples or oral swabbings, and the infecting microorganisms were unequivocally recovered. The colonial morphology and physiological characteristics of the recovered WT strain and mutants could not be distinguished from those of the microorganisms used as infectious challenges.

DISCUSSION

Of the several aspects of *S. mutans* physiology thought to be related to its pathogenicity, the ability to synthesize cell surface and extra-

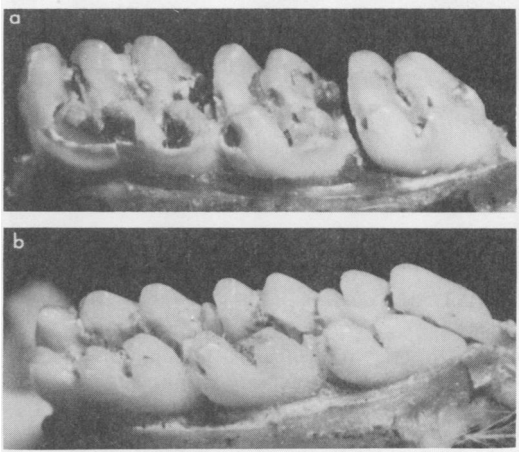

FIG. 2. *Comparison of mandibular teeth from S. mutans 6715-13 WT-infected (a) and mutant 33-infected (b) SPFOM rats after defleshing and plaque removal. Teeth have been sliced to reveal sulcal carious lesions. Buccal view shows extensive smooth surface and sulcal caries in typical WT-infected animal dentition (a) but essential absence of smooth surface caries and reduction of sulcal caries in typical mutant-infected animal dentition.* ×8.

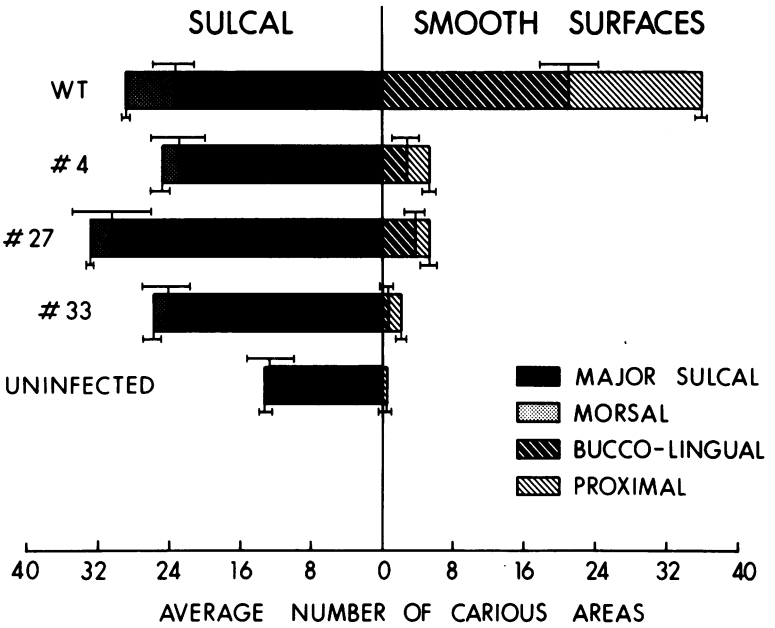

FIG. 3. *Graph of average number of carious enamel areas of SPFOM rats infected by strain 6715-13 WT or the mutants, or, uninfected. The mean and standard error of the mean are plotted. There were four rats per experimental group.*

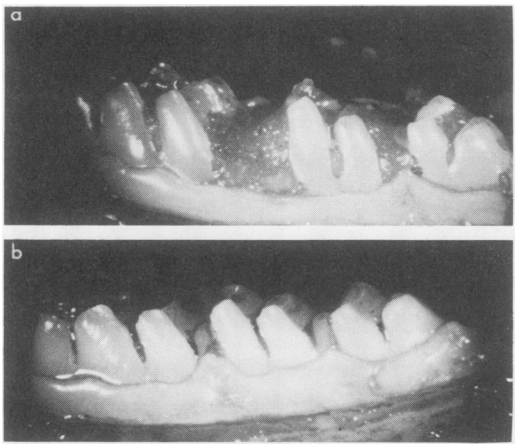

FIG. 4. *Comparison of mandibular teeth from S. mutans 6715-13 WT-infected (a) and mutant 27-infected (b) gnotobiotic rats with plaque in situ. Lingual view shows safranin-stained heavy plaque on surface of teeth of WT-infected animal (a) but essential absence of plaque from surfaces of teeth of mutant-infected animal (b). Specimen (a) is typical of those from WT-infected animals; specimen (b) is typical of those from animals infected by any of the mutants or uninfected.* ×8.

cellular glucans from sucrose has aroused considerable interest. These glucans are hypothesized to be the cause of cohesion of the cells and their adhesion to surfaces such as those of glass, stainless steel, and, of course, teeth.

Studies with glucanases demonstrated their ability to detach preformed plaques of *S. mutans* from surfaces in vitro and in vivo, as well as their ability to inhibit caries development (22, 24, 30). The present experiments directly establish that cell surface-associated glucan is required for the expression of virulence on the smooth surfaces of the crowns of the teeth of rats. Three colonially and metabolically distinct mutants, all defective in the formation of cell surface-associated glucans, albeit to different degrees (25), each failed to form plaque on the teeth of both conventionalized SPFOM and gnotobiotic rats. The absence of smooth surface coronal caries corresponded to the failure of plaque formation.

In addition, the ability of *S. mutans* to synthesize cell surface-associated glucans augmented the virulence of these cells in the molar sulci of the gnotobiotic rats. Also, the augmentation of caries in the sulci of conventionalized SPFOM animals upon infection by either the WT or mutant 27 indicates that *S. mutans* is potent in causing caries at sulcal tooth sites. Other recent data also support this contention (39, 51). However, this expression of virulence in sulci by cell surface-associated, glucan synthesis-defective mutant 27, which is defective in plaque formation both in vitro (25) and, as shown here, in vivo, indicates that additional determinants of virulence exist at the sulcal surfaces. Although with diminished intensity,

the three mutants all retain some measure of sulcal surface virulence in the gnotobiote. Thus, cell surface-associated glucan synthesis apparently contributes to the local conditions which foster the expression of virulence at these tooth sites as well, perhaps as a result of the glucan's imposition of a barrier to the diffusion of the metabolically produced lactic acid hypothesized to be critical to the process of demineralization of the teeth (9, 10, 49). The expression of virulence in the sulci by the nonadhering mutants probably results from their mechanical implantation and retention in the deep molar fissures. Other microorganisms (e.g., enterococci and lactobacilli) which do not form appreciable glucans from sucrose (19) also produce sulcal lesions in gnotobiotic rats but, like the mutants in point, do not cause smooth surface lesions.

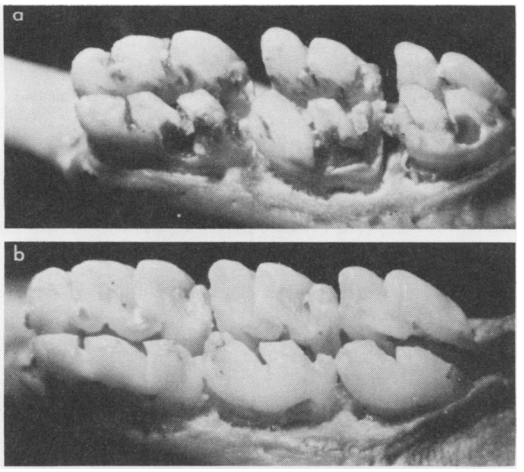

FIG. 5. *Comparison of mandibular teeth from S. mutans 6715-13 WT-infected (a) and mutant 4-infected (b) gnotobiotic rats after defleshing and plaque removal. Teeth have been sliced to reveal sulcal carious lesions. Buccal view shows extensive smooth surface and sulcal caries in typical WT-infected animal dentition (a) but absence of smooth surface caries and reduction of sulcal caries in typical mutant-infected animal dentition. Uninfected gnotobiote jaws (not shown) are totally caries free.* ×8.

According to the scoring system of Keyes (35), lesions placed in the "morsal" category include those found on the occlusal surfaces of the molar cusps as well as those in the shallow sulci separating major and minor cusps. In this study, lesions originating on the occlusal surfaces of the molar cusps were not seen, and so the morsal category represents only lesions originating in the shallow minor sulci. Lesions of this type were completely absent in the gnotobiotic rats infected with the mutants but were present in WT-infected gnotobiotes. In conventionalized rats, their presence paralleled that of plaque-associated lesions on the smooth

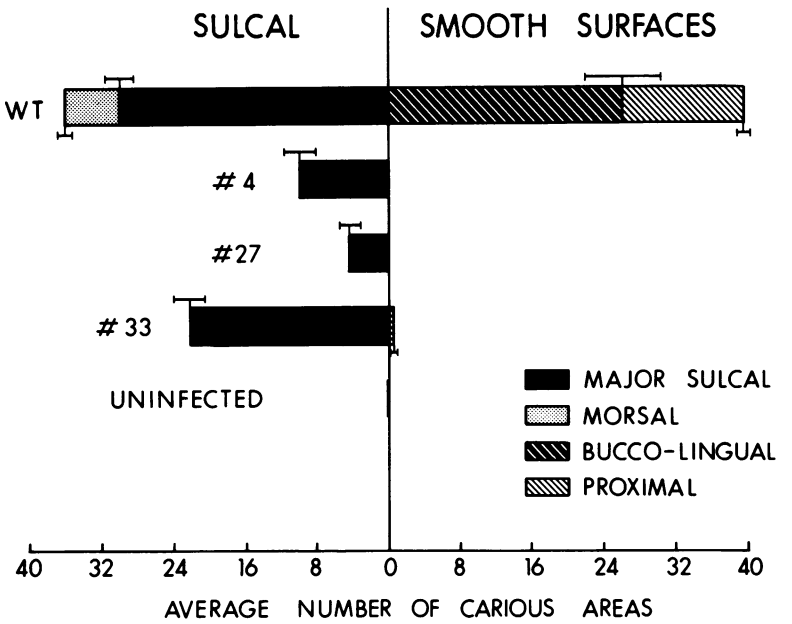

FIG. 6. *Average number of carious enamel areas of uninfected gnotobiotic rats or those infected by strain 6715-13 WT or mutants. The mean and standard error of the mean are plotted. There generally were six rats per experimental group. The uninfected group can be considered to comprise thousands of rats that have been studied for a number of years in our laboratory.*

surfaces. It therefore appears that caries in the minor sulci is etiologically more closely related to smooth surface caries and, thus, more adhesion dependent than sulcal caries, a conclusion that is supported by other experimental evidence (23, 37, 38).

At least four and perhaps five distinct genetic groups of *S. mutants* occur in nature (4–7, 12, 13, 16). Representative strains of all of these groups form plaque and are virulent (17, 20, 21, 26, 29, 41, 51). Edwardsson (18) has described a representative strain (NCTC 10449) of one of these groups which spontaneously produced colonial variants whose morphologies changed from the typical rough, frosted-glass appearance of *S. mutans* on MS agar to smooth and mucoid. However, virulence tests of these variants in hamsters showed no difference in their disease-causing abilities. In addition, these spontaneous variants were notably unstable as to their colonial morphologies, such that when one colonial type was used to infect animals a high proportion of the other colonial types was recovered. In separate studies, a spontaneous change of strain GS-5, a strain of the same genetic type as NCTC 10449, from rough to smooth morphology occurred, but no concomitant change of its virulence was seen (R. J. Fitzgerald, unpublished data).

de Stoppelaar et al. (15) described a colonially smooth, ethyl methane sulfonic acid-induced mutant of a strain of the same genetic group as NCTC 10449 and GS-5. Under the conditions of their study, the mutant was shown not to form plaque in vitro nor to agglutinate in the presence of exogenous glucan at pH 7.2. The formation and distribution of extracellular and cell surface glucans were not reported. However, the mutant was shown to have diminished virulence in both gnotobiotic rats and hamsters. In our report the three mutants of strain 6715-13 WT, a representative of one of the other genetic groups of *S. mutans*, showed failure to form plaque in vitro, diminished ability to form cell surface-associated glucan, essentially normal agglutination in the presence of exogenous glucans, failure to form smooth surface plaque in vivo in both conventionalized SPFOM and gnotobiotic rat caries test models, failure to cause decay of the smooth surfaces of the teeth, and diminished virulence at sulcal tooth sites. Clearly this trait of cell surface glucan-associated adhesion is germane to virulence.

A note of caution is required. It is well known that other oral streptococci, for example *Streptococcus sanguis* (53, 54), are found adhering to the surfaces of the teeth. Although it might be assumed that this trait of adherence to surfaces is the sole determinant of virulence, epidemiological data indicate that *S. sanguis*, although found in large numbers adhering to many sites of the dentition, is not strongly associated with caries activity either in humans or experimental animals (2, 14). In fact, the numbers of *S. mutans* and *S. sanguis* at tooth sites of humans appears inversely related. It is with the presence of *S. mutans*, however, that caries activity correlates. Thus, adhesion of *S. mutans* per se cannot be the sole determinant of its virulence.

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