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Streptococcus mutans Adherence: Presumptive Evidence for Protein-Mediated Attachment Followed by Glucan-Dependent Cellular Accumulation

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Adherence of *Streptococcus mutans* to smooth surfaces has been attributed to the production of sucrose-derived D-glucans. However, several studies indicate that the bacterium will adhere in the absence of sucrose. The present data confirmed that *S. mutans* adherence to saliva-coated hydroxyapatite beads in the absence of sucrose is described by the Langmuir equation. The nature of the sucrose-independent adherence was studied with the *Persea americana* agglutinin as a selective adherence inhibitor. Pretreatment of the bacterium with *P. americana* agglutinin caused a 10-fold reduction in adherence, and the inhibition was not reversed with the addition of sucrose. Pretreatment of *S. mutans* with proteases also reduced adherence, regardless of the sucrose content, whereas periodate oxidation and glucanohydrolase treatment of the bacteria reduced sucrose-mediated adherence to the levels found for sucrose-independent adherence. The *P. americana* agglutinin, glucanohydrolase, and pepsin pretreatment of the cells did not eliminate sucrose-induced agglutination. Scanning electron microscopy showed that short streptococcal chains were bound to saliva-coated hydroxyapatite crystals in the sucrose-independent system, whereas the presence of sucrose caused larger bacterial clumps to be found. A two-reaction model of *S. mutans* adherence was developed from these data. It is proposed that one reaction is attachment to the tooth pellicle which is mediated by cell-surface proteins rather than glucans or teichoic acids. The other reaction is cellular accumulation mediated by sucrose-derived D-glucans and cell surface lectins. A series of sequential adherence experiments with *P. americana* agglutinin as a selective inhibitor provided presumptive evidence for the validity of our model of *S. mutans* adherence.

Streptococcus mutans is an oral bacterium capable of initiating dental caries in experimental animals and has been implicated as a causative agent of the same disease in humans. Virulence of *S. mutans*, as with most other pathogenic organisms, is ascribed to their ability to adhere and maintain themselves on selected host tissues (8, 25). The traditional view in oral microbiology is that adherence of *S. mutans* to smooth surfaces is dependent on sucrose-derived α-1,6- and α-1,3-linked water-insoluble D-glucans produced by a group of glucosyltransferases (GTF). However, physicochemical data do not totally support the foregoing premise, and there are several examples of *S. mutans* adherence in the absence of sucrose (4, 5, 15, 27, 28). Results of in vivo experiments demonstrated that *S. mutans* was recovered from the dental plaque of children who had metabolic deficiencies that forced them to eat diets deficient in sucrose (28). It was also shown that laboratory animals were susceptible to infection by various *S. mutans* regardless of the dietary sucrose content (27). Moreover, in most instances, the minimal infective dose was equivalent for sucrose- or glucose-supplemented animals, although sucrose enhanced the final bacterial population. In vitro adherence studies indicated that *S. mutans* cells were adsorbed to saliva-coated hydroxyapatite (SHA) beads or SHA crystals in the absence of sucrose (4, 5, 15). One of the most provocative experiments which suggested that sucrose-derived D-glucans were not solely responsible for *S. mutans* adherence was reported by Kuramitsu and Ingersoll (14). They attached GTF from *S. mutans* GS-5 onto the surface of *Streptococcus sanguis* ST-3, an oral microbe that does not utilize sucrose for adherence, and found that the synthesis of *S. mutans* glucans only slightly increased the sucrose-dependent adherence of *S. sanguis* ST-3 to glass surfaces. Adherence levels analogous to those of the GTF donor, *S. mutans* GS-5, were not found.

There has been some speculation on the na-

ture of the nonglucan molecules that participate in the adherence of *S. mutans*. Gibbons' laboratory suggested that cellular components which interact with blood group-reactive (BGR) salivary mucins could play a role in adherence (10, 11). Rolla et al. (19, 20) implicated teichoic acids (TA) in the adherence of *S. mutans*. The Rolla model states that adherence occurs via the electrostatic interactions of acid salivary glycoproteins, calcium ions, and the negatively charged TA molecules.

In this report, presumptive evidence is presented which shows that the *S. mutans* cell surface molecule responsible for sucrose-independent adherence is protein in nature. Furthermore, the data suggest that the role of GTF in adherence is to promote cell-cell interactions rather than attachment to the saliva-derived tooth pellicle. From these observations, we propose that *S. mutans* adherence consists of two discrete and separable reactions, attachment and accumulation.

MATERIALS AND METHODS

Bacterial growth conditions. *S. mutans* 6715 was used throughout the study and was grown anaerobically overnight in sucrose-free brain heart infusion broth (Difco, Detroit, Mich). The medium was supplemented with 2 μCi of [^{3}H]thymidine per ml (Research Products International; Elk Grove, Ill., specific activity, 20.1 Ci/mmol). The stock culture was plated monthly on streptomycin-containing (200 μg/ml) mitis-salivarius agar (Difco) to verify the strain (7). After overnight growth, the *S. mutans* 6715 cells were recovered by centrifugation and washed three times in phosphate buffer (0.001 M, pH 6.0) with added KCl (0.05 M) and sodium azide (0.04%, wt/vol). The washed cells were suspended to an optical density (500 nm, 1 cm) of 1.0 in buffer. Typically, 10 μl of the ^{3}H-labeled cell suspension gave 3×10^3 to 4×10^3 cpm.

Adherence assay. The adherence assay that we employed is modeled after the SHA bead assay described by Clark et al. (5). The adherence surface support is a porous hydroxyapatite bead (BDH Chemicals, Ltd., Poole, England) with a diameter of 125 ± 50 μm. The beads were volumetrically (42 ± 1 mg) dispensed into 4-ml plastic scintillation vials (Beckman Instruments, Fullerton, Calif.) and equilibrated for 18 h at room temperature in 1.5 ml of buffer before saliva coating. The vials were continuously inverted at a rate of 12 times per min for all bead equilibrations and incubations. Whole saliva for coating the beads was routinely gathered from four laboratory employees. The saliva was pooled, clarified by centrifugation (14,000 × *g*, 30 min), and then heated to 60°C for 30 min. The saliva was again centrifuged, and 0.04% (wt/vol) sodium azide was added to prevent microbial metabolism. Control experiments indicated that the heating and sodium azide, as well as the use of parotid saliva, had no effect on the adherence assay. The equilibrated beads were coated by aspirating off the buffer and adding 1.5 ml of processed saliva to the beads followed by overnight, room temperature incubation. Unadsorbed salivary components were removed by washing the beads three times with buffer. The SHA beads were then incubated with 1.5 ml of ^{3}H-labeled *S. mutans* for 1.5 h. Bacteria which were not adsorbed to the SHA beads were removed with two rapid washes of buffer. After aspiration of the last buffer wash, the beads were dried at 60°C overnight and then assayed for radioactivity with a scintillation spectrometer. Bacterial adherence to the plastic vials in the absence of SHA beads was determined for all experiments, and the data were corrected to reflect only binding to SHA beads. The binding to the vials was usually 10% or less of the amount bound to the SHA beads.

Agglutinin preparation. Seeds of *Persea americana* (avocado) were washed, quartered, and lyophilized (N. A. Meade, R. H. Staat, S. D. Langley, and R. J. Doyle, Carbohydr. Res., in press). The dried sections were then ground to a coarse powder. The agglutinin was extracted from the powder by adding 500 ml of potassium phosphate (0.05 M, pH 6.5) buffered saline per 100 g of powder and stirred overnight at 4°C. The particulate material was then discarded, and the supernatant was dialyzed against distilled water. After dialysis, the material was lyophilized, with a typical yield of 1 to 2 g/100 g of seed powder. The dried agglutinin maintained the original titer of 128 (human type A erythrocytes) at a concentration of 1 mg/ml for over 6 months. Buffered solutions of the agglutinin showed a decrease in activity after 1 week of storage at 4°C.

GTF and glucan production. The *S. mutans* GTF used in these studies were prepared by ammonium sulfate precipitation of the cell-free supernatant from glucose-grown brain heart infusion broth cultures of *S. mutans* 6715 (23). The *S. mutans* glucan used in the inhibition study was made by incubating 1 ml of GTF in 10 ml of 5% (wt/vol) sucrose buffered to pH 5.7 with sodium acetate (0.05 M) and supplemented with 0.007 M NaF. After 24 h of incubation at 37°C, four parts methanol were added, and the precipitate was recovered by centrifugation. The precipitated polysaccharide was washed three times with methanol, and then the precipitate was resuspended in water and freeze-dried.

Scanning electron microscopy. Hydroxyapatite crystals (Bio Rad, Richmond, Calif.) were washed and saliva coated by the same procedure as for the SHA beads. The SHA crystals were incubated with a 2.0-optical density suspension of *S. mutans* 6715 either in the absence of sucrose or with 1% sucrose added. After washing, the crystals were air dried and sifted onto aluminum specimen studs which had just been painted with polyvinylchloride cement. The samples were then gold coated. A Cambridge Stereoscan MK II A scanning electron microscope equipped with a Polaroid camera was used for examination. The micrographs presented in this paper were taken at an original magnification of 6,000× with a 20 kV filament voltage.

RESULTS

Characterization of sucrose-independent adherence. Other investigators (5) determined

that adherence of *S. mutans* to SHA beads in the absence of any exogenous carbohydrate was described by the classical Langmuir adsorption isotherm. We have confirmed these results in our sucrose-independent assay of *S. mutans* 6715 adherence to SHA beads. In our assay, the sucrose-independent binding of *S. mutans* to SHA beads reaches equilibrium in less than 15 min and does not appear to require metabolically active cells.

Major groups on the *S. mutans* cell surface which could function in cellular attachment are proteins, carbohydrates, and TA. The data in Table 1 indicate that pretreatment of *S. mutans* 6715 with either sodium periodate, to oxidize vicinal hydroxyl groups found in many carbohydrates, or glucanohydrolytic enzymes had no effect on the sucrose-independent adherence. Although periodate pretreatment did not inhibit sucrose-independent attachment, it apparently destroyed the glucan receptor activity because no cellular agglutination could be detected when either sucrose or Dextran T 10 was added. On the other hand, the presence of sucrose and Dextran T 10 resulted in elevated adherence levels and agglutination of the cells pretreated with glucanohydrolase. Pretreatment of the cells with several proteolytic enzymes markedly reduced *S. mutans* 6715 adherence to SHA beads regardless of the sucrose content. However, cells treated with pepsin still agglutinated in the presence of added sucrose.

The proposed adherence mechanism involving TA suggests calcium-dependent chelation, or bridging, of the acidic salivary glycoproteins which constitute the tooth pellicle and the bacterial TA extending to the cell surface (19, 20). The data in Table 2 reveal that the binding of *S. mutans* 6715 to SHA beads was not reduced by elimination of Ca^{2+}. In fact, at levels above 10 mM Ca^{2+}, adherence was reduced. Interestingly, the elevated concentrations of Ca^{2+} tended to cause the *S. mutans* cells to aggregate in the suspending medium.

TABLE 1. *Effect of glucanohydrolase, periodate oxidation, or protease pretreatment of S. mutans 6715 cells on sucrose-dependent and independent adherence of the cells to SHA beads*

S. mutans pretreatment[a]	Adherence[b]	
	No sucrose added	1% Sucrose added
None (control)	28.6 ± 2.0	81.0 ± 7.9
Glucanohydrolase (dextranase)	31.7 ± 1.3	52.3 ± 1.4
Sodium periodate	25.7 ± 1.7	31.5 ± 1.8
Pronase	6.6 ± 0.3	6.5 ± 0.3
Trypsin	12.4 ± 1.1	13.1 ± 0.8
Pepsin	13.5 ± 1.3	12.8 ± 1.0

[a] Pretreatment was with 0.05 U of *Penicillium* dextranase for 2.0 h, 1.0 mg of sodium periodate per ml, for 4.0 h, 1 mg of pronase and trypsin per ml for 1.5 h at pH 6.0 and 22°C. Pepsin was used at pH 4.5 for 1.5 h at a concentration of 1 mg/ml. All reagents were from Sigma Chemical Co. After pretreatment, the cells were washed three times in buffer and then used in the adherence assay.

[b] Mean of three experiments expressed as ^3H counts per minute × 10^3 ± standard error.

Visualization of sucrose-independent and -dependent adherence. Scanning electron micrographs of *S. mutans* 6715 cells bound to SHA crystals in the absence of sucrose indicated that the short chains were bound as discrete units (Fig. 1a). Additional cells adsorbed to the SHA crystals as a result of added sucrose (Fig. 1b) did not appear to be bound as multiples of the discrete units seen in Fig. 1a, but rather, as larger, contiguous masses with numerous points of cell-cell contact. Although less consistent in in vitro adherence assays, SHA crystals rather than SHA beads were used for the scanning electron micrographs because the beads are extremely porous with many bacteria-like artifacts on the surfaces. Visual interpretation of *S. mutans* binding to the artifact-laden SHA beads was difficult at best.

Separation of adherence into attachment and accumulation reactions. Our recent work with lectins and agglutinins was used to further the concept that nonglucan cell surface molecules are involved in the attachment of *S. mutans* to the tooth surface (26). We found that the agglutinin extracted from seeds of *P. americana* (PAA) was a potent inhibitor of sucrose-dependent adherence of *S. mutans* 6715 to glass surfaces and that the PAA was interacting with the bacterial surface. Data in Table 3 show that PAA was an equally effective adherence inhibitor in the sucrose-independent SHA bead assay system. The significant aspects of these data are (i) that the sucrose-independent adherence was reduced approximately 10-fold by treatment of the cells with PAA and (ii) that the effect of the attachment inhibitor (PAA) was not overcome by sucrose-derived D-glucans. An uncharacter-

TABLE 2. *Effect of Ca^{2+} on the sucrose-independent adherence of S. mutans 6715 to SHA beads*

$CaCl_2$ (mM)	Adherence[a]
0.0	23.5 ± 0.9
1.0	22.1 ± 0.4
10.0	23.5 ± 2.0
50.0	15.2 ± 1.9
100.0	9.1 ± 1.8

[a] Mean of two experiments expressed as ^3H counts per minute × 10^3 ± standard error.

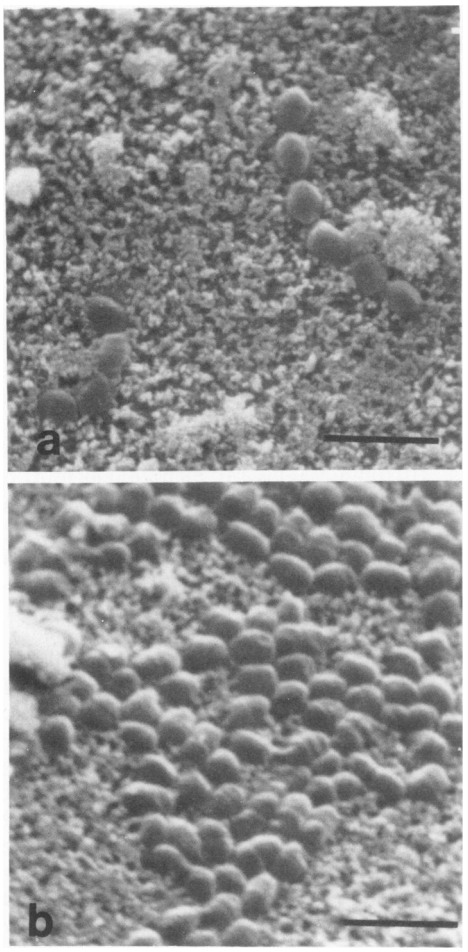

FIG. 1. *Scanning electron micrographs of S. mutans 6715 attached to SHA crystals (a) in the absence of sucrose and (b) in the presence of 1% sucrose. Bar, 2 μm.*

ized strain of *S. mutans* which was freshly isolated from a pedodontic patient produced a similar pattern of results (data not shown).

Although PAA inhibits adherence of *S. mutans* 6715, previous data from our laboratory (26) demonstrated that PAA did not reduce the rate of total glucan formation or the amount of water-insoluble polysaccharide formed by *S. mutans* 6715 GTF. Inhibition experiments provided further evidence that PAA was not modifying D-glucan metabolism. In these experiments, incubation of PAA with several glucans did not moderate the adherence inhibitory property of PAA. Additionally, pretreatment of *S. mutans* 6715 with PAA had no demonstrable effect on the sucrose-dependent agglutination of the cells (26).

One interpretation of these data is that the primary role of glucans in *S. mutans* colonization is to facilitate cellular aggregation rather than adherence while a separate, sucrose-independent, reaction initiates attachment. If this reasoning is valid, then experimentally it should be possible to adsorb PAA-treated *S. mutans* 6715 (inhibited attachment mechanism) via the glucan-dependent cell-cell interaction to *S. mutans* cells which were previously attached to the surface of SHA beads in the absence of sucrose. Table 4 illustrates the results of this sequential experiment. It is evident from these data that the sucrose promoted the binding of additional microbes to the *S. mutans*-SHA bead complex even though the bacterial attachment receptor for SHA was blocked. No increased cell accumulation was noted in the absence of sucrose. Experimentation with [^{14}C]sucrose indicated that the GTF associated with the cells were active in this series of assays.

DISCUSSION

Several pieces of evidence have been presented and interpreted by others to imply that attachment of *S. mutans* to surfaces is dependent on nonglucan factors (4, 5, 14, 15, 19, 20, 27,

TABLE 3. *Effect of sucrose and PAA on adherence of ^{3}H-labeled S. mutans 6715 to SHA beads*

Pretreatment of cells with PAA[a]	1% Sucrose supplementation	Adherence[b]
−	+	105.1 ± 9.4
−	−	22.5 ± 1.9
+	+	3.8 ± 0.3
+	−	2.3 ± 0.6

[a] Pretreatment was 1 h at 1 mg of PAA per ml in buffer followed by three washings with buffer.

[b] Mean of three experiments expressed as ^{3}H counts per minute × 10^{3} ± standard error.

TABLE 4. *Demonstration that cell-cell accumulation of S. mutans is dependent on the presence of sucrose and previously adsorbed S. mutans cells*

Addition to the ^{3}H-labeled *S. mutans*-SHA bead complex	Adherence[a]	% Increase
None[b]	22.5 ± 1.9	
PAA-treated, ^{3}H-labeled *S. mutans*	22.9 ± 2.0	1.8
PAA-treated, ^{3}H-labeled *S. mutans* and 1% sucrose	53.9 ± 4.9	139.6

[a] Mean of three experiments expressed as ^{3}H counts per minute × 10^{3} ±

[b] The experimental control was the amount of sucrose-independent adherence of ^{3}H-labeled *S. mutans* 6715 cells to SHA beads before the addition of PAA-treated, ^{3}H-labeled *S. mutans* 6715 cells.

28). These reports generally focus on one of two conceptually different mechanisms of attachment. The first involves electrostatic interactions between the cell, inorganic ions, and host receptors, whereas the second suggests specific molecular configuration or structure recognition between the cell and host receptors.

The electrostatic theory utilizes the negatively charged TA on the cell surface as the selective attachment molecules. The TA are bound to the negatively charged salivary glycoproteins of the pellicle by chelation with calcium ions. The evidence presented to support this theory is that Ca^{2+} promotes the agglutination of *S. mutans*, and that ethylenediaminetetra-acetic acid, an effective calcium chelator, rapidly disrupts dental plaque (19, 20). We find that Ca^{2+} promotes agglutination of *S. mutans*; however, our data indicate that low levels of added Ca^{2+} did not effect adherence of *S. mutans* 6715 to SHA beads, although Ca^{2+} bound to the salivary glycoproteins may have influenced these results. The bacterial binding was inhibited at levels above 10 mM Ca^{2+} rather than enhanced as the electrostatic model of adherence predicts. In addition, we have not been able to demonstrate any interaction between PAA, an adherence inhibitor, and various TA molecules (Meade et al., in press). Although TA and other amphipathic molecules may play a role in dental plaque formation, particularly in light of the paper of Ofek et al. (18), which showed that TA were involved in group A streptococcal adherence to epithelial cells, we feel that the evidence does not support TA as the non-glucan binding moiety in *S. mutans* adherence. Similar interpretations have recently been offered in a review by Gibbons (9).

Cell surface carbohydrates, including glucans, do not appear to be involved in the sucrose-independent attachment of *S. mutans* to SHA beads because of the ineffectiveness of either periodate oxidation or dextranase to interfere with the binding of cells to SHA beads. These experiments do not rule out the involvement of α-1,3-linked D-glucans as the adherence moiety because they are refractory to both treatments. However, Ebisu et al. (6) prepared purified α-1,3 D-glucans from *S. mutans* and showed that these glucans were nonadherent. In addition, PAA is not bound by *S. mutans* glucans, and adsorption of PAA with *S. mutans* glucans does not moderate the effect of PAA (26). Of the reagents tested, only the proteases and PAA had significant inhibitory effects on *S. mutans* 6715 adherence. Others have noted the sensitivity of *S. mutans* adherence to proteases (15).

Although the structure and affinity of PAA are not completely understood, it appears that the agglutinin interacts with proteins rather than carbohydrates (Meade et al., in press). Although this is unique, an agglutinin from *Arion empericorum* was recently described which also has an affinity for proteins rather than carbohydrates (12). Evidence to indicate that protein is closely associated with the cell wall of oral streptococci is beginning to accumulate. Bleiweis et al. (1, 2) and Rosan (21) demonstrated that amino acids of non-peptidoglycan origin can be detected in washed cell wall preparations from *S. mutans* and *S. sanguis*, respectively. In our preliminary experiments, we showed that after a series of rigorous extractions of *S. mutans* 6715 cell-wall preparations with concentrated salts, denaturants, and detergents, several amino acids were detected in the purified preparation which are not normally thought to be associated with peptidoglycan (Nesbitt et al., J. Dent. Res. **58** (Special Issue A):631, 1979). The adherence inhibition and non-peptidoglycan cell wall amino acids data may be interpreted to suggest that proteins associated with the cell surface are responsible for attachment of *S. mutans* to SHA beads.

The nature of either the bacterial protein or host salivary receptor involved in adherence is unknown. Potential host receptors include the BGR salivary mucins found in the acquired enamel pellicle. Gibbons and Qureshi recently demonstrated that *S. mutans* will bind BGR-reactive substances (10, 11). It is interesting to note that they used cells grown in chemically defined medium because cells grown in complex media, such as we used, were not reactive in the BGR mucin studies. Gibbons and Qureshi also indicated that pretreatment of the BGR salivary mucins with several compounds containing free amino groups inhibited bacterial binding. Even though preliminary findings on the specificity of PAA suggest that it reacts with basic proteins, pretreatment of SHA beads with compounds such as lysine, putrescine, spermidine, and amino sugars did not markedly affect *S. mutans* 6715 adherence in our system. However, we have found that pretreatment of SHA beads with the basic molecule chlorhexidine effectively inhibits *S. mutans* 6715 adherence (data not shown). This supports the premise that *S. mutans* adherence is mediated by basic groups on the surface of the bacterium, although there appears to be host salivary receptors additional to the BGR mucins.

Unquestionably, the presence of sucrose enhances the apparent adherence of *S. mutans*. The enhancement is visualized in the scanning electron micrographs and can be readily detected in the typical SHA bead assay. Exami-

nation of numerous scanning electron microscopy fields suggested to us that the main difference between the non-sucrose- and sucrose-enhanced adherence was the size of the individual aggregates of bacteria.

Recently, McCabe et al. (16, 17) demonstrated the existence of *S. mutans* proteins with the capacity to bind glucans. These proteins may be analogous to lectins, such as concanavalin A (24). Although the binding protein was recovered from culture supernatants, it is conceivable that the *S. mutans* "lectin" is a cell surface protein which recognizes D-glucans. There appear to be several of these lectins on the surface of *S. mutans* with specificities for a variety of D-glucans (29). The lectin-glucan interaction readily explains sucrose-dependent agglutination and accumulation of *S. mutans* cells on surfaces coated with D-glucans (13), as well as the interaction of dextran-coated *Actinomyces* species with *S. mutans* (3) and the heterotropic binding of *Streptococcus sanguis* to *S. mutans* cells (22). The glucan-binding lectins appear to be distinct from the cellular attachment proteins based on our findings that sucrose-induced agglutination is not affected by the attachment inhibitors pepsin, PAA, and chorhexidine.

It is evident that reagents that interfere with or alter the sucrose-derived glucans reduce the sucrose-enhanced binding of *S. mutans* 6715 to the approximate levels found for the sucrose-independent assays. Additionally, sucrose does not reverse the adherence inhibition caused by reagents which interact with cellular proteins. It should be recalled that adherence inhibition by PAA does not interfere with either the sucrose-dependent agglutination or glucan synthesis by *S. mutans* 6715. Therefore, it can be suggested that the adherence enhancement effects of sucrose are secondary to, or dependent on, the attachment of the cells to SHA surfaces. From the foregoing, we have developed the hypothesis, presented schematically in Fig. 2, that adherence of *S. mutans* is a two-reaction process in which initial attachment is accomplished via adsorption of nonglucan cell surface products, possibly tightly bound cell wall proteins, to the glycoprotein pellicle. This is followed by a cellular accumulation reaction mediated by the sucrose-derived glucans and the cell surface lectins. The data in Table 4 support the validity of this concept in sequential experiments with PAA as the tool for selective attachment inhibition.

At the present time, measures aimed at in vivo control of *S. mutans* have focused on interference with the sucrose-glucan system. These efforts have only been partially successful at controlling the disease. The hypothesis that we present in this report suggests that attachment

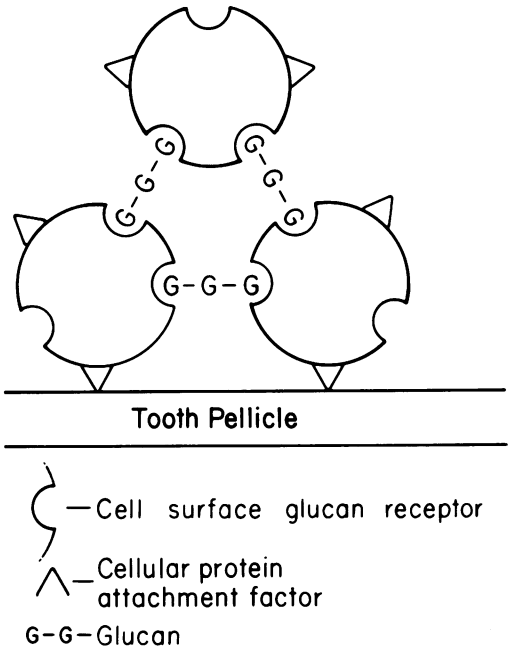

FIG. 2. *Schematic diagram of proposed model of S. mutans adherence which illustrates the cell surface protein-salivary pellicle attachment phase and the glucan-mediated cell-cell accumulation phase.*

of the cells is independent of the glucan system. This model may be extrapolated to explain the maintenance and opportunistic character of *S. mutans* as a member of the normal oral flora regardless of the host diet. If our hypothesis is valid, a new approach to dental caries prevention will be blockage of the first reaction, or attachment phase, by either chemical or immunological techniques. This would eliminate the bacterium from the oral cavity rather than just suppressing the sucrose-dependent cellular accumulation.

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