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Glucan-Binding Factor in Saliva

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High-molecular-weight polymers of α-1,6-linked D-glucans are insoluble in alcohol solutions. Whole, but not parotid, saliva prevented the precipitation of D-glucans by 80% (vol/vol) ethanol, showing that the whole saliva contained a factor which complexed with the glucan to render it alcohol soluble. The glucan-binding factor was retained on a column of Sephacryl S-200 which had been preequilibrated with 80% ethanol. The factor was then eluted with water. Passive hemagglutination assays revealed that the glucan-binding factor could sensitize erythrocytes to agglutination with anti-poly(glycerolphosphate), suggesting that the active glucan-binding component was lipoteichoic acid. The glucan-solubilizing factor was resistant to heat (100°C), proteases, sialidase, lysozyme, lactoperoxidase, trichloroacetic acid, and Triton X-100. When sucrose was added to saliva, a suspension of *Streptococcus cricetus* AHT, or a suspension of *Streptococcus sanguis* 10556, relatively large amounts of glucan-binding factor were released in a soluble form. In addition, penicillin G caused the release of the glucan-solubilizing component from a suspension of *S. cricetus* AHT. It is suggested that whole saliva contains a component, tentatively identified as lipoteichoic acid, which can complex with glucans in a relatively hydrophobic solvent. This type of complex formation may be important in the adhesion of oral streptococci to saliva-coated surfaces.

For several years, it has been clear that glucans derived from sucrose play a significant role in oral disease. Oral streptococci such as *Streptococcus mutans* synthesize glucosyltransferases (GFT) that convert sucrose into water-soluble or water-insoluble glucan. The exact role that glucan may have in promoting dental caries is not clear. Some streptococci possess glucan-binding proteins (22), possibly enabling the bacteria to accumulate in plaque when glucan is present. Similarly, initial adhesion of streptococci to teeth may involve GTF and GTF-glucan complexes on pellicle (3, 29–31).

Nesbitt et al. (25) in 1982 proposed that the adhesion of oral streptococci to artificial pellicle was partially dependent on hydrophobic forces. Later, Doyle et al. (9) suggested that multiply interacting sites stabilized by the hydrophobic effect could result in a high-affinity association between the bacteria and the pellicle. One corollary for the model proposed by Doyle et al. (9) for the streptococcal adhesion to pellicle was that hydrophilic molecules may be able to form stable complexes in media with a low dielectric constant.

Results of this study revealed that there is a glucan-binding factor in whole saliva but not in parotid saliva. This factor prevents the ethanol-induced precipitation of glucan and is microbially derived. Not only does saliva possess a factor which can complex with glucans in solvents with a low dielectric constant, but bacteria such as *Streptococcus cricetus* and *Streptococcus sanguis* were also shown to contain alcohol-soluble glucan-binding molecules.

MATERIALS AND METHODS

Reagents, chemicals, and polymers. A linear α-1,6-linked glucan with an average molecular weight of 186,000 was obtained from Pharmacia, Inc. (Piscataway, N.J.). Lipoteichoic acid (LTA) from *Bacillus subtilis* 168 was purified by the method of Thaniyavarn et al. (37). Streptococcal LTA was purified as described previously (17). One sample of *S. mutans* LTA was purchased from Sigma Chemical Co. (St. Louis, Mo.). Rabbit antisera to LTA from *Staphylococcus aureus* ATCC 6538P (5) gave an hemagglutination titer greater than 8,192 with human erythrocytes coated with *S. mutans* LTA. Crude GTF was obtained from culture supernatants (4) of *Streptococcus sobrinus* 6715. The fraction precipitating between 20 and 50% saturation of ammonium sulfate was dialyzed against 50 mM acetate buffer (pH 5.6) and used without further purification. All salts and solvents were from Fisher Chemical Co. (Cincinnati, Ohio). Enzymes were from Sigma.

Insoluble glucan preparation. The enzyme preparation from *S. sobrinus* 6715 was allowed to incubate with 5% (wt/vol) sucrose in 50 mM sodium acetate (adjusted to pH 5.5 with acetic acid) for 4 days at 37°C. The insoluble material was removed by centrifugation and washed three times in hot water (100°C for 15 min, followed by centrifugation). The precipitate was then freeze-dried.

Saliva collection. Whole human saliva was collected without stimulation over ice to an average volume of 5.0 ml from at least two donors. Parotid saliva was obtained by use of plastic collection cups fitted over the duct and held in place by suction. The average volume collected was 0.5 ml per duct (1.0 ml per person). All saliva samples were centrifuged at 14,000 × *g* for 10 min, and any insoluble material was discarded.

Glucan binding assay. The formation of a complex between saliva or LTA and glucan was assayed as follows. Whole human saliva was collected without stimulation over ice to a volume of 5 ml from at least two donors, centrifuged at 14,000 × *g* for 10 min at 4°C, and pooled. A quantity of glucan (usually 500 μg) in 0.5 ml of distilled water was added to 4.0 ml of absolute ethanol and 0.5 ml of water containing 50 μl of saliva or 50 μl of purified LTA. The tube was shaken vigorously, vortexed, and monitored turbidimetrically at a wavelength of 500 nm for a period of up to 20 min. Solubi-

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lization was quantitated by the following equation: percent soluble glucan = [1 − (OD sample/OD control)] × 100, where control = glucan + ethanol + water and OD is the optical density. In some experiments, the noncomplexed, insoluble glucan was separated by centrifugation, washed twice with 5.0-ml volumes of 80% (vol/vol) ethanol, and assayed for hexose with the anthrone reagent (23). The results obtained from chemical assays were in agreement with results obtained by turbidimetric readings.

Chemical analyses. Phosphorus was determined by the ashing procedure described by Ames (2). Protein was quantitated by the method of Lowry et al. (20), using bovine albumin as the standard. Glycerol was determined with a glycerol test kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) after acid hydrolysis of the saliva sample in 4 N HCl at 110°C for 4 h (3), followed by freeze-drying. Ketose and hexose were determined by the anthrone procedure of van Handel (38), using fructose and D-glucose standards.

Hemagglutination and hemagglutination inhibition. The titer of antibody directed against the poly(glycerolphosphate) backbone of LTA was determined by incubating serial dilutions of the antiserum (heated first at 56°C for 30 min) with equal amounts of a 2% suspension of human group O erythrocytes. The erythrocytes had previously been incubated with equal amounts of 0.1-mg/ml LTA from *B. subtilis* 168 and washed twice with 0.85% NaCl (13, 37). The antibody and sensitized cells were mixed together at 37°C for 3 h, at which time the reaction was read. The titer was taken as the reciprocal of the highest dilution showing agglutination.

To test various fractions for their ability to inhibit hemagglutination, we sensitized erythrocytes as above and suspended them to a 2% density. Heated, clarified whole saliva and peak B crude material (Fig. 6 and accompanying results describe these peaks of fractionated saliva) were serially diluted three times in 0.85% NaCl, and each dilution was added to 20 μl of the antibody diluted to one dilution higher than the previously identified titer. LTA and wall teichoic acid preparations (7) were also used as control inhibitors and mixed with the antibody. In all cases, the final antibody concentration was 1:250. Equal volumes of the 2% erythrocyte suspension and the antibody plus inhibitor were mixed and incubated at 37°C for 3 h.

RESULTS

Interaction between saliva and glucan. Solutions of glucan were mixed with parotid or whole saliva. The mixtures were then made 80% with respect to ethanol. As expected, the control glucan precipitated in the alcohol solution (Fig. 1). In contrast, when whole saliva was used in the mixture, the glucan did not precipitate. Parotid saliva did not solubilize the glucan, suggesting that a microbial product was involved in the complex formation. It is interesting that the parotid saliva-glucan mixture in alcohol gave a higher turbidity than the glucan alone. This is because parotid saliva partially precipitates in 80% ethanol, but whole saliva does not. The whole saliva therefore contains a component that not only complexes with glucan in alcohol, but also solubilizes protein molecules as well. Maximal solubilization of 1,000 μg of glucan in 80% ethanol required approximately 100 μl of whole saliva in a reaction volume of 5.0 ml (Fig. 2).

Alcohol-soluble complexes of LTA and glucans. Previous work from this laboratory has shown that both cell wall teichoic acid and LTA can form soluble complexes with glucans in alcohol solutions (8). In addition, because whole, but not parotid, saliva could form similar types of complexes with glucans, it was considered that the polysaccharide-binding factor in the saliva may indeed be LTA. Small amounts of an LTA preparation could readily solubilize the glucan in 80% ethanol (Fig. 3). Only 3.0 μg of the LTA was able to complex with and prevent the precipitation of 500 μg of the glucan. Similar results (Fig. 4) were obtained from several LTA preparations, the glycerol residues of which were substituted with D-glucose, D-alanine, or hydrogen (17, 19). Neither the extent nor the nature of the substitution seemed to affect the ability of the LTA to complex with glucan (Fig. 4).

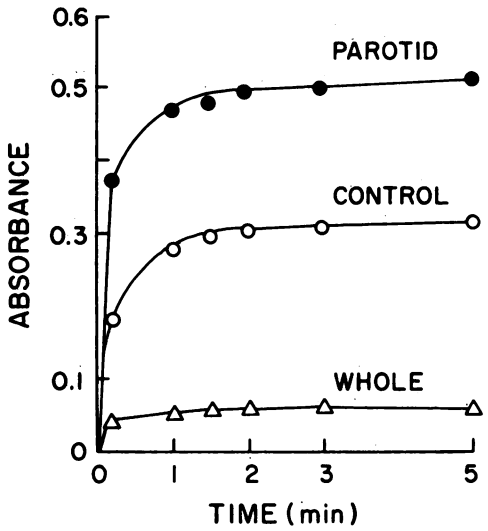

FIG. 1. Interaction between parotid or whole saliva and glucan. Complex formation was monitored turbidimetrically. Final ethanol concentration was 80% (vol/vol). A 5.0-ml reaction volume contained 1.0 mg of glucan and 100 μl of saliva. The control sample contained glucan, but no saliva.

Affinity purification of glucan-binding factor(s) in saliva. A column of Sephacryl S-200 was preequilibrated with 80% ethanol, and a sample of whole saliva in the alcohol was applied to the column. The column was then eluted with 80% ethanol. Results (Fig. 5) revealed that a part of the 280-nm-absorbing material was eluted by the alcohol (peak A). Another fraction (peak B) was eluted by water. Both fractions were freeze-dried and used in the glucan-binding assay. The ethanol-eluted fraction (A) was incapable of complexing with the glucan (Fig. 6), but the water-eluted fraction (B) possessed strong glucan-binding properties.

Preliminary results of chemical analyses of the water-eluted fraction revealed that the fraction contained glycerol

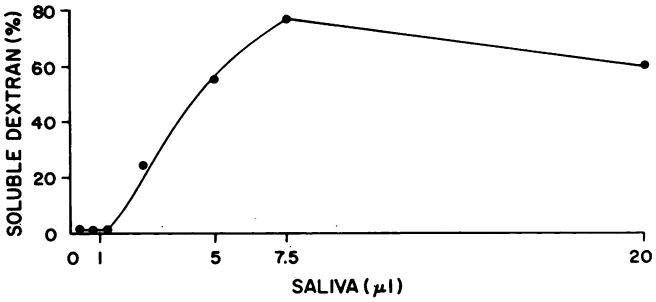

FIG. 2. Solubilization of glucan in alcohol by whole saliva. Conditions are described in the legend to Fig. 1.

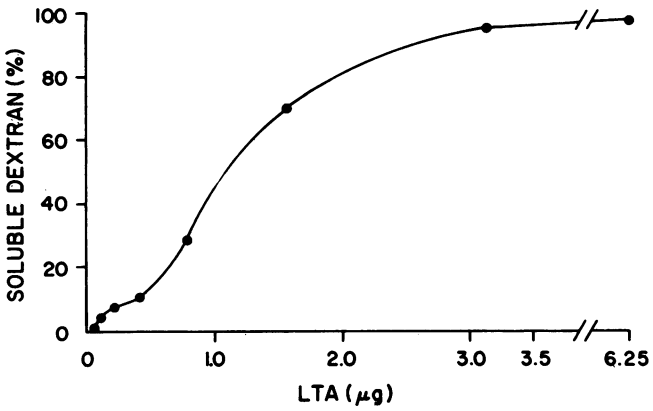

FIG. 3. Binding of glucan by *Streptococcus faecium* 9790 LTA. A total volume of 5.0 ml contained 500 μg of glucan, LTA as shown, and a final ethanol concentration of 80%.

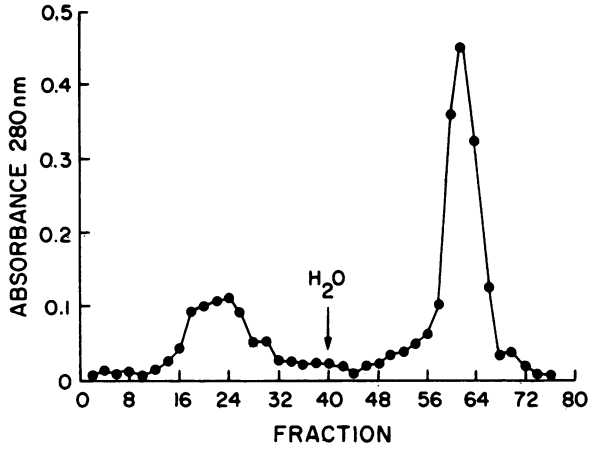

FIG. 5. Chromatography of saliva on Sephacryl. Saliva was treated for 5 min at 100°C in 80% ethanol, and insoluble material was removed by centrifugation. The supernatant in 80% ethanol was applied to a column of Sephacryl S-200 preequilibrated with alcohol. Elution was with 80% ethanol and water as indicated.

and phosphorus in equimolar ratios, as well as protein. When the water-eluted fraction was subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis, only a single band of approximately 60,000 molecular weight could be detected (stained with Coomassie brilliant blue R-250). Parotid saliva did not contain a protein capable of binding to the Sephacryl column.

When the water-eluted fraction was used in passive hemagglutination reactions (13), the anti-LTA preparation agglutinated the sensitized erythrocytes. In addition, inhibition of passive hemagglutination was successful when the water-eluted fraction was the sensitizing agent and cell wall poly(glycerolphosphate) was the inhibitor. At present, because of insufficient quantities of material, it is impossible to quantitate the relative effectiveness of the water-eluted fraction in comparison to pure wall teichoic acid or LTA preparations in promoting hemagglutination inhibition.

Reversibility of alcohol-soluble glucan complexes with saliva or LTA. Samples of glucan (1,000 μg) in water were brought to 80% in ethanol in a final volume of 5.0 ml. When turbidity rapidly developed (Fig. 7), LTA or saliva was added and the mixture was vortexed (at arrow 1 in Fig. 7). No significant additional turbidity developed, but in addition, the previously precipitated glucan was not solubilized. When LTA or saliva was added to aqueous glucan followed by the addition of ethanol, very little glucan was precipitated (as shown by the control saliva-glucan or LTA-glucan in Fig. 7). An important question was whether the LTA or salivary glucan-binding factor could actually complex with the alcohol-precipitated glucan. At arrow 2, the insoluble materials were removed by centrifugation, washed twice in 80% ethanol, and then dissolved in water. Finally, the aqueous solutions were brought to 80% in ethanol (arrow 3). The results showed that very little glucan became insoluble. It therefore appears that glucan precipitated by alcohol can still bind LTA or the glucan-binding factor of saliva but that the LTA or saliva cannot reverse the precipitation.

Binding of LTA to water-insoluble glucan from *S. sobrinus*. A sample of water-insoluble glucan was prepared from an *S.*

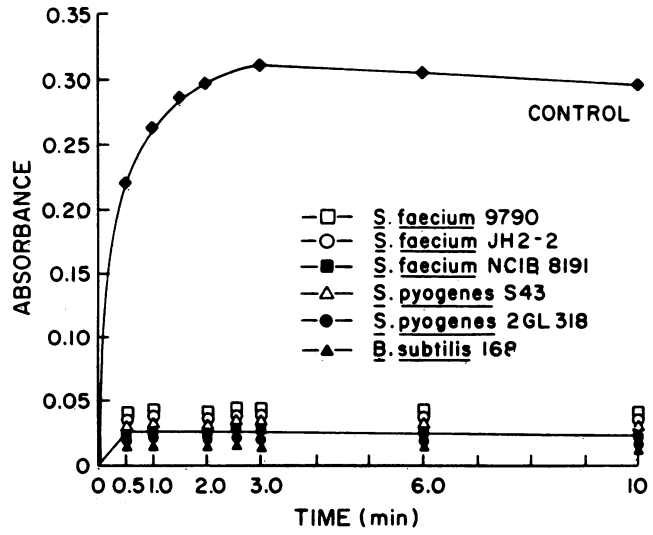

FIG. 4. Dextran-solubilizing activities of LTA preparations. Details are given in the legend to Fig. 1.

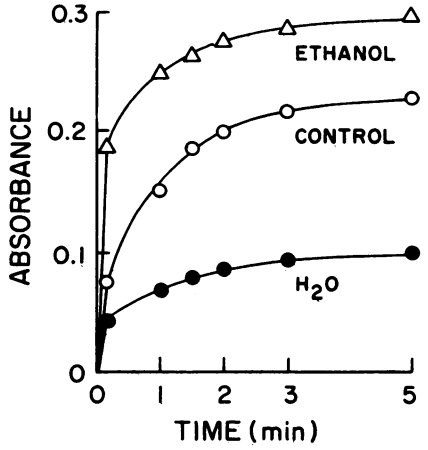

FIG. 6. Dextran-solubilizing capabilities of Sephacryl column eluants. The column run was described in the legend to Fig. 5. Elution with 80% ethanol yielded the first fraction, whereas elution with water yielded the second fraction. Amount of glucan in the assay was 500 μg. Conditions were described in the legend to Fig. 1 for assay of complex formation in 80% ethanol. Control was glucan in 80% ethanol.

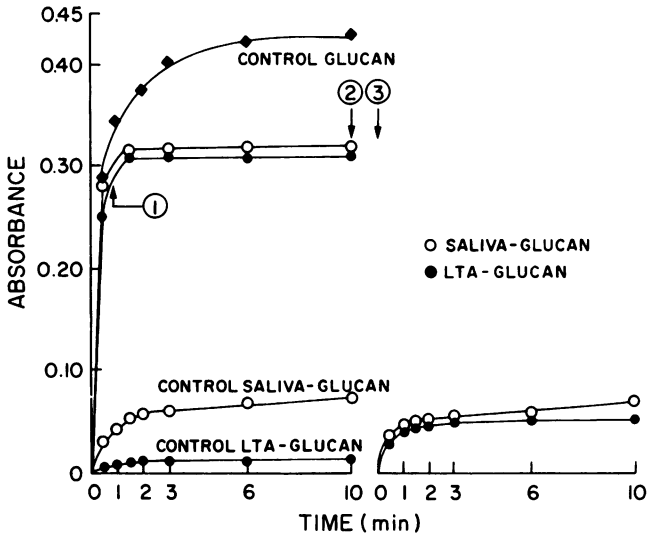

FIG. 7. Reversibility of complex formation between glucan and LTA or saliva. Glucan (1,000 μg in 0.5 ml of H_2O) was brought to 80% in ethanol. Quantities of LTA (5.0 μg in 50 μl) or saliva (50 μl) were added at arrow 1. At arrow 2, insoluble materials were removed by centrifugation, washed twice in 80% ethanol, and dissolved in 0.5 ml of H_2O. At arrow 3, enough ethanol was added to make the final concentration 80%.

sobrinus 6715 enzyme preparation and sucrose solution. The glucan was free of ketose, as determined by the cold anthrone procedure (38). When an *S. mutans* LTA preparation was mixed with the glucan, the glucan remained insoluble in 80% ethanol (Fig. 8), but the LTA bound strongly to the glucan. In fact, most of the LTA could be recovered in the insoluble fraction, as determined by phosphorus analyses. In contrast, when water-insoluble glucan was mixed

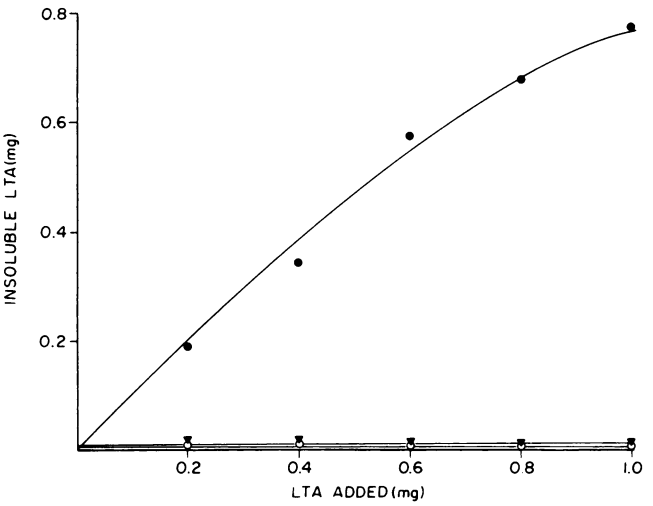

FIG. 8. Binding of LTA to water-insoluble glucan. Samples of water-insoluble glucan (500 μg each) in 0.5 ml of water were mixed with 0.5 ml of *S. mutans* LTA solution. The mixture was vortexed, and 4.0 ml of absolute ethanol was added. The polysaccharide remained insoluble and was washed twice with 5.0 ml of 80% ethanol. Insoluble LTA was determined by phosphorus analyses. Symbols: ●, glucan-associated LTA in alcohol; ○, alcohol-insoluble LTA in the absence of glucan; ▼, insoluble LTA associated with glucan in water suspension without added alcohol.

TABLE 1. Stability of salivary glucan-binding factor[a]

Treatment	Soluble glucan (%)
None, untreated control	83.3
Heat, 100°C, 15 min	80.5
Trichloroacetic acid (5%, wt/vol)	71.5
Triton X-100 (1%)	80.7
Trypsin	74.0
Heat (100°C, 15 min), then trypsin	68.4
Pronase	70.2
Subtilisin	72.5
Lactoperoxidase	74.7
Lysozyme	72.3
Sialidase	76.5

[a] The reaction mixtures were monitored by turbidity measurements after the addition of ethanol (80% [vol/vol] final concentration). Final volume was 5.0 ml. Glucan (1,000 μg) and saliva (100 μg) were the reactants. Enzyme concentrations were 50 μg/ml, and incubations were at 37°C for 1 h. Enzyme incubations were in 40 mM phosphate buffer (pH 7.3). Following incubation, salts were removed by dialysis against water. No effort was made to remove the trichloroacetic acid or the Triton X-100.

with LTA in a water solvent, there was very little phosphorus in the insoluble fraction. The results showed that LTA can directly bind to water-insoluble glucan in 80% ethanol but not in aqueous solution.

Stability of glucan-binding factor of saliva. Samples of clarified whole saliva were subjected to various enzymes, protein precipitants, a detergent, or high temperatures. The treated saliva samples were then mixed with glucan, and alcohol was subsequently added. The extent of complex formation was then determined by turbidity measurements as described above. The results (Table 1) revealed that the glucan-binding factor was relatively resistant to the heat, enzymes, detergent, and acid.

Release of glucan-binding factor from saliva and streptococci. It is known that streptococci may release LTA when penicillin or sucrose is added (1, 16, 18, 21). If the glucan-binding factor in saliva is LTA from streptococci or other bacteria, then incubation of the saliva in penicillin or sucrose may result in an increased glucan-binding activity. Suspensions of *S. cricetus* AHT and *S. sanguis* along with samples of unclarified whole saliva were subjected to additions of water, 20 mM sucrose, or 50 μg of penicillin G per ml. After a 10-min incubation at 3°C, the suspensions were clarified by centrifugation and the resulting supernatants were used in glucan-binding assays. The results (Table 2) show that sucrose released glucan-binding activity from both streptococcal species as well as from the saliva sample. Penicillin released glucan-binding factor only from *S. cricetus*.

DISCUSSION

The results of this work showed that saliva contains a glucan-binding factor capable of forming stable complexes in ethanol. Based on passive hemagglutination, hemagglutination inhibition with a wall poly(glycerolphosphate) teichoic acid, and direct chemical analyses, it is suggested that the glucan-complexing molecule is LTA. Several investigators have reported that LTA can form complexes with microbially derived protein (28, 37) and components of dental plaque (32).

Proteases and other enzymes had no effect on the glucan-binding factor. In addition, agents such as trichloroacetic acid and heat treatment at 100°C did not reduce the capacity of saliva to solubilize the glucan in 80% ethanol. If the glucan-complexing component in saliva was protein, one or

TABLE 2. Release of glucan-binding factor from saliva and streptococci[a]

Source	Soluble glucan (%)[b]		
	H_2O	Sucrose	Penicillin
S. cricetus AHT (serotype a)	6.6	43.3	46.7
S. sanguis 10556	6.3	25.0	6.3
Saliva	28.6	40.0	31.4

[a] Bacteria were obtained from overnight growth in brain heart infusion broth, harvested by centrifugation, and washed three times in distilled deionized water. The cells were suspended to an optical density of 1.5 at 500 nm with a 1-cm light path. Saliva was from a single donor and was obtained by Parafilm stimulation. To the cells or saliva was added either water or a final concentration of 20 mM sucrose or 50 μg of penicillin G per ml. Dilution of cells or saliva with water, sucrose, or penicillin G was 10%. The suspensions and saliva were held at 2 to 4°C for 10 min and centrifuged. The supernatants were used in the glucan-binding assays.

[b] Reaction conditions were 4.0 ml of ethanol, 200 μl of glucan (400 μg), and 200 μl of supernatant from cells or saliva. Final ethanol concentration was 90%.

more of the above treatments would have probably destroyed its activity.

When saliva was fractionated on alcohol-equilibrated Sephacryl S-200, the glucan-complexing fraction was eluted by water. The fraction consisted mainly of protein, but contained glycerol and phosphorus as well. The glycerol and phosphorus were probably due to LTA as this fraction could sensitize erythrocytes to hemagglutination by an anti-poly(glycerolphosphate) antibody preparation. It is unknown if the protein that adhered to the affinity column, along with LTA, was derived from oral microbiota or from saliva. In control experiments, it was shown that components of parotid saliva would not bind the alcohol-equilibrated Sephacryl. LTA (or deacylated LTA), however, may complex with salivary components, thereby promoting the binding of a protein from saliva to the Sephacryl.

The observation that penicillin or sucrose could release additional glucan-complexing activity from saliva and from oral streptococci (Table 2) may have relevance in oral health. The presence of sucrose promotes the colonization of oral streptococci on the tooth surface (11). Small amounts of LTA liberated from oral streptococci by sucrose may later bind with glucans produced from the sucrose. Ciardi et al. (5, 6) and Rolla (28) believe that extracellular LTA may be important in the adhesion of oral streptococci to both saliva-coated and saliva-free surfaces, such as glass or hydroxylapatite. Furthermore, Rolla et al. (32) showed that when sucrose was added to a rich growth medium, *S. mutans* strains produced glucans and LTA molecules that could not be separated by exclusion chromatography. Horne and Tomasz (16) and Kessler and van de Rijn (18) have noted that the addition of penicillin to streptococci results in increased levels of LTA (and deacylated LTA) in supernatants from cell suspensions. We could observe no increased release of glucan-binding factor after treatment of *S. sanguis* 10556 with penicillin. This could be due to lack of release of LTA under the conditions used in this study.

Rolla and colleagues (28–32) and Schilling and Bowen (35) have proposed an interesting mechanism for adhesion of *S. mutans* to pellicle. They believe that GTF from salivary microbiota form a part of the pellicle. These enzymes could then synthesize glucans in the presence of sucrose and provide a substrate for attachment of streptococci. Conversely, glucans already associated with streptococci may bind to GTF on pellicle. An important aspect of the mechanism proposed by these workers is that LTAs have been suggested to complex with glucan on pellicle. The results described in this report show that under the proper conditions, small amounts of LTA molecules can combine with much greater quantities of glucan. Other polyelectrolyte-glucan complexes may also be amplified by low-polarity solvents (12, 14, 15, 33, 39).

Exactly how glucan, LTA, salivary protein, and microbial surfaces may interact to form plaque is unknown. Streptococcal cell surfaces may be hydrophobic (25, 26, 34); salivary proteins have hydrophobic domains (10). Saliva also contains glycolipids (36). It may be that these hydrophobic components provide the proper environment to stabilize glucan-LTA complexes. This would be consistent with the view that the adhesion of streptococci to pellicle involves multiply interacting sites (9, 25). Ofek et al. (27) found that LTA could form alcohol-soluble complexes with the M protein of *Streptococcus pyogenes*. Ofek et al. (27) proposed that hydrophobic domains of the M protein could stabilize ionic contacts between LTA and charged amino acid side chains.

Mooser and Wong (24) have recently found that a domain of α-1,6 glucan synthase (dextransucrase) could complex with D-glucans. It is presently unknown if their enzyme fragment forms alcohol-soluble complexes with glucans.

ACKNOWLEDGMENT

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