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Antibody-Mediated Inhibition of Dextran/Sucrose-Induced Agglutination of *Streptococcus mutans*

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Immune sera to strains of *Streptococcus mutans* were found to inhibit agglutination of bacterial suspensions to which either high-molecular-weight dextran or sucrose was added. This inhibitory activity was shown to be mediated by antibody of the immunoglobulin G class. A semiquantitative assay was developed which demonstrated cross-inhibition of dextran/sucrose-induced agglutination among several strains of *S. mutans*. Antiserum to a partially purified glucosyltransferase was found to lack agglutination inhibition activity, consistent with the hypothesis that the dextran-binding antigen detected by the assay is immunologically distinct from the glucan-synthetic enzyme. A model for glucan synthesis and binding consistent with the reported data is described.

Bacterial strains of *Streptococcus mutans* share the ability to synthesize high-molecular-weight extracellular glucan polymers from sucrose through the synthetic action of cell-bound and cell-free glucosyltransferase enzymes (6, 8). Glucan production is closely associated with the ability of these bacteria to adhere to tooth surfaces as dental plaque (6).

Some glucan-producing bacteria may be detected by observing their agglutination after the addition of sucrose (9) or dextran (7) to bacterial suspensions grown in the absence of sucrose. This agglutination results from the binding of dextran molecules by multiple bacterial cells (7). Agglutination in sucrose occurs after enzymatic synthesis of glucan molecules that are then bound (9). These bacteria may also be identified by their ability to adhere to smooth surfaces, such as wire (11) or glass (13), when grown in media containing sucrose. Adherence ability is susceptible to inhibition by serum antibody (13). This report describes the similar inhibition of dextran- or sucrose-induced agglutination of *S. mutans* strains.

MATERIALS AND METHODS

Bacteria. Bacterial strains used for immunization were grown in tryptic soy broth (Difco). Subsequent difficulty with bacterial agglutination (probably from contaminating sucrose) prompted a change to Todd-Hewitt broth medium (Difco). After 12 to 18 h of growth at 37 C, bacteria were harvested by centrifugation, washed once in 0.9% saline, and suspended in 0.15 M phosphate-buffered saline, pH 7.0. Bacteria for immunization were killed by suspending in 0.6% Formalin-0.9% saline for 48 h. They were then washed twice in 0.9% saline to remove the Formalin.

Glucosyltransferase purification. Glucosyltransferase enzymes from *S. mutans* OMZ 176 were partially purified by hydroxyapatite adsorption of culture fluid supernatant (8). The enzymes were eluted between 0.2 and 0.5 M phosphate buffer and produced insoluble glucan (Fig. 1). Some non-enzyme contaminants were present in this preparation. It contained 12.6 glucosyltransferase units per mg of protein.

Antisera. Adult New Zealand white rabbits were inoculated intravenously three times per week, on alternate days, for 3 weeks with 1 ml of the bacterial immunogens (OD_{540} = 2.0). Rabbits were bled 4 to 6 days after the final injection. Glucosyltransferase antiserum was obtained from rabbits injected subcutaneously with 1.2 mg of glucosyltransferase in 0.5 ml of complete Freund adjuvant. Two injections were given at 3-week intervals, and rabbits were bled 3 weeks after the second injection. Sera were collected and frozen at −20 C until used. All antisera were examined for anti-dextran activity by gel diffusion against Dextran 80 and Dextran 150 (Pharmacia Fine Chemicals, Inc.) (1). Only antisera not showing precipitin bands with dextran were used. OMZ 176 antiserum was also examined for anti-glucan activity by using soluble glucan extracted from strain OMZ 176 (9). No precipitin line was observed.

Agglutination inhibition assay. A 0.15 M phosphate-buffered saline, pH 7.0, was used as the standard buffer for the assay. Bacteria to be used in the assay were suspended in this buffer at an optical density of 0.45 (540 nm, 1-cm light path). Volumes of 1.5 ml were added to a series of test tubes. A 0.1-ml volume of antiserum or serial twofold antiserum dilutions in the phosphate buffer were then added. A

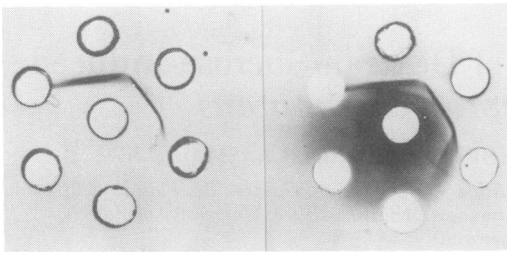

FIG. 1. *Left, agar gel diffusion of partially purified glucosyltransferase of strain OMZ 176 (1 mg, center well) with IgG from the 0.33 saturated ammonium sulfate precipitate of a rabbit antiserum to strain OMZ 176. Clockwise, from the top, wells contain undiluted IgG, 1:4 IgG, 1:16 IgG, 1:64 IgG, normal rabbit serum, and 0.9% saline. Right, the same plate after 12 h of incubation in 25% sucrose.*

15-min period was allowed for incubation at room temperature. Tubes were then checked for the presence of macroscopic direct agglutination by serum antibody. A previous study (13) showed that serum antibody causes direct microscopic agglutination that is not always visible macroscopically. Strains showing direct macroscopic agglutination could not be used to determine agglutination inhibition. However, it was sometimes possible to observe agglutination inhibition in the prozone. Then 0.05 ml of a high molecular weight (2×10^6) dextran (2.5 mg/ml) (Sigma Chemical Co., type 2000) or sucrose (150 mg/ml) was added. Tubes were observed for agglutination and scored on a visual basis as – (no agglutination), + (fine agglutination), ++ (intermediate agglutination), or +++ (coarse agglutination). The titer was defined as the reciprocal of the serum dilution in the last tube showing negative agglutination. Serum dilution referred to the final dilution, i.e., after addition of serum to the bacterial suspension. Preimmune rabbit sera were shown to have no agglutination inhibition activity. Assay tubes to which 0.9% saline was added served as controls. Titers below 16 were designated as negative (0).

Adherence inhibition assay (13). This assay detects in vitro inhibition of adherence of *S. mutans* to glass. It is quantitative for antibody inhibition, with serum titer indicating the reciprocal of the serum dilution at which 50% of the bacteria are adherent and 50% are nonadherent.

Ion-exchange chromatography (14) and gel filtration (5). A 10-ml amount of immune serum was fractionated on a column (2 by 20 cm) of diethylaminoethyl cellulose (Whatman DE-32). A 0.025 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.3) was used to elute the sample, and the unadsorbed material was collected and pooled. The protein was concentrated by negative-pressure dialysis. Gel diffusion analysis with specific antisera revealed the presence of both immunoglobulin IgG and IgM. These immunoglobulins were separated on a Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) column (2.5 by 100 cm with a void volume of 156 ml). The elution buffer was 0.1 M Tris-hydrochloride-0.2 M NaCl (pH 8.0) containing 0.2% sodium azide. Fraction volumes of 5.8 ml were collected, and light absorbance of the eluate was determined at 280 nm. Enough soluble dextrans were present in the eluate from the Sephadex column to interfere with the assay. Dextrans were removed by precipitating the globulins and washing the precipitate twice in 0.5 saturated ammonium sulfate. Excess salt was removed by dialysis against 0.9% saline. Immunoelectrophoresis using anti-whole rabbit serum revealed single precipitin lines with electrophoretic mobility characteristic of IgG and IgM. Gel diffusion analysis using antisera specific for heavy chains of rabbit immunoglobulins indicated them to be uncontaminated.

Serum adsorption. Bacteria were grown aerobically at 37 C for 18 h and washed in 0.9% saline. A 0.1-ml volume of whole packed bacteria cells was used to adsorb 1.0 ml of serum diluted 1:4 in 0.9% saline for 0.5 h at room temperature and 24 h at 4 C. Serum was recovered by centrifugation at 4 C.

RESULTS

Agglutination inhibition activity in purified immunoglobulins of immune rabbit serum. Activity in immune serum was found to be present in the IgG fraction (Table 1). Immunoglobulin concentration was adjusted to the approximate concentration in whole serum. Titers for whole serum and the IgG fraction were equivalent. No activity was found in the IgM fraction.

Anti-glucosyltransferase activity in purified IgG of glucosyltransferase antiserum. Glucosyltransferase antiserum was found to form precipitin lines with the glucosyltransferase immunogen (Fig. 1). When a sucrose solution was allowed to incubate over the agar containing the enzyme, insoluble glucan was synthesized. Glucan synthesis did not occur beyond the precipitin lines, confirming the presence of antibody specific for glucosyltransferase. IgG from normal rabbit serum did not form precipitin lines or prevent migration of enzyme away from the center well. The adherence inhibition titer of the antiserum was greater than 100, and the agglutination inhibi-

TABLE 1. *Agglutination inhibition activity in purified immunoglobulins from an immune serum*

Immunoglobulin	Concentration (mg/ml)[a]	Agglutination inhibition titer[b]
IgG	10	256
IgM	2	0

[a] Assuming an extinction coefficient of 13.5 for IgG and IgM.

[b] Inhibition of dextran-induced agglutination at 10 min when using strain 6715 as test organism and antiserum to strain 6715; titer of unfractionated antiserum was 256.

tion titer was 0. Similar reactions were observed when anti-glucosyltransferase was replaced with antiserum to whole OMZ 176 cells.

Comparison of agglutination inhibition activity in antisera to whole bacteria and glucosyltransferase. Antiserum to whole OMZ 176 bacteria was found to be active in both adherence inhibition and agglutination inhibition assays. Antiserum to glucosyltransferase of the same strain was active only in the adherence inhibition assay (Table 2). Glucosyltransferase antiserum was unable to inhibit synthesis of glucan because the bacteria agglutinated in the presence of antiserum.

Cross-reactions among S. mutans strains in the agglutination inhibition assay. Strains 6715, OMZ 176, and AHT are good test bacteria for the assay. Titers of antisera to these *S. mutans* strains are shown in Table 3. The strongest reaction occurred with homologous antiserum for both dextran- and sucrose-induced agglutination inhibition. Strains 6715 and OMZ 176 showed good cross-reactions to each other, and strain AHT showed weak cross-reactions to the other two strains.

TABLE 2. *Comparison of antisera activity in the adherence and agglutination inhibition assays*

Immunogen	Serum titers[a]		
	Adherence inhibition	Agglutination inhibition[b]	
		Dextran	Sucrose
OMZ 176 whole bacteria	48	256	2,048
OMZ 176 glucosyltransferase	65	0	0

[a] OMZ 176 as test strain.
[b] Dextran- and sucrose-induced agglutination read at 10 and 30 min, respectively.

TABLE 3. *Agglutination inhibition titers to homologous and heterologous S. mutans strains*

Strain antiserum	Agglutination inhibition titers			
	Agglutination inducer[a]	Assay strains		
		6715	OMZ 176	AHT
6715	Dextran	256	64	0
	Sucrose	1,024	256	0
OMZ 176	Dextran	64	256	[b]
	Sucrose	256	2,048	[b]
AHT	Dextran	16	16	16[c]
	Sucrose	16	16	16[c]

[a] Dextran- and sucrose-induced agglutination read at 10 and 30 min, respectively.
[b] Direct agglutination interfered with assay.
[c] Determined in prozone of direct agglutination caused by antiserum.

Removal of agglutination inhibition activity by adsorption to whole bacteria. Adsorption of OMZ 176 antiserum with whole OMZ 176 bacteria grown in the absence of sucrose resulted in the loss of more than 90% of the agglutination inhibition activity (Table 4). Both dextran- and sucrose-induced agglutination were inhibited.

Comparison of antiserum incubation with dextran and whole bacteria. Incubation of antiserum with dextran prior to addition to whole bacteria did not result in agglutination inhibition (Table 5). Bacteria in all tubes, regardless of antiserum dilution, agglutinated to the same extent at the same time. On the other hand, incubation of bacteria with antiserum prior to addition of dextran allowed interaction of antibody with bacterial antigens and resulted in agglutination inhibition.

DISCUSSION

The agglutination of *S. mutans* by the addition of sucrose was first observed by Guggenheim and Schroeder (9). Gibbons and Fitzgerald subsequently reported dextran-induced agglutination by high-molecular-weight exogenous dextran and suggested the existence of receptor sites on the bacterial surface which could bind dextran and thereby cause bacterial agglutination (7). This reaction is depicted schematically in Fig. 2, no. 1. In the case of sucrose-induced agglutination, glucan synthesis by glucosyltransferases would be necessary prior to binding and resultant agglutination, as in Fig. 2, no. 2.

This study described the ability of serum antibody to inhibit both sucrose- and dextran-induced agglutination. Antiserum to whole bac-

TABLE 4. *Effect of adsorption with whole bacterial cells on agglutination inhibition*

Strain antiserum	Agglutination inhibition titer[a]	
	Dextran[b]	Sucrose[b]
OMZ 176	256	2,048
OMZ 176 adsorbed with whole OMZ 176 cells grown in absence of sucrose	<64	128

[a] Strain OMZ 176 was the test organism.
[b] Dextran- and sucrose-agglutination read at 10 and 30 min, respectively.

TABLE 5. *Effect of incubation of antiserum with dextran on agglutination inhibition*

Antiserum incubation	Bacterial agglutination[a]							
	Normal rabbit serum	Antiserum dilution[b]						
	1:16	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
Dextran	+++	+++	+++	+++	+++	+++	+++	+++
Bacteria	+++	–	–	–	–	–	+	+++

[a] Dextran-induced agglutination read at 10 min; strain 6715.
[b] Antiserum to strain 6715.

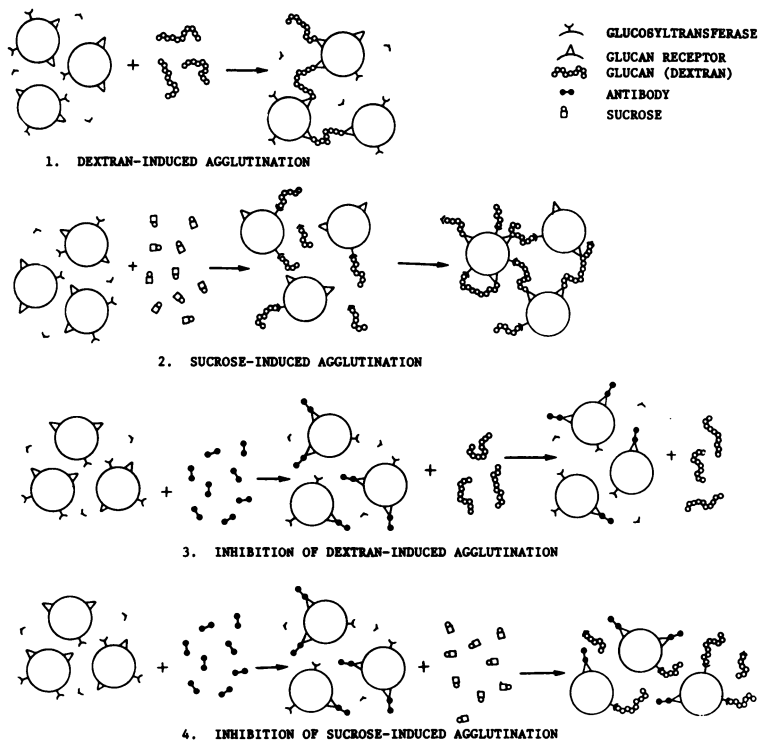

FIG. 2. *Model for dextran/sucrose-induced agglutination of S. mutans and its inhibition by antibody specific for a dextran binding site.*

teria of strain OMZ 176 was found to inhibit agglutination, but antiserum to partially purified cell-free glucosyltransferases of the same strain did not. Thus, it appears that the dextran binding site is a cell-associated molecule other than these glucosyltransferases. Antibody reacting with the binding site would inhibit dextran-induced (Fig. 2, no. 3) and sucrose-induced agglutination (Fig. 2, no. 4). In both cases the binding site is blocked, and it does not matter whether or not glucan synthesis by glucosyltransferase has occurred.

Mutants of *S. mutans* have been reported to lose their ability (i) to adhere to smooth surfaces and (ii) to agglutinate in sucrose or dextran, even though normal amounts of extracellular polysaccharide are present in culture fluid when grown in sucrose (3). The behavior of these mutants may be explained as the result of a genetic alteration in the dextran receptor site, so that despite normal glucan synthesis, glucan binding and subsequent bacterial agglutination and adherence cannot occur.

Other mutants have been reported to lose their ability to adhere to smooth surfaces but to retain their ability to agglutinate in exogenous

sucrose or glucan (M. L. Freedman and J. M. Tanzer, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 183, 1972). In this case, it would appear that the dextran receptor is functional but that some additional process is affected.

The finding (Table 2) that antiserum to cell-free glucosyltransferases were able to inhibit adherence to glass while being unable to inhibit sucrose-induced agglutination parallels the observations with these mutants. These observations are not explained by the simple model proposed in Fig. 2. It is possible that there is a difference in the structure or quantity of glucan required for adherence as opposed to agglutination. Adherence may require the interaction of several types of glucan synthesized by different populations of glucosyltransferases, whereas the product of any single glucosyltransferase may be sufficient to induce bacterial agglutination. The observation of antigenic and physicochemical differences in glucosyltransferases from single strains of *S. mutans* (8) is consistent with this hypothesis. The proposed model can be modified by showing additional types of polymer-producing enzymes on the cell surface, but at this time it is too speculative for inclusion.

What is the nature of the dextran-glucan binding site? Mukasa and Slade (12) have recently reported the existence of a polysaccharide antigen on the cell surface of *S. mutans* group a and d strains which is necessary for bacterial adherence to glass. Specific antiserum to this antigen inhibits bacterial adherence without inhibiting synthesis of insoluble polysaccharide. They suggest that this antigen may be a dextran receptor. At first glance, our own work rules out glucosyltransferase as the binding site, and, hence, is consistent with the Mukasa and Slade hypothesis. But because the antibody was produced by immunization with cell-free glucosyltransferases, it was possible that antibody was not reacting with cell-bound enzyme which was serving as a dextran receptor. Therefore, we can draw no conclusions from our own data relative to the nature of the dextran binding site.

The cross-reactions observed in the inhibition of dextran-induced agglutination indicate antigenic homogeneity of the dextran binding sites among several of the *S. mutans* strains. Cross-reactions were observed in strains 6715, OMZ 176, and AHT. These strains also cross-react in the adherence inhibition assay (G. A. Olson, A. S. Bleiweis, P. E. Mahan, and P. A. Small, Jr., Int. Ass. Dent. Res. Abstr. 1973, no. 868) and have been demonstrated to have immunologically identical glucosyltransferases (G. A. Olson and B. Guggenheim, unpublished data). The low agglutination inhibition cross-reactivity of strain AHT suggests antigenic differences from strains OMZ 176 and 6715 in its dextran binding site.

Antibody directed to dextran could complicate the interpretation of our data. Hence, it was important to show that the antisera were free of anti-dextran antibody. No precipitin lines were detected in the antisera by gel diffusion analysis when using commercial dextran or glucan extracts from *S. mutans* strain OMZ 176. Adsorption of antiserum with bacteria grown in the absence of sucrose removed most of the inhibitory activity. Finally, incubation of antiserum with the dextran preparation used to induce agglutination did not inhibit bacterial agglutination.

Agglutination inhibition is a good assay for monitoring the serum antibody response to those bacterial strains which agglutinate in the presence of dextran or sucrose. It is rapid and requires only small quantities of serum. Live bacteria refrigerated in buffer will retain their ability to agglutinate for over 6 months, allowing one to keep bacterial reagents in storage. Several investigations have shown that antibody will inhibit bacterial adherence to smooth surfaces (2, 4, 10, 13). Such inhibition could be the result of antibody interaction with either glucan-synthesizing enzymes or glucan-binding molecules, or both. It is necessary to define the specific target of inhibitory antibody in such studies. The dextran/sucrose-induced agglutination inhibition assay should prove useful in detecting inactivation of the dextran binding function.

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