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Characterization of an Anti-Glucosyltransferase Serum Specific for Insoluble Glucan Synthesis by *Streptococcus mutans*

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An anti-glucosyltransferase serum, which synthesized 96% insoluble glucans, was prepared against a purified enzyme preparation from *Streptococcus mutans* strain HS6 (serotype *a*). This serum was examined for its effects on glucan synthesis by crude enzyme preparations from eight strains (four serotypes) of *S. mutans* and for the ability of these preparations to promote adherence of *S. mutans* to a smooth surface. Glucosyltransferase activity was assayed by measuring the incorporation of glucose from [^{14}C]glucose-labeled sucrose into water-insoluble and water-soluble (ethanol-insoluble) glucans. Anti-glucosyltransferase serum inhibited insoluble glucan synthesis by crude enzyme preparations from cells of the four serotypes of *S. mutans*. Enzymes from strains of types *a*, *b*, and *d* were inhibited between 70 to 90%; enzymes from type *c* strains were inhibited from 45 to 60%. The adherence to a glass surface of heat-killed cells from these four serotypes was likewise inhibited. Soluble glucan synthesis was not inhibited by the serum, and in some cases its synthesis increased as insoluble glucan synthesis decreased.

Water-insoluble glucans produced by strains of *Streptococcus mutans* and *Streptococcus sanguis* appear to play a key role in the streptococcal colonization of smooth tooth surfaces (12, 16). Mutants of *S. mutans* that fail to synthesize insoluble glucans have been shown to lack plaque-forming ability on wire, glass, or tooth surfaces and to possess a greatly reduced caries potential (3, 7, 30).

Structurally, insoluble glucans contain high concentrations of α-1,3 linkages and 1,3 branch points, whereas water-soluble glucans are composed mainly of α-1,6 linkages (1, 5, 13, 26). During the purification of culture fluids from *S. mutans*, multiple glucosyltransferase fractions have been identified (2, 15, 24). Serological studies should contribute to the elucidation of structural relationships among the enzymes both within and between the various serotypes of this streptococcus and aid in examining their functions.

Initial studies with whole cell antisera and antiserum to crude enzyme preparations have reported inhibition of total or cell-associated glucan synthesis and inhibition of cell adherence to wire and glass surfaces (6, 10, 22, 27). Fukui et al. have prepared an antiserum to a highly purified α-1,6-glucan:D-fructose 2-glucosyltransferase and utilized it to examine cross-reactions among the enzymes synthesizing soluble glucans from the various serotypes of *S. mutans* (9). The present report describes an antiserum specific for the glucosyltransferases which synthesize insoluble glucans. The anti-glucosyltransferase serum (anti-GT) was examined with respect to (i) its effects on soluble versus insoluble glucan synthesis, (ii) its effects on the ability of glucosyltransferase preparations to promote cell adherence to a smooth surface, and (iii) its effect on enzyme preparations from strains representing four serotypes of *S. mutans*.

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MATERIALS AND METHODS

Streptococcal strains. *S. mutans* strains HS6, AHT, FA1, BHT, GS5, NCTC 10449, B13, and 6715 were received from sources previously described (19). The cells were grown to stationary phase in Todd-Hewitt broth (Difco Laboratories, De-

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troit, Mich.) supplemented with 1.8% glucose and salts (18).

Preparation of enzymes. Crude glucosyltransferase preparations were obtained from the culture supernatant by ammonium sulfate precipitation. The supernatants from overnight cultures (4 liters) were saturated 45% with ammonium sulfate and incubated at 5 C overnight. The precipitate was collected by centrifugation, dissolved in 100 ml of distilled water, and dialyzed for 24 h against 4 liters of 0.05 M phosphate buffer, pH 6.8. The buffer was changed at 18 h. The preparations were centrifuged, and the supernatant fraction was used as the enzyme source. The protein contents of the preparations were determined by the method of Lowry et al. (20). The preparations were stored frozen and diluted with buffer to 10 mg/ml of protein prior to use. A single glucosyltransferase preparation from each strain was used throughout the study.

Preparation of antisera. Anti-B13 and anti-HS6 whole cell sera were prepared in New Zealand white rabbits as previously described (19). These antisera have been characterized and shown to contain globulins against the serotype-specific cell wall polysaccharides (19, 21). These anti-B13 and anti-HS6 sera do not contain anti-teichoic acid or anti-dextran globulins.

Anti-GT was prepared in rabbits by the intravenous injection of a mixture containing equal volumes of enzyme preparation (0.5 mg/ml) and Freund incomplete adjuvant (Difco). The glucosyltransferase preparation used for these inoculations had been purified by H. Mukasa from *S. mutans* strain HS6 (type *a*) using Bio-Gel A-0.5 m and Bio-Gel HTP filtrations (24). The enzyme was purified 190-fold from the culture supernatant. It converted 3 mg of sucrose to glucan per h per mg of protein and synthesized 96% insoluble versus soluble glucans. Three injections of 0.5 ml were administered the first week, and six injections of 1 ml were given during weeks 2 and 3. The rabbit was given a 1-ml booster injection after 4 months and was exsanguinated the following week. Serum from the final bleeding was used throughout the study.

Normal rabbit serum was obtained from Grand Island Biological Co. (Grand Island, N.Y.)

Total proteins in the sera were determined by the method of Lowry et al. (20).

Assay for glucosyltransferase activity. The procedure for measuring glucosyltransferase activity was a modification of the assay by Robrish et al. (28) for total polysaccharide synthesis. Enzyme preparation (1 mg) was preincubated at 37 C for 30 min in the presence or absence of antiserum, 2 μl of 1% merthiolate, and 0.05 M phosphate buffer, pH 6.8, to a volume of 200 μl. After preincubation, 12.5 μmol of sucrose ([U-^{14}C]glucose) (New England Nuclear Corp., Boston, Mass.; final specific activity, 1 μCi/mmol) and buffer were added to a final volume of 0.5 ml. The reaction mixture was incubated at 37 C for 2 h (or as indicated). Assays measuring water-insoluble glucan synthesis were stopped by the addition of 1 ml of cold buffer and immediately filtered by vacuum through 2.4-cm glass-fiber disks (Reeve Angel 984H, ultrafine). The filter disks were washed twice with 1.5 ml of cold buffer. Assays measuring total glucan synthesis were stopped by the addition of 1 ml of 95% ethanol. The reaction tubes were mixed thoroughly on a Vortex mixer and allowed to stand at 5 C for 15 min prior to filtration. The filters were washed twice with 1.5 ml of cold 60% ethanol. The air-dried filters were placed in vials with 10 ml of scintillation fluid [5.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per liter of toluene] and counted. All experiments were performed in duplicate, and zero-time assays were used to determine radioactivity backgrounds. Soluble glucans were calculated as the difference between total and insoluble glucans.

Cell-associated glucosyltransferase activity was measured as follows. Cells were grown to late log phase in Todd-Hewitt broth (unsupplemented), harvested by centrifugation, and washed with 0.05 M phosphate buffer, pH 6.8. The freshly washed cells were resuspended to one-tenth of their original volume (optical density at 550 nm = 3.0) in buffer. The cell suspension (100 μl) was used as the enzyme source in the assay described above. Aliquots of cell-associated enzymes were incubated for 20 h.

Assay of adherence activity. The ability of the enzyme preparations to promote the adherence of heat-killed cells to a glass surface was measured as follows. Enzyme preparations (5 mg) and serum (1.5 mg) were preincubated with 0.05 M phosphate buffer, pH 6.8, to a volume of 1.5 ml. After 10 min at 37 C, 0.5 ml of a 0.25% cell suspension, 1.0 ml of 5% sucrose, and 2 μl of 10% merthiolate were added. The reaction tubes (12 by 75 mm) were incubated at a 30° angle for 16 h at 37 C. After incubation, the nonadhering cells were poured off and the tubes were rinsed twice; the adhering cells were resuspended, and their optical densities were measured at 550 nm, as previously described (22). The nonadhering suspensions from certain reactions were centrifuged to remove cells, and the supernatants were precipitated with 2 volumes of 95% ethanol (5 C, overnight). The ethanol-insoluble fractions were assayed for total hexoses (4) and fructose (29).

Materials. Dextranase (α-1,6-glucan 6-glucanohydrolase, EC 3.2.1.11) was obtained from Sigma Chemical Co., St. Louis, Mo. Dextran T10 was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J.

RESULTS

Effects of anti-GT on soluble versus insoluble glucan synthesis. Since the anti-GT had been prepared against an enzyme that synthesized 96% insoluble glucans, the effects of this antiserum on soluble versus insoluble glucan synthesis were studied. In Fig. 1, the effects of increasing concentrations of anti-GT on enzyme preparations from strains HS6 and B13 are shown. Considering total glucan synthesis, anti-GT appeared to be specific in inhibiting enzymes from strain HS6 (serotype *a*) and

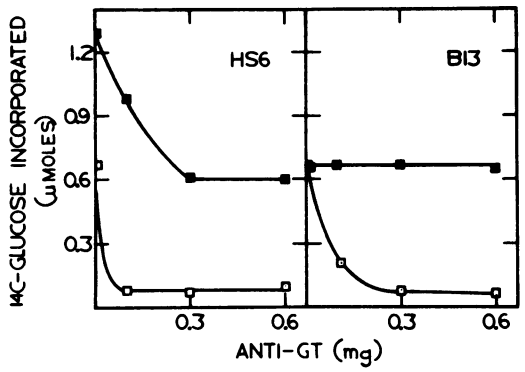

FIG. 1. *Effects of increasing anti-GT concentrations on glucan synthesis. Enzyme preparations from S. mutans strains HS6 and B13 were assayed for total (■) and water-insoluble (□) glucan synthesis. A 0.6-mg sample of anti-GT is equivalent to approximately 10 μl of serum.*

had no apparent effect on enzymes from strain B13 (serotype *d*). However, when insoluble glucans were measured, anti-GT was found to strongly inhibit synthesis by both strains. In the absence of antiserum, soluble glucans (i.e., the difference between total and insoluble glucans) represented about 50% of the glucans synthesized by the HS6 system, whereas no soluble glucans were recovered in the B13 system. As anti-GT was added to the HS6 enzyme preparation, soluble glucan synthesis remained essentially constant. In the B13 system, recovery of soluble glucans increased as synthesis of insoluble glucans decreased such that total glucan synthesis remained constant. From the data in Fig. 1, it is suggested that anti-GT is specific for enzymes that synthesize insoluble glucans.

The B13 system with its shift from insoluble to soluble glucan synthesis in the presence of anti-GT appeared a suitable system for further characterization work. Figure 2 reports glucan synthesis by the enzyme preparation from strain B13 during a 4-h incubation in the absence (panel A) and presence (panel B) of anti-GT. In Fig. 2A, 95% or more of the glucans synthesized by B13 enzymes are recovered in the water-insoluble fraction after 2 h of incubation. In the presence of anti-GT (Fig. 2B), insoluble glucan synthesis represented only 10% of the total synthesis and increased linearly at this rate over the 4-h period.

The nature of the products of the reactions in the presence and absence of antiserum was compared by treatment of these glucans with an α-1,6-dextranase. The synthesis was stopped after 4 h by incubating the tubes at 100 C for 2 min. After cooling, the reactions were titrated to pH 6, and 10 μg (ca. 2 units) of dextranase was added. Incubation in the presence of dextranase was continued for 2 h. After dextranase treatment, 54 to 60% of the insoluble glucans synthesized in the absence of anti-GT were recovered in the insoluble or total glucan fractions (Table 1). In contrast, only 10% of the glucans synthesized in the presence of antiserum were recovered, and one-half of these glucans could be attributed to the low concentration of insoluble polymer present in the reaction. Therefore, in the presence of anti-GT, 90% or more of the product was α-1,6-dextran.

To determine if the inhibition of insoluble glucan synthesis was a nonspecific serum effect, the enzyme preparation from strain B13 was assayed in the presence of several sera. The sera used were normal, commercially available rabbit serum and sera prepared against whole cells of *S. mutans* strains B13 and HS6. The anti-B13 and anti-HS6 sera contained antibodies to their respective serotype antigens, which have been identified as wall polysaccharides (19, 21). None of these sera inhibited glucan synthesis (Fig. 3). On the con-

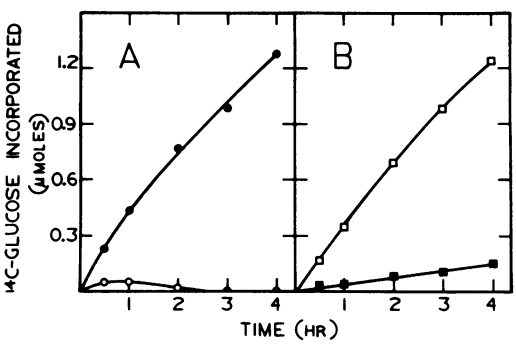

FIG. 2. *Effects of anti-GT on glucan synthesis as a function of time. Enzyme preparation from S. mutans strain B13 was assayed for water-soluble (○, □) and -insoluble (●, ■) glucan synthesis (A) in the absence and (B) in the presence of anti-GT (0.3 mg).*

TABLE 1. *Dextranase treatment of glucans produced by glucosyltransferases of S. mutans B13*

Addition	Glucan synthesis[a]			
	Control		Anti-GT	
	Insoluble	Total	Insoluble	Total
None	1.03	1.08	0.09	1.25
Dextranase	0.56	0.68	0	0.13

[a] Micromoles of [^{14}C]glucose incorporated.

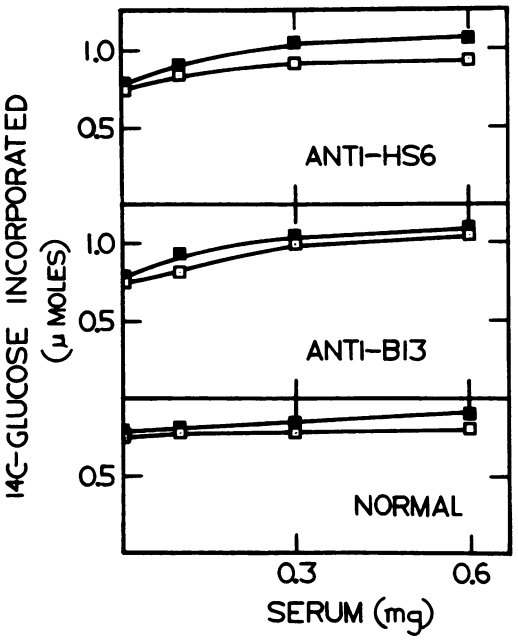

FIG. 3. *Effects of various sera on glucan synthesis. Enzyme preparation from S. mutans strain B13 was assayed for total (■) and water-insoluble (□) glucan synthesis in the presence of increasing concentrations of the indicated sera.*

trary, some stimulation of synthesis was found with the addition of serum.

Purified glucosyltransferases from *S. mutans* have been shown to contain about 30% carbohydrate (11, 24). The carbohydrate component of the HS6 enzyme used to prepare anti-GT was shown to be made up of glucose (24). The configuration of this polymer is not known. It is conceivable that it is a dextran and that part of the anti-GT activity could be directed against it. Therefore, the effects of primer dextran on the inhibitory activity of the antiserum and on the B13 enzyme system in general were studied. T10-dextran was used as a primer. Anti-GT was incubated with 200 μg of the primer dextran for 5 min at 25 C prior to the addition of the enzyme preparation and sucrose. Total glucan synthesis was stimulated 110% by the presence of primer (Fig. 4). Insoluble glucan synthesis was also stimulated by the presence of primer. However, anti-GT was as effective an inhibitor of insoluble glucan synthesis in the presence of primer as in its absence. Therefore, the action of the antiserum is not due to anti-dextran globulins.

Cell-associated glucosyltransferases from strains HS6 and B13 were assayed in the presence of anti-GT. Recovery of insoluble, cell-associated glucans was drastically decreased by the antiserum (Table 2). Binding of soluble glucans to dextran receptors on the cell may account for part of the cell-associated radioactivity, although incubating and washing the cells in the absence of a divalent metal ion may have minimized this effect (7). Recovery of soluble glucans increased in the B13 system when anti-GT was present. These results agree with the data in Fig. 1 for the soluble enzyme preparations. With HS6 enzymes, soluble glucan synthesis was inhibited by anti-GT in the cell-associated enzyme system, whereas this inhibition was not observed with the soluble enzymes.

Inhibition of cell adherence by anti-GT. The effects of anti-GT on the ability of the glucosyltransferase preparations to promote the adherence of *S. mutans* cells to a smooth surface were examined. The adherence assay was performed in the presence of normal serum or

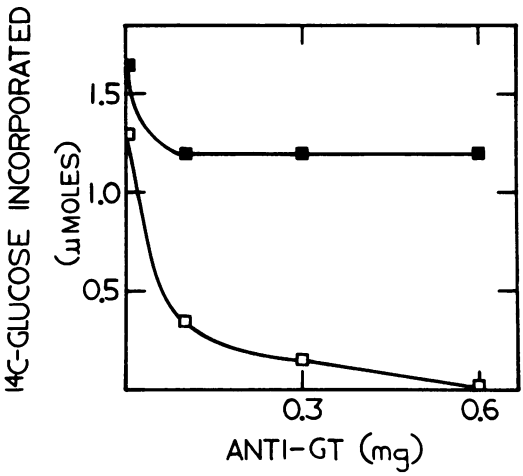

FIG. 4. *Effects of increasing anti-GT concentrations on glucan synthesis in the presence of dextran primer. Dextran T10 (200 mg) was incubated with anti-GT prior to adding B13 enzyme and assaying for total (■) and water-insoluble (□) glucan synthesis.*

TABLE 2. *Effect of anti-GT on activity of cell-associated glucosyltransferases*

Cells	Addition (1 mg each)	Glucan synthesis[a]	
		Soluble	Insoluble
HS6	NRS[b]	1.10	0.85
	Anti-GT	0.32	0.18
B13	NRS	0.54	3.37
	Anti-GT	2.55	0.47

[a] Micromoles of [^{14}C]glucose incorporated.
[b] NRS, Normal rabbit serum.

anti-GT as described in Materials and Methods. The HS6 enzyme preparation effected 45 to 60% cell adherence in the presence of normal rabbit serum, and the B13 enzyme produced 90% cell adherence under these conditions (Table 3). In the presence of an equal concentration of anti-GT, the adherence-promoting ability of both preparations was completely inhibited. The nonadhering suspensions from the reaction tubes with B13 cells and anti-GT serum were centrifuged, precipitated with ethanol, and assayed for hexoses and fructose. The tests showed that, in the presence of anti-GT, the HS6 enzyme synthesized 1.8 mg of soluble glucan per ml and the B13 enzyme synthesized 0.9 mg of soluble glucan per ml (no levan was detected). Therefore, in the presence of anti-GT, which inhibits insoluble glucan synthesis, cells failed to adhere to a glass surface even though high concentrations of soluble glucans were being synthesized.

Serotype specificity of the anti-GT. The effects of the anti-GT serum on glucosyltransferase preparations from various serotypes of *S. mutans* were examined. The eight strains studied represent four serotypes. In Table 4, the synthesis of soluble and insoluble glucans in the absence and presence of anti-GT and the percentage of inhibition of insoluble glucan synthesis are recorded. Anti-GT significantly inhibited insoluble glucan synthesis by the enzyme preparations from all eight strains. Enzymes from serotypes *a* and *d* were most strongly inhibited (90% in three of four strains); enzymes from serotype *b* strains were inhibited 72%; and enzymes from serotype *c* strains were inhibited 45 to 58%. The effect of inhibition of insoluble glucan synthesis on soluble glucans varied. In type *b* strains, soluble glucan synthesis remained essentially constant. Serotype *c* strains showed a 30 to 38% increase in soluble glucans. In serotype *d* strains, recovery of soluble glucans that were essentially absent in the controls increased rapidly to keep pace with the decrease in insoluble glucans.

The enzyme preparations were also examined for α-1,6-dextranase activity. However, this activity appeared essentially absent in most of the preparations, as measured by an assay of reducing power (25). The GS5 preparation demonstrated the greatest increase in reducing power in a 4-h assay (0.13 μmol/ml). Neither the GS5 dextranase activity nor that of a fungal dextranase (Sigma) was affected by the addition of anti-GT.

TABLE 3. *Inhibition of cell adherence by anti-GT*[a]

Cells	HS6 enzyme		B13 enzyme	
	Control	Anti-GT	Control	Anti-GT
HS6	0.207	0.014	0.321	0.017
BHT	0.179	0.005	0.345	0.018
10449	0.168	0.006	0.280	0.012
B13	0.160	0.012	0.332	0.013

[a] Values are in optical density units at 550 nm.

TABLE 4. *Inhibition of insoluble glucan synthesis by anti-GT*

Source of enzyme		Addition	Glucan synthesis[a]		Inhibition of insoluble glucans (%)
Serotype	Strain		Soluble	Insoluble	
a	HS6	Control	0.63	0.66	
		Anti-GT	0.54	0.07	90
	AHT	Control	0	0.54	
		Anti-GT	0.13	0.15	72
b	FA1	Control	0.90	0.60	
		Anti-GT	0.89	0.17	72
	BHT	Control	0.82	0.90	
		Anti-GT	0.79	0.25	73
c	GS5	Control	0.26	0.66	
		Anti-GT	0.37	0.28	58
	10449	Control	0.20	1.24	
		Anti-GT	0.32	0.68	45
d	B13	Control	0	0.67	
		Anti-GT	0.57	0.07	90
	6715	Control	0.06	0.49	
		Anti-GT	0.36	0.04	92

[a] Micromoles of [^{14}C]glucose incorporated.

DISCUSSION

Antiserum was prepared against a purified glucosyltransferase preparation from *S. mutans* strain HS6. The anti-GT serum was assayed for its effects on the glucosyltransferase that synthesizes water-soluble and -insoluble glucans. The antiserum was found to specifically inhibit insoluble glucan synthesis (Fig. 1 and 2). In studies with an enzyme preparation from *S. mutans* strain B13, inhibition of insoluble glucan synthesis resulted in increased recovery of soluble glucans. These soluble glucans were readily digested by an α-1,6-dextranase (Table 1). However, 54 to 60% of the insoluble glucans synthesized in the absence of antiserum were recovered after dextranase digestion (Table 1). These data agree with the reports of other investigators, namely, that the soluble glucans are α-1,6-linked polymers (1, 5, 13, 26). Since anti-GT inhibited only insoluble glucan synthesis, it would appear to be specific for 1,3-glucan:D-fructose 2-glucosyltransferase.

The absence of soluble glucans in the B13

system probably indicates that any α-1,6 polymers synthesized were rapidly incorporated into insoluble polymers by the enzymes that synthesized 1,3 linkages (see Fig. 2A). In the presence of anti-GT, the 1,3-glucan:D-fructose 2-glucosyltransferase was inhibited, and soluble α-1,6 polymers were recovered (Fig. 2B). In the presence of exogenous dextran primer (Fig. 4), some incorporated label was recovered as soluble glucan. This would indicate that the 1,3 enzymes were not able to utilize all of the available primer within the 2-h assay period.

Normal rabbit serum, and whole cell antiserum that contained antibodies to the polysaccharide serotype antigens of *S. mutans* strains HS6 and B13 (19, 21), did not inhibit glucan synthesis (Fig. 3). On the contrary, some stimulation of synthesis was found with the addition of serum. Similar nonspecific stimulation by serum and albumin of glucan synthesis has been previously reported (8, 23).

Primer dextran (T10) stimulated both soluble and insoluble glucan synthesis (Fig. 4). However, anti-GT was equally effective in inhibiting insoluble glucan synthesis in the presence and absence of primer. Therefore, its action is not due to anti-dextran globulins, which might react with primer present in the enzyme preparations, or dextran-like moieties of the glucosyltransferases (11, 24). Specificity to a nondextran, glucan moiety of the enzyme cannot be ruled out. The structure of the glucan portion of the glucosyltransferases remains undetermined. It might be proposed that anti-GT functions as an anti-dextranase, hence the increase in soluble glucans recovered from the B13 system in the presence of anti-GT. However, dextranase activity appeared negligible in these preparations (vide infra). Also, soluble glucan synthesis by enzyme preparations from strains HS6, FA1, and BHT remained constant in the presence of anti-GT, although insoluble glucan synthesis was inhibited 72 to 90% (see Table 4). Therefore, anti-GT is not considered to be an anti-dextranase.

Anti-GT appeared to inhibit cell-associated glucosyltransferase synthesis of insoluble glucans in a manner similar to its inhibition of soluble enzymes (Table 2).

In an in vitro assay, the ability of enzyme preparations from strains HS6 and B13 to promote cell adherence to a glass surface was examined in the presence and absence of anti-GT. Although the enzymes synthesized high concentrations of soluble glucans in the presence of antiserum, the anti-GT completely inhibited the cell adherence-promoting ability of both preparations (Table 3). Therefore, inhibition of insoluble glucan synthesis, rather than total glucan synthesis, is sufficient to inhibit artificial plaque formation.

Several whole cell antisera have been characterized with respect to inhibition of cell-associated or total glucan synthesis (6, 10, 27). These preparations have shown marked serotype specificity with significant cross-reaction between serotypes *a* and *d*. The limited cross-reactions have not been explained but may be related to the presence of additional anti-polysaccharide and anti-dextran globulins in the whole cell antisera. The anti-GT serum used in this study was prepared against a highly purified glucosyltransferase, which synthesized 96% insoluble glucans (21). In a study of eight strains of *S. mutans* representing four serotypes (Table 4), enzymes from serotypes *a* and *d* were inhibited 90% by anti-GT (three of four strains); enzymes from serotype *b* were inhibited 72%; and enzymes from serotype *c* were inhibited 45 to 58%. This significant inhibition of insoluble glucan synthesis by anti-GT represents substantially less serotype specificity than that demonstrated by previous preparations.

Correlations between insoluble glucan synthesis, plaque formation, and dental caries have been confirmed in many studies (3, 7, 12, 16, 30). However, most studies examining antibody inhibition of glucan synthesis by whole cell antisera have not distinguished between the enzymes that synthesize soluble and insoluble glucans (6, 27). The present study has shown that an antiserum specific for the enzyme from a serotype *a* strain, which synthesizes insoluble glucan, inhibits the synthesis of insoluble glucan by *b*, *c*, and *d* serotypes as well as the *a* serotype of *S. mutans*. The enzyme also completely inhibited the adherence of heat-killed cells of these serotypes to a glass surface. The present results thus illustrate a correlation between insoluble glucan synthesis and the adherence of *S. mutans*. These results also indicate that purified enzyme preparations may be useful in caries prevention. Results with crude enzyme preparations as immunizing agents in animals have not been conclusive (14, 17).

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