INFECTION AND IMMUNITY, Feb. 1973, p. 237-241

Vol. 7, No. 2
Printed in U.S.A.

Inhibition of Glucosyltransferase Activity by Antisera to Known Serotypes of *Streptococcus mutans*

R. T. EVANS AND R. J. GENCO

Department of Oral Biology, School of Dentistry, State University of New York at Buffalo, Buffalo, New York 14226

Received for publication 16 August 1972

Using a recently developed assay for glucosyltransferase activity based on ^{14}C-glucose incorporation into an alcohol-insoluble polysaccharide, we were able to study inhibition by antibody of this enzyme activity. Rabbit antibody was relatively specific for the strain of *Streptoccus mutans* from which the enzyme was obtained. Absorption studies showed that neither removal of antibodies directed to dextran nor absorption with intact bacteria offset the enzyme-inhibitory capacity of these sera, whereas absorption with partially purified enzyme did result in removal of the inhibitory capacity.

An adhesive cell-associated polysaccharide is one of the important determinants of virulence of cariogenic streptococci. This polysaccharide in *Streptococcus mutans* is mainly composed of a mixture of dextrans exhibiting α 1-6 linkage between the constituent glucose units with α 1-3 and 1-4 branches (11). The various dextrans are formed by enzymes classified as glucosyltransferases (8, 11, 16). These enzymes, also termed dextransucrases, function by producing the dextrans from sucrose with release of fructose (3, 5). Restriction in the formation of the adhesive dextran results in a diminution of the cariogenic potential of these organisms (9). A mutant of *S. mutans* lacking the ability to produce cell-associated dextran was found to have lost its cariogenic potential (4), further indicating the importance of this polysaccharide in adhesion to smooth surfaces.

Studies from this laboratory (7) have shown that rabbit antisera directed to *S. mutans* inhibit the formation of in vitro plaque by these organisms. In addition, it was found in our studies that *S. mutans* cells grown in homologous antiserum had much less cell-associated dextran than cells grown in normal rabbit serum. Our results have recently been confirmed (14). These studies (7, 14) suggest that one mechanism by which antibodies may interfere with colonization of smooth surface by *S. mutans* is by inhibiting the enzymatic activity of glucosyltransferases responsible for production of adhesive polysaccharides. Strains of *S. mutans* have been found by Bratthall (1) to belong to one of five serological groups. If antibodies directed to *S. mutans* glucosyltransferases are important as determinants of plaque formation, it is important to know the antigenic relationship among the glucosyltransferases of each of the five serotypes of *S. mutans*. Previously, Carlsson and Krasse (2) showed that homologous rabbit antisera would inhibit the activity of partially purified glucosyltransferases from one serotype of *S. sanguis*. The experiments reported here were undertaken to study the inhibition by antibody of glucosyltransferases obtained from cariogenic *S. mutans* of the various known serotypes. The aims of this study were (i) to adopt an assay for glucosyltransferase activity which would be useful in studying the effects of antibody on the enzyme and (ii) to study the immunological specificity of glucosyltransferase enzymes obtained from strains representing the various known serotypes of *S. mutans*.

MATERIALS AND METHODS

Enzyme sources and extraction. Cultures for enzyme production were grown in Trypticase soy broth (BBL) for 48 hr at 37 C in 9-liter volumes. Strains of *S. mutans* used as enzyme sources were 6715, E-49, FA-1, GS-5, and LM-7. These belong to Bratthall's groups d, a, b, and c, and to Lancefields's group E, respectively (1). The original cultures were kindly provided by Robert Fitzgerald, Veterans Administration Hospital, Miami, Fla., and Ronald Gibbons, Forsyth Dental Center, Boston, Mass. The cultures were maintained in Eugonagar deeps (BBL) at -70 C and were tested periodically for their ability to form plaque on nichrome wire by use of the procedures of McCabe et al. (13). Only cultures that

were able to form in vitro plaque were used as a source of glucosyltransferases. The glucosyltransferases were partially purified from the culture fluids by the method of Wood (15). Briefly, the method consists of precipitation of culture supernatant fluids with 60% ammonium sulfate followed by centrifugation. The precipitate was dissolved in distilled water and extensively dialyzed to remove salt. The dialysate was lyophilized and stored. Under these conditions the glucosyltransferases were found to remain active for up to 1 year.

Enzyme assay. Glucosyltransferase activity was measured by determining ^{14}C-glucose incorporation into an ethanol-insoluble polysaccharide. This procedure allows a rapid and precise quantitation of the activity present. A typical reaction mixture determined by previous experiments to give easily measured activity contained 375 μg of enzyme, 50 μg of primer dextran, (average molecular weight, 20,000; Pharmacia Fine Chemicals, Inc.), and 0.01 μg of uniformly labeled ^{14}C-sucrose (specific activity, 346 mCi/mmole; New England Nuclear Corp.) in a reaction volume of 200 μliters. The buffer was 0.01 M sodium citrate-sodium phosphate, pH 5.0. Incubation temperature was 30 C, and reaction times were varied. The reaction was stopped by precipitation with the addition of ethanol to a final concentration of 70% (v/v). To ensure complete precipitation of the labeled product, 24 mg of unlabeled dextran (molecular weight, 20,000) was added as carrier. The precipitate was centrifuged at 9,000 $\times$ g, redissolved in distilled water, and reprecipitated with ethanol again at a final concentration 70%. The precipitate was dissolved in 0.25 ml of distilled water, mixed with the scintillation fluid described by Garvey (6), containing 4% Cab-O-Sil (Packard Instrument Co., Inc.), and counted in a liquid scintillation counter (Packard Instrument Co., Inc.). Under these conditions of sample preparation and counting, no quenching of added known amounts of ^{14}C was detected.

Critical assessment of the assay was made to determine its specificity in detecting dextran synthesis. Preparations of sucrose labeled with ^{14}C in the glucosyl moiety and with ^{3}H in the fructosyl moiety (New England Nuclear Corp.) were used in the assay under conditions similar to those described for the uniformly labeled sucrose. Concentrations for the substrates were 0.018 μg for the ^{14}C-glucose-labeled sucrose (specific activity, 243 mCi/mmole) and 0.0084 μg for the ^{3}H-fructose-labeled sucrose (specific activity, 5.21 Ci/mmole).

Immunization procedures. Antisera to *S. mutans* were prepared in rabbits by intravenous injection of 10^{8} saline-washed cells per ml based on McFarland turbidity standards. The immunizations were repeated for a total of six injections over a 2-week period. The rabbits were bled 1 week after the final injection, and the sera were separated and stored at −20 C. Booster immunizations were given with a single injection of the same dose, and the animals were bled 1 week later.

Inhibition assay. Inhibition of enzyme activity was measured by incubating antisera at the desired dilution with the enzyme at 30 C for 30 min prior to the addition of the dextran primer and radioactive substrate. The procedure described above was then followed to determine residual enzyme activity. Under these conditions of sample preparation and counting, no detectable quenching of added known amounts of isotope occurred.

Absorption of antisera. Absorption of the antisera was carried out by adding heat-killed cells (1 ml of packed cells per ml of serum), G-25 Sephadex, an insoluble form of dextran (1 ml of hydrated Sephadex G-25 slurry per ml of serum; Pharmacia Fine Chemicals, Inc.), or various amounts of glucosyltransferase. The glucosyltransferase added (amounts given in Table 2) did not give visible reactions with the antisera. The mixtures were incubated at 37 C for 1 hr with continuous gentle mixing, followed by 1 to 2 hr at 4 C. The cells and G-25 Sephadex were removed by centrifugation. The activity of glucosyltransferase added to the sera as absorbing antigen was subtracted when necessary. Gel diffusion analyses of the sera were done in 1.2% agarose made in 0.07 M sodium barbital buffer, pH 8.6.

RESULTS

Rate studies with the radiolabeled assay. Figure 1 depicts a typical curve seen when uniformly labeled sucrose was used as substrate. Polysaccharide formation by enzymes from *S. mutans* strain 6715 in the absence of antibody was followed as a function of time at 30 C. This reaction plateaus at 4 to 6 hr and again at 14 to 18 hr. Figure 2 gives the results of an experiment in which the incorporation of glucose from ^{14}C-glucose-labeled sucrose and of fructose from ^{3}H-fructose-labeled sucrose into ethanol-insoluble product was measured. It can be seen that there was no detectable ^{3}H-fructose incorporation from 0 to 5 hr of incubation under conditions of the assay. From 5 to 18 hr, there was an increasing amount of ^{3}H-fructose incorporation. On the other hand, ^{14}C-glucose was incorporated throughout the period studied and appeared to reach plateau levels at 12 to 14 hr. From this experiment, it is clear that under the conditions of this assay only glucosyltransferase enzymes were assayed during the first 5 hr of incubation. From 5 to 18 hr, levansucrase activity as well as glucosyltransferase activity was detected.

Inhibition of the radiolabeled assay with immune sera. The effects of rabbit antiserum to *S. mutans* strain 6715 on the incorporation of radioactive sucrose moieties into ethanol-insoluble products by homologous enzyme is shown in Table 1. After 2 hr of incubation, a substantial amount of the uniformly labeled sucrose and the ^{14}C-glucose-labeled sucrose was incorporated into product. Essentially no differences were seen in the results between the two glucose labeled isotopes. Nonimmune rabbit sera showed little or no effect. None of the tritiated fructose moiety was incorporated into product during this time period.

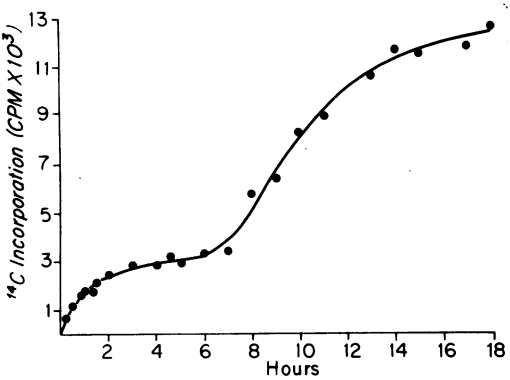

FIG. 1. *Incorporation of radioactivity from uniformly labeled sucrose into ethanol-insoluble product by supernatant enzymes from S. mutans strain 6715. The reaction mixture contained 375 μg of enzyme, 50 μg of dextran (molecular weight, 20,000), and 0.01 M sodium citrate-sodium phosphate buffered at pH 5.0 in a reaction volume of 200 μliters. Incubation was carried out at 30 C for various times.*

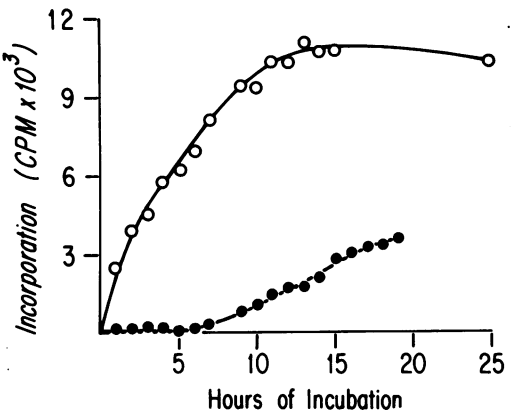

FIG. 2. *Incorporation of radioactivity from ^{14}C-glucose-labeled sucrose and ^{3}H-fructose-labeled sucrose into ethanol-insoluble products by supernatant enzymes from S. mutans strain 6715. The conditions of assay were identical to those used in Fig. 1, except that either 0.018 μg of ^{14}C-glucose-labeled sucrose (○) or 0.0084 μg of ^{3}H-fructose-labeled sucrose (●) was substituted for the uniformity-labeled sucrose.*

This experiment demonstrates that the use of uniformly labeled sucrose or ^{14}C-glucose-labeled sucrose at 2 hr of incubation provides a good assay for glucosyltransferase activity. In addition, this experiment shows that marked inhibition of the activity of glucosyltransferases occurs in homologous rabbit antisera obtained by extensive immunization with washed bacteria.

Figure 3 shows the results of an experiment in which the glucosyltransferases of *S. mutans* strains 6715, E-49, GS-5, and FA-1 were tested for inhibition by homologous rabbit antiserum.

TABLE 1. *Antiserum inhibition of glucosyltransferase obtained from S. mutans strain 6715 with various labeled sucrose substrates*[a]

Serum	Uniformly labeled sucrose		^{14}C-glucose-labeled sucrose	
	Counts/min[b]	Inhibition (%)[c]	Counts/min	Inhibition (%)
No serum (control)	1,998 ± 95	0	1,255 ± 111	0
Immune serum[d]				
1:10	551 ± 17	72	330 ± 12	74
1:20	631 ± 16	68	503 ± 34	60
1:100	1,450 ± 84	27	959 ± 59	24
1:200	1,598 ± 131	20	946 ± 69	25
Normal serum				
1:10	1,762 ± 30	12	1,174 ± 48	6
1:20	1,882 ± 65	6	1,222 ± 40	3
1:100	1,870 ± 38	6	1,300 ± 28	0
1:200	1,935 ± 72	3	1,325 ± 34	0

[a] In addition to the two labeled sucrose substrates shown in the table, ^{3}H-fructose-labeled sucrose was also tested, but none was incorporated into product during the 2-hr period of the test.

[b] Counts were corrected for radioactivity precipitated at zero incubation time. Averages were obtained from triplicate samples and standard deviations are given. Assay was carried out for 2 hr at 30 C.

[c] Percent inhibition calculated as 100% activity of control minus activity in presence of immune or normal serum.

[d] Rabbit antiserum directed to *S. mutans* strain 6715 whole-cell vaccine.

Rabbit antisera directed to strains 6715, E-49, and GS-5 showed marked inhibition of homologous glucosyltransferase activity. These three antisera appeared to reach characteristic maximal inhibition ranging from 50 to 95%, which may reflect the antibody content of each serum. Antiserum directed to strain FA-1 showed detectable but low levels of inhibitory activity against the homologous glucosyltransferases. Using absorbed antisera specific for strain FA-1, we obtained titers of 1:80 by indirect fluorescent-antibody procedures, indicating reactivity of the antisera to antigens on the cell surface. Similar titers were obtained with antisera specific for the other serotypes.

Table 2 shows that the glucosyltransferase-inhibitory capacity of rabbit antiserum to strain 6715 can be diminished by prior absorption of the antiserum with small quantities of the homologous enzyme. Absorption of the antiserum with washed homologous cells of *S. mutans* did not result in reduction of inhibitory capacity. This antiserum was also found to react with dextran by gel diffusion experiments. Absorption of the antidextran antibodies with an excess of Sephadex G-25 failed to reduce the glucosyltransferase inhibitory ac-

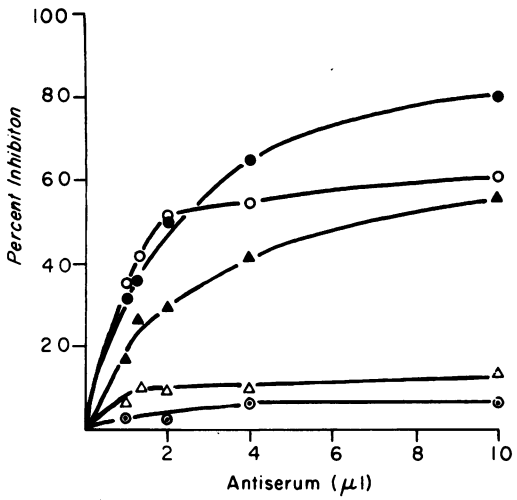

FIG. 3. *Inhibition of S. mutans glucosyltransferase activity by homologous rabbit antisera.* (●) *S. mutans strain 6715 (enzyme and antisera to strain 6715),* (○) *strain E-49,* (▲) *strain GS-5,* (△) *strain FA-1,* (⊙) *nonimmune rabbit serum. Appropriate dilutions of each rabbit antiserum were incubated with its homologous enzyme for 30 min at 30 C prior to addition of uniformly labeled sucrose substrate and dextran primer.*

tivity of the antiserum, although the reaction of this antiserum with dextran in gel was eliminated.

Glucosyltransferase inhibition by heterologous antisera was tested in an experiment shown in Table 3. As expected, antisera against homologous (strain 6715) glucosyltransferase showed marked inhibition of enzyme activity. However, antisera directed to strains LM-7, GS-5, and FA-1 showed low levels of inhibition of strain 6715 enzyme, suggesting limited antigenic cross-reactivity among glucosyltransferases of the serotypes of *S. mutans.*

DISCUSSION

Inhibition of glucosyltransferase activity by antisera obtained from animals injected with whole cells appears to be relatively specific for the enzyme obtained from the immunizing organism. This finding suggests that, although soluble glucosyltransferases from different strains have similar catalytic activities, they differ antigenically.

A surprising finding was the inability of homologous whole cells to absorb inhibiting antibody from the immune serum. It has been reported that these are cell-associated glucosyltransferases (9, 11) which might compose a portion of the cell surface. However, since the cells did not remove significant amounts of antibody, several possibilities may be considered. The cell-bound glucosyltransferase enzymes may be masked by the dextran capsule or removed during the washing procedure, or the supernatant enzymes used in this study may be antigenically distinct from cell-associated enzymes. A possibility also exists that the procedure used to heat-kill the absorbing cells denatured the enzyme and thus altered its antigenic character. This possibility is considered to be unlikely because of the minimal heat treatment used. Further studies are currently planned to determine the specificity and localization of supernatant and cell-associated enzymes.

It should be noted that numerous attempts in our laboratories to inhibit the activity of partially purified cell-associated glucosyltransferases with antisera have shown enhance-

TABLE 2. *Glucosyltransferase inhibition by rabbit antiserum to S. mutans strain 6715 after absorption with homologous glucosyltransferases*[a]

Absorbing enzyme added[b]	Enzyme inhibition at 2 hr (%)[c]
0	81 ± 2
18.75	74 ± 3
37.5	68 ± 3
75	62 ± 3
150	58 ± 2
300	55 ± 4[d]

[a] This serum was absorbed with Sephadex G-25, with no change in enzyme-inhibiting capacity, prior to absorption with enzyme.

[b] Micrograms per 100 μliters of serum.

[c] Average of duplicate assays ± standard deviation.

[d] This value was corrected by 8% to account for activity of absorbing enzyme added. Lower concentrations of absorbing enzyme showed no added activity.

TABLE 3. *Inhibition of S. mutans strain 6715 glucosyltransferase activity by homologous and heterologous antisera*

Serum[a]	Enzyme inhibition (%)[b]
None	0
Rabbit anti-6715	95 ± 2
Rabbit anti-LM-7	16 ± 4
Rabbit anti-GS-5	11 ± 3
Rabbit anti-FA-1	22 ± 6
Nonimmunized rabbit serum	6 ± 4

[a] Antisera were at 1:10 final dilution in the reaction mixture.

[b] The enzyme was assayed with uniformly labeled sucrose as substrate. The reaction was assayed after 2 hr of incubation. Percent inhibition was calculated on duplicate samples and the standard deviation is given.

ment, no effect, or inhibition. It may be that the cell-associated glucosyltransferases are particulate and that the effect of antibody on these particulate enzymes is not simple but depends on factors such as the size of the particles and the spectrum of antibodies present in the serum.

It is premature to consider immunological control of plaque-forming organisms in humans. However, it is necessary to examine closely the parameters such as the antigens which might be involved in such control. It would appear from this study that the glucosyltransferases from *S. mutans* are antigens whose inhibition might be a controlling factor in antibody-mediated inhibition of plaque. It should be noted that antibodies directed to other antigens on *S. mutans* may also function in preventing the adherence of these microorganisms to smooth surfaces of the teeth and to one another in dental plaque.

Several investigators have examined the possible effect of antibodies on plaque formation in vivo. Hayashi et al. (12) demonstrated that rats were less susceptible to caries when challenged with cariogenic bacteria in the diet after immunization intraperitoneally with dextransucrase fractions obtained in a fashion similar to those used in the present study. Guggenheim (10), also using a partially purified enzyme preparation as well as bacterial cells given rats intravenously, could not show a difference in plaque formation or caries incidence. Others (Gaffer et al., Int. Ass. Dent. Res. Abstr. no. 304, 1970; Tanzer et al. Int. Ass. Dent. Res. Abstr. no. 466, 1970), using bacterial cells as immunogens, have had variable results. It would appear to be important to study further the route of immunization, the form of the antigen administered, and the type of response evoked.

It is clear that antigenic analysis of the various glucosyltransferase enzymes is important for an understanding of the enzymes as well as in determining whether naturally occurring enzyme-inhibiting antibodies in the human play a role in smooth surface caries resistance. These studies are made difficult by the inability to obtain cell-associated glucosyltransferases in soluble form and by the difficulty of demonstrating glucosyltransferase enzymes in gel-diffusion experiments. Hence, it is necessary to study the antigenicity of these enzymes indirectly through the antibody-mediated inhibition of enzyme activity.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants DE-03000, DE-167, and DE-02814 from the National Institute of Dental Research.

We express our appreciation to Judy Castine for excellent technical assistance.

LITERATURE CITED

1. Bratthall, D. 1970. Demonstration of serologic groups of streptococcal strains resembling *Streptococcus mutans*. Odont. Revy **21**:143-152.
2. Carlsson, J., and B. Krasse. 1968. Inhibition of streptococcal dextransucrase by sera of rabbits infected with *Streptococcus sanguis*. Arch. Oral Biol. **13**:849-852.
3. Carlsson, J., E. Newburn, and B. Krasse. 1969. Purification and properties of dextransucrase from *Streptococcus sanguis*. Arch. Oral Biol **14**:469-478.
4. deStoppelaar, J. D., K. G. Konig, A. J. M. Plasschaert, and J. S. van der Hoeven. 1971. Decreased cariogenicity of a mutant of *Streptococcus mutans*. Arch. Oral Biol. **16**:971-975.
5. Fitzgerald, R. J., and H. V. Jordon. 1968. Polysaccharide producing bacteria and caries, p. 79-86. *In* R. S. Harris (ed.), Art and science of dental caries research. Academic Press Inc., New York.
6. Garvey, J. S. 1968. Radioisotopes and their applications, p. 216. *In* C. A. Williams and M. W. Chase (ed.), *In* Methods in immunology and immunochemistry, vol. 2. Academic Press Inc., New York.
7. Genco, R. J., and R. T. Evans. 1969. Discussion, p. 253-254. *In* D. Dayton, Jr., P. A. Small, Jr., R. M. Chanock, H. E. Kaufman, T. B. Tomasi, Jr. (ed.), The Secretory Immunologic System, Proceedings of Conference. U.S. Government Printing Office, Washington, D.C.
8. Gibbons, R. J., R. J. Banghart. 1967. Synthesis of extracellular dextran by cariogenic bacteria and its presence in human dental plaque. Arch. Oral Biol. **12**:11-24.
9. Gibbons, R. J., and M. Nygaard. 1968. Synthesis of insoluble dextran and its significance in the formation of gelatinous deposits by plaque-forming streptococci. Arch. Oral Biol. **13**:1249-1263.
10. Guggenheim, B., H. R. Muhlemann, B. Regolati, and R. Schmid. 1970. The effect of immunization against streptococci or glucosyltransferases on plaque formation and dental caries in rats, p. 287-296. *In* W. D. McHugh (ed.), Dental plaque. E & S Livingston Ltd. Edinburgh.
11. Guggenheim, B., and H. E. Schroeder. 1967. Biochemical and morphological aspects of extracellular polysaccharides produced by cariogenic streptococci. Helv. Odont. Acta **11**:131-152.
12. Hayashi, J. A., I. L. Shklair, and A. N. Bahn. 1972. Immunization with dextransucrase and glycosidic hydrolases. J. Dent. Res. Suppl. **51**:436-442.
13. McCabe, R. M., P. H. Keyes, and A. Howell. 1967. An *in vitro* method for assessing the plaque forming ability of oral bacteria. Arch. Oral Biol. **12**:1653-1656.
14. Olson, G. A., A. S. Bleiweis, and P. A. Small, Jr. 1972. Adherence inhibition of *Streptococcus mutans*: an assay reflecting a possible role of antibody in dental caries prophylaxis. Infect. Immunity **5**:419-427.
15. Wood, J. M. 1967. A dextransucrase activity from Streptococcus FA-1. Arch. Oral Biol. **12**:1659-1660.
16. Wood, J. M., and P. Critchley. 1966. The extracellular polysaccharide produced from sucrose by a cariogenic streptococcus. Arch. Oral Biol. **11**:1039-1042.