Effects of Dextranases on Attachment of *Streptococcus mutans* to Hydroxyapatite

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A *Fusarium* dextranase and a *Penicillium* dextranase were compared for their relative ability to quantitatively reduce the adsorption of ^3H -labeled *Streptococcus mutans* cells onto hydroxyapatite. *Fusarium* dextranase-treated hydroxyapatite disks caused a statistically significant decrease in the hydroxyapatite adsorption of both the OMZ 176 and NCTC 10449 strains of *S. mutans* relative to untreated control disks. The extent of initial bacterial adsorption was not promoted by sucrose-dependent glucan synthesis. Since the *Fusarium* dextranase has a much greater affinity for hydroxyapatite than the *Penicillium* dextranase, it could represent an enzyme with improved decay-preventive therapeutic properties. This was concluded because the *Fusarium* dextranase may interfere with both the initial attachment and later glucan-dependent accumulation of dental plaque microorganisms.

Dextranase (EC 3.2.1.11) has usually been successful in preventing dental caries in animal experiments (1, 2, 8, 12, 13, 23). Conflicting results, however, have been reported when the enzyme was used in human clinical trials (3, 7, 16, 19, 20). This lack of success in humans may have been due to the relatively short period that the enzyme was retained orally. We have found that a dextranase isolated from *Fusarium moniliforme* (*Fusarium* dextranase [FD]) (27) has a much greater affinity for hydroxyapatite (HA), the principal mineral component of human teeth, than a *Penicillium* dextranase (PD). A commercial PD (Worthington Biochemicals Corp.) had no detectable binding to HA at pH 6.8 in 0.01 M phosphate buffer, whereas the FD adhered so strongly that 0.2 M phosphate buffer was required for its elution. Therefore, the FD could bind to HA as enamel pellicle does and could be slowly released or interfere directly with bacterial attachment, or both.

Recently, the FD was also observed to reduce dental caries in hamsters to a greater extent than PD (L. G. Simonson, D. Jackola, B. L. Lamberts, and E. P. Leonard, Pharmacol. Ther. Dent., in press). In the present study we compared the efficacy of the two dextranases to prevent adherence of *Streptococcus mutans* onto HA disks in vitro. Inhibition of initial bacterial adherence to tooth surfaces and subsequent aggregation processes could eventually be a method for controlling dental caries.

MATERIALS AND METHODS

The adsorption of ^3H -labeled *S. mutans* NCTC 10449 (serotype c) and OMZ 176 (serotype d) cells to

disks of HA was measured by a modification of the procedure of Clark and Gibbons (6). Briefly, ^3H -labeled cells were prepared by growing the organisms in Todd-Hewitt broth (Difco) with 0.5% glucose and 2.5 μCi of [*methyl*- ^3H]thymidine per ml (New England Nuclear Corp.). Cells from overnight cultures were harvested by centrifugation, washed twice with 0.85% NaCl, and suspended in phosphate-buffered saline (PBS) (pH 7.2).

Both dextranases were prepared to be identical in specific activity (800 U/ml and 0.25 mg of protein per ml). The FD was prepared from *F. moniliforme* ATCC 32426 as described previously (27), except that it was concentrated by ultrafiltration (Millipore Corp.) in the 10^3 to 10^6 molecular weight range. The PD was purchased as a lyophilized powder (Worthington Biochemicals Corp.). Both dextranases were subjected to gel chromatography on a Bio-Gel P-150 column as described previously (27). The active fractions were pooled and dialyzed against 0.01 M potassium phosphate buffer (pH 6.8). HA disks, 1.3 cm in diameter, were prepared by compressing 150 mg of HA powder (Bio-Rad Labs) for 15 min at 8,400 pounds (591 kg/cm²) of pressure in a KBr dye (Perkin-Elmer) under vacuum. The disks were slowly hydrated by storing in a desiccator jar over water. The incubation buffer consisted of 0.01 M potassium phosphate with 0.08% sodium azide (pH 6.8), with or without sucrose. The disks were soaked in the incubation buffer without sucrose for at least 10 min prior to experimental pretreatment. The disks were pretreated with either PBS, FD, or PD for 10 min. After treatment, the disks were washed in the phosphate buffer for 10 min and then flooded with 12 ml of incubation buffer, with or without sucrose. A 2-ml sample of the washed, PBS-suspended cells was then added, and the mixture was incubated at 37°C for 24 h with gentle shaking (100 rpm). The disks were then rinsed twice in deionized water, 10 min each, and once in methanol. The disks were air-dried and placed in 15 ml of liquid scintillation

cocktail (Filter-Solv; Beckman). The position of the upper surfaces was maintained throughout the experiments, and the triplicate samples were counted with a scintillation counter (LS-133; Beckman). Total colony-forming units per milliliter of bacterial suspension was found by performing serial dilutions and plating on mitis salivarius agar (Difco). The colony-forming units per milliliter were related to the radioactivity in 50- μ l volumes of the cell suspensions by collecting the cells on 0.45- μ m membrane filters (Millipore) and counting as described above. The total colony-forming units per disintegrations-per-minute count were then calculated.

Additional experiments were performed to reduce the possibility that either the 0.08% sodium azide present in the incubation fluid or the washing of the cells before incubation adversely affected glucan synthesis in this system. The effects of the sodium azide and of cell washing were determined, respectively, on the cell-free supernatant and the whole cells. The two *S. mutans* cultures were incubated for 18 h in a chemically defined medium (22). The cells were harvested by centrifugation, and the supernatant was filtered with a 0.45- μ m membrane filter (Gelman). One half of the cells were immediately resuspended in PBS, and the other half were washed twice in 0.85% NaCl and then suspended in PBS. Either 5 ml of cell-free supernatant or 2.5 ml of suspended cells was incubated with 5 ml of a [14 C]sucrose buffer for 6 h at 37°C. The sucrose buffer consisted of 2% sucrose in 0.01 M potassium phosphate (pH 7.0), with 0.16 μ Ci of [*glucose-U- 14 C*]sucrose per ml, with or without 0.08% sodium azide. Washed and unwashed cells were compared for cell-associated 14 C-glucan synthesis by first precipitating the glucans with 2 volumes of methanol. The precipitate was collected on a 0.45- μ m membrane filter (Millipore) and washed with 50% methanol. The filters with their samples were dried thoroughly, placed in scintillation fluid, and counted as described above. The effect of sodium azide on cell-free glucan synthesis was determined from the amounts of 14 C-glucan, which was precipitated by methanol, collected, washed, and counted as described for the cells.

We also determined FD activity while the enzyme was adsorbed on HA disks. The disks were treated with an FD solution as above, rinsed with deionized water, and assayed for activity. The dextran substrate

was suspended in 0.02 M sodium acetate buffer (pH 5.5) to prevent elution of the FD from the disk during the assay procedure. The disks were assayed directly by placing them in aliquots of the buffered substrate. Other HA disks were eluted with 0.2 M potassium phosphate buffer after FD treatment. These disks were also assayed for any residual adsorbed dextranase activity. The 0.2 M phosphate eluates were dialyzed against deionized water and assayed for dextranase activity.

RESULTS

The two strains of *S. mutans* were found to adsorb differently to the HA disks (Table 1) under the conditions of this investigation. This is shown by the total colony-forming units per square centimeter of the PBS controls, which for strain NCTC 10449 was approximately threefold greater than for strain OMZ 176. The presence or absence of sucrose in the incubation mixture had no significant effect on the quantitative adsorption of the bacteria during the 24-h incubation period. The FD-treated disks were found to have significantly less ($P < 0.05$, Student's *t* test) bacteria adsorbed than the PBS-treated control disks. However, the presence or absence of sucrose, a required substrate for glucan synthesis, was again not a factor during the 24-h incubation. The FD was noted to cause a greater decrease in the percentage of OMZ 176 adsorbed relative to NCTC 10449. The PD also caused a reduction in bacterial adsorption, but it was not a significant decrease ($P > 0.05$) relative to the PBS controls.

Both strains were found to have glucan-synthesizing activity (Table 2). The effect of sodium azide and cell washing on glucosyl transferase activity of the two *S. mutans* strains was also studied. This was done to decrease the possibility that these factors could be responsible for the lack of a sucrose influence on bacterial adsorption. The glucan synthesis by cell-free supernatants of both strains was not adversely

TABLE 1. Effect of pretreating HA disks with dextranases on adsorption of *S. mutans*

HA disk pretreatment	<i>S. mutans</i> NCTC 10449		<i>S. mutans</i> OMZ 176	
	Total CFU $\pm$ SE ^a per cm ² ($\times 10^6$)	Adsorption (% of control)	Total CFU $\pm$ SE per cm ² ($\times 10^6$)	Adsorption (% of control)
Sucrose absent:				
PBS	11.84 $\pm$ 2.05	100.0	3.76 $\pm$ 0.34	100.0
FD	5.28 $\pm$ 0.25	44.6 ^b	0.64 $\pm$ 0.31	16.7 ^b
PD	10.64 $\pm$ 0.95	89.8	2.07 $\pm$ 0.26	55.2
Sucrose present:				
PBS	11.45 $\pm$ 0.99	100.0	3.76 $\pm$ 0.17	100.0
FD	5.18 $\pm$ 0.62	43.8 ^b	0.48 $\pm$ 0.06	12.6 ^b
PD	8.31 $\pm$ 0.97	72.6	3.24 $\pm$ 0.40	86.4

^a CFU $\pm$ SE, Colony-forming units $\pm$ standard error of the mean.

^b Significantly lower than PBS control, $P < 0.05$.

TABLE 2. Effect of sodium azide and cell washing on glucan synthesis

Sample	<i>S. mutans</i> NCTC 10449 (CPM $\pm$ SE) ^a	<i>S. mutans</i> OMZ 176 (CPM $\pm$ SE)
Cell-free supernatant		
Control	16,398 $\pm$ 82	23,262 $\pm$ 70
0.08% NaN ₃	18,960 $\pm$ 95	25,103 $\pm$ 75
Cells		
Control (unwashed)	5,365 $\pm$ 38	2,831 $\pm$ 28
Washed	5,614 $\pm$ 39	3,328 $\pm$ 33

^a CPM $\pm$ SE, Counts per minute $\pm$ standard error of the mean.

affected by 0.08% sodium azide, since the counts per minute of ¹⁴C-glucan incorporation were observed to be slightly higher in its presence. The cell-associated glucan synthesis (measured as the amount of labeled glucan formed) of both strains was found to be nearly equivalent, regardless of the repeated washings. The OMZ 176 strain even showed a slight but nonsignificant increase in glucan synthesis as a result of cell washing. A difference in the ratio of cell-associated to cell-free glucan synthesis was observed for the two strains of *S. mutans*. Since the radioactive sucrose employed was labeled only in the glucosyl moiety, the methanol precipitation of labeled levans was eliminated.

The FD enzyme adsorbed on HA disks remained active in this state. The FD enzymatic activity adsorbed onto HA disks was found to be 56% of the total dextranase activity released from the disks by the 0.2 M phosphate elution buffer. Nearly 100% of the adsorbed activity was eluted from the disks by the elution procedure.

DISCUSSION

Studies using both in vitro and in vivo models have indicated that dextranases can interfere with artificial and natural plaque accumulation (1, 2, 8, 9, 11-13, 15-17, 19, 20, 23, 25, 26, 28). The therapeutic importance of interfering with the adsorption of oral bacteria has been previously reviewed (24). However, the use of a PD mouthwash in a human clinical trial was found to be ineffective in reducing dental plaque (3). This lack of clinical success may have been due to the physical properties of the enzyme and/or the method of delivery used. In the present investigation, an FD with a high affinity for HA was found to interfere with the adsorption of *S. mutans* onto HA disks. The PD-treated and washed disks were not capable of causing a statistically significant reduction in bacterial adsorption. These effects were nearly identical both in the presence and in the absence of sucrose, which could indicate that the physical affinity of the protein for HA, and not its enzy-

matic activity, was responsible. The increased reduction of bacterial adsorption by FD over PD directly correlates with a similar increased reduction in dental caries scores using hamsters (Simonson et al., Pharmacol. Ther. Dent., in press).

Other studies (5, 6, 10, 14, 17, 18, 21, 29) have also shown that glucan formation is not a necessary prerequisite for attachment. Furthermore, Van Houte et al. (30) have shown that all the serogroups of *S. mutans* are capable of colonizing the teeth of rats in the complete absence of sucrose, and that the minimum infective dose of bacteria required for implantation was similar for rats on diets containing either glucose or sucrose. The sucrose-dependent glucan formation process has been suggested as being important after initial attachment (6, 29, 30) by creating increased bacterial adhesiveness and glucan-associated aggregation and accumulation. Our results suggest that the presence or absence of cell-associated glucans does not influence the total number of cells initially adhering, although the strength of adherence could have been affected. The possibility that the glucan synthetic system was somehow impaired by the conditions of the study was greatly reduced by our findings that 0.08% sodium azide and also cell washing did not adversely affect glucan synthesis.

Our finding that more cells of the serogroup c strain of *S. mutans* consistently adsorbed to HA than cells of the serogroup d strain has been similarly observed by others (4, 29). This differential adsorption between the strains may explain the differential effect of the FD on their adsorption. Since *S. sanguis* has been shown to adsorb to HA and salivary pellicle components to a greater extent than *S. mutans* (4, 18), the FD could also have a direct effect on clinical mixed-culture plaque formation. We suggest that FD might provide a unique additive effect over other dextranases. Our findings of a pronounced increase in activity after the release of the FD from the HA disks by a phosphate buffer suggested that the binding of the dextranase interfered with its access to the substrate. We envision a novel approach to caries prevention involving a dextranase with a high affinity for HA, which could replace the salivary pellicle by exposing tooth surfaces to the enzyme immediately following mechanical pellicle removal. This preparation could then be released over a long time period while interfering directly with the number of available binding sites. It could act as a bifunctional agent both by causing direct interference with organisms that adsorb to a salivary pellicle and by enzymatically interfering with glucan synthesis associated with plaque accumulation.

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LITERATURE CITED

- Block, P. L., C. L. Dooley, and E. E. Howe. 1969. The retardation of spontaneous periodontal disease and the prevention of caries in hamsters with dextranase. *Periodontics* 40:41-46.
- Bowen, W. H. 1971. The effect of dextranase on caries activity in monkeys (*Macaca irus*). *Br. Dent. J.* 131: 445-449.
- Caldwell, R. C., H. J. Sandham, W. V. Mann, S. B. Finn, and A. J. Formicola. 1971. The effect of a dextranase mouthwash on dental plaque in young adults and children. *J. Am. Dent. Assoc.* 82:124-131.
- Clark, W. B., L. L. Bammann, and R. J. Gibbons. 1978. Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. *Infect. Immun.* 19:846-853.
- Clark, W. B., L. L. Bammann, and R. J. Gibbons. 1978. Ability of *Streptococcus mutans* and a glucosyltransferase-defective mutant to colonize rodents and attach to hydroxyapatite surfaces. *Infect. Immun.* 21: 681-684.
- Clark, W. B., and R. J. Gibbons. 1977. Influence of salivary components and extracellular polysaccharide synthesis from sucrose on the attachment of *Streptococcus mutans* 6715 to hydroxyapatite surfaces. *Infect. Immun.* 18:514-523.
- Duany, L. F., D. D. Zinner, J. C. Mena, and J. M. Gonzalez. 1975. The effect of dextranase on oral health in children. *J. Prev. Dent.* 2:23-27.
- Fitzgerald, R. J., P. H. Keyes, T. H. Stoudt, and D. M. Spinell. 1968. The effects of a dextranase preparation on plaque and caries in hamsters, a preliminary report. *J. Am. Dent. Assoc.* 76:301-304.
- Fitzgerald, R. J., D. M. Spinell, and T. H. Stoudt. 1968. Enzymatic removal of artificial plaques. *Arch. Oral Biol.* 13:125-128.
- Gibbons, R. J., E. C. Moreno, and D. M. Spinell. 1976. Model delineating the effects of salivary pellicle on the adsorption of *Streptococcus mitior* onto hydroxyapatite. *Infect. Immun.* 14:1109-1112.
- Hamada, S., T. Ooshima, N. Masuda, J. Mizuno, and S. Sobue. 1976. Inhibition of rat dental caries by dextranase from a strain of *Spicaria violacea*. *Jpn. J. Microbiol.* 20:321-330.
- Hamada, S., T. Ooshima, N. Masuda, and S. Sobue. 1976. Effect of dextranase prepared from *Spicaria violacea* on dental caries in hamsters. *J. Dent. Res.* 55: 552.
- Hillman, J. D., J. van Houte, and R. J. Gibbons. 1970. Sorption of bacteria to human enamel powder. *Arch. Oral Biol.* 15:899-903.
- Hoffman, S., H. D. Tow, and J. S. Cole. 1973. Scanning electron microscope studies of dextranase-treated plaque streptococci. *J. Dent. Res.* 52:551-557.
- Keyes, P. H., M. A. Hicks, B. M. Goldman, R. M. McCabe, and R. J. Fitzgerald. 1971. Dispersion of dextranous bacterial plaque of human teeth with dextranase. *J. Am. Dent. Assoc.* 82:136-141.
- Liljemark, W. F., and S. V. Schauer. 1975. Studies on the bacterial components which bind *Streptococcus sanguis* and *Streptococcus mutans* to hydroxyapatite. *Arch. Oral Biol.* 20:609-615.
- Liljemark, W. F., and S. V. Schauer. 1977. Competitive binding among oral streptococci to hydroxyapatite. *J. Dent. Res.* 56:157-165.
- Lobene, R. R. 1971. A clinical study of the effect of dextranase on human dental plaque. *J. Am. Dent. Assoc.* 82:132-135.
- Murayama, Y., H. Wada, H. Hayashi, T. Uchida, I. Yokomizo, and S. Hamada. 1973. Effects of dextranase from *Spicaria violacea* (IFO 6120) on the polysaccharides produced by oral streptococci and on human dental plaque. *J. Dent. Res.* 52:658-667.
- Orstavik, D., F. W. Kraus, and L. C. Henshaw. 1974. In vitro attachment of streptococci to the tooth surface. *Infect. Immun.* 9:794-800.
- Osborne, R. M., B. L. Lamberts, and A. H. Roush. 1975. Invertase in cell-free culture fluids of *Streptococcus mutans* strain SL-1. *Experientia* 31:1399-1401.
- Poulsen, S., R. H. Larson, and R. S. Senning. 1978. Effect of dextranase on plaque formation and caries development in the rat. *Scand. J. Dent. Res.* 86:231-236.
- Rolla, G. 1976. Inhibition of adsorption—general considerations, p. 309-324. In H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), *Proceedings, Microbial Aspects of Dental Caries*, Spec. Suppl. Microbiol. Abstr., vol. 2. Information Retrieval, Inc., Washington, D.C.
- Schachtele, C. F., S. K. Harlander, D. F. Fuller, P. K. Zollinger, and W.-L. S. Leung. 1976. Bacterial interference with sucrose-dependent adhesion of oral streptococci, p. 401-412. In H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), *Proceedings, Microbial Aspects of Dental Caries*, Spec. Suppl. Microbiol. Abstr., vol. 2. Information Retrieval, Inc., Washington, D.C.
- Schachtele, C. F., R. H. Staat, and S. K. Harlander. 1975. Dextranases from oral bacteria: inhibition of water-insoluble glucan production and adherence to smooth surfaces by *Streptococcus mutans*. *Infect. Immun.* 12:309-317.
- Simonson, L. G., A. E. Liberta, and A. Richardson. 1975. Characterization of an extracellular dextranase from *Fusarium moniliforme*. *Appl. Microbiol.* 30:855-861.
- Slade, H. D. 1976. In vitro models for the study of adherence of oral streptococci, p. 21-38. In W. Bowen, R. Genco, and T. O'Brien (ed.), *Immunological aspects of dental caries*, vol. 1. Information Retrieval, Inc., Washington, D.C.
- Tinanoff, N., J. M. Tanzer, and M. L. Freedman. 1978. In vitro colonization of *Streptococcus mutans* on enamel. *Infect. Immun.* 21:1010-1019.
- Van Houte, J., R. C. Burgess, and H. Onose. 1976. Oral implantation of human strains of *Streptococcus mutans* in rats fed sucrose or glucose diets. *Arch. Oral Biol.* 21:561-564.