JOURNAL OF BACTERIOLOGY, May 1969, p. 341-346
Vol. 98, No. 2

Dextran-induced Agglutination of *Streptococcus mutans*, and Its Potential Role in the Formation of Microbial Dental Plaques

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Received for publication 13 January 1969

Glucose-grown washed cells of streptococci similar to *Streptococcus mutans*, which contain cell-bound dextransucrase, have been observed to agglutinate upon the addition of high molecular weight dextran. Low molecular weight dextran or unrelated polysaccharides were ineffective. Agglutination also occurred upon addition of sucrose, which can be converted into dextran, but not with other mono- and disaccharides. Other bacteria, including species capable of synthesizing dextrans, were not observed to exhibit this phenomenon. Cells of *S. mutans* agglutinated upon addition of dextran over a wide *p*H range, but maximal sensitivity to dextran occurred at *p*H 8.5. At this *p*H, such cells can be used for a simple, specific, and exquisitely sensitive qualitative assay for high molecular weight dextran, for addition of 6 ng of dextran with a molecular weight of 2×10^6 (i.e., approximately three molecules per cell) caused detectable agglutination. High concentrations of glucose, levan, and dextran of molecular weight of 2×10^4 inhibited the reaction. Fluorescein-labeled cells of *S. mutans* were observed to adhere to dextran-containing plaques and dextran-treated teeth, suggesting that this phenomenon may be of importance in the formation of streptococcal dental plaques. The mechanism responsible for dextran-induced agglutination appears to involve the affinity of a receptor site, possibly dextransucrase, on the surface of several cells for common dextran molecules.

Streptococci similar to *Streptococcus mutans* (2, 6) have been associated with microbial dental plaques and multisurface carious lesions in both rodents and humans (18). These organisms synthesize extracellular gummy dextrans from sucrose, and it has been postulated that such polymers are responsible for the ability of these streptococci to form gelatinous plaques on the surfaces of teeth (7, 11). The enzyme system responsible for the synthesis of dextran (dextransucrase) is formed constitutively by *S. mutans*, and is present in a cell-associated and a soluble form (15, 16). Guggenheim and Schroeder (16) found that washed *S. mutans* cells obtained from glucose broth cultures aggregated and clumped together within a few seconds after the addition of sucrose, owing to the rapid synthesis of insoluble dextran which coated the organisms. The present report describes the agglutination of *S. mutans* by high molecular weight dextrans, and indicates the potential of this phenomenon in the formation of gelatinous dental plaque.

MATERIALS AND METHODS

Cultures and cultural conditions. *Streptococcus* strains resembling *S. mutans* have been previously characterized, and represent isolates from humans (10, 14, 22), hamsters (9), and rats (8). These isolates were known to form gelatinous plaques in vitro and to induce dental caries in experimental animals. *S. mutans* strain GS5-MR was obtained from W. C Bowen. This strain was a streptomycin-resistant mutant [parent strain GS5 (14)] which was reisolated from a caries lesion in an infected monkey. *Streptococcus* strains 2M2 and 4M4 were hamster isolates which have not been observed to induce plaque formation or dental caries in experimental animals (9). *Streptococcus* strain SS2 is a levan-forming organism known to possess plaque-forming and cariogenic potential (13). Other organisms studied were obtained from the culture collection of the Laboratory of Microbiology, National Institute of Dental Research. All strains were maintained by weekly transfer in National Institutes of Health thioglycolate broth. For experimental purposes, the organisms were cultivated in a medium of the following composition: Trypticase (BBL), 2%; NaCl, 0.2%; KH_2PO_4, 0.4%;

Na_2HPO_4, 0.2%; K_2CO_3, 0.1%; $MgSO_4$, 0.012%; $MnSO_4$, 0.0015%; and glucose, 0.2%. The glucose was autoclaved separately and added aseptically to complete the medium. All cultures were incubated anaerobically at 37 C in Brewer jars filled with 95% nitrogen and 5% carbon dioxide.

Source of materials. Dextrans of known average molecular weight were obtained from Pharmacia A. B., Uppsala, Sweden. Levan was prepared from *Streptococcus* strain SS2 as previously described (13). Samples of dextrans were prepared from *Leuconostoc* strains ATCC 10830 and ATCC 14935 according to the methods of Jeanes et al. (17). Preparations from strain 14935 have been reported to contain 66% α-1,6, 10% α-1,4, and 24% α-1,3 bonds; those from strain 10830 contain 95% α-1,6 and 5% α-1,4 bonds (17). The preparations used contained over 95% total carbohydrate when analyzed with anthrone reagent. Samples of dextranase were generously provided by T. H. Stoudt of Merck Sharp and Dohme Research Laboratories, Rahway, N.J.

Agglutination testing. Cell suspensions for agglutination testing were prepared from 18-hr cultures grown in 0.2% glucose broth. The organisms were harvested by centrifugation, washed twice with saline, and suspended in buffer to give a cell density of approximately 2×10^9 organisms per ml, as determined by microscopic count. The buffers used were 0.1 M acetate for *p*H 4.5 to 5.5, 0.067 M phosphate for *p*H 6 to 7.5, and 0.1 M glycyl-glycine for *p*H 8 to 10.0. Tris(hydroxymethyl)aminomethane buffer was found to be inhibitory. Reaction mixtures containing 0.3 ml of the cell suspension and 0.2 ml of additives and buffer were mixed and incubated in a water bath at 35 C. The suspensions were examined for agglutination after 2 hr., and in some cases 18 hr, and were scored as 0 (no agglutination) to 4+ (marked agglutination). Control cell suspensions incubated without additives were always included.

Adherence of cells to artificial plaque and dextran-treated teeth. Washed-cell suspensions were labeled with fluorescein by the methods generally outlined by Cherry et al. (3). Twice-washed, glucose-grown cells were suspended in chilled saline to give a cell density of approximately 2×10^{10} organisms per ml. One-tenth volume of 0.5 M carbonate buffer (*p*H 9.0) and 20 μg of fluorescein isothiocyanate (BBL) per ml were added with stirring to the cell suspension. The suspension was stirred overnight at 4 C, and the organisms were then washed with cold saline by centrifugation to remove unconjugated fluorescein. Washing was continued until the supernatant liquor no longer fluoresced in ultraviolet light. The labeled organisms were then diluted 10-fold and suspended in phosphate buffer (*p*H 7.0). They were either used immediately or lyophilized.

In vitro plaques were formed on wires by use of *S. mutans* strains GS5 and 6715 according to the methods described by McCabe et al. (19). The resulting plaques were washed free from residual sucrose by immersion in phosphate buffer (*p*H 7.0), and then were placed into tubes containing fluorescein-labeled cell suspensions. These were incubated at 35 C for 20 to 40 min, and then the plaques were removed, washed several times by immersion in phosphate buffer, and examined macroscopically for a coating of fluorescent cells under ultraviolet light.

The adherence of *S. mutans* to dextran-treated teeth was determined with both pumiced and unpumiced extracted human teeth. A surface of intact enamel was walled off with wax, and 1 μliter containing either 10 or 1 μg of dextran obtained from *Leuconostoc* ATCC 14935 was applied to the surface. The teeth were air-dried and incubated in buffer at *p*H 7.0 with fluorescein-labeled cells for 30 to 60 min. They were then washed by immersion in several changes of phosphate buffer (*p*H 7.0), air-dried, and examined for a coating of labeled organisms by use of a dissecting microscope illuminated by ultraviolet light (15 times magnification).

RESULTS

Dextran-induced agglutination of S. mutans. Washed-cell suspensions prepared from cultures of *S. mutans* grown for several transfers in glucose broth can be uniformly dispersed. When soluble dextran with a molecular weight of 2×10^6 was added to such cell suspensions in phosphate buffer (*p*H 7.0), marked agglutination occurred with several strains (Table 1). With many strains, agglutination occurred within seconds after the addition of dextran, whereas other strains of *S. mutans* agglutinated after minutes or hours. Dextran-induced agglutination was not observed with other bacteria tested (Table 1), including dextran-forming strains of *S. bovis*, *S. sanguis*, or *L. mesenteroides*.

S. mutans strain 6715 was selected for further study. Dextran-induced agglutination occurred over the range from *p*H 5 to 10.0 (Table 2). Spontaneous agglutination occurred at acidities greater than *p*H 5, and no agglutination was observed above *p*H 10.0. The reaction was remarkably sensitive to dextran between *p*H 8.0 and 9.0, and agglutination was detected at *p*H 8.5 upon the addition of only 6 ng of dextran with a molecular weight of 2×10^6. This corresponds to approximately three molecules of dextran per streptococcus cell in the reaction mixture. Lyophilized cells of *S. mutans* strain 6715 responded to dextran equally as well as freshly prepared cells, but no agglutination was observed if the organisms were heated at 100 C for 10 min.

Specificity of agglutination. The ability of high molecular weight dextran to induce agglutination of *S. mutans* strain 6715 appears to be specific, for other polysaccharides, including starch and levan, were ineffective (Table 3). Dextrans of high molecular weight were more effective than dextrans of low molecular weight at both *p*H 7.0 and 8.5. The organisms responded with equal sensitivity to highly branched dextran

TABLE 1. *Induction of agglutination by high molecular weight dextran*

Organism	Agglutination[a]	
	2 hr	18 hr
Streptococcus mutans 6715	4+	4+
S. mutans K1-R	±	4+
S. mutans AHT	3+	4+
S. mutans BHT	±	2+
S. mutans 6927	3+	4+
S. mutans HS6	1+	4+
S. mutans SL	±	3+
S. mutans LM7	±	2+
S. mutans GS5	−	±
S. mutans GS5-MR	3+	4+
S. mutans E49	4+	4+
S. mutans OMZ-61	4+	4+
S. sanguis 10558	−	−
S. bovis ATCC 9809	−	−
Leuconostoc mesenteroides	−	−
S. faecium	−	−
S. faecalis	−	−
Streptococcus 2M2	−	−
Streptococcus 4M4	−	−
Streptococcus SS2	−	−
Serratia marcescens	−	−
Escherichia coli	−	−
Lactobacillus casei	−	−

[a] After addition of 100 μg of dextran with a molecular weight of 2×10^6 to buffered (*p*H 7.0) cell suspensions.

TABLE 2. *Effect of pH on dextran-induced agglutination of S. mutans strain 6715*

Dextran (mol wt 2×10^6) added/ml	*p*H[a]									
	5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
None	−	−	−	−	−	−	−	−	−	−
100 μg	3+	3+	3+	3+	4+	4+	4+	4+	3+	2+
20 μg	2+	3+	3+	3+	4+	4+	4+	4+	3+	1+
4 μg	2+	2+	2+	3+	4+	4+	4+	4+	3+	1+
0.8 μg	1+	1+	1+	2+	3+	4+	4+	4+	3+	−
160 ng	−	−	−	1+	2+	3+	3+	3+	2+	−
30 ng	−	−	−	−	1+	2+	3+	2+	±	−
6 ng	−	−	−	−	−	±	1+	−	−	−
1.2 ng	−	−	−	−	−	−	±	−	−	−
0.24 ng	−	−	−	−	−	−	−	−	−	−

[a] Agglutination determined after 2 hr of incubation.

from *Leuconostoc* ATCC 14935 and to linear dextran from *Leuconostoc* ATCC 10830. Agglutination of strain 6715 also occurred after the addition of sucrose but not with glucose or fructose, as has been reported by Guggenheim and Shroeder (16). However, the agglutination reaction was approximately 2×10^4 times more sensitive to high molecular weight dextran (molecular weight of 2×10^6) than to sucrose on a weight basis, and approximately 6×10^7 times more sensitive on a molar basis. The addition of dextranase to cells agglutinated by dextran with a molecular weight of 2×10^6 redispersed the organisms.

TABLE 3. *Agglutination of S. mutans strain 6715 upon addition of various carbohydrates*

Additive	Concn (μg/ml)	Agglutination after 2 hr	
		*p*H 7.0	*p*H 8.5
None	—	−	−
Dextran, mol wt 2×10^6	100	4+	4+
Dextran, mol wt 2×10^5	100	2+	3+
Dextran, mol wt 2×10^4	100	−	±
Levan	100	−	−
Starch	100	−	−
Dextrin	100	−	−
Inulin	100	−	−
Agar	100	−	−
Agarose	100	−	−
Sucrose	1,000	4+	3+
Sucrose	100	±	−
Glucose	1,000	−	−
Fructose	1,000	−	−
Maltose	1,000	−	−
α-Methyl glucoside	1,000	−	−

Inhibition of agglutination. Several carbohydrates were tested for their ability to inhibit agglutination of *S. mutans* strain 6715 induced by addition of 1 μg of dextran with a molecular weight of 2×10^6. These were incubated with the cell suspensions at 35 C for 30 min prior to the addition of dextran. It was found that high concentrations of levan, glucose, and dextran with a molecular weight of 2×10^4 inhibited agglutination, whereas the other carbohydrates tested were without effect (Table 4). Though fructose at a concentration of 60 mg/ml did not affect agglutination induced by dextran addition, this sugar was found to inhibit agglutination induced by addition of 400 μg of sucrose per ml. Thus, fructose appears to interfere with the enzymatic synthesis of dextran but not with the binding site for dextran.

Adherence of S. mutans to plaques and dextran-treated teeth. The apparent affinity of cells of *S. mutans* for dextran could be an important factor in the buildup of gelatinous dental plaque. To determine whether *S. mutans* strain 6715 could adhere to dextran in an insoluble form, spheres of Sephadex G-25 (Pharmacia) were incubated with washed cells of the organism for

TABLE 4. *Ability of carbohydrates to inhibit agglutination of S. mutans strain 6715 by dextran with a molecular weight of 2 × 10⁶*

Inhibitor added	Concn (mg/ml)	Agglutination after 2 hr[a]	
		pH 7.0	pH 8.5
None	—	3+	4+
Levan	10	±	2+
Levan	1	1+	2+
Levan	0.1	2+	3+
Dextran, mol wt 2 × 10⁴	10	−	−
Dextran, mol wt 2 × 10⁴	1	1+	1+
Dextran, mol wt 2 × 10⁴	0.1	1+	1+
Glucose	60	±	ND[b]
Glucose	6	2+	ND
Glucose	1	3+	ND
Maltose	60	3+	ND
Fructose	60	3+	ND
α-Methyl glucoside	2	3+	ND
Starch	2	3+	ND

[a] All reaction mixtures contained 1 μg of dextran per ml.
[b] Not determined.

30 min and then examined by light microscopy. Many cells of strain 6715 were observed to adhere to the dextran spheres, but few, if any, cells of *Lactobacillus casei* or *Streptococcus* strain 4M4 were observed to adhere to Sephadex spheres when tested similarly. Analogous results were obtained with dextran-containing plaques formed in vitro on wires by cultures of *S. mutans* strain GS5 or 6715. Plaques of these organisms fluoresced intensely when they were incubated with fluorescein-labeled suspensions of active *S. mutans* strain 6715, but not when incubated with heat inactivated cells or with labeled suspensions of *L. casei* or *Streptococcus* strain 4M4 (Fig. 1A).

When teeth were treated with either 1 or 10 μg of high molecular weight dextran and then incubated with fluorescein-labeled cells of *S. mutans* strain 6715, patches of fluorescing cells were observed to adhere to the tooth surface (Fig. 1B). Comparable patches of fluorescent cells were not observed on untreated teeth. No significant adherence was observed when dextran-treated teeth were incubated with labeled cells of *L. casei* or with *Streptococcus* strain 4M4. On teeth which possessed cracks or other defects in the enamel surface, fluorescence was observed in the area of the defect with all labeled strains studied. This was evidently nonspecific in nature, and it occurred on untreated as well as dextran-treated teeth. Pumiced teeth were observed to act comparably to unpumiced teeth for all studies reported.

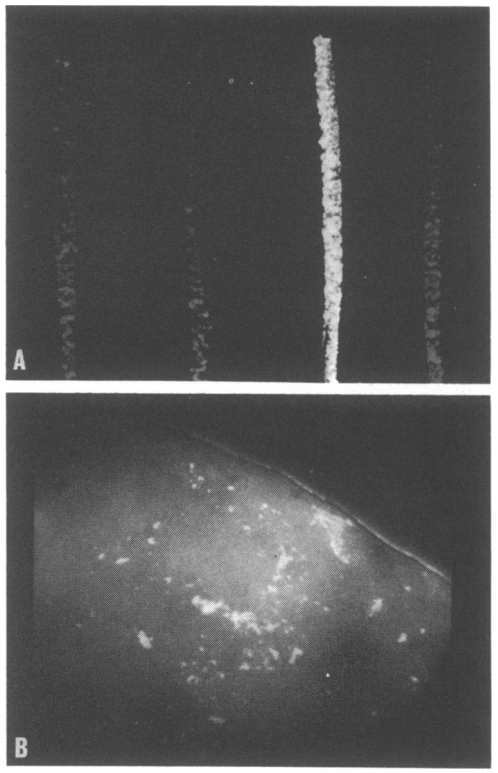

FIG. 1. *(A) Appearance in ultraviolet light of in vitro-grown plaques of Streptococcus strain GS5 after immersion in fluorescein-labeled washed cells of (from left to right) L. casei, Streptococcus strain 4M4, active strain 6715, and heat-inactivated strain 6715. (B) Appearance in ultraviolet light of a sound tooth surface treated with 1 μg of dextran, and immersed in fluorescein-labeled washed cells of Streptococcus strain 6715.*

DISCUSSION

Agglutination induced by high molecular weight dextran appears to be a characteristic of streptococci resembling *S. mutans*, but we have not observed it in other bacterial species, including other dextran-forming organisms. The ability of *S. mutans* to agglutinate in the presence of dextran appears to be due to the presence of specific receptor sites on the surface of the organisms which are capable of binding dextran molecules. If the molecules are sufficiently large to permit more than one cell of *S. mutans* to bind to the same molecule, this would initiate the observed agglutination. The overall specificity and sensitivity of the reaction are consistent with this mechanism. The failure of dextrans with a

molecular weight of 2×10^4 to induce agglutination suggests that these molecules are not large enough to be bound by two cells simultaneously. The ability of *S. mutans* to agglutinate in the presence of high molecular weight dextrans provides the basis for a simple, rapid, specific, and exquisitely sensitive assay for the qualitative detection of these polysaccharides. For example, we have detected dextran in exhaustively dialyzed solutions of reagent-grade sucrose.

The nature of the receptor sites involved in dextran-induced agglutination is not yet clear. These sites may be specific loci on the cell surface, or they may be identical with the reactive sites for dextran possessed by dextransucrase. This enzyme is recognized to have a high affinity for dextrans (5, 19, 20), a fact which has made it difficult, if not impossible, to obtain preparations of the enzyme from *Leuconostoc* species which are completely free from dextran. Dextransucrase is known to be present in a cell-bound form in *S. mutans* (15, 16), and is presumably located on or near the surface of the organisms. Thus, the affinity of this enzyme for dextran could initiate the observed agglutination. The finding that glucose and low molecular weight dextran in high concentration are capable of inhibiting the reaction is in accord with this view, since these substances are known to act as acceptors for dextransucrase (21). In addition, dextransucrase preparations derived from *S. mutans* have been observed to synthesize maximal quantities of high molecular weight insoluble dextrans in the range of *p*H 7 to 8.5 (15). These enzyme preparations differ in this respect from those obtained from *Leuconostoc* species, which exhibit maximal activity at *p*H 5 to 5.5 (21). This implies that the enzyme from *S. mutans* has a higher affinity for dextran in this elevated *p*H range, a finding which is consistent with the observed effects of *p*H on dextran-induced agglutination.

The mechanism of the observed agglutination could have application to other bacterial systems. Washed cells of certain strains of *Staphylococcus aureus* agglutinate upon the addition of fibrinogen, and the "clumping factor" responsible is believed to be coagulase present in a cell-bound form (1). This system is therefore analogous in several ways to dextran dependent agglutination by *S. mutans*, and a mechanism, possibly enzymatic in nature, involving receptor site-polymer binding would seem to be involved.

Recent interest in *S. mutans* is due to this organism's ability to form gelatinous bacterial deposits on the surfaces of teeth in experimental animals and to initiate rampant dental caries (7, 11, 18). The formation of these streptococcal plaques is thought to be dependent upon the synthesis of dextrans from sucrose. Consequently, the ability of *S. mutans* to bind dextran can be visualized as playing an important role in this process. The present investigation has shown that 1 μg of insoluble dextran deposited on the surface of a tooth tends to bind cells of *S. mutans* to the tooth surface. Dextran-producing organisms growing on the tooth surface would therefore be able continually to select additional cells of *S. mutans* from the oral environment. This could at least partially explain the preferential localization of these organisms on the surfaces of teeth in the oral cavity. Since part of the insoluble matrix present in some types of human dental plaque consists of dextran (4, 12), the ability of *S. mutans* to bind to this polysaccharide explains in part the cohesive, aggregated nature of this material.

Certain strains of *S. mutans* have been observed to lose their plaque-forming and cariogenic potential upon continued laboratory culture. The basis of this loss in virulence is not understood at the present time, but it may be speculated that the ability of the organisms to bind to dextran is involved. For example, *S. mutans* strain 6715 is a highly cariogenic strain which has been maintained by repeated animal passage. This strain exhibits dextran-induced agglutination more readily than the laboratory-cultured parent (strain K1-R) from which it was derived (10). Similarly, *S. mutans* strain GS5 has been laboratory-cultured for several years and appears to have lost virulence. This strain was not observed to exhibit significant dextran-induced agglutination; yet, strain GS5 MR, which was derived from strain GS5 after passage in monkeys, readily agglutinated with dextran. A correlation between virulence for the hamster (22) and ability to exhibit dextran-induced agglutination also exists between strains AHT and BHT. Thus, it is conceivable that the relative ability of strains of *S. mutans* to bind dextran may be one of the factors affecting their virulence in relation to plaque formation and cariogenic potential.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants DE-01471 and DE-02847 from the National Institute of Dental Research. One of the authors (R.J.G.) was supported by a career development award from the U.S. Public Health Service, and participated in this study as a Visiting Scientist at the National Institute of Dental Research.

We acknowledge the technical assistance of Patricia K. Smiley.

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