INFECTION AND IMMUNITY, Apr. 1976, p. 1228-1234

Vol. 13, No. 4
Printed in U.S.A.

Dextran-Mediated Interbacterial Aggregation Between Dextran-Synthesizing Streptococci and *Actinomyces viscosus*

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Received for publication 18 November 1975

Streptococcus sanguis and *Streptococcus mutans* bind to the surface of *Actinomyces viscosus*, producing large microbial aggregates. Aggregates form rapidly and are not easily dissociated by vigorous mixing. The binding is mediated by dextran. Glucose-grown streptococci will not aggregate unless they are first mixed with high-molecular-weight dextran. Aggregation is induced with dextrans isolated from *Leuconostoc*, *S. sanguis*, or *S. mutans*. Sucrose-grown streptococci will adhere to *A. viscosus* without the addition of an exogenous source of dextran. *A. viscosus* will bind dextran and then bind glucose-grown streptococci. Aggregation occurs over a wide pH range and is dependent on cations. The aggregating activity of *A. viscosus* is both protease and heat sensitive. The aggregating activity of *S. sanguis* is heat stable but sensitive to dextranase.

Human dental plaque is a complex assortment of bacteria held together by host-synthesized (7) and bacterially synthesized polymers (6). Although the flora is complex, it is at the same time a specific association of microorganisms (9). This specificity is in part a consequence of the ability of bacteria to adhere to host tissue or to other bacteria. Gibbons and Nygaard (8) reported that certain oral organisms would aggregate with one another when pure cultures were mixed together. They suggested that this aggregating capability represented a mechanism that would allow bacteria to bind into plaque. Specific interspecies aggregation of bacteria has been observed by microscopic study of dental plaque. The most striking example is the "corncob" association of cocci and rods (12, 13).

In recent publications (14, 14a), we reported that high-molecular-weight dextran will induce aggregation of the filamentous organism *Actinomyces viscosus*. The reaction is sensitive, requiring as few as three molecules of dextran per bacterial cell, and it is specific for dextran. The association is strong, as evidenced by the fact that cells will bind to Sephadex beads (dextran) and resist removal by vigorous washing. In the presence of sucrose *Streptococcus sanguis* and *Streptococcus mutans* will synthesize dextran that is bound to the cell and dextran that is released into the media (5, 11, 18). We postulated that the *Actinomyces* could use the cell-free dextran to form aggregates in plaque. In this report we will provide evidence that cell-bound dextran will mediate the attachment of *A. viscosus* to *S. sanguis* and *S. mutans*.

MATERIALS AND METHODS

Organisms. *A. viscosus* (15987) was obtained from the American Type Culture Collection. It was grown in brain heart infusion broth (BHI) in static culture, aerobically at 37 C. Growth on a solid medium required incubation in a 10% CO_2 atmosphere. *S. mutans* was isolated from human carious lesions as described by Gold et al. (10). *S. sanguis* was isolated from human dental plaque as described by Carlsson (1). These organisms were grown in Trypticase soy broth (TSB).

Stock cultures were stored at −70 C in growth medium supplemented with enough sterile glycerol or dimethyl sulfoxide to give a final concentration of 10%. It was found that *A. viscosus* cells that were repeatedly subcultured gradually lost the ability to aggregate. Because of this, cells were grown from the stock cultures once a month.

Dextran. Dextrans of known molecular weight were purchased from Pharmacia (Montreal, Quebec, Canada). Samples of dextran were isolated from various *S. sanguis* and *S. mutans* strains by the procedure of Sidebotham et al. (7).

Analysis of the isolated dextrans. Total carbohydrate was measured by the anthrone method as described by Scott and Melvin (16) or by the phenol-sulfuric acid method of Dubois et al. (3), using dextran T2000 as the standard. Ketohexose was determined by the cysteine-sulfuric acid method of Dische and Devi (2), using fructose as a standard. Protein was measured by the Lowry method, using bovine serum albumin as a standard. The purity of the dextran samples was checked by thin-layer chroma-

tography. Dextran samples were dissolved in distilled water to a final concentration of 2.5 mg/ml. One hundred microliters of the sample was mixed with 100 μl of 4 N HCl and sealed in a glass ampoule. The mixture was hydrolyzed in a boiling water bath for 1 h. The hydrolyzed samples were then freeze-dried in the presence of NaOH pellets. The residue was resuspended in 100 μl of distilled water, and 10 μl was chromatographed on a cellulose thin-layer chromatography sheet (Polygram Cell 300; Macherey-Nagel and Co., Düren, Germany) in *n*-butanol-acetic acid-water (3:1:1). The chromatogram was developed using an aniline diphenylamine spray followed by heating at 100 C for 2 min. Dextran T2000, glucose, and fructose were used as standards.

Enzymes. Trypsin (type XI), chymotrypsin (type III), subtilisin (type VIII), and hyaluronidase (type III) were purchased from Sigma. Dextranase, purchased from Sigma, was found to be contaminated with protease and numerous glycosidases. The glycosidase activities detected are reported in Table 1. Protease was measured using the casein assay described by Rick (15).

Interbacterial aggregation. Bacterial cells were harvested by centrifugation, washed three times in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, and resuspended in Tris buffer to give the desired number of cells per milliliter. To insure that the streptococcal cells were free of soluble dextran, the supernatant from the third wash was assayed to determine whether it could induce aggregation of *A. viscosus*. The required numbers of cells were then mixed together with enough buffer to give a final volume of 1.1 ml. The mixture was incubated at room temperature for 1 h. At 15, 30, and 45 min the tubes were agitated gently. The extent of aggregation was measured after 1 h of incubation by either scoring aggregation on a 0 to +4 basis or by counting the nonaggregated bacteria. In the former procedure the size of the aggregates was estimated visually, using a stereomicroscope; a score of zero indicated no visible aggregation and a score of +4 indicated that the majority of bacteria had formed a single large clump. Only a limited amount of information can be obtained in the visual assay. Therefore, a second more precise measure of activity was made by counting the numbers of organisms remaining in suspension after the aggregates had settled to the bottom of the tube. Fifteen minutes after the final shaking, bacterial aggregates had settled to the bottom of the tube and it was possible to remove a sample from the top of the reaction mixture. The sample was diluted in exponential steps into sterile buffer and replicate samples were plated on Mitis-Salivarius agar to determine the number of streptococci and onto BHI agar to determine total counts. The numbers of *A. viscosus* were determined by the difference. The relative recovery rates of each organism on the two types of media were also determined.

RESULTS

Interbacterial aggregation. When *A. viscosus* was mixed with *S. sanguis*, the cell suspension rapidly became granular and soon large clumps of bacteria formed (Fig. 1). Microscopic examination of these clumps showed that they were a mixture of the two organisms. The specimen used for photomicrography was prepared by sonicating clumps of aggregated bacteria. This procedure dispersed the organisms well enough to permit photographs to be taken. Before sonication, the organisms were all present in large refractile clumps and it was difficult to distinguish rods from cocci. The visibly obvious aggregation could be measured as a decrease in the numbers of bacteria that remained in suspension in the reaction mixture (Table 2). There was no observable decrease in the numbers of cells in suspension in control reactions in which each organism was incubated alone. The colony-forming units (CFU) of *A. viscosus* were estimated by difference as described above. The accuracy of this procedure was verified in an experiment in which each colony on a BHI plate was identified by microscopy as consisting of either coccal or filamentous bacteria. There was excellent correlation between the

TABLE 1. *Contaminating glycosidase activity in commercial dextranase*

PNP substrate	Temp (C)	pH	Buffer (molar concn of sodium citrate)	Substrate hydrolyzed (μmol) per min by 0.5 mg of dextranase	Enzyme U per mg of dextranase[a]
β-D-Galactopyranoside	30	4.6	0.05	2.35×10^{-3}	2.12×10^{2}
α-D-Galactopyranoside	30	4.6	0.05	3.66×10^{-3}	1.36×10^{2}
β-D-Glucoside	25	4.8	0.10	3.30×10^{-3}	1.51×10^{2}
α-L-Fucoside	25	6.0	0.05	7.70×10^{-6}	6.50×10^{4}
α-D-Glucoside	25	4.8	0.10	1.15×10^{-3}	4.35×10^{2}
N-acetyl-β-D-galactosaminide	30	4.6	0.05	3.90×10^{-3}	1.28×10^{2}
N-acetyl-β-D-glucosaminide	30	4.6	0.05	8.00×10^{-4}	6.25×10^{2}
β-Glucuronide	37	5.0	0.20	9.00×10^{-5}	5.60×10^{3}
α-D-Mannoside	25	4.5	0.05	1.70×10^{-4}	2.93×10^{3}

[a] One unit is the amount of enzyme needed to hydrolyze 1 μmol of substrate per min under the conditions cited.

TABLE 2. *Interbacterial aggregation of S. sanguis and A. viscosus*

Bacterial mixture	Cells/ml	Decrease in CFU (%)	Aggregation
S. sanguis	10^9	0	0
A. viscosus	10^9	0	0
S. sanguis +	10^9	57	+4
A. viscosus	10^9	86	
S. sanguis +	10^9	15	+3
A. viscosus	10^8	84	
S. sanguis +	10^9	84	+3
A. viscosus	10^7	90	
S. sanguis +	10^9	0	0
A. viscosus	10^6	0	

difference method and the colony identification method; therefore the less time-consuming difference procedure was used for all experiments. Aggregates could be partially dispersed by vigorous mixing, but they rapidly reformed upon standing.

S. mutans also co-aggregates with *A. viscosus*, but because *S. mutans* self-aggregates the assay for co-aggregation is more difficult. For this reason *S. sanguis* was used for the majority of experiments reported in this paper.

Co-aggregation is dependent on the presence of ions. The requirement is not specific and may be satisfied by either 5×10^{-3} M divalent or 10^{-2} M monovalent cations. Ions tested included Na, K, Mg, Mn, Ca, Ba, and Cs. This is analogous to dextran-induced aggregation of *A. viscosus*. Co-aggregation was not affected by altering the pH between 5.0 and 8.5.

Role of sucrose. Dextran induces aggregation of *A. viscosus*, and it seemed reasonable that dextran on the cell surface of the streptococci might be the factor that mediates co-aggregation. To test this hypothesis, *S. mutans* and *S. sanguis* were grown in TSB and TSB supplemented with one of eleven different sugars to a final concentration of 1%. The cells were harvested and washed three times in Tris buffer and then tested for their ability to co-aggregate with *A. viscosus*. To insure that there was no soluble dextran, the streptococci were washed with buffer until the washings no longer aggregated *A. viscosus*. The *Actinomyces* and one of the streptococci were mixed and incubated, and aggregation was measured. Streptococci grown in TSB supplemented with glucose did not co-aggregate with *A. viscosus*, whereas cells grown in the presence of sucrose aggregated strongly with *A. viscosus*. No other sugar supplement resulted in aggregation. The ability of *A. viscosus* to aggregate was not affected by the type of sugar in its growth medium.

The importance of sucrose was indicated in another experiment in which *S. sanguis* was grown to the stationary growth phase in TSB. Sucrose was then added to give a final concentration of 5%. The culture was incubated at 37 C, and cells were removed at various times, washed, and then assayed for aggregating activity. After incubation for 4 h in the presence of sucrose, the cells were able to aggregate (Table 4). Maximum activity was observed after 12 h of incubation. Control cells that were not exposed to sucrose did not aggregate at any time during the 12-h incubation period. In this experiment there was a small amount of background aggregation; this was observed sporadically and can probably be attributed to the small amount of sucrose that is found as a contaminant in TSB.

Effect of cell concentration. In vitro aggregation is dependent on the number of organisms in the assay mixture. When the *S. sanguis* concentration was kept constant at 10^9 CFU/ml, the *A. viscosus* concentration could be dropped to as low as 10^7 CFU/ml before there was a large loss in aggregating activity (Table 2). In the reverse experiment (Table 3), the *A.*

TABLE 3. *Interbacterial aggregation of A. viscosus and glucose or sucrose-grown S. sanguis*

Bacterial mixture	Cells/ml	Decrease in cfu (%)		Aggregation	
		glucose[a]	sucrose[a]	glucose[a]	sucrose[a]
A. viscosus	10^{10}	0	0	0	0
S. sanguis	10^{10}	0	0	0	0
A. viscosus +	10^{10}	32	95	+1	+4
S. sanguis	10^{10}	35	95		
A. viscosus +	10^{10}	0	79	0	+3
S. sanguis	10^9	0	99		
A. viscosus +	10^{10}	0	74	0	+2
S. sanguis	10^8	0	99		
A. viscosus +	10^{10}		0	0	0
S. sanguis	10^7		0	0	0

[a] *S. sanguis* grown in TSB supplemented with either glucose or sucrose.

TABLE 4. *Effect of length of incubation with sucrose on interbacterial aggregation*

Reaction mixture	Sucrose	Length of incubation (h)			
		0[a]	4	8	12
A. viscosus	–	0	0	0	0
S. sanguis	+	0	0	0	0
A. viscosus +	–	+1	+3	+3	+4
S. sanguis	+				
A. viscosus +	–	+1	+1	+1	+1
S. sanguis	–				

[a] Sucrose was added and then immediately removed by washing.

viscosus concentration was kept constant and the numbers of *S. sanguis* were varied. As can be seen, there was no demonstrable aggregation when the streptococcal population fell below 10^8 CFU/ml. These results indicate that the relative proportions of the organisms are important in the in vitro assay.

Effect of cell age. Cell surfaces can change with the age of the cells and culture conditions, and such changes may affect the ability of cells to aggregate. To determine whether this was occurring, an experiment was designed in which the age of the *S. sanguis* culture was kept constant at 18 h and the age of the *A. viscosus* culture was varied from 1 to 4 days. Cells were washed and resuspended to a concentration of 10^{10} cells/ml in buffer. There was no change in the ability to aggregate. When 4-day-old *A. viscosus* cells were incubated with *S. sanguis* cells that varied in age between 8 and 48 h, there was no change in the ability to co-aggregate although there appeared to be a slight increase in the tendency to self-aggregate on the part of the *S. sanguis* cells at 48 h. If *A. viscosus* was grown in TSB rather than BHI, a different picture emerged. Aggregating activity was most pronounced during late log phase and disappeared rapidly during stationary phase. Thus cultural conditions have a significant effect on the nature of the *Actinomyces* cell surface.

Effect of exogenously added dextran. In an experiment designed to prove that dextran mediates aggregation, glucose-grown *S. sanguis* was incubated with dextran (5 mg/ml) for 30 min. Dextran not bound to the cell surface was removed by washing the cells in Tris buffer. The dextran-treated *S. sanguis* cells were mixed with *A. viscosus* and aggregation was measured (Table 5). Dextran T2000 or dextran isolated from *S. sanguis* bound to the streptococcal cell surface and promoted binding to *A. viscosus*. Cells that were not treated with dextran did not aggregate. Dextran T10, molecular weight 10,000, was not large enough to promote aggregation. A similar experiment was performed with *S. mutans* (Table 6). The results were the same, but there was aggregation of the *S. mutans* in the presence of dextran, which made it necessary to disrupt these aggregates by vigorous agitation immediately before mixing the cells with *A. viscosus*. When this procedure was followed, most of the *A. viscosus* aggregated with the *S. mutans* as evidenced by the loss of CFU from the reaction mixture. This was not caused by release of dextran from the streptococci since the supernatant from an *S. mutans* control would not aggregate *A. viscosus*.

In the reverse experiment (Table 5) *A. viscosus* was incubated with dextran and then mixed with glucose-grown *S. sanguis* or *S. mutans*. Interbacterial aggregation was induced by *S. mutans*, *S. sanguis*, or dextran T2000 or dextran isolated from *S. sanguis* or *S. mutans*. Dextran T10 had no effect.

Cell modification. Treatment of *A. viscosus* with protease severely impaired or eliminated its ability to aggregate with dextran T2000. If dextran is the link between *A. viscosus* and the

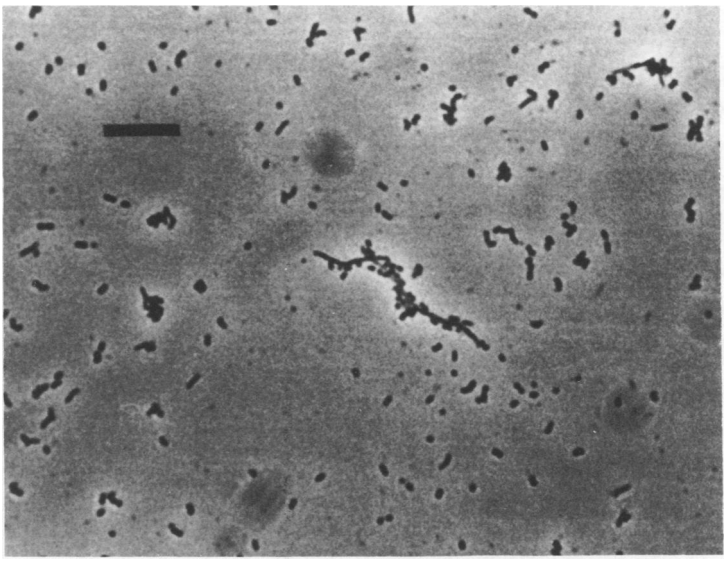

FIG. 1. *Photomicrograph of S. sanguis bound to A. viscosus. Bar represents 10 μm.*

streptococci, such treatment should have a similar effect on co-aggregation. The enzymes listed in Table 7 were incubated with *A. viscosus* cells (10^{10} cells/ml) for 1 h. The final concentration of enzyme was 2 mg/ml. The cells were then washed three times in Tris buffer and resuspended to the original concentration. *A. viscosus* and *S. sanguis* were mixed in a 1:1 ratio. As can be seen in Table 7, protease destroyed the ability to aggregate. Heating the *A. viscosus* to 90 C for 15 min eliminated aggregating activity. Dextranase destroyed the ability of *A. viscosus* mixed with dextran to bind to glucose-grown *S. sanguis*.

The effect of treating *S. sanguis* cells with dextranase (2 mg/ml) and various proteases (2 mg/ml) is shown in Table 8. The cells were incubated with the enzyme for 1 h, washed three times with Tris buffer, and assayed. Dextranase completely eliminated aggregation, suggesting that a dextran molecule is involved. Since the dextranase is badly contaminated with other glycosidases, this is not a conclusive experiment. To try to get around this problem, the cells were mixed with dextranase in the presence of low-molecular-weight dextran. The dextran protected the cells from the enzyme, providing more evidence that the dextranse is

TABLE 5. *Interbacterial aggregation mediated by dextran bound to S. sanguis or A. viscosus*

Expt[a]	Aggregation mixture	Decrease in CFU (%)		Aggregation
		A. viscosus	*S. sanguis*	
1	*A. viscosus*	0	–	0
2	*S. sanguis*	–	0	0
3	*S. sanguis* + dextran T2000	–	0	+1
4	*S. sanguis* + *S. sanguis* dextran	–	0	+1
5	*A. viscosus* + *S. sanguis*	10	0	+1
6	*A. viscosus* + S. sanguis + dextran 10	0	0	0
7	*A. viscosus* + *S. sanguis* + dextran T2000	99	97	+2
8	*A. viscosus* + *S. sanguis* + *S. sanguis* dextran	99	99	+3
9	*A. viscosus* + dextran[b]	80	–	+3
10	*A. viscosus* + dextran[b] + *S. sanguis*	99	99	+3

[a] *S. sanguis* (expt. 3-8) or *A. viscosus* (expt. 9, 10) was incubated with dextran for 60 min and then washed to remove unbound dextran.

[b] Dextan T2000 or *S. sanguis* dextran.

TABLE 6. *Interbacterial aggregation mediated by dextran bound to S. mutans*

Aggregation mixture	Decrease in CFU (%)		Aggregation
	A. viscosus	*S. mutans*	
S. mutans		0	+1
S. mutans + dextran 10		0	+1
S. mutans + dextran T2000		25	+2
S. mutans + Mutans dextran		98	+4
A. viscosus + *S. mutans*	33	50	+2
A. viscosus + *S. mutans* + dextran 10	0	0	+1
A. viscosus + *S. mutans* + dextran T2000	99	90	+3
A. viscosus + *S. mutans* + mutans dextran	99	99	+4

TABLE 7. *Co-aggregation after modification of the A. viscosus cell surface*

Aggregation mixture	Aggregation					
	Trypsin	Hyaluronidase	Subtilisin	Heat	Dextranase	None
A. viscosus	0	0	0	0	0	0
A. viscosus + dextran T2000	0	+2	0	0	0	+2
A. viscosus + dextran T2000 + *S. sanguis*[a]	0	+4	0	+1	0	+4
A. viscosus + *S. sanguis*[b]	0	+4	0	+1	+3	+4

[a] *S. sanguis* was grown in glucose-supplemented TSB broth.

[b] *S. sanguis* was grown in sucrose-supplemented TSB broth.

TABLE 8. *Co-aggregation after modification of the S. sanguis cell surface*

Aggregation mixture	Aggregation					
	Trypsin	Hyaluronidase	Subtilisin	Dextranase	Heat	None
S. sanguis	0	0	0	0	0	0
S. sanguis[a] + *A. viscosus*	+4	+4	+4	0	+3	0
S. sanguis[b] + *A. viscosus* + dextran T2000	+4	+4	+4	+4	+3	0

[a] *S. sanguis* grown in glucose supplemented TSB broth.
[b] *A. viscosus* grown in sucrose supplemented TSB broth.

the active enzyme. Boiling the streptococcal cells for 20 min or treating them with protease did not affect the ability of the cells to aggregate.

DISCUSSION

The experiments described in this paper prove that the dextran-synthesizing organisms *S. sanguis* and *S. mutans* will bind to the surface of *A. viscosus*. In the in vitro assay the consequence is the formation of clumps of bacteria; in vivo this would result in interbacterial aggregations within the plaque matrix.

Dextran appears to be the molecule that binds the organisms together. A summary of the evidence follows. (i) Only those streptococci that have been grown in the presence of sucrose will aggregate. This is to be expected because sucrose is required for dextran synthesis. (ii) The addition of dextran to streptococci grown in the absence of sucrose creates a cell that can aggregate. In other words, the streptococci can bind dextran to the cell surface and this dextran can then be used to bind to *A. viscosus*. The reverse is also true—dextran bound to the *Actinomyces* is available for binding to the streptococci. (iii) An enzyme mixture containing dextranase will destroy the ability of sucrose-grown streptococci to aggregate.

The source of dextran does not seem important; linear *Leuconostoc* dextran (dextran T2000) works as well as the soluble and insoluble dextrans isolated from *S. sanguis* and *S. mutans*. Thus dextrans that would be synthesized within the plaque matrix are capable of mediating interbacterial aggregation.

Low-molecular-weight dextran does not mediate binding. This is in keeping with the observations that this compound will not induce aggregation of *S. mutans* (6) or *A. viscosus* (14a). *A. viscosus* loses the ability to bind to dextran and therefore to bind to the streptococci after continuous subculture on laboratory medium. In some instances the organism will regain its binding abilities, but more often the binding activity is permanently lost. Therefore, it was necessary to continually go back to the stock cultures to obtain reactive cells. The lability of surface properties is a problem that is often encountered in studies of cell recognition systems. It is advisable that those who study microbial adherence use freshly isolated strains in their experiments. The receptor on the *Actinomyces* cell wall is a protein or is associated with a protein as it is sensitive to protease and heating. It is unstable when the organism is removed from its growth medium. Cells lose the ability to aggregate if they are incubated at room temperature for 6 h, incubated at 4 C overnight, or frozen. The dextran-binding unit on the *S. sanguis* cell wall is probably not a protein since it is insensitive to protease or heat. It could be a carbohydrate polymer or the murein in the cell wall.

We do not wish to suggest that dextrans are the only mechanism by which *A. viscosus* binds into a plaque matrix, but the ability of the organism to bind to dextran makes it a very plausible mechanism and one that could be used in an appropriate environment. The ability of *A. viscosus* to adsorb soluble dextran means that it could use the dextran in an ecosystem removed from the streptococci; alternatively it could bind directly to dextran-synthesizing organisms.

It is possible that the dextran is a model of the in vivo receptor. The natural receptor system could possibly consist of a number of α-1,6 glucose units linked to a host glycoprotein or cell wall of some other bacteria. If this is the case, then dextran is a model compound. However, it seems likely that given its dextran-binding capability the organism would use it if the occasion arose. It is possible that the "corncobs" (13) present in plaque are streptococci bound to an *Actinomyces* by dextran.

ACKNOWLEDGMENTS

The assistance of Mary Gisslow is gratefully acknowledged.

This work was supported by the Canadian Medical Research Council.

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