INFECTION AND IMMUNITY, Oct. 1977, p. 35-40
Vol. 18, No. 1

Printed in U.S.A.

Role of Sialic Acid in Saliva-Induced Aggregation of *Streptococcus sanguis*

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Received for publication 29 September 1976

The ability of saliva to induce aggregation of *Streptococcus sanguis* was destroyed by treating the saliva with protease or neuraminidase. Loss of aggregating activity could be correlated with the appearance of free sialic acid. Clarified saliva contains an endogenous neuraminidase that modifies aggregating activity. Aggregation was inhibited by mixed ganglioside preparations but less effectively by acid-hydrolyzed gangliosides. The aggregating activity of *S. sanguis* was not related to the rhamnose or phosphorous content of the cell wall or to antigen a, b, c, d, or e.

Specific salivary glycoproteins have been postulated to bind oral bacteria into human dental plaque (7). Gibbons and Spinell (7) reported that whole saliva or cannulated secretions from the parotid or submaxillary glands induced aggregation in 28 of 62 plaque organisms tested. Studies performed with a coccobacillus isolated from the human oral cavity indicated that the salivary aggregating factor was a high-molecular-weight, heat-stable aggregate (7, 9). Activity was Ca^{2+} dependent and occurred optimally between pH 5 and 7.5 (7). The factor was found to contain 33% protein, 19% anthrone-positive carbohydrate, and 2.9% sialic acid (9). Different organisms reacted differently to parotid or submaxillary saliva, suggesting that there is more than one aggregating system. Separate aggregating factors have been found for *Streptococcus sanguis* and *Streptococcus mitis* (11). There is some evidence that enzymatic modification of saliva reduces its aggregating potential for *S. sanguis* (8). Recently Kashket and Hankin (12) reported that the *S. sanguis* aggregating factor secreted by some individuals is sensitive to sulfhydryl reagents, whereas saliva from other individuals is not affected.

S. sanguis has been the subject of a number of studies designed to define its taxonomic status (4, 16, 17, 19). Rosan (16) has described five antigens (a, b, c, d, and e) found in autoclaved extracts of whole cells. Cole et al. (4) correlated serological specificity with bacteriophage adsorption, susceptibility to group C phage lysin, lysogeny, and the presence of certain neutral sugars in the cell wall. A number of other workers have attempted to relate the chemical composition of the cell wall to serological activity (19; J. M. Hardie, Ph.D. thesis, University of London, London, England, 1975). The result of these efforts has been to subdivide *S. sanguis* into two subspecies: type I contains high levels of rhamnose and low levels of phosphorous and ribitol, and type II contains low levels of rhamnose and higher levels of ribitol and phosphorous.

The study reported here is concerned with identifying the components of the salivary aggregating factor that are responsible for binding to *S. sanguis*.

MATERIALS AND METHODS

Bacteria. *S. sanguis* strains 10556, 10557, and 10558 were obtained from the American Type Culture Collection. *S. sanguis* strains 12, 14, and 15 were obtained from human dental plaque. *S. sanguis* strain 12 na is a variant of strain 12 that has lost the ability to aggregate with saliva. Inocula were stored at −70°C in growth medium supplemented with 7% dimethyl sulfoxide. The organisms were grown in Trypticase soy broth at 37°C for 24 h and then harvested by centrifugation. Cells to be used in aggregation assays were resuspended in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride–0.005 M $CaCl_2$ buffer (pH 7.2). Unless indicated otherwise, all experiments were done with *S. sanguis* 12.

Cell wall analysis. Purified cell walls were prepared essentially as described by Rosan (17). Four liters of a 24-h Trypticase soy broth culture of *S. sanguis* were harvested by centrifugation and washed three times in distilled water. Four grams of cell paste was mixed with 5 g of glass beads (75 to 150 μm) and 10 ml of distilled water, and the cells were broken in a Mini-Mill (Gifford-Wood Co.). Intact cells and cell walls were separated from glass beads by filtration through a sintered glass filter. The crude walls in the filtrate were separated from soluble cellular material by centrifugation at 15,000 × *g* for 15 min. The majority of whole cells were separated by gently resuspend-

ing the upper cell wall layer of the pellet. The cell walls were purified further by repeated centrifugation at 3,000 × *g* for 5 min. The whole cells formed a firm pellet, whereas the walls did not sediment or formed a soft pellet that was removed with the supernatant. The low-speed centrifugations were repeated until there were no unbroken cells in the preparation when visualized by phase-contrast microscopy. The walls were washed twice in 0.15 M NaCl and then treated with ribonuclease, deoxyribonuclease, and trypsin as described by Cummins and Harris (5). The purified walls were washed four times with distilled water and lyophilized. Rhamnose was measured by the Dische and Shettles technique (6); phosphate was determined by the method of Chen et al. (3).

Salivary aggregating factor. Paraffin-stimulated saliva was collected at 4°C and clarified by centrifugation at 20,000 × *g* for 15 min. At this point, subsequent steps in the preparation of saliva varied, depending on the experiment for which it was intended. For short-term studies of 20 min or less or for studies on endogenous inactivators, no further treatment was necessary. For experiments lasting longer than 20 min, the saliva was placed in a boiling water bath for 15 min to destroy enzymes that inactivated the aggregating factor. Occasionally, it was necessary to centrifuge the sample to remove a precipitate that formed during heating. Many of the modification studies were performed on a more highly purified aggregating factor obtained by centrifuging the heat-treated clarified saliva at 100,000 × *g* for 1 h. Ninety percent of the aggregating activity and 70% of the protein sedimented and was subsequently resuspended in buffer by brief (5 s) sonic treatment.

Chemicals. Unless noted otherwise, all enzymes and chemicals were purchased from Sigma Chemical Co. Ganglioside type III is a mixed ganglioside preparation isolated from bovine brain. It contains mono-, di-, and trisialoganglioside in the approximate ratio of 1:2:2. Acid-hydrolyzed ganglioside was prepared by incubating type III ganglioside in 0.05 N HCl at 80°C for 90 min. This procedure selectively removes sialidase-sensitive sialic acid, yielding the monosialo derivative (GM_1). The hydrolysis products were analyzed by chromatography of the hydrolysate in $CHCl_3$-MeOH-water (60:35:8). Compounds were visualized by spraying with resorcinol reagent according to the method of Penick et al. (15). Analysis of the chromatogram indicated that at least 95% of the ganglioside was in a form that migrated with an R_f identical to that of purified GM_1. Trypsin type III is the 2× crystallized product from bovine pancreas. Neuraminidase type VI from *Clostridium histolyticum* was determined to be free of proteolytic activity by assaying its activity against Azocoll (10) and casein (13). Pronase lot no. 30014 and Azocoll were purchased from Calbiochem. Dextran T2000 was purchased from Pharmacia.

Modification studies. Modification studies were performed on the pellet obtained from centrifugation of 30 ml of clarified saliva at 100,000 × *g*.

(i) Neuraminidase. The pellet was resuspended in 3 ml of 0.05 M sodium acetate–0.005 M $CaCl_2$, pH 5.5, together with the required amount of neuraminidase, and the mixture was incubated at 37°C. At designated intervals samples were removed, placed in boiling water for 5 min, and then assayed for free and total sialic acid and for aggregating activity. Free sialic acid was measured by the method of Warren (22). Total sialic acid was measured by the procedure of Svennerholm (20).

(ii) Protease. The pellet was mixed with 0.05 M Tris-hydrochloride buffer, pH 7.6, and 1.2 mg of proteolytic enzyme and incubated at room temperature for 1 h. The proteases were inactivated by heating the mixture in a boiling water bath for 15 min. The effectiveness of the heat inactivation step was assayed by incubating a sample of the reaction mixture with Azocoll at room temperature. After 1 h, the unreacted substrate was removed by filtration and the filtrate was examined spectroscopically at 520 nm for the presence of solubilized chromophore.

(iii) Glycosidase. One unit each of β-galactosidase, α-mannosidase, β-*N'*-acetylhexosaminidase purified from jack bean meal (14), and β-galactosidase and α-mannosidase purified from pinto beans (1) was mixed with the pellet in 0.05 M citrate buffer, pH 4.6, and incubated at room temperature for 48 h. A drop of $CHCl_3$ was added to prevent growth of bacteria. The activity of the enzymes was measured by incubating them with their appropriate *p*-nitrophenyl glycopyranoside or *p*-nitrophenyl *N*-acetylglycosaminide substrates. The enzymes were determined to be free of other glycosidases and protease by measuring their ability to hydrolyze a number of glycosidic substrates, Azocoll, and casein.

(iv) Alkaline phosphatase. The pH of 3 ml of clarified saliva was adjusted to 8.0 by the dropwise addition of 1 N NaOH. Forty units of enzyme was added, and the mixture was incubated for 2 h at room temperature. The activity of the enzyme was determined by measuring its ability to hydrolyze *p*-nitrophenyl phosphate.

(v) Acid hydrolysis. Five milliliters of saliva was mixed with 5 ml of 0.1 N H_2SO_4 and placed in an 80°C water bath. At designated intervals samples were removed and assayed for free sialic acid and for aggregating activity.

Aggregation assays. Aggregation was measured in a reaction mixture containing: *S. sanguis* (absorbance at 660 nm = 10), 50 μl; Tris-hydrochloride (0.05 M, pH 7.2, containing 0.005 M $CaCl_2$), 50 μl; and serially diluted saliva, 50 μl. The reaction mixtures were shaken at room temperature for 30 min. The last tube containing visually detectable aggregation was taken as the end point of the reaction. In some instances undiluted saliva was used, and aggregation was scored on a 0 to +4 basis.

RESULTS

Characterization of *S. sanguis* strains. The different chemical and antigenic components present in the cell walls of *S. sanguis* isolates prompted us to determine whether saliva-induced aggregation was associated with specific subspecies of the organism. Aggregation with heat-treated saliva was not restricted to either the Rosan group I or II (Table 1).

Aggregation was not related to the antigens a,

TABLE 1. *Relationship between cell wall structure and aggregating ability*

Strain	Source	Antigenic composition[a]	Rhamnose (%)	Phosphorus (%)	Group	Aggregation
10556	ATCC[b]	b	18	0.8	I	−
10557	ATCC	c, d	2	2.3	II	+
10558	ATCC	a, b, c, d, e	23	0.6	I	−
12	Plaque		24	0.6	I	+
12na	Plaque		24	0.7	I	−
14	Plaque		18.5	1.1	I	+
15	Plaque		1.3	2.3	II	−

[a] Data taken from Rosan (16).
[b] ATCC, American Type Culture Collection.

b, c, d, and e. Strain 10558 possessed all five antigens but was aggregation negative; strain 10557 possessed only antigens c and d and was positive. Apparently the quantity of rhamnose or phosphorous was not related to the ability to bind the salivary factor. Strain 10558 had high levels of rhamnose and did not aggregate, whereas strain 12 with similar levels of rhamnose aggregated strongly. The nonaggregating variant strain 12 na had levels of phosphorous and rhamnose similar to those of the parent strain. Strains 10556, 10558, and 15 did not aggregate with heated saliva but did aggregate weakly with undiluted, unheated saliva. Strains 12 and 10557 aggregated strongly with clarified saliva from eight different donors.

Inhibition of saliva-induced aggregation. *S. sanguis* was incubated with a number of sugars, glycoproteins, and glycolipids in an attempt to find a compound that would act as an analog of the salivary aggregating factor and bind to the saliva-specific receptor site on the bacterial cell wall. Cells were incubated with the test material for 30 min at room temperature and then assayed for their ability to aggregate with clarified saliva. None of the sugars tested had an appreciable inhibitory effect on aggregation, nor did dextran or concanavalin A (Table 2). Some inhibition was observed when the cells were preincubated with lecithin. Pretreatment of the bacterial cells with a mixed ganglioside preparation had a marked inhibitory effect on saliva-induced aggregation. As little as 20 μg of ganglioside per ml resulted in a decrease in aggregation when preincubated with *S. sanguis* cells (Table 3). Ganglioside preparations containing predominantly GM_1 were less effective inhibitors, suggesting that removal of neuraminidase-sensitive sialic acid residues created a molecule with a reduced capacity to bind to the saliva receptor sites on the cell surface.

Modification of the aggregating factor. To gain further insight into the nature of the aggregation reaction, selective biochemical and chemical probes were used to modify the salivary aggregating factor (Table 4). Proteolytic enzymes and neuraminidase were effective in destroying the aggregating factor. Collagenase, alkaline phosphatase, α-mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase, and lipase were without effect. Incubation of boiled clarified saliva with 0.1 M β-mercaptoethanol for 8 h did not affect aggregating activity.

TABLE 2. *Effect of various compounds on the aggregating activity of S. sanguis*

Compound tested	Concn (mg/ml)	Aggregation titer
Glucose	9.0	16
Galactose	9.0	16
Glucosamine	9.0	16
N-acetylglucosamine	22.0	8
Lecithin	0.25	4
Mucin[a]	4.25	16
Dextran T2000	0.2	16
Ganglioside	0.2	0
Concanavalin A	0.2	8
Buffer control		16

[a] Bovine submaxillary mucin type I (Sigma Chemical Co.).

TABLE 3. *Effect of ganglioside on aggregation*

Concn of inhibitor (μg/ml)	Aggregation titer[a]	
	Ganglioside	AHG[b]
500	0	4
250	0	4
125	2	8
50	4	16
25	4	8
20	8	16
15	16	16
0	16	16

[a] Time of incubation, 60 min.
[b] AHG, Acid-hydrolyzed ganglioside.

Role of neuraminic acid. The pronounced effect of neuraminidase on aggregating activity suggested that sialic acid was an important component of the reaction, and therefore the role of this molecule was investigated in more

detail. The enzymatic or chemical release of sialic acid from the salivary factor was closely related to the loss of aggregating activity (Table 5). The control retained its aggregating activity and its sialic acid during the 60-min incubation period. Saliva from eight different donors examined reacted identically. Weak aggregation, in undiluted saliva only, was observed when strains 10556, 10558, 12 na, and 15 were mixed with unheated saliva. This weak aggregating activity was destroyed by heat but not by neuraminidase.

Endogenous neuraminidase. Clarified saliva that has not been boiled loses aggregating activity if incubated at room temperature. The loss of aggregating activity was shown to parallel the release of free sialic acid (Table 6). Clarified saliva was incubated at 30°C; portions were taken at intervals and assayed for aggregating activity and for free sialic acid. The loss of aggregating activity paralleled the release of sialic acid (Table 6). No protease activity could be detected in clarified saliva incubated with Azocoll or casein for 4 h at 37°C.

Enzyme treatment of aggregated cells. *S. sanguis* was aggregated with boiled clarified saliva and then incubated with proteolytic enzymes or neuraminidase, and the reaction mixture was observed visually for dispersion of the aggregates. The enzymes were capable of disrupting preformed aggregates (Table 7). Neuraminidase reduced aggregation from 4 to 0 within 2 min at room temperature.

TABLE 4. *Effect of hydrolytic enzymes on aggregating activity*

Enzyme	Enzyme activity (U/ml)	Aggregation titer
Chymotrypsin	19	2
Trypsin	4,460	2
Subtilisin	3.4	0
Pronase	78	8
Collagenase	180	16
Alkaline phosphatase	40	16
Glycosidases[a]		16
Neuraminidase[b]	1.0	0
None	0.004	16

[a] Includes treatment with β-galactosidase, α-mannosidase, β-*N'*-acetylhexosaminidase.

[b] Activity determined by measuring the hydrolysis of *N*-acetylneuraminyl-lactose.

TABLE 5. *Effect of neuraminidase treatment and acid hydrolysis on the aggregating activity of boiled saliva*

Treatment	Time (min)	Aggregation titer	Free sialic acid (μmol × 10^2/ml)	% of total sialic acid
Neuraminidase	0	32	0	0
	10	16	1.6	4
	30	2	11.6	31
	60	0	30.4	82
None	60	32	0	0
Acid hydrolysis	0	32	0	
	15	2	5.8	
	30	0	17.4	

DISCUSSION

It has been suggested (6, 8) but not proven that the salivary aggregating factor is a glycoprotein. The experiment described in Table 4 substantiates this hypothesis and extends the original observations to indicate that sialic acid is a critical component of the binding reaction. This conclusion is supported by the observation that removal of sialic acid by neuraminidase or mild acid hydrolysis correlates with a loss of aggregating activity. Further support is supplied by the observation that mixed gangliosides are potent inhibitors of aggregation, whereas acid-hydrolyzed ganglioside (GM_1), which contains fewer sialic acid residues, has a reduced ability to inhibit the reaction. Sialic acid is known to function as a receptor for virus particles in virus-induced hemagglutination (2) and has been implicated in the attachment and consequent infectivity of virus particles (18). The inability of the sialic acid-containing proteins mucin and fetuin to inhibit aggregation may be due to the fact that the molecules do not form large aggregates analogous to the salivary aggregating complex. Ganglioside is effective possibly because it forms micelles, which present a concen-

TABLE 6. *Endogenous neuraminidase activity in clarified saliva*

Time at 30°C (min)	Aggregation	Free sialic acid (μmol × 10^2/ml)
0	16	0
30	8	2.8
60	4	4.2
90	4	5.0
120	2	5.8
180	2	6.0
240	2	6.8

TABLE 7. *Enzymatic disruption of S. sanguis-saliva aggregates*

Enzyme	Enzyme activity (U/ml)	Aggregation	
		0 min	45 min
Trypsin	19,000	4	2
Chymotrypsin	80	4	2
Subtilisin	145	4	1
Subtilopeptidase	17	4	1
Pronase	16	4	1
Neuraminidase	0.53	4	0
Control		4	4

trated array of sialic acid groups. This would be particularly true of mixed ganglioside and less so with micelles composed primarily of GM_1. The salivary aggregating factor used in these studies elutes in the V_0 from a Sepharose 4B column and is readily sedimented at 100,000 × g. Presumably the factor is a complex of many molecules that may present a concentrated array of sialic acid residues on its surface. This hypothesis assumes that it is the number of sialic acid molecules that is important. The other possibility is that binding is dependent on the specific environment surrounding the sialic acid molecule; presumably a ganglioside would mimic this particular arrangement, whereas the sialic acid in mucin is not present in an environment conducive to binding.

It is possible that removal of sialic acid results in a conformational change in the aggregating factor that masks the binding site, thus making it unavailable to the bacteria. However, the fact that nonhydrolyzed ganglioside interferes with binding argues for a direct role for sialic acid.

Lecithin has a slight inhibitory effect, but at concentrations which probably reflect a nonspecific effect (21) and thus are not of interest in defining the biochemistry of binding.

Aggregation is dependent on Ca^{2+} ions, suggesting that aggregation results when negatively charged groups on the bacterial cells and glycoprotein are bridged by a divalent ion. Aggregation is not affected by pH as low as 3.9 (11). This is consistent with the idea of an ionic interaction involving sialic acid, which has a pK of 2.6.

Salivary glycoprotein-induced aggregation of oral bacteria could be caused by either a salivary immunoglobulin or a non-immunoglobulin protein. The presence of salivary immunoglobulins in plaque suggests that some organisms may utilize these proteins to adhere to oral surfaces. It seems unlikely that the salivary glycoprotein which binds *S. sanguis* is an immunoglobulin because of its size, resistance to heat, and dependency on Ca^{2+} and neuraminic acid. If the binding molecule is an immunoglobulin, then the receptor activity must reside in a sialic acid-containing region and not with the Fab region of the molecule. The weak neuraminidase-insensitive aggregation observed with strains 10556 and 10558 in unheated saliva suggests that there are at least two binding reactions occurring in untreated saliva. Possibly the neuraminidase-resistant, heat-sensitive system is associated with salivary immunoglobulins. Saliva-induced aggregating activity for different bacterial species and strains probably resides in a number of molecules (12); thus it would be unwise to generalize our results to include organisms other than *S. sanguis*. On the other hand, the results indicate that all strains of *S. sanguis* which react with the heat-sensitive aggregating factor bind to a component of the molecule that is inactivated by treatment with neuraminidase.

Saliva-induced aggregates of *S. sanguis* are disrupted by proteolytic enzymes and neuraminidase (Table 4). Both classes of enzymes are present in plaque, suggesting that the salivary glycoprotein may not be a very stable cementing substance and therefore is not used by *S. sanguis* to adhere to the tooth surface. Gibbons and Spinell (7) extracted an aggregating factor from human dental plaque and suggested but did not prove that this aggregating component was similar to the one present in saliva. If their suggestion is correct, then the salivary aggregating protein must be resistant to enzymatic modification when it is in the plaque environment because, as shown in Table 6, there is endogenous neuraminidase in saliva that will modify the salivary aggregating factor.

The potential instability of the surface components that confer binding ability on bacteria is a factor that must be taken into consideration in any adherence experiment. Once removed from their natural environment, bacteria can readily dispense with adherence functions that are not required for survival in vitro. The *S. sanguis* strain (12 na) used in this study is an excellent example of this phenomenon. When first isolated, the organism bound avidly to the salivary factor, but on repeated subculture this property was lost. The rate at which binding ability is lost varies with the isolate. Some isolates will lose activity on the second subculture, whereas others maintain activity through 30 to 40 subcultures. The awkward question of whether any isolate truly presents a cell surface similar to that seen in vivo is important and bears on the validity of any adherence study. Needless to say, it is wisest to use fresh isolates and to preserve the characteristics of isolates by storing them frozen. In this way, it is possible to continually use cultures of organisms that have been subcultured no more than two times.

ACKNOWLEDGMENT

This investigation was supported by the Medical Research Council.

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