INFECTION AND IMMUNITY, Dec. 1983, p. 1073-1080
0019-9567/83/121073-08$02.00/0

Vol. 42, No. 3

Aggregation of *Streptococcus sanguis* by a Neuraminidase-Sensitive Component of Serum and Crevicular Fluid

E. JANE MORRIS AND BARRY C. McBRIDE*

Departments of Microbiology and Oral Biology, University of British Columbia, Vancouver, British Columbia, V6T 1W5, Canada

Received 17 May 1983/Accepted 21 September 1983

A number of strains of *Streptococcus sanguis* were found to aggregate in nonimmune serum and in crevicular fluid. All strains which aggregated in serum also aggregated in saliva, but some strains which aggregated in saliva did not aggregate in serum. Aggregation was destroyed by treatment of serum or crevicular fluid with neuraminidase and was inhibited by gangliosides. Treatment of serum with proteases reduced aggregating activity. Adsorption of serum to hydroxyapatite did not reduce the aggregating activity. The aggregating factor was partially purified by gel filtration and polyacrylamide gel electrophoresis and was found to be an acidic glycoprotein with a molecular weight of >200,000, comprised of subunits with molecular weights of approximately 100,000. It did not appear to be an immunoglobulin and could not be identified with any other serum component tested. The possible role of the aggregating factor in providing nonimmune protection against colonization of *S. sanguis* in the gingival crevice and blood is discussed.

Streptococcus sanguis is an oral microorganism which is found in large numbers on the tooth surfaces and in the gingival sulci of humans. It may make up 15% of the organisms in coronal plaque and 8% of organisms in gingival crevices (16).

The ability of salivary components to aggregate a variety of oral streptococci is well known (1, 15, 16). Such reactions may promote the accumulation of bacteria in the plaque matrix as well as causing the removal of unattached bacteria by masking bacterial adhesins and blocking attachment to immobilized receptors (3, 39).

Several salivary components have been implicated in the aggregation of *S. sanguis*. These include lysozyme (21), immunoglobulin A (IgA) (23), and mucinous glycoproteins (18, 22). Salivary mucins involved in aggregation carry blood group reactivity, although the bacterial receptor sites are apparently distinct from the sugar sequences responsible for the A and B antigenic determinants (14, 18). Eggert (8–11) demonstrated that streptococcal aggregation could also be caused by nonmucinous glycoproteins, although this may not apply to *S. sanguis*. IgA and other salivary components have been found to form complexes with mucins (2; A. Prakobphol, M. J. Levine, and L. A. Tabak, Annu. Meet. Complex Carbohydr. 1981, abstr. no. 81) as well as with high-molecular-weight nonmucin glycoproteins (7). However, at least in the case of the association between IgA and agglutinins in parotid saliva, aggregation of *S. mutans* is apparently due to the high-molecular-weight portion of the complex rather than to the IgA (30).

The aggregation of *S. sanguis* can be differentiated from that of other streptococci by its sensitivity to neuraminidase treatment of the saliva (22, 25). Aggregation appears to be caused by one or more sialic acid-containing high-molecular-weight mucinous glycoproteins (17, 18) as well as by a sialic acid-containing monomeric mucin with a molecular weight of 200,000 to 250,000 (32). Murray et al. (28) have recently reported the isolation of a sialic acid-binding lectin from *S. sanguis*.

A chance observation has recently revealed that nonimmune serum and crevicular fluid can cause neuraminidase-sensitive aggregation of certain strains of *S. sanguis*. This paper describes some of the characteristics of the aggregation of *S. sanguis* in serum and crevicular fluid as well as the partial purification of the aggregating factor from serum.

(A preliminary account of this work has been published elsewhere [E. J. Morris and B. C. McBride, Abstr. Annu. Meet. Am. Assoc. Dent. Res. 1983, 792, p. 257].)

MATERIALS AND METHODS

Bacteria. *S. sanguis* strains 12 and 12na were described by McBride and Gisslow (25), strains 10556 and 10558 were obtained from the American Type Culture Collection (Rockville, Md.), and strains NY101 and OMZ9 were obtained from J. S. van der

Hoeven, University of Nijmegen, The Netherlands. Other *S. sanguis* strains were isolated in our laboratory from human dental plaque. *Streptococcus salivarius* HB and HB-7 were described by Weerkamp and McBride (38). *Streptococcus mitior* was a human oral isolate. *Streptococcus mutans* C67-1 and LM-7 were obtained from J. S. van der Hoeven. *S. mutans* LK2 was obtained from B. Krasse, University of Goteborg, Sweden.

Bacteria were grown in tryptic soy broth supplemented with 0.3% yeast extract or (where stated in the text) in the chemically defined medium of Terleckyj et al. (37). Unless indicated otherwise, *S. sanguis* 12 was used for all experiments.

Aggregation assays. Overnight cultures of bacteria were harvested by centrifugation, washed once in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2, and suspended in HEPES buffer (absorbance at 660 nm, 3.0). Samples to be tested for aggregating activity were serially diluted in HEPES. An equal volume of bacterial suspension was added, and the mixture was shaken at room temperature for 5 min. Results are expressed in terms of the aggregation titer; this is the highest dilution giving macroscopically visible aggregation. Specific aggregating activity is defined as the aggregation titer divided by the protein concentration (milligrams per milliliter). Protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) against a standard of bovine serum albumin.

Serum, saliva, and crevicular fluid. For isolation of the aggregating factor, serum was pooled from several New Zealand white rabbits. Serum from a number of human donors (including blood groups A, B, and O, Rh positive and negative) was also tested for ability to aggregate *S. sanguis*. In one instance when the aggregating factor from human serum was partially purified, serum obtained from a single individual (group B, Rh negative) was used.

Parafilm-stimulated saliva was collected at 0°C, clarified by centrifugation at 20,000 × *g* for 10 min, and then heated at 60°C for 30 min to inactivate endogenous enzymes. Saliva was pooled from a number of donors.

Crevicular fluid was obtained from the gingival sulci of two dental patients with inflamed gingivae. The sampling area was isolated by using cotton wool plugs, rinsed with water to remove saliva, and dried thoroughly. A microcapillary tube was placed at the gingival margin, and approximately 20 μl of the exudate was collected. The fluid was diluted 1:500 in HEPES buffer and then treated as for saliva.

Neuraminidase treatment. The sample to be treated was adjusted to pH 5.0. Neuraminidase was dissolved as required in 0.05 M acetate buffer, pH 5.0, and added to the sample. The mixture was routinely incubated overnight at 37°C (although a strong effect could be seen in 1 to 2 h), and the pH was readjusted to 7.0 before assaying for aggregating activity. A control sample was treated identically, except for the omission of neuraminidase.

Treatment with other enzymes. Samples were incubated at room temperature for 3 h with chymotrypsin, trypsin, papain, pronase, subtilisin, phospholipase A, phospholipase C, and mixed glycosidases at concentrations of 1 mg/ml.

Incubations were carried out in HEPES buffer at pH 7.2 except in the case of the mixed glycosidases, in which 0.05 M potassium biphthalate buffer (pH 4.0) was used. Subsequent aggregation assays were carried out at 0°C to prevent the action of enzymes on the bacteria.

Inhibition studies. A variety of sugars and other compounds were tested for their ability to inhibit the aggregation reaction. Glucose, galactose, glucosamine hydrochloride, galactosamine hydrochloride, *N*-acetylglucosamine, and *N*-acetylgalactosamine were added to the bacterial suspension to give a 0.05 M solution. Sialic acid was tested at 1 mg/ml, mixed gangliosides were tested at various concentrations up to 500 μg/ml, and lecithin was tested at 100 μg/ml. Bovine submaxillary mucin and concanavalin A both caused aggregation of the bacteria in the absence of serum or saliva and so could not be tested as inhibitors. Potential inhibitors were incubated with the bacterial suspension for 30 min before addition to serial dilutions of saliva or serum.

Haptoglobin. The presence or absence of haptoglobin was determined qualitatively by the method of Connell and Smithies (4). Any increase in absorbance at 470 nm after 30 min of reaction time was taken to indicate the presence of haptoglobin.

Fibronectin and C-reactive protein. Fibronectin and C-reactive protein were tested for their ability to aggregate *S. sanguis* 12. Solutions of these compounds were made up at concentrations of 0.3 and 0.1 mg/ml, respectively, and were serially diluted as in the standard aggregation assay. C-reactive protein was tested both before and after dialysis against water.

Adherence of serum aggregating factor to hydroxyapatite. Whole serum (2 ml) was applied to a column containing 350 mg of hydroxyapatite powder. The column eluate was tested for any reduction in aggregating activity. The eluate was then mixed with a further amount (approximately 100 mg) of hydroxyapatite powder for 30 min and again was tested for aggregating activity.

Gel filtration. Serum (20 ml) was fractionated on a column (2.5 by 85 cm) containing Sephacryl S-400 equilibrated with 0.05 M HEPES buffer (pH 8.0) containing 1 M sodium chloride and 0.02% sodium azide. Fractions of 10 ml were collected.

PAGE. Nondenaturing gels containing 5% polyacrylamide were run in the absence of sodium dodecyl sulfate (SDS) or 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis (PAGE) was run with 10% polyacrylamide. Molecular weight standards used were myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), and ovalbumin (45,000). Gels were stained for protein with Coomassie blue and for carbohydrate with periodic acid-Schiff reagent, or else they were silver stained (29).

To locate aggregating activity, the gels were cut into 0.5-cm slices and eluted with HEPES buffer, pH 7.2. SDS was removed where applicable by extensive dialysis.

Immunological procedures. Standard immunoelectrophoretic and immunodiffusion techniques were performed with agarose gels in 0.1 M barbital buffer, pH 8.6.

Chemicals. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise specified. Neuraminidase type VI from *Clostridium perfrin-*

gens was stated to contain protease activity of <0.001 U/mg of protein, using casein, and no protease activity was found with azocoll or azocasein as substrates. Phospholipase C was type I from *C. perfringens*, trypsin was type XI from bovine pancreas, and subtilisin was type VII from *Bacillus amyloliquefaciens*. α-Chymotrypsin (code CDI) was obtained from Worthington Diagnostics, Freehold, N.J., and both pronase and phospholipase A (*Crotalus terr. terr.*) were obtained from Calbiochem-Behring, La Jolla, Calif. Mixed glycosidases (from *Turbo cornutus*) were supplied by Miles Laboratories, Rexdale, Ontario, Canada.

Fibronectin was from human plasma. C-reactive protein (hemagglutinin free) was from *Limulus polyphemus*. Goat anti-rabbit whole serum and anti-rabbit IgG, IgA, and IgM were obtained from Miles Laboratories. Molecular weight standards for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories.

RESULTS

Characteristics of *S. sanguis* aggregation. *S. sanguis* 12 was found to aggregate in all samples of serum tested (Table 1). The aggregation titer was remarkably constant, no significant difference being found among species or blood groups. Sera from 10 human group O donors and from a similar number of rabbits gave almost identical results. In contrast, the aggregating activity of salivary secretions showed considerable variation among species and individuals (Table 1) (34). Aggregation in all samples of rabbit, mouse, and human serum was completely destroyed by the action of neuraminidase, and aggregation in rat serum was reduced by over 90%. Aggregation in all saliva samples was also destroyed by neuraminidase. Neuraminidase acted on the serum or saliva rather than on the bacteria in the aggregation assay. This could be demonstrated by the fact that after neuraminidase treatment, no aggregation was seen, even when the assay was carried out at 0°C, when enzyme activity over a short time period would be negligible.

Two samples of human crevicular fluid caused neuraminidase-sensitive aggregation up to a dilution of approximately 1:500. Although the possibility of contamination of the crevicular fluid with saliva cannot be completely excluded, the saliva could only form a small part of the sample. It is, therefore, unlikely that such a high aggregation titer (higher than that normally found with saliva alone) could be due to such contamination.

TABLE 1. Aggregation of *S. sanguis* 12 in serum and saliva

Source of samples	Aggregation titer	
	Serum	Saliva
Rabbit	2,056	ND[a]
Rat	1,024	4
Mouse	1,024	4
Human		
Group O	1,024	64
Group A	2,056	32
Group B	2,056	128

[a] ND, Not determined.

A variety of bacterial strains were examined for their ability to aggregate in saliva and serum (Table 2). Aggregation titers in serum were lower than those shown in Table 1 since the serum was predialyzed; the resulting dilution reduced the aggregation by one to two tubes. All strains of *S. sanguis* which were found to aggregate in serum also aggregated in saliva. However, two of the strains tested (OMZ9 and 7) showed neuraminidase-sensitive aggregation in saliva but did not aggregate in serum.

Of the other streptococci tested, *S. salivarius* HB and *S. mutans* LK2 showed some aggregation in serum (Table 2). Unlike aggregation in *S. sanguis*, aggregation in these strains was not destroyed by neuraminidase treatment, although there was a small decrease in aggregation in the case of *S. salivarius* HB.

After four transfers in chemically defined medium, the aggregation titer for *S. sanguis* remained the same as when the cells were grown in tryptic soy broth. This suggests that aggregation is a property of the cells themselves rather than of any component adsorbed from the growth medium.

Although aggregation in serum occurred to a high dilution, aggregation was weaker and somewhat variable in undiluted serum. This effect seemed more noticeable in animal than in human serum. It was not established whether the same occurred in crevicular fluid since insufficient material was obtained to permit determination of aggregating activity in the undiluted sample. Aggregation in serum was usually enhanced after dialysis or dilution of the sample. However, if the assay was carried out at 0°C, large aggregates were formed even in the undiluted serum, and the aggregation titer was increased. A similar increase in the aggregation titer in saliva was found at 0°C. This effect was reversible since samples cooled to 0°C after initial shaking at room temperature also formed large aggregates; these aggregates tended to disperse on warming to room temperature.

The serum aggregating factor was found to be stable to heat (60°C for 60 min or 100°C for 5 min). The aggregation reaction appeared similar to aggregation in saliva in that the addition of 0.1 M sodium chloride or 0.025 M EDTA completely inhibited aggregation, whereas 0.1 M 2-mercaptoethanol was without effect.

A number of compounds were tested for their ability to inhibit aggregation. Glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine at concentrations of 0.05 M had no effect. Glucosamine hydrochloride and galactosamine hydrochloride (0.05 M) both reduced aggregation by over 90% in both serum and saliva, but the same effect could be seen upon the addition of an equivalent amount of sodium chloride to the reaction mixture. Sialic acid was without effect. However, a mixed ganglioside preparation caused a marked inhibition of aggregation, the aggregation titer being reduced by 75% in the presence of 100 μg of ganglioside per ml and by 95% with 500 μg of ganglioside per ml. After mild acid hydrolysis to remove sialic acid (25), ganglioside did not inhibit aggregation, even at a concentration of 500 μg/ml, implying an important role for sialic acid in the aggregation reaction. Lecithin also reduced aggregation (aggregation titer reduced from to 512 to 64) as was seen for saliva (25), but this may be a nonspecific effect.

To gain more information about the nature of the aggregating factor, the effects of a number of enzymes on aggregation activity were determined (Table 3). In comparison with the salivary aggregating factor, aggregation in serum was affected relatively little by the action of proteolytic enzymes. However, after partial purification of the serum aggregating factor (eluate from nondenaturing gel; see below), a considerable reduction in aggregation was achieved by treatment with chymotrypsin, papain, and trypsin. In whole serum, the aggregating factor appeared to be to some extent protected from enzyme action. Both phospholipase C and neuraminidase caused complete loss of aggregating activity; however, the phospholipase C was found to contain neuraminidase as a major contaminant. The reason for the increase in aggregation in saliva after treatment with steapsin or subtilisin is not known. Neither enzyme caused aggregation of *S. sanguis* on its own; possibly the enzyme action uncovered chemical groups which were previously masked, or perhaps it destroyed some inhibitory activity.

Aggregation with fibronectin and C-reactive protein. No aggregation of *S. sanguis* occurred in the presence of fibronectin or C-reactive protein, either before or after dialysis.

Adherence to hydroxyapatite. The aggregation titer of the serum was not reduced, either by passing the serum through a column of hydroxyapatite or by incubation with hydroxyapatite for 30 min.

Partial purification of the aggregating factor from serum. Results obtained with rabbit serum are described. This was used for the majority of experiments since no evidence of *S. sanguis* was found in the normal oral flora of the rabbits, and it was therefore hoped that the level of natural antibody to the organism would be low. On one occasion when human serum was fractionated, the results obtained were similar to those observed with rabbit serum.

TABLE 2. Bacterial aggregation in saliva and serum

Species	Strain	Aggregation titer			
		Saliva		Serum[a]	
		Untreated	Neuraminidase treated	Untreated	Neuraminidase treated
S. sanguis	12	256	0	512	0
	12na	0	0	0	0
	10556	0	0	0	0
	10558	0	0	0	0
	NY101	128	0	64	0
	OMZ9	64	0	0	0
	N	256	0	512	0
	7	128	0	0	0
	M	128	0	512	0
	L	512	0	256	0
S. mitior		4	4	0	0
S. salivarius	HB	128	128	64	32
	HB-7	0	0	0	0
S. mutans	LM-7	0	0	0	0
	C67-1	8	8	0	0
	LK2 (serotype c)	8	8	4	4

[a] Dialyzed against water before use.

TABLE 3. Effects of hydrolytic enzymes on aggregating activity

Enzyme	Aggregation titer		
	Saliva	Serum	Gel eluate[a]
Control	256	1,024	4,096
Chymotrypsin	128	1,024	512
Pronase	128	512	2,048
Papain	256	2,048	4
Trypsin	64	2,048	128
Subtilisin	1,024	1,024	4,096
Phospholipase C	0	0	0
Phospholipase A	256	1,024	4,096
Steapsin	1,024	2,048	2,048
Neuraminidase	0	0	0

[a] Eluate from nondenaturing gel (partially purified serum aggregating factor).

Serum was initially fractionated by gel filtration on Sephacryl S-400 (Pharmacia Fine Chemicals, Piscataway, N.J.) (Fig. 1). Aggregating activity was recovered in the first major protein peak (absorbance at 280 nm) from both rabbit and human serum. However, the peak of aggregating activity sometimes eluted slightly behind the protein peak. In certain instances, a preliminary precipitation of the serum in 2% boric acid was carried out; aggregating activity precipitated under these conditions. When the redissolved precipitate was fractionated on Sephacryl S-400, the activity again eluted slightly behind the first major protein peak. In initial experiments when a column of Sephadex G-200 was used, the aggregating activity eluted at the void volume, suggesting a molecular weight of 200,000 or higher.

Fractions containing activity from the Sephacryl column were combined, concentrated by ultrafiltration on an Amicon XM-50 membrane, and dialyzed against water. The specific aggregating activity of the combined fractions increased to 483 from 32 in the original serum. Haptoglobin, which was present in the original serum, was not found in the concentrated combined fractions.

A further 20-fold increase in specific aggregating activity was obtained by PAGE on nondenaturing gels (Fig. 2). Aggregating activity with a specific activity of 8,830 was recovered from an area of the gel which stained weakly for both protein and carbohydrate. Crossed immunoelectrophoresis indicated that immunoglobulins traveled a considerably shorter distance than the aggregating factor in the gel. Immunoelectrophoresis and radial immunodiffusion of the gel slices with aggregating activity failed to show any evidence of IgA, IgG, or IgM. This does not, however, rule out the possibility that small amounts of immunoglobulins could be associated with the aggregating factor and would only be detectable by more sensitive methods.

Aggregating activity was recovered from the nondenaturing gel in a discrete band. The distance traveled suggests that the aggregating factor may be acidic in character. When the nondenaturing gel eluate was applied to an isoelectric focusing gel (pH 4 to 9), aggregating activity could be eluted from the acidic end of the gel, implying an isoelectric point of 4 or lower. Isoelectric focusing could not be carried out on whole serum or on the Sephacryl column eluate since the aggregating factor precipitated in the ampholyte. Before substantial purification was achieved, precipitation of activity was a major problem, particularly when the pH was reduced to 4 to 5 or after the sample was frozen for any length of time.

Less than 10% of the aggregating activity was recovered from the nondenaturing gel. All methods of isolation tested suffered from the problem of losses of activity once a certain level of purification was obtained, although the activity which was recovered appeared very stable.

The aggregating activity from the nondenaturing gel was further fractionated by SDS-PAGE (Fig. 3). Although only a single band was eluted from the nondenaturing gel, multiple bands were seen on SDS-PAGE. However, when gel slices were eluted and dialyzed to remove SDS, aggregating activity for *S. sanguis* was recovered from the gel. The peak of activity corresponded to a subunit molecular weight $\approx$ 100,000, but the activity could not be related with certainty to a particular gel band. The recovered activity retained its sensitivity to neuraminidase.

DISCUSSION

The aggregation of *S. sanguis* in serum and crevicular fluid appears to fall into the class of short-circuit reactions (12) which may provide nonimmune protection against bacterial colonization in the gingival crevice or circulatory system. The importance of the reaction in vivo remains uncertain since aggregation was weaker in undiluted serum at room temperature. However, the extent of inhibition was variable, apparently occurring to a lesser degree in human than in animal serum. If activity does occur in vivo, it could provide an important host defense mechanism against streptococcal infections; *S. sanguis* is the causative agent in 30 to 40% of cases of subacute bacterial endocarditis (5). Conversely, it might promote the disease through the accumulation of bacterial aggregates on the endocardial lesion. The presence of aggregating activity in crevicular fluid points to a role in clearance of *S. sanguis* from the gingival crevice. Crevicular fluid bathes enamel, cementum, and epithelial surfaces in the sulcus, all of

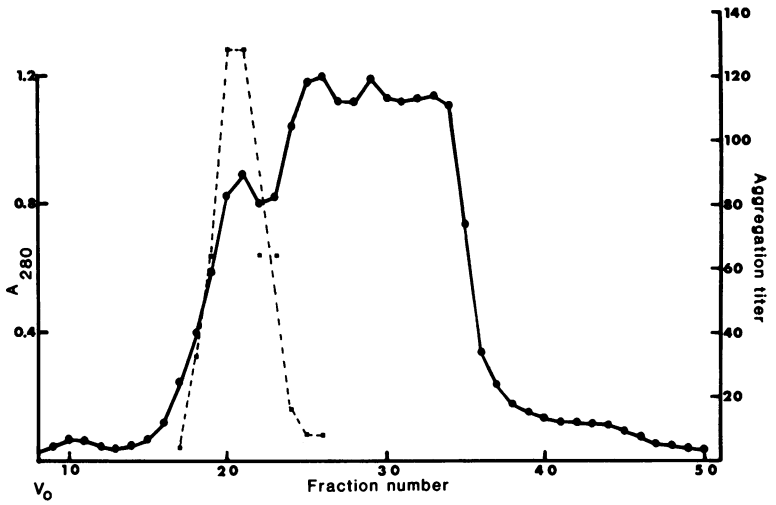

FIG. 1. Fractionation of serum on Sephacryl S-400. Symbols: ●—●, absorbance at 280 nm (A_{280}); ■- - -■, aggregation titer.

which provide important adherence sites for bacteria. In addition, the gingival exudate may come into contact with supragingival plaque. The salivary aggregating factor is capable of binding to hydroxyapatite (13) to form one of the components of the tooth pellicle. It may thus be involved in the adherence of *S. sanguis* to the tooth surface, although the aggregation and adherence reactions show some characteristic differences (34). In contrast, no evidence was found for binding of the serum aggregating factor to hydroxyapatite. Assuming that the factor in crevicular fluid is similar to that in serum, this suggests that it is primarily involved in protection against colonization and does not play a part in the adherence of *S. sanguis* to the tooth enamel. Suspension of *S. sanguis* in saliva affects the ability of the cells to attach to saliva-coated enamel surfaces (31) and to epithelial cells (40). Similarly, human serum blocks adherence to saliva-coated hydroxyapatite (24) and might also play a role in preventing adherence to enamel cementum and to the gingival epithelium.

Although serum aggregation shares a number of properties with salivary aggregation, there are some obvious differences. Both appear to be due to sialic acid-containing glycoproteins, but the subunit molecular weight of the serum factor (approximately 100,000) is approximately half that of the monomeric low-molecular-weight salivary factor (32), whereas the high-molecular-weight salivary mucin has resisted attempts to deaggregate it into subunits (17). Some strains of *S. sanguis* were found which aggregated in saliva but not in serum, suggesting that different sites on the cell surface may be involved.

It was of interest to determine whether the serum aggregating factor could be identified with any known serum component or whether it was a previously unidentified protein. A number of mechanisms whereby streptococci may be aggregated in serum are known. As in the case of salivary aggregation of *S. sanguis*, it seems unlikely that the serum aggregating factor is an immunoglobulin because of its size, resistance to heat, and sensitivity to neuraminidase; in addition, no immunoglobulins were detected in a partially purified preparation of the aggregating

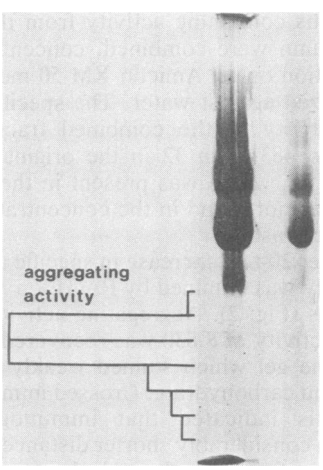

FIG. 2. Nondenaturing PAGE of active fractions from Sephacryl S-400 column. Left, Stained with Coomassie blue; right, stained with Schiff periodate. Aggregating activity in eluted slices is shown. Only fractions with the highest aggregating activity were retained.

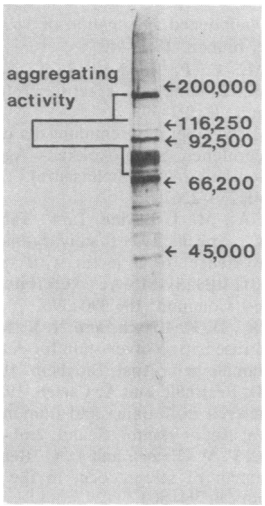

FIG. 3. SDS-PAGE of active fractions from nondenaturing gel. Silver stained. Aggregating activity in eluted slices and positions of molecular weight markers are indicated.

factor. Its resistance to heat and the fact that aggregation is apparently caused by a single molecule also suggest that complement activation is not involved.

Haptoglobin has been found to aggregate streptococci from groups A, C, and G (20) by a mechanism which is inhibited by sialic acid (27). However, it is not thought likely that haptoglobin is involved in the present instance since aggregation to a high titer is caused only by haptoglobin types 2-1 and 2-2 (19). These are present in human serum, but other animals possess only type 1-1. Ericson et al. (12) found no binding of haptoglobin to *S. sanguis*, and in the present instance, no haptoglobin was found in fractions with aggregating activity.

Simpson and Beachey (36) have shown that group A streptococci adhere to fibronectin on the surface of oral epithelial cells. This adhesion could be inhibited by soluble plasma fibronectin. Although fibronectin is a glycoprotein carrying terminal sialic acid residues, its subunit molecular weight of 200,000 to 220,000 (41) is higher than that found for the *S. sanguis* aggregating factor. Unlike the *S. sanguis* aggregating factor, the agglutinating activity of fibronectin is destroyed by heat. In addition, a commercially obtained preparation of fibronectin caused no aggregation of *S. sanguis* 12.

Binding of C-reactive protein to *S. sanguis* has been demonstrated by Mold et al. (26). However, this has a subunit molecular weight of 21,500 and is probably free of carbohydrate (33). Commercially obtained C-reactive protein did not aggregate *S. sanguis*.

Fibrinogen (6) and its degradation products (35) have been shown to bind to β-hemolytic streptococci and to *Staphylococcus aureus*. However, two α-hemolytic streptococci isolated from subacute bacterial endocarditis patients showed no reaction with fibrinogen (6). A laboratory strain of *S. aureus* which was known to clump in fibrinogen up to a concentration of 10 μg/ml showed no reaction with serum under conditions which produce strong aggregation of *S. sanguis*. This suggests that fibrinogen is not involved in *S. sanguis* aggregation.

Ericson et al. (12) tested a variety of serum proteins for binding to *S. sanguis*. Of those examined, only aggregated β_2-microglobulin (but not the normal monomeric form) showed positive binding. β_2-Microglobulin contains no carbohydrate, and so sialic acid is, therefore, not involved in its binding.

It seems possible that the aggregation reaction described in this paper involves some previously unknown serum component with a high specificity towards *S. sanguis*. This type of nonimmune reaction has apparently not been previously described but may involve a protein with reactivity similar to that in the salivary mucinous glycoprotein. Further studies would be required to confirm this.

ACKNOWLEDGMENT

This investigation was supported by the Medical Research Council.

LITERATURE CITED

1. **Bowden, G. H. W., D. C. Ellwood, and I. R. Hamilton.** 1979. Microbial ecology of the oral cavity. Adv. Microb. Ecol. **3**:135–217.
2. **Clamp, J. R.** 1977. The relationship between secretory immunoglobulin A and mucus. Biochem. Soc. Trans. **5**:1579–1581.
3. **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.
4. **Connell, G. E., and G. Smithies.** 1959. Human haptoglobins: estimation and purification. Biochem. J. **72**:115–121.
5. **de Moor, C. E., J. van Houte, and J. D. de Stoppelaar.** 1968. Endocarditis lenta en caries streptokokken—*Streptococcus mutans*. Versl. Meded. Volksgezond. **20**:325–336.
6. **Duthie, E. S.** 1955. The action of fibrinogen on certain pathogenic cocci. J. Gen. Microbiol. **13**:383–393.
7. **Eggert, F. M.** 1979. Salivary and amniotic-fluid agglutinin and aggregating factors: evidence for complexing between high-molecular-weight non-mucin glycoproteins and immunoglobulins. Biochem. Soc. Trans. **7**:194–196.
8. **Eggert, F. M.** 1980. The nature of secretory agglutinins and aggregating factors. I. Secretory conglutinin-like factor, secretory bacterial aggregating factors and secretory IgA antibody in human saliva and amniotic fluid. Int. Arch. Allergy Appl. Immun. **61**:192–202.
9. **Eggert, F. M.** 1980. The nature of secretory agglutinins and aggregating factors. II. Biochemical and immunochemical properties of factors in human saliva and amniotic fluid. Int. Arch. Allergy Appl. Immun. **61**:203–212.
10. **Eggert, F. M.** 1980. The nature of secretory agglutinins and aggregating factors. III. Secretory conglutinin-like factor SKF detects a cross-reaction between bacteria and

complement component C3. Int. Arch. Allergy Appl. Immun. **62**:34–45.
11. **Eggert, F. M.** 1980. The nature of secretory agglutinins and aggregating factors. IV. Complexing between non-mucin glycoproteins, immunoglobulins and mucins in human saliva and amniotic fluid. Int. Arch. Allergy Appl. Immun. **62**:46–58.
12. **Ericson, D., D. Bratthall, L. Björck, E. Myhre, and G. Kronvall.** 1979. Interactions between human serum proteins and oral streptococci reveal occurrence of receptors for aggregated β_2-microglobulin. Infect. Immun. **25**:279–283.
13. **Ericson, T., and I. Magnusson.** 1976. Affinity for hydroxyapatite of salivary substances inducing aggregation of oral streptococci. Caries Res. **10**:8–18.
14. **Gibbons, R. J., and J. V. Qureshi.** 1978. Selective binding of blood group-reactive salivary mucins by *Streptococcus mutans* and other oral organisms. Infect. Immun. **22**:665–671.
15. **Gibbons, R. J., and D. M. Spinell.** 1970. Salivary induced aggregation of plaque bacteria, p. 207–215. *In* W. B. McHugh (ed.), Dental plaque. Churchill Livingstone, Ltd., Edinburgh.
16. **Gibbons, R. J., and J. van Houte.** 1975. Bacterial adherence in oral microbial ecology. Annu. Rev. Microbiol. **29**:19–44.
17. **Hay, D. I., R. J. Gibbons, and D. M. Spinell.** 1971. Characteristics of some high molecular weight constituents with bacterial aggregating activity from whole saliva and dental plaque. Caries Res. **6**:111–123.
18. **Hogg, S. D., and G. Embery.** 1979. The isolation and partial characterization of a sulphated glycoprotein from human whole saliva which aggregates strains of *Streptococcus sanguis* but not *Streptococcus mutans*. Arch. Oral Biol. **24**:791–797.
19. **Kohler, W., and O. Prokop.** 1977. Haptoglobintyp und G-Streptokokken. Dtsch. Gesundheitswes. **32**:1248.
20. **Kohler, W., O. Prokop, and G. Uhlenbruck.** 1977. Zur Agglutinin-Natur der menschlichen Haptoglobine gegenuber Streptokokken mit dem T4-Antigen. Naturwissenschaften **64**:538.
21. **Laible, N. J., and G. R. Germaine.** 1982. Adsorption of lysozyme from human whole saliva by *Streptococcus sanguis* 903 and other oral microorganisms. Infect. Immun. **36**:148–159.
22. **Levine, M. J., M. C. Herzberg, M. S. Levine, S. A. Ellison, M. W. Stinson, H. C. Li, and T. Van Dyke.** 1978. Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. Infect. Immun. **19**:107–115.
23. **Liljemark, W. F., C. G. Bloomquist, and J. C. Ofstehage.** 1979. Aggregation and adherence of *Streptococcus sanguis*: role of human salivary immunoglobulin A. Infect. Immun. **26**:1104–1110.
24. **Liljemark, W. F., S. V. Schauer, and C. G. Bloomquist.** 1978. Compounds which affect the adherence of *Streptococcus sanguis* and *Streptococcus mutans* to hydroxyapatite. J. Dent. Res. **57**:373–379.
25. **McBride, B. C., and M. T. Gisslow.** 1977. Role of sialic acid in saliva-induced aggregation of *Streptococcus sanguis*. Infect. Immun. **18**:35–40.
26. **Mold, C. M., C. P. Rodgers, R. L. Kaplan, and H. Gewurz.** 1982. Binding of C-reactive protein to bacteria. Infect. Immun. **38**:392–395.
27. **Montag, T.** 1979. Über die Hemmung der durch Haptoglobin hervorgerufenen Streptokokken—Agglutination mit *N*-Acetyl-Neuraminsaure (Sialinsaure). Dtsch. Gesundheitswes. **34**:2266–2267.
28. **Murray, P. A., M. J. Levine, L. A. Tabak, and M. S. Reddy.** 1982. Specificity of salivary-bacterial interactions. II. Evidence for a lectin with specificity for a NeuAcα2,3Galβ1,3GalNAc sequence. Biochem. Biophys. Res. Commun. **106**:390–396.
29. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361–363.
30. **Olsson, J., D. Bratthall, and A. Carlen.** 1981. Association between bacterial agglutinins and immunoglobulin A in human saliva. Acta Odontol. Scand. **2**:61–66.
31. **Ørstavik, D., F. W. Kraus, and L. C. Henshaw.** 1974. In vitro attachment of streptococci to the tooth surface. Infect. Immun. **9**:794–800.
32. **Prakobphol, A., M. J. Levine, L. A. Tabak, and M. S. Reddy.** 1982. Purification of a low-molecular-weight, mucin-type glycoprotein from human submandibular-sublingual saliva. Carbohydr. Res. **108**:111–122.
33. **Putnam, F. W.** 1975. The roster of the plasma proteins, p. 57–131. *In* F. W. Putnam (ed.), The plasma proteins. Academic Press, Inc., New York.
34. **Rosan, B., D. Malamud, B. Appelbaum, and E. Golub.** 1982. Characteristic differences between saliva-dependent aggregation and adhesion of streptococci. Infect. Immun. **35**:86–90.
35. **Runehagen, A., C. Schonbeck, U. Hedner, B. Hessel, and G. Kronvall.** 1981. Binding of fibrinogen degradation products to *S. aureus* and to β-hemolytic streptococci group A, C and G. Acta Pathol. Microbiol. Scand. Sect. B **89**:49–55.
36. **Simpson, W. A., and E. H. Beachey.** 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. **39**:275–279.
37. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11**:649–655.
38. **Weerkamp, A. H., and B. C. McBride.** 1980. Characterization of the adherence properties of *Streptococcus salivarius*. Infect. Immun. **29**:459–468.
39. **Weerkamp, A. H., and B. C. McBride.** 1980. Adherence of *Streptococcus salivarius* HB and HB-7 to oral surfaces and saliva-coated hydroxyapatite. Infect. Immun. **30**:150–158.
40. **Williams, R. C., and R. J. Gibbons.** 1975. Inhibition of streptococcal attachment to receptors on human buccal epithelial cells by antigenically similar salivary glycoproteins. Infect. Immun. **11**:711–718.
41. **Yamada, K. M., and K. Olden.** 1978. Fibronectins—adhesive glycoproteins of cell surface and blood. Nature (London) **275**:179–184.