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Characteristic Differences Between Saliva-Dependent Aggregation and Adhesion of Streptococci

BURTON ROSAN,* DANIEL MALAMUD, BENJAMIN APPELBAUM, AND ELLIS GOLUB

Departments of Microbiology and Biochemistry and Center for Oral Health Research, University of Pennsylvania, School of Dental Medicine, Philadelphia, Pennsylvania 19104

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Comparison of saliva-mediated aggregation of *Streptococcus sanguis*, *Streptococcus mitis*, and *Streptococcus mutans* and adhesion of these organisms to saliva-coated hydroxyapatite showed that there was no relationship between these two activities. Adsorption of salivary aggregating activity to bacteria appears to have little effect on the ability of the residual saliva to support adherence; conversely, adsorption of salivary adherence factors to hydroxyapatite does not affect aggregation. Although heating saliva significantly reduces bacterial aggregation, it has little or no effect on adherence. A comparison of aggregation and adhesion with serial dilutions of saliva demonstrated that adhesion could still be detected at 100 to 500-fold-lower concentrations of salivary protein than bacterial aggregation. These findings support the concept that aggregation and adherence involve two distinct mechanisms of microbial clearance in the oral cavity.

The adhesion of oral streptococci to hydroxyapatite (HA) appears to be mediated by salivary proteins which form a coating of pellicle on the tooth surfaces (7, 25). These interactions represent the initial phase of plaque formation and appear to be specific with respect to both bacterial receptor(s) and the salivary protein(s). Because many of the streptococci which interact with the pellicle also aggregate with saliva, it has been widely assumed that the same bacterial surface components and salivary proteins were involved in both phenomena (3, 6, 10, 14, 15, 20, 21). Thus, saliva-mediated aggregation of bacteria has often been used as a correlate of bacterial adhesion. However, there are no clear-cut data to verify that the two processes are mediated by the same components (25), since neither the bacterial nor the salivary factor has yet been isolated. Mandel (17, 18) has suggested that the two processes may have a reciprocal relationship to one another; adhesion may represent the initial phase of plaque formation by small numbers of bacteria, whereas aggregation may be responsible for clearing large numbers of organisms from the oral cavity by forming clumps of bacteria that are swallowed. Recent studies in our laboratory (1, 8, 16) have utilized precise assays to measure both bacterial adherence to saliva-coated HA (a model for adhesion to tooth surfaces) and aggregation. The present study suggests that saliva-induced aggregation and adherence are distinct processes.

MATERIALS AND METHODS

Organisms. The strains used in this study are listed in Table 1. *Streptococcus sanguis* strain H311 was obtained from Pauline Handley (University of Manchester, Manchester, England), *Streptococcus mutans* BHT was from Arnold Bleiweis (University of Florida, Gainesville, Florida), and *S. mutans* KPSK-2 was from Peter Berthold (University of Pennsylvania, Philadelphia). Stock cultures were kept both in the lyophilized state and frozen at −70°C; the latter were used for starter cultures. Physiological characteristics of all strains were determined by modifications of the methods of Carlsson (2) and Facklam (4); all *S. sanguis* strains used were biotype 1 (4). Serological identification of *S. sanguis* strains was based on the criteria of Rosan (22, 23); criteria for identification of *Streptococcus mitis* have also been published previously (23).

Cell growth and labeling conditions. Cells for adherence assays were grown at 37°C to stationary phase in 100 ml of brain heart infusion broth (Difco Laboratories) containing 2 μCi of [*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear Corp.) per ml; the cells were harvested, washed, and suspended in buffered KCl (containing 2 mM potassium phosphate buffer [pH 6.8], 1 mM $CaCl_2$, and 50 mM KCl) to a turbidity of 300 Klett units (6×10^9 cells per ml) as previously described by Appelbaum et al. (1). If not used immediately, washed cell pellets were stored on ice at 4°C for up to 1 week. Cells for aggregation studies were also grown to stationary phase in brain heart infusion broth containing 0.5 μCi of [*methyl*-^{3}H]thymidine per ml (specific activity, 6.7 Ci/mmol) and were harvested and washed with phosphate-buffered saline containing 0.01 M potassium phosphate and 0.15 M NaCl (pH 7); the cells were

TABLE 1. Streptococcal strains used in this study

Strain	Serotype
S. sanguis[a]	
G9B	1
38	1
47A2	1
CC5A	1
CC6	1
H311	1
M-5	2
10558	2
Challis-6	2
10556	2
S. mitis	
9811	
S. mutans	
OMZ61	a
BHT	b
GS-5	c
Ingbritt	c
KPSK-2	c
OMZ176	d
LM-7	e
6715	g

[a] Data from reference 22.

resuspended in phosphate-buffered saline and aliquoted to contain 10^{10} cells per ml as described by Malamud et al. (16) and stored frozen at −20°C.

Adherence assay. The protocol for the adherence assay was essentially the same as that described previously (1), except that the assay was scaled down to use 10 mg of HA beads (Gallard-Schlessinger Chemical Corp., Carle Place, N.Y.). Unless otherwise indicated, the beads were incubated with 1 ml of a 1:4 dilution of saliva in buffered KCl for 1.5 h at room temperature (1). The beads were washed three times with buffered KCl followed by the addition of 5×10^8 to 4×10^9 streptococci per ml.

Adherence was expressed either as the product of the association constant (K_a) and the number of binding sets (N) as calculated from the equation for the Langmuir adsorption isotherm (1) or as the percentage of available organisms adhering. In one experiment (see Table 3), adherence was expressed in terms of activity. All experiments included a control of uncoated HA beads.

Aggregation assay. Bacterial stock suspension (0.1 ml, approximately 200,000 cpm/10^{10} cells per ml) was mixed with 0.1 ml of clarified saliva and incubated for 5 min in a 37°C water bath with shaking (350 rpm). The reaction was terminated by the addition of 0.2 ml of 2.5% phosphate-buffered glutaraldehyde (in 0.1 M potassium phosphate, pH 7.0) and shaken for 5 min. This mixture was layered on 1 ml of 25% sucrose and centrifuged at 1,100 × g for 5 min; free cells were found in the supernatant fluids, and aggregated cells were found in the pellet. The percent aggregation, expressed as the percentage of total counts found in the pellet (aggregated bacteria) was determined as described previously (8, 16). In all cases, the percent aggregation of a blank reaction (bacteria plus phosphate-buffered saline) was subtracted.

Saliva. Whole paraffin-stimulated saliva was collected into ice-chilled tubes and clarified by centrifugation as described previously (1, 8). For most adherence assays, samples from five individuals were pooled, aliquoted, and frozen at −20°C. The effects of temperature on aggregation and adherence were tested by treating 3.5 ml of pooled saliva for 30 min at 0, 37, 50, 60, 70, and 80°C. Immediately after treatment the saliva was chilled in ice. When the adherence and aggregation of a given strain were compared, the same saliva preparation was used for both experiments.

Saliva depletion experiments. Frozen cultures containing 8×10^{10} cells in 8 ml were thawed, and after centrifugation at 3,500 rpm for 10 min, the cells were resuspended in 8 ml of saliva. After 15 min of incubation in an ice bath, the cells were removed by centrifugation. This was repeated three times to insure maximum depletion. Triplicate samples of 0.1 ml of the saliva supernatant were assayed for their ability to induce bacterial aggregation, and another 4-ml sample was diluted with buffered KCl and used to coat HA and tested for adherence assays using strain G9B. Saliva was also adsorbed with spheroidal HA beads for 1.5 h at room temperature, and the supernatant fluids were removed and tested for aggregating activity. A similar protocol was used to adsorb saliva with hydroxyapatite powder (Sigma Chemical Co., St. Louis, Mo.).

RESULTS

Comparison of streptococcal species and strains in adherence and aggregation assays. In Table 2, the degree of adherence (K_aN) and the percent aggregation of several strains of *S. sanguis*, *S. mitis*, and *S. mutans* with the same saliva pool

TABLE 2. Comparison of adherence and aggregation of *S. mutans*

Strain	Adherence ($K_aN \times 10^{-2}$)	% Aggregation
S. mutans		
OMZ61	71	45
6715	5.8	1
GS-5	4.1	1
BHT	4.0	5
Ingbritt	3.5	1
LM-7	0.5	63
KPSK-2	0.6	67
OMZ176	1.4	2
S. sanguis		
47A2	219	17
G9B	192	40
38	167	17
CC5A	137	17
CC6	118	3
H311	30	58
M-5	74	67
10556	59	49
Challis-6	31	40
S. mitis		
9811	146	37

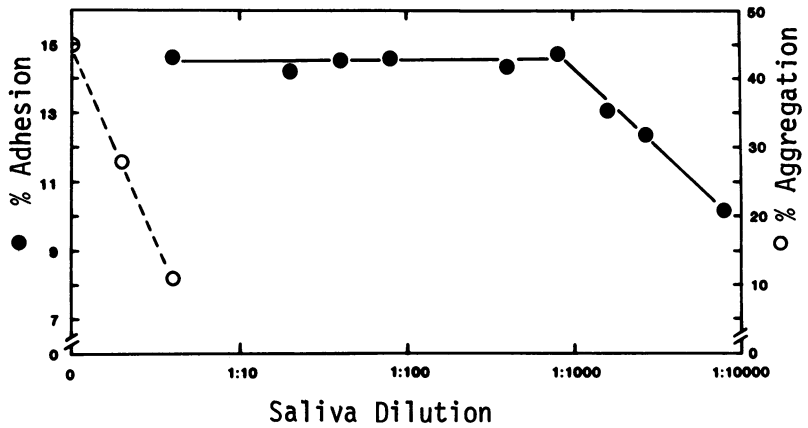

FIG. 1. Titers of adherence (●) and aggregation (○) factors for *S. sanguis* G9B in pooled saliva. Adhesion to uncoated HA is 2.9%. The data are plotted as net percent adherence.

are shown. A comparison of the adhesion and aggregating activities of these strains reveals no relationship between adherence and aggregation. With the exception of strain OMZ 61, the K_aN of *S. mutans* was 10- to 100-fold less than those previously reported for *S. sanguis* and *S. mitis* (1, 3).

Titer of salivary adherence and aggregation activity. To study the relative levels of *S. sanguis* G9B aggregating and adhesion activities in saliva, a dilution analysis was carried out. Aggregating activity rapidly decreased in the range between whole saliva and a 1:4 dilution (Fig. 1). In contrast, adhesion to saliva-coated HA did not significantly decrease until saliva had been diluted more than 1:1,000. Aggregation of other bacterial species (e.g., *S. mutans* KPSK-2) could still be detected with saliva dilutions as great as 1:100; however, adherence activity was always maintained at higher dilution levels than aggregating activity.

TABLE 3. Comparison of adherence and aggregation activities for saliva from different individuals

Sample no.	Adherence activity[a]	Aggregation activity[b]
1	40	61.6
2	40	11.0
3	400	48.5
4	1,000	19.4
5	1,000	4.2
6	2,000	28.8
7	2,000	13.1
8	>4,000	0.5
9	>4,000	25.8

[a] Adherence activity for *S. sanguis* G9B to saliva-coated HA beads was assayed at a series of saliva dilutions from 1:4 to 1:4,000. The dilution at which adherence was 50% of the 1:4 value is shown.

[b] Aggregating activity was tested with undiluted saliva and *S. sanguis* M5; the results are expressed as percent aggregation after subtraction of the blank value (bacteria plus buffer). Similar results were obtained with aggregation of G9B.

In Table 3, a comparison of adherence activity of individual salivas as measured with strain G9B and aggregating activity of the same salivas as measured with strain M-5 is shown. In earlier studies adherence of G9B was shown to be typical of many *S. sanguis* serotype 1 strains (1). Strain M-5 was chosen as a reference strain for aggregation because its behavior was representative of many *S. sanguis* strains. It was observed that although individual saliva samples varied in absolute amounts of aggregating activity, samples which were high for the reference strain M-5 were also high for other aggregating *S. sanguis* strains. In these strains, the data in Table 3 indicate that there was no correlation between adherence and aggregation in these individual saliva samples, although both activities showed a great range among individuals. To our knowledge, this individual variation in adherence activity has not been reported previously.

Cross-depletion experiments. Adsorption of saliva with HA beads did not deplete saliva of aggregating activity. This was probably due to the low surface area of HA beads, because saliva treated with HA powder lost all aggregating activity as has been reported previously (9). Preabsorption of saliva with G9B depleted saliva of more than 80% of the aggregating activity for G9B, but did not significantly reduce the adherence activity of the saliva (data not shown). These observations suggest that it is feasible to study adherence and aggregation with the same saliva sample, since coating of the HA beads for adherence assays does not deplete aggregating activity, nor does coating of bacteria with aggre-

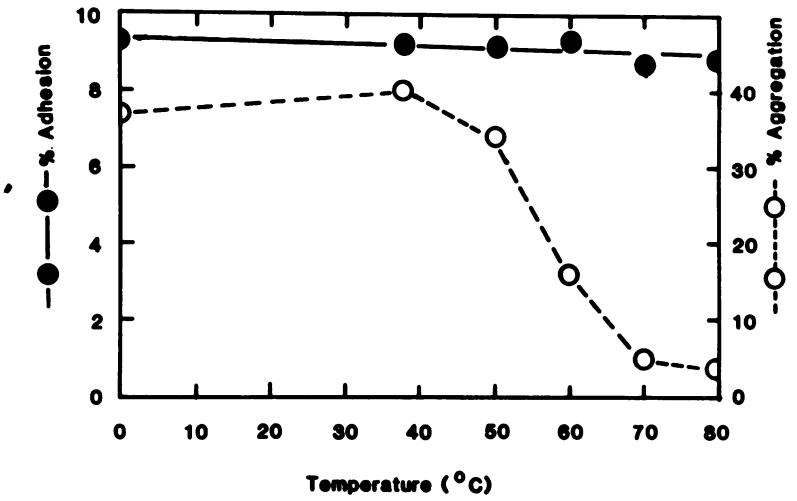

FIG. 2. Effects of temperature on salivary aggregation of strain M-5 and adherence of strain G9B. Adhesion to uncoated HA is 9.2%. The data are plotted as gross percent adherence.

gating factors (8, 16) reduce adherence activity.

Effect of heating saliva on aggregation and adhesion. Saliva was heated at 37 to 80°C for 30 min and then tested for its ability to promote aggregation of M-5 and adhesion of G9B (Fig. 2). At temperatures above 50°C, aggregating activity was lost, whereas adherence activity was maintained through 80°C. A similar loss of aggregating activity was observed when strain G9B was used as the test organism. In contrast, saliva heated at 60°C for 30 min and diluted 1:4,000 (the midpoint of linear decrease in Fig. 2) showed no decrease in adherence activity.

DISCUSSION

Our findings indicate that the processes of streptococcal adhesion and aggregation demonstrate strikingly different properties. The most surprising observation in these studies was the extremely high activity of salivary adherence factor(s). The serial dilution tests used to determine the titer of adherence activity show that the linear range for adherence is ca. 0.1 to 1.0 μg of protein per ml. In contrast, the linear range for salivary aggregating factors is 100 to 1,000 μg of protein per ml (8). Such differences signify that the salivary adherence factor is 100 times more active than the aggregating factor(s). The distinction between these two phenomena was also reported recently by Liljemark et al. (13), using an assay which more closely mimics in vivo adherence and aggregation. They suggest that salivas which promote aggregation show less adherence and vice versa. In our studies no significant correlations were observed between adherence and aggregation either in terms of individual saliva samples or in terms of bacterial specificity. These results are consonant with Mandel's ideas (17, 18) concerning the bacterial clearance mechanisms of the oral cavity, although clear indications of reciprocity were not demonstrated.

The observation that heating saliva reduces aggregation would explain why other investigators using heated saliva samples found little or no aggregation activity with some *S. sanguis* strains (11, 12, 19). No effects of heating were noted for the adherence of G9B or most other strains tested (unpublished data). Although this observation suggests that adherence-promoting activity in saliva is not sensitive to heat inactivation, the result may also reflect the small amount of protein required for adherence.

The hypothesis that different salivary factors are responsible for aggregation and adherence is consistent with the idea that there may be a repertoire of salivary proteins which can react with specific bacterial surface receptors. Cross-reactions among these proteins and different bacteria would insure efficient removal of bacteria from the mouth through aggregation or deposition on the tooth surface via the adherence mechanism. Clearly, purification of both the salivary factors as well as the bacterial surface receptors will be required to understand the mechanism involved in these processes.

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