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Aggregation and Adherence of *Streptococcus sanguis*: Role of Human Salivary Immunoglobulin A

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Fourteen freshly isolated strains of *Streptococcus sanguis* were obtained from the dental plaque of five healthy adults. Whole saliva was collected concomitant with the plaque isolates from the five subjects, and a second whole saliva sample was collected 10 weeks later. All possible combinations of the first five saliva samples, the second five saliva samples, and 14 strains of bacteria were tested for aggregation. Of the 140 combinations examined, 108 of 140 (77%) of the strains aggregated with the first saliva samples and 95 of 140 (68%) aggregated with the second saliva samples. Overall, 72% of the strains aggregated with both the first and second saliva samples. Removal of immunoglobulin A (IgA) from these same salivas resulted in 38 of 108 (35%) of the aggregates decreasing in intensity with the first saliva samples and 27 of 95 (29%) of the aggregates decreasing in intensity with the second saliva samples. No aggregates increased in intensity with saliva samples when IgA had been removed. Removal of IgA from saliva also resulted in a mean decrease of 46% in adherence of *S. sanguis* to hydroxyapatite coated with the IgA-deficient saliva. Several strains of *S. sanguis* were shown to aggregate strongly with human salivary and colostral IgA. In addition, *S. sanguis* strain S7 showed a 31% stimulation of adherence to hydroxyapatite precoated with human salivary IgA over the uncoated controls. Stepwise removal of IgA from saliva resulted in a decrease in aggregation intensity from strong (4+) to weak (1+ to 2+). Similarly, the adherence of *S. sanguis* to hydroxyapatite coated with these saliva samples decreased linearly as the salivary IgA was depleted. Alternatively, the addition of a small quantity of salivary IgA (20 μg/ml) to progressively diluted saliva maintained a high level of adherence and strong aggregation until the saliva dilutions reached between 1:8 in the adherence experiments and 1:32 for the aggregations. These data indicate that salivary IgA may play an important role in the microbial ecology of human dental plaque formation.

Saliva and various salivary components have been recognized for several years to play an important role in the initial colonization of the tooth surfaces by indigenous oral bacteria and the accumulation of these bacteria resulting in the formation of human dental plaque (12, 13, 15, 30, 36). These same salivary components (i.e., the mucinous glycoproteins, agglutinins, lysozyme, and the immunoglobulins) have also been shown to be a major controlling factor(s) in the indigenous microbial ecology of the oral cavity (11, 17, 19). Numerous reports have implicated one of these factors (salivary immunoglobulin A [IgA]) as a potentially important component in this ecosystem (4, 11, 22, 37).

Although small quantities of serum IgG, IgA, and IgM are found in the oral cavity, their presence is usually attributed to leakage through the gingival crevice (5, 25, 33, 35). However, secretory IgA has been shown to be the major immunoglobulin and is secreted primarily by the salivary glands (2, 5, 9, 33). It has been shown to comprise approximately 2% of the dry weight of human dental plaque and has been estimated to be 1.6 to 2.7% of the total protein found in plaque (33). It has also been shown by several investigators that IgA is found in the salivary pellicle in considerable quantities (18, 29) and that it is present in a biologically active form (33). The possibility that salivary aggregation of oral bacteria is influenced by an immunoglobulin was first suggested by Hay (16) when he reported a low-molecular-weight fraction from the gel filtration of saliva, thought to be immunoglobulin, caused the aggregation of *S. sanguis*. McBride and Gisslow (23) have also suggested that the neuraminidase-resistant, heat-sensitive system they observed with *S. sanguis* aggregation may be due to a specific IgA antibody effect. In addition, studies by Brandtzaeg et al. (5) and

Arnold et al. (3) have shown that bacteria found in saliva and plaque are coated with salivary IgA. Tomasi (35) has also reported that secretory IgA antibodies are capable of specifically binding to antigenic components on the surfaces of bacteria and of causing their agglutination. Finally, two reports by Arnold et al. (2, 4) have indicated that parotid saliva from immunodeficient patients containing no secretory IgA failed to agglutinate certain strains of *S. mutans*. This further suggests a potential antibody effect, specifically salivary IgA.

As an example, salivary IgA has been found to play a key role in the defense of the host against the colonization of mucosal tissues by oral streptococci (11, 37). Since secretory IgA can prevent the colonization of bacteria, a concerted effort has been and is being made to use this ability to develop an effective caries vaccine (24, 26, 34). This approach has been shown to be partially effective in the control of *S. mutans* populations in various animal caries vaccine models (26, 34). However, very limited knowledge is available concerning its "normal" role in the ecology of dental plaque formation. Thus, the primary aim of this study was to attempt to further elucidate the role of salivary IgA on the aggregation of *S. sanguis* and its role in the adherence of *S. sanguis* to saliva coated hydroxyapatite.

MATERIALS AND METHODS

Cultures and cultural conditions. Fourteen strains of *S. sanguis* were isolated from the plaque of five healthy adult subjects (two males, three females) ranging in age from 21 to 35 years. Supragingival plaque samples were obtained with sterile McCall curettes, placed in Ringer's solution, and sonified in a Branson Sonifier model 185S at a number 2 setting for 15 s. The samples were then serially diluted and plated on mitis salivarius agar (Difco Laboratories). Approximately three strains were isolated from each individual. Strains which differed in colonial morphology and corresponded with the criteria of Carlsson (7) for the identification of *S. sanguis* were chosen. After isolation, the freshly isolated strains were stored frozen on glass beads (27) at −100°C in a Revco Ultra-low freezer. The strains were grown in Todd-Hewitt broth (Difco) at 37°C in anaerobic jars (BBL Microbiology Systems) in an atmosphere containing 80% N_2–10% CO_2–10% H_2. To keep laboratory transfers to a minimum, each strain was grown from frozen or lyophilized stocks weekly. Additional strains of *S. sanguis* used in these studies included a well-characterized strain M5, courtesy of B. Rosan, strain H7PR5 from the Forsyth Dental Center culture collection, and strains S7, S18, J4, LT, and SH from our own collection. All the laboratory strains were handled and stored in the manner described above.

Saliva collection and removal of salivary IgA. Whole saliva for use in the aggregation experiments was collected from each of the five subjects concomitant with the plaque collections and again 10 weeks later. Saliva for the preparation of purified salivary IgA was collected from two of these five subjects, and that for use in the adherence assays was collected from several healthy adults, including the initial five subjects and other adults.

All whole saliva samples were Parafilm-stimulated, pooled, and clarified at 10,000 × *g* for 10 min, and the supernatants were heated at 60°C for 30 min and stored at −100°C in a Revco freezer. Whole saliva samples used within 3 h were kept ice cold and not heat treated. Parotid saliva was collected by lemon drop stimulation and a Curby cup. This saliva was processed similarly to the whole saliva. The IgA from all saliva samples was removed by gradually adding rabbit anti-human IgA specific for the α-chain (Miles Laboratories) to the saliva. The saliva-anti-IgA mixture was incubated at 37°C for 60 min and stored for 48 h at 4°C. The complexed IgA was removed by slow centrifugation at 2,500 × *g* for 1 h. Control rabbit sera not immunized against human IgA were also mixed with the saliva samples under the same conditions and protein concentration and served as controls. Protein was quantified, using the Bio-Rad protein assay with bovine serum albumin as standard. Removal of the IgA was monitored with radial immunodiffusion plates (Behring Diagnostics, LC Partigen plates; Human colostral 11S IgA was generously donated by J. R. McGhee and used as the standard). Levels of salivary IgM were also monitored but were undetectable (Behring Diagnostics, S. Partigen plates). Human serum IgG was obtained from Miles Laboratories.

Preparation of salivary IgA. Salivary IgA was separated from pooled whole saliva which had been extensively dialyzed against water and lyophilized, by gel filtration, utilizing Sepharose 4B (Pharmacia Fine Chemicals) by the method of Hay (16). The lyophilized saliva was rehydrated in 2-ml quantities representing a 5× concentrated solution. Fractions (2 ml) were collected and monitored at 280 nm by using an LKB fraction collector and the Uvicord II system. Five peaks were identified and lyophilized (Fig. 1). Fraction A, the void volume, contained no IgA; fraction AA contained both IgA (42% of dry weight) and other proteins. Fraction B was predominantly IgA (92% of dry weight). Fractions C and D contained IgA levels of 12 and 1%, respectively. A quantitative comparison of protein obtained from one of the IgA-containing peaks (peak B) by using the Bio-Rad protein assay as well as dry weight, with IgA content as measured by radial immunodiffusion, yielded an almost 1:1 relationship.

Aggregation and adherence assays. Bacterial aggregations were studied using a mixture containing 0.1 ml of 0.01 M phosphate buffer with 0.05 M KCl and 0.001 M $CaCl_2$ at pH 6.0; 0.1 ml of washed bacteria suspended in this buffer to an optical density of 1.0; and 0.1 ml of saliva or other material to be tested. The reaction mix was blended in a Vortex mixer and slowly shaken in a water bath for 1 h at 37°C. Mixtures lacking either saliva or bacteria were routinely prepared and served as controls. Aggregations were scored visually and independently by two persons. Numerical scores from 0 to +4 were assigned to designate samples

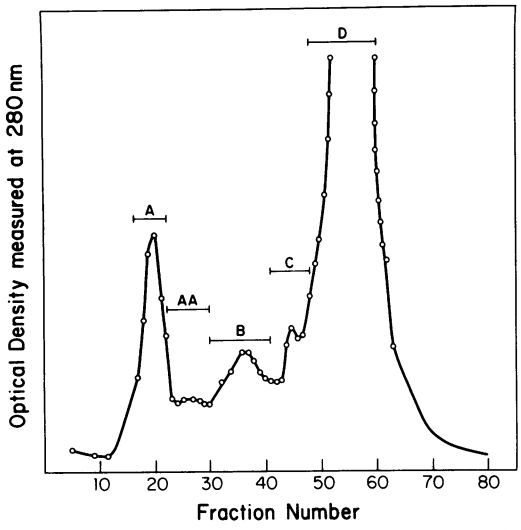

FIG. 1. *Sepharose 4B gel filtration of human whole parafilm-stimulated saliva. Five fractions, A, AA, B, C, and D, were obtained.*

ranging from no aggregation to complete aggregation with large aggregates and no turbidity of the supernatant fluid. Changes in the intensity of aggregations between individuals, and over time (i.e., at a 10-week interval), were recorded only if the change was at least 2 full units (e.g., +1 to +3). All aggregations were repeated at least once.

The adherence assay which detects the attachment of bacteria to spheroidal hydroxyapatite (SHA; Gallard-Schlessinger) follows the method described by Liljemark et al. (J. Dent. Res. Abstr. 1978, p. 418) and is similar to the one described by Clark et al. (8). Cell suspensions were prepared from stationary-phase cultures grown in Todd-Hewitt broth containing a final concentration of 10 μCi of [*methyl*-^{3}H]thymidine (Research Products International Corp.) per ml. The bacteria were harvested by centrifugation (10,000 × *g* for 10 min), washed twice with saline, and suspended in the phosphate buffer previously described to a concentration of 2.0×10^9 cells per ml. These suspensions were routinely sonicated for 10 to 15 s with a Branson Sonifier, model 185S, at a number 2 setting to eliminate any chains of bacteria. Before assaying bacterial adherence, 10-mg quantities of SHA were placed into separate culture tubes and equilibrated overnight with the phosphate buffer. Collection and preparation of saliva for the coating of SHA have been previously described. However, when possible, saliva was collected and used the day of the experiment. Before coating with saliva, the SHA was washed once with the phosphate buffer to remove any "fines," and the excess buffer was aspirated. Each 10 mg of SHA was mixed with 1.0 ml of saliva and incubated from 30 to 60 min at 37°C on a Roto-Torque (Cole Parmer Instruments, Chicago, Ill.) to ensure uniform coating. After coating, the excess saliva was aspirated and the SHA was washed with buffer into a clean reaction tube (50-ml Pyrex round-bottomed centrifuge tubes), and the excess buffer was aspirated. To test for adsorption of bacteria to this saliva-coated SHA, 1.0 ml of the washed radiolabeled bacterial suspension was added to each reaction tube and incubated in a water bath at 37°C for at least 1 h. The SHA bacterial mixture was sufficiently agitated to keep the SHA in suspension. After the incubation the SHA and bacteria were allowed to settle for 30 s, and unattached bacteria were removed by aspiration. The SHA-bacteria mixture was washed into a second 50-ml tube. The SHA-bacteria were washed three more times in this tube. The SHA and adsorbed bacteria then were finally washed into a scintillation vial; the excess buffer was removed and dried in a 37°C incubator overnight. The radioactivity was monitored in a Packard liquid scintillation spectrometer. The number of bacteria adsorbed to the SHA was expressed as the number of bacteria adhering to 10 mg of SHA. The specific activity of the radiolabeled *S. sanguis* was generally between 3.0×10^3 to 9.0×10^3 bacteria per cpm. No quenching of counts occurred with this amount of SHA. All experiments were run under saturating conditions of bacteria to SHA and always included a bacterial control minus the SHA, which was usually less than 1% of the bacteria adherent to the SHA. Changes in adherence levels from untreated controls of greater than 20% reach a level of statistical significance of $P > 0.005$ (Student's *t* test).

RESULTS

Characteristics of the aggregating activity of fresh oral isolates of *S. sanguis*. The initial aggregation experiments were designed to observe the interactions between fresh oral isolates of *S. sanguis* and saliva; the 14 strains of *S. sanguis* and the saliva samples that were twice collected from the same five individuals (see Materials and Methods) and 140 aggregations examined (all possible combinations between both saliva samples and strains). Less than 100% of *S. sanguis* strains aggregated with the saliva samples; 108 of 140 (77%) of the 14 strains aggregated with the first five saliva samples, and 95 of 140 (68%) of the strains aggregated with the second five saliva samples. However, 2 of the 14 strains did not aggregate with any of the saliva samples and were removed from the study.

Comparing the aggregations of the remaining 12 strains and first saliva samples with the second saliva samples, it was found that 202 of 220 (84%) of the possible combinations aggregated with both the first saliva samples collected and the saliva samples of the same individuals collected 10 weeks later (second saliva samples). No change occurred between the aggregations with the first saliva samples and second saliva samples 73% of the time, which suggests a generally stable and reproducible system. It was also observed that no specific patterns of aggregation existed. The two nonreactive strains eliminated from the study were obtained from dif-

ferent subjects. The saliva samples from two of the subjects tended to be more highly reactive toward all strains than the others, and that of one subject was very unreactive. No exclusive intrasubject aggregations were observed. It is now clear that no inter- or intrabacterial relationships between the aggregations or intensity of aggregations of a subject's strains and saliva samples were predictable.

Influence of salivary IgA removal on *S. sanguis* aggregation. Because salivary IgA is known to aggregate oral bacteria (3, 37), we examined its relationship in the aggregations discussed above. This large number of bacteria-saliva interactions was examined to be certain that any effect discerned would be meaningful.

The combinations which aggregated were examined with the same saliva samples treated to remove IgA. Aggregations of *S. sanguis* strains in saliva samples with IgA were greater than aggregations in the same saliva samples 38 of 108 times (35%) in the first and 27 of 95 times (29%) in the second saliva samples. In contrast, none of the aggregations in saliva samples without IgA was greater than saliva samples with IgA. Generally, only the 3+ or 4+ aggregations were affected and were reduced to 1+ or 2+. Occasionally a 2+ or 1+ aggregation was affected, but almost never was a 3+ or 4+ aggregation not affected. Thus, removal of IgA did not drastically affect aggregation, but the effect was consistent. None of the aggregations utilizing saliva samples without IgA was greater than 2+. These results suggested a potentially important role of IgA in these interactions with saliva.

Aggregation of *S. sanguis* strains with immunoglobulins and salivary fractions. Aggregation of *S. sanguis* strains, including both fresh oral isolates and several well-characterized strains, was tested directly with the various fractions obtained from the gel filtration of whole saliva, as well as 11S human colostral IgA, human serum IgG, and whole saliva (Table 1). Aggregation with whole saliva was strong with all but two of the strains tested, (M5, SH) (Table 1). The void volume fraction and fractions AA, B, and colostral IgA mimic the results obtained in aggregations with whole saliva, but with less intensity. In contrast, serum IgG does not aggregate these *S. sanguis* strains. Aggregation of the strains with fractions C and D was variable.

Effect of salivary IgA on the adherence to saliva-coated SHA. Experiments designed to measure the role of salivary IgA on the adherence of *S. sanguis* to saliva-coated SHA were done several ways. Except for comparative purposes, only strains of *S. sanguis* which showed reactivity with IgA in the aggregation assay were chosen for use in the adherence assays, because nonaggregating strains do not show a stimulated adherence to saliva-coated SHA.

Table 2 shows the effect of selective removal of IgA from saliva samples, collected from three individuals, that were used to coat the SHA. Antisera to IgA were added to slight excess to assure complete removal, and rabbit sera were added in identical amounts and concentration based on total protein. Whereas saliva treated with rabbit anti-human IgA decreased adherence an average of 46%, the saliva treated with nonspecific rabbit sera showed no change.

To test the direct effect of salivary IgA and serum IgG on the adherence of *S. sanguis* S7 to SHA, similar amounts of IgA from fraction B and IgG were used to coat the SHA directly (i.e., 100 μg of each per ml). This direct coating of SHA by IgA and IgG affected the adherence of *S. sanguis* differently. Based on adherence to uncoated SHA as 100%, the adherence to SHA coated with IgA and with IgG was at 131 and 38%, respectively.

The effect of partial removal of IgA from the saliva with rabbit anti-human IgA was next observed. This stepwise removal of salivary IgA, which ranged from no removal to complete removal, resulted in a linear decrease in *S. sanguis*

TABLE 1. *Aggregation of S. sanguis with saliva, saliva fractions, and immunoglobulins*

Strain	Control	Saliva	Fractions[a]					Colostral IgA[b]	Serum IgG[c]
			A	AA	B	C	D		
M5	–	+/–	–	–	–	–	–	–	–
H7PR5	+/–	4+	2+	3+	4+	3+	2+	2+	1+
SH	–	1+	+/–	+/–	+/–	–	–	1+	–
J4	–	3+	2+	2+	3+	–	–	3+	–
LT	–	3+	2+	2+	1+	–	–	2+	–
S7	–	4+	4+	4+	4+	–	2+	3+	–
S18	–	4+	3+	3+	3+	2+	–	2+	–

[a] Equal protein amounts used, final concentration 20 μg/ml
[b] Final concentration, 83 μg/ml
[c] Final concentration 67 μg/ml

TABLE 2. *Adherence of S. sanguis J-4 to SHA coated with saliva without salivary IgA*

Saliva	Treated with[a]	% Adherence	No. of bacteria adhering ($\times 10^8$)
A	NT	100	2.55
	Anti-IgA	62	1.59
	Control sera	100	2.54
B	NT	100	2.30
	Anti-IgA	51	1.17
	Control sera	98	2.26
C	NT	100	2.17
	Anti-IgA	48	1.03
	Control sera	113	2.45

[a] NT, No treatment. Equal protein amounts of rabbit anti-human IgA and control rabbit sera were used.

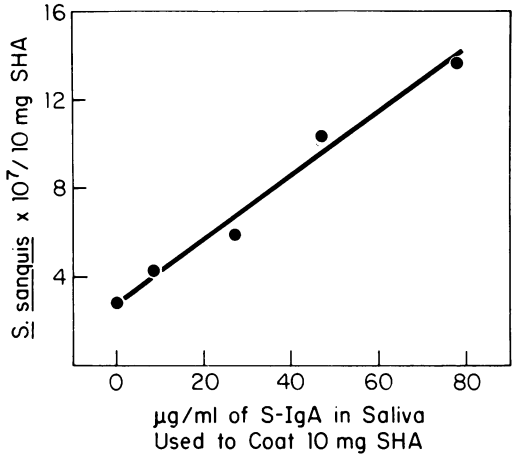

FIG. 2. *Adherence of S. sanguis strain J4 to SHA coated with saliva containing variable amounts of salivary IgA.*

adherence to treated saliva-coated SHA (Fig. 2). The stepwise removal of IgA from parotid saliva followed a similar pattern (data not shown).

To further quantify the effect of salivary IgA on the adherence of *S. sanguis* to SHA, a constant amount (20 µg/ml) of salivary IgA was added to $\log_2$ dilutions of saliva from 0 dilution to 1:32 before coating the SHA. Similarly, using the same saliva samples and extending the dilution to 1:128, *S. sanguis* was incubated in the aggregation assay conditions. Both aggregation and adherence levels decreased at a much slower rate when IgA was added (Fig. 3).

DISCUSSION

It is known that several substances found in saliva are involved both in aggregation and in the adherence of the bacteria to the pellicle and the subsequent formation of dental plaque. The agglutinating and adherence factors found in saliva appear to be numerous. Mucinous glycoproteins (10, 12, 16), including those containing sialic acid (19, 23) and blood group reactivity (14), lysozyme (22) and immunoglobulins (2, 3, 32), have all been implicated. Alternatively, the ecological importance of agglutination in the oral cavity has been proposed as a possible mechanism of protection by removal of bacteria from the oral cavity through the swallowing of large aggregates (37, 38). The role of both agglutinin and adherence factor may be a body defense system of antigen disposal, countering bacterial adherence to the pellicle. As an example, whole stimulated saliva both aggregates *S. sanguis* and causes this bacterium to show enhanced adherence to SHA coated with it in vitro. The mucinous glycoproteins, when isolated from saliva, play this dual role. Another substance found in significant quantities in the pellicle is salivary IgA (33). The salivary IgA causes bacterial aggregation and could also be involved in adherence and in the accumulation and cohesiveness of plaque, as indicated by the results of this report. However, the importance of glycoprotein-mediated aggregation and adherence of *S. sanguis* should not be discounted.

It has been widely reported that *S. sanguis* strains aggregate with whole human saliva (10,

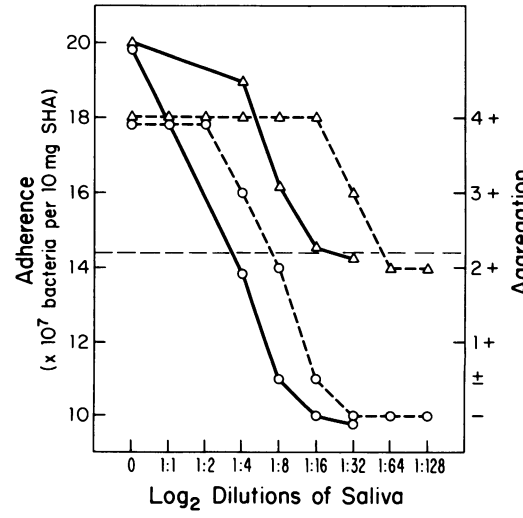

FIG. 3. *Aggregation of S. sanguis strain S-7 with dilutions of whole saliva supplemented with salivary IgA at a final concentration of 20 µg/ml (Δ- - -Δ), and without salivary IgA supplementation (O- - -O). The adherence of strain S-7 to SHA coated with the same salivary IgA-supplemented saliva (Δ—Δ) and unsupplemented (O—O). Broken horizontal line indicates level of S. sanguis adherence to SHA coated with salivary IgA alone.*

12, 17). Our results indicate that most, but not all, *S. sanguis* strains were found to aggregate with whole saliva. These aggregations with the saliva samples suggest a less than universal relationship in the aggregation of *S. sanguis* strains with whole saliva than is generally implied in the literature. However, since this aggregation phenomena is frequent among *S. sanguis* strains and between the saliva samples from various individuals, a certain commonality may exist among the strains of *S. sanguis* and the response of the host to them. For example, it has been shown that *S. mutans* strains possess multiple antigens that correspond to serogroups, which elicit a secretory IgA response both in colostrum and in saliva (3). It also has been reported by Rosan (31) and Applebaum and Rosan (1) that *S. sanguis* strains possess a variety of antigens on their cell surfaces. Furthermore, Bratthall and Gibbons (6) have shown that strains of *S. sanguis* and IgA preparations from the saliva from the same individual exhibited varying degrees of agglutinating activity, which they related to the difference in antigenic composition of the different isolates. The pattern of aggregations observed in this study could also be similarly explained. The freshly isolated *S. sanguis* strains that did not aggregate with whole saliva and the strains SH and M5 (Table 1) might not have the specific antigens necessary for aggregation. The difference in intensity of aggregations may also be due to the amount of antigen present on the various streptococcal strains surfaces, or it may be due to the type of antigen, whether a common one or one not occurring as frequently. Thus, the pattern of aggregations observed in this study could be explained by the antigenic diversity of the species.

It is even more significant, therefore, if no overall predictable model of aggregation occurred within our experiments, that the one pattern we did observe was the consistent decrease in aggregations with *S. sanguis* upon removal of IgA from whole saliva. Compared with aggregations with the same saliva samples with IgA, approximately one-third of the aggregations showed a significant decrease in intensity.

Furthermore, the results of this study suggest that salivary IgA plays a role in the adherence of *S. sanguis* to the salivary pellicle. Although the adherence assay system is more a model for colonization than accumulation, by varying the sequence of events in the assay selectively, the role of salivary IgA in the initial adherence to the saliva-coated SHA can be studied. First, the simple direct coating of SHA with salivary IgA resulted in enhanced adherence of *S. sanguis* strains. This contrasts strongly with IgG, albeit from sera, which strongly inhibits the adherence of *S. sanguis*. Furthermore, serum IgG does not aggregate *S. sanguis* (salivary IgG is difficult, if not impossible, to obtain; see reference 31 and Table 1). However, the contrast is apparent. Second, it was also observed that removal of IgA from whole saliva before coating the SHA effects a significant decrease in the adherence of *S. sanguis*. The decrease was generally of the order of magnitude of 50% (Table 2) and was not as drastic a decrease as has been observed in various adherence blocking experiments (20, 21) but was nonetheless significant. Third, the stepwise removal of IgA from saliva effected a linear decrease in adherence. The removal of IgA from parotid saliva also resulted in a similar decrease in the adherence of *S. sanguis*. Finally, the addition of constant small amounts of salivary IgA to progressively diluted saliva before coating the SHA with the saliva enhanced the ability of the saliva to maintain a relatively high level of adherence of *S. sanguis* (Fig. 3). A parallel phenomenon was also observed with the aggregation of *S. sanguis*.

In a complex ecosystem such as the human oral cavity, attempting to study any single factor (i.e., salivary IgA) amid the huge number of components in saliva poses many problems both in experimental design and in data interpretation. Although we have not proven that immune IgA is required for the aggregation and adherence of *S. sanguis*, the evidence presented here and in reports from other investigators shows that salivary IgA is present in a biologically active state in the salivary pellicle and in plaque, which strongly suggests that certain microorganisms, e.g., *S. sanguis*, may utilize, in part, this immunoglobulin to colonize and accumulate on tooth surfaces.

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