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Interference of Salivary Immunoglobulin A Antibodies and Other Salivary Fractions with Adherence of *Streptococcus mutans* to Hydroxyapatite

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The adherence of *Streptococcus mutans* to hydroxyapatite was studied in the presence of salivary fractions with varying activity of naturally occurring immunoglobulin A (IgA) antibodies. Human parotid saliva from different donors was fractionated by chromatography and compared. Salivary IgA antibodies had no decisive effect on the adherence of the *S. mutans* strain used. High-molecular-weight salivary components from some subjects had an adherence-promoting effect, whereas fractions collected after the void volume of a Sepharose 2B column always inhibited adherence. The data indicate that the influence of unfractionated saliva on adherence is dependent on the net effect of adherence-promoting and adherence-inhibiting components. This principle has to be considered when the effect of human saliva on microbial adherence is studied.

The role of *Streptococcus mutans* for the initiation of dental caries has stimulated considerable interest in the development of a caries vaccine. A basis for this work is the observation that salivary immunoglobulin A (IgA) antibodies can inhibit the adherence of streptococci to buccal epithelial cells (27). The use of streptococcal vaccines can influence the implantation of *S. mutans*, both in animals and in humans (12, 17, 23; J. Huis in't Veld and E. M. Kamp, abstr. 1292. J. Dent. Res. 60, special issue A. 1981, p. 632; M. F. Cole, C. G. Emilson, J. E. Ciardi, and W. H. Bowen, Abstr. 798. J. Dent. Res. 60, Special Issue A. 1981, p. 509). This effect can be explained by different mechanisms. Salivary IgA antibodies may interfere with the initial adherence and accumulation of *S. mutans* to teeth. Another possibility is that specific agglutination facilitates the elimination of *S. mutans* from the oral cavity. However, in addition to IgA antibodies, other salivary components, such as high-molecular-weight glycoproteins, can agglutinate *S. mutans* (11, 15). Furthermore, such glycoproteins can form a complex with salivary IgA (9, 24). The size of microbial aggregates due to variations of the concentration of the agglutinin may affect the adherence of bacteria to hydroxyapatite (HA) (18). It has also been reported that salivary IgA may promote the adherence of oral streptococci to HA (19, 25). Thus various possibilities exist with regard to the effect of salivary IgA antibodies and other components on *S. mutans* adherence.

In the present study we examined the effect of IgA containing salivary fractions on *S. mutans* adherence. This effect has been compared with that of other salivary fractions in parotid saliva from the same individual. Furthermore, saliva from various persons have been compared.

MATERIAL AND METHODS

Saliva. Parotid saliva, stimulated with citric acid, was collected by means of Lashley cups from two male and five female subjects. One of the females was selected from a group of IgA-deficient patients studied by J. Björkander, Department of Allergology, First Medical Service, Sahlgren's Hospital, Göteborg, Sweden. Twelve to fifteen milliliters of saliva was collected each time from every person. Ten milliliters of the saliva was immediately transferred to a Sepharose 2B column for fractionation. The remaining saliva was diluted in half in 0.010 M Tris-hydrochloride (pH 8) with 0.05 M NaCl and 0.01% NaN_3 (Tris buffer). The diluted saliva was kept at 4°C for later experiments.

Gel permeation chromatography. The parotid saliva was applied to a column (2.5 by 30 cm) of Sepharose 2B and eluted at 4°C with Tris buffer by the method of Kashket and Guilmette (15). Fractions of 3 ml were collected at an elution rate of 5 ml/h. The absorbance at 230 nm was determined in a Beckman DB-GT spectrophotometer (Beckman Instruments AB, Stockholm, Sweden). From every second fraction a 0.3 ml sample was analyzed for IgA and aggregating activity. The remainder of the fractions were kept cold until used.

Microorganisms. *S. mutans* strain KPSK2 (= JC2), serotype *c* (3), used for the aggregation test, was grown anaerobically in Jordan broth (14) for 16 h at 37°C. The bacteria were washed twice in 0.010 M sodium phosphate buffer (pH 7.2) containing 0.154 M NaCl and 0.02% NaN_3 (PBS). The cells were suspended in PBS to an optical density at 720 nm of 1.5 in a Vitatron colorimeter (UC 200; Dieren, Holland).

For the adherence studies, the bacteria were grown in a dialyzed tryptose-yeast extract medium (6), supplemented with 10 μCi of [*methyl*-^{3}H]thymidine (The Radiochemical Centre Ltd., Amersham, England) per ml. Standardized volumes of PBS-washed bacteria were lyophilized. Before use, the lyophilized cells were washed once and suspended to 20 ml in Tris buffer. The number of cells in this suspension was determined by counting in a Petroff-Hausser counter and found to vary between 6.4×10^8 to 9.4×10^8 cells per ml in different batches of growth.

Bacteria used in the absorption experiments were grown anaerobically for 16 h in a defined medium by the method of Carlsson [5; medium D1-D20 with the exception that $(NH_4)_2SO_4$ was replaced by NH_4HCO_3 in a concentration of 2 g/liter]. The cells were washed twice in PBS before use.

Tracing of aggregating activity. Samples of 0.2 ml from every second fraction from the Sepharose column were mixed with 0.2 ml of the bacterial suspension, optical density at 720 nm of 1.5, in polystyrol tubes (70 by 11 mm; Labassco, Göteborg, Sweden). A 0.2-ml amount of Tris buffer alone was also mixed with bacteria as a control. After 2 and 24 h at room temperature, the extent of aggregation was estimated with the aid of a stereomicroscope by the method of Olsson et al. (24).

Tracing of IgA. The IgA was traced by single radial immunodiffusion (22), using rabbit anti-human IgA (α-chain specific) serum and stabilized human serum (Beringwerke AG, Marburg, W. Germany) as standard.

Analysis of IgA antibody activity. Salivary IgA antibody activity to whole cells of *S. mutans* KPSK2 was determined with an enzyme-linked immunosorbent assay (ELISA) (10) as previously described (13). Briefly, whole cells of *S. mutans* KPSK2 were used to coat the microtiter plates (Microelisa plates, M 129 A, Dynatech, Novakemi AB, Enskede, Sweden). Two step dilutions (PBS + 0.05% Tween 20) of IgA-containing fractions of parotid saliva were added. IgA antibodies bound to the bacterial cells were assayed with anti-human IgA specific for α-chains (Dakopatts A/S, Copenhagen, Denmark) conjugated to alkaline phosphatase (~400 IU/mg of protein at 25°C, Sigma Chemical Co., St. Louis, Mo.). Enzyme activity retained in the wells was measured by adding the substrate, *p*-nitrophenyl phosphate dissolved in diethanolamine buffer (1 M, pH 9.8), and recording the optical density at 405 nm. IgA antibody activity is expressed as average optical density for duplicate samples multiplied by 100/t, where t = the number of minutes when the color development was stopped.

Absorption experiments. After chromatography on Sepharose 2B, IgA-containing fractions from 20 to 30 ml of parotid saliva from each of five subjects was individually pooled. The samples were then dialyzed repeatedly against distilled water and lyophilized. Before use, each pool was redissolved in 6 ml of Tris buffer. A pellet of washed cells, from growth in 20 ml of defined medium, was used to absorb 1.0 ml of concentrated IgA-containing fractions. The mixtures were kept at room temperature for 60 min and, to assure a uniform absorption, the mixtures were occasionally inverted several times. After centrifugation for 10 to 15 min at 6,000 × *g*, the supernatants were collected to be used in the adherence study. The choice of growth medium could have an effect on the ability of the bacteria to bind IgA antibodies. This was tested by absorbing saliva from one of the participants in the study with *S. mutans* KPSK2 grown in the three different media described above. Protein content in the unabsorbed and absorbed salivary IgA preparations was determined by the procedure of Lowry et al. (20) with tyrosine as a standard.

Adherence experiments with HA beads. The adherence experiments were performed as described by Clark et al. (7) with a few modifications. To remove "fines," a batch of spheroidal HA beads (BDH Chemicals Ltd., Poole, England) were washed with 0.2 M NaOH and rinsed with distilled water. The beads were then dried at 37°C. Before an experiment 40-mg quantities of beads in polystyrol tubes were washed once with Tris buffer. The number of ^{3}H-labeled bacteria added to each assay varied from 3.2×10^8 to 4.7×10^8 cells. In this method, a deviation in adherence level from buffer control of more than 20% reach a level of statistical significance. This is in accordance with other investigators (19).

Three sets of experiments were performed. (i) The HA was pretreated with the test solution before the ^{3}H-labeled cells were added; (ii) the test solution and the bacterial suspension were simultaneously added to the beads; and (iii) the bacteria were pretreated with the test solution before adding them to the beads.

(i) Pretreatment of HA. For pretreatment, 0.5 ml of test solution and 0.5 ml of Tris buffer were added to 40 mg of beads. As a control, 1.0 ml of buffer alone was added. The tubes were slowly and continuously inverted for 60 min at room temperature. The beads were washed once with Tris buffer before they were incubated with 1.0 ml of the ^{3}H-labeled bacterial suspension diluted in half with buffer. After this incubation the beads were washed twice.

To facilitate the transfer of the HA beads into scintillation vials and to reduce quenching, 0.2 ml of 6 M HCl was added to each tube which was blended in a Vortex mixer for 20 s. After 15 min the acid sample solutions were poured into the vials, and each tube was rinsed twice with 0.5 ml of buffer also added to the vial.

(ii) Simultaneous incubation. For the simultaneous incubation, 0.5 ml of bacterial suspension and 0.5 ml of test solution were concomitantly added to 40 mg of HA beads. Buffer was used as a control.

(iii) Pretreatment of bacteria. To pretreat bacteria, pellets from samples of the ^{3}H-labeled bacterial suspension were suspended in the test substances or Tris buffer. The mixtures were inverted several times during 60 min at room temperature. The cells were then washed once and restandardized to equal activities with buffer, and 1.0 ml was incubated as described above under (i) with 40 mg of untreated beads.

All experiments were performed in duplicate, and the samples were counted in a liquid scintillation counter (1215 Rackbeta, Wallac, LKB, Sweden, Sverige AB, Bromma, Sweden).

RESULTS

The relative adherence varied between 10 and 38% of the buffer control when the HA was pretreated with the IgA-containing samples from five individuals (Table 1). Absorption of these

TABLE 1. Adherence of *S. mutans* KPSK2 to HA beads pretreated with salivary fractions[a] with varying IgA activity to *S. mutans* KPSK2

Subject	No. of cells (10^7) bound per 40 mg of HA[b]		% of buffer control[c]		Measurement by ELISA[d] (absorbance at 405 nm × 100/*t*)		Protein (g/liter)	
	IgA	IgA_{abs}[e]	IgA	IgA_{abs}	IgA	IgA_{abs}	IgA	IgA_{abs}
a	4.1	3.7	27	24	1.25	0.12	1.3	1.3
b	4.8	4.0	31	27	0.50	0.07	0.9	1.0
c	3.6	3.5	24	23	0.91	0.18	1.2	1.0
e	1.6	3.5	10	23	1.76	0.25	3.2	3.3
f	5.8	5.0	38	33	1.95	0.27	0.8	0.8

[a] Concentrated IgA-containing fractions from parotid saliva separated on Sepharose 2B.
[b] 4.7×10^8 cells were added in the assay.
[c] The coefficient of variation from the numbers under this heading ranges from 1 to 5%.
[d] IgA antibody activity to *S. mutans* KPSK2 as determined in a dilution of 1/4.
[e] Absorbed with *S. mutans* KPSK2.

fractions with *S. mutans* KPSK2 had no decisive influence on adherence in four of the subjects. In one subject (e), approximately a twofold increase in the adherence of KPSK2 was observed after absorption of the IgA-containing salivary fraction. Repeating the experiment confirmed the deviating pattern for this subject.

In contrast to what was found with regard to adherence, absorption of the IgA-containing fractions resulted in an almost complete loss of specific IgA activity, as determined by the ELISA technique. Absorption with cells grown in the different media gave almost identical ELISA values. The absorption procedure had no measureable influence on the total protein content of the various fractions.

In another experiment pretreatment of the bacteria instead of HA was examined (Table 2). Also in this experiment the specific IgA activity was largely reduced by absorption although higher ELISA values were obtained in this series of salivas than in the preceding. The absorption did not markedly affect the relative adherence, nor was the relative adherence affected by an originally high or low activity of antibodies in the fraction. It should be observed that relatively more bacteria adhered when the bacteria were pretreated and the HA was not pretreated (74 to 118%) compared with the reverse (10 to 38%) (Tables 1 and 2).

The IgA-containing salivary fractions studied above were obtained by gel filtration of parotid saliva on Sepharose 2B. The elution profiles from six individuals were very similar (Fig. 1). A small protein peak was detected in the void fractions. Except for the IgA-deficient subject (d), IgA was always detected in the fractions collected before the maximum of the major protein peak.

Figure 1 also shows the adherence of *S. mutans* KPSK2 to HA beads when fractions of or unfractionated parotid saliva and the bacteria were added concomitantly to the beads. Relative to the buffer control, unfractionated saliva from all subjects but one (f) inhibited adherence by 70% or more. Different fractions of parotid saliva had different effects on adherence. In all subjects, fractions collected just before the maximum of the major protein peak were the most powerful inhibitors of *S. mutans* adherence to HA. In two of the subjects (e and f), fractions with aggregating activity promoted the adherence of *S. mutans* KPSK2.

In other experiments the HA beads were pretreated with unfractionated parotid saliva or with certain salivary fractions before incubation with the bacterial suspension. Pretreatment of the HA beads with unfractionated parotid saliva resulted in a relative adherence ranging from 24 to 222%. In all subjects fractions with aggregating activity increased and IgA-containing fractions decreased the adherence as compared with the buffer controls (Table 3).

DISCUSSION

In this study naturally occuring salivary IgA antibodies reacting with *S. mutans* did not have a decisive adherence-inhibiting effect. Other salivary fractions affected the adherence in different ways, but the pattern from different individuals showed a striking similarity (Fig. 1). An adherence-inhibiting effect was observed when IgA-containing salivary fractions were used for pretreatment of HA (Tables 1 and 3) but not when the bacteria were pretreated with these fractions (Table 2). This observation indicates that the adherence-inhibiting effect by the IgA-containing fractions shown in Fig. 1 is the result of a binding of these fractions to HA and not to the bacteria.

In the experiments only one strain of *S. mutans* was used. Both the aggregating and the IgA

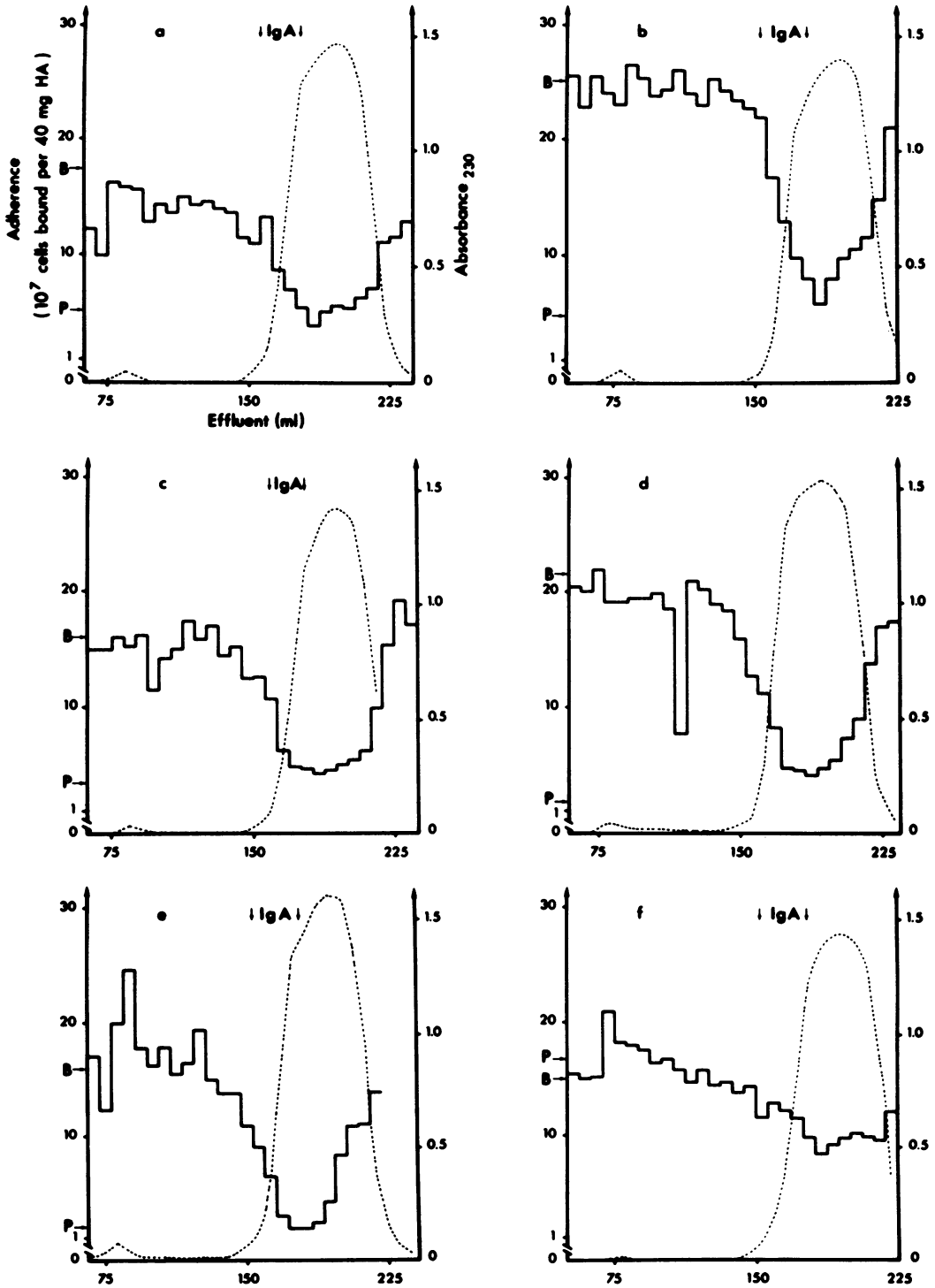

FIG. 1. Sepharose 2B chromatography of parotid saliva from six subjects (a through f). Dotted curve indicates absorbance at 230 nm. P indicates the adherence of *S. mutans* to HA when unfractionated parotid saliva was added concomitantly to the HA. B indicates the adherence of *S. mutans* to HA when buffer was added concomitantly to the HA. Adherence of *S. mutans* in the presence of fractions of parotid saliva is shown by the solid curve. The interval of IgA-positive fractions is indicated by ↓IgA↓. The numbers of cells added in the adherence assays were 3.2×10^8 for subjects a, c, and f; 4.7×10^8 for subject b; 4.3×10^8 for subjects d and e.

TABLE 2. Adherence to HA beads of *S. mutans* KPSK2 pretreated with parotid saliva fractions[a] with varying IgA activity to *S. mutans* KPSK2

Subject	Measurement by ELISA[b] (absorbance at 405 nm × 100/*t*)		Protein (g/liter)		No. of cells (10^7) bound per 40 mg of HA[c]		% of buffer control[d]	
	IgA	IgA_{abs}[e]	IgA	IgA_{abs}	IgA	IgA_{abs}	IgA	IgA_{abs}
a	5.78	0.57	1.7	2.0	15.0	14.5	94	91
b	2.79	0.20	1.7	1.6	18.4	16.5	115	103
c	3.78	0.36	0.9	1.0	18.8	11.8	118	74
e	5.16	0.69	2.4	2.5	15.0	16.9	94	106
g	3.98	1.10	1.6	1.9	16.5	17.4	103	109

[a] Concentrated IgA-containing fractions from parotid saliva separated on Sepharose 2B.
[b] IgA antibody activity to *S. mutans* KPSK2 as determined in a dilution of 1/4.
[c] 4.7×10^8 cells were added in the assay.
[d] The coefficient of variation from the numbers under this heading ranges from 1 to 10%.
[e] Absorbed with *S. mutans* KPSK2.

activity in parotid saliva vary against different *S. mutans* strains (2, 4, 21). These facts must be borne in mind in the discussion below where the possible influence on adherence of salivary IgA antibodies and other salivary non-immunoglobulin components is analyzed.

It is conceivable that salivary IgA antibodies can both promote and inhibit adherence to the HA surface. By binding unspecifically to HA, salivary IgA antibodies would expose the Fab fragment and thereby promote the adherence of specific bacteria. On the other hand, specific antibodies would block adherence if they bound specifically to adhesins on the bacterial surface. No such effect was, however, observed in the present study although bacteria were pretreated with various amounts of IgA antibodies. Still, an inhibitory effect of salivary IgA antibodies can not be completely ruled out. The lack of adherence-inhibiting effect could be explained by lack of antibodies specific for adhesins or by lack of adhesins on the surface of this laboratory strain. Consequently, more detailed knowledge has to be obtained about the adhesins of *S. mutans* before the biological effect of salivary IgA antibodies can be resolved.

In our experiments salivary antibodies did not markedly interfere with the initial adherence of *S. mutans* to HA. Unfractionated parotid saliva, however, decreased the relative adherence in all but one of the subjects. Furthermore, salivary fractions with nondetectable amounts of IgA had a pronounced inhibitory effect on adherence (Fig. 1). These observations indicate that other salivary components must be more important than IgA for the adherence-inhibiting effect of saliva observed in this and other studies (1, 7, 8, 16, 26). Olsson et al. (J. Olsson, T. Ericsson, J. Ciardi, and W. Bowen, abstr. 1048, J. Dent. Res. 60, special issue A, 1981, p. 571) recently reported that saliva contains at least two classes of components, which can either promote or inhibit adherence of *S. mutans* to HA. Our results support these observations. Thus high-

TABLE 3. Adherence of *S. mutans* KPSK2 to HA beads pretreated with parotid saliva, aggregating, or IgA-containing fractions from parotid saliva[a]

Subject	No. of cells ($\times 10^7$) bound per 40 mg of HA			% of buffer control[b]		
	Parotid[c] saliva	Aggregation fraction	IgA[d] fraction	Parotid saliva	Aggregating fraction	IgA[d] fraction
a	10.7	13.0	7.9	96	117	71
b	20.9	25.5	15.4	94	115	69
c	14.3	13.7	8.3	128	122	74
d[e]	4.7	23.1	6.7	24	117	34
e	19.0	14.6	3.4	222	170	40
f	18.2	13.9	9.3	163	125	83

[a] Number of cells added in the assays: 3.2×10^8 for subjects a, c, and f; 4.7×10^8 for subject b; 4.3×10^8 for subjects d and e.
[b] The coefficient of variation for the numbers under this heading ranges from 1 to 14%.
[c] Diluted 1/2.
[d] IgA-containing fractions from parotid saliva separated on Sepharose 2B.
[e] IgA-deficient subject.

molecular-weight components in saliva from subject e and f promoted adherence, whereas in all subjects salivary components of lower molecular weight inhibited the adherence of *S. mutans* KPSK2 to HA (Fig. 1). These findings indicate that the influence of unfractionated parotid saliva depends on the net result of adherence-promoting and adherence-inhibiting factors. This principle is clearly demonstrated by the observation of an increased adherence of *S. mutans* to HA in the presence of unfractionated parotid saliva from subject f. Fractionation of parotid saliva from this subject revealed an adherence-promoting capacity by the high-molecular-weight components and a relatively weak adherence-inhibiting effect by components of lower molecular weight (Fig. 1). These findings do not necessarily mean that the saliva from subject f has the same effect on other bacterial strains. Consequently, general conclusions concerning the effect of saliva on adherence should not be drawn from studies using saliva from only one or a few persons. Our results illustrate, however, a principle which has to be considered when the effect of human saliva on microbial adherence is studied.

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