Association between bacterial agglutinins and immunoglobulin A in human saliva

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Two distinct peaks were obtained when human parotid saliva was separated on a Sepharose 2B column. The bacterial agglutinating activity was concentrated to the void volume fractions whereas the IgA was found in the beginning of the second, large peak. Unfractionated saliva as well as the pooled agglutinin fractions, or a mixture of agglutinin and IgA, all induced the aggregation of KPSK2, a *Streptococcus mutans* serotype c strain. By adding anti-human-IgA antiserum to the whole saliva or to the mixture of agglutinin and IgA, the aggregation reaction could be eliminated. In order to achieve this effect the agglutinin and IgA had to be mixed prior to the addition of anti-IgA. Addition of anti-IgA antiserum to the agglutinin fraction only did not impair the aggregation of bacteria. The homologous reactions with anti-IgG antiserum did not give any inhibition effect. However, when human IgG was added to the saliva, or to the agglutinin, before the addition of anti-IgG, the aggregation of KPSK2 was again impaired. The data in this paper indicate that the agglutinins and the IgA antibodies in saliva may be normally associated with each other.

Key-words: Salivary agglutinins; microbiology; *Streptococcus mutans*

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High molecular weight substances in saliva with the capacity to agglutinate bacteria may have a decisive influence on the bacterial colonization of the human mouth. Such substances, including mucins or glycoproteins, can mediate cell to cell cohesion or prevent cell to surface adherence (6, 10).

Moreover, secretory immunoglobulins (s-IgA) may have the potential to govern bacterial ecology in the mouth. Williams & Gibbons (15) showed that s-IgA could inhibit adherence to buccal epithelial cells and proposed that a similar mechanism fostered the clearance of specific bacterial serotypes from the mouth.

Several studies have established that the effect of the agglutinins is different from that of the s-IgA, indicating that these substances are discrete entities and separately distributed in the saliva (8, 11, 12, 16). However, a peculiar observation was recently reported by Ericson, Bratthall & Rundegren (9), who showed that the agglutinating activity of parotid saliva was completely abolished after the addition of anti-IgA

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antiserum. This fact raises a few pertinent questions: Is IgA responsible for all the agglutinating activity in the saliva? Do anti-IgA antibodies react with salivary agglutinins in such a way that agglutination is prevented? Do IgA-anti-IgA-precipitates interfere with the reactivity of the agglutinins? Is IgA associated with salivary agglutinins in such a way that a reaction with anti-IgA will also include the agglutinins with a concomitant reduction of the capacity to induce agglutination?

In order to answer these questions the following series of experiments were performed.

MATERIAL AND METHODS

Microorganisms

Streptococcus mutans strain KPSK2, serotype c (3) was used. The strain was kept on blood agar plates and transferred to a defined medium (medium D 1 – D 20 according to Carlsson (4) with the exception that $(NH_4)_2\ SO_4$ was replaced by NH_4CHO_3 in a concentration of 2 g/l) for anaerobic growth at 37°C for 16 hours.

The bacteria were washed in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.154 M NaCl and 0.02 NaN_3 (PBS) and resuspended in PBS to an optical density (OD) of 1.5 units as measured in Vitatron colourimeter (UC 200: Dieren, Holland) at 720 nm. These suspensions were then used for the aggregation experiments.

Saliva

Parotid saliva was collected by means of Lashley cups from two male and two female subjects. The secretion was stimulated slightly by putting citric acid crystals on the tip of the tongue. Thirteen ml of saliva was collected at each time. Ten ml of this saliva was immediately transferred to a Sepharose 2 B column for fractionation. The remaining saliva was kept at +4°C (2–3 days) to be used in later experiments together with the eluates from the Sepharose column.

Gel permeation chromatography of saliva

Ten ml of parotid saliva was applied on top of a 2.5 x 38 cm column of Sepharose 2 B and eluted at +4° C with 0.01 M Tris-HCl, pH 8.0, with 0.5 M NaCl and 0.01 % NaN_3 in accordance with Kashket and Guilmette (11). 3 ml fractions were collected at an elution rate of 5 ml per hour. Occurrence of protein in the fractions was determined by OD measurements at 230 nm (Beckman DB-GT spectrophotometer).

IgA determination

Every second fraction was assayed for IgA content by means of single radial immunodiffusion (14) using a human serum standard and a specific rabbit antiserum (Behringwerke A G Marburg, Germany). All fractions positive for IgA were pooled and stored at 4°C and will be denoted «IgA» below. At the same time the IgA content in the original unfractionated parotid saliva was determined.

Tracing of aggregating activity in fractions

Two tenths of every second fraction was mixed with 0.2 ml of the washed bacterial suspenion ($OD_{720\ nm}$ = 1.5) in 70 x 11 mm polystyrol tubes (Labassco, Göteborg, Sweden). As controls 0.2 ml of whole parotid saliva diluted 1:2 with Tris-buffer, and 0.2 ml of Tris-buffer alone was mixed with 0.2 ml of bacterial suspension. After 3 and 24 hours at room temperature, aggregation was estimated by the aid of a stereomicroscope. Fractions showing aggregating

capacity were pooled and kept cold and will be referred to as «agglutinins» although these fractions certainly might contain other substances as well.

Antisera and IgG standard

Rabbit antisera to human IgA (α-chain) and human IgG (γ-chain) (Behringwerke A G) were used, diluted 1:2 with PBS containing 0.02 % NaN_3. In some experiments, 15μg of human IgG-standard (Behringwerke A G) was added to 0.5 ml of parotid saliva or «agglutinin» while 5 μg IgG was added to the «IgA» which was diluted three times during gel filtration

Assay for measuring aggregating activity

A spectrophotometrical method was used which continously records the progressive decrease in OD caused by the formation and setting of bacterial aggregates (7). To a test solution at 0.6 ml (see below), 1.2 ml of the bacterial suspension was added and the OD recorded for 90 minutes in a Vitatron (UFD 100 Dieren, Holland) spectrophotometer at 720 nm).

Test solutions

The 0.6 ml test solution consisted of two main groups of components. The first part was of salivary origin: 1. Whole parotid saliva. 2. «Agglutinins». 3. «IgA». 4. «Agglutinins» + «IgA» (1/1, v/v). This part accounted for 0.5 ml of the test solution. The second part, which accounted for 0.1 ml of the test solution, consisted of PBS-buffer or of anti-Ig antiserum components diluted 1:2 with PBS.

The protein content of the pooled agglutinin fraction was about 0.015 g/l and that of the IgA about 0.2 g/l. Whole parotid saliva contained around 2 g/l according to the method of Lowry et al. (13) with Tyrosine standard.

In further studies of the mechanisms involved in bacterial aggregation the agglutinating potential of various combinations of salivary and anti-Ig antiserum components were examined. The contents of the mixtures for these experiments can be seen in Table 1–3. In one series of experiments, the IgA-anti-IgA complexes were eliminated by centrifugation (2000 x g, 15 min), and the supernatant fluid was used for aggregation experiments.

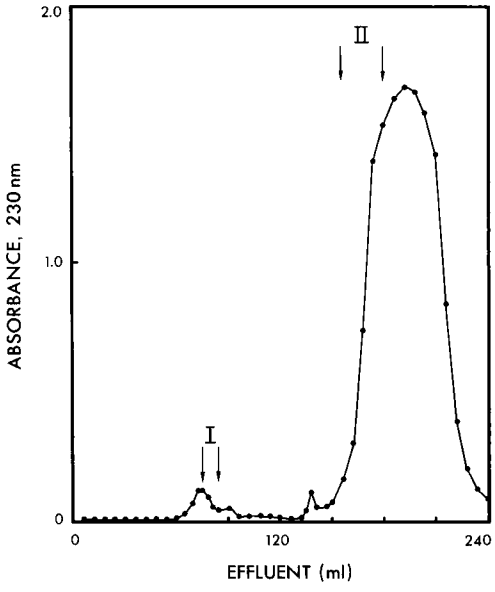

Fig. 1. Elution profile of human whole parotid saliva from a Sepharose 2B column. Fractions containing agglutinins (I) and IgA (II) are indicated.

RESULTS

In Fig 1, a typical profile is shown of parotid saliva eluted from a Sepharose 2B column. Aggregating activity was concentrated in the first peak and IgA was found in the beginning of the second large peak. No aggregating activity could be found in the latter. The lack of IgA in the first peak was confirmed by

control experiments using a modified ELISA technique (2). Bacteria were covered with agglutinins and tested with enzyme-linked anti-IgA. No reaction occurred when the substrate for the enzyme was added. Further control experiments showed that components of the anti-IgA or anti-IgG antisera did not react directly with strain KPSK2 either in such a way that the effect of subsequent added agglutinins was affected or that these anti-sera themselves induced agglutination.

Control experiments confirmed that addition of anti-IgA but not anti-IgG antiserum to parotid saliva eliminated aggregating activity of the saliva (Fig 2, Table 1). When the same antiserum was added to the pooled agglutinin fraction only, the aggregating activity was not affected (Fig 3, Table 1). When anti-IgA was added to a mixture of agglutinin and IgA, the same effect as with whole parotid saliva was obtained.

These experiments were repeated by adding various components of the mixtures in different sequences. The results are summarized in Table 2. It is evident that the order by which the components were mixed was of critical importance. To eliminate the aggregating capacity, IgA had to be mixed with agglutinin before anti-IgA was added. Further experiments showed that the same results were obtained even if the agglutinin and IgA had been mixed only for a few minutes.

It was considered of interest to study whether an IgG-anti-IgG reaction could influence the aggregating pattern of saliva similar to the IgA-anti-IgA system. Table 3 shows that anti-IgG could eliminate the aggregation of strain KPSK2. However, it was again found that IgG had to be added to the agglutinin, or the saliva, prior to the anti-IgG to obtain this effect.

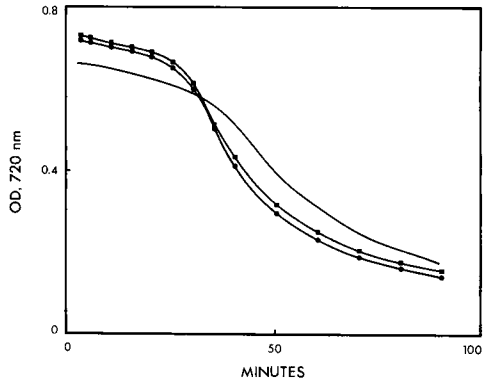

Fig. 2. Aggregation of *S. mutans* KPSK 2, induced by parotid saliva (——) or by parotid saliva in the presence of anti-IgG (•——•) in the reaction mixture. If anti-IgA (▪——▪) was present, no aggregation was obtained.

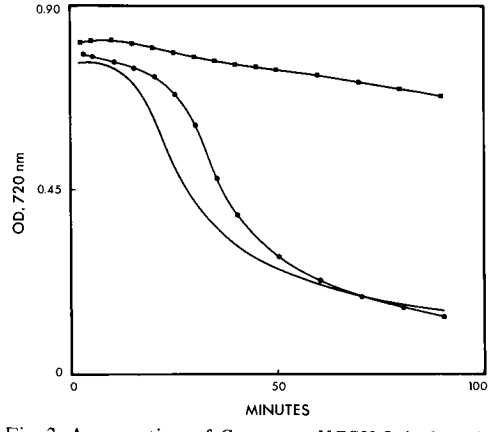

Fig. 3. Aggregation of *S. mutans* KPSK 2, induced by the agglutinin fraction of parotid saliva (——) or by the agglutinin fraction in the presence of anti-IgA (▪——▪) or anti-IgG (•——•) in the reaction mixture.

DISCUSSION

Two distinct peaks were obtained when parotid saliva was separated in a Sepharose 2B column. The factor responsible for aggregation of *S. mutans* strain KPSK2 was shown to be in the first peak while the IgA was found in the second peak. These results, confirming the study of Kaskhet & Guilmette (11), indicated that IgA in this

Table 1. *Effect on the aggregating activity of whole parotid saliva, «agglutinin» or «IgA» fractions after treatment with a control buffer, anti-IgA or anti-IgG*

	Buffer (PBS)	Anti-IgA	Anti-IgG
Saliva (unfract.)	+[a)]	–	+
«Agglutinin»	+	+	+
«IgA»	–	–	–

[a)] + denotes aggregating activity
– denotes no aggregating activity

Table 2. *Effect on the aggregating activity of mixing fractions of parotid saliva with IgA-antiserum in different orders*

Components	Aggregation
/«agglutinin» + anti-IgA/ + «IgA»	+
/«IgA» + anti-IgA/ + «agglutinin»	+
/«agglutinin» + «IgA»/ + anti-IgA	–

/ / denotes 70 minutes of preincubation at 20°C.

Table 3. *Effect on the aggregating activity of mixing saliva or fractions of parotid saliva with IgG-antiserum in different orders*

Components	Aggregation
/Saliva + IgG/	+
(Saliva + IgG) + anti-IgG	–
(«Agglutinin» + IgG) + anti IgG	–
/IgG + anti-IgG/ + saliva	+
(IgG + anti-IgG) + «agglutinin»	+

/ / denotes 70 minutes of preincubation at 20°C
( ) denotes that the two components within brackets were mixed directly before the third component was added

particular saliva-bacteria system did not induce the aggregation.

The authors behind the present study were also able to confirm that anti-IgA added to parotid saliva inhibited the reaction (9). Conversely, this antiserum had no effect when added to the agglutinin fraction only (Table 1), suggesting that antiserum components did not react directly with the agglutinin. When, however, the agglutinin and IgA fractions were mixed before anti-IgA was added, a type of reaction similar to that obtained with whole parotid saliva was recorded.

These findings suggested that IgA-anti-IgA complexes might have prevented the agglutinins from inducing the aggregation. However, results outlined in Table 2 showed that this was not the case. When the IgA and anti-IgA reaction took place before the agglutinin was added, no inhibition of the aggregation occurred. It was necessary that the agglutinin and the IgA were mixed first to get the aggregation inhibition effect of anti-IgA added subsequently. When, in these cases, IgA-anti-IgA complexes were eliminated through centrifugation, no aggregation could be obtained with the supernatant fluid.

Parotid saliva contains IgA but not IgG, at least only trace amounts of it (1, 5). Anti-IgG did not affect the aggregation reaction of either whole parotid saliva or the agglutinin fraction. Nevertheless, when human IgG was added to the saliva, or to the agglutinin, the subsequent addition of anti-IgG impaired the aggregation reaction. The results were quite similar to those obtained with IgA in that the mixing of the components had to be performed in a distinct order (Table 3). Collectively, the data seem to suggest that IgA – or IgG – can interfere with the agglutinin to form a complex whose aggregating capacity is destroyed by anti-immunoglobulin. If this is the case, the reaction between the agglutinin and the IgA is apparently not very strong as they are so easily separated by gel permeation chromatography. The observations raise the question if in fact the agglutinins and the IgA antibodies normally are associated with each other in the saliva.

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