

Anti-*Streptococcus mutans* antibodies in saliva of children with different degrees of dental caries

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The aim of this study was to evaluate the relationship between the secretory immune system and dental caries. Forty-nine 3–5-year-old children with primary dentition were classified into three groups according to their caries indices: no caries (group I), one or two surfaces with caries lesions (group II) and rampant caries (group III). Lower numbers of *mutans* streptococci were found in group I in relation to groups II and III. Secretory IgA and anti-*S. mutans* IgA, IgM and IgG antibody levels were not significantly different among the groups. Western blotting analysis showed that some *S. mutans* proteins, including the 39, 59, 97 and 150 kDa molecular mass bands, were recognized by almost all the saliva samples. Antibodies against the 185 kDa band, known as antigen I/II, were present in all adults' saliva and in only one child in group III. The absence of antibodies to the 185 kDa band in children's saliva suggest a specific immunologic immaturity. Further prospective studies will be necessary to establish the possible effect of reactivity to this antigen on the *S. mutans* colonization in this age group.

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Streptococcus mutans is the major etiological agent in human dental caries (1). The mechanisms by which these bacteria initialize the colonization of tooth surfaces are not fully understood (2,3). The initial *S. mutans* binding to the salivary pellicle may be mediated by a streptococcal cell surface protein with a molecular mass of approximately 185 kDa, termed streptococcal antigen I/II (4–6). Important protective effects including inhibition of bacterial adherence, acid production, neutralization of toxins and prevention of antigen absorption on oral surfaces have been ascribed to salivary IgA (7–10). Several investigators have found a negative correlation between salivary anti-*S. mutans* IgA levels and caries activity (11–13), while others reported no correlation (14). Any functional activity that salivary IgA antibodies may have in human caries remains questionable, since numerous conflicting studies have been reported. In the present work, we have evaluated the oral immune response in

Brazilian children aged 3–5 years old with different degrees of caries.

Materials and methods

Subjects

Forty-nine healthy 3–5-year-old children exhibiting deciduous dentition were included in the study, after receiving informed consent from their parents. They were assigned to three groups: children without caries (group I, n = 20), children with 1 or 2 surfaces with caries lesion (group II, n = 15), and children with rampant caries (group III, n = 14). Saliva samples from four healthy adults were used as controls in the assays.

Saliva samples

For the microbiological analysis, paraffin-chewing- stimulated saliva was collected (15) and transported to the laboratory in sterile flasks

containing transport medium ('Viability Medium of Göteborg Saliva', VMGAIIS), where they were utilized within a 24 h period (16). For the immunologic tests, samples of unstimulated saliva were collected, placed into plastic tubes and stored at -70°C until analysis. The saliva was collected after a fasting period of at least 3 h after the last meal.

Microbiological analysis

A volume corresponding to 25 μl of each saliva sample was cultivated over selective MSB agar (17), in serial dilutions (from 1:10), as described by Westergren & Krasse (18). After 48 h incubation, the number of colony forming-units (c.f.u.) was determined.

Secretory IgA measurement

The secretory IgA levels were measured by an enzyme-linked immunosorbent assay (ELISA), using an anti-secretory component as capture antibody (19). Polystyrene microplates (Costar, USA) were coated with 100 μl of anti-secretory component (5 mg/l in 0.1 M bicarbonate buffer, pH 9.0) during 16 h at 4°C . After washing with phosphate-buffered saline (PBS–0.05% Tween-20), increasing amounts of purified secretory IgA (Sigma, USA) and saliva samples diluted in PBS–0.2% Tween-20 were incubated in triplicate for 24 h at 4°C . After washing, horseradish peroxidase (HRPO) anti-IgA conjugate (Sigma, USA) was added to the plates and incubated for 2 h at 37°C . After additional washing, substrate solution containing *o*-phenylenediamine (at 0.4 mg/ml) and H_2O_2 at 0.01% of final concentration in 0.1 M citrate sodium phosphate buffer, pH 5.0, was added to the plates. After 30 min at room temperature, the reaction was stopped with 25 μl of 1.0 N H_2SO_4 . The absorbance was read in a plate reader (Labsystems Multiskan, USA) at 492 nm. The results were expressed as an index, defined as: (absorbance of test saliva sample – absorbance of diluent (PBS-T))/absorbance of a positive control, where the positive control was a pool of colostrum.

Extraction of antigens

Crude extract from *S. mutans* serotype c (strain ATCC 25175) was obtained by sonication, as previously described (20). Briefly, *S. mutans* was grown in 5 litre culture. After growth, the bacteria were washed with PBS, pH 7.2, and sonicated at 75 W for 30 min (Labsonic 150 Braun Melzengel Ultrasonicator). After centrifugation for 30 min at 9000 g, the supernatant and

the pellet were kept separately and stored at -20°C . The pellet was resuspended in a 1.0 ml volume and used as antigen in the ELISA and Western blotting assays.

Salivary antibody titer determination

Anti-*S. mutans* antibodies titers (IgM, IgA, IgG isotypes) were evaluated by the ELISA method, as described previously (21). Polystyrene microplates (Costar, USA) were coated with 100 μl of crude bacterial extract (0.500 optical density at 660 nm) (22) in 0.1 M bicarbonate buffer, pH 9.0, for 18 h at 4°C . After washings with PBS–0.05% Tween-20, test and control samples were diluted in sample buffer (PBS–0.2% Tween-20) and added to the plates in triplicate for 24 h at 37°C . The plates were washed and incubated with peroxidase-conjugated anti-IgA, anti-IgM or anti-IgG (Sigma, USA) for 2 h at 37°C . After additional washing, the substrate solution was added to the plates, as described previously.

SDS–PAGE and Western blotting analysis

Salivary IgA and IgG antibodies from a subgroup of 14 children and four adults were evaluated by the Western blotting technique. Polyacrylamide gels (7.5% concentration) were used for the electrophoretic separation of the antigens. The bacterial extract was boiled for 5 min in buffer solution (1.5% SDS, 3.5% β -mercaptoethanol, 0.007% bromophenol blue) and a volume of 100 μl was applied to the gel. Electrophoresis was carried out for 1 h at 150 V (Mini Protean II apparatus, BioRad). High-

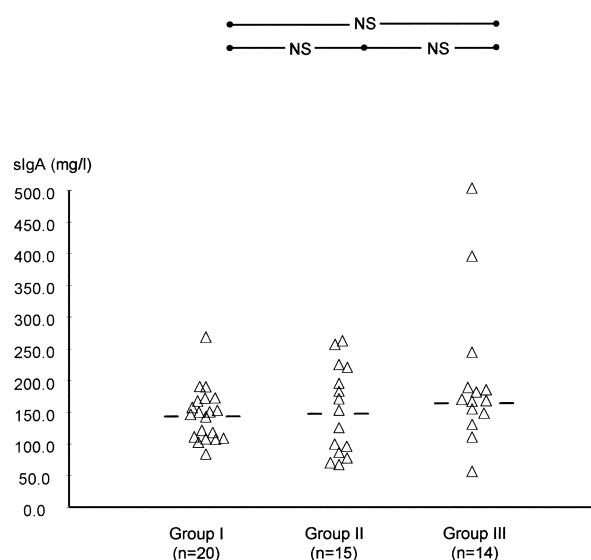


Fig. 1. Total salivary levels of secretory IgA (mg/l) in children of groups I (without caries), II (one or two caries) and III (rampant caries). The horizontal lines represent the medians.

Table 1. *Mutans* streptococci counts in saliva samples from the three groups of children studied

	n	Age (years)	dmfs	Number of <i>mutans</i> streptococci (c.f.u. $\times 10^3$)	
				Range	Median
Group I	20	3–5	0 ^a	0–252 ^b	12
Group II	15	3–5	1–2	0.9–1600	430
Group III	14	3–5	12–46	29–7600	848

Differences: Kruskal–Wallis test, $p < 0.005$ (^agroup I and II $<$ III; ^bgroup I $<$ II and III). dmfs: decayed, missing or fully filled surfaces.

molecular-weight standard markers (Gibco, USA) were included in the SDS–PAGE. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (BioRad, USA). The membranes were cut into strips and incubated with 5% bovine serum albumin at 37°C for 2 h. The strips were then incubated overnight with the saliva samples diluted 1/5 in 0.01 m Tris/1% BSA/0.05% Tween-20. After washing with Tris–0.05% Tween-20 (six times), peroxidase-conjugated anti-human IgA (Sigma, USA) or alkaline phosphatase (AP)-conjugated anti-human IgG (Sigma, USA), both diluted to 1:500, were added, and the mixture incubated for 2 h. Finally, the strips were washed and added to the peroxidase–substrate solution (diaminobenzidine 50 mg/ml, 0.25% hydrogen peroxide in 0.05 m Tris, pH 7.4) or the AP–substrate solution (1% 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.1 m Tris, pH 9.5).

Statistical analysis

Total salivary secretory IgA levels, anti-*S. mutans* IgA, IgM and IgG titers were compared by the non-parametric Kruskal–Wallis test (for comparison of three or more groups).

Results

Culture of *mutans* streptococci

Table 1 shows the numbers of *mutans* streptococci colony-forming units in saliva samples of the children. Group I (children without caries) had significantly lower numbers of colony-forming units of *mutans* streptococci/ml in saliva compared to the other two groups. There was no difference in *mutans* streptococci counts between groups II and III. Results are shown as median and range, due to the wide range of individual values.

Secretory IgA

No significant differences in secretory IgA levels were observed among the groups (Fig. 1).

Salivary antibody titers

No significant differences were found among the three groups with respect to anti-*Streptococcus mutans* IgA, IgM and IgG antibodies (Fig. 2).

Western blotting

Recognition of the *S. mutans* antigens by salivary IgA and IgG antibodies using the Western blotting technique is demonstrated in Figs 3 and 4, respectively. Some bands represent proteins that are important for the adherence of *S. mutans* to the tooth surface, including the 185 kDa band (I/II antigen), which is responsible for the initial adherence of *S. mutans* to the hydroxyapatite. Anti-185 kDa IgA antibodies were not found in saliva samples obtained from children; however, the adult salivas had detectable IgA antibodies to this protein (Fig. 3). One child from group III had IgG antibodies to the 185 kDa band (Fig. 4). Antibodies to the 150 kDa band were recognized in all samples (Figs 3 and 4).

Discussion

Several authors have studied the possible protective role of the immune system in relation to dental caries (8,13,23). It is known that IgA is the main isotype present in secretions, and acts in agglutination of bacteria, neutralization of toxins and enzymes, having an important role in mucosal homeostasis (8). Before tooth eruption, the presence of *S. mutans* is transitory and can induce salivary antibodies (23). In conditions of immune deficiency, such as primary IgA deficiency, absence of IgA antibodies is compensated by IgM (21).

Some authors also postulate a protective role for IgA antibody in caries development. An investigation evaluating 3–7-year-old children revealed a positive correlation between high total salivary IgA levels and low caries activity (24); nevertheless, only total IgA levels were measured. The same authors further reported a negative correlation between salivary IgA levels,

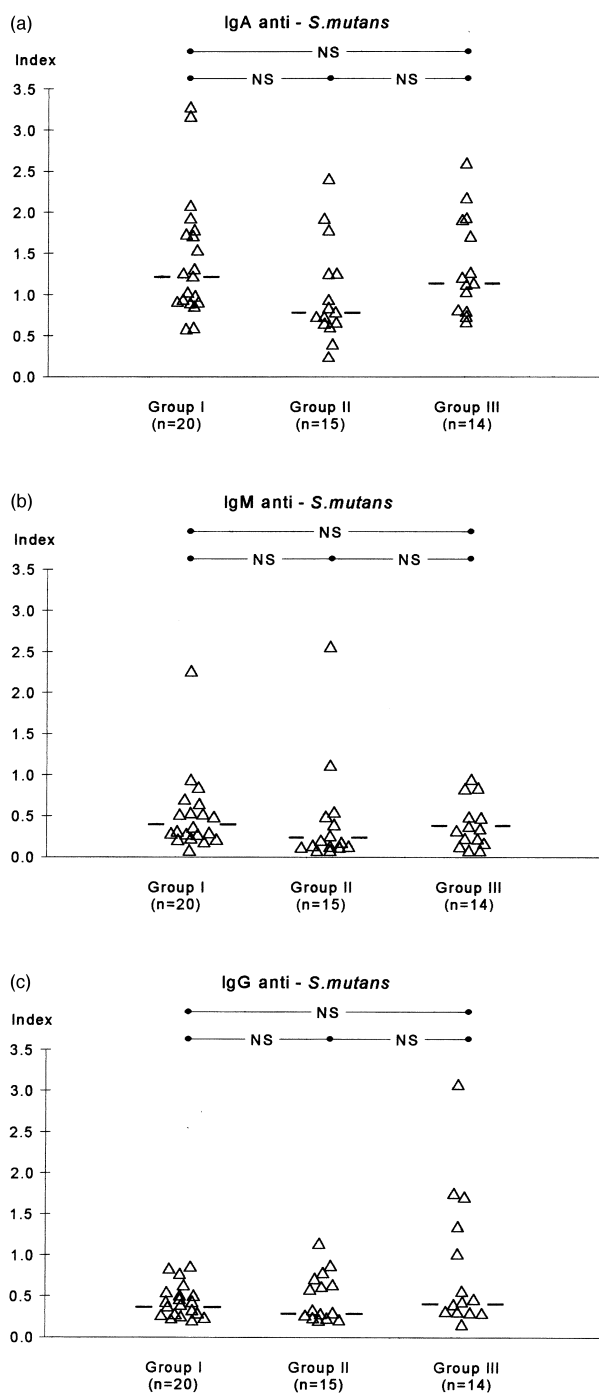


Fig. 2. Anti-*Streptococcus mutans* titers (IgA, IgM and IgG antibodies) in saliva of children of group I, II and III, respectively. The horizontal lines represent the medians.

anti-*S. mutans* levels and caries activity (25). Rose et al. (9), evaluating groups of children with low and high caries susceptibility, observed that higher secretory IgA levels and lower numbers of *mutans* streptococci were found in the former group, suggesting a natural protective role for salivary anti-*S. mutans* IgA.

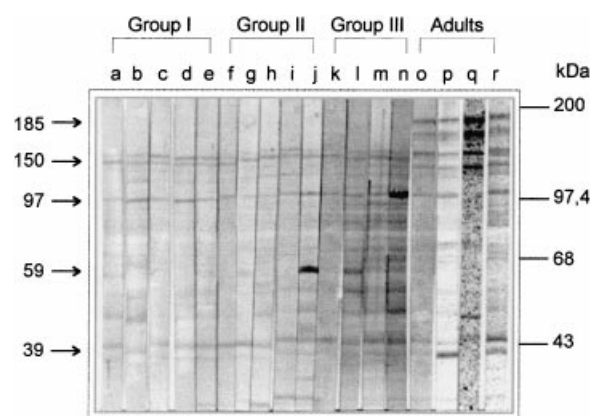


Fig. 3. Western blotting analysis of IgA salivary antibodies against *S. mutans* antigens. Antibodies to the 39, 59, 97 and 150 kDa molecular mass bands were recognized in all saliva samples. Antibodies to the 185 kDa band were found only in adults' saliva.

Our data did not show any correlation between anti-*S. mutans* salivary antibody titers and caries activity, although higher numbers of bacteria were found in saliva samples from children with high activity of caries. This higher number of bacteria was also observed by several investigators (15,26,27). Widerström et al. (28), analyzing the presence of antibodies anti-*S. sobrinus*, *S. mutans* and *S. parasanguis* in total saliva from parents and their sons by immunoblot technique, verified a similarity of the salivary IgA response within the families. In this longitudinal study, the authors verified that the number of bands increased with age. However, these authors were careful to state a real protective role for

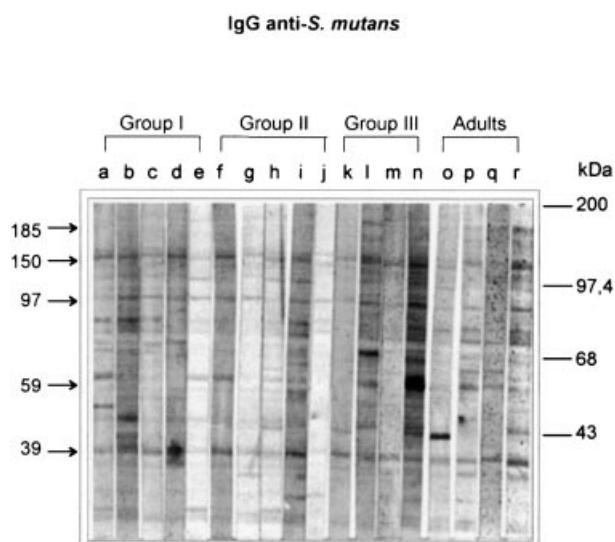


Fig. 4. Western blotting analysis of IgG salivary antibodies against *S. mutans* antigens. Antibodies to the 39, 59, 97 and 150 kDa molecular mass bands were recognized in all saliva samples. Antibodies to the 185 kDa band were found only in adults saliva and in one child in group III (rampant caries).

anti-*S. mutans* antibodies in caries. It is likely that salivary antibodies are not the only factor in caries prevention, and that additional factors may contribute, including frequent fluoride application, tooth anatomy, timing of infection, low sucrose diet and low exposure to *S. mutans*. Probably these additional factors could also explain the higher number of *mutans* streptococci in the children with caries.

In Brazil, high sucrose ingestion and poor buccal hygiene are common among children with low socio-economic status. These are factors of susceptibility in caries progression. Despite the introduction of fluoride in running water, the various factors mentioned above caries continues as an unsolved public health problem.

The influence of antibodies to glycosyltransferase, glucan binding protein and antigen I/II in the natural colonization or accumulation process of *mutans* streptococci on erupting tooth surfaces is not fully understood. The glycosyltransferase is an enzyme constitutive to the *S. mutans* cellular wall and acts in the polymerization of sugars. Salivary antibodies directed to this protein are considered to be effective in protection against caries. Experimental immunization with glycosyltransferase and antigen I/II has suggested that the presence of antibody to these antigens in the oral cavity can decrease *mutans* streptococci infection and disease (1).

In our study, analysis of anti-*S. mutans* IgA and IgG by the immunoblot technique revealed recognition of various bands, such as 150, 74 and 59 kDa, except the 185 kDa band. Antibodies to the 185 kDa band were found only in adults' saliva, probably suggesting a late appearance of this antibody (specific immunologic immaturity?). The absence of antibodies to this band in saliva samples obtained from children raises the question of whether reactivity to this antigen is a crucial step in the prevention of *S. mutans* colonization in this age group, once the various other antigens are recognized, including the glycosyltransferase. Further prospective studies will be necessary to evaluate the role of salivary antibodies to antigen I/II in children with different activities of caries.

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