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Immunoglobulin A Antibodies Reactive with *Streptococcus mutans* in Saliva of Adults, Children, and Predentate Infants

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Immunoglobulin A (IgA) antibodies reactive with *Streptococcus mutans* MT3 cells (serotype *c*) were sought, using an indirect enzyme-linked immunosorbent assay, in the saliva of humans who either harbored or did not harbor detectable levels of this organism. Samples of unstimulated whole saliva from three adults and one child who were infected with *S. mutans* contained IgA which bound to MT3 cells. Saliva samples of two adults studied also contained IgA which reacted with *S. mutans* strains of serotypes *e*, *g*, *a*, and *b*, the latter two of which are rarely isolated from humans. The saliva of three children who did not harbor detectable levels of *S. mutans* and of three of five predentate infants also contained IgA reactive with MT3 cells. The latter observation is of special interest since *S. mutans* does not colonize the mouth before eruption of teeth. Thus, the presence of salivary IgA reactive with *S. mutans* cells is not necessarily related to present or past infection by this organism. Absorption with MT3 cells markedly reduced the reactivity of adult saliva without greatly altering the total concentration of IgA present; this suggests that the IgA was not binding to *S. mutans* MT3 cells via Fc receptors. The possibility that the antibodies which reacted with *S. mutans* MT3 may have been induced to other bacteria with cross-reactive antigens was supported by the finding that absorption of saliva with mixed bacterial growth derived from common dairy products significantly reduced its reactivity. Absorption experiments further suggested that a significant portion of the salivary IgA antibodies was binding to glucans on the cell surface.

Organisms of the *Streptococcus mutans* group are associated with the etiology of certain types of dental caries (18). Strains derived from humans and various animals have been divided into seven serotypes, designated *a* through *g*, on the basis of cell wall antigens (4, 24). Several cultural surveys have indicated that strains of serotype *c* are dominant in most human populations, whereas serotype *a* and *b* strains are rarely isolated (5, 21, 25). However, Arnold et al. (1) observed that samples of human colostrum and parotid saliva contained agglutinating immunoglobulin A (IgA) antibodies reactive with *S. mutans* strains of serotypes *a* through *e*, respectively. Everhart et al. (15), using fluorescent-antibody techniques, and Bratthall et al. (6), using an enzyme-linked immunosorbent assay (ELISA), also detected IgA antibodies which reacted with *S. mutans* strains of various serotypes in samples of saliva from children and adults.

The presence of salivary IgA antibodies reactive with *S. mutans* serotypes which are only rarely isolated from humans could be due to cross-reactions among strains of different serotypes or between strains of *S. mutans* and other organisms. In the present study, the distribution of salivary IgA antibodies reactive with a serotype *c* strain of *S. mutans* was determined, by an indirect ELISA technique, in humans who were either infected or not infected with these streptococci.

MATERIALS AND METHODS

Cultures and cultural conditions. *S. mutans* strains AHT, LB1, MT3, H12, GS5, LM7, and 6715 (serotypes *a*, *b*, *c*, *c*, *c*, *e*, and *g*, respectively) were obtained from the culture collection of the Forsyth Dental Center. The organisms were maintained in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and in fluid thioglycolate medium (BBL Microbiology Systems). All cultures were incubated anaerobically in Brewer Anaerobic Jars (Becton, Dickinson & Co.) filled with 80% N_2, 10% H_2, and 10% CO_2 for 24 to 48 h at 35°C.

Saliva collection. To determine whether individuals were infected with *S. mutans*, a sample of whole unstimulated saliva was collected, and 0.1 ml was cultured directly on mitis salivarius agar (Difco Laboratories, Detroit, Mich.) and on mitis salivarius-bacitracin medium, which is highly selective for this organism (19). *S. mutans* colonies which develop on these media can be identified by their characteristic

morphology due to glucan synthesis (19). Samples of unstimulated saliva used for antibody measurements were collected in containers chilled in ice. The saliva was heated at 60°C for 30 min to inactivate degradative enzymes and then clarified by centrifugation. Sodium azide (0.04%) was added as a perservative, and the samples were stored at −20°C.

Samples of unstimulated whole saliva were also obtained from 4- to 7-month-old predentate infants by placing Calgiswabs (Inolex, Inc., Glenwood, Ill.) in their mouths. The swabs, which absorbed approximately 0.1 ml of saliva, were immediately placed in 0.9 ml of 0.01 M phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN_3. After standing for 5 min at room temperature, the samples were dispersed for 1 min with a Vortex mixer, heat-inactivated, clarified, and stored as described above.

Antigen preparation. Whole *S. mutans* cells served as antigen for the indirect ELISA technique. The organisms were harvested from 36-h Trypticase soy broth cultures, washed twice with saline, and suspended in saline containing 0.6% Formalin. The suspensions were permitted to stand overnight at 4°C; the streptococci were then washed twice with saline and once with 0.1 M Na_2CO_3 buffer (pH 9.6) containing 0.02% NaN_3 and finally suspended in this buffer. The suspensions were standardized to contain approximately 6×10^9 organisms per ml and then heated at 60°C for 30 min to inactivate phosphatases. Samples (0.25 ml) of the standardized suspensions were applied to each well of microtiter plates (Cook Laboratories Products, Alexandria, Va.). The plates were incubated on a shaker for 3 h at 35°C and then stored at 4°C. For use, the suspensions were removed, and the plates were washed three times with "washing" saline, which consisted of 0.9% NaCl and 0.05% Tween 20. Plates treated with sodium carbonate buffer alone were washed in a similar manner to serve as antigen-free controls. For streptococci grown in broth supplemented with 10 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml, it was found that $(2.4 \pm 0.4) \times 10^8$ *S. mutans* MT3 cells adsorbed to each microtiter well under the conditions used; this corresponds to approximately 200 to 250 μg, dry cell weight.

Indirect ELISA. IgA antibodies reactive with strains of *S. mutans* were determined by an indirect ELISA technique (14) as modified by Ebersole et al. (13). Rabbit anti-human IgA (RAHA) specific for heavy chains (Miles Laboratories, Inc., Kankakee, Ill.), containing 0.5 mg of albumin per ml, and the gamma globulin fraction of goat anti-rabbit IgG (Miles Laboratories) conjugated to alkaline phosphatase (GARG-P) (type VII, Sigma Chemical Co., St. Louis, Mo.) as described by Engvall and Perlmann (14) were used. The RAHA and GARG-P were absorbed with 10% (vol/vol) human type A_1 and type B erythrocytes (Hyland Laboratories, Costa Mesa, Calif.) and with Formalin-killed *S. mutans* cells for 1 h at 35°C to reduce nonspecific binding. Optimal dilutions of each reagent (RAHA; GARG-P) in phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN_3 were determined by checkerboard titrations; they were found to be 1:200 and 1:500 for RAHA and GARG-P, respectively. Dilutions of each saliva sample were analyzed in triplicate. ELISA values were considered to be the absorbance at 400 nm due to the release of *p*-nitrophenol by phosphatase from *p*-nitrophenylphosphate $\times$ $(60/t)$, where t was the reaction time (in minutes). Samples of adult saliva used as standards were considered to contain 1,000 ELISA units per ml of antibody reactive with *S. mutans* MT3 cells.

Bacterial absorption of saliva. Saliva samples were absorbed by cells of various *S. mutans* strains or with mixed cultures derived from pasteurized milk, yogurt, and cottage cheese. The latter were prepared by inoculating approximately 5 ml of the dairy product into 2 liters of Trypticase soy broth. Packed Formalin- and heat-treated bacterial cells were added to samples of whole undiluted saliva, and the mixtures were agitated for 1 h at 35°C; they were then permitted to stand overnight at 4°C and clarified by centrifugation. The total concentration of IgA in samples of saliva from subject no. 1 was also determined by using radial immunodiffusion plates (Behring Diagnostics, Somerville, N.J.; assay range 0.5 to 7.9 IU/ml) before and after absorption with strain MT3.

Inhibition by bacterial products. The ability of certain sugars and bacterial products to inhibit the binding of salivary IgA antibodies to *S. mutans* cells was determined by adding them to 1:4 dilutions of saliva before assay. Bacterial products studied included dextran of molecular weight 2×10^4 (Pharmacia Fine Chemicals, Uppsala, Sweden) and soluble and insoluble glucans prepared from cultures of *S. mutans* GS5 grown in Trypticase basal broth containing 5% sucrose as previously described (17). Purified *c* antigen was prepared from cells of *S. mutans* GS5 grown in a chemically defined medium (Socransky et al., Annu. Meet. Int. Assoc. Dent. Res. 1973, abstr. 120, p. 88) by the method of Linzer et al. (22). This material formed a single precipitin band when analyzed immunoelectrophoretically using an anti-*S. mutans* serum which produced multiple precipitin bands with Rantz-Randall antigen extracts of strain GS5.

Crude glucosyltransferase was prepared from cultures of *S. mutans* MT3 grown in a chemically defined medium devoid of sucrose; exhaustively dialyzed and lyophilized culture liquor served as a source of enzyme. The protein content of the crude glucosyltransferase was determined by the method of Lowry et al. (23), with bovine albumin as a standard.

RESULTS

Salivary IgA antibodies reactive with *S. mutans* strains in human saliva. The ELISA values for *S. mutans* MT3 cells obtained for twofold serial dilutions of whole saliva were linearly related to the $\log_2$ of the saliva dilutions, at least over the range of 1:4 to 1:32 (Fig. 1). Saliva samples from two adults studied were also found to contain IgA reactive with *S. mutans* strains AHT, LB1, LM7, and 6715, which belong to serotypes *a*, *b*, *e*, and *g*, respectively (data not shown). These observations are therefore consistent with previous reports that human saliva contains IgA antibodies which react with serotypes of *S. mutans* which are only rarely isolated from humans (1, 6, 15).

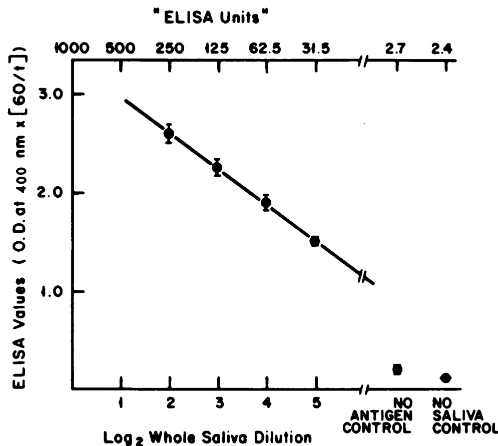

FIG. 1. *ELISA values of twofold dilutions of whole saliva IgA reactive with S. mutans MT3 (serotype c). Mean values are indicated by solid dots, and the standard errors of the means are indicated by bars. The relation between ELISA values and ELISA units is also shown.*

Salivary IgA antibodies reactive with *S. mutans* MT3 in the saliva of adults, children, and predentate infants. The subjects selected included three adults and one child who were infected with *S. mutans* and three children whose saliva did not contain detectable levels of this organism when cultured on mitis salivarius-bacitracin medium (Table 1). Various levels of IgA reactive with strain MT3 (serotype *c*) were detected in saliva samples from all subjects studied, irrespective of whether they harbored detectable levels of *S. mutans* or not (Table 1). Samples from at least three of five predentate infants examined also contained IgA antibodies that reacted with *S. mutans* MT3 cells (Table 2). These observations are of special interest because *S. mutans* does not colonize the mouth before eruption of teeth (2, 8). In general, the levels of IgA antibodies reactive with this organism appeared to be higher in saliva from the three adults than from children and infants (Tables 1 and 2).

Effect of bacterial absorption on the reactivity of salivary IgA with *S. mutans* MT3. Absorption of adult saliva with homologous *S. mutans* MT3 cells greatly reduced its reactivity (Table 3). However, the total quantity of IgA removed by absorption was not found to be significantly reduced as determined by radial immunodiffusion. This suggests that the salivary IgA was not binding to *S. mutans* cells by Fc receptors. Absorption of the saliva with *S. mutans* H12 and GS5, which are also of serotype *c*, was somewhat less effective in reducing its reactivity; this was especially evident with strain GS5 (Table 3). Samples absorbed with mixed bacterial growth derived from milk, yogurt, and cottage cheese were also much less reactive with cells of *S. mutans* MT3, indicating that a major fraction of the IgA antibodies present were also reactive with other bacterial species.

Effect of bacterial products on the reactivity of salivary IgA with *S. mutans* MT3. To obtain clues as to the nature of the antigenic components of *S. mutans* cells which were reacting with the salivary IgA, the ability of some bacterial products to inhibit antibody binding was studied. Purified preparations of *c* antigen reduced the reactivity of one of two saliva samples studied (Table 4). Similarly, a mixture of glucose and rhamnose, the sugar constituents of *c* antigen (22), weakly inhibited the IgA reactivity of this sample but not of two others studied (Table 4).

Preparations of soluble and insoluble glucans derived from *S. mutans* GS5, and commercial

TABLE 1. *ELISA units of IgA reactive with S. mutans MT3 in whole saliva of adults and 6- to 9-year-old children*

Subject	Age	Infected with *S. mutans*	ELISA units[a] in relation to saliva no. 1	% Relative to saliva no. 1
1	37	+	1,000 ± 128[b]	100.0
2	23	+	712 ± 52	71.2
3	44	+	1,300 ± 360	130.0
4	9	+	308 ± 92	30.8
5	6	−	652 ± 188	65.2
6	6	−	108 ± 16	10.8
7	7	−	148 ± 52	14.8

[a] Undiluted saliva from subject 1 (saliva no. 1) was considered to contain 1,000 ELISA units per ml; saliva samples were tested at a 1:4 dilution.
[b] ± Standard error of the mean.

TABLE 2. *ELISA units of IgA reactive with S. mutans MT3 in whole saliva of predentate infants*

Subject	ELISA units[a] in relation to saliva no. 1	% Relative to saliva no. 1
1 (standard)	1,000 ± 440[b]	100.0
8	240 ± 49	24.0
9	110 ± 13	11.0
10	390 ± 111	39.0
11	30 ± 4	3.0
12	160 ± 43	16.0

[a] Undiluted saliva from subject no. 1 (saliva no. 1) was considered to contain 1,000 ELISA units per ml; saliva samples were tested at a 1:10 dilution.
[b] ± Standard error of mean.

dextran, strongly blocked IgA binding to cells of strain MT3 (Table 4). This suggests that a significant portion of the IgA antibodies which react with this organism are directed to glucans. A crude glucosyltransferase preparation also was effective in inhibiting binding.

DISCUSSION

IgA antibodies which react with *S. mutans* strains of various serotypes have previously been detected in samples of parotid or whole saliva by agglutination (1), ELISA (6), or fluorescent-antibody techniques (15), and it has been suggested that indigenous *S. mutans* cells selectively stimulate IgA antibody-producing cells (1). The present study also detected IgA antibodies in samples of adult saliva which reacted with *S. mutans* cells, including strains of serotypes *a* and *b* which are infrequently isolated from humans (5, 21, 25). In addition, IgA antibodies reactive with strain MT3 were detected in the saliva of children who appeared to be uninfected with *S. mutans*, based upon analyses of saliva cultured on mitis salivarius-bacitracin agar. The presence of *S. mutans* in saliva was used as an indicator of infection because its salivary concentrations are thought to reflect its presence on the teeth (7, 9). Furthermore, although the organism primarily colonizes the teeth, it does so in a highly localized manner (18; J. van Houte, *in* H. M. Stiles, W. J. Loesche, and T. C. O'Brien, ed., *Proceedings, Microbial Aspects of Dental Caries, Special Supplement,* Microbial Abstracts, vol. 1, p. 3-32, 1976); consequently, it may not be present in all or even most plaque samples derived from infected individuals.

The reactivity of the salivary IgA with *S. mutans* MT3 did not appear to be due to Fc binding because absorption of the saliva with MT3 cells markedly reduced ELISA values without significantly affecting the total quantity of IgA present. Thus, IgA binding to this organism appeared to be a manifestation of antibody activity. Because the children who did not harbor detectable levels of *S. mutans* may have been previously infected, salivary IgA antibodies were sought in samples of saliva collected from predentate infants, since several investigators have shown that this organism does not colonize the mouth before eruption of teeth (2, 8). Saliva samples from at least three of the infants studied contained IgA antibodies which reacted with *S. mutans* MT3 cells. This observation is of special interest because one can assume that the infants were not previously infected with *S. mutans*, although they may have been exposed to small numbers of the organism from others in their immediate environment. Therefore, it seems clear that the presence of salivary IgA antibodies which react with *S. mutans* cells is not necessarily related to past or present infection by this organism. The observations also suggest that the IgA antibodies which reacted with *S. mutans* cells probably represent cross-reactive antibodies induced to other bacteria. This was supported by the observation that mixed cultures derived from common dairy products absorbed

TABLE 3. *Effect of bacterial absorption on the reactivity of salivary IgA with S. mutans MT3*

Bacteria used for absorption[a]	% ELISA units relative to untreated saliva		
	Saliva no. 1[b]	Saliva no. 2[b]	Saliva no. 3[b]
None	100 ± 29[c]	100 ± 34	100 ± 32
S. mutans MT3	3 ± 0.3	15 ± 2	6 ± 3
S. mutans H12	4 ± 0.3	17 ± 1	10 ± 4
S. mutans GS5	9 ± 1	22 ± 5	62 ± 27
Mixed milk culture	23 ± 6	14 ± 3	16 ± 5
Mixed yogurt culture	21 ± 2	9 ± 2	22 ± 9
Mixed cottage cheese culture	22 ± 2	11 ± 2	17 ± 6

[a] Packed bacteria (10%, vol/vol) used for absorption.
[b] Saliva no. 1, no. 2, and no. 3: Saliva from subjects (adults) 1, 2, and 3.
[c] ± Standard error of the mean.

TABLE 4. *Effect of sugars and bacterial products on the reactivity of salivary IgA with S. mutans MT3*

Substance added	% ELISA units relative to untreated saliva		
	Saliva no. 1[a]	Saliva no. 2[a]	Saliva no. 3[a]
None	100 ± 18[b]	100 ± 20	100 ± 19
c Antigen (1 mg/ml)	54 ± 4	108 ± 14	ND[c]
Glucose-rhamnose	72 ± 4	119 ± 14	158 ± 28
GS5 soluble glucan (2 mg/ml)	27 ± 2	32 ± 7	30 ± 5
GS5 insoluble glucan (2 mg/ml)	37 ± 2	40 ± 4	31 ± 6
Dextran, mol wt, 2×10^4 (2 mg/ml)	38 ± 3	39 ± 11	59 ± 13
Crude GTF[d] (0.5 mg of protein/ml)	26 ± 1	44 ± 6	54 ± 10

[a] Saliva no. 1, no. 2, and no. 3: Saliva from subjects (adults) 1, 2, and 3.
[b] ± Standard error of the mean.
[c] ND, Not determined.
[d] GTF, Glucosyltransferase.

significant quantities of the antibodies present in adult saliva.

Unstimulated whole saliva was used for study because it contains secretions from all of the salivary glands, particularly the minor glands, which are major sources of IgA in the mouth (12). However, some of the antibody may have been bound to salivary bacteria and, therefore, lost upon clarification. Since whole streptococcal cells were used as a source of antigen, the salivary IgA antibodies could have reacted with several possible antigenic components. *S. mutans* cells grown in Trypticase soy broth, which contains small quantities of sucrose, are known to contain the serotype antigen, glucan, and glucosyltransferases on their surface (3, 4, 26, 27). Therefore, preparations of these components were tested for their ability to inhibit the reactivity of salivary antibodies with this organism. Purified preparations of *c* antigen reduced binding of one of two samples studied, whereas a mixture of glucose and rhamnose exerted a weak effect on one of three samples; glucose is the determinant sugar of *c* antigen, which is composed of glucose and rhamnose (22). Challacombe (10) also recently noted that addition of *c* antigen or teichoic acid caused little or no reduction in the agglutination titers of human salivary antibodies reactive with a serotype *c* strain of *S. mutans*. Glucans derived from sucrose broth cultures of *S. mutans* GS5 and commercial preparations of dextran significantly inhibited the binding of IgA to MT3 cells with all three saliva samples studied. Both soluble and insoluble glucans appeared to inhibit to a comparable extent. This observation is consistent with the recent report that glucans containing different proportions of α-1:6 and α-1:3 linkages may exhibit considerable cross-reactivity when measured by ELISA (16).

Crude glucosyltransferase was also highly effective in inhibiting IgA antibodies reactive with *S. mutans* MT3 cells. Since the glucosyltransferase preparations were derived from streptococcal cultures grown in a chemically defined medium devoid of sucrose, it is not likely that they contained appreciable quantities of glucan. However, glucosyltransferase is known to be elaborated as aggregates (20), probably complexed with lipoteichoic acid, phospholipids, and other cell components (11, 20). Consequently, the nature of the antigenic component(s) present in these preparations to which the IgA antibodies reacted is not clear.

The apparent presence of significant concentrations of anti-glucan antibodies in samples of human saliva is of interest, since glucans are being investigated as potential immunogens in vaccination studies designed to control dental caries (3, 16). The cross-reactions of salivary IgA antibodies with mixed bacteria derived from common dairy products and *S. mutans* cells are also of interest. They could be due to lipo- or wall teichoic acids present in many gram-positive bacteria (28) or to glucans or glucosyltransferases synthesized by *Leuconostoc* species, which are common in dairy products. However, they raise the possibility that antigens contained in certain foods might either induce or inhibit antibodies which cross-react with *S. mutans* cells, and thereby influence dental caries.

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