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Antibodies to indigenous and laboratory strains of *Streptococcus mutans* in saliva from children with dental caries and from caries-free children

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Abstract

Whole, unstimulated saliva and dental plaque were collected from 63 children ages 6-13 years, 29 with dental caries and 34 without. If a child was infected with Streptococcus mutans, it was isolated from dental plaque and characterized. Saliva was tested for IgM, IgG, and IgA that would bind indigenous S. mutans, if present, and for antibodies that would bind 6 laboratory strains of S. mutans, each representing distinct antigen types. The results show that there were no significant differences in antibody levels for any type of S. mutans between children of different caries experience. The data do not support a protective role for salivary antibodies to S. mutans.

Most investigators agree that the etiologic agent of many types of dental caries is *Streptococcus mutans* (Loesche, 1986). *S. mutans* is actually a group of bacteria that are very much alike except that they have, among other characteristics, distinct antigens called serotypes (Brathall, 1970; Perch et al. 1974) and biochemical properties (Perch et al. 1974; Shklair and Keene 1976). All types can cause dental decay.

It is not known why most people are susceptible to dental caries nor is it known why those people who do not have dental caries are apparently resistant. It is extremely difficult to conduct research with humans because there are so many unknown and uncontrollable factors. For this reason, most investigators use laboratory animals to study dental caries.

Experiments with laboratory rats have shown that daily oral administration of dead *S. mutanss*, mimicking the route of natural immunization from dental infections, leads to increased levels of salivary *S. mutans* antibodies, decreased colonization of teeth by *S. mutans*, and decreased dental decay (Michalek et al. 1978; Michalek et al. 1983). Thus, it appears that the body defends itself against dental caries as it does other microbial diseases, i.e., by means of the immune system.

However, if these animal studies are relevant to man, then children without dental caries ought to have more salivary antibodies to *S. mutans* than children with caries. Furthermore, the highest level of antibody activity ought to be against the bacteria that induced the immune response, that is, indigenous *S. mutans*, the presumptive cause of decay. This is expected because it generally is recognized from the study of other diseases that the highest levels of antibodies are found against the causative organism.

However, antibodies are not entirely specific. Antibodies also will bind related but different microorganisms. For example, antibodies to *S. mutans* also will bind *Streptococcus sanguis* (Riviere, unpublished observations). This phenomenon is called cross-reaction and it occurs because similar antigens can be found on many bacteria. These shared antigens are rarely the basis for protective immunity.

Nevertheless, it has been difficult to demonstrate an association between immunity and dental caries in man (Lehtonen et al. 1984; Pucci et al. 1986). Everhart et al. (1978) studied saliva of children for antibodies to 4 types of *S. mutans*, not including the children's own indigenous *S. mutans*. They were unable to find a correlation between IgA, the most common type of antibody in saliva, and serotype c *S. mutans*, the most common type of *S. mutans* in people with caries.[1] However, they did report a significant negative correlation between antibodies to serotype b *S. mutans* and dental decay. This led them to conclude that although IgA to serotype b was found in the lowest concentration in saliva, it was protective against *S. mutans*. The authors suggested that dental caries is caused by serotype b *S. mutans*. The type of *S. mutans* carried by the subjects was not determined.

Most children are reported to be infected with serotype c *S. mutans*[2] and most children have antibodies that bind this serotype.[3] The assumption generally is made that serotype c represents indigenous *S. mutans* and that

[1] Loesche and Grenier 1976; Perch et al. 1974; Walter and Shklair 1982.
[2] Berkowitz et al. 1975; Bright et al. 1977; Thompson et al. 1980.
[3] Bammann and Gibbons 1979; Bolton and Hlava 1982; Everhart et al. 1978.

S. mutans antibodies were induced by resident bacteria. However, Bammann and Gibbons (1979) found that salivary IgA to serotype c *S. mutans* were found whether or not *S. mutans* could be found in saliva and that serotype c antibodies could be removed by absorption with oral bacteria that were not *S. mutans*. Bratthall and Pettersson (1976) also removed *S. mutans* antibodies by absorption with other oral streptococci.

Three questions arise from this perusal of the literature. First, has *S. mutans* induced protective immunity in children who are caries free but not in children who have dental caries? Second, do antibody levels to indigenous *S. mutans*, or some other type of *S. mutans*, correlate with the disease state of the child? Third, are the highest antibody levels against indigenous *S. mutans* the presumptive cause of decay in the subject under study?

The purpose of this investigation was to characterize the relative levels of salivary IgM, IgG, and IgA that bound indigenous *S. mutans* as well as 6 laboratory strains of *S. mutans* representing 6 distinct antigens or serotypes. If salivary *S. mutans* antibodies played a role in acquired resistance to dental caries, then the children with the highest antibody levels ought to have the fewest lesions. Furthermore, the highest levels of antibodies ought to be bound by indigenous bacteria or a related strain, assuming that resident *S. mutans* were responsible for dental caries.

Methods and Materials

Subjects. Sixty-three children ages 6-13 years were studied. They were examined using a dental probe, mouth mirror, and recent radiographs. Children with no detectable caries and no fillings or history of fillings were classified as caries free (CF). Children who had 1 or more carious lesions, fillings, or a history of fillings were classified as caries prone (CP).

Saliva. Whole, unstimulated saliva was collected on ice, heat inactivated at 60° C for 10 min, centrifuged at 10K x G for 4 min and stored at -70° C. Approximately 3-5 ml were collected from each child. Not every child donated enough saliva to test every variable.

Indigenous *S. mutans*. Plaque was collected from the interproximal, buccal, and lingual surfaces of the posterior molar in each quadrant and was placed in 10 ml reduced transport medium (Rundell et al. 1973). Dispersed plaque was streaked on mitis salivarius agar[a] supplemented with bacitracin (MSB, Gold et al. 1973) and incubated anaerobically[b] for 48 hr and in air for 48 hr at 37° C. Colonies of *S. mutans* (broken glass appearance) were picked and cultured for 24 hr in broth.[c] An aliquot was biotyped using the micromethod of Oldershaw et al. (1982).

[a] Difco; Detroit, MI.
[b] BBL GasPak — Becton Dickinson; Cockeysville, MD.
[c] Todd Newitt broth — Difco; Detroit, MI.

Representative strains bearing distinct cell wall carbohydrate antigens designated as serotypes b, c, d, e, f, and g (Fa-1, Ingbritt, OMZ-176, LM-7, OMZ-175, and 6715, respectively)[d] were maintained by serial passage in broth. Serotype a could not be detected because it is susceptible to bacitracin.

Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA is a technique to detect antibody binding to antigen and to determine which class of antibody is bound. In general, bacteria in excess are attached to the insides of plastic wells and then the rest of the plastic surface is blocked with an unrelated protein so that nothing else will attach to the plastic. Human antibodies are added and, after incubation, unbound antibodies are washed away. Their presence on antigen is detected by adding an excess of a second antibody made in goats against human immunoglobulins and purified so that the goat antibodies only bind one class of human antibody, IgM, IgG, or IgA. An enzyme is attached to the goat antibody so that when substrate is added a color change is produced indicating the presence of the human immunoglobulin on antigen. The more color, the more human antibody is present.

Subjects from both CF and CP groups were tested simultaneously and about 20 subjects were tested each day and all subjects were tested within 1 week to minimize the effects of day-to-day variation on the ELISA.

An 18-hr culture of each indigenous *S. mutans* isolate and each serotype strain were killed by washing cells in saline and incubating overnight in 1% formalin-saline (v/v) at room temperature. Killed cells were washed and resuspended to a 1% (v/v) suspension in carbonate buffer, pH 9.8. Seventy-five µl of each suspension were placed in each well of microtiter plates[e] and incubated overnight at 26° C in a humidified incubator. The next day unbound bacteria were removed with 3 washes of saline containing 0.05% (v/v) Tween 20[f] and remaining reactive sites in wells were blocked by the addition of 1% (w/v) bovine serum albumin[g] (BSA) for 4 hr at 26° C. Plates were washed 3 times with saline-Tween.

Saliva was placed in 9 wells, 50 µl/well, for each of 7 bacteria; each subject was tested against his/her own indigenous *S. mutans*, if present, and against each of the 6 serotype strains. BSA was placed in the 10th well as a blank. Undiluted saliva was incubated 4 hr at 26° C and then plates were washed. Fifty µl of alkaline phosphatase-conjugated, rabbit, heavy chain-specific sera for human IgM, IgG, and IgA were placed in 3 wells of each suspension for each subject. IgA is the most common immunoglobulin in saliva while IgM and IgG are the predominant immunoglobulins in serum but are found in low concentrations in saliva. Plates were incu-

[d] The generous gift of I Shklair; Great Lake, IL.
[e] EIA plates — Flow Laboratories; McLean, VA.
[f] Sigma Chemical Co; St. Louis, MO.
[g] Cohn fraction V — Sigma Chemical Co; St. Louis, MO.

bated overnight at 26° C and then were washed as before.

Substrate[h] was added, 200 µl/well, and the plates were incubated 100 min. The reaction was stopped with 100 µl 1 N NaOH. Absorbance at 405 nm was determined.

The average of triplicate readings for each variable was calculated for each subject. Results are expressed as the mean ± one standard deviation for each variable. The significance of difference between means was determined by means of a *t* test (Dunn 1977). Significance was set at $P \leq 0.05$.

Results

Children in this study ranged in age from 6 to 13 years with the majority between 6 and 10. There were 29 children (12 males, 17 females) with caries or a history of caries and all carried detectable *S. mutans*. Their mean age (± one SD) was 8.5 ± 1.8 years and they had 5.8 ± 3.1 (mean + one SD) decayed or filled teeth per child at the time of examination. There were 15 children (5 males, 10 females) with no caries or history of caries who carried *S. mutans*. Their age was 9.1 ± 1.9 years. There were 19 children (8 males and 11 females) who had neither caries nor a history of caries and who failed to demonstrate detectable *S. mutans*. Their age was 9.3 ± 2.0 years. Not every child donated enough saliva to test for every variable. Numbers of subjects studied for each variable are given parenthetically in the tables.

Table 1 gives levels of IgM, IgG, and IgA in saliva that bound indigenous and laboratory strains of *S. mutans* in ELISA for subjects grouped by caries status and *S. mutans* carrier status. There were no significant differences among groups for any bacteria type. Comparisons between bacteria types cannot be made because there was no way to assure that equivalent numbers of bacteria adhered to wells in EIA plates, even though an attempt was made to prepare equivalent cell suspensions. It is striking, however, that in each group the highest mean absorbance values were against serotype c and the lowest values were almost always against indigenous bacteria. It is also important to note that all children had salivary antibodies that bound each bacteria tested.

TABLE 1. Antibody Levels to *S. mutans*

Antibody Type	S.m. Type	Mean ± 1 SD Absorbance (# Subjects)		
		Caries and S.m.	No Caries but S.m.	No Caries or S.m.
IgM	I	0.13 ± 0.10 (26)	0.19 ± 0.13 (12)	No S.m.
	b	0.60 0.30 (29)	0.62 0.48 (13)	0.57 ± 0.24 (18)
	c	0.95 0.37 (29)	0.93 0.40 (13)	0.95 0.23 (18)
	d	0.28 0.18 (28)	0.32 0.50 (13)	0.29 0.11 (18)
	e	0.68 0.30 (29)	0.79 0.44 (13)	0.66 0.24 (18)
	f	0.57 0.27 (29)	0.58 0.40 (12)	0.57 ± 0.22 (18)
	g	0.48 0.27 (29)	0.40 0.17 (12)	0.50 ± 0.20 (18)
IgG	I	0.09 0.06 (28)	0.09 0.04 (14)	No S.m.
	b	0.46 0.24 (29)	0.46 0.47 (14)	0.42 ± 0.21 (18)
	c	0.75 0.33 (29)	0.67 0.44 (13)	0.77 0.26 (18)
	d	0.07 0.05 (28)	0.06 0.02 (14)	0.08 0.04 (18)
	e	0.29 0.14 (29)	0.37 0.48 (14)	0.28 0.11 (18)
	f	0.27 0.13 (29)	0.19 0.11 (13)	0.29 0.14 (18)
	g	0.27 0.16 (29)	0.21 0.08 (13)	0.32 ± 0.19 (18)
IgA	I	0.67 0.49 (26)	0.62 0.31 (14)	No S.m.
	b	0.52 0.38 (28)	0.50 0.24 (14)	0.62 ± 0.45 (17)
	c	1.03 0.59 (29)	1.11 0.41 (14)	1.01 0.49 (16)
	d	0.44 0.37 (29)	0.46 0.28 (14)	0.46 0.26 (17)
	e	1.07 0.59 (29)	1.12 0.47 (14)	1.14 0.58 (17)
	f	0.81 0.57 (29)	0.78 0.48 (13)	0.84 0.49 (17)
	g	0.61 ± 0.50 (27)	0.51 ± 0.30 (14)	0.59 ± 0.37 (17)

I = Indigenous *S.m.* Each subject was tested against his/her own *S.m.* as well as representatives of each serotype listed.

[h] p-nitrophenyl phosphate — Sigma Chemical Co; St. Louis, MO.

Biotypes represent the ability of *S. mutans* to ferment a defined group of sugars in vitro. Each biotype of *S. mutans* is distinguished from other biotypes by the pattern of sugar fermentation (Shklair and Keene 1976). Biotype I represents serotypes c, e, and f. Twenty-two/29 CP subjects carried this biotype and had 5.2 ± 1.7 decayed or filled teeth per subject. Twelve/15 CF subjects carried biotype I *S. mutans*. No child in this study carried biotype II which represents serotype b. Biotype III represents serotype a *S. mutans*, but is not detectable because these bacteria are sensitive to the bacitracin in the selective agar. Biotype IV represents serotypes d and g. There were 7/29 CP and 3/15 CF children with this biotype. The CP children had 7.7 ± 3.8 decayed or filled teeth per subject. There were more lesions per child when biotype IV was found than when biotype I was found ($P < 0.025$, *t* test).

Table 2 shows the distribution of mean absorbance values based on biotype of *S. mutans* isolated from carriers and caries experience. Although the volume of saliva collected sometimes limited the number of variables that could be tested, it can be seen that there were no significant differences between groups for either IgM or IgA. There was a significant difference ($P < 0.025$, *t* test) for IgG in subjects with caries who carried either biotype I or IV when tested against serotype c *S. mutans* (Ingbritt). There were no other significant differences for IgG.

Discussion

The distribution of biotypes reported in this population of children closely approximates the distribution of serotypes of *S. mutans* reported by Keene et al. (1973). They found that serotype c was the most common form of *S. mutans* and that serotypes a (biotype III) and b (biotype II) were extremely rare and never were found alone in subjects from North America. There were no carriers of biotype II in the present study population of 63 children from the Los Angeles area. This argues against an important role for serotype b *S. mutans* in the pathogenesis of dental caries as suggested by Everhart et al. (1978).

The first question raised at the onset of this work was whether or not *S. mutans* had induced protective immunity in CF subjects but not CP subjects. If, as animal experiments suggest (Michalek et al. 1978; Michalek et al. 1983), resistance correlates with increased levels of salivary antibodies to *S. mutans*, then CF children should

TABLE 2. Antibody Levels Based on Biotype of *S. mutans*

Anti-body	Subject Biotype	Mean + 1 SD Absorbance (# Subjects)			
		Subjects with Caries		Subjects without Caries	
		Serotype c	Indigenous	Serotype c	Indigenous
IgM	I	0.95 ± 0.36 (22)	0.14 ± 0.11 (19)	0.93 ± 0.41 (12)	0.21 ± 0.14 (10)
	IV	0.91 0.25 (7)	0.10 0.07 (6)	Not done	Not done
	None	—	—	0.96 0.27 (16)	—
IgG	I	0.83 0.32 (22)*	0.09 0.06 (20)	0.67 0.46 (12)	0.09 0.04 (12)
	IV	0.54 0.21 (7)	0.09 0.06 (7)	Not done	Not done
	None	—	—	0.77 0.26 (18)	—
IgA	I	1.02 0.61 (21)	0.75 0.56 (17)	1.09 0.38 (10)	0.64 0.32 (10)
	IV	1.05 ± 0.53 (7)	0.67 ± 0.39 (7)	0.96 0.50 (3)	0.56 ± 0.19 (2)
	None	—	—	1.05 ± 0.50 (17)	—

* Biotype I > IV for IgG from subjects with caries against serotype c *S.m.*, $P < 0.025$, *t* test (Dunn, 1977).

have had demonstrably higher levels of antibodies than CP children. Data from this study cannot support that assumption. Indeed, children had similar levels of each antibody isotype against each bacteria type irrespective of the biotype of indigenous *S. mutans* or caries status. Even children without demonstrable *S. mutans* in saliva had equivalent levels of antibodies to each of the laboratory strains. These observations support the conclusions of Bammann and Gibbons (1979) and Bratthall and Pettersson (1976) that salivary antibodies to *S. mutans* may be due, in part, to immune responses generated by other bacteria bearing cross-reacting antigens. Thus, it appears that *S. mutans* did not induce protective immunity in saliva of CF children that could be distinguished from immunity in CP children.

The second question raised at the onset of this study was whether or not salivary antibody levels to *S. mutans* correlated with disease status. Everhart et al. (1978) suggested that although antibody levels to serotype c *S. mutans* were higher than any other serotype tested, these antibodies did not correlate with caries experience of children studied. The present study confirms their observations and supports their conclusion regarding serotype c. However, they also reported that antibodies to serotype b, found in lowest concentration, had a significant negative correlation with disease status. The present study cannot confirm those observations; its authors found no difference for any variable relative to serotype b and salivary antibody levels to serotype b were far from the lowest values that they observed. The authors of the present study also were unable to find a correlation of any kind between salivary antibodies to indigenous or laboratory types of *S. mutans* and disease status.

It should be noted that children with caries had nearly the same levels of salivary antibodies to their indigenous *S. mutans* as did children who carried *S. mutans* but did not have caries. Caries-free children without apparent (detectable) *S. mutans* also had equivalent antibody levels to laboratory strains as did children with caries. These observations cast serious reservations on the protective value of salivary antibodies that bind *S. mutans* and suggest that animal studies may not always have direct bearing on human experience with dental caries.

The third question raised at the start of this work was whether or not antibodies to indigenous *S. mutans* were greater than to laboratory strains. The conceptual basis for this question is the "doctrine of original antigenic sin" which states that antibodies bind best to antigens that stimulated their formation and that the closer a given antigen is to the original antigen, the better antibodies will bind to it. The most dramatic observation that can be made from this study is that antibodies to indigenous *S. mutans* were almost always lower than any other type of *S. mutans* tested, even though the authors attempted to apply them in the same concentration as other types.

A word of caution must be added at this point. The work of Bammann and Gibbons (1979) and Bratthall and Pettersson (1976) clearly show that much, if not all, antibody in saliva binds to antigens on *S. mutans* that are shared by other types of oral bacteria. It is possible that critical antigen-antibody interactions are present but are masked by these cross reactions. Other experimental approaches must be utilized before any final conclusions can be reached about the role of immunity in resistance to dental caries.

It may be, for example, that children who respond to *S. mutans* with a particular form or variety of antibody molecule are better able to protect themselves against dental caries. For instance, it could be of great importance to have antibodies that can resist degradation by proteases in saliva (Kilian et al. 1980) or to have antibodies that can mediate the aggregation of *S. mutans* (Rosan et al. 1982). There may be other characteristics of immunoglobulins that contribute to protection that are not disclosed by simple, direct binding to bacteria cells.

In summary, this study has demonstrated that children with caries and children without caries, including those with and without indigenous *S. mutans*, had similar levels of salivary IgM, IgG, and IgA antibodies to 6 serotypes of *S. mutans* as well as the indigenous type. If the immune system is involved in protection against dental caries, it must be by mechanisms that are not associated with the binding of immunoglobulins to bacterial cells.

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Dr. Riviere was a pediatric dentist and professor, pediatric dentistry, UCLA School of Dentistry, and in microbiology and immunology, UCLA School of Medicine at the time the study was conducted. Dr. Papagiannoulis, a pediatric dentist in community dentistry, University of Athens, Greece, collaborated on this research as a visiting scholar at UCLA. Reprint requests should be sent to: Dr. George R. Riviere, Dept. of Oral Biology, School of Dentistry, University of Missouri, 650 E. 25th St., Kansas City, MO 64108-2795.

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