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Salivary Immunoglobulin A Antibodies and Recovery from Challenge of *Streptococcus mutans* After Oral Administration of *Streptococcus mutans* Vaccine in Humans

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Heat-killed *Streptococcus mutans* was administered orally in two periods of 1 week to six subjects in an attempt to affect the salivary immunoglobulin A (IgA) response to this bacterium. Enzyme-linked immunosorbent assays were used to detect specific IgA antibody activity, and an immunofluorescent assay was used for measurement of total IgA in parotid saliva. The salivary IgA response to *S. mutans* was compared with that against a noncross-reacting antigen preparation from *Escherichia coli* and with antibody responses in five sham-immunized subjects. No change in salivary IgA response to *S. mutans* was observed after oral administration of this organism. Significantly less streptomycin-resistant *S. mutans* could be recovered from the six test subjects than from the five controls after the first of two challenges with streptomycin-resistant microorganisms. At the day of the first challenge, a significantly higher IgA antibody response to all tested antigens was observed in the test group than in the control group. The data show that this difference was not related to the oral administration of *S. mutans* but rather was an occasional finding. The coincidence of a rapid elimination of the challenge strain and a high IgA antibody response to *S. mutans* supports the concept that salivary IgA antibodies could have a biological significance in the human defense against cariogenic microorganisms.

The central role of *Streptococcus mutans* in the etiology of dental caries (18, 27) has stimulated attempts to increase the immune response in saliva to this group of bacteria (23).

Naturally occurring immunoglobulin A (IgA) antibodies to *S. mutans* are in saliva and in colostrum (2). In colostrum, increased IgA antibody activity has been reported after antigenic stimulation of gut-associated lymphoid tissues (17). In saliva, an increase of the IgA antibody activity to *S. mutans* was observed after peroral administration of this organism (24; M. F. Cole, C. G. Emilson, J. E. Ciardi, and W. H. Bowen, J. Dent. Res. **60** [Special Issue A]:509, abstr. no. 798, 1981). In other studies, however, no clear-cut effect of peroral administration of an *S. mutans* vaccine was observed in humans (C. Y. Bonta, R. Linzer, F. Emmings, R. T. Evans, and R. J. Genco, J. Dent. Res. **58** [Special Issue A]:143, abstr. no. 204, 1979) or in primates (21, 28). Topical application of an *S. mutans* vaccine also did not evoke a definite immune response in minor salivary glands in humans (19).

In a longitudinal study, we found that variations in total salivary IgA have a decisive effect on specific antibody activity (14). This study showed that both a reference antigen and an accurate determination of total IgA have to be used when studying the salivary response to immunization. By using this principle, we have examined the salivary IgA antibody response after peroral administration of an *S. mutans* vaccine. Furthermore, the recovery after challenge with streptomycin-resistant *S. mutans* was studied.

MATERIALS AND METHODS

Eleven subjects, nine males and two females, ages 23 to 48, volunteered for the study. All subjects were students at the Faculty of Odontology of Göteborg, Sweden, and free from active caries or periodontal disease. None was tonsillectomized. The subjects were divided at random into a control group of five and a test group of six.

Bacteria. *S. mutans* strain B13 serotype *d* (5), originally isolated from human plaque (9), was used for peroral immunization. A vaccine was prepared by growing *S. mutans* B13 in a dialyzed yeast extract medium (8) for 20 h at 37°C in an atmosphere of 95% N_2 and 5% CO_2. The cells were harvested by centrifugation, washed three times with 0.01 M potassium phosphate-buffered saline, pH 7.1 (PBS), and suspended to a final concentration of approximately 6×10^8 colony-forming units (CFU) per ml in PBS. The bacteria were then heat killed at 60°C for 30 min.

A streptomycin-resistant mutant, *S. mutans* B13 (Str^r), was used for implantation experiments. This strain was capable of growing on mitis salivarius

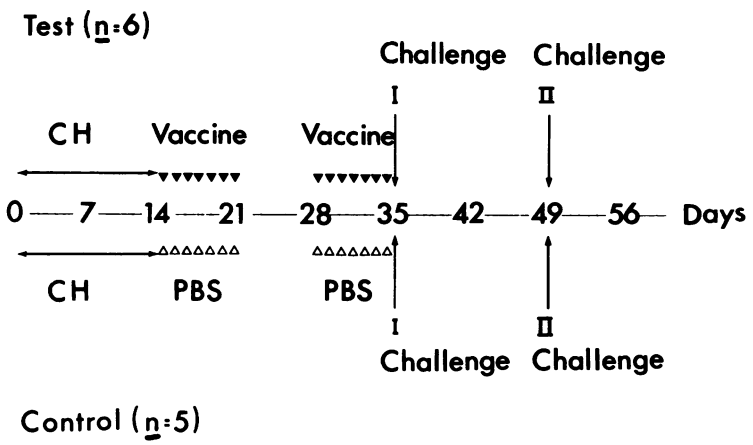

FIG. 1. Experimental design. All subjects rinsed three times per day with 0.2% chlorhexidine digluconate (CH) during the first 14 days. In two periods (days 14 to 21 and 28 to 35), the test group was daily (▼) orally administered an *S. mutans* vaccine. The control group followed the same routine (△), but was administered PBS. At two occasions (I and II), all subjects were challenged with streptomycin-resistant *S. mutans*.

(Difco Laboratories, Detroit, Mich.) agar plates containing 2 mg of streptomycin sulfate per ml (745 U/mg, Galaxo Laboratories Ltd., Greenford, England). *S. mutans* B13 (Str^r) was cultivated as *S. mutans* B13 and used viable in the same concentration (6×10^8 CFU/ml of PBS).

Experimental design. The experiment was performed over 53 days. As shown in Fig. 1, all subjects rinsed with 10 ml of 0.2% chlorhexidine digluconate (Hibitane Dental, ICI Ltd., London, England) three times per day during the first 14 days. The 2 weeks of treatment with chlorhexidine was performed to eliminate or decrease the number of indigenous *S. mutans*. In two periods (days 14 to 21 and 28 to 35), the experimental group rinsed for 1 min and then swallowed 10 ml of a suspension of heat-killed *S. mutans* B13 (i.e., ca. 6×10^9 cells) once per day. The control group followed the same scheme but rinsed and swallowed 10 ml of PBS. At days 35 and 49, all subjects rinsed for 1 min with 10 ml of viable *S. mutans* B13 (Str^r) suspended in PBS.

Saliva samples. Citric acid-stimulated parotid saliva was collected from all subjects at days 0, 7, 14, 21, 28, 35, and 49. The parotid saliva was collected in ice-chilled glass tubes by Lashley cups and immediately frozen. Before the study, a reference pool of parotid saliva was collected from 16 subjects, and parotid saliva from a male suffering from hypogammaglobulinemia (14) was used as a negative reference. Before use, the saliva samples were thawed, heated at 56°C for 30 min to inactivate degradative enzymes, and centrifuged at $12,500 \times g$ for 20 min.

Bacterial samples. Directly after the sampling of parotid saliva, a stimulated whole saliva sample was collected by chewing on a piece of paraffin wax. Whole saliva was also sampled at days 36, 37, 50, 51, 52, and 53. All samples were cultivated within 1 h from sampling by the method described by Westergren and Krasse (29). The CFU of *S. mutans* were enumerated after cultivation on mitis salivarius-bacitracin agar (16), which was incubated in an atmosphere of 95% N_2 and 5% CO_2 for 48 h at 37°C. Samples collected at day 35 and thereafter were also cultivated on mitis salivarius agar supplemented with streptomycin (2 mg/ml). Serotyping of *S. mutans* was performed according to Bratthall (5).

Total amount of IgA. The total amount of IgA was determined by a commercially available immunofluorescent assay (Immunofluor IgA, Bio-Rad Laboratories, Richmond, Calif.). The procedure was scaled down according to the manufacturers instruction manual. Fluorescence was measured in an Aminco fluorocolorimeter (Aminco J4-7439, American Instrument Co., Silver Spring, Md.).

ELISA. Salivary IgA antibodies reacting with whole cells of *S. mutans* strain B13 serotype *d* antigen from *S. mutans* and a pool of antigens from *Escherichia coli* were detected by an enzyme-linked immunosorbent assay (ELISA) (10). The ELISA was performed as previously described (14). All readings were made at 405 nm with a Titertek Multiskan photometer (Flow Laboratories Svenska AB, Solna, Sweden). All saliva samples were analyzed in dilutions of 1/2, 1/8, and 1/16 (PBS plus 0.05% Tween 20). The average optical density for duplicate samples was multiplied by $100/t$, where t is the number of minutes after which the color development was stopped. The salivary IgA antibody activity was expressed as a percentage of the reference pool run on each microtiter plate. The immune response in saliva was expressed as salivary IgA antibody activity per milligram-percent of total IgA as suggested by Gahnberg and Krasse (14).

Antigen preparations. *S. mutans* strain B13 was cultivated in the dialyzed yeast extract medium as described above and used at a turbidity of 75% of one optical unit (650 nm) to coat the solid phase in ELISA (6). Serotype *d* antigen was prepared from *S. mutans* B13 with a slight modification (14) of the technique described by Linzer and Slade (22). Optimal coating concentration for the serotype *d* extract was 6.5-μg/ml coating buffer (PBS, pH 7.1, containing 0.02% NaN_3), incubating the microtiter plates for 96 h at 37°C (14).

As a reference, a pool of antigens from *E. coli* was used. This antigen pool was prepared by boiling the

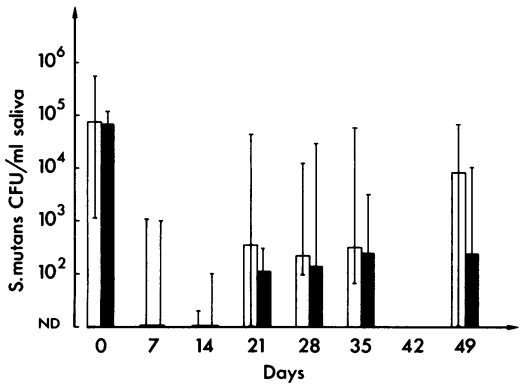

FIG. 2. Median CFU of *S. mutans* over the experimental period. ND indicates nondetectable (<40 CFU/ml). Open bars indicate numbers for the control group, and solid bars indicate numbers for the test group. Range is also indicated.

bacterial suspension of eight strains of *E. coli* frequently found in a Swedish population (serogroups O1, O2, O4, O6, O7, O8, O18, and O75) (7). The *E. coli* antigen pool was a kind gift of B. Carlsson, Department of Immunology, Institute of Medical Microbiology, University of Göteborg, Göteborg, Sweden. The same concentration as used by Ahlstedt et al. (1) was used for coating the microtiter plates.

Inhibition test. Cross-reactivity between the *E. coli* antigen preparation and antigens from *S. mutans* B13 was tested by an inhibition test. Before ELISA analyses, the reference pool of saliva was incubated for 3 h at 37°C with different dilutions (1/10 to 1/2,560) of the stock solution of *E. coli* antigen (8 mg of protein per ml).

Statistics. Statistical analyses were performed by using the Wilcoxon rank-sum test for two samples (30).

RESULTS

In all subjects, the CFU of *S. mutans* in saliva decreased during the 2 weeks of rinsing with 0.2% chlorhexidine digluconate. At day 14, *S. mutans* was detected in two individuals and only in low numbers. No statistically significant difference was observed between test and control groups at the sampling occasions shown in Fig. 2. Thus, oral administration of the *S. mutans* vaccine did not affect the recolonization of indigenous *S. mutans*.

Samples collected on day 35 just before challenge with *S. mutans* B13 (Str^r) showed that no subject harbored streptomycin-resistant *S. mutans* (Fig. 3I). On day 1 after challenge with streptomycin-resistant *S. mutans*, the median CFU of *S. mutans* (Str^r) was lower in the test group than in the control group (day 36) and significantly lower ($P < 0.05$) after 2 days (day 37). A significant difference between the groups ($P < 0.05$) was also observed on day 49, when samples were collected before the second challenge with *S. mutans* B13 (Str^r). In saliva samples collected on the following 4 days, however, no significant differences could be observed between the two groups (Fig. 3II).

In the antibody analyses, the antigen preparation from *E. coli* was used as a reference antigen. Cross-reactivity between *S. mutans* B13 antigens and *E. coli* antigens was tested by inhibition experiments. Salivary IgA antibodies to *S. mutans* B13 antigens were not affected by preincubation of saliva with various amounts of *E. coli* antigens. IgA antibody reactions with the *E. coli* antigens, on the other hand, decreased considerably by preincubation with increasing concentrations of this antigen preparation (Fig. 4).

The parotid salivary IgA immune response to whole cells of *S. mutans* B13, serotype *d*-antigen from *S. mutans* B13, and to antigens from *E. coli* was followed over the experimental period. As shown in Fig. 5, a covariation in the salivary immune response to these antigens was observed in both the test and control groups. No statistically significant difference was observed at any of the sampling occasions with the excep-

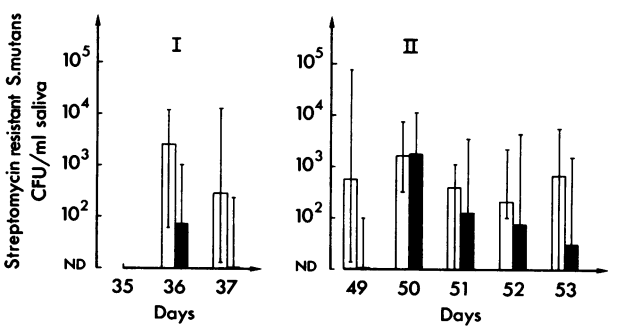

FIG. 3. Median CFU of streptomycin-resistant *S. mutans* at the first (I) and second (II) challenges with *S. mutans* B13 (Str^r). ND indicates nondetectable (<40 CFU/ml). Open bars indicate numbers for the control group, and solid bars indicate numbers for the test group. Range is also indicated.

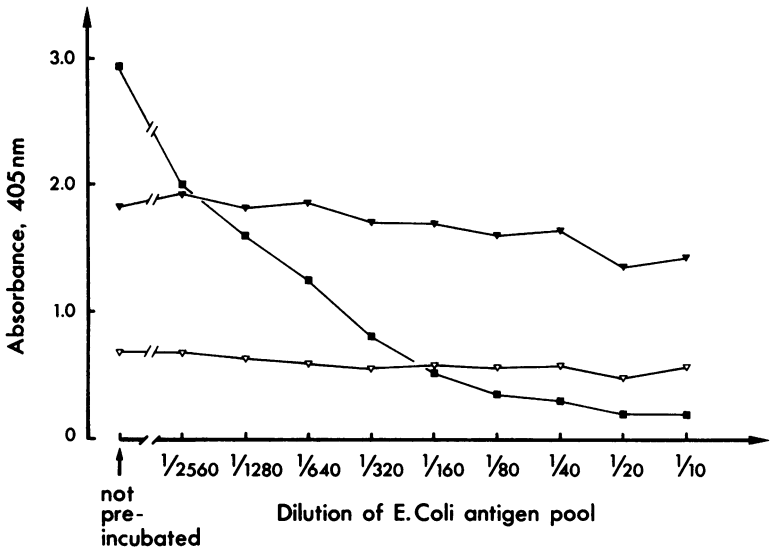

FIG. 4. Inhibition experiment for test of cross-reactivity between the antigens from *S. mutans* B13 and the *E. coli* antigen pool. A pool of parotid saliva (diluted 1/2) from 16 adults was analyzed for IgA antibody activity (absorbance at 405 nm) to: (■) a pool of *E. coli* antigens; (▼) whole cells of *S. mutans* B13; (△) a serotype *d* antigen preparation from *S. mutans* B13. The saliva pool was analyzed after preincubation with various amounts of the *E. coli* antigen pool.

tion of day 35. At this occasion, i.e., the day when *S. mutans* B13 (Str^r) first was implanted, a significantly higher activity ($P < 0.05$) to all three antigen preparations was observed in the test group. No difference in the salivary secretion rate was found between the groups at this occasion.

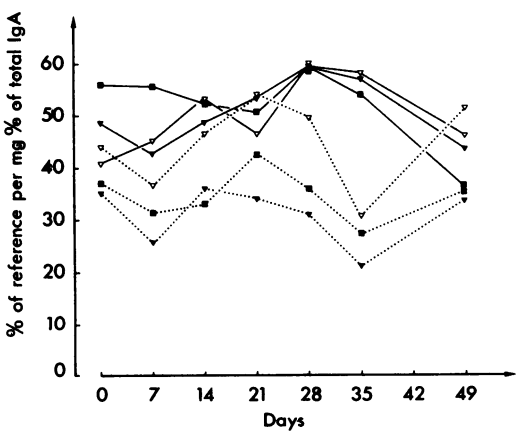

FIG. 5. Mean IgA antibody response in parotid saliva (diluted 1/2) over the experimental period. Mean values are indicated by solid curves (–) for the test group and dotted curves (···) for the control group. IgA antibodies reacting with: (■) a pool of *E. coli* antigens; (▼) whole cells of *S. mutans* B13; and (△) a serotype *d* antigen preparation from *S. mutans* B13.

DISCUSSION

In the present study, the peroral administration of heat-killed *S. mutans* B13 did not affect the parotid salivary IgA response to whole cells or serotype antigen of this bacterial strain. This was proved by the covariation between the immune response to antigens from *S. mutans* B13 and the noncross-reacting reference antigen preparation from *E. coli*. The immune response also was not affected by the reduction of indigenous *S. mutans* by means of chlorhexidine digluconate. A difference was, however, observed between the test and control groups with regard to recovery after challenge with *S. mutans* B13 (Str^r) at the first implantation experiment.

A positive effect of peroral immunization with *S. mutans* in humans has been reported in two studies (24; Cole, Emilson, Ciardi, and Bowen, J. Dent. Res. **60**[Special Issue A]:509, abstr. no. 798, 1981; Cole, Hsu, and Bowen, J. Dent. Res. **61**[Program and Abstracts of Papers]:250, abstr. no. 642, 1982). Our negative result seems irrefutable, and it is therefore of interest to discuss the reason for the difference between our findings and those of the others.

In the study by Mestecky et al. (24), the immune response was measured by agglutination. At the start of the study, the four subjects examined showed no or a very slight agglutinating titer to the strain used. Our subjects, on the contrary, all had a definite initial antibody immune response as measured by the ELISA tech-

nique. The difference in initial immune response cannot, however, explain the different outcome as Mestecky et al. observed a definite booster effect at a second immunization. To test whether the choice of method for antibody analysis could have been of importance for our results, we also analyzed our samples with the agglutination method used by Mestecky et al. (24). With this method, we could not use the serotype *d* antigen extract or the antigen preparation from *E. coli*. When whole cells of *S. mutans* B13 were used, no differences between the test group and the control group could be observed with regard to the agglutinating properties of parotid saliva. Furthermore, Cole et al. (J. Dent. Res. **61**[Program and Abstracts of Papers]:250, abstr. no. 642, 1982), using techniques similar to ours for determining the immune response, also found a positive effect.

An apparent difference between our study and the others is the mode of application of the vaccine. In the other studies, the antigen was administered in capsules and was thus not exposed to the oral fluid. Our antigen, on the other hand, was administered as a suspension directly in the mouth and swallowed after 1 min. Our aim was to stimulate not only gut-associated lymphoid tissues but also oral lymphoid tissues, thus mimicking the natural situation.

It is possible that salivary components capable of binding to oral streptococci (4, 11, 15) prevented the microorganisms from coming in close contact with the lymphoid tissues. This assumption is supported by the observations made by Williams and Gibbons (31), who showed that naturally occurring salivary antibodies could serve as a mechanism for antigen disposal. If our assumption is correct, it would be necessary to exceed the blocking capacity to stimulate an immune response in saliva.

In our experiment, the number of cells in the vaccine was estimated to be 1,000 to 1,000,000 times the number of *S. mutans* which can be found per milliliter of whole saliva in a Swedish population (C. G. Emilson, Scand. J. Dent. Res., in press). Apparently, this was not sufficient to overcome the neutralizing capacity of the defense mechanisms in our adults. The consequence of this is that if the natural route is used for immunization, the dose of antigen must be adjusted to the actual defense mechanisms. Thus, the insufficient dose used in this study might have been quite sufficient in another population, e.g., children.

Reduced recovery of implanted *S. mutans* after administration of *S. mutans* vaccines has also been reported by other investigators (12, 20, 25, 26; Bonta et al., J. Dent. Res. **58**[Special Issue A]:143, abstr. no. 204, 1979; Cole, Emilson, Ciardi, and Bowen, J. Dent. Res. **60**[Special Issue A]:509, abstr. no. 798, 1981; Cole, Hsu, and Bowen, J. Dent. Res. **61**[Program and Abstracts of Papers]:250, abstr. no. 642, 1982). It cannot be excluded that the reduced recovery of implanted *S. mutans* in our test group at days 36 and 37 was the result of an induction of specific antibodies. If so, these antibodies were secreted into the oral cavity from sources other than the parotid gland. Since no difference in elimination of implanted bacteria was observed after the second challenge, such an immune response would have been of very short duration. This fact excludes the possibility of antibodies secreted in the crevicular fluid since these are mainly IgG antibodies derived from serum (3). Little is known about the duration of an antibody response in human saliva but available data suggest such a response to be at least longer than 2 weeks (13, 25).

There is, however, another possibility. A statistically significant difference in the parotid salivary immune response to all tested antigens was observed between the test and control groups at day 35 (Fig. 5), i.e., the day for the first implantation experiment. If the immune response in parotid saliva reflects the response in the mixture of oral secretions, this difference in the overall salivary immune response could have influenced the recovery of *S. mutans* B13 (Str^r) at days 36 and 37 (Fig. 3).

In our opinion, the difference in the parotid salivary immune response at day 35 is not a result of the peroral administration of *S. mutans* in the test group but rather an occasional difference between the two groups. This chance observation supports the opinion that salivary IgA antibodies could be of biological significance in the human defense against cariogenic microorganisms. It also justifies further studies on the possibilities to stimulate the immune response against *S. mutans*.

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