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Effect of Peroral Immunization of Humans with *Streptococcus mutans* on Induction of Salivary and Serum Antibodies and Inhibition of Experimental Infection

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Naturally occurring antibodies reactive with *Streptococcus mutans* whole cells were assayed in whole saliva, parotid saliva, and blood samples collected from eight human volunteers. The levels and serotypes of indigenous *S. mutans* in plaque and whole saliva samples were also determined. After baseline sampling the teeth were cleaned and the subjects were inoculated with streptomycin-resistant *S. mutans* strains Ingbritt (serotype *c*) and OMZ65 (serotype *g*). The level of implantation and duration of colonization were determined in plaque and saliva, and antibodies reactive with these strains were monitored in saliva and serum. After the implanted bacteria were shed, the subjects were immunized by the daily ingestion of an enteric-coated capsule containing 25 mg of Formalin-killed, freeze-dried OMZ65 cells for 3 days and inoculation was repeated. The levels of antibodies and of implantation and the duration of colonization were monitored as before. One month after the bacteria could no longer be detected, the immunization and inoculation cycle was repeated except that the subjects were immunized for 7 days. Five of the eight subjects were successfully colonized by strains Ingbritt and OMZ65. The remaining three did not become colonized with either strain. Strain OMZ65 implanted at a higher level than did strain Ingbritt. Oral immunization did not result in a detectable antibody response in saliva or serum to whole bacterial cells. However, after both the first and second immunizations there were marked reductions in the peak levels of infection and the duration of colonization of both OMZ65 and Ingbritt.

The identification of *Streptococcus mutans* as a principal etiological agent of dental caries (14, 27) has led to attempts to control this disease by active immunization. Because secretory immunoglobulin A (SIgA) is the predominant immunoglobulin isotype in saliva as well as in other external secretions (26), efforts have centered in some laboratories on the most effective methods of inducing salivary IgA antibodies directed against *S. mutans* antigens (17).

Observations of the high numbers of plasma cells synthesizing IgA in salivary glands, lacrimal glands, and the lamina propria of the small intestine, together with the finding that local immunization results in the production of SIgA antibody restricted to that specific site (11), suggested that SIgA antibody responses were generated entirely locally. Thus, injection of *S. mutans* emulsified in adjuvant into, or in the vicinity of, the major salivary glands (18, 23) or the retrograde instillation of *S. mutans* into the parotid salivary gland (8) resulted in the induction of salivary IgA antibody. However, the concept of local induction of IgA responses was difficult to reconcile with the detection of SIgA antibodies reactive with the enteric bacterium *Escherichia coli* (13) and the oral bacterium *S. mutans* (1) in human colostrum where local antigenic stimulation would not be expected to occur.

Evidence for a common mucosal immune system comprising the gut-associated lymphoid tissue (GALT), particularly the Peyer's patches but also the appendix and tonsils, and the bronchial-associated lymphoid tissue (BALT) resulted from findings that these tissues contain percurser IgA B cells that migrate to various glandular epithelia and, after differentiation, synthesize SIgA antibodies (3, 6). Thus stimulation of Peyer's patches after ingestion of *S. mutans* by rats induced specific SIgA antibody in saliva and colostrum which correlated with a reduction in dental caries in rats challenged with the homologous bacterium and fed a caries-promoting diet (19). In addition, it has been reported that when four human volunteers were immunized by ingesting enteric-coated capsules containing Formalin-killed *S. mutans*, specific SIgA antibodies were induced in saliva and lacrimal fluid (16). The parallel appearance of SIgA antibodies at anatomically remote secretory sites was suggested as providing supporting evidence for a common mucosal immune system.

The purpose of the present study was to confirm and extend these preliminary findings obtained with human subjects and to determine whether specific salivary SIgA antibody induced by peroral immunization would impair implantation and colonization of tooth surfaces with *S. mutans*. To examine whether peroral immunization with one serotype would protect against infection with a second serotype (cross-protection), the subjects were immunized with a serotype *g* strain but infected with both the homologous bacterium and a serotype *c* strain.

MATERIALS AND METHODS

Experimental design. The design of the experiment is shown in Fig. 1. At the baseline, samples of dental plaque and saliva were collected from each subject to determine the level and serotype of their indigenous *S. mutans* and to ensure that they did not harbor streptomycin-resistant *S. mutans*. Samples of whole and parotid saliva and serum were collected

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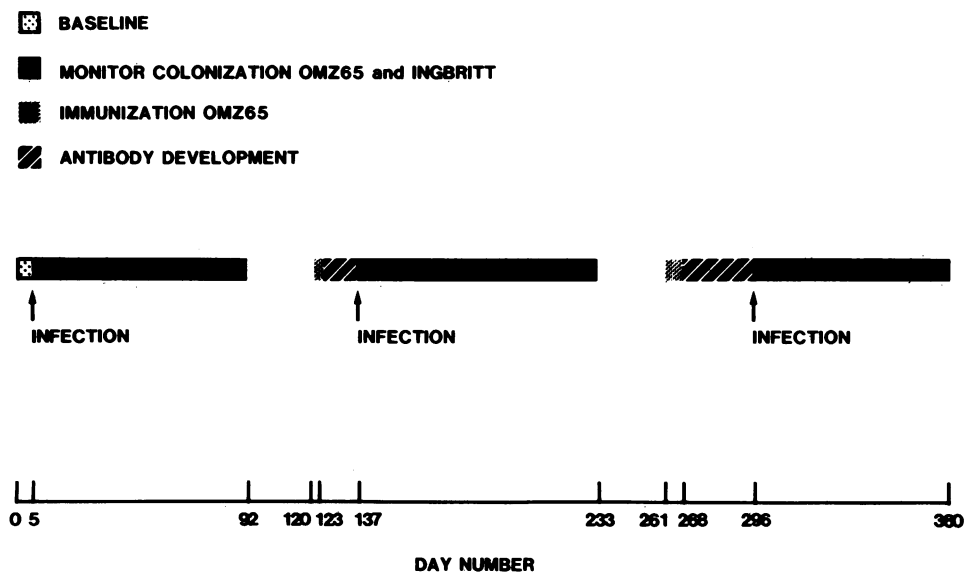

FIG. 1. Experimental design; see key and refer to text.

daily to determine the levels of naturally occurring antibodies reactive with *S. mutans* and *E. coli* K1 polysaccharide.

After baseline sampling, the teeth were cleaned with a rubber cup, pumice, and dental floss. The subjects rinsed with deionized water and were infected with streptomycin-resistant *S. mutans* strains Ingbritt (IB; serotype *c*) and OMZ65 (serotype *g*) by rinsing for 2 min with each bacterial suspension and then expectorating. Each subject then sucked a piece of hard candy. The subjects were instructed to continue their regular dietary and oral hygiene practices. The level of implantation and duration of colonization were monitored in plaque and saliva on alternate days, and samples of saliva and serum were collected for antibody assay twice weekly until the labeled bacteria were no longer detected. One month thereafter the subjects were immunized by ingesting an enteric-coated capsule containing 25 mg (approximately 2.5×10^{10} CFU) of Formalin-killed, freeze-dried OMZ65 for 3 successive days. Fourteen days after the final capsule was ingested infection was repeated. The level of implantation and duration of colonization and antibody levels were monitored as before. One month after the implanted bacteria could no longer be detected, the immunization and implantation cycle was repeated except that immunization was conducted for 7 days, and infection was effected 28 days after immunization was completed. In this experimental design each subject served as his own control.

Subjects. Eight male subjects, 32 to 50 years old, volunteered for the study. All had natural teeth and a mean dental caries experience of 20.5 (range, 16 to 27) decayed, missing, and filled teeth and 56.7 (range, 25 to 73) decayed, missing, and filled surfaces.

Saliva and blood collection. All samples of saliva, blood, and plaque were collected in the laboratory in midmorning, 2 to 3 h after breakfast. Unstimulated parotid saliva was collected with plastic intraoral cups (22). Unstimulated whole saliva was collected by allowing the subjects to drool into sterile 25-ml beakers held on crushed ice. One milliliter of whole saliva was removed from each sample for bacteriology, and the remainder was centrifuged at 10,000 × *g* for 20 min at 4°C to remove debris. The parotid and whole saliva samples were then frozen at −70°C. Blood was obtained by venipuncture and allowed to clot at room temperature, and the serum was separated by centrifugation at 2,000 × *g* for 15 min at 4°C and stored at −70°C.

Determination of salivary IgA concentration. The concentration of IgA in whole and parotid saliva was determined in duplicate samples, using a modified solid-phase immunofluorescence assay (Immuno-Fluor; Bio-Rad Laboratories, Richmond, Calif.) (5). SIgA purified from human colostrum was used as a standard. The limit of detection was 200 ng.

Determination of antibody reactive with *S. mutans* and *E. coli* K1 polysaccharide. IgA antibody in whole and parotid saliva and IgG and IgM antibody in serum reactive with *S. mutans* whole cells were determined with an enzyme-linked immunosorbent assay (ELISA) as described previously (4). IgA antibody reactive with the K1 polysaccharide from *E. coli* (obtained from R. Schneerson, Food and Drug Administration) was also measured in parotid saliva to examine the variation in salivary antibody to an antigen structurally unrelated to *S. mutans* (7).

Preliminary titration experiments determined the optimal dilutions for the ELISA to be 1:40 for whole saliva, 1:80 for parotid saliva, and 1:100 (IgM) and 1:400 (IgG) for serum. All samples of whole or parotid saliva or serum collected throughout the experiment were assayed at the same time.

To examine the specificity of binding to the immobilized *S. mutans* in the ELISA and to ensure that antibodies reactive with *S. mutans* were not cross-reactive with the K1 polysaccharide, samples of parotid saliva from each subject were assayed for IgA antibodies reactive with *S. mutans* OMZ65 and K1 polysaccharide both before and after absorption with *S. mutans* OMZ65. Parotid saliva samples were diluted 1:80 in phosphate-buffered saline (pH 8.2) containing 0.1% Tween 20 and gently mixed overnight at 4°C with packed cells. The bacteria were removed by centrifugation at 17,000 × *g* for 20 min at 4°C and the parotid saliva samples were assayed by the ELISA as described earlier. Antibodies

reactive with OMZ65 were almost completely removed from six of eight parotid saliva samples by prior absorption with homologous bacteria. However, antibody reactive with K1 polysaccharide remaining after absorption with OMZ65 cells averaged approximately 60% of preabsorption values (data not shown).

To control for the effect of normal day-to-day variation in salivary IgA concentration on IgA antibody activity, IgA antibody activity (optical density at 450 nm per milliliter) was normalized with respect to IgA immunoglobulin concentration (micrograms per milliliter) and expressed as optical density at 450 nm per microgram of IgA. Serum IgM and IgG antibodies were expressed as optical density at 450 nm per milliliter.

Antibody response to immunization was evaluated by comparing (i) the mean antibody levels in saliva and serum reactive with OMZ65 and IB at the baseline period with that of the first and second periods of antibody development (ii) the mean antibody level at the initial infection period with that of the infection periods after the first and second immunizations, and (iii) the mean antibody level of the baseline and initial infection periods with that after the first immunization period (combined first antibody development and second infection period) and the second immunization period (combined second antibody development and third infection period) (see experimental design in Fig. 1).

As essentially the same trends were observed for each of the three comparisons, only the results of the combined periods (iii) are presented.

Bacteriological sampling procedures. All subjects refrained from brushing their teeth 24 h before sampling. Whole saliva was collected as described earlier and 1 ml was immediately transferred to a vial containing 1 ml of reduced transport fluid (21). Approximal plaque samples were collected from between the lower right and left second premolar and first molar, using sterile unwaxed dental floss, and were pooled in 2 ml of reduced transport fluid. In addition, each subject brushed his teeth with sterile disposable toothbrushes (Oral-B 20-child size; Cooper Laboratories, Inc., Wayne, N.J.); the head of each toothbrush was cut off and placed in 10 ml of reduced transport fluid. The toothbrushes were weighed before and after brushing so as to obtain the weight of the material representing mostly saliva and pooled, smooth surface plaque. All samples were kept on ice and were cultured within 1 to 3 h. The samples were dispersed by ultrasound for 30 s at 30-W power. Serial 10-fold dilutions of the samples were made in 0.01 M phosphate buffer, pH 7.0, and aliquots of 0.1 ml of appropriate dilutions were plated in duplicate on the following media: tryptic soy agar with 5% sheep blood and 5% sucrose to determine viable counts; mitis salivarius-bacitracin agar (MSB) (12) for determination of total *S. mutans* counts; and mitis salivarius agar (Difco Laboratories, Detroit, Mich.) supplemented with 200 μg of streptomycin per ml (MSS) for enumeration of streptomycin-resistant *S. mutans*. All plates were incubated at 37°C under anaerobic conditions in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.); MSB and MSS plates were incubated for 48 h and blood agar plates were incubated for 7 days. The prevalence of *S. mutans* was enumerated on MSB and MSS by their typical colonial morphology, expressed as the number of CFU per milliliter of saliva or as a percentage of the total CFU on blood agar. Representative and questionable colonies were isolated and examined with fluorescent antisera to *S. mutans* serotypes *a* through *g*, using methods and antisera described previously (25, 26).

Counts in whole saliva correlated well with those in approximal dental plaque or those recovered from the debris obtained from the tared toothbrushes. Moreover, streptomycin-resistant *S. mutans* were detected in whole saliva samples even when corresponding floss and toothbrush samples were negative (data not shown). Therefore, the results of infection with the streptomycin-resistant *S. mutans* strains OMZ65 and IB are expressed as the median number of CFU per milliliter of whole saliva for each infection period.

Growth conditions of bacteria used for implantation and immunization. (i) Implantation. Stock cultures of streptomycin-resistant *S. mutans* strains IB (serotype *c*) and OMZ65 (serotype *g*) were grown separately at 37°C for 17 h in 50 ml of the ultrafiltrate (PM10 membrane; Amicon Corp., Lexington, Mass.) of Jordan's streptococcal broth (15) containing 0.5% glucose in an atmosphere of 95% N_2–5% CO_2. The bacteria were centrifuged at 16,000 × *g* for 20 min, washed twice in phosphate-buffered saline (pH 7.0), and suspended to a concentration of approximately 2×10^8 to 3×10^8 CFU/ml in phosphate-buffered saline (pH 7.0). A 10-ml rinse of each strain containing 2×10^9 to 3×10^9 CFU was used. Purity of the suspensions was confirmed by plating on blood agar.

(ii) Immunization. *S. mutans* OMZ65 was grown to substrate exhaustion in the ultrafiltrate (PM10) of Jordan's streptococcal broth containing 1.5% glucose maintained at pH 6.8. The bacteria were harvested by centrifugation at 16,000 × *g* for 20 min, washed twice in deionized water, and killed by the addition of Formalin to a final concentration of 0.1%. The killed cells were washed three times in deionized water and lyophilized. Gelatin capsules (no. 2; Eli Lilly & Co., Indianapolis, Ind.) were filled with 25 mg of the lyophilized bacteria. The capsules were enteric coated by applying eight coats of polyvinylacetate phthalate (Opaseal; Colorcon Inc., West Point, Pa.) diluted 60:40 with 95% ethanol. Each coat was dried for 15 min at 50°C.

Statistical analysis. The nonparametric Spearman rank correlation coefficient (*r*) was used to evaluate correlations between the level of infection versus the level of indigenous *S. mutans* and the level of infection versus the level of naturally occurring antibody. The levels of infection are represented by the peak number of implanted bacteria for each period for individual subjects. The levels of indigenous *S. mutans* are represented by the median number for each period for individual subjects. Since the variation of *S. mutans* for each individual in each period is large, the median is more resistant to extreme large or small variation than the mean and is used as the representative value for the whole period for individual subjects. The levels of naturally occurring antibody are represented by the mean for each period for individual subjects.

The duration of colonization as well as the peak level of *S. mutans* strains IB and OMZ65 were used as criteria to evaluate the effect of immunization on implantation. The nonparametric Wilcoxon signed-rank test was performed to evaluate the reduction in implantation. The differences of peak and duration between the baseline and the first or second immunization for each individual were considered as the basic unit of observation for statistical tests. Since only five of the eight subjects were successfully implanted with IB and OMZ65, the three subjects who resisted infection were excluded from the statistical analysis.

The paired *t*-test was used to evaluate the different antibody responses to immunization. The differences in the mean antibody level between the baseline and the first or

TABLE 1. Median levels of indigenous *S. mutans*

Subject no.	CFU per ml of whole saliva		
	Baseline	1st immunization	2nd immunization
1	300,000	128,000	440,000
2	105	0	0
3	16,050	4,200	700
4	1,820,000	2,285,000	2,450,000
5	329,000	83,000	265,000
6	37,500,000	10,500,000	1,105,000
7	332,000	338,000	240,000
8	8,000	5,000	6,000

second immunization for each individual were considered as the basic unit of observation in the paired *t*-test. The correlations between the different antibody responses were evaluated by the nonparametric Spearman rank correlation coefficient. The mean levels of antibody responses for each individual for each period were used as the basic unit of observation. An increase in antibody level after immunization was considered significant only if it exceeded the mean baseline value plus 2 standard deviations.

RESULTS

Microbiology. All eight subjects harbored indigenous *S. mutans* which were exclusively serotype *c*. However, the level varied markedly from one subject to another. The number of indigenous *S. mutans* encompassed a range between 37.5×10^6 and 1.05×10^2 CFU per ml of whole saliva (Table 1). No streptomycin-resistant *S. mutans* cells were detected.

At the baseline five of the eight subjects were successfully infected by streptomycin-resistant *S. mutans* strains OMZ65 and IB. The other three subjects failed to become infected with either strain despite an additional implantation attempt. Two of the subjects that resisted infection harbored the lowest numbers of indigenous *S. mutans*. There was a positive correlation between the level of indigenous *S. mutans* and the ability of strain OMZ65 to colonize. The correlation coefficient was 0.690 ($P = 0.058$). Also, the level of infection of strain OMZ65 was negatively correlated with the level of naturally occurring antibody in whole saliva. The correlation coefficient was −0.786 ($P = 0.021$).

Strain OMZ65 implanted at a higher level than strain IB (Table 2). After both the first and second immunization periods there were marked reductions in the peak levels of infection and the duration of colonization of both *S. mutans* strains compared with baseline values. These reductions were statistically significant for strain IB after the first immunization and for strain OMZ65 after the second immunization ($P = 0.03$).

No statistically significant change in the level of indigenous *S. mutans* was observed throughout the experiment (Table 1).

TABLE 3. Mean level of IgA antibody reactive with *S. mutans* strains IB and OMZ65 in whole saliva

Subject no.	$OD_{450}/\mu g$ of IgA[a]					
	Baseline		1st immunization		2nd immunization	
	IB	OMZ65	IB	OMZ65	IB	OMZ65
1	0.93 (1.78)[b]	0.56 (0.77)	1.31	0.51	0.92	0.45
2	1.10 (2.10)	0.60 (0.95)	1.61	0.73	1.36	0.58
3	0.36 (0.47)	0.14 (0.25)	0.32	0.14	0.29	0.13
4	1.24 (2.34)	0.53 (0.86)	1.34	0.48	1.04	0.34
5	0.19 (0.31)	0.07 (0.18)	0.39	0.04	0.21	0.00
6	0.64 (1.03)	0.12 (0.24)	0.80	0.18	1.36	0.10
7	0.81 (1.52)	0.63 (0.86)	1.06	0.66	1.01	0.57
8	0.25 (0.40)	0.23 (0.32)	0.18	0.23	0.17	0.19

[a] OD_{450}, Optical density at 450 nm.
[b] Numbers in parentheses are the baseline values plus 2 standard deviations.

Immunology. The mean antibody levels in saliva and serum for the baseline and first and second immunization periods for each subject are tabulated in Tables 3 to 6.

All subjects exhibited naturally occurring IgA antibody in saliva and IgG and IgM antibody reactive with *S. mutans* strains OMZ65 and IB. The level of this natural antibody differed markedly between subjects. The level of antibody reactive with both serotypes in parotid saliva was generally greater than that in whole saliva (Tables 2 and 3). The mean level of natural antibody activity in parotid saliva reactive with strains IB and OMZ65 was directly correlated with the numbers of indigenous *S. mutans*. The correlation coefficient for strain IB was 0.905 ($P = 0.002$) and that for strain OMZ65 was 0.714 ($P = 0.046$). No relationship was apparent between the level of parotid IgA antibody reactive with K1 polysaccharide and the numbers of indigenous *S. mutans*. In addition, no relationships between the levels of IgA antibodies in whole saliva and IgG and IgM antibodies in serum reactive with OMZ65 and IB and indigenous *S. mutans* were observed.

Although changes in the mean antibody levels were observed after the immunization periods, the magnitude of

TABLE 2. Peak level of implantation and duration of colonization of *S. mutans* strains IB and OMZ65

Level	Strain	Subject 3[a]		Subject 4		Subject 5		Subject 6		Subject 7	
		Peak (CFU)[b]	Duration (days)	Peak (CFU)	Duration (days)	Peak (CFU)	Duration (days)	Peak (CFU)	Duration (days)	Peak (CFU)	Duration (days)
Baseline	IB	27,000	87	2,900	21	26,000	87	600	14	500	10
	OMZ65	51,000	87	14,900	14	432,000	42	91,300	73	14,700	35
1st immunization	IB	180	21	40	5	320	37	0	0	50	14
	OMZ65	800	28	10	7	1,200	28	3,360,000	98	3,200	42
2nd immunization	IB	830	21	190	14	3,000	14	400	21	1,300	28
	OMZ65	360	14	50	7	3,900	14	2,580	37	1,800	21

[a] Subjects 1, 2, and 8 failed to become implanted with either strain.
[b] Per milliliter of whole saliva.

TABLE 4. Mean level of IgA antibody reactive with *S. mutans* strains IB and OMZ65 and *E. coli* K1 polysaccharide in parotid saliva

Subject no.	OD_{450}/μg of IgA[a]								
	Baseline			1st immunization			2nd immunization		
	IB	OMZ65	K1	IB	OMZ65	K1	IB	OMZ65	K1
1	1.24 (1.79)[b]	0.37 (0.49)	0.26 (0.37)	1.05	0.40	0.37	0.79	0.38	0.39
2	0.92 (1.73)	0.37 (0.66)	0.43 (1.04)	0.94	0.21	0.47	0.52	0.13	0.26
3	0.98 (2.61)	0.28 (0.55)	1.01 (2.71)	0.60	0.21	0.35	0.41	0.12	0.53
4	2.60 (4.67)	0.82 (1.39)	0.46 (0.96)	2.84	1.15	0.39	1.71	0.41	0.31
5	1.83 (4.77)	0.55 (1.94)	1.43 (4.15)	1.38	0.39	0.97	1.15	0.26	0.78
6	37.63 (76.81)	3.28 (11.26)	3.51 (11.48)	14.46	1.37	1.32	6.46	1.43	1.46
7	16.60 (24.41)	5.76 (9.73)	2.61 (5.71)	12.94	4.86	1.98	10.94	3.79	1.80
8	0.18 (0.34)	0.15 (0.21)	0.21 (0.36)	0.21	0.12	0.16	0.11	0.15	0.13

[a] OD_{450}, Optical density at 450 nm.
[b] Numbers in parentheses are the baseline values plus 2 standard deviations.

these differences in the means fell within the confines of the mean baseline value plus 2 standard deviations and were, therefore, not regarded as significant.

DISCUSSION

It was interesting that the subjects, all of whom harbored serotype *c S. mutans*, were much more resistant to infection by a strain of the homologous serotype (IB) than by OMZ65, a serotype *g* isolate. Also, there was a statistically significant positive correlation between the level of indigenous *S. mutans* and the level of infection of OMZ65. When the relationships between the level of indigenous *S. mutans* and the levels of naturally occurring IgA antibody in parotid and whole saliva reactive with IB and OMZ65 were examined, statistically significant positive correlations were observed between the level of indigenous *S. mutans* and the level of natural antibody in parotid saliva reactive with both strains. The correlation for serotype *c* strain IB was particularly strong ($r = 0.905$; $P = 0.002$). In contrast, when the correlations between the levels of naturally occurring IgA antibody reactive with IB and OMZ65 in whole and parotid saliva and the level of infection were examined, a statistically significant inverse correlation was observed for the level of IgA antibody in whole saliva reactive with OMZ65 and the level of infection of this strain. One possible explanation for the disparate relationships between the levels of naturally occurring antibody in whole and parotid saliva and the levels of indigenous *S. mutans* and the levels of implantation of IB and OMZ65 is that antibody in the whole saliva of subjects harboring high numbers of indigenous *S. mutans* becomes bound to these bacteria and is thus unavailable for assay.

The peak levels and duration of colonization of both implanted strains of *S. mutans* were reduced after peroral immunization with *S. mutans* OMZ65. Although it would be tempting to suggest that these reductions were the result of cross-protective salivary immunity, the results of this study fail to support the assertion that the ingestion of enteric-coated gelatin capsules containing Formalin-killed *S. mutans* by humans results in the induction of specific salivary or serum antibodies against *S. mutans* whole cells. Although there were changes in the mean levels of salivary and serum antibodies after immunization suggestive of antibody induction, these changes rarely exceeded the mean absorbance plus 2 standard deviations of the baseline samples. Changes of similar magnitude were observed for antibody reactive with the K1 polysaccharide reference antigen. The covariation observed between antibody reactive with *S. mutans* and *E. coli* K1 polysaccharide suggests that the differences in antibody levels reactive with *S. mutans* postimmunization resulted from normal variation and not from a specific antibody response, although the possibility of cross-reactivity between *S. mutans* and *E. coli* K1 polysaccharide cannot be excluded.

The finding of a reduction in the level of infection and duration of colonization of implanted strains of *S. mutans* in humans after peroral immunization in the absence of a detectable specific antibody response is consistent with the results of studies described by Gahnberg and Krasse (10) and Bonta et al. (J. Dent. Res. **58**[Special Issue A]:143, abstr. no. 204, 1979). In contrast, Mestecky et al. (16) reported the selective induction of an immune response in

TABLE 5. Mean level of IgG antibody reactive with *S. mutans* strains IB and OMZ65 in serum

Subject no.	OD_{450}/ml[a]					
	Baseline		1st immunization		2nd immunization	
	IB	OMZ65	IB	OMZ65	IB	OMZ65
1	720.80 (993.29)[b]	2,852.00 (3,331.93)	752.89	2,640.44	1,511.33	2,581.33
2	1,133.33 (1,884.75)	821.33 (968.12)	874.22	841.33	359.20	770.40
3	1,332.80 (1,985.44)	1,821.60 (2,263.69)	1,031.56	1,992.44	1,060.67	1,708.00
4	1,708.80 (3,030.92)	1,041.60 (1,364.47)	1,559.11	1,059.56	2,038.67	922.00
5	1,096.00 (1,988.42)	1,356.80 (1,488.06)	936.50	1,738.00	1,142.00	1,524.00
6	1,489.33 (2,160.62)	1,367.20 (1,774.67)	1,184.44	1,689.33	655.33	1,368.00
7	487.20 (583.77)	1,860.80 (2,477.85)	511.56	1,611.56	304.80	1,517.60
8	1,710.67 (2,332.34)	2,255.33 (2,558.00)	1,237.78	2,210.22	1,284.00	2,316.67

[a] OD_{450}, Optical density at 450 nm.
[b] Numbers in parentheses are the baseline values plus 2 standard deviations.

TABLE 6. Mean level of IgM antibody reactive with *S. mutans* strains IB and OMZ65 in serum

Subject no.	OD_{450}/ml					
	Baseline		1st immunization		2nd immunization	
	IB	OMZ65	IB	OMZ65	IB	OMZ65
1	165.60 (206.63)[b]	88.60 (171.90)	118.67	113.22	102.33	112.17
2	146.50 (193.38)	34.33 (59.94)	140.44	44.56	153.60	42.40
3	202.00 (233.62)	137.00 (179.26)	174.00	100.56	150.50	104.50
4	256.00 (353.82)	334.50 (388.18)	215.22	365.11	285.67	351.67
5	78.80 (98.11)	34.80 (60.51)	68.13	50.88	73.00	55.33
6	288.50 (383.70)	159.00 (218.65)	271.78	159.33	228.50	165.17
7	173.40 (219.20)	135.00 (177.33)	143.00	78.22	158.60	80.20
8	106.50 (132.04)	93.50 (111.33)	93.44	63.56	65.50	60.17

[a] OD_{450}, Optical density at 450 nm.
[b] Numbers in parentheses are the baseline values plus 2 standard deviations.

external secretions by ingestion of gelatin capsules containing Formalin-killed *S. mutans*, although the effect on implantation was not determined. It is unlikely that the failure to induce a salivary IgA response in the present study resulted simply from differences in immunization dosage or schedule because Bonta et al. used an identical dosage, schedule, and method of antibody determination to that reported by Mestecky et al. (16) but failed to induce salivary IgA antibody by peroral immunization. Also, Walker (28) has reported the failure of oral immunization with enteric-coated capsules containing viable *S. mutans* to induce salivary or serum antibodies in monkeys.

In the study reported here and in that described by Gahnberg and Krasse (10), salivary antibodies reactive with *S. mutans* and either *E. coli* K1 polysaccharide or an antigen pool from eight strains of *E. coli* used as a reference were measured by using an ELISA. In addition, antibody activity was normalized with respect to total IgA concentration to control for differences in salivary flow rate (9). In the studies described by Mestecky et al. (16) and Bonta et al. (J. Dent. Res. **58**[Spec. Issue A]:143, 1979), antibody was determined by bacterial agglutination. Despite the high sensitivity of the ELISA, the samples of parotid saliva analyzed by Gahnberg and Krasse (10) for antibodies reactive with *S. mutans* were assayed at dilutions of 1:2, 1:8, and 1:16 ($\log_2 = 1$ to $\log_2 = 4$). In the present study samples of parotid saliva were diluted 1:80 ($\log_2 = 6.3$). In marked contrast, using the far less sensitive technique of whole-cell agglutination, Mestecky et al. (16) reported peak agglutinin titers in parotid saliva of between $\log_2 = 7$ and $\log_2 = 10$ (1:128 to 1:1,024), although Bonta et al., using an identical method of antibody determination, detected low parotid agglutinin titers of only $\log_2 = 1$ to $\log_2 = 2$ (1:2 to 1:4).

Saliva is known to contain naturally occurring SIgA antibodies reactive with the various serotypes of *S. mutans* (1). Such natural antibodies reactive with a serotype *c* and *g* strain were observed in the whole and parotid saliva and serum of the subjects in the present study and naturally occurring antibodies reactive with a serotype *d* strain (B13) were detected in the parotid saliva of the subjects studied by Gahnberg and Krasse (10) and Bonta et al. In contrast, no naturally occurring antibody reactive with another serotype *d* strain (OMZ176) was observed in whole, parotid, or submandibular-sublingual saliva, lacrimal fluid, or serum from the adult volunteers studied by Mestecky et al. (16), although there is no reason to believe that this is an atypical serotype *d* strain (20).

Despite the apparent failure to reproducibly induce a salivary IgA antibody response in humans by peroral immunization and the discrepant antibody levels reported, the studies conducted by Bonta et al., Gahnberg and Krasse (10), and that described here, in which immunization was followed by challenge with homologous bacteria, have consistently demonstrated a marked reduction in the level of implanted strains of *S. mutans* after peroral immunization. Whether this inhibition of colonization after peroral immunization results from the induction of specific salivary antibody to a specific antigen or antigens which cannot be detected against a continuously changing background awaits further studies that will use strategies capable of delineating specific antibody induction from normal variations in the level of naturally occurring salivary antibody.

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