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Protective Secretory Immunoglobulin A Antibodies in Humans following Oral Immunization with *Streptococcus mutans*

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Ingestion of a vaccine containing killed *Streptococcus mutans*, originally isolated from each volunteer, daily for 10 consecutive days induced increased levels of specific secretory immunoglobulin A (sIgA) antibodies to *S. mutans* cells and two cell surface proteins, glucosyltransferase and surface antigen I/II, in parotid saliva and tears of four healthy males and in parotid saliva, tears, colostrum, and milk of a pregnant woman. In addition, these antibodies inhibited glucosyltransferase activity. Both IgA1 and IgA2 antibodies were induced. The levels of IgA antibodies in all secretions remained significantly above preimmunization levels for more than 50 days after oral administration of antigen. A second series of immunizations for 7 consecutive days resulted in even higher levels of sIgA antibodies, which peaked earlier and persisted longer than those observed after the primary immunizations. No increase in levels of antibodies in serum were detected in any subject. Antibodies reactive with human heart and kidney antigens could not be detected in saliva, tears, colostrum, milk, or serum samples collected at any time during the immunization regimen. The numbers of viable *S. mutans* organisms in dental plaque and whole saliva decreased after each series of immunizations, which correlated with increased levels of IgA antibodies in saliva, suggesting that IgA antibodies in saliva were responsible for the reduced adherence of this bacterium. These results indicate that ingested *S. mutans* antigen induces secretion of specific IgA1 and IgA2 antibodies in saliva, tears, colostrum, and milk, providing further evidence for the existence of a common mucosal immune system.

Secretory immunoglobulin A (sIgA) has been shown to be the predominant immunoglobulin produced at mucosal surfaces (4, 9, 53). Two subclasses of IgA (IgA1 and IgA2) exist in approximately equal proportions in secretions (see reference 32 for a review). Naturally occurring sIgA antibodies to many different antigens (e.g., oral, ocular, and respiratory microorganisms) are present in mucosal fluids (1, 3, 19) and may serve as the major immunological defense against infection by many pathogens. Immunization with pathogenic agents may induce protective effects against infection of mucosal surfaces. However, parenteral immunization induces systemic IgG and IgM antibodies, and little or no sIgA antibody is induced (12, 14, 40, 54). Ingestion of antigen stimulates the synthesis and release of specific sIgA antibodies into saliva, tears, colostrum, milk, and gastrointestinal, respiratory, and cervical mucosal fluids (11, 14, 19, 36, 40; see references 5 and 32 for reviews). Thus, oral administration of vaccines composed of pathogenic agents may be efficacious for the prevention of many infectious diseases. In this regard, Sabin live attenuated oral poliovirus vaccine induces an sIgA antibody response that protects against both the disease and infection by the virulent virus (42). A common mucosal immune system (CMIS) has been postulated to consist of the inductive sites, gut-associated lymphoreticular tissue, i.e., Peyer's patches (PP), and bronchus-associated lymphoreticular tissue and the effector sites, laminae propriae of the gastrointestinal, upper respiratory, and genitourinary tracts and the salivary, lacrimal, and mammary glands. Oral administration of antigen stimulates IgA precursor B and regulatory T cells in PP, which then migrate through the circulation to distant mucosal sites and differentiate into IgA-producing plasma cells which secrete polymeric IgA antibodies (11, 26, 30, 31; see references 6 and 37 for reviews).

Streptococcus mutans has been implicated as the major etiological agent of human dental caries (29; see reference 33 for a review). The mutans streptococci have been divided into seven serotypes (a to g), of which serotype c *S. mutans* is predominant in the United States (33). Injection of rats and hamsters in the region of the salivary glands with *S. mutans* antigens induced good IgA antibody responses in saliva and protection against dental caries after challenge with virulent *S. mutans* (34, 51). Reports from this and other laboratories have shown that ingestion of bacterial antigen induced high levels of sIgA antibodies in mucosal secretions of experimental animals and provided protection from challenge with virulent bacteria (24, 38, 44). Ingestion of killed *S. sobrinus* by four human volunteers induced increased levels of sIgA antibodies to *S. sobrinus* in saliva and tears but not serum. Numbers of *S. sobrinus* organisms in dental plaque or saliva were not determined (36). A recent study by this group showed that after oral administration of *S. mutans*, antigen-specific IgA precursor plasma cells appear in peripheral blood preceding sIgA antibodies in secretions (11). Recently, Gahnberg and Krasse (13) reported that oral immunization of six human subjects with killed *S. sobrinus* did not consistently induce IgA antibody responses in saliva, but significantly fewer *S. sobrinus* organisms could be recovered from immunized individuals than from unimmunized individuals after challenge with streptomycin-resistant bacteria. This laboratory also reported that subjects with high levels of naturally occurring IgA antibodies in mouth rinses cleared an implanted *S. sobrinus* strain faster than did individuals with low levels of antibody (25).

We present data which show that ingestion of a vaccine containing killed *S. mutans* originally isolated from each

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subject induced high levels of sIgA antibodies to *S. mutans* whole cells and purified cell surface antigens in saliva, tears, colostrum, and milk and reduced the number of *S. mutans* in dental plaque and whole saliva. In addition, both IgA1 and IgA2 antibodies were induced. These results provide further evidence to support the concept of a CMIS and suggest that oral administration of microbial antigens may be efficacious against many pathogenic agents.

MATERIALS AND METHODS

Subjects and sample collection. Five healthy individuals (four males [ages, 24, 25, 42, and 42 years] and a pregnant female [age, 31 years]) volunteered for this study. Unstimulated parotid saliva samples were collected with a plastic intraoral cup (48). Tears were collected after irritation with a mist expressed from lemon rind. Colostrum and milk were collected from the pregnant woman before and after parturition. All samples were clarified by centrifugation at 10,000 × *g* for 30 min. All accessible dental plaque present on the most posterior molar without crown restoration in each oral quadrant was collected with a periodontal probe. Plaque and unstimulated whole saliva samples were diluted in sterile saline, vortexed for 30 s, and plated in triplicate on mitis salivarius agar (Difco Laboratories, Detroit, Mich.) and mitis salivarius agar supplemented with bacitracin and sucrose (MSB; 15), for enumeration of total oral streptococci and *S. mutans*, respectively, after incubation for 3 days at 37°C in an atmosphere of 5% CO_2 in air. Blood samples (5 ml) were obtained by venipuncture and allowed to clot. Serum was separated from the clot by centrifugation (5,000 × *g*; 10 min), and saliva, tear, colostrum, milk, and serum samples were stored at −20°C until assayed for antibody activity in an enzyme-linked immunosorbent assay (ELISA) described below.

Preparation of antigens. The *S. mutans* whole cell preparations used for oral immunization were selected by screening of plaque samples on MSB agar. The most predominant *S. mutans*, as determined by colony morphology for each subject, was isolated and identified as *S. mutans* strains by using fluorescein-labeled anti-*S. mutans* typing serum (kindly provided by Ariel Thomson, National Institute of Dental Research, Bethesda, Md.). Glucosyltransferase (GTF) was prepared by the method of Smith et al. (49), and surface antigen (sAg) I/II was a kind gift from Michael W. Russell (Department of Microbiology, University of Alabama at Birmingham).

To prepare large amounts of bacterial antigens for immunization, 8 liters of dialyzed medium (7) was inoculated with log-phase cultures of the *S. mutans* isolates and incubated at 37°C with shaking for 24 h. Periodically during incubation, the acid formed was neutralized by adding sterile 2 N NaOH. Bacteria were harvested by centrifugation (10,000 × *g* for 10 min), washed five times with pyrogen-free saline, and killed by suspension in 0.5% Formalin–saline. After 3 days, sterility was tested by culturing on blood agar. The bacteria were extensively washed with pyrogen-free saline and then lyophilized.

Gelatin capsules (no. 000; Parke, Davis & Co., Detroit, Mich.) were filled with 100 mg of lyophilized *S. mutans* (representing approximately 10^{11} bacteria) and rinsed in tap water to avoid contact of the *S. mutans* antigen with oral lymphoid tissues. Capsules were swallowed daily at specified times. Capsules were taken for 10 consecutive days during primary immunizations and for 7 consecutive days in secondary immunizations. The woman delivered on day 10 after initial ingestion of antigen. Saliva, tears, colostrum or milk, whole blood, and dental plaque were collected at weekly or bimonthly intervals.

ELISA and GTF neutralization antibody assay. The ELISA used was a modification of methods described previously (17, 18, 39). An *S. mutans* whole cell preparation (100 μl) from each subject (diluted in 0.1 M carbonate buffer [pH 9.6] to an optical density of 0.500 at 660 nm) or 1 μg of GTF or sAg I/II per ml was added to wells of flat-bottom polystyrene microtiter plates (EIA, Linbro; Flow Laboratories, Inc., McLean, Va.). The plates were incubated for 3 h at 37°C and overnight at 4°C and then washed three times with 0.9% NaCl containing 0.05% Tween 20 to remove unbound antigen. After the final wash, 10 μg of human serum albumin (globulin free; Sigma Chemical Co., St. Louis, Mo.) per ml in carbonate buffer was added to each well (200 μl) to block unreacted sites, and the plates were incubated at 25°C for 1 h. The plates were washed, and then a previously optimized dilution of human parotid saliva (1:4), colostrum or milk (1:50), tears (1:50), or serum (1:100) was added to each well (100 μl) in triplicate and incubated at 37°C for 1 h. The plates were washed, and rabbit IgG anti-human IgA, IgG, or IgM heavy chain-specific reagent (Meloy Laboratories, Inc., Springfield, Va.) or rabbit IgG anti-human IgA1 or IgA2 (Fc specific; Nordic Immunological Laboratories, Tilburg, The Netherlands) was added to appropriate wells (100 μl) and incubated at 37°C for 1 h. The plates were washed, and alkaline phosphatase (Sigma)-labeled goat IgG anti-rabbit IgG heavy chain-specific reagent (Behring Diagnostics, Div. of American Hoechst Corp., Somerville, N.J.) or horseradish peroxidase-labeled goat IgG anti-rabbit IgG heavy chain-specific reagent (Cappel Scientific Division, Cooper Biomedical, Inc., Malvern, Pa.) was added to each well (100 μl). After incubation at 37°C for 3 h and then at 4°C overnight, the plates were washed and alkaline phosphatase substrate (*p*-nitrophenyl phosphate; Sigma), dissolved in 10% diethanolamine buffer (1 mg/ml; pH 9.8) or horseradish peroxidase substrate (0.4 mg of orthophenylene diamine hydrochloride [Sigma] per ml in citrate buffer [pH 5.0] containing 0.025% H_2O_2), was added to each well (100 μl) and reacted at 30°C for 1.5 h or 30 min, respectively. The amount of color which developed was measured at 405 or 492 nm, respectively, in the microtiter plate by using a Titertek Multiskan photometer (Flow Laboratories). The individual ELISA absorbances were reduced by computing means and standard errors of the means of the absorbances of triplicate determinations, and some were logarithmically transformed into ELISA units as previously described (17). The data are expressed as absorbances, ELISA units, or ELISA units per milligram of IgA. The levels of antibodies in parotid saliva capable of GTF inhibition were determined by the method of Montville et al. (41) and are reported as percent inhibition from untreated controls.

Immunoglobulin measurement. Immunoglobulin levels in saliva, tears, colostrum, milk, and serum were measured by radial immunodiffusion with commercial antisera to human immunoglobulin (heavy chain specific; Meloy) for determining normal and low levels of IgA, IgG, and IgM. Dilutions of commercial preparations of 7S IgA, IgG, and IgM were used as standards (Meloy).

Determination of heart-reactive antibodies. Selected saliva, tear, colostrum, milk, and serum samples were assessed for the presence of antibodies reactive with human heart and kidney antigens by ELISA. Briefly, fresh human heart and kidney tissues obtained at autopsy were extensively washed with saline to remove extraneous erythrocytes, homoge-

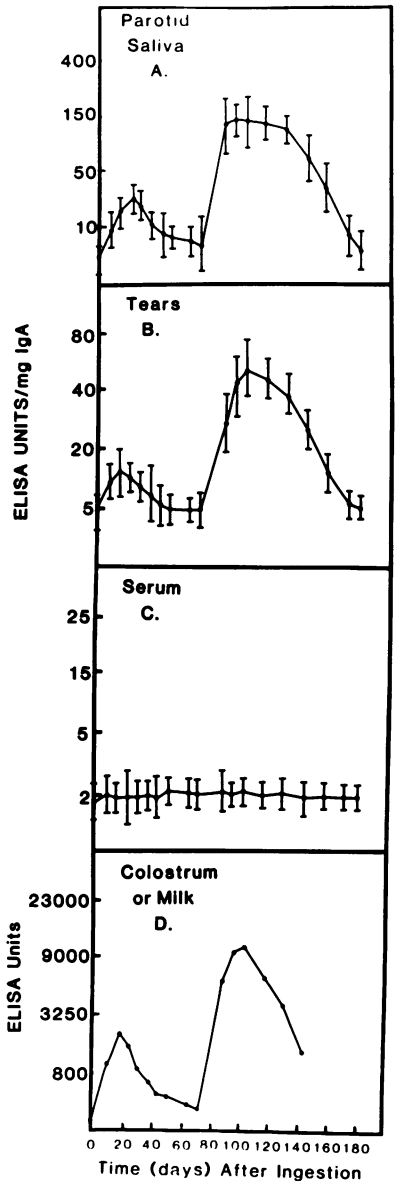

FIG. 1. Parallel dynamics of mean IgA antibody responses to *S. mutans* serotype c whole cells in parotid saliva (A), tears (B), serum (C), and colostrum or milk (D) following primary and secondary oral immunization of four male volunteers and a pregnant woman. Parturition was on day 10. Vertical bars represent one standard error above and below the mean. Details are in the text.

nized in a tissue homogenizer, suspended to an optical density of 0.500 at 660 nm in 0.1 M carbonate buffer (pH 9.6), and used in an ELISA as described previously (20). Positive controls included mouse anti-human heart antisera and serum from a patient with a high titer of anti-streptolysin O antibody.

RESULTS

Immune response to *S. mutans* antigens in saliva, tears, colostrum, milk, and serum. The levels of sIgA antibodies to the appropriate *S. mutans* whole cell preparation in saliva and tears but not serum samples from the five volunteers increased significantly above ($P \leq 0.05$) preimmunization titers after the primary immunization (Fig. 1). IgA antibodies in colostrum or milk paralleled the levels in saliva and tears. All five volunteers had significantly increased IgA antibody levels in secretions following immunization. Maximum levels of sIgA antibody were observed approximately 20 days after the initial immunization and decreased to preimmunization titers about 40 days later. At 80 days after the initial immunization, a second series of seven consecutive daily immunizations was given. Following this second immunization, sIgA antibody levels increased more rapidly and reached higher peak titers than those obtained after the primary immunizations ($P \leq 0.05$). High levels of sIgA antibodies remained in secretions for a longer period of time (approximately 80 days) than after the primary series of immunizations (approximately 30 days). No increase in the levels of IgG and IgM antibodies to *S. mutans* in serum, saliva, and tears was observed. In addition, increased titers of IgA antibodies in serum were not seen. The concentration of total IgA, IgG, and IgM in saliva, tears, and serum did not increase significantly above preimmunization levels, although the concentration of total IgA in colostrum and milk rose from 62.5 mg/100 ml (prepartum) to 500 mg/100 ml (2 weeks postpartum) and then decreased to 100 mg/100 ml at 140 days after parturition, when lactation was terminated. Antibodies reactive with human heart and kidney antigens could not be detected by ELISA in saliva, tears, or serum samples collected at any time during the experiment. The levels of IgA1 and IgA2 antibodies to *S. mutans* whole cells in colostrum or milk paralleled the levels of sIgA antibodies (Fig. 2). Both IgA1 and IgA2 antibodies increased in titer following immunization. sIgA antibodies to sAg I/II and GTF in parotid saliva from one volunteer also increased following immunization, paralleling the levels of anti-*S. mutans* whole cell antibodies (Fig. 3). Furthermore, the levels of parotid salivary GTF-neutralizing antibodies from one of the subjects paralleled sIgA antibody levels to *S. mutans* whole cells (Fig. 4).

Reduction of *S. mutans* in dental plaque. The proportions of *S. mutans* in dental plaque from the four males and the pregnant female decreased significantly ($P \leq 0.01$) following both immunizations and paralleled the levels of IgA antibodies to *S. mutans* in saliva (Fig. 5). The males and the pregnant woman had similar reductions in the proportions of *S. mutans*. The proportion of *S. mutans* in whole saliva also decreased following immunization (data not shown). The percentage of *S. mutans* in the total oral streptococci in dental plaque ranged from 2.5 to 4.5% in preimmunization samples and was reduced to as low as 0.1% in several of the subjects following the secondary immunizations ($P \leq 0.005$). The proportion of *S. mutans* in the five individuals returned to preimmunization numbers by approximately 60 days after the initial immunization and 100 days after the secondary administration of antigen. The parallel dynamics between the proportions of *S. mutans* in dental plaque and the levels of IgA antibodies to *S. mutans* in saliva indicate that immunization with indigenous *S. mutans* induces specific IgA antibodies in saliva which reduce colonization of *S. mutans* in situ.

DISCUSSION

This study indicates that oral administration of a vaccine containing killed *S. mutans* antigen to four human males and a pregnant female induced high levels of sIgA antibodies to *S. mutans* whole cells, GTF, and sAg I/II in parotid saliva, tears, colostrum, and milk. Following a second series of

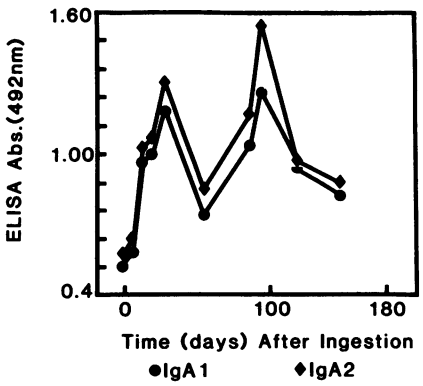

FIG. 2. Parallel dynamics of IgA1 (●) and IgA2 (◆) antibody responses to *S. mutans* serotype c whole cells in colostrum or milk following primary and secondary oral immunization of a pregnant female. Abs., Absorbance.

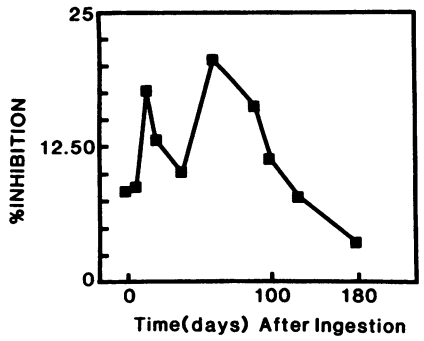

FIG. 4. Parallel dynamics of the inhibitory effects of parotid saliva from one of the volunteers on GTF activity following primary and secondary oral immunization.

immunizations, higher levels of sIgA antibodies were reached faster and persisted longer than those induced by the primary administration of antigen. This implies that an anamnestic sIgA immune response was induced by this immunization regimen. These results are in agreement with previous studies (11, 36). Furthermore, both IgA1 and IgA2 antibodies to *S. mutans* whole cells were induced. The proportion of *S. mutans* among the total oral streptococci in dental plaque (and saliva) was significantly reduced ($P \leq 0.01$) following each immunization regimen. In addition, the numbers of viable *S. mutans* in dental plaque were negatively correlated with the levels of IgA antibodies to *S. mutans* in saliva, suggesting that the antibodies inhibited adherence and colonization of *S. mutans*. These antibodies were found to neutralize GTF activity, implying that anti-GTF antibody is responsible for the reduced numbers of plaque-adherent *S. mutans* organisms by inhibiting adherence. Taken together, these data provide further support for the existence of a CMIS in humans, in that following ingestion of *S. mutans* vaccine, specific IgA antibodies were secreted into saliva, tears, colostrum, and milk.

In this regard, other investigators have described a CMIS in experimental animals, and migratory and homing pathways for antigen-committed IgA precursor B cells have been postulated (10, 22, 30, 31; see reference 52 for a review). Ingested antigens are taken up by microfolding and membranous cells (termed M cells; 43) lining the PP and are presented to underlying IgA precursor B and regulatory T cells. IgA precursor B cells leave the PP and migrate through the mesenteric lymph node, the thoracic duct, and the circulation (11) and home to the lamina propria regions of the gastrointestinal, upper respiratory, and genitourinary tracts and the salivary, lacrimal, and mammary glands. There, IgA precursor B cells undergo differentiation into IgA-producing plasma cells which secrete specific IgA antibodies into saliva, tears, colostrum, milk, and gastrointestinal, respiratory, and cervical mucosal fluids. Weisz-Carrington et al. (57) orally immunized mice with ferritin and found IgA antiferritin antibody-producing plasma cells in the laminae

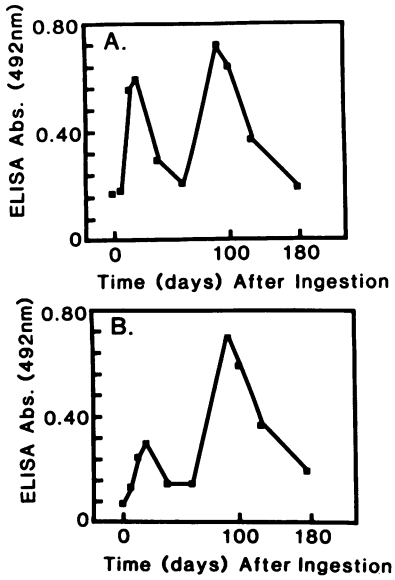

FIG. 3. Parallel dynamics of mean IgA antibody responses in parotid saliva from one of the volunteers to *S. mutans* sAg I/II (A) and GTF (B) following primary and secondary oral immunization. Abs., Absorbance.

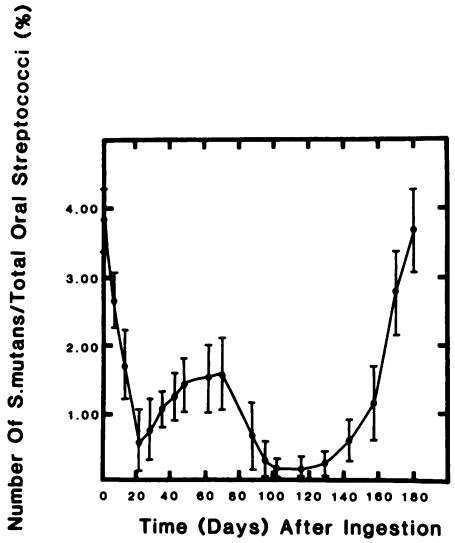

FIG. 5. Mean proportion (%) of *S. mutans* among the total oral streptococci in dental plaque following primary and secondary oral immunization of four male volunteers and a pregnant woman. Vertical bars represent one standard error above and below the mean.

propriae of the gastrointestinal and upper respiratory tracts and in the salivary and mammary glands. In addition, antigen-sensitized mesenteric lymph node IgA-bearing B cells migrated to these mucosal sites following adoptive cell transfer (23, 57). Other studies have shown that oral immunization leads to IgA antibody-forming cells and specific IgA antibodies in colostrum from humans (16) and mice (46). Cells migrating from the mesenteric lymph node following oral immunization are already committed to IgA synthesis and bear surface IgA before reaching their destination (35, 46). Additional work has shown that transferred PP and bronchial lymphocytes repopulate the lamina propria regions of the upper respiratory and gastrointestinal tracts (47). Pierce and Gowans (45) reported that intraintestinal immunization of rats with cholera toxoid induced B cells bearing IgA antibodies to the toxoid in thoracic duct lymph, which then homed to the portion of the gut closest to the site of immunization. Furthermore, antigenic challenge of the lamina propria of Thiry-Vella small intestinal loops indicated that antibody-bearing B cells were more prevalent in challenged loops than in nonchallenged loops (24). These studies show that antigen-committed B cells migrate to mucosal immune sites independently of antigen, but the presence of antigen at a site has a great influence on the degree of the immune response.

The results of the present study show that oral administration of killed-*S. mutans* vaccine reduces the numbers of *S. mutans* in dental plaque and saliva, suggesting that oral immunization with *S. mutans* may be an efficacious method of preventing human dental caries. The ability of a mucosal immune response following oral immunization to protect against microbial colonization has been examined previously in experimental animals. Pierce and colleagues (44) have shown that dogs orally immunized with cholera toxin and challenged with virulent *Vibrio cholerae* were markedly protected from disease. Oral immunization of rats with *S. mutans* has been reported to induce good IgA antibody responses in saliva and caries immunity (38). In other studies, Allardyce (2) showed that ingestion of rat sperm by female rats induces a significant sIgA antisperm antibody in genital fluid and reduces the incidence of pregnancy. These studies indicate that oral or intraintestinal administration of antigen induces a number of effects, such as prevention of infection by microbial pathogens or pregnancy. Although we showed reductions in the proportion of *S. mutans* in dental plaque following oral immunization, protection against dental caries could not be assessed because of the age of the subjects. Future studies must evaluate oral vaccines against human dental caries in children.

Our studies, which showed that orally immunized human subjects developed higher, faster, and more persistent sIgA antibody responses following a secondary administration of antigen than those found after initial ingestion of *S. mutans*, clearly suggest that memory cells are induced after primary oral immunization. In support of this was the recent demonstration of an sIgA memory response in gut secretions (24). Rabbits were orally immunized three times with *Shigella flexneri* and allowed to rest for 60 days. When another oral immunization was given, a high sIgA anamnestic response was obtained.

Several laboratories have reported no obvious effect of oral immunization with *S. sobrinus* or *S. mutans* on levels of IgA antibodies in saliva of humans (13; C. Y. Bonta, R. Linzer, F. Emmings, R. T. Evans and R. J. Genco, J. Dent. Res. **58**:143, abstr. no. 204, 1979) or monkeys (28, 55). In other studies, however, increased IgA antibody levels in saliva were found in humans following oral administration of *S. mutans* or *S. sobrinus* (11, 36; M. F. Cole, C. G. Emilson, J. E. Ciardi, and W. H. Bowen, J. Dent. Res. **60**:509, abstr. no. 798, 1981). More recently, Cole et al. (8) reported that individuals perorally immunized with an enteric coated capsule containing 25 mg of Formalin-killed lyophilized *S. sobrinus* whole cells did not have increased levels of specific antibodies in saliva but had significant reductions in the subsequent colonization of challenge streptomycin-resistant *S. mutans* and *S. sobrinus*. Lehner and colleagues recently presented an interesting method of inducing an immune response and protection from dental caries in rhesus monkeys (27). They directly applied a small (molecular weight, 3,800) *S. mutans* surface protein to the gingival crevices 10 times over a 1-year period. There were increases in specific IgG antibodies in crevicular fluid and IgA antibodies in saliva, which correlated with significantly lower levels of caries and colonization of *S. mutans*. This immunization approach induces antibodies in both crevicular fluid and saliva, both of which may be very important in mediation of dental caries. The present study clearly shows that oral immunization of humans with killed, indigenous *S. mutans* induced increased levels of specific sIgA antibodies in all of the secretions examined, and the numbers of *S. mutans* organisms in dental plaque and saliva were negatively correlated with the levels of IgA antibodies to *S. mutans* in saliva. The reduced numbers of *S. mutans* in dental plaque suggest that oral administration of *S. mutans* antigens may be an effective route of immunization against dental caries in humans. Furthermore, since it has been suggested that antibodies to *S. mutans* components may cross-react with human heart and kidney tissues (21, 50), it was important to show that oral administration of *S. mutans* whole cells to human volunteers does not induce heart-reactive antibodies.

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