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Intranasal Immunization against Dental Caries with a *Streptococcus mutans*-Enriched Fimbrial Preparation

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Streptococcus mutans **has been identified as the major etiological agent of human dental caries. The first step in the initiation of infection by this pathogenic bacterium is its attachment (i.e., through bacterial surface proteins such as glucosyltransferases, P1, glucan-binding proteins, and fimbriae) to a suitable receptor. It is hypothesized that a mucosal vaccine against a combination of *S. mutans* surface proteins would protect against dental caries by inducing specific salivary immunoglobulin A (IgA) antibodies which may reduce bacterial pathogenesis and adhesion to the tooth surface by affecting several adhesins simultaneously. Conventional Sprague-Dawley rats, infected with *S. mutans* at 18 to 20 days of age, were intranasally immunized with a mixture of *S. mutans* surface proteins, enriched for fimbriae and conjugated with cholera toxin B subunit (CTB) plus free cholera toxin (CT) at 13, 15, 22, 29, and 36 days of age (group A). Control rats were either not immunized (group B) or immunized with adjuvant alone (CTB and CT [group C]). At the termination of the study (when rats were 46 days of age), immunized animals (group A) had significantly ($P < 0.05$) higher salivary IgA and serum IgG antibody responses to the mixture of surface proteins and to whole bacterial cells than did the other two groups (B and C). No significant differences were found in the average numbers of recovered *S. mutans* cells among groups. However, statistically fewer smooth-surface enamel lesions (buccal and lingual) were detected in the immunized group than in the two other groups. Therefore, a mixture of *S. mutans* surface proteins, enriched with fimbria components, appears to be a promising immunogen candidate for a mucosal vaccine against dental caries.**

The first step necessary for any pathogenic bacterium to initiate infection is its attachment to a suitable receptor. Several different attachment mechanisms have been identified for oral bacteria (i.e., through surface proteins, such as glucosyltransferases [GTF] and glucan-binding proteins, by sucrose-dependent mechanisms and through surface antigen P1 and/or fimbriae in sucrose-independent functions). Bacterial fimbriae have been defined as small (100 to 300 nm), nonflagellar, filamentous, proteinaceous surface appendages that do not participate in the transfer of bacterial or viral nucleic acids (1). *Streptococcus mutans* has been identified as the major etiological agent in human dental caries and comprises a significant percentage of the oral streptococci in carious lesions (16). Fimbriae have been identified on numerous gram-negative microorganisms as long fibrillar structures but have been reported for only a limited number of gram-positive microorganisms, including some oral streptococci, in which they typically appear as a much shorter fuzzy coat (4, 21). It is our belief that fimbriae are important virulence factors for *S. mutans* and are at least partially responsible for *S. mutans* sucrose-independent adherence to enamel surfaces. We have isolated a mixture of *S. mutans* surface proteins, which contained fimbria components (fimbria-enriched preparation), as demonstrated by immunostaining and electron microscopy, and have elicited antibodies in rabbits against this preparation (7).

An essential goal in the development of a vaccine for dental caries is to induce antibodies that block bacterial adhesion and, therefore, prevent bacterial colonization. This should then affect the formation of carious lesions. A number of studies with experimental animals and humans have shown that active and passive immunizations with *S. mutans*, either with whole cells or with different cellular components, inhibit *S. mutans* colonization and the subsequent formation of dental caries (8, 14, 18, 29). An in vitro microbial model (5) was used to demonstrate, for the first time, the efficacy of antibodies against the fimbria-enriched preparation in preventing the formation of carious lesions (6).

The association of *S. mutans* soluble cell protein antigens (e.g., P1) or dextran preparations with cholera toxin (CT) and the B subunit of CT (CTB) has been shown to increase the immunogenicity (salivary immunoglobulin A [IgA] antibody responses) of many antigens given perorally, intragastrically, or intranasally without causing toxic effects (2, 3, 11, 26, 28, 30). However, only two studies have addressed the role of salivary antibodies elicited intranasally by an antigen linked to CTB in protection against dental caries (9, 10). CT is an exceptionally immunogenic antigen. This is attributed to the immunopotentiating (or adjuvant) property of CT, as well as to the ability of nontoxic CTB to bind to cell surface GM_1 ganglioside and act as a carrier protein (3, 26).

The purpose of this study was to test the hypothesis that conventional rats which are intranasally immunized with a mixture of fimbria-enriched preparation of *S. mutans* surface proteins conjugated with CTB exhibit a higher salivary IgA response to the fimbria-enriched preparation, have fewer *S. mutans* organisms adhered to the teeth, and develop fewer caries than do control animals. The combination of surface antigens used as the immunogen in this study was expected to elicit a mucosal immune response that would affect *S. mutans* cariogenicity by inhibiting several adhesion mechanisms simultaneously.

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MATERIALS AND METHODS

Vaccine preparation. The isolation of a mixture of *S. mutans* surface proteins enriched for fimbria components has been previously described by Fontana et al. (7). Furthermore, Perrone et al. (23) characterized two of the bands seen in the mixed protein preparation as GTF and P1. In this study, we isolated a mixture of fimbria-enriched proteins from *S. mutans* A32-2 (serotype c) by using a 10 mM sodium phosphate saline solution (pH 7.2), containing 1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (fimbria buffer).

The fimbria-enriched preparation was chemically conjugated to CTB as previously described (11, 25, 26, 30). Briefly, equal amounts of fimbria-enriched preparation and low-salt CTB (List Biological Laboratories, Inc., Campbell, Calif.) were coupled by using *N*-succinimidyl-(3-[2-pyridyl]-dithio)propionate (SPDP) (Pharmacia LKB Biotechnology, Piscataway, N.J.). The precipitate that formed in the CTB derivative was dissolved by adding 10 μl of ethanolamine (Sigma Chemical Company, St. Louis, Mo.), and both preparations were dialyzed separately against 0.01 M phosphate-buffered saline (pH 7.4) overnight at 4°C, to remove excess SPDP. The fimbriae derivative was reduced with 50 mM dithiothreitol (Pharmacia) for 30 min at room temperature, passed over a Sephadex G-25 column (Pharmacia), added to the unreduced CTB derivative, and kept overnight at 4°C. The final conjugate was dialyzed against phosphate-buffered saline (0.01 M, pH 7.4) and stored in aliquots at −80°C. Enzyme-linked immunosorbent assay (ELISA) of plates coated with GM_1 ganglioside (Sigma) followed by the vaccine conjugate and probed with antibodies to CTB and the fimbria-enriched preparation demonstrated that both the receptor binding ability of CTB and the antigenicity of the fimbria-enriched preparation were preserved in the conjugate.

General experimental design. The study had three groups labeled A, B, and C. Twenty-eight conventional rats (Harlan Sprague-Dawley) were used per group. From their arrival in our laboratory, the dams and pups were given Diet MIT 305 (containing 5% sucrose) and deionized water ad libitum until the pups were weaned (18 days old). The animals were then provided Diet MIT 200 (containing 67% sucrose) ad libitum throughout the challenge period (18 to 46 days old).

Group A was intranasally given an *S. mutans* fimbria-enriched preparation–CTB vaccine (50 μg, containing 37.5 μg of fimbria-enriched preparation and 12.5 μg of CTB) together with a small dose (5 μg) of free (azide-free) CT (List Biological) with a pipettor adapted with a sterile tip, on day 13 of age and again on days 15, 22, 29, and 36. The dose volume was divided between the two nostrils (14 μl in each) and administered twice. Five days after the first immunization (at 18 days of age) the rat pups were challenged with 0.2 ml of an overnight, stationary-phase culture of streptomycin-resistant *S. mutans* A32-2 serotype c (10^8 CFU/ml) for three consecutive days (18, 19, and 20 days of age). This involved placing 0.1 ml of the *S. mutans* culture on the occlusal surfaces of each of the mandibular molar quadrants (for a total of 0.2 ml of culture/animal) with a 1,000-μl micropipettor. Colonization was confirmed at day 25 of age (5 days after the last bacterial challenge) by culturing oral swab samples on mitis salivarius agar supplemented with streptomycin (0.04 g/ml) (MS-S). The test isolate (*S. mutans* A32-2) used in this study was made streptomycin resistant by stepwise isolation on MS-S. Stability of the streptomycin resistance was tested by passaging the culture for 12 consecutive days in Todd-Hewitt broth without streptomycin and plating on MS-S plates on days 6 and 12.

Group B served as a positive unimmunized control and was challenged with *S. mutans* A32-2 at days 18 through 20 but did not receive the fimbria-enriched preparation–CTB vaccine. Group C was infected and received the CTB (12.5 μg) and CT (5 μg) adjuvants only.

The animals were euthanized at 46 days of age during blood collection by intracardiac puncture. Death was verified by the detection of pneumothorax. Saliva and serum samples were collected to determine the levels of salivary IgA and serum IgG antibody to *S. mutans* A32-2 whole cells, the *S. mutans* A32-2 fimbria-enriched preparation, and CTB as described below. Following termination, the right mandibular hemijaw quadrant of each rat was placed in a tube containing 3 ml of sterile saline. Plaque was disrupted from the molar surfaces by vortexing for 20 s, followed by sonication for 20 s at a setting of 20 (50 Sonic Dismembrator; Fisher), and finally vortexing again for 20 s. The number of *S. mutans* cells adhered to the teeth on one hemijaw quadrant was determined by culturing undiluted and 1:10-diluted (double-plated) aliquots of each sample by using a spiral plater (Spiral Systems) on MS-S plates and incubating the plates at 37°C in 5% CO_2 and 95% air for 72 h. All four hemijaws were then stained overnight with a murexide (Sigma) solution (0.3 g of murexide, 300 ml of distilled H_2O, and 700 ml of ethanol) for caries scoring. The jaws were rinsed, allowed to dry, examined for smooth-surface caries, sectioned, and then microscopically examined for sulcal and interproximal caries by using the Keyes method (12, 22).

Collection of saliva and serum samples. At the termination of the study, the rats were injected intramuscularly with ketamine-xylazine (9:5 vol/vol; 0.14 ml/100 g of body weight), and individual saliva samples (approximately 1 ml/animal) were collected with a capillary Pasteur pipette after pilocarpine stimulation over a 15-min interval. Pilocarpine (5 μg/ml in sterile saline; 0.1 ml/100 g of body weight) was given intraperitoneally between 3 and 5 min after anesthesia. The saliva samples were centrifuged (735 × *g* for 30 min, 4°C) and stored at −20°C until assayed for IgA antibody activity against *S. mutans* whole cells, the fimbria-enriched preparation, and CTB by using ELISA as described below. After the collection of saliva, all available blood was collected by cardiac puncture, allowed to clot at room temperature for 1 h, and stored overnight at 4°C. Serum was separated from the clot by centrifugation (3,210 × *g* for 30 min, 4°C) and stored at −20°C until it was assayed for IgG antibody activity by ELISA.

Determination of antibody activities against the fimbria-enriched preparation, whole cells, and CTB by ELISA. Polystyrene microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.) were coated (100 μl/well) with either the fimbria-enriched preparation (1 μg/ml diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6), formaldehyde-killed bacteria (diluted to an optical density at 540 nm of 0.5 in carbonate-bicarbonate buffer), or CTB (1 μg/ml diluted in carbonate-bicarbonate buffer) and incubated at 37°C for 3 h. Coated plates were washed three times in Tween-saline (TS) (0.9% NaCl containing 0.05% Tween 20) to remove unbound antigen. Free sites on the plates were blocked by reaction for 1 h with 200 μl of a solution containing 10 μg of bovine serum albumin (Sigma) per ml at 25°C. Diluted rat serum or saliva samples (diluted 1:100 or 1:10, respectively, in TS) were added (100 μl/well) to the wells, in triplicate, and the plates were incubated for 2 h at 37°C. Antigen added without serum or saliva, but with TS, served as the negative control. A saliva or serum sample from an immunized rat (group A) served as a reference control. The plates were washed three times with TS and incubated for 3 h at 37°C with 100 μl of a reagent specific for the heavy chain of either horseradish peroxidase-labeled anti-rat IgA (for saliva samples) or IgG (for serum samples) (1:1,000; Sigma) per well. After the plates were washed three times with TS, orthophenylenediamine dihydrochloride (0.5 mg/ml) in 0.05 M citrate buffer (pH 5.0) containing 0.7 μl of 30% H_2O_2/ml of substrate was added (100 μl) to every well. Color development was monitored between 10 and 30 min, and the reaction was stopped with 2 N H_2SO_4 (100 μl/well). The amount of color that developed in the microtiter plate was measured at 490 nm with a Titertek Multiscan spectrophotometer (Flow). The background values were automatically subtracted from the values for the experimental samples. The data were reduced by computing the means and standard errors of the means (SEM) of the absorbances of each sample, determined in triplicate.

Determination of antibody specificity to the fimbria-enriched preparation by electrophoretic techniques. To confirm antibody specificity to fimbria-associated components, the fimbria-enriched preparation was electrophoresed by reducing sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Molecular weight standards were included in the gel (Rainbow colored protein molecular weight markers; Amersham, Arlington Heights, Ill.). Proteins were transferred electrophoretically to nitrocellulose paper for immunoblotting. Two blots were prepared. Each blot was cut into strips with a sharp scalpel, so that it could be probed with saliva or serum from different groups. Strips from one blot were each probed with the pooled serum (diluted 1:50) from rats in either group A, B, or C (adjuvant), while strips from the second blot were each probed with the pooled saliva (diluted 1:4) from rats in either group A, B, or C. Negative control strips from both blots were incubated with washing buffer (Trizma base, NaCl, Tween-20 [pH 7.4]). Proteins which reacted with serum antibodies were visualized on nitrocellulose by alkaline phosphatase-labeled anti-rat IgG heavy chain antibody (Sigma) followed by nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) (Bio-Rad Laboratories, Richmond, Calif.). Proteins which reacted with salivary antibodies were visualized on nitrocellulose by applying anti-rat IgA biotin-labeled antibody (Zymed Laboratories Inc., San Francisco, Calif.) followed by alkaline phosphatase-labeled ExtrAvidin (Sigma) and NBT-BCIP. Molecular weights and percentages of total antibody binding were determined by comparison to protein standards with an UltroScan XL laser densitometer and GelScan XL software (Pharmacia).

Data analysis. Means and variances were calculated for each measured parameter and treatment group. If the variances of any of the response variables appeared to be unequal, an appropriate transformation (e.g., logarithmic or square root) was done prior to analysis. The statistical analysis of ELISA salivary IgA and serum IgG antibody data, bacterial numbers, and smooth-surface and total smooth-surface caries scores was done on the logarithmic scale because the means and standard deviations had a positive association (i.e., standard deviations increased as the means increased). However, original scores were used in all tables for presentation purposes. The caries scores, antibody data, and bacterial counts were analyzed with separate single-factor analysis-of-variance models. A multiple-factor analysis-of-variance model was used to compare the treatment groups for differences in caries measurements. The type of experimental or control group was assigned as the fixed effect, and litter was designated the random effect. Multiple comparisons were made by using Tukey's method at a 95% overall confidence level.

RESULTS

In general, the group A animals demonstrated significantly increased ($P < 0.05$) levels of IgA and IgG antibodies in saliva and serum, respectively (Tables 1 and 2), against the *S. mutans* A32-2 surface proteins, fimbria-enriched preparation, and whole cells. Significantly higher levels of antibodies against CTB were present in the saliva of all group A rats. The immunoblot of the *S. mutans* fimbria-enriched preparation probed with the pooled saliva from group A rats demonstrated only two bands,

TABLE 1. Salivary IgA antibody to *S. mutans* A32-2 fimbriae, *S. mutans* A32-2 whole cells, and CTB

Group	Immunization treatment	ELISA absorbance value[a]		
		S. mutans fimbriae	CTB	WC
A	Vaccine and adjuvant	0.246 ± 0.025	0.040 ± 0.005	0.139 ± 0.008
B	None	0.064 ± 0.010	0.016 ± 0.003	0.110 ± 0.004
C	Adjuvant alone	0.055 ± 0.005	0.024 ± 0.003	0.109 ± 0.004

[a] Mean ± SEM ($n = 28$). P values are as follows: for *S. mutans* fimbriae, A versus B = 0.0001, A versus C = 0.0001, and B versus C = 0.9999; for CTB, A versus B = 0.0001, A versus C = 0.0187, and B versus C = 0.1362; and for whole cells (WC), A versus B = 0.0022, A versus C = 0.0019, and B versus C = 0.9985. Results for groups B and C were not significantly different ($P > 0.05$) as determined by Tukey's procedure.

at approximately 59 and 190 kDa (Fig. 1), while the immunoblot probed with the pooled serum from group A rats demonstrated only one band, at 59 kDa. The band at 59 kDa is believed to be a fimbrial component, distinct from Smith and Taubman's 59-kDa glucan-binding protein (reference 27 and unpublished data), whose role is currently being investigated; the band seen at 190 kDa has been previously shown to be P1 (23). Negative-control blot strips for both serum- and saliva-probed blots, as well as strips probed with saliva or serum from group B rats, showed no response. The blot strip probed with serum from group C rats showed no response, while the strip probed with saliva from group C rats showed very faint bands at 190 and 66 kDa.

The increase in antibodies to the fimbria-enriched preparation did not result in a decrease of bacteria adhered to the teeth, since the three groups were not significantly different from each other. The *S. mutans* A32-2 plaque counts (mean ± SEM) for groups A, B, and C were $(6.62 \pm 5.24) \times 10^4$, $(8.85 \pm 3.52) \times 10^4$, and $(5.16 \pm 2.11) \times 10^4$ CFU/ml, respectively. The obtained P values were 0.3760 for group A versus B, 0.6588 for group A versus C, and 0.8857 for group B versus C. On the other hand, significantly fewer smooth-surface enamel lesions (Table 3) were detected in group A rats than in animals from the other two groups. No significant difference was found between groups B and C. Group A rats had the lowest total-enamel caries scores of the three groups analyzed (Table 4). However, there were no significant differences in carious lesions in the interproximal or sulcal enamel or in the dentin among the treatment groups.

DISCUSSION

Our laboratory has been extensively involved in establishing the role *S. mutans* fimbriae play in adherence to and colonization of the tooth surface by this bacterium and testing if antibodies against *S. mutans* fimbria components reduce the adherence of *S. mutans* to the tooth surface, thereby inhibiting the development of primary dental caries (6, 23, 24). Caries-free (CF) adult individuals have higher levels of salivary IgA antibodies to fimbria-enriched preparation of *S. mutans* than do caries-active (CA) individuals (7). These results suggest that CF subjects may be protected immunologically from dental caries in part by salivary IgA antibody against *S. mutans* fimbrial antigens. Perrone et al. (23) demonstrated, with immunoblot analyses and ELISA techniques with antibody to fimbria-enriched preparations, GTF, and P1 antigen, that the levels of fimbria components, GTF, and P1 antigen were higher in fimbria-enriched preparations from *S. mutans* isolates from CA subjects than in preparations from CF individuals. These results suggest that the differences between the composition of *S. mutans* fimbriae in isolates from CA and CF subjects may play an important role in the virulence of this microorganism in dental caries. Our laboratory has also reported that a 52-kDa salivary protein, identified as amylase, is the major protein in human saliva which binds *S. mutans* fimbria-enriched preparations (24). In addition, results obtained with an in vitro bacterial model demonstrated the efficacy of antibodies against *S. mutans* fimbria-enriched surface components in decreasing caries development (6).

Decisions regarding the use of a conventional, rather than a gnotobiotic, rat model and the immunization regimen used in this study were partially based on the intranasal CTB vaccine study of Katz et al. (11). Although they demonstrated that the

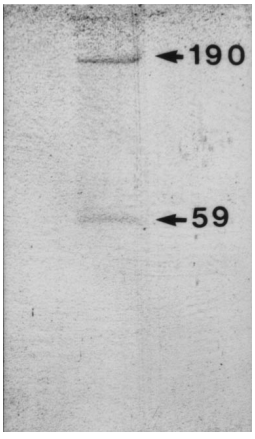

FIG. 1. Representative immunoblot of *S. mutans* A32-2 fimbria-enriched preparation probed with the pooled saliva from rats in group A, followed by anti-rat IgA biotin-labeled antibody, alkaline phosphatase-labeled ExtrAvidin, and NBT-BCIP. Numbers at the right are molecular masses, in kilodaltons.

TABLE 2. Serum IgG antibody to *S. mutans* A32-2 fimbriae, *S. mutans* A32-2 whole cells, and CTB

Group	Immunization treatment	ELISA absorbance values[a]		
		S. mutans fimbriae	CTB	WC
A	Vaccine and adjuvant	0.722 ± 0.047	0.644 ± 0.038	1.183 ± 0.108
B	None	0.370 ± 0.032	0.060 ± 0.014	0.717 ± 0.062
C	Adjuvant alone	0.379 ± 0.034	0.480 ± 0.041	0.760 ± 0.072

[a] Mean ± SEM ($n = 28$). P values are as follows: for *S. mutans* fimbriae, A versus B = 0.0001, A versus C = 0.0001, and B versus C = 0.9793; for CTB, A versus B = 0.0001, A versus C = 0.0451, and B versus C = 0.0001; and for whole cells (WC), A versus B = 0.0001, A versus C = 0.0001, and B versus C = 0.9023. Results with *S. mutans* fimbriae and WC for groups B and C were not significantly different ($P > 0.05$) as determined by Tukey's procedure.

TABLE 3. Smooth-surface (buccal and lingual) enamel and dentinal caries scores

Group	Immunization treatment	Caries score[a]	
		Enamel	Dentin
A	Vaccine and adjuvant	8.61 ± 0.77	0.36 ± 0.15
B	None	10.32 ± 0.86	0.68 ± 0.17
C	Adjuvant	11.75 ± 0.82	0.61 ± 1.31

[a] Mean ± SEM ($n = 28$). P values are as follows: for enamel, A versus B = 0.0472, A versus C = 0.0326, and B versus C = 0.3417; for dentin, A versus B = 0.0239, A versus C = 0.0502, and B versus C = 0.6843. Results for groups B and C were not significantly different ($P > 0.05$) as determined by Tukey's procedure.

TABLE 4. Total (smooth-surface, interproximal, and sulcal) enamel and dentinal caries scores

Group	Immunization treatment	Caries scores[a]	
		Enamel	Dentin
A	Vaccine and adjuvant	22.96 ± 1.25	1.82 ± 0.37
B	None	23.18 ± 1.12	1.68 ± 0.37
C	Adjuvant	25.18 ± 1.29	1.93 ± 0.50

[a] Mean ± SEM ($n = 28$). P values are as follows: for enamel, A versus B = 0.9898, A versus C = 0.3446, and B versus C = 0.4178; for dentin, A versus B = 0.9585, A versus C = 0.9764, and B versus C = 0.8785. Results for groups A, B, and C were not significantly different ($P > 0.05$) as determined by Tukey's procedure.

magnitude of the salivary IgA response in conventional animals was significantly lower than that in gnotobiotic rats, antibody levels increased in conventional rats after the second and third immunizations and reached their highest titers after the fourth immunization. Wu and Russell (30) have also demonstrated that mice required three immunizations before substantial elevations of antibody levels were obtained; however, monkeys responded after the second immunization (25). Furthermore, in the study by Katz et al. (11), immunized conventional rats had a 38% reduction of *S. mutans* cells in their plaque and a 64% reduction in buccal-enamel caries activity, and the levels of caries activity on sulcal surfaces were also significantly reduced, supporting the effectiveness of an intranasal CTB vaccine in these rats. In addition, since the conventional rat model is more similar to humans, it was selected for use in the present study. However, our data failed to demonstrate a decrease in the number of *S. mutans* cells adhered to the teeth or a statistically significant decrease in caries score categories in the vaccinated group other than smooth surfaces (e.g., sulcal caries and interproximal caries). A possible significant difference between our study and that of Katz et al. (11) was that the latter coupled a single protein (antigen I/II [AgI/II]) to CTB, while a combination of proteins (fimbria-enriched preparation) was coupled to CTB in the present study. This may have led to a dilution of immunoprotective antigens coupled to CTB, which was not expected initially. Alternatively, the coupling technique may not have been as effective. In rhesus monkeys, the coupling or mixing of antigen with CTB seemed not to make a great difference (25). The fact that intranasal immunization is an effective route for generating mucosal immune responses in the nonhuman primate, particularly when the vaccine includes CTB, is promising for humans (25).

In addition, although the immunization protocol in this study was similar to that of Katz et al. (11), the rats used in this investigation were much younger when antigen administration began. This might have affected the animals' immunocompetence status at the beginning of the study. Michalek et al. (20) demonstrated that significant antibody responses occurred in the saliva of gnotobiotic rats 5 or 6 days after gastric intubation of *S. mutans*. However, those animals were initially immunized at 19 days of age. The results obtained by Michalek et al. (20) clearly indicated that local antibodies were present in the saliva at the time of *S. mutans* challenge (i.e., 5 days after initial immunization). Based on this, conventional rats in the present study were immunized 5 days prior to bacterial challenge. Because the first and second molars of the rat erupt between 16 and 21 days of age, animals are usually challenged with cariogenic bacteria when they are between 19 and 24 days of age (19). After tooth eruption, enamel maturation occurs and indigenous plaque microorganisms colonize the teeth, which then become more resistant to specific bacterial colonization and to caries attack. If Harlan Sprague-Dawley rats (the rat model used for this experiment) are not challenged with a cariogenic strain of bacteria at the time their molars erupt, they will not develop any significant caries in the study time frame, even if put on a highly cariogenic diet. Additionally, the superinfection at the time of molar eruption ensures the colonization of the surface of the newly erupted tooth mainly with the superinfecting bacteria, so that the colonization of the teeth by indigenous bacteria is greatly decreased. However, although the potential role of the indigenous flora in caries development is greatly minimized, it should not be completely ignored. The first two immunizations in the present study were done anticipating the presence of antifimbria antibodies in saliva during mineralization of the newly erupted molars. Theoretically, the antibodies, by binding the bacteria and inhibiting colonization, could block the subsequent attachment of *S. mutans* A32-2 to the teeth. In mice, at least two to three intragastric doses of more than 15 μg of AgI/II coupled to CTB plus free CT were required to induce salivary IgA antibody responses, which peaked at 35 days and persisted at lower levels for 5 to 6 months (26). However, the use of 50 μg of AgI/II produced maximal responses (26) and was effective in eliciting protection against dental caries in rats (11). Therefore, we decided to use a similar dose in this study.

Salivary IgA and serum IgG antibody levels were significantly increased in the vaccinated group. These data indicated that the immunization protocol used was effective in producing a mucosal and systemic immune response against an *S. mutans* fimbria-enriched surface protein preparation and, therefore, whole cells which have these same cell surface components. This is not surprising, since the immunization regimen with CTB and CT is known to result not only in mucosal responses but also in systemic responses (3). Previous studies with mice (30) and monkeys (25) intranasally immunized with AgI/II coupled to CTB demonstrated that this route was highly effective at inducing secretory IgA in saliva and other secretions, as well as IgG in plasma. However, the previous studies did not investigate the level of antibodies sufficient to protect against dental caries. Furthermore, intranasal immunization has been reported to induce stronger antibody responses in saliva and serum than does intragastric immunization (30). A possible explanation is that intranasal cavities contain fewer proteolytic enzymes than the intestinal lumen; therefore, antigen administered intranasally may be more effective at stimulating the mucosal immune system than comparable amounts of antigen delivered by the intragastric route (30). Although it is known that immunization protocols which elicit only salivary IgA antibodies are successful in reducing dental caries (18), parenteral immunization, in which serum IgG is the main antibody elicited, has also been shown to confer partial immunity against dental caries (15). Therefore, eliciting both mucosal and systemic responses may be beneficial (3). While IgA antibody would offer protection against a mucosal pathogen by preventing colonization at the mucosal surface, serum IgG antibody might act against organisms that evade the mucosal defenses and invade the tissues or colonize subgingival sites. The immunoblot results demonstrate that antibodies against the 59-kDa protein were successfully elicited in both saliva and serum. Furthermore, pooled saliva from group A rats strongly reacted with purified 59-kDa protein during immunoblotting (data not shown). However, antibodies were also elicited against P1 in saliva. This is not surprising, since it has been suggested that P1 forms part of the fimbriae (or fuzzy coat) surrounding *S. mutans* cells, since P1 mutants lack a fuzzy coat (13). P1 has been shown to be protective (11); future studies will address the role of the 59-kDa protein. Therefore, in the

present study, salivary antibodies were directed against a mixture of *S. mutans* surface proteins and were expected to offer better protection than each antigen alone. It was evident that the amount of antibodies elicited in this study was not sufficient to produce an overwhelming reduction in all caries scores. However, the data for smooth-surface caries definitely indicated a trend in caries reduction in the vaccinated group. The fact that antibodies were not protective against sulcal caries may be a consequence of sulcal anatomy and the inaccessibility of sulci to antibodies. That specific salivary IgA antibodies might affect bacteria not only by agglutinating them but also by neutralizing enzyme activities (17) may explain the effect seen on caries in spite of no observable reduction in the number of adherent bacteria. Another possible explanation is that enumerated bacteria were recovered from various sites, but caries protection was observed only for specific sites. Site-specific sampling might have shown a difference in colonization. The present study demonstrated that either the dose of fimbria-enriched preparation used has to be increased or the immunization protocol used has to be changed in future studies in order to increase the level of caries-protective antibodies.

Dietary factors critically influence the composition and pathogenic potential of *S. mutans*-infected animal models by affecting the implantation, colonization, and metabolic virulence of the bacterium. Sucrose has been demonstrated to be extremely cariogenic and to support rapidly progressive pathogenesis (10). In the present study, mean weight gains among treatment groups were not significantly different, indicating that all groups consumed the same amount of food and that none of the treatment regimens had an adverse effect on growth. However, the presence of such a large amount of dietary sucrose (67%) probably supported the action of GTF in mainly inducing a glucan-adhered plaque. This may additionally explain why no differences in the numbers of bacteria were observed among the treatment groups in this study, although an antibody effect on cell surface protein or sucrose-independent attachment was anticipated. Future investigators should consider using a diet lower in sucrose.

The ultimate goal in the prevention of bacterial adhesion is a long-lasting protection conferred by an appropriate vaccine. A mixture of *S. mutans* surface proteins, enriched with fimbria components, coupled to CTB was used in this study. It was concluded that the intranasal immunization route successfully raised antibody levels in the saliva and serum of vaccinated rats, which was subsequently reflected in a decrease in smooth-surface caries scores. However, further studies are being conducted to characterize and sequence the 59-kDa protein and to compare the effect of specific antibodies to this protein to the effects of antibodies to P1 or a mixture of both proteins.

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