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

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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.

**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 af-

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MATERIALS AND METHODS

Vaccine preparation. The isolation of a mixture of S. mutans surface proteins enriched for fimbrin components has been previously described by Fontana et al. (7). A representative consomer sample of about 100 mg of whole cells was used in the present study. We used two of the bands that were detected in a mixed protein preparation as GTF and PI. In this study, we isolated a mixture of fimbrin-enriched proteins from S. mutans A32-2 (serotype c) by using a 10 mM sodium phosphate saline solution (pH 7.2), containing 1 mM CaCl2 and 1 mM phenylmethylsulfonyl fluoride (fimbrina buffer). The fimbrin-enriched preparation was chemically conjugated to CTB as previously described (11, 25, 26, 30). Briefly, equal amounts of fimbrin-enriched preparation and SRBC (Compu-Blood, Innovex, Inc., Campbell, Calif.) were coupled by using N-succinimidyl 3-[2-pyridylidy]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 fimbrin derivative was reduced with 50 mM dithiothreitol (Sigma Chemical Company, St. Louis, Mo.) and both preparations were dialyzed against 0.01 M NaCl, 0.05 M sodium phosphate, 0.1% Tween-20 [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 were probed with antibodies to CTB and the fimbrin-enriched preparation demonstrated that both the receptor binding ability of CTB and the antigenicity of the fimbrin-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 MFT 305 (containing 5% sucrose) and deionized water ad libitum until the pups were weaned (18 days old). The animals were then provided Diet MFT 200 (containing 67% sucrose) ad libitum throughout the challenge period (18 to 46 days old). Group A was intranasally given an S. mutans fimbrin-enriched preparation–CTB vaccine (50 μg, containing 57.5 μg of fimbrin-enriched preparation and 12.5 μg of CTB) together with a small dose (5 μg) of free (azide-free) CT (List Biological Laboratories, Campbell, Calif.) by using N-succinimidyl 3-[2-pyridylidy]propionate (SPDP) (Pharmacia LKB Biotechnology, Piscataway, N.J.) 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 were probed with antibodies to CTB and the fimbrin-enriched preparation demonstrated that both the receptor binding ability of CTB and the antigenicity of the fimbrin-enriched preparation were preserved in the conjugate.

Collection of saliva and serum samples. 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 fimbrin-enriched preparation, and CTB as described below. Following termination of the experiment, the right mandibular hemijaw quadrant of each rat was placed in a tube containing 0.1 ml 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. The final conjugate was encapsulated 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 GM1 ganglioside (Sigma) followed by the vaccine conjugate were probed with antibodies to CTB and the fimbrin-enriched preparation demonstrated that both the receptor binding ability of CTB and the antigenicity of the fimbrin-enriched preparation were preserved in the conjugate.

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, fimbrina-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 fimbrina-enriched preparation probed with the pooled saliva from group A rats demonstrated only two bands,
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 28). 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 B 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 ± 5.24) × 10⁴, (8.85 ± 3.52) × 10⁴, and (5.16 ± 2.11) × 10⁴ 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 coloni-

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization treatment</th>
<th>ELISA absorbance value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. mutans fimbriae</td>
<td>CTB</td>
</tr>
<tr>
<td>A</td>
<td>Vaccine and adjuvant</td>
<td>0.246 ± 0.025</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>0.064 ± 0.010</td>
</tr>
<tr>
<td>C</td>
<td>Adjuvant alone</td>
<td>0.055 ± 0.005</td>
</tr>
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</table>

\(^a\) 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 = 0.0001.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization treatment</th>
<th>ELISA absorbance values(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. mutans fimbriae</td>
<td>CTB</td>
</tr>
<tr>
<td>A</td>
<td>Vaccine and adjuvant</td>
<td>0.722 ± 0.047</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>0.370 ± 0.032</td>
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<tr>
<td>C</td>
<td>Adjuvant alone</td>
<td>0.379 ± 0.034</td>
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</table>

\(^a\) Mean ± SEM (n = 28). P values are as follows: for enamel, A versus B = 0.0326, A versus C = 0.072, and B versus C = 0.17; for dentin, A versus B = 0.480, A versus C = 0.004, and B versus C = 0.0136. Results for groups A and C were not significantly different (P > 0.05) as determined by Tukey’s procedure.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization treatment</th>
<th>Caries score(^a)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Enamel</td>
</tr>
<tr>
<td>A</td>
<td>Vaccine and adjuvant</td>
<td>8.61 ± 0.77</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>10.32 ± 0.86</td>
</tr>
<tr>
<td>C</td>
<td>Adjuvant alone</td>
<td>11.75 ± 0.82</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SEM (n = 28). P values are as follows: for enamel, A versus B = 0.0326, 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 = 0.6834. Results for groups 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization treatment</th>
<th>Caries scores*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Enamel</td>
<td>Dentin</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Vaccine and adjuvant</td>
<td>22.96 ± 1.25</td>
<td>1.82 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>23.18 ± 1.12</td>
<td>1.68 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Adjuvant</td>
<td>25.18 ± 1.29</td>
<td>1.93 ± 0.50</td>
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* Mean ± SEM (n = 28). P values are as follows: for enamel, A versus B = 0.9898, A versus C = 0.8446, and B versus C = 0.4178; for dentin, A versus B = 0.9585, A versus C = 0.9784, 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.
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 smooth 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 the fimbria 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.

REFERENCES


