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 GM1 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 fimbrin components has been previously described by Fontana et al. (7). Bacterial surface proteins were separated by two-dimensional gel electrophoresis of a mixed protein preparation as GTF and P1. 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 (fimbrin buffer). The fimbrin-enriched preparation was chemically conjugated to CTB as previously described (11, 25, 2b, 30). Briefly, equal amounts of fimbrin-enriched preparation and fimbria buffer were mixed and allowed to react for 15 min at room temperature. The mixture was then added to the reduced CTB derivative, 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 fimbrinaceous preparation was reduced with 50 mM dithiothreitol (Pharmacia) for 30 min at room temperature, passed over a Sephadex G-25 column, and added to the unreacted 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 GM₁ ganglioside (Sifag) followed by the vaccine conjugate was 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 dam and pups were given Diet MTT 305 (containing 5% sucrose) and deionized water ad libitum until the pups were weaned (18 days old). The animals were then provided Diet MTT 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 (axide-free) CT (List Biological) with a pipettor adapted with a sterile tip, on day 15 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), 0.1 ml of the gel (g/mouse) was placed on the mandibular molar quadrants (for a total of 0.2 ml of culture/animal) with a 15-min interval. The vehicle groups were administered 0.2 ml of 0.01 M phosphate buffer, pH 7.4, followed by sonication for 20 s at a setting of 20 (50 Sonic Dismembrator; Fisher), and finally vortexing for 20 s. The number of viable cells was determined by comparison to protein standards with an UltroScan XL laser densitometer and GelScan XL software (Pharmacia).

Collection of saliva and serum samples. At the termination of the study, the rats were killed intracardially with ketamine-xylazine (9.5:vd/vol, 0.14 ml/100 g body weight), and individual saliva samples (approximately 1 ml/animal) were collected in 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 x g for 30 min, 4°C) and stored at −20°C until they were used for SDS-PAGE analysis and ELISA. The fimbria-enriched preparation, and CTB were determined by comparison to the background values was automatically subtracted from the values for the experimental samples.

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 saliva IgA and serum IgG antibody data, bacterial numbers, and smooth-surface and total smooth-surface caries scores were 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, fimbrin-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 fimbrin-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 unpublished data), whose role is currently being investigated. A band seen at 190 kDa has been previously shown to be P1 major protein in human saliva which binds to the tooth surface, thereby inhibiting the development of 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

**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&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. mutans fimbriae</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>
</tbody>
</table>

<sup>a</sup> 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 vs C = 0.9793, 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 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&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. mutans fimbriae</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>
</tr>
<tr>
<td>C</td>
<td>Adjuvant alone</td>
<td>0.379 ± 0.034</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 = 0.9793, 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**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization treatment</th>
<th>Caries score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<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>

<sup>a</sup> Mean ± SEM (n = 28). P values are as follows: for enamel, A versus B = 0.0372, A versus C = 0.3026, and B versus C = 0.3417; for dentin, A versus B = 0.0239, A versus C = 0.0302, 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.
magnitudes 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 the present study, 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>Fimbria &amp; CTB</td>
<td></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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</table>

* 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 = 0.9784, 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 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.

REFERENCES