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Immune response in humans to a nasal boost with *Streptococcus mutans* antigens

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We previously reported that a *Streptococcus mutans* enriched-glucosytransferase (E-GTF) preparation induces an immune response following intranasal, but not tonsillar, immunization of humans. In this study, we determined whether intranasal immunization of these subjects 2 years later resulted in augmented immune responses compared to those seen in control subjects. Subjects previously immunized via the intranasal (IN, $n = 7$) or tonsillar (IT, $n = 7$) route and control ($n = 12$) subjects were immunized via the intranasal route with E-GTF. Nasal wash, saliva, and serum were collected before immunization and then weekly for 3 months after immunization. Significant ($P < 0.05$) mucosal and serum immunoglobulin A (IgA) anti-E-GTF responses were observed in all three groups. Nasal and serum IgA anti-E-GTF responses were significantly higher ($P < 0.05$) in the IN group. The salivary responses in the three groups were, in general, similar. These results indicate that intranasal immunization primes the immune system for a localized secondary response to *S. mutans* antigens.

Key words: dental caries; nasal immunization; secretory immunoglobulin A; *Streptococcus mutans*

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Dental caries is an infectious disease and members of the mutans streptococci, i.e. *Streptococcus mutans* and *Streptococcus sobrinus*, have been implicated as the major etiological agents (16). It is difficult to control this disease because the mutans streptococci become established in the indigenous oral flora and efforts to clear them from the oral cavity have been unsuccessful. In this regard, present clinical preventive approaches (e.g. toothbrushing, toothpaste, and antimicrobial rinses and varnishes) reduce, but do not eliminate, mutans streptococci from the oral cavity (22). Therefore, an important approach to preventing dental caries would be to interfere with the initial colonization of the tooth surface by the mutans streptococci.

Secretory immunoglobulin A (IgA) has been shown to be the predominant immunoglobulin in the major and minor salivary gland secretions (2, 27). Studies have also shown that immunization via inductive sites of the common mucosal immune system results in the presence of specific secretory IgA antibodies in the secretions, such as saliva (17). Therefore, animal and clinical studies have focused on the development of a vaccine against dental caries using a mucosal immunization approach (20, 23).

Animal studies have shown that the induction of specific antibodies to virulence factors of mutans streptococci such as AgI/II, glucosyltransferases (GTF), and glucan-binding protein, as well as to whole cells can inhibit the attachment of the bacteria to the tooth surface and reduce the incidence of dental caries (7, 14, 19, 24). Clinical studies in humans to develop a caries vaccine have used various antigens of mutans streptococci and various formulations (23). Smith & Taubman (25, 26) observed that oral or local (to the minor salivary glands) administration of GTF from *S. sobrinus*, when combined with an aluminum-based adjuvant, resulted in salivary IgA antibody responses and was associated with a decrease in the re-accumulation of indigenous mutans

streptococci following dental prophylaxis. Our previous studies have shown the induction of specific immune responses in volunteers when an enriched-GTF preparation (E-GTF) from *S. mutans* GS-5 was administered by a mucosal route (i.e. oral or intranasal) (4–6, 8, 15). These studies provided evidence that mucosal immunization is a promising approach to the control of infection by mutans streptococci and thus the prevention of dental caries in humans. However, little information is available regarding the duration and recall of the salivary antibody response (20). The purpose of the present study was to determine the effect of a previous immunization with E-GTF given via the intranasal or tonsillar route (4) on a subsequent intranasal immunization with E-GTF given 2 years after the initial immunization compared to a control group of individuals who were receiving the immunization for the first time.

Materials and methods

Antigen preparation

E-GTF used for immunization and enzyme-linked immunosorbent assay (ELISA) was derived as previously described (8). Briefly, *S. mutans* GS-5 (a serotype c isolate, obtained from F. Macrina, VCU, Richmond, VA) was cultured in chemically defined medium (J.R.H. Biosciences, Lenexa, KS). Cells were removed by centrifugation, the culture supernatant was concentrated using a PLGC Pellicon Cassette System (10,000 MW cutoff, Millipore Inc., MA). Proteins were precipitated from the supernatant using ammonium sulfate and then dialyzed. The purity of the E-GTF preparation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis.

Liposomal E-GTF (L-E-GTF) was prepared by sonication of the aqueous antigen preparation and membrane filtration as previously reported (5, 7). The components used for the production of liposomes consisted of D-L-α-dipalmitoyl phosphatidyloholine, cholesterol (Avanti Polar Lipid, Birmingham, AL), and dicetylphosphate (Sigma Chemical Company, St Louis, MO) in a molar ratio of 8.0 : 3.5 : 0.5.

Human immunization

Fourteen of 21 healthy adult subjects (age 30–50 years) who were previously immunized (4) were available to participate in this study. These individuals were originally immunized 2 years previously with an intranasal (IN group; $n = 7$) or a tonsillar (IT group; $n = 7$) spray of soluble E-GTF ($n = 5$ IN and $n = 3$ IT) or L-E-GTF ($n = 2$ IN and $n = 4$ IT). Twelve similarly aged subjects who had not been immunized were recruited to participate in this study as the control (C group). In compliance with guidelines established by the University of Alabama at Birmingham (UAB) Institutional Review Board, informed consent was obtained from all subjects.

The 14 previously immunized subjects were assigned to two groups according to their previous immunization regimen (i.e. individuals were boosted with either soluble E-GTF or L-E-GTF as used for the primary immunization). The subjects in the control group were randomly assigned to be immunized with soluble ($n = 6$) or L-E-GTF ($n = 6$) preparations. Each subject was immunized twice (on day 0 and day 7) via the intranasal route with 62.5 μg soluble E-GTF or L-E-GTF delivered in a total volume of 240 μl. While the subject was in a reclined position, 120 μl (31.25 μg) of soluble E-GTF or L-E-GTF was delivered into each nostril using a Bi-Dose System nasal spray (Pfeiffer, Princeton, NJ). Both the subject and the clinician were blinded to the group assignment.

Sample collection

Unstimulated parotid saliva, nasal wash, and serum samples were collected weekly for 2 weeks before immunization (baseline) and then on days 7, 14, 21, 28, 35, 42, 56, and 90 following the initial immunization (day 0). Parotid saliva samples were collected using Schaefer cups (21). Nasal wash samples were obtained by depositing 1.5 ml sterile saline into each nostril while the subject was reclined and instructed to hold the saline in their nose for 10 s. The subject was then instructed to sit up and lean forward so the nasal wash solution could be collected into a specimen cup. Saliva and nasal wash samples were immediately clarified by centrifugation at 14,000 **g** for 2 min. Serum was obtained by centrifugation of blood collected by finger stick into a microvette tube with clotting activator (Sarstedt, Numbrecht, Germany) and stored frozen until analyzed by ELISA.

ELISA methods

The levels of total immunoglobulin and the relative concentration of antibodies to E-GTF were determined by ELISA as previously described (4). Briefly, optimal dilutions of saliva, serum, and nasal wash samples were added in duplicates of four 2-fold serial dilutions to designated microtiter plate wells. A purified human colostrol IgA (provided by J. Mestecky, UAB) was used as the immunoglobulin standard for saliva and nasal wash samples; and a human serum pool (Moni-trol, Baxter Diagnostic Inc., Deerfield, IL) of known isotype concentrations was used as the immunoglobulin standard for serum samples. After sample incubation, biotin-conjugated goat antiserum to human IgA or IgG (Biosource Inc., Burlingame, CA) was added to the appropriate wells followed by color development using streptavidin horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL), substrate, and recorded at 414 nm (Molecular Devices, Molecular Devices Corporation, Sunnyvale, CA). A four-parameter curve-fitting program (Softmax; Molecular Devices) was used to construct reference curves for each ELISA plate from optical density readings of the standard wells. Saliva and nasal wash results were converted to a ratio of anti-E-GTF antibody activity per total isotype antibody concentration to normalize the variation in total immunoglobulin content in the samples, while serum results were reported as ng/ml of anti-E-GTF antibody activity.

Statistical analysis

The results were analyzed by multivariate analysis with a mixed linear model for differences in route of initial immunization (i.e. IN, IT, or none), delivery system (soluble E-GTF vs. L-E-GTF) and time (e.g. pre-immunization vs. post-immunization). Group, time, and group × time were treated as fixed effects and subject-to-subject variation (repeated measurement) and its interactions were treated as random effects. Antibody levels were log-10 transformed to normalize for variance with results reported as geometric mean and asymptotic standard error (ASE, the geometric mean multiplied by the SE of log-transformed data). Baseline (pre-immunization) was defined as the average levels of antibody from days −14, −7, and 0, and was adjusted in the analysis. Post-immunization was defined as the average levels of antibody from days 7, 14, 21, 28, 35, 42, 56, and 90. Type I error probability (P-value) <0.05 was designated as the accepted level of significance.

Results

Twenty-six subjects completed the study (three dropped out for circumstances unrelated to the study). All subjects reported no serious adverse symptoms throughout the study. The only minor symptoms (10 out of 26 subjects) reported during or after immunization were transient nasal congestion (four subjects), mild nausea (four subjects), headache (five subjects), and 'sore gums' (one subject); these could not be directly related to effects caused by the study. There were no statistically significant differences in the antibody levels among groups from the baseline samples. Three of the seven subjects who were previously immunized with E-GTF by tonsillar spray were boosted by soluble E-GTF, while the other four subjects received L-E-GTF. Five of the seven subjects who were previously immunized with E-GTF by IN spray were boosted with L-E-GTF, whereas two subjects were boosted with soluble E-GTF. The subjects in the control group were evenly assigned to receive either soluble E-GTF or L-E-GTF by the intranasal route. Since no significant differences were found between the L-E-GTF-immunized and E-GTF-immunized subjects within a group, the results of these subgroups were combined for each experimental group. When pre-immunization samples were compared with post-immunization samples, a significant increase in the anti-E-GTF response ($P < 0.0001$) was seen in nasal wash, saliva, and serum samples from all three groups (Figs 1–3, respectively).

Nasal immune responses

The increase in the mean nasal wash IgA anti-E-GTF activity was more than 1.5-fold over baseline (i.e., baseline plus 1.5 times baseline) between days 21 and 56 in the IN group, with a peak increase 2-fold over baseline on day 28 (Fig. 1). Six of the seven subjects in the IN group had more than a 1-fold increase in antibody activity over baseline (data not shown), which persisted through day 90. In the IT group, the increase over baseline in the IgA anti-E-GTF was more than 0.5-fold between day 21 to day 42 (peak mean increase of 1.1-fold on day 42), with four of seven subjects having more than a 1-fold increase in anti-E-GTF antibody activity over baseline (data not shown). In the C group, the increase in mean IgA anti-E-GTF activity over baseline ranged from 0.7-fold to 1.4-fold between days 21 and 90, with eight of 12 subjects having more than a 1-fold increase over baseline (data not shown). Nasal wash IgA anti-E-GTF antibody levels (Fig. 1) in the IN group were significantly higher than those seen in controls on day 7 ($P = 0.0423$), day 14 ($P = 0.0168$), day 28 ($P = 0.0096$), and day 56 ($P = 0.0093$). Similarly, the IN group had significantly higher levels of IgA anti-E-GTF antibody activity than the IT group on days 14 ($P = 0.02$), 28 ($P = 0.0187$), and 56 ($P = 0.0211$). These results indicate that a local memory-recall response occurred in the IN group.

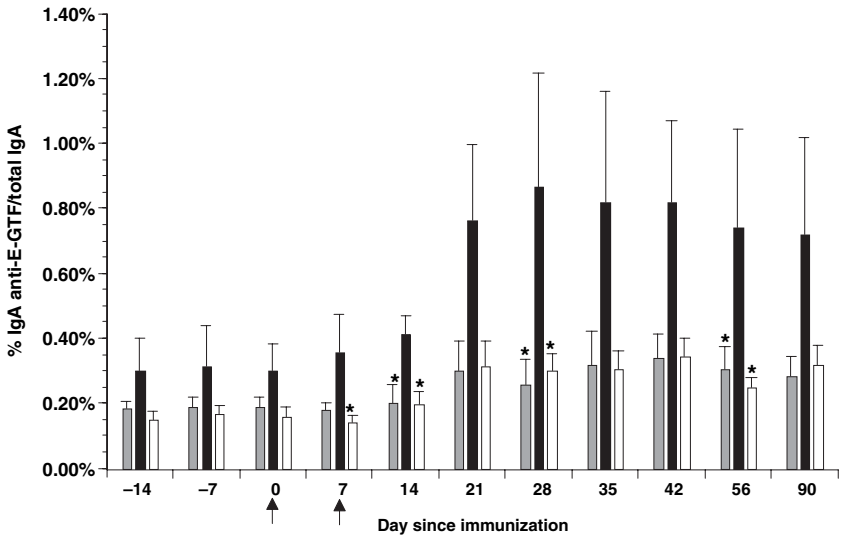

Fig. 1. IgA anti-E-GTF response in nasal wash from intranasally immunized subjects. Nasal wash samples were collected before and after intranasal immunization with 62.5 μg E-GTF on days 0 and 7 (arrows). The results presented are the geometric mean (plus asymptotic standard error; ASE) of the % IgA anti-E-GTF/total IgA in nasal wash from intranasally immunized subjects initially immunized by tonsillar (IT group, ▩, $n = 7$) or nasal (IN group, ■, $n = 7$) spray with the same antigen 2 years earlier and non-immunized controls (C group, □, $n = 12$). The asterisks indicate days on which the geometric mean antibody activity was significantly ($P < 0.05$) less than in the IN group.

Saliva immune responses

The IN group showed a significantly higher IgA anti-E-GTF antibody level than the IT group (but not the C group) on day 42 ($P = 0.0113$) (Fig. 2). However, salivary IgA anti-GTF antibody activity was significantly higher in the IT group than the IN group ($P = 0.0343$) and almost significantly higher than the C group ($P = 0.0697$) on day 28. Although the geometric mean of the IgA anti-E-GTF antibody response in the IN group was higher than in the other groups (i.e. significantly higher than the IT group on day 42, Fig. 2), because of the relatively high baseline, the increase in the IN group was less than 0.35-fold over baseline (data not shown). In the IT group, the peak IgA anti-E-GTF antibody level occurred on day 28. The increase in the mean IgA anti-E-GTF antibody levels ranged from 0.3-fold to 1-fold over baseline (increase sustained through day 90). Two of seven subjects in the IT group had an increase of greater than 1-fold antibody activity over baseline at different time-points (data not shown). In the C group subjects, the geometric mean of the IgA anti-E-GTF antibody activity was less than 0.3-fold increased over baseline at all time-points with only one of the 12 subjects having an increase in baseline greater than 1-fold (data not shown).

Serum immune response

The serum IgA anti-E-GTF antibody level (Fig. 3A) in the IN group was significantly higher than in the C group on days 21 ($P = 0.044$), 56 ($P = 0.021$); and significantly higher that seen in the than IT group on day 21 ($P = 0.02$). The level of serum IgA anti-E-GTF antibody activity in IT group was significantly higher than that seen in C group on days 56 ($P = 0.028$) and almost significantly higher on day 90 ($P = 0.065$). Three of the seven individuals in the IN group, two of the seven in the IT group, and two of the 12 in the C group had more than a 1-fold increase over baseline of their serum IgA anti-E-GTF response.

A significant increase ($P < 0.001$) was observed in the serum IgG anti-E-GTF activity after immunization in all three groups; however, no significant difference

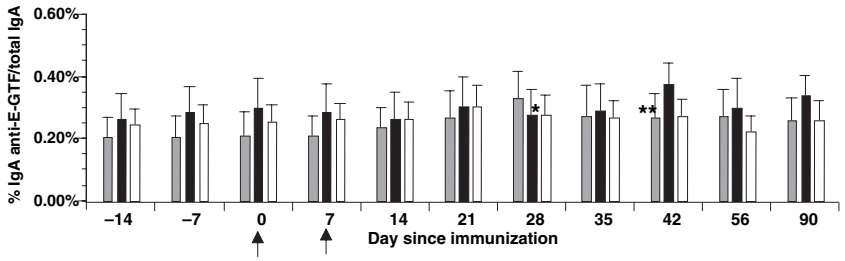

Fig. 2. IgA anti-E-GTF response in saliva from intranasally immunized subjects. Parotid saliva samples were collected before and after intranasal immunization with 62.5 μg E-GTF on days 0 and 7 (arrows). The results presented are the geometric mean (plus ASE) of the % IgA anti-E-GTF/total IgA in parotid saliva from intranasally immunized subjects who had initially been immunized by tonsillar (IT group, ■, $n = 7$) or nasal (IN group, ■, $n = 7$) spray with the same antigen 2 years earlier and non-immunized controls (C group, □, $n = 12$). The results were subjected to mixed model analysis. A single asterisk indicates the day that the geometric mean antibody activity was significantly ($P < 0.05$) less than the IT group and the double asterisk indicates the day that the geometric mean antibody activity was significantly ($P < 0.05$) less than the IN group.

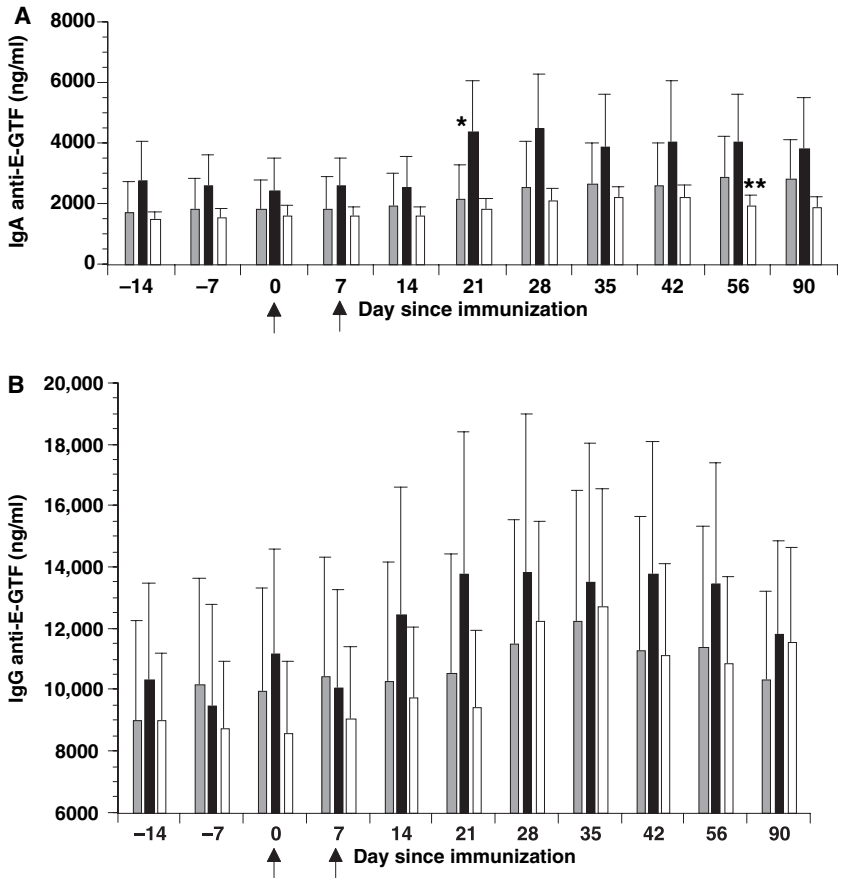

Fig. 3. Anti-E-GTF response in serum from intranasally immunized subjects. Serum samples were collected before and after intranasal immunization with 62.5 μg E-GTF on days 0 and 7 (arrows). The results are the geometric mean (plus ASE) of serum IgA (A) and IgG (B) anti-E-GTF antibody activity from intranasally immunized subjects that had been initially immunized by tonsillar (IT group, ■, $n = 7$) or nasal (IN group, ■, $n = 7$) spray and boosted intranasally with the same antigen 2 years earlier and non-immunized controls (C group, □, $n = 12$). A single asterisk indicates IgA antibody activity (A) that was significantly higher than the other two groups ($P < 0.05$); a double asterisk indicates significantly lower IgA antibody activity than the other two groups ($P < 0.05$).

was observed among the three groups (Fig. 3B). Two of the individuals in the IN group, none in the IT group, and four in the C group had more than a 1-fold increase over baseline of their serum IgA anti-E-GTF response.

Discussion

Our previous studies have indicated that intranasal immunization induces higher immune responses in mucosal secretions than immunization via the oral or tonsillar route (3, 4, 6, 15). We therefore used the intranasal route to immunize subjects who had been previously immunized by the nasal or tonsillar route and compared their responses to those in subjects who were immunized for the first time in this study to determine the effectiveness of this immunization regimen in boosting mucosal responses. The highest secretory IgA response was observed in nasal wash samples from the IN group who had been immunized via the intranasal route 2 years previously. These results indicate that intranasal, but not tonsillar, immunization can prime the host for a subsequent local IgA nasal response to the antigen. Interestingly, the IT group had significantly higher salivary IgA responses than the IN group (and almost significantly higher responses than the C group), but only on day 28. Although the IN group showed a higher salivary IgA response than the IT group at a later time (i.e. day 42), the response in the IT group (compared to baseline antibody activity) was maintained through to day 90. The initial tonsillar immunization (4) did not result in a salivary immune response; however, it may have primed for a salivary IgA response following intranasal immunization (i.e. day 28). Therefore, it appears that the second series of mucosal immunizations resulted in a response that was localized to the site of original immunization (i.e. nasal and possibly even tonsillar). Previous studies in rabbits (9–12) have indicated that nasal or tonsillar applied mutans streptococci antigens resulted in higher nasal or salivary responses, respectively. Future studies will be required to confirm that the inductive site for an IgA response in saliva might be localized to immunizing within the oral cavity (i.e. tonsil, gingiva, or salivary glands).

A memory mucosal immune response will be important for the development of mucosal vaccines against infectious diseases including dental caries. Focusing on an immunization regimen that will maximize the induction of specific antibodies in saliva should optimize the potential success of a vaccine to control an infection with cariogenic mutans streptococci. The results of the present study did not reveal an immunization regimen that effectively primed for a salivary IgA response; however, we did demonstrate priming with

intranasal immunization for nasal IgA responses. These results support earlier findings showing memory (18) as well as compartmentalization (1, 13) within the mucosal immune system.

Clinically, dental caries often occurs early in childhood (28). Therefore, early intervention against the initial attachment of mutans streptococci on the tooth surface is critical. The study reported herein and others (4–6, 8, 15) provide important safety and immunogenicity data (i.e. Food and Drug Administration Phase 1) that support the initiation of studies in children. Future clinical studies are planned to examine the induction of salivary immune responses in children and its protective effects against colonization with cariogenic bacteria such as *S. mutans*.

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