Glucosyltransferase-I is Capable of Functioning as an Adjuvant to Promote Immune Responses Against the Co-administered Protein Glucosyltransferase-B

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Abstract
The purpose of this study is to evaluate the efficacy of the vaccine using the glucan-producing enzymes glucosyltransferase-B (GTF-B) produced by Streptococcus mutans as antigen and glucosyltransferase-I (GTF-I) produced by Streptococcus sobrinus as adjuvant for protection against dental caries. Nasal immunization with GTF-B plus GTF-I enhanced significant levels of GTF-B–specific secretory IgA antibody in saliva as well as IgG and IgA in serum, which were equivalent to those of mice immunized with GTF-B plus CpG oligodeoxynucleotides (ODN) 1826. Antibody-forming cell (AFC) analysis revealed high numbers of GTF-B–specific IgA AFCs in salivary glands, nasal passages, and spleen in mice immunized with GTF-B plus GTF-I. IgG subclass detection showed that administration of GTF-B plus GTF-I mainly induced IgG1 production followed by IgG2a and IgG2b. Serum IgG purified from mice given GTF-B plus GTF-I inhibited biofilm formation by S. mutans. These results suggest that nasal immunization with GTF-B plus GTF-I elicits efficient protective mucosal immune responses against S. mutans infection.

Keywords:
dental caries, nasal immunization, glucosyltransferase-B, Streptococcus mutans

Introduction
Dental caries, a widespread chronic infectious disease, results from the formation of biofilm on tooth surface followed by acid fermentation of cariogenic pathogens. Mutans streptococci are thought to be as important members of etiological agent in dental caries (5, 15). Streptococcus mutans and S. sobrinus are major causative agents of dental caries (15). The both strains produce water–soluble and water–insoluble glucans from sucrose by the combined action of glucosyltransferases (GTFs). Glucans synthesized from dietary sucrose by GTFs are of central importance in adhesive interactions and in the expression of virulence by these bacteria (6). Glucans enhance the pathogenic potential of dental biofilm by promoting the adherence and accumulation of cariogenic streptococci on the tooth surface, and by contributing to the bulk and structural integrity of dental plaque (6). GTF facilitate the sucrose-dependent accumulation of S. mutans on tooth surfaces, which has been reported as an important cariogenic property of these organisms (11, 15, 23). S. sobrinus and S. mutans produce GTF-I and GTF-B respectively. Both glucan–producing enzymes consist of two functional domains, which are an N–terminal sucrose-binding domain and a C–terminal glucan–binding domain (1, 3, 14). The activities of these GTFs are mediated through both catalytic and glucan–binding functions (12, 17).

If an effective vaccine for the oral cavity is to be designed, careful consideration must be given to the various immune responses and antigen–delivery systems. Because the risk of needle–borne diseases is associated with reuse and improper disposal of needles, needle–free delivery has become a global priority. Nasal administration of vaccine has been widely used for mucosal immunization because it delivers antigen directly to IgA–inductive sites termed nasal-
associated lymphoid tissues without the influence of enzymes and acids in the gastrointestinal tract. Furthermore, this form of immunization is capable of inducing both mucosal and systemic immune responses, which result in two layers of host protection against infectious diseases (8, 9).

Previous study has reported that GTF-I is a potent candidate for vaccination to prevent dental caries (19). Because nasal administration with GTF-I alone elicited significant serum IgG and IgA as well as salivary IgA, we considered the possibility that GTF-I acts as an adjuvant. Indeed, previous study has demonstrated that oral immunization of hamster with GTF complex of \textit{S. mutans} without adjuvant induces serum and salivary antibody responses (18). Furthermore, nasal immunization with the glucan-binding domain of \textit{S. mutans} GTF-I elicits protective antibody responses against \textit{S. mutans} infection even in the absence of adjuvant (10). These studies suggest that GTF-I possesses both the characteristic of the antigenicity and the adjuvanticity. Moreover, another study has reported that co-administered dual antigens strongly enhanced the immune responses compared with single administration of antigen (19), suggesting double administration of antigen might suitable for augmentation of the immune responses.

Therefore, the major aim of this study is to assess the potential of a nasal vaccine using GTF-B plus GTF-I to prevent oral infection by \textit{S. mutans} and \textit{S. sobrinus} followed by dental caries. We also applied CpG ODN 1826 via nasal route for comparison in efficacy of adjuvanticity.

\textbf{Materials and Methods}

\textit{Mice}

Female 8–11 week-old BALB/c mice were purchased from Sankyo Laboratory Services (Tokyo, Japan) and maintained under pathogen-free conditions in the experimental facility of Nihon University School of Dentistry at Matsudo. Mice received sterile food and water. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals (Nihon University School of Dentistry at Matsudo).

\textit{Antigen}

GTF-B was prepared as described previously (4), with minor modification. Briefly, \textit{Streptococcus milleri} transformant KSB8 expressing the gtfb were grown in Todd–Hewitt broth (THB, BD, Difco, Franklin Lakes, NJ) containing erythromycin anaerobically. Cells were removed by centrifugation and the supernatant was mixed with an equal volume of chilled ethanol. The precipitate was collected by centrifugation, and dissolved in distilled water. Insoluble materials were removed by centrifugation and the supernatant was used as the crude enzyme preparation. This preparation was supplemented with loading buffer, and applied to a preparative PAGE gel column as previously described (7). Eluted fractions were subjected to mini-slab SDS–PAGE, and the gels were stained with Coomassie brilliant blue or analyzed by Western blotting to confirm. The active fraction were pooled and mixed with chilled ethanol to remove the detergent. The precipitate was collected by centrifugation, and dissolved in distilled

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Fig1.png}
\caption{SDS–PAGE analysis of purified GTF-B. The purified GTF-B was subjected to SDS–PAGE. After electrophoresis, the gel was stained with Coomassie Blue Brilliant (lane 2), silver (lane 3), or incubated with sucrose to detect insoluble glucan synthesis (lane 4). Lane 1 shows a protein standard.}
\end{figure}
water, and dialyzed in PBS. The purity of the protein was determined by SDS-PAGE with silver staining, and no contaminating protein bands were noted. Furthermore, this band displayed GTF activity and synthesized insoluble glucan after incubation with sucrose (Fig. 1). Possible residual endotoxin was assessed in the preparation with an LAL pyrochrome kit (Associates of Cape Cod Inc., Woods Hole, MA). The purified GTF-B contained no detectable endotoxin.

**Immunization and sample collection**

Mice were immunized nasally on days 0, 7, and 14 with 20 μg of GTF-B alone or 20 μg of GTF-B plus 10 μg of CpG ODN, or 20 μg of GTF-B and 20 μg of GTF-I, suspended in 20 μl of PBS (twice of 5 μl of antigen into respective nostril). Non-immunized group was sham-immunized with PBS. CpG ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3') was purchased from Coley Pharmaceutical Group, Inc. (Wellesley, MA). The antigen was delivered with a micropipette applied against the both nostril. Serum and saliva samples were collected from each group in order to examine GTF-B-specific antibody responses.

**Antigen-specific antibody responses**

Antibody titers in serum and saliva were detected by ELISA (16). Briefly, plates were coated with GTF-B (5 μg/ml) and blocked with PBS containing 1% bovine serum albumin. After blocking, serial dilutions of serum or saliva samples were added in duplicate. Following incubation, plates were washed and peroxidase-labeled goat anti-mouse α, γ, γ1, γ2a, γ2b, and γ3 heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham AL) were added to the appropriate wells. Finally, 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) with H₂O₂ was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 415 nm of 0.1 greater than non-immunized control sample after 15 min of incubation.

**Measurement of antibody-forming cells**

Single-cell suspensions were obtained from the salivary glands, nasal passages, and spleen 7 days after the last immunization. Briefly, salivary glands were carefully extracted, teased apart, and dissociated using 0.3 mg/ml collagenase (Nitta Gelatin Co. Ltd., Osaka, Japan). Mononuclear cells were obtained at the interface of the 40% and 55%, 55% and 75% layers of a discontinuous Percoll gradient (GE Healthcare UK, Ltd., Little Chalfont, United Kingdom) (16). To assess the numbers of antigen-specific antibody-forming cells (AFCs), an enzyme-linked immunospot (ELISPOT) assay was performed as described previously (22). Briefly, 96-well nitrocellulose plates (BD Biosciences, Franklin Lakes, NJ) were coated with GTF-B (5 μg/ml), incubated at 4 °C for 20 h, and then washed extensively before being blocked with RPMI 1640 containing 10% fetal calf serum. After 30 min, the blocking solution was discarded, and cell suspensions at various dilutions were added to wells and incubated at 37 °C for 4 h under 5% CO₂ in moist air. The cells were washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse γ or α heavy chain specific antibodies (Southern Biotechnology Associatees) at 4 °C for 20 h. Following incubation, the plates were washed with PBS and were developed by the addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H₂O₂ (Moss, Inc., Pasadena, MD). Plates were incubated at room temperature for 30 min and were washed with water, and antibody-forming cells were then counted with the aid of a stereomicroscope (Olympus, Tokyo, Japan).

**GTF-B-specific CD4⁺ T cell proliferation**

CD4⁺ T cells were isolated 7 days after the final immunization from spleen, cervical lymph nodes (CLNs), and submandibular lymph nodes (SMLs) of the immunized mice and purified using a magnet-actuated cell sorter system (Miltenyi Biotec, Auburn, CA), as described elsewhere (21). Cell suspension was adjusted at the concentration of 2.5×10⁶ cells/ml and co-cultured with 2.5×10⁶ cells/ml of naïve splenic feeder cells in presence of 5 μg/ml of GTF-B in
RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml of recombinant IL-2 (Genzyme, Cambridge, MA). To measure GTF-B-specific cell proliferation, 1.0 μCi of [3H] thymidine was added to the culture 18 h before harvesting, and incorporated radioactivity was measured by scintillation counting.

Quantitative biofilm assay

Serum IgG antibodies from immunized mice were purified using a HiTrap™ protein G HP column (Amersham Biosciences, Piscataway, NJ). Biofilm formation by S. mutans was quantitatively measured using a modified version of the microplate adherence assay as described previously (2), with minor modification. Briefly, S. mutans cells (approximately 2.5 × 10⁶ CFU/ml in BHI broth) were pre-incubated with purified IgG antibodies at 37°C for 1 h and then plated with 1% sucrose in a 96-well flat-bottomed tissue culture plate (Life Technologies, Paisley, UK). After 12 hours of incubation, each well was stained with 0.1% safranine (Sigma, St. Louis, MO) for 30 min. The wells were washed with distilled water and adherent material was solubilised through incubation with 200 μl of 0.2 M sodium hydroxide for 1 h at 80°C. The absorbance for each well was measured at 540 nm.

Statistical analysis

Results are expressed as means±standard deviation (SD) and were compared using an unpaired Student’s t test.

Result

Nasal GTF-B plus GTF-I immunization enhances mucosal and systemic immunity

To determine the effectiveness of the nasal immunization, mice were immunized with GTF-B alone, GTF-B plus CpG ODN 1826, GTF-B plus GTF-I, or PBS. Nasal immunization with GTF-B plus GTF-I induced significant higher salivary IgA 7 days after last immunization which was comparable to that in mice immunized with GTF-B plus CpG ODN 1826 (Fig. 2A). By contrast, GTF-B alone-immunized and non-immunized mice induced no detectable titers. The results of salivary antibody titers were con-

![Graph A](image)

![Graph B](image)

**Fig. 2.** Mucosal immune responses after nasal immunization. GTF-B–specific IgA antibody titer in saliva (A) and IgA AFCs in SG and NP. Groups of mice were nasally immunized with 20 μg of GTF-B alone (vertical bars), 20 μg of GTF-B plus CpG ODN (dotted bars), 20 μg of GTF-B plus 20 μg of GTF-I (filled bar), or PBS (open bar) on days 0, 7, and 14. Saliva samples and sample tissues were collected at day 21 and were assessed for GTF-B–specific antibody titer by ELISA and antibody-forming cells by ELISPOT respectively. The results were expressed as the mean±S.D. obtained for five mice per group. The P values for antigen–specific salivary IgA antibody titer and number of IgA AFCs with GTF-B alone, GTF-B plus CpG ODN 1826, or GTF-B plus GTF-I were <0.01 when compared with PBS given group.
firmed by antibody-forming cell responses which indicated significant numbers of GTF-B-specific IgA Antibody-forming cells (AFCs) in salivary glands (SGs) and nasal passages (NPs) of mice given GTF-B plus GTF-I (Fig. 2B). In addition, level of serum IgG and IgA after nasal immunization were significantly increased in mice immunized with GTF-B plus GTF-I (Fig. 3A). Significantly higher numbers of GTF-B-specific IgA AFCs were also detected in the spleen of mice given GTF-B plus GTF-I via nasal route (Fig. 3B).

In order to confirm the cross reactivity between GTF-B and GTF-I, serum samples from mice given GTF-B- and GTF-I were subjected to GTF-I- and GTF-B-specific ELISA respectively. As expected, although the cross reaction was detected, titers were negligible (data not shown). When the subclasses of GTF-B-specific IgG antibodies induced by nasal GTF-B plus GTF-I challenge were determined, the dominant subclasses were IgG1 and IgG2b, and relatively lower IgG2a (Fig. 3C).

**GTF-B-specific T cells responses**

To determine the T cell proliferation by GTF-B-
specific CD4+ T cells in mucosal as well as systemic compartment following nasal immunization, CD4+ T cells from CLN, SML, and spleen were cultured with GTF-B in the presence of mitomycin–treated splenic feeder cells (Fig. 4). Re-stimulation with GTF-B significantly enhanced T cell proliferation in spleen, CLN and SML of mice nasally immunized with GTF-B plus GTF-I (Fig. 4). Mice immunized with GTF-B alone showed equivalent proliferative capacity to that of GTF-B plus GTF-I group in spleen, CLN and SML.

Fig. 4. GTF-B-specific cell proliferative responses in mice immunized nasally with GTF-B. Groups of mice were nasally immunized with 20 μg of GTF-B alone, 20 μg of GTF-B plus 10 μg of CpG ODN 1826, 20 μg of GTF-B plus 20 μg of GTF-I, or PBS on days 0, 7 and 14. CD4+ T cells were isolated from spleen, CLNs, and SMLs. The results were expressed as the mean±S.D. obtained for eight mice per group. The P values for mice immunized with GTF-B alone, GTF-B plus CpG ODN 1826, or GTF-B plus GTF-I were <0.01 when compared with PBS.

Fig. 5. Inhibition of S. mutans biofilm accumulation by GTF-B-specific IgG antibodies. S. mutans cells were pre-incubated with 0.5 μg, 2 μg, and 4 μg of IgG antibodies purified from serum of mice immunized with GTF-B alone, GTF-B plus CpG ODN 1826, GTF-B plus GTF-I, or PBS and then incubated in the presence of sucrose. The results were expressed as the mean±S.D. obtained for six mice per group. The P values for mice immunized with GTF-B alone, GTF-B plus CpG ODN 1826, or GTF-B plus GTF-I were <0.01 when compared with PBS.
Nasally GTF-B–induced antibodies suppress biofilm formation by S. mutans

Next we sought to determine whether antibodies induced by nasally administered GTF–B plus GTF–I were capable of interfering biofilm formation by S. mutans. In this regard, S. mutans was pre–treated with serum IgG from mice given GTF–B alone, GTF–B plus CpG ODN 1826, GTF–B plus GTF–I or PBS as control and then incubated with 1% sucrose. The results showed that biofilm formation of S. mutans was inhibited by IgG antibodies purified from mice given GTF–B alone, GTF–B plus CpG ODN 1826 or GTF–B plus GTF–I in a dose–dependent manner (Fig. 5). Non–specific serum IgG slightly inhibited biofilm formation.

Discussion

Protein antigen given via the mucosal route without adjuvant has been generally reported to be only a weak immunogen and therefore requires a mucosal adjuvant to induce antigen–specific immune responses (9). Previous study has demonstrated that nasal immunization of GTF–I with or without CpG ODN 1826 induced protective immunity against dental caries caused by S. sobrinus which is caries–inducing etiological agents (19). Another studies also showed the immunogenicity of GTF–I without any mucosal adjuvant (10, 18). Taking account of these facts, GTF–I might possesses the adjuvanticity to promote immune responses against the co–administered protein. Since S. mutans and S. sobrinus are two major causative agents of dental caries in human, the induction of protective immune responses against S. mutans as well as S. sobrinus infection is indispensable. Previous study has demonstrated that administration of dual vaccine augmented antigen–specific Ab titers and survival rate of mice following influenza virus infection, which were similar to those with antigen plus adjuvant (20). When the effective vaccine for dental caries prevention is considered, it is most ideal to immunize mucosally GTF–B and GTF–I together. GTF–B which is generated by S. mutans is virulence factor in dental caries by synthesizing the water–insoluble glucan. Moreover, since GTF–B and GTF–I have high homology in amino acid sequence, it is predictable to occur the cross reaction to synergize the immune responses. Therefore, we immunized these two proteins as mixed antigens via nasal route.

As expected, nasal immunization with GTF–B plus GTF–I induced significantly higher GTF–B–specific salivary IgA as well as serum IgG and IgA antibody titers comparable level to those of mice immunized with GTF–B plus CpG ODN 1826. A significant level of antigen–specific IgA AFCs were also detected in salivary glands and nasal passage compared to non–immunized and GTF–B alone–immunized groups. However, mice nasally immunized with GTF–B alone also produced significantly higher IgG antibody in serum, which suggest GTF–B has immunogenicity as well as GTF–I. Possible explanations of the strong immunogenicity of GTF–B and GTF–I might be that the large size of these molecules persists in the body to be continuously uptaken by macrophages or other immune cells, or that these enzymes are key virulence factors and synthesize water–insoluble glucans (14). However, although the nasal vaccination with GTF–I alone induced salivary IgA in previous study, nasal immunization with GTF–B alone failed to induce Ag–specific salivary IgA Ab in this study, suggesting that GTF–B needs GTF–I as adjuvant for seretory IgA production. Although, cross reaction between GTF–B and GTF–I in serum was detected predictably (data not shown), the Ab titers were relatively lower, which suggest that the epitope for adjuvanticity may be discriminated from homologic site of these two GTFs.

Salivary secretary IgA is considered to act as important first–line of defense mechanism within the oral cavity. Previous studies have shown a positive correlation between salivary IgA antibody levels to S. mutans and caries resistance in adults (13). This suggests that prevention of dental caries requires the mucosal immunity represented by secretary IgA rather than systemic immune system. Although mice immunized with GTF–B alone also induced serum IgG which can interfere the biofilm formation by S. mutans, salivary IgA, which has the key role in
inhibition of dental caries, was not induced in these mice. In contrast, GTF-B plus GTF-I-immunized mice showed significant serum IgG and IgA as well as salivary IgA antibody responses. Further, in order to induce the protective immunity against dental caries in oral cavity, co-administration of mucosal adjuvant is indispensable. Given those, GTF-I has not only antigenicity but adjuvanticity equivalent to CpG ODN 1826.

In summary, our present study provides evidence that nasal administration with GTF-B plus GTF-I augmented significant GTF-B–specific salivary IgA compared to GTF-B alone group, and directed Th1– and Th2–type cytokine responses equivalent to GTF–B plus CpG ODN 1826–immunized group in both systemic and mucosal lymphoid tissues. Since antibodies from mice immunized with GTF-B plus GTF–I reduced biofilm formation by S. mutans at levels comparable to that of GTF-B plus CpG ODN 1826–immunized mice, dual vaccine that consists of GTF–B plus GTF–I can effectively promote protective immune responses. Taken together, GTF–I is capable of acting as an effective mucosal adjuvant, and therefore nasal immunization with mixed antigen GTF–B plus GTF–I may act as a potent candidate for safe and effective vaccine for protection from dental caries caused by mutants streptococci infection.

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