Correlation between the Amount of Glucosyltransferase-B and the Decayed, Missing and Filled Teeth Index in Oral Samples

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Abstract
Glucosyltransferase B (GTF–B) secreted from Streptococcus mutans (S. mutans) is considered to be the most important virulence factor of cariogenic biofilm formation for its high enzymatic activity in the synthesis of water-insoluble glucans. The quantity of GTF–B protein in the saliva or plaque is readily measurable for the estimation of caries risk in an individual. However, previous reports have indicated that GTF–B-secreting ability varies among S. mutans strains harbored by subjects. In this study, we examined the correlation among the quantity of GTF–B protein, the quantity of S. mutans, and the decayed, missing and filled teeth (DMFT) index in oral samples. Our results showed a clear positive correlation between GTF–B quantity and S. mutans quantity in oral samples. Furthermore, a brushing-plaque sample was more useful than a stimulated saliva sample for the estimation of caries risk. In addition, in the brushing-plaque samples, the caries-susceptible group was infected with S. mutans strains that had a higher ability to secrete GTF–B enzyme than those of the caries-free and low-level caries groups. These results suggested that GTF–B-producing ability differed among individuals and the risk of caries predicted from the amount of GTF–B seemed to be proportional to the DMFT index. Taken together, the quantity of GTF–B protein is useful for the estimation of caries risk.

Keywords:
glucosyltransferase-B, Streptococcus mutans, caries risk, sandwich-ELISA

Introduction
Dental caries is a chronic infectious disease caused by the formation of biofilm on tooth surfaces. Streptococcus mutans (S. mutans) is a major cariogenic organism in the development of human dental caries (1, 2). S. mutans is well known to synthesize adhesive water-soluble glucans (WSG) and water-insoluble glucans (WIG) from dietary sucrose by the combined action of glucosyltransferases (GTFs) (1, 2). S. mutans secretes three types of GTFs: GTF–B and GTF–C for the synthesis of WIG and GTF–D for the synthesis of WSG (3). The synthesis of WIG is necessary for the accumulation of bacteria on the tooth surface and the induction of dental caries (4, 5). Furthermore, GTF–B is considered to be an important virulence factor due to its high enzymatic activity for synthesizing sticky WIG (6). Thus, the determination of the quantity of GTF–B present in saliva and plaque samples might contribute to evaluating the caries risk in individuals. Moreover, previous studies have reported that a positive proportion has been observed between S. mutans quantity and the decayed, missing and filled teeth (DMFT) index in humans (7, 8). However, other studies have pointed out that the cariogenicity of S. mutans was distinct from oral samples (9) and that the extent of GTF–B production varied among S. mutans strains harbored by individuals (10–12).

In this study, we measured the GTF–B quantities in saliva and plaque samples and assessed the rela-
tionship among the quantity of GTF-B, the quantity of *S. mutans*, and the DMFT index in individuals to develop a useful system for the estimation of caries risk.

**Materials and Methods**

**Strains and culture conditions**

Recombinant *Streptococcus milleri* (*S. milleri*) strain KSB8 (13) expressing the *gtfB* gene from *S. mutans* GS5 (14) produces the GTF-B enzyme into culture fluid and was used as the source of the GTF-B preparation. KSB8 was grown anaerobically in Todd–Hewitt broth (THB; BD Difco, Franklin Lakes, NJ) containing erythromycin (10 µg/ml).

**Subjects and collecting samples**

A total of 146 healthy human volunteers (students of the Nihon University School of Dentistry at Matsudo; ages 20 to 27 years) participated in this study. This study was carried out in accordance with the Ethical Review Board of Nihon University School of Dentistry at Matsudo (ECO 2-015). Oral samples of stimulated saliva and brushing-plaque were collected from each subject. After the subject gargled once, stimulated saliva was collected by chewing paraffin gum. The mouth was then washed with drinking water before plaque was taken. The brushing-plaque sample was collected following vigorous brushing for 1 min with a sterile toothbrush and a mouth rinse for 30 sec with 5 ml phosphate-buffered saline (PBS), with collection of the plaque-containing rinse in a sterile bottle. One part 10% skim milk and one part 50% dimethyl sulfoxide were added and the sample was kept at −80 °C until usage.

**Extraction of glucosyltransferases from oral samples**

In the preliminary experiments, we assessed extraction methods to find the optimal experimental conditions and we selected 0.5 N NaOH as the best solvent and 1 min as the optimal extraction time of GTFs from the oral samples (Fig. 1). The stimulated saliva or brushing-plaque samples (600 µl) were centrifuged (6,000 × g, 5 min). After the supernatant was removed, 50 µl of 0.5 N NaOH was added to the precipitate, followed by mixing for 1 min for extraction, and then 50 µl of 1M MOPS was added for neutralization. Finally, 25 µl PBS was added and the sample was centrifuged (6,000 × g, 5 min). The supernatant was kept on ice, the precipitate was extracted again, and the second extraction was mixed with the first extraction and used as the GTF extraction.

**Microbiological analysis**

*Mitis Salivarius* agar (MS agar, BD Difco) supplemented with 0.2 units/ml bacitracin and 15% sucrose (MSB) was used to grow *mutans streptococci* and MSB agar containing 0.2 mg/ml aztreonam (MSBA) was used to grow *S. sobrinus*. Frozen culture suspension samples were immediately dissolved at 37 °C following dispersion by sonication (50 W, 20 sec) and were diluted tenfold by adding chilled brain heart infusion broth (BHI, BD Difco). Then, 50-µl
aliquots of diluents were inoculated onto the plates of selective media using a spiral system (Model-D, Gunze Sangyo, Inc., Tokyo, Japan). After anaerobic incubation for 48 h, the number of mutans streptococci colonies on the MSB plates and the number of S. sobrinus colonies on the MSBA plates were counted under a microscope. The difference in colony numbers between selective plates represents the colony number of S. mutans.

Purification of recombinant glucosyltransferase-B

Extracellular GTF-B was purified from the culture fluids of KSB8 (13) as reported earlier, with slight modification (15). In brief, cells were grown overnight anaerobically at 37 °C for 18 h, followed by centrifugation (10,000×g, 15 min). The supernatant was mixed with an equal volume of chilled ethanol and then left at 4 °C for 2 h. The precipitate was collected by centrifugation (20,000×g, 20 min) 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 [50 mM Tris–HCl, pH 6.8 containing 1% lithium dodecyl sulfate (LDS)], filtered (0.22 mm), and applied to a preparative polyacrylamide gel column, as previously described (16). Eluted fractions were subjected to mini–slab LDS–polyacrylamide gel electrophoresis and the gels were either stained with Coomassie brilliant blue (CBB) R–250 or analyzed by Western blotting to confirm the presence of GTF-B. The active fractions were pooled and mixed with chilled ethanol to remove the detergent. The precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against PBS.

Sandwich enzyme–linked immunosorbent assay

To quantitate the GTF-B protein in each sample, sandwich enzyme–linked immunosorbent assay (sandwich–ELISA) was carried out. Anti-GTF-B monoclonal antibody P136 developed by Fukushima et al. (16) was used in this study and was kindly provided by Dr. Fukushima. Briefly, plates were coated with P136 monoclonal antibody (10 μg/ml) (17, 18) for detecting GTF-B. After incubation overnight, the plates were washed with PBS containing 0.05% Tween 20 and blocked with PBS containing 0.05% Tween 20, 1.0% bovine serum albumin, 10% sucrose, and 0.1% Proclin 300 for 1 h at room temperature. After blocking, the plates were kept in a chamber (dry module, AS–ONE) for 2 days, and then stored at 4 °C until usage. Extracts of oral samples (50 μl) were added to each well and incubated for 2 h. After washing, rabbit IgG anti–recombinant GTF-B polyclonal antibody was added and incubated for 1 h. After washing, the plates were incubated with goat anti–rabbit IgG horseradish peroxidase conjugate (-Life technologies, Japan) for 1 h at room temperature followed by washing with PBS containing 0.05% Tween 20. Following the addition of chromogenic substrate (1.5% citric acid monohydrate, 5.3% trisodium citrate dehydrate, 0.0015% thiourea, 0.2% 1.2–phenylenediamine dihydrochloride, and 0.02% hydrogen peroxide in distilled water), the plates were incubated for 15 min at room temperature. The amount of GTF-B protein was quantitated by measuring the absorbance at 492 nm with a microplate reader (model MTP–32, Corona, Japan).

Results

Standard curve for quantity of GTF–B protein in subjects

To avoid the overestimation of GTF–B protein due to its reaching a plateau, an optical density less than 0.6 was employed in the ELISA (data not shown).

Correlation between S. mutans and GTF–B quantities in oral samples

In the initial study, we analyzed the relationship between the S. mutans quantity and GTF–B quantity in each sample. From 146 healthy subjects, samples of stimulated saliva and brushing–plaque were collected and the quantity of S. mutans in each sample was determined by the cultivation method as outlined in the Material and Methods section using selective media, MSB and MSBA plates. Simultaneously, the amount of GTF–B protein in each sample was assessed by the sandwich–ELISA method. By
the amount of GTF-B protein as zero when the CFU concentration of *S. mutans* was less than $10^8$ per ml. Furthermore, 146 volunteers were expediently divided into four groups depending on the following criteria: caries-free group (DMFT: 0), low-level caries group (DMFT: 1–5), middle-level caries group (DMFT: 8–12), and caries-susceptible group (DMFT: more than 15). Our results showed that GTF-B production tended to be higher in the brushing-plaque samples of the caries-susceptible group (Fig. 3B), whereas no significant relationship was noted in the stimulated saliva samples (Fig. 3A).

Furthermore, the percentage of samples containing a high amount of GTF-B (>1 ng/10⁶ CFU) in the subjects harboring more than $10^8$ CFU of *S. mutans* per ml in both stimulated saliva and brushing-plaque samples was higher in the high DMFT groups (Fig. 4). An increase in the proportion of high-GTF-B-containing samples was shown in both the stimulated saliva and brushing-plaque samples, although a marked tendency was noted in the brushing-plaque samples. These results suggested that the GTF-B-producing ability of *S. mutans* possessed by the combining these results, it was demonstrated that the quantities of GTF-B and *S. mutans* (measured as colony-forming units, CFU) in both stimulated saliva (Fig. 2A) and brushing-plaque samples (Fig. 2B) showed a clear positive correlation, with correlation coefficients of 0.75 in saliva and 0.88 in plaque. These results indicated that the quantitative determination of GTF-B protein in subjects is useful in the evaluation of caries risk.

**Relationship between GTF-B and DMFT index**

In the next study, we analyzed the relevance between GTF-B quantity and DMFT index. For this purpose, we calculated the amount of GTF-B protein per $10^8$ CFU of *S. mutans* in each sample. We defined
caries-susceptible group was stronger than those of the caries-free and low-level caries groups. Furthermore, brushing-plaque samples were more useful than stimulated saliva samples for estimating the risk of caries.

Discussion

It is well established that S. mutans has been detected from the oral cavity of most adults. Previous studies have shown that mutans streptococci infection is initiated by transmission from a mother to her infant (19, 20) and the quantity of oral bacteria increases with age. Therefore, the S. mutans quantity in the oral cavity is one of the risk factors for the formation of caries. Furthermore, using GTF-B secreted by S. mutans as a predictor of caries risk is rational due to its important role in the cariogenicity of S. mutans. In addition, a strong correlation has been observed between GTF-B quantity and DMFT values in salivary samples (7) and between GTF-B quantity and DT and DFT scores in plaque samples (8). However, the cariogenicity of S. mutans strains reportedly differs among clinical isolates (9). In addition, the ability to produce GTF-B varies among S. mutans strains isolated from clinical subjects (12, 13). These findings imply that further study is required to clarify the correlation between the cariogenicity of S. mutans strains and their ability to secrete GTF-B.

In this study, we investigated the relationship among the quantity of GTF-B protein, the quantity of S. mutans, and the DMFT index in oral samples. Our results indicated that a positive proportion was clearly observed between S. mutans quantity and the quantity of GTF-B protein in oral samples. Furthermore, a brushing-plaque sample was more effective than a stimulated saliva sample for estimating caries risk. In addition, the GTF-B-producing ability of S. mutans varied and the caries-susceptible group (DMFT: more than 15) tended to have high-GTF-B-secreting strains, whereas low-GTF-B-secreting strains seemed to be detected in the caries-free group. These findings suggest that cariogenicity depends on not only the quantity of bacteria harbored by subjects but also on the GTF-B-secreting ability of the strains. This result is supported by that of a previous report (17).

In the present study, we detected GTF-B protein by the sandwich-ELISA method with monoclonal and polyclonal antibodies specific for GTF-B. Other approaches such as polymerase chain reaction (PCR) have also been established to be useful for estimating GTF-B or bacterial quantity for estimating caries risk (8, 21). The PCR method is known to be more sensitive and more specific than other techniques, including colony morphology and immunological methods. However, the PCR method cannot detect expressed proteins. Furthermore, our sandwich-ELISA method could be used chairsise for assessment within hours and it requires no special technique to perform. In addition, the sensitivity and specificity of the sandwich-ELISA method for the detection of the GTF-B protein are satisfactory and this method was not affected by other components such as plaque and saliva left in the samples (Shinzaki-Kuwahara et al., unpublished). Therefore, we think that the sandwich-ELISA method have many advantages and is convenient for assessing caries risk.

In summary, a strong correlation was observed between the amount of GTF-B and the DMFT index as well as between S. mutans quantity and the DMFT index in both stimulated saliva and brushing-plaque samples isolated from subjects, although brushing-plaque samples showed a higher correlation than that of stimulated saliva samples. Further-
more, the _S. mutans_ strains harbored by the caries-susceptible group showed higher cariogenicity than those in the caries-free group. These results indicate that the quantitative determination of GTF-B protein in oral samples is practical for the estimation of caries risk. Furthermore, an epidemiological study revealed that the risk of caries is increased by an increasing DMFT index.

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**References**