Anti–cell-associated glucosyltransferase immunoglobulin Y suppression of salivary mutans streptococci in healthy young adults

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ABSTRACT

Background. The authors evaluated the suppressive effects of lozenges containing egg yolk antibodies (that is, immunoglobulin Y [IgY]) against Streptococcus mutans cell-associated glucosyltransferase (CA-gtf) on oral colonization by mutans streptococci (MS) in healthy young adults.

Methods. In a five-day double-masked placebo-controlled trial, young adult participants self-administered lozenges containing anti–CA-gtf IgY (Ovalgen DC, GHEN, Gifu-City, Japan) or a placebo at prescribed times each day. On the basis of bacterial colony counts of saliva cultures, the authors analyzed the pretrial and posttrial differences in levels of MS and total anaerobic bacteria among participants in the treatment (anti–CA-gtf IgY) and placebo groups and a control group.

Results. Salivary MS scores in participants in the treatment group decreased significantly (P < .001), and the mean anaerobic bacterial count in the treatment group was not statistically different before and after the trial. In the placebo and control groups, posttrial changes in median MS scores and total salivary anaerobic bacterial counts were not statistically significant.

Conclusions. The results of the study show that lozenges containing anti–CA-gtf IgY can suppress oral colonization by MS in healthy young adults.

Clinical Implications. Lozenges containing anti–CA-gtf IgY may help reduce dental caries risk in humans.

Key Words. Egg antibody; glucosyltransferase; Streptococcus mutans; dental caries.

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teins may linger. Although caries vaccines are considered to be effective in animal models, no caries vaccines are available commercially for human use to date, and few companies are willing to underwrite the investment for this type of vaccine development, ostensibly owing to the cost considerations and the rigorous requirements for vaccine safety.

Given the hurdles involved in caries vaccine development, passive immunization has received attention. This strategy, which involves the introduction of exogenous antibodies to the oral cavity, has fewer safety issues than do parenteral caries vaccines. The repackaging of antibodies as antinfective agents in place of antibiotic agents has received critical scrutiny owing to the general trend in antimicrobial resistance by human and animal pathogens. Depending on the targeted bacterial component, passive immunity against caries may be sufficient to retain all the functional mechanisms of antibodies generated during vaccination, including immunological interception, bacterial agglutination and abrogation of dental plaque–building enzymes. With this approach, the results of earlier investigations have shown the potential value of using oral monoclonal and polyclonal antibodies to reduce the extent or frequency of carious lesions or decrease the level of oral \textit{S. mutans} in rats or humans.

Passive immunization against dental caries will not become a practical reality unless an inexpensive method for mass production of antibodies is available. To this end, poultry eggs have been found to be a convenient source of polyclonal antibodies in the form of egg yolk immunoglobulins (immunoglobulin Y [IgY]). The results of passive immunization trials in rats in which IgY was used against different antigens from \textit{S. mutans} showed varying degrees of success. Among these antigens, CA-gtf is a target for immunological intervention in humans because antibodies to CA-gtf do not to cross-react with other human tissues. The safety of the oral administration of IgY has been documented.

Considering these findings, we conducted a study to determine whether anti–CA-gtf IgY (Ovalgen DC, GHEN, Gifu-City, Japan) administered via lozenges is effective in suppressing \textit{oral mutans streptococci} (MS) colonization in humans.

**PARTICIPANTS, MATERIALS AND METHODS**

**Purification of CA-gtf from \textit{S. mutans}**. We used \textit{S. mutans} strain MT 8148 to produce the CA-gtf enzyme. To isolate and purify CA-gtf from this strain, we followed a procedure described previously and used ion exchange chromatography (DEAE Sephacel, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and hydroxylapatite (HA) chromatography. This purification process yielded a protein of approximately 156 kilodaltons as determined by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown).

**Production of IgY**. To produce anti–CA-gtf IgY, we administered immunizations to 18-week-old poultry hens intramuscularly by using an oil-in-water emulsion of purified CA-gtf or, in cases of mock-immunized poultry, oil-in-water emulsion of phosphate buffered saline (PBS). We harvested all eggs laid by these hens between three and 10 weeks after the immunization, and we isolated the yolks. We then pooled and spray-dried the yolk in accordance with a method described previously. The spray-dried egg yolk underwent further extraction by means of hexane and ethanol at 50°C followed by vacuum drying to produce egg yolk powder with less than 2 percent fat content. We designated the defatted egg yolk powder containing IgY derived from CA-gtf-immunized poultry (anti–CA-gtf IgY) as the treatment and the defatted egg yolk powder containing IgY from mock-immunized poultry as the placebo.

**Preparation of lozenges**. We mixed anti–CA-gtf IgY or the placebo IgY with tablet excipients and processed them into 9-millimeter lozenges by means of direct compression. Each treatment lozenge contained 72 milligrams of anti–CA-gtf IgY plus excipients (4 mg aspartame, 80 mg microcrystalline cellulose, 50 mg eggshell calcium, 16 mg calcium bicarbonate, 148 mg maltose, 6 mg silica, 8 mg fermented milk powder, 10 mg fruit powder and flavoring). Each placebo lozenge contained 72 mg of the placebo IgY plus the same excipients as in the anti–CA-gtf IgY lozenges. We stored the lozenges at room temperature in airtight amber glass bottles for one to two weeks until we used them for the clinical trial.

**Extraction and analysis of IgY**. To conduct in vitro reactivity assays, we extracted IgY from the anti–CA-gtf IgY or the placebo by dissolving 1 gram of the sample in 9 milliliters of PBS, mixing them with an equal volume of chloroform and incubating them at 20°C while stirring.
gently for 30 minutes. We centrifuged the mixture at 2,000 gravity for 10 minutes, collected the supernatant water-soluble fraction containing IgY, lyophilized the sample and analyzed it by means of enzyme-linked immunosorbent assay (ELISA) as described previously and by using an IgY quantitation kit.

**Reactivity of IgY to different MS serotypes.** To determine the activity and specificity of the anti–CA-gtf IgY or the placebo IgY, we performed an indirect ELISA by using the following bacteria strains as coating materials: *Streptococcus cricetus* American Type Culture Collection (ATCC) (Manassas, Va.) 19642 (serotype a), *Streptococcus rattus* ATCC 19645 (serotype b), *Streptococcus downei* ATCC 33748 (serotype h), *S. mutans* MT 8148 (serotype c), *S. mutans* Ingbritt (serotype c), *S. mutans* P-4 (serotype e) and *S. mutans* SE 11 (serotype f).

We cultivated the bacteria in brain heart infusion (BHI) medium, centrifuged and washed the bacterial cell pellets with 0.05 molar carbonate buffer (pH 9.6) and disrupted the pellets by means of ultrasonication. We used these bacterial sonicates (absorbance at 600 nanometers = 1.0) to coat ELISA plates (5 micrograms/mL, 100 microliters/well) and performed an ELISA titer as described previously with anti–CA-gtf IgY as a reactive antibody. We considered samples yielding 0.2 optical density (OD) or less to be negative and the highest OD to be the ELISA titer.

**HA adherence assay.** To determine whether the anti–CA-gtf IgY could inhibit bacterial adherence in vitro, we performed an HA adherence assay according to the method used by Otake and colleagues. To prepare bacterial sonicates for the assay, we cultured *S. mutans* strains MT 8148, P-4 and SE 11 and *S. cricetus* strain ATCC 19642 for 20 hours at 37°C in BHI medium. On the day we performed the assay, we collected the cells, washed and resuspended the cells with Hanks’ balanced salt solution (pH 7.4) and resuspended the cells in Hanks’ balanced salt solution at a final concentration of 1.0 to 1.5 × 10⁷ colony-forming units (CFUs)/mL. We mixed 100 µL of the anti–CA-gtf IgY or the placebo predissolved in Hanks’ balanced salt solution (10.0 mg/mL) and incubated the mixture with 1.0 mL of the bacterial suspension for one hour at 37°C before incubating the mixture with HA squares. We used Hanks’ balanced salt solution (10.0 mg/mL) that did not contain any IgY as a buffer control. We performed all other procedures, including saliva coating of HA squares, HA-bacteria incubation and enumeration of adherent bacteria, as described by Otake and colleagues.

**Intervention study.** To test the efficacy of lozenges containing anti–CA-gtf IgY, we designed a five-day double-masked placebo-controlled intervention study. The Ethical Committee of the School of Dentistry, Health Sciences University of Hokkaido (Ishikari-gun, Japan), approved our clinical research protocol. We recruited a convenience sample population of 99 healthy male (n = 76) and female (n = 23) (average age, 23 years ± standard deviation 2 years) dental students at the university. All of the participants provided written informed consent before participating in the study. The participants had unknown MS or MS species infection levels or statuses in their oral cavities. Without considering their sexes, ages or MS statuses, we assigned the participants to one of three groups: treatment (n = 49), placebo (n = 19) or control (n = 31). We assigned more participants to the treatment group on the basis of the assumption that the results of testing would be more conclusive than if there had been fewer participants in this group.

We provided lozenges that contained either anti–CA-gtf IgY or the placebo to participants in the treatment or placebo groups. We instructed them to take one lozenge after every meal, at 3 p.m. and before bedtime for five days but to take only one lozenge the morning of the last day. Participants in the control group did not self-administer lozenges. We instructed the participants who were taking lozenges to let them dissolve slowly in the oral cavity while sucking gently on them. We provided each participant with a toothbrush and toothpaste that did not contain fluoride or xylitol and instructed them to maintain their routine personal hygiene and dietary habits during the test period except for the following: to avoid receiving antibiotic or dental treatment, to brush their teeth only in the morning and evening, and to avoid eating cheese, yogurt or natto (a traditional Japanese fermented-soybean food). After the trial was concluded, we asked the participants to provide written feedback regarding any adverse or unusual signs or symptoms they experienced during the trial and two weeks after the trial.
ended, as well as to assess their adherence to the prescribed frequency and timing of lozenge intake.

To create a double-masked trial, we did not inform the clinical research supervisor (I.C.) about the content of each randomly coded set of lozenges containing either the anti–CA-gtf IgY or the placebo. We also did not reveal to the laboratory technicians conducting the microbiological testing the group to which each saliva sample belonged. For five minutes, we collected into a cooled sterile tube 2 mL of saliva from each participant the day before the start of the trial and again two hours after the last lozenge was taken on day five. We kept the saliva samples on ice and processed them within two hours of collection.

**Microbial count.** We used a test kit (Mucount, Showa Pharmacological Industry, Tokyo) and a scoring system to assess the level of MS in the saliva samples. The kit allows MS cells, but no other bacteria cells found in saliva samples, to grow and form CFUs on the glass surface of the kit in a sucrose-containing selective medium. Immediately after sampling, we vortexed the saliva samples for at least 30 seconds and inoculated 100 µL of each sample into each Mucount kit. After incubating the samples at 37°C for 24 hours according to the manufacturer’s instructions, we counted the number of CFUs on the glass wall of the kit and scored the samples as follows: 0 = no CFUs, 1 = one to 15 CFUs, 2 = 16 to 30 CFUs, 3 = 31 to 45 CFUs, 4 = 46 to 60 CFUs, 5 = 61 to 75 CFUs, 6 = 76 to 90 CFUs and 7 = 91 or more CFUs. To determine total anaerobic bacterial counts, we inoculated 100 µL of the same saliva into BHI agar plates, incubated them at 37°C anaerobically for two days and then counted the CFUs.

To compare in-group differences in MS scores and total anaerobic bacterial counts (shown in log_{10}), we used the Wilcoxon matched-pair signed rank test and paired t test, respectively. To compare differences in MS scores and CFU counts among groups, we used the Kruskal-Wallis test and analysis of variance methods, respectively.

**RESULTS**

**Reactivity and analysis of anti–CA-gtf and placebo IgY.** Table 1 shows the reactivity of anti–CA-gtf IgY to different MS serotypes. The anti–CA-gtf IgY reacted with *S. mutans* (serotypes c, e and f) but not with *S. cricetus* (serotype a), *S. rattus* (serotype b) or *S. downei* (serotype h). The placebo IgY had a negative reaction with all of the MS strains tested. The ELISA titers of IgY extracted from anti–CA-gtf IgY and the placebo were 128,000 and less than 100, respectively. The total IgY content in anti–CA-gtf IgY was 30.0 mg/g. The average anti–CA-gtf IgY content at three separated ELISA measurements was 3.0 mg/g.

**Effect of IgY on the adherence of *S. mutans*.** Table 2 shows the results of the HA adherence assay. The anti–CA-gtf IgY inhibited adherence of *S. mutans* serotype c, e and f by 87.8, 87.1 and 83.6 percent, respectively, and the placebo IgY inhibited adherence of the same serotypes by 5.7, 11.6 and 1.3 percent, respectively. The anti–CA-gtf IgY content at three separated ELISA measurements was 3.0 mg/g.

**Intervention study.** In the treatment group, six of the 49 participants had no detectable MS before and after the trial. The salivary levels of MS were reduced significantly, with the median scores decreasing from 7 to 1 after the trial (P < .001; Wilcoxon matched-pair signed rank test) (Table 3). In this treatment group, 36 of the 43 participants with MS had reduced scores,
and seven had unchanged scores at the end of the trial. Fifteen participants (34.9 percent) had a zero MS score at the end of the trial. In the placebo and control groups, posttrial changes in median MS scores were not statistically significant ($P > .05$; Wilcoxon matched-pair signed rank test) (Table 3). Only one participant in each group who had an initial MS score of 1 had a zero score at the end of the trial. Total salivary anaerobic bacteria counts in all groups were not significantly different before and after the trial ($P > .05$; paired $t$ test) (Table 4). The median MS scores before the trial were not significantly different among the three groups (median score, 7, 5 and 7 for the treatment, placebo, control groups, respectively), but the scores were significantly lower after the trial for the treatment group (median score, 1) ($P < .01$; Kruskal-Wallis test) compared with the scores for the placebo and control groups (median score, 7 for each group). We also found no significant differences in mean CFUs of total anaerobic bacteria among the three groups before and after the trial ($P > .05$; $t$ test) (Table 4). None of the written feedback from the participants revealed any adverse reactions or signs during and at the end of the clinical trial.

**DISCUSSION**

An important event in the formation of dental plaque is the adherence of MS to the tooth surface and subsequent synthesis of water-insoluble glucan from dietary sucrose mediated by the CA-gtf enzyme. Although MS can colonize the tooth surface directly, the results of a recent study suggested that most tooth colonization by MS occurs after other oral bacteria have colonized the tooth. The buildup of water-insoluble glucan polymers converts dental plaque into microaerobic acidified matrices. These matrices are conducive for growth of MS and lactobacilli whose lactic acid products dissolve the carbonated HA mineral of teeth, which may lead to overt carious lesions. Disrupting the initial events leading to bacterial adherence by means of passive immunization has had positive results in the effort to shift the use of caries-fighting antibodies from the laboratory to mainstream use in oral hygiene.

Antibodies in the saliva resulting from passive antibody transfer may interrupt the initial stage of dental plaque formation by means of specific mechanisms: aggregation of the bacteria before colonization, inactivation of enzymes necessary for metabolically important functions or blockage of surface receptors necessary for colonization. In our study, the anti–CA-gtf IgY

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### TABLE 2

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>MUTANS STREPTOCOCCI CELLS (SEROTYPES) (NO. OF COLONY-FORMING UNITS OF BACTERIAL CELLS PER MILLILITER OF DISPERSED BUFFER [MEAN ± STANDARD DEVIATION (% INHIBITION OF ADHERENCE)])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Cell-Associated Glucosyltransferase Immunoglobulin Y</td>
<td>Streptococcus mutans MT 8418 (c) 175 ± 17 (87.8) S. mutans P-4 (e) 157 ± 41 (87.1) S. mutans SE 11 (f) 293 ± 59 (83.6) Streptococcus cricetus (a) 933 ± 18 (2.7)</td>
</tr>
<tr>
<td>Placebo Immunoglobulin Y</td>
<td>1,348 ± 123 (5.7) 1,077 ± 117 (11.6) 1,760 ± 132 (1.3) 941 ± 19 (1.9)</td>
</tr>
<tr>
<td>Buffer Control</td>
<td>1,429 ± 84 (0) 1,219 ± 82 (0) 1,798 ± 400 (0) 959 ± 24 (0)</td>
</tr>
</tbody>
</table>

### TABLE 3

Changes in salivary mutans streptococci scores in the participants during the trial.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>SALIVARY MUTANS STREPTOCOCCI SCORES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before (Median Mutans Streptococci Scores [25th, 75th Percentiles])</td>
</tr>
<tr>
<td>Treatment (n = 49)</td>
<td>7 (1, 7)</td>
</tr>
<tr>
<td>Placebo (n = 19)</td>
<td>5 (1, 7)</td>
</tr>
<tr>
<td>Control (n = 31)</td>
<td>7 (1, 7)</td>
</tr>
</tbody>
</table>

* Significant difference compared with placebo and control groups ($P < .01$; Kruskal-Wallis test).
Total anaerobic bacteria in saliva collected from the participants before and after intervention.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TOTAL COLONY-FORMING UNITS OF BACTERIA CELLS (LOG₁₀) PER MILLILITER OF SALIVA</th>
<th>BEFORE (MEAN ± STANDARD DEVIATION)</th>
<th>AFTER (MEAN ± STANDARD DEVIATION)</th>
<th>PAIRED t TEST (P VALUE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (n = 49)</td>
<td></td>
<td>7.4 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>Placebo (n = 19)</td>
<td></td>
<td>7.2 ± 0.6</td>
<td>7.4 ± 0.5</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>Control (n = 31)</td>
<td></td>
<td>7.3 ± 0.7</td>
<td>7.5 ± 0.6</td>
<td>&gt; .05</td>
</tr>
</tbody>
</table>

may have suppressed *S. mutans* colonization at all three points of bacterial vulnerability. First, the dimeric Fab domain of IgY may have aggregated the target bacterial cells into larger clusters, making them susceptible to displacement by salivary movement and rendering them unable to anchor to and colonize tooth surfaces. The results of the HA adherence assay (Table 2) show such inhibitory action by IgY. Second, the polyclonal IgY of anti–CA-gtf IgY may have abolished the glucosyltransferase catalytic site. We have observed the abrogation of *S. mutans* glucan synthesis by anti–CA-gtf IgY in a dose-dependent manner (data not shown) in our laboratory. This may result in the inability of *S. mutans* to adhere to tooth surfaces by means of glucan-dependent adherence or the inability of adherent *S. mutans* to initiate dental plaque deposition or to build on existing dental plaque. This view is supported by the results of studies in rats using glucosyltransferase-negative,²⁹ or glucosyltransferase-defective ³⁰ *S. mutans* that failed to colonize the oral cavity. Third, as streptococcal CA-gtf has a natural glucan-binding domain at the carboxyl-terminal portion,³¹,³² the IgY in anti–CA-gtf IgY may have blocked sterically multiple epitopes on the CA-gtf molecule, including the glucan-binding site, resulting in a failure of *S. mutans* cells to supercolonize existing dental plaques and thereby limiting the number of *S. mutans* in the oral cavity. Thus, the suppressive effect of anti–CA-gtf IgY appears to strike at the incentive mechanisms of dental plaque formation.

A direct correlation exists between elevated proportions of *S. mutans* and a higher frequency of people with active caries.³⁰ Salivary levels of *S. mutans* reflect the number of colonies formed by the bacterium on the tooth surface,³⁴,³⁶ which means that saliva sampling can be used to monitor dental plaque formation and evaluate caries risk.³⁵,³⁶ The results of studies involving the use of different scoring categories have shown that salivary MS levels or scores are correlated significantly with decayed, missing and filled teeth status.³⁷–³⁹ In our study, we used a 0 to 7 scoring system, because it allowed us to detect changes in MS levels across time better than the common 0 to 3 or 0 to 4 scoring systems. The treatment group exhibited significantly reduced salivary MS scores after the trial, and the placebo and control groups had virtually unchanged salivary MS scores after the trial (Table 3). Such reduction in salivary MS scores as in the treatment group may have had a negative effect on the initiation and frequency of dental plaque formation and an indirect effect on cariogenesis; however, the actual extent of this effect needs to be studied in a long-term trial. Nevertheless, the overall data presented in Table 3 provide enough proof of concept regarding the feasibility of significantly suppressing MS colonization through a daily intake of lozenges containing anti–CA-gtf IgY. About a one-third (34.9 percent) of the posttrial saliva samples in the treatment group had a zero MS score at the end of the trial, compared with one sample each in the placebo (5.3 percent) and control (3.3 percent) groups.

Although we could not rule out possible changes in the proportion of some anaerobic species in the background oral microflora, reduction in the salivary MS level in the treatment group did not cause significant changes in the total count of anaerobic bacteria (Table 4). Specific IgY in anti–CA-gtf IgY binds only to *S. mutans* of various serotypes but not to *S. cricetus, S. rattus* or *S. downei* (Table 1). These results indicate a high level of selectivity by anti–CA-gtf IgY, which contrasts with the “shotgun effect” of antibiotic agents that can eliminate bacteria indiscriminately in the oral cavity. Furthermore, none of the participants in the three groups reported any adverse events in their overall health condition during the trial and two weeks after the trial ended. Taken together, these findings indicate that lozenges containing anti–CA-gtf IgY can be taken safely. The use of a lozenge as a vehicle for IgY delivery is novel and has the advantage of releasing antibodies slowly into the oral cavity. Moreover, IgY powder compressed directly into a lozenge or a tablet is more stable and more convenient for long-term storage,⁴⁰ oral dosing and handling.
CONCLUSIONS
The results of this study provide proof of concept that lozenges containing specific egg yolks antibodies (IgY) against CA-gtf of S. mutans exerted a suppressive effect on S. mutans colonization of the oral cavity in healthy young adults and that such a suppressive effect did not destabilize quantitatively normal oral microflora. A long-term intervention study that would allow investigators to evaluate changes in dental plaque levels and caries scores among participants may provide more information regarding the benefits and safety of the use of anti-CA-gtf IgY in caries control.

Disclosures. Dr. Nguyen works for Immunology Research Institute in Ghent (IRIG) (Gifu-City, Japan), and Dr. Icatlo worked for IRIG when the research reported in this article was conducted. IRIG is the in-house research institute of GHEN (Gifu-City, Japan), which manufactures Ovalgen DC, and is part of EW Group (Visbek, Germany). The EW Group also includes EW Nutrition (Visbek, Germany), which markets all of the immunoglobulin Y-based products that have been developed by IRIG, including Ovalgen DC. Dr. Nakano works for Bean Stalk Snow (Kawagoe-city, Japan) a company that manufactures products that contain Ovalgen DC. Drs. Emiko Isogai, Hirose, Mitsuaki Isogai, Kobayashi-Sakamoto, Hiroshi Isogai and Chiba did not report any disclosures.

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37. Hamada S, Horikoshi T, Minami T, et al. Oral passive immuno-

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