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The Effects of Egg-Derived Antibodies to Glucosyltransferases on Dental Caries in Rats

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Chicken antibodies · Glucosyltransferase(s) · Immunotherapy · *Streptococcus mutans*

Abstract
The role of *Streptococcus mutans* in the development of dental caries is well recognized. Important virulence factors include the glucosyltransferases (gtf), essential for production of glucans. We evaluated the anticariogenic effects of orally administered chicken anti-cell-associated (CA) Gtf antibodies in desalivated rats. The animals were infected with *S. mutans* MT8148R and treated with chicken anti-CA-Gtf egg yolk antibodies (IgY) or nonimmune egg yolk powder. Smooth surface lesions were significantly lower in the anti-CA-Gtf-treated group in comparison to the control groups. Sulcal surface caries was also decreased and of less severity. Our study suggests that chicken anti-CA-Gtf antibodies may have promise as a prophylaxis for high caries risk patients.

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Dental caries results from interaction between the host, diet and the microflora on the tooth surface. Virulence factors of *Streptococcus mutans* include aciduránce, acidogenicity and the ability of the bacteria to adhere to and accumulate on the tooth surfaces [Loesche, 1986; Marcotte and Lavoie, 1998]. Glucosyltransferases (Gtfs) have been shown to be one of the major virulence factors in the pathogenesis of dental caries [Tanzer, 1979; Yamashita et al, 1993]. The Gtfs produced by *S. mutans* are present in whole saliva from humans and are incorporated into the initial salivary pellicle in active form [Rølla et al., 1983; Schilling and Bowen, 1992] and result in production of glucan from sucrose. *S. mutans* harbors at least three gtf genes that encode GtfB, GtfC, and GtfD [Aoki et al., 1986; Honda et al., 1990; Shiroza et al., 1987; Ueda et al., 1988]. GtfB primarily synthesizes water-insoluble glucans from sucrose, while GtfD is responsible for the formation of soluble glucans. GtfC synthesizes predominantly insoluble glucans but also significant amounts of soluble glucans [Kopec et al., 2001; Hanada and Kuramitsu, 1989; Tsumori and Kuramitsu, 1997]. In addition, GtfB, GtfC and GtfD are active in an adsorbed state and the glucans produced by GtfB or GtfC can influence the adherence of cariogenic oral streptococci to an experimental pellicle [Venkitaraman et al., 1995]. Mutants of *S. mutans*, defective in either or both *gtfB* and *gtfC* genes, induce markedly reduced levels of

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smooth and sulcal surface carious lesions in rats, relative to that of the parental strains [Yamashita et al., 1993].

The effects of passive immunization against *S. mutans* has previously been explored using topical oral administration of antibodies derived from animals vaccinated with whole bacteria or purified components such as Gtf, streptococcal antigen I/II or serotype carbohydrate [Loimaranta et al., 1999; Hamada et al., 1991; Ma et al., 1998; Otake et al., 1991]. In humans, a mouth rinse containing bovine antibodies against *S. mutans* has also been claimed to decrease the population of *S. mutans* in dental plaque [Filler et al., 1991] and antibodies from chicken directed against *S. mutans* bacteria have also been shown to decrease the number of *S. mutans* in the saliva of healthy volunteers [Hatta et al., 1997].

Although the incidence of caries has been reduced in some sections of the population due to prophylactic measures during the past decades, there are groups of patients for whom caries remains a major clinical problem. These include individuals with hyposalivation due to adverse effects of certain pharmaceutical products, systemic diseases such as Sjögren's syndrome, and head and neck tumor patients receiving heavy local irradiation therapy [Spak et al., 1994].

When the major salivary glands are surgically removed in rats, both coronal and root surface caries develop rapidly [Bowen et al., 1988]. This clearly shows the critical importance of saliva in maintaining the oral health and protection against dental caries.

In an attempt to mimic the clinical caries situation in patients with reduced salivation, our study was designed to evaluate the use of chicken-derived anti-Gtf antibodies given in the drinking water to desalivated rats.

Materials and Methods

Bacterial Strains and Cultivation

S. mutans MT8148R, a clinical isolate resistant to streptomycin, and *S. sobrinus* 6715 were cultured in low-molecular-weight medium and grown to exponential phase. Establishment of successful infection in the rats and quantitative analyses of the number of *S. mutans* and the total cultivable flora after termination were checked by oral swabbing and culturing on blood agar plates and Mitis salivarius (Difco) and streptomycin (Sigma) (MSS) agar plates.

Preparation of Gtf

The cell-associated Gtf used for immunization was prepared as previously described [Hamada et al., 1989]. Briefly, cell-associated (CA) Gtf of *S. mutans* MT8148 (serotype c) was extracted by treatment with 8 *M* urea at 25°C for 1 h. The crude extract was purified by DEAE-Sephacel and hydroxyapatite column chromatography. The molecular mass of CA-Gtf was determined by SDS-PAGE. The GtfB, GtfC and GtfD used for the in vitro experiments were prepared as previously described [Vacca-Smith et al., 1996].

Immunization of Hens

Two hundred white Leghorn hens (18 weeks old) were used and divided into two groups. The hens in group 1 were immunized intramuscularly with CA-Gtf (0.4 mg/0.5 ml) mixed with Freund's complete adjuvant (0.5 ml). Group 2 was immunized with an emulsion of saline (0.5 ml) and Freund's complete adjuvant (0.5 ml). After the initial immunization two booster injections of the emulsions were given intramuscularly 7 and 13 weeks following initial injection. Eggs were collected and kept at 4°C until used.

Preparation of Egg Yolk Antibodies and Control IgY

The preparation of antibodies and control powder from egg yolk was performed as previously described [Hamada et al., 1991]. Briefly, the yolks were separated from the eggs of immunized or sham-immunized hens, mixed with saline and chloroform and incubated at 20°C for 30 min. After centrifugation, the water-soluble fraction was separated and lyophilized. The preparation was further purified by ammonium sulfate precipitation and DEAE-Sephacel column chromatography. The product contained 98% of IgY.

Agglutination Assay

S. mutans MT8148R (serotype c) was cultivated in brain-heart infusion broth (Difco) and harvested by centrifugation after entering the stationary phase. The bacteria were washed 3 times in phosphate-buffered saline, pH 7.0, and the optical density was adjusted to OD_{600} 1.0. Twenty-five microliters of *S. mutans* suspension was placed on a glass slide (76 × 26 mm, Menzel-Glaser) after which 25 µl of either chicken anti-Gtf or nonimmunized egg yolk (10 mg dissolved in 1ml of sterile water) was added. The slide was then rotated vertically by hand and agglutination was evaluated visually after 2 min.

Effect of Chicken Anti-Gtf on Streptococcal Gtf

Solution Assay. A solution of immunoglobulins was prepared in adsorption buffer (50 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* potassium phosphate, 0.1 m*M* $MgCl_2$, pH 6.5) at 1 mg/ml concentration. Two hundred microliters of the antibody preparation was mixed with 2.5 µg of either GtfB, GtfC, or GtfD, to reach a final volume of 400 µl. In the control experiments, Gtfs were mixed with 200 µl of adsorption buffer which included nonimmunized egg yolk powder instead of antibody. All samples were then exposed to ^{14}C-glucosyl-sucrose (100 m*M*, final concentration) for 4 h, at 37°C. The reaction was stopped by addition of 1.0 ml of ice-cold ethanol. Glucans were precipitated overnight at 4°C, collected on glass-fiber filters, and quantified by liquid scintillation spectrometry [Schilling and Bowen, 1992].

Surface Assay. Ten milligrams of hydroxyapatite beads (Integration Separation Systems, Hyde Park, Mass., USA; surface area 0.24 m^2, size 60.0–100.0 µm) was coated with human clarified whole saliva (Gtf-free) supplemented with the protease inhibitor phenylmethylsulfonylfluoride (1.0 m*M*) and sodium azide (0.02%) for 30 min at 37°C. After incubation, the beads were washed 3 times with 1.0 ml of adsorption buffer. The beads were then exposed to 2.5 µg of GtfB, GtfC or GtfD, prepared as previously described [Vacca-Smith et al., 1996] for 30 min at 37°C, then washed and exposed to 300 µl of egg antibody in adsorption buffer at 1 mg/ml concentration, or 300 µl of nonimmunized egg yolk powder in adsorption buffer for 30 min at 37°C. Sufficient enzyme was always included to ensure satura-

tion kinetics. After incubation, the beads were washed as described above and exposed to ^{14}C-glucosyl-sucrose (100 mM final concentration) for 4 h. The reaction was stopped with ice-cold methanol, glucans were precipitated overnight, collected on glass-fiber filters and quantified by liquid scintillation spectrometry [Schilling and Bowen, 1992].

Preparation of Experimental Drinking Solutions

The IgY and the nonimmunized egg yolk powder were dissolved in sterile water in a concentration of 10 mg/ml which was freshly prepared 3 times per week.

Animals

Specific pathogen-free Sprague-Dawley (SPF) female rats at the age of 14 days were purchased from Charles River Breeding Laboratories, Kingston, N.Y., USA. The dams were screened for the presence of sialodacryoadenitis virus and mutans streptococci using methods described by Bowen et al. [1988]. At the age of 20 days the animals were weaned from their dams.

Caries Studies

The first experiment was performed to compare the effect of polyclonal egg derived immunoglobulins in a desalivated rat model. Thirty-two female SPF rats, 23 days of age, were infected with *S. mutans* MT8148R. They were then randomly divided into two groups of 16 which received Diet 2000 and distilled sterile water ad libitum. Successful infection was verified using oral swabs plated on MSS and blood agar media. At 26 days of age, they were all desalivated surgically [Bowen et al., 1988]. The first group received 10 mg/ml of chicken anti-Gtf immunoglobulins dissolved in the drinking water and the control groups received 10 mg/ml of albumin in the drinking water. After 17 days the rats were killed using carbon dioxide and the left mandible was dissected aseptically, sonicated in 5 ml sterile saline and the resulting suspension cultured for *S. mutans* [Bowen et al., 1988]. The jaws were evaluated for dental caries [Keyes, 1958, modified by Larson, 1981].

In the second experiment, we increased the number of rats per group to 20 and added a nonimmunized egg yolk control and an intact control group. Female Sprague-Dawley SPF rats aged 18 and 19 days were fed Diet 2000 ad libitum and infected with an active culture of *S. mutans* MT8148R. At 20 days of age, 80 of the rats were desalivated and randomly divided into four different groups. Group 1 received 10 mg/ml of chicken anti-Gtf immunoglobulins in their drinking water, and group 2 received an egg yolk preparation from nonimmunized hens. The control groups received sterile distilled water. The intact control group drank sterile distilled water. All rats received a volume of 50 ml/rat per day. Twenty days after desalivation, the animals were killed using carbon dioxide. Quantitative culturing procedures and evaluation of dental caries were carried out as described above [Bowen et al., 1988].

Statistical Analyses

The outcome measures are performed using transformed values of the measures. Smooth surface and sulcal surface caries scores are expressed as proportions of their maximum possible values (124 and 56, respectively), and the arcsine transformation was applied. The total flora was counted and *S. mutans* counts were replaced by their logarithms, using Software for Statistical Visualization on the Apple Macintosch from SAS Institute Inc., version 2 (SAS Institute Inc., Cary, N.C., USA). Analysis of variance and the Tukey-Kramer HSD for all comparisons were performed.

Table 1. Effect of chicken anti-Gtf antibodies on *S. mutans*

Experimental group	n	*S. mutans* MT8148R % of total cultivable flora
First experiment		
Chicken anti-Gtf 10 mg/ml	16	7.0±1.5
Albumin 10 mg/ml	16	11.0±1.5
Second experiment		
Chicken anti-Gtf 10 mg/ml	19	9.0±2.6
Nonimmunized egg yolk 10 mg/ml	20	10.4±2.6
Sterile water	20	12.2±2.6
Intact rats	20	32.0±2.6

Mean values (MSD) expressed as percentage of *S. mutans* MT8148R of total cultivable flora.

Results

Inhibition of GtfB and GtfC by Chicken Antibodies

The chicken anti-CA-Gtf IgY effectively agglutinated *S. mutans* MT8148R in contrast to the nonimmunized egg yolk control (data not shown). In the presence of chicken anti-CA-Gtf immunoglobulin, the activities of both GtfB and GtfC in solution were reduced by 87.3 ± 3.7 and 85.3 ± 34.6%, respectively. Chicken anti-CA-Gtf antibodies were without effect on the activity of GtfD in solution. The activities of GtfB and GtfC bound on the surface of saliva-coated hydroxyapatite beads were reduced by 32 ± 17 and 39 ± 13% by exposure to egg antibody. Chicken anti-CA-Gtf IgY was without effect on surface-bound GtfD.

Effect of Anti-CA-Gtf Antibodies on Caries Development

In the first experiment, the animals receiving the anti-CA-Gtf immunoglobulin preparation showed significantly fewer *S. mutans* bacteria, and the percent of *S. mutans* was lower but not statistically different from the albumin control group ($p > 0.05$; table 1).

The IgY group displayed a lower development of smooth surface lesions and sulcal caries lesions than the control group; the anti-CA-Gtf group also developed lower and less extensive slight and moderate dentinal caries than the control group ($p < 0.05$; table 2).

In the second experiment, three groups of desalivated rats drank solutions containing either chicken anti-CA-Gtf immunoglobulins, egg yolk powder from nonimmunized hens, or sterile water only, while one group of intact

Table 2. Effect of chicken anti-Gtf antibodies on rat caries (experiment 1)

Experimental group	Total number of rats	Total smooth caries	D_s	D_m	D_x	Total sulcal caries	D_s	D_m	D_x
Chicken anti-Gtf 10 mg/ml	16	43.0 (13.4)[a]	22.6 (11.1)[a]	3.0 (3.6)	0.4 (0.6)	40.9 (6.8)[a]	31.8 (4.8)[a]	13.3 (5.9)[a]	0.4 (0.9)[a]
Albumin 10 mg/ml	16	59.6 (14.3)	43.8 (19.7)	5.9 (5.0)	1.6 (2.6)	48.5 (2.2)	40.6 (3.5)	23.9 (6.3)	2.4 (2.4)

Smooth surface caries and severity scores (means, SD in parentheses) on molar teeth of desalivated rats given chicken anti-CA-Gtf antibodies in the drinking water. Severity scores are expressed as: D_s = dentinal slight, D_m = dentinal moderate, D_x = dentinal extensive caries.

a Significantly lower than albumin group ($p < 0.05$).

Table 3. Effect of chicken anti-Gtf antibodies on development of dental caries (experiment 2)

Experimental group	Total number of rats	Total smooth caries	D_s	D_m	D_x	Total sulcal caries	D_s	D_m	D_x
Desalivated, chicken anti-Gtf, 10 mg/ml	19	50.2 (6.3)[a]	16.7 (6.3)[b]	2.1 (1.3)[c]	0.2 (0.5)[d]	45.9 (2.6)[e]	35.3 (3.8)[f]	12.2 (4.2)[g]	1.5 (1.6)[h]
Desalivated, non-immunized egg, 10 mg/ml	20	64.2 (6.4)[a]	36.7 (7.0)[b]	8.1 (6.4)[c]	1.5 (3.4)	50.6 (3.2)[e]	41.7 (4.1)[f]	23.1 (6.0)[g]	6.1 (4.0)[h]
Desalivated, sterile water	20	67.6 (8.8)[a]	44.4 (8.6)[b]	11.2 (5.9)[c]	2.2 (2.0)[d]	51.5 (2.0)[e]	42.7 (3.7)[f]	23.7 (6.1)[g]	6.4 (4.8)[h]
Intact, sterile water	20	1.1 (2.1)	0.5 (1.8)	0.0 (0.0)	0.0 (0.0)	28.5 (10.5)	21.3 (8.4)	1.8 (2.1)	0.1 (0.3)

Smooth surface caries and severity scores (means, SD in parentheses) on molar teeth of desalivated rats given chicken anti-CA-Gtf antibodies in the drinking water. Severity scores are expressed as: D_s = dentinal slight, D_m = dentinal moderate, D_x = dentinal extensive caries.

a Significantly lower than all other groups except intact: $p < 0.05$.
b Significantly lower than all other groups except intact: $p < 0.05$.
c Significantly lower than nonimmunized egg and sterile water groups: $p < 0.05$.
d Significantly lower than sterile water group: $p < 0.05$.
e Significantly lower than nonimmunized egg and sterile water groups: $p < 0.05$.
f Significantly lower than nonimmunized and sterile water groups: $p < 0.05$.
g Significantly lower than all other groups except intact: $p < 0.05$.
h Significantly lower than nonimmunized and sterile water groups: $p < 0.05$.

rats drank sterile water. No statistically significant differences were observed in the population of *S. mutans* among the groups (table 1).

The animals receiving IgY developed less extensive and less severe smooth surface lesions. Severity of the lesions was significantly lower in the anti-CA-Gtf group than in all the other groups ($p < 0.05$)

A significantly lower development of sulcal surface caries was observed in rats receiving anti-CA-Gtf antibodies in comparison to the nonimmunized and sterile water groups ($p < 0.05$). Significantly less severe sulcal caries lesions were found compared to the nonimmunized egg yolk and sterile water group ($p < 0.05$; table 3).

Discussion

Passive immunization using antibodies from a number of different species has previously been used successfully as immunotherapy against infections in the oral and gastrointestinal tract in animal models [Weiner et al., 1999]. Topical (passive) administration of antibodies has several advantages compared with systemic induction of antibodies. It is a safe procedure and high local concentrations of specific antibodies can be achieved. Production of polyclonal antibodies in eggs is convenient and economical as up to 40 mg of IgY can be obtained from a single egg.

The Gtfs are especially important virulence factors for development of smooth and sulcal surface caries lesions [Yamashita et al., 1993]. The egg-derived antibodies (IgY) against Gtf used in the present study [Hamada et al., 1991] have not previously been checked for which particular Gtf enzyme they are active against. In the inhibition

assays, the chicken anti-Gtf antibodies reduced the activity of both GtfB and GtfC in solution. The inhibition was less pronounced when the enzyme was bound to the surface of hydroxyapatite beads, supporting previous results showing a difference in antibody activity in solution and on a surface, e.g. artificial pellicle [Vacca-Smith et al., 1996; Wunder and Bowen, 2000].

In earlier studies [Jespersgaard et al., 1999], antibodies have been raised against soluble antigens without considering whether the antibodies will react against the antigen adsorbed to a surface. Dental caries results from a series of interactions, which occurs on a surface. It is therefore desirable to determine whether antibodies that affect Gtf in solution can also influence enzymes bound to the tooth surface. Results from previous studies have shown that Gtf enzyme adsorbed to pellicles displays several distinct properties different from those of the soluble enzyme [Venkitaraman et al., 1995; Vacca-Smith et al., 1996; Wunder and Bowen, 2000].

The present study was conducted to determine the effectiveness of chicken egg yolk antibodies against Gtfs administered as a drinking solution in a desalivated animal model. This is in contrast to previous animal experiments where the antibody preparations have been included in the food [Michalek et al., 1987; Otake et al., 1991], or both food and water [Smith et al., 2001].

In the second animal experiment, there were surprisingly no differences in the number or proportion of *S. mutans* between the groups. The methods used are, however, not particularly sensitive and are heavily dependent on effective dispersion techniques, which in turn will be affected by the composition of the plaque. The antibody was effective against GtfB, which in turn will affect the matrix of plaque. Plaque with less B glucan will be easier to disperse.

The use of the desalivated rat model represents a very severe cariogenic challenge. Preliminary results have shown that the *S. mutans* strain MT8148R was equal in cariogenicity in intact and desalivated rats compared to the *S. sobrinus* 6715, which had been used in previous studies [Bowen et al., 1988]. This is the first study evaluating passive immunization against caries in an animal model mimicking to some extent the clinical situation for dry mouth patients. In patients irradiated for head-neck tumors, the salivary flow rate may decrease to 5% of the preirradiation level [Cheng et al., 1981], resulting in a dramatic change in the oral microbiological flora and a high caries activity [Brown et al., 1978; Keene et al., 1981]. We wanted to evaluate the possibility of using egg-derived anti-CA-Gtf antibodies as a rinsing solution for selected patient groups. A rinsing solution may be preferable to tablets due to the low salivary flow and the fragility of the oral mucosa in hyposalivatory patients. In humans, egg-derived anti-Gtf immunoglobulins have previously been utilized in mouth rinsing solutions leading to reduced population of *S. mutans* in dental plaque [Hatta et al., 1997]. However, their ability to prevent development of caries lesions has not been investigated to date.

In contrast to previous published studies [Otake et al., 1991; Smith et al., 2001] we have also analyzed the severity of dental caries as this is of clinical importance. In our experiment, rats receiving chicken anti-CA-Gtf antibodies developed fewer and less extensive caries lesions, demonstrating that the anti-CA-Gtf antibodies not only prevent the initiation of caries but also reduce the progression of dental caries.

A future strategy to control dental caries may include the choice of target antigen, immunization route, and decision whether to use polyclonal or monoclonal antibodies. Monoclonal antibodies against the different Gtfs or conserved regions of the enzyme have been evaluated in vitro [Nanbu et al., 2000], but have not yet been tested in animal models.

Further evaluation of both polyclonal and monoclonal antibody therapy is needed in clinical trials for patients with high caries activity. There are as yet no studies where passive immunization has been evaluated in patients with a reduced salivary flow. A future strategy could be to evaluate the efficacy of anti-Gtf antibodies in patients with head-neck tumors.

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