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Oral Passive Immunization against Dental Caries in Rats by Use of Hen Egg Yolk Antibodies Specific for Cell-Associated Glucosyltransferase of *Streptococcus mutans*

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The effect of polyclonal egg yolk immunoglobulin G antibodies (yIgG) raised against whole cells, cell-free (CF) glucosyltransferase (GTase), or cell-associated (CA) GTase of serotype c *Streptococcus mutans* was examined in terms of in vitro inhibition of virulence-related factors of *S. mutans* and protection of *S. mutans*-infected rats against the development of dental caries. Hens (18 weeks old) were immunized with formalin-treated whole cells, purified CF-GTase, or CA-GTase together with Freund's complete adjuvant. In addition, yIgG to surface protein antigen was used in some in vitro experiments for comparison with other antibodies. yIgG was purified by ammonium sulfate precipitation followed by DEAE-Sephacel column chromatography or fractional precipitation with ethanol. Purified yIgG was found to be a 220-kDa protein, which was dissociated into heavy and light chains upon addition of 2-mercaptoethanol. yIgG to whole cells and surface protein antigen gave a heavy aggregation of *S. mutans* organisms, while yIgG to CF- and CA-GTase specifically inhibited the enzymatic activity of the respective GTase. yIgG to CA-GTase and whole cells was found to clearly suppress the adherence of *S. mutans* cells to a glass surface. Specific-pathogen-free Sprague-Dawley rats that had been infected heavily and repeatedly with *S. mutans* and fed diet no. 2000 developed severe dental caries, while rats fed diet 2000 containing ≥0.1% yIgG to CA-GTase showed a statistically significant reduction in dental plaque accumulation and caries development. Administration of yIgG to CF-GTase and whole cells failed to protect against caries. These results clearly suggest that yIgG to *S. mutans* CA-GTase specifically inhibited a virulence factor of this organism, i.e., insoluble glucan-synthesizing CA-GTase, resulting in a significant reduction in the development of dental caries.

It has been found that mutans streptococci possess virulence factors which serve in dental caries development in experimental animals and most probably in humans. The abilities of *Streptococcus mutans* and other species of mutans streptococci to adhere firmly to the tooth surface in the presence of sucrose and to release acids by fermenting a variety of sugars from the diet, among other abilities, have been associated with the cariogenic potential of mutans streptococci (10). The adherence of *S. mutans* to the tooth surface is mediated by the enzymatic action of the glucosyltransferases (GTases) of the organism. Although initial attachment of *S. mutans* to the saliva-coated enamel surface occurs through the surface protein of *S. mutans*, synthesis of water-insoluble, adherent glucan from sucrose by the GTases is essential for dental caries development (17, 19). *S. mutans* produces both cell-associated (CA) and cell-free (CF) forms of GTase; the former primarily synthesizes water-insoluble glucan, while the latter produces water-soluble glucan (9). The combined action of these two GTases on the cell surface of *S. mutans* during its growth in the presence of sucrose is critically important in allowing firm adherence. The GTase system of *S. mutans* has therefore been considered an important virulence factor promoting caries development.

One of the major strategies is to combat *S. mutans* by immunological procedures such as active immunization or passive oral administration of preformed antibodies to *S. mutans* (35). Oral passive immunization with polyclonal antibodies from the milk of the immunized cow (25) or mouse monoclonal antibodies (MAb) specific for SA I/II, an *S. mutans* protein antigen (21), was shown to decrease colonization by *S. mutans* and caries development in *S. mutans*-infected rats and monkeys. The bovine milk antibodies were elaborated by use of a multivalent vaccine consisting of whole-cell antigens of mutans streptococci, but the active entities in the antibodies were not elucidated (8, 25). Ma et al. (22, 23) recently reported that an MAb to SA I/II significantly prevents colonization of the tooth surface in humans by *S. mutans*, and this protection was found to last up to 2 years, although the MAb was given only over a period of 3 weeks. These authors considered that this effect was possibly attributable to a shift in the ecological balance of the oral flora which discouraged specific colonization of *S. mutans* on teeth. It should be mentioned that passive immunization has been used successfully to prevent gastrointestinal bacterial, viral, and parasitic infections (5, 6, 26).

It has been found that antibodies are actively transported to the egg yolks from serum in large quantities during gestation in immunized hens (3). Evidence indicated that oral administration of hen egg yolk antibodies (yAbs) prevented experimental rotavirus infection in mice (4, 42). These successful trials with yAbs raise the possibility of conferring passive protection against *S. mutans*-induced dental caries by using antibody prepared from the eggs of the hens hyperimmune to *S. mutans* antigen. In this study, we have prepared yAbs elaborated against some *S. mutans* antigens and examined their immunological specificity and

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effects on dental caries development in *S. mutans*-infected rats.

MATERIALS AND METHODS

Bacteria. Organisms of *S. mutans* MT8148R (serotype c) were usually used. They were maintained on brain-heart infusion agar slants at 4°C and transferred once a month. For comparison, organisms of other serotypes of mutans streptococci found in the human oral cavity were used in some experiments. These were *S. mutans* MT4245 (serotype e) and OMZ175 (serotype f) and *S. sobrinus* B13 (serotype d) and 6715 (serotype g).

Preparation of GTases and PAc. CF-GTase was purified from the culture supernatant of *S. mutans* MT8148R by 50% saturated ammonium sulfate precipitation, followed by chromatofocusing (36). CA-GTase was isolated from whole cells of *S. mutans* MT8148R as described previously (9). Surface protein antigen (PAc) of *S. mutans* MT8148R was purified chromatographically from the culture supernatant as described by Russell et al. (34) for determination of the immunological specificity of antibodies.

Immunization of hens. For in vitro studies and experiment I of experimental caries in rats (see below), 40 White Leghorn hens (18 weeks old) were used and divided into four groups. The hens in group 1 (10 hens) were immunized by intramuscular injection of an emulsified mixture (1 ml) of whole-cell suspension (10^9 cells per 0.5 ml) and 0.5 ml of Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, Mich.). A booster injection was administered 8 weeks after the initial injection. Group 2 hens (10 hens) were similarly immunized and boosted with an emulsified mixture (1 ml) of CF-GTase (0.4 mg/0.5 ml) and FCA (0.5 ml), while group 3 hens were immunized with an emulsified mixture of CA-GTase (0.4 mg/0.5 ml) and FCA (0.5 ml). Group 4 hens (10 hens) were sham-immunized with an emulsion of saline (0.5 ml) and FCA (0.5 ml). Eggs were collected and stored at 4°C until processed. In addition, five hens were injected intramuscularly with an emulsion (1 ml) of PAc (0.3 mg/0.5 ml) and FCA (0.5 ml), mainly for in vitro studies. For the caries study with rats (see below), a total of 1,160 hens (18 weeks old) were immunized intramuscularly with CA-GTase (0.4 mg/0.5 ml) emulsified with an equal volume (0.5 ml) of FCA. Two booster injections of the emulsions were done intramuscularly 7 and 13 weeks after the initial immunization. Eggs were collected and kept at 4°C until use.

Preparation of antibodies from egg yolks. In experiment I, yAb was prepared by the modified method of Aulisio and Shelokov (1). In brief, egg yolks were separated from the eggs of immunized or sham-immunized hens, mixed with an equal volume of saline and 2 volumes of chloroform, and incubated at 20°C for 30 min. The mixture was then centrifuged at 2,000 × *g* for 10 min at 20°C, and the supernatant, i.e., the water-soluble fraction (WSF), was separated and lyophilized. The WSF was calculated to be 10 to 12% pure yIgG by densitometric analysis (2222 Ultroscan; Pharmacia-LKB Biotechnology, Uppsala, Sweden) of the protein profiles after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the WSF preparations (12). The WSF was used as a source for further purification of the antibodies and for the caries immunization study in rats. Purified antibodies were obtained by one-third-saturated ammonium sulfate precipitation of WSF and DEAE-Sephacel column chromatography. Fractions possessing antibody activity were obtained as a single protein peak by linear gradient elution with NaCl (0.05 to 0.3 M). These purified yIgG preparations were used for in vitro experiments.

In experiment II, yAb was prepared by the ethanol precipitation method as follows. Collected yolks were diluted with 8 volumes of distilled water, mixed, and kept at 10°C for 4 h. The upper layer of the mixture was transferred to a container. Chilled ethanol (−20°C) was added to the solution to a final ethanol concentration of 60% (vol/vol) and mixed by stirring for 30 min at 4°C. The resultant precipitate was removed by centrifugation at 12,000 × *g* at 4°C, dissolved in 30 mM NaCl solution, and cleared by filtration through filter paper (no. 2; Advantec Toyo, Tokyo, Japan) to obtain the water-soluble protein fraction containing antibodies. This was further purified by ethanol precipitation; 50% ethanol was added to the soluble protein fraction to a final ethanol concentration of 30% (vol/vol) and stirred for 30 min at 4°C. The resultant precipitate was collected by centrifugation and dissolved in 30 mM NaCl solution at a concentration of 2 mg of protein per ml. To purify the antibodies further, the above solution was adjusted to pH 7.4 by adding 0.5 M Na_2HPO_4 solution and then by adding 50% ethanol to give a final ethanol concentration of 25%. The mixture was incubated for 30 min at 4°C and centrifuged at 12,000 × *g* at 4°C. The precipitate, dissolved again in 30 mM NaCl solution, was found to give a single protein peak on DEAE-Sephacel and Sephacryl S-300 chromatography and was strongly reactive with rabbit anti-chicken IgG, forming a single precipitin band in an agar immunodiffusion assay. Thus, it was concluded that the purified antibodies from hen egg yolks were composed of IgG, and the preparation was designated yIgG thereafter. For the in vivo study in rats, the yIgG solution in 30 mM NaCl solution was lyophilized.

SDS-PAGE and Western immunoblotting. SDS-PAGE was carried out as described by Laemmli (20). Protein specimens were treated at 100°C for 5 min in 50 mM Tris-HCl (pH 6.8) containing 2% SDS, 1% 2-mercaptoethanol, 20% glycerol, and 0.01% bromphenol blue. 2-Mercaptoethanol was omitted when the specimens were treated for SDS-PAGE in nonreducing gels. The reduced specimens were electrophoresed on a 12.5% polyacrylamide gel, and the nonreduced specimens were done similarly with a 7.5% gel. The gels were immersed in Coomassie brilliant blue R-250 for visualization of protein bands.

To determine the immunological specificity of yIgG, the concentrate of culture supernatant of *S. mutans* MT8148R and the 8 M urea extract of whole cells of *S. mutans* were electrophoresed in SDS-PAGE, and electrophoretic transfer (160 mA at 4°C for 2 h) of proteins from the SDS-PAGE gels onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass.) was carried out (24). The membranes were reacted in a solution of yIgG at a concentration of 5 μg/ml. The antibody bound to a protein band(s) was detected by a solid-phase immunoassay with horseradish peroxidase-conjugated rabbit anti-chicken IgG (1:1,000 dilution; Cappel Laboratories, Cochranville, Pa.) for 1 h. The membrane was rinsed in phosphate-buffered saline (PBS; pH 7.4)–0.02% Tween solution and developed in 12 mM Tris-HCl (pH 8.0) containing 0.05% 4-chloro-1-naphthol and 0.012% H_2O_2 for 5 min at 20°C. The membranes visualized were washed with a destaining solution (7.5% acetic acid, 25% ethanol).

ELISA. The immunological specificity of yAbs elaborated against whole cells, CA-GTase, CF-GTase, and PAc was examined by enzyme-linked immunosorbent assay (ELISA) as described previously (9). In brief, wells of microtiter plates were coated with 100 μl of antigen solution appropri-

ately diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed, and 150 μl of PBS (pH 7.4) containing bovine serum albumin (30 mg/ml) was added to the wells in order to block the uncoated surface. Then, the wells were reacted with serially diluted yIgG solution for 1 h at 37°C and washed extensively. Peroxidase-labeled rabbit anti-chicken IgG (heavy- and light-chain specific; Cappel Laboratories, Cochranville, Pa.) antibody was added, and the plates were incubated for 30 min at 25°C. After the wells were washed, *o*-phenylendiamine and 0.02% H_2O_2 in citrate-phosphate buffer (pH 4.8; 100 μl per well) was added. The reaction was stopped after 20 min with 3 N H_2SO_4 (100 μl per well), and the intensity of color developed was measured at 492 nm with a microplate reader (model MTP-32; Corona Electric Co., Katsuta, Japan). The ELISA titer was defined as the dilution giving an A_{492} of 0.2.

Agglutination of *S. mutans* cells by yIgG. yAbs to *S. mutans* antigens were titrated in terms of whole-cell agglutination. Formalin (0.3%)-treated whole cells were suspended in saline (2 mg [dry weight] per ml). The cell suspension (50 μl) was mixed with an equal volume of twofold-diluted yIgG and incubated for 2 h at 37°C and then overnight at 4°C, and agglutination was determined visually. The antibody titer was expressed as the minimum concentration of yIgG in the reaction mixture that gave positive agglutination.

Effect of yIgG on GTase- and sucrose-dependent cell adherence. To assess the effect of yIgG to *S. mutans* antigens on CA-GTase activity, a reaction mixture containing CA-GTase (2 mU) in 0.1 M sodium phosphate buffer (pH 6.8) and yIgG (0 to 1 mg/ml) was preincubated for 30 min at 37°C, and sucrose was added to a 1% (final) concentration and incubated for 2 h at 37°C. The amount of insoluble glucan was quantitated turbidimetrically by measuring the OD_{550}. The effect of yIgG on CF-GTase was also determined by the method of Koga et al. (17) by quantitating the radioactivity of the [^{14}C]glucose residues into methanol-insoluble material from [^{14}C]glucose-labeled sucrose (New England Nuclear Corp., Boston, Mass.), with preincubation of CF-GTase and yIgG to *S. mutans* antigens.

S. mutans MT8148R was grown at 37°C at a 30° angle to the horizontal for 18 h in 3 ml of brain-heart infusion broth containing 1% sucrose and filter-sterilized yIgG (0 to 1 mg/ml). The number of adherent cells was determined turbidimetrically and expressed as a percentage of the total cell mass (18).

Cariogenicity. The ability of *S. mutans* MT8148R to induce dental caries in specific-pathogen-free Sprague-Dawley rats fed diet no. 2000 with or without yIgG was determined as described previously (27). Accumulation of dental plaque was evaluated according to the description of Regolati and Hotz (31). The caries lesions were scored by the modification by Ooshima et al. (27) of the method of Keyes (16). Statistical analysis of the results was done by the Student *t* test.

RESULTS

We found that immunoglobulins were purified from egg yolks to a single peak of protein by saline-chloroform extraction followed by ammonium sulfate precipitation and DEAE-Sephacel chromatography. The class of immunoglobulin was identified as IgG (yIgG). yIgG was also purified by the differential ethanol precipitation method. SDS-PAGE of yIgG indicated that purified yIgG gave a single protein band of 220 kDa (Fig. 1). Chicken serum IgG gave a protein band of the same molecular mass, 220 kDa, while rabbit serum IgG, used as a reference, gave a 165-kDa band. When yIgG was reduced by 2-mercaptoethanol treatment, a heavy 76-kDa and a light 28-kDa band appeared (Fig. 1). These bands were considered to be IgG heavy and light chains, respectively.

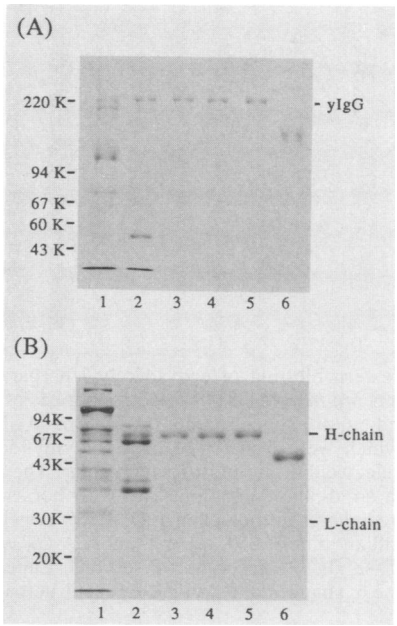

FIG. 1. SDS-PAGE of egg yolk IgG. (A) Nonreducing gel. Protein samples were prepared in the absence of 2-mercaptoethanol. Lane 1, yolk (4.5 μg); lane 2, WSF (3 μg); lane 3, column-purified yIgG (anti-CA-GTase, 0.75 μg); lane 4, ethanol-purified yIgG (anti-CA-GTase, 0.75 μg); lane 5, purified chicken serum IgG (0.75 μg); lane 6, purified rabbit serum IgG (0.75 μg). (B) Reducing gel. Protein samples were prepared in the presence of 2-mercaptoethanol. Lane 1, yolk (6 μg); lane 2, WSF (4 μg); lane 3, column-purified yIgG (anti-CA-GTase, 1 μg); lane 4, ethanol-purified yIgG (anti-CA-GTase, 1 μg); lane 5, purified chicken serum IgG (1 μg); lane 6, purified rabbit serum IgG (1 μg). Heavy (H) and light (L) chains are indicated. Sizes are shown in kilodaltons.

The immunological specificity of yIgG preparations was assessed by ELISA. Table 1 shows that yIgG to CA-GTase reacted strongly with the homologous antigen, CA-GTase, as well as with whole cells of *S. mutans* MT8148R but not with PAc. yIgG to PAc was found to be reactive with PAc and whole cells but not with CA-GTase. yIgG to CF-GTase reacted specifically with CF-GTase, while yIgG to whole cells gave a marked reaction with whole cells, CA-GTase, and PAc, all of which are cell associated. yIgG from sham-

TABLE 1. ELISA of various antigens of *S. mutans* MT8148R with yIgG to *S. mutans* antigens

yIgG	ELISA titer[a] (10^4)			
	CA-GTase	CF-GTase	Whole cells	PAc
CA-GTase	7.55	0.32	2.09	0.36
CF-GTase	0.30	2.79	0.12	0.08
Whole cells	2.40	0.45	4.67	1.51
PAc	0.09	0.23	1.24	3.06
Sham-immune	NR[b]	NR	NR	NR

[a] ELISA titer was defined as the dilution giving an A_{492} of 0.2.
[b] NR, not reactive.

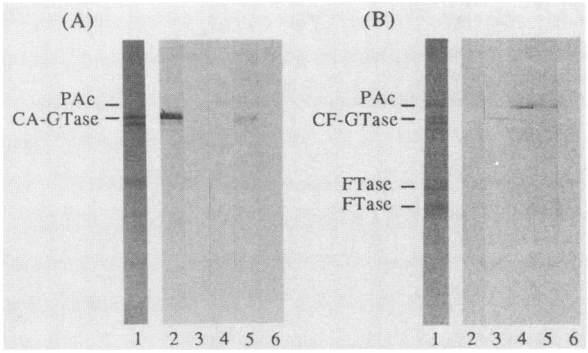

FIG. 2. Western blotting of egg yolk IgG preparations against antigens from *S. mutans* MT 8148. Electrophoresis of the 8 M urea extract (A) and culture supernatant (B) was done in SDS-polyacrylamide gels, which were transferred to a polyvinylidene difluoride sheet by the electrophoretic blotting technique. The antibody was detected by a solid-phase immunoassay with horseradish peroxidase-conjugated rabbit anti-chicken IgG. Lane 1, gel stained with Coomassie brilliant blue R-250. Lane 2, anti-CA-GTase yIgG. Lane 3, anti-CF-GTase yIgG. Lane 4, anti-PAc yIgG. Lane 5, anti-whole cell yIgG. Lane 6, sham-immune yIgG. FTase, fructosyltransferase.

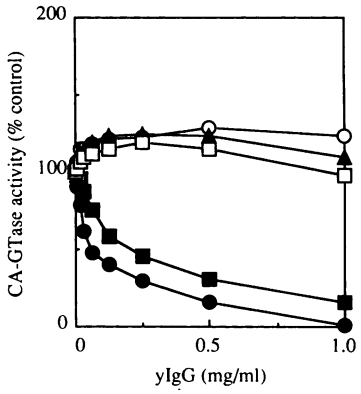

FIG. 3. Inhibitory effects of yIgG on glucan synthesis by *S. mutans* CA-GTase. yIgG to CA-GTase (●), CF-GTase (▲), PAc (□), and whole cells (■) and sham-immune yIgG (○) were preincubated with CA-GTase for 30 min at 37°C, after which sucrose substrate was added. The activities are expressed relative to the activity in the absence of yIgG.

immunized hens did not react with CA-GTase, CF-GTase, whole cells, or PAc.

Western blotting clearly shows that yIgG to CA-GTase recognized a component with a relative molecular mass of 156 kDa (i.e., CA-GTase) in the 8 M urea extract of whole cells, while yIgG to CF-GTase reacted with a 156-kDa (i.e., CF-GTase) component in the culture supernatant. On the other hand, yIgG to *S. mutans* whole cells bound to a 185-kDa component (i.e., PAc) found in the culture supernatant and to CA-GTase and PAc in the 8 M urea extract of whole cells (Fig. 2). yIgG to whole cells, PAc, and CA-GTase was found to agglutinate whole cells of *S. mutans* but not *S. sobrinus*, while yIgG to CF-GTase did not agglutinate the cells (Table 2). The minimum agglutinating concentration was considerably different and was lowest for yIgG to whole cells and PAc and highest for yIgG to CA-GTase.

yIgG to CA-GTase and whole cells markedly inhibited CA-GTase activity, resulting in reduced synthesis of insoluble glucan from sucrose (Fig. 3). On the other hand, CF-GTase was inhibited by yIgG to CF-GTase only. yIgG to other antigens or sham-immunized yIgG instead increased the synthesis of glucan from sucrose by CF-GTase (Fig. 4).

As shown in Table 3, yIgG to CA-GTase and whole cells inhibited the adherence of cells of *S. mutans* to the glass surface in the presence of sucrose. However, yIgG to CF-GTase and PAc and yIgG from sham-immunized hens did not have any significant effect on sucrose-dependent cell adherence to the glass surface in vitro. It was noted that yIgG to whole cells at lower concentrations enhanced cell adherence but inhibited adherence at higher concentrations. Only yIgG to CA-GTase gradually inhibited cell adherence as the concentration of yIgG in the reaction mixture increased.

In the experimental dental caries model with *S. mutans*-infected rats fed diet 2000, WSF yIgG to CA-GTase significantly inhibited plaque accumulation and caries development compared with the control group that was given no yIgG (Table 4). yIgG to CF-GTase or whole cells or sham-immune yIgG did not inhibit plaque formation and caries development. In the second series of rat experiments, increasing concentrations (0 to 1%) of yIgG to CA-GTase were added to diet 2000 and the anticaries effect of the yIgG was examined. Statistically significant reductions in both plaque accumulation and caries development were noted when the yIgG was incorporated at concentrations of ≥0.1% (wt/wt)

TABLE 2. Minimum agglutinating concentration of yIgG against whole cells of mutans streptococci

yIgG	Min agglutinating concn (mg/ml)				
	S. mutans			*S. sobrinus*	
	MT8148 (c)	MT4245 (e)	OMZ175 (f)	B13 (d)	6715 (g)
CA-GTase	1.00	1.00	1.00	NA	NA
CF-GTase	NA[a]	NA	NA	NA	NA
Whole cells	0.125	0.25	0.50	NA	NA
PAc	0.125	0.25	0.50	NA	NA
Sham-immune	NA	NA	NA	NA	NA

[a] NA, not agglutinated by yIgG tested up to a concentration of 2.0 mg/ml of reaction mixture.

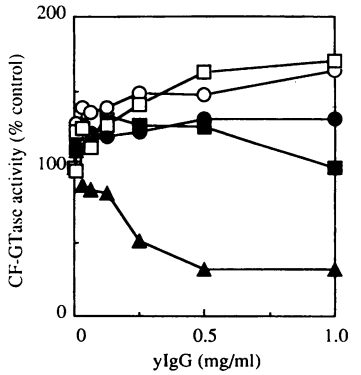

FIG. 4. Inhibitory effects of yIgG on glucan synthesis by *S. mutans* CF-GTase. yIgG to CA-GTase (●), CF-GTase (▲), PAc (□), and whole cells (■) and sham-immune yIgG (○) were preincubated with CF-GTase for 30 min at 37°C, after which [^{14}C]sucrose substrate was added. The activities are expressed relative to the activity in the absence of yIgG.

TABLE 3. Inhibitory effect of egg yIgG on sucrose-dependent adherence of *S. mutans* MT8148R

yIgG	Concn (mg/ml)	% Adherence (mean ± SD)	
		Loose	Firm
None (control)		82.4 ± 1.6	72.7 ± 1.2
CA-GTase	0.2	76.7 ± 0.3	63.7 ± 0.9
	0.4	77.3 ± 1.2	52.8 ± 2.8
	0.6	67.4 ± 6.4	26.2 ± 7.6
	0.8	55.8 ± 4.7	17.4 ± 3.4
	1.0	32.4 ± 5.2	5.2 ± 0.2
CF-GTase	0.2	88.7 ± 0.7	77.8 ± 1.5
	0.4	85.5 ± 1.1	73.8 ± 3.0
	1.0	79.6 ± 1.9	55.1 ± 2.8
PAc	0.2	89.8 ± 0.4	81.4 ± 1.2
	0.4	88.7 ± 0.9	81.4 ± 0.7
	1.0	85.5 ± 1.1	73.8 ± 3.0
Whole cells	0.2	93.0 ± 0.2	86.1 ± 0.1
	0.4	82.0 ± 1.9	48.7 ± 8.2
	0.6	71.6 ± 3.2	20.1 ± 3.4
	0.8	50.9 ± 2.5	11.4 ± 1.2
	1.0	19.1 ± 3.6	1.8 ± 0.2
Sham-immune	0.2	79.2 ± 1.2	69.3 ± 0.8
	0.4	76.8 ± 1.5	67.1 ± 2.0
	1.0	70.7 ± 2.0	61.7 ± 1.9

in diet 2000 compared with control group A. Inhibition rates increased gradually as the concentration of yIgG in the diet was increased up to 1%. When the caries score of the smooth surface of the rat molars was evaluated specifically, the inhibitory effect of yIgG became more evident (Table 5).

DISCUSSION

The principal strategy in this study was to inhibit the function of the GTase system of *S. mutans* by use of specific antibodies to enzyme protein(s) and to achieve suppression of experimental dental caries in rats. Our previous studies clearly indicate that the GTase system of *S. mutans*, by promoting firm cell adherence to the tooth surface in the presence of sucrose, is a major virulence factor causing dental caries (17, 19). We have found that among the GTases produced by *S. mutans*, CA-GTase is the most important (9). It synthesizes water-insoluble glucan, which appears to be essential for sucrose-dependent adherence of this organism. CA-GTase can be extracted with 8 M urea from cultured whole cells and purified to a 156-kDa protein (9). We tried to

TABLE 4. Effect of immune yIgG WSF preparations on plaque deposition and caries development in specific-pathogen-free rats infected with *S. mutans* MT8148R

Rat group	yIgG WSF (concn [%])	Plaque index (mean ± SD)	Caries score (mean ± SD)
A	None (control)	0.66 ± 0.22	55.8 ± 23.0
B	CA-GTase (0.8)	0.49 ± 0.08[a]	32.5 ± 8.2[b]
C	CF-GTase (0.8)	0.62 ± 0.22	53.0 ± 12.2
D	Whole cells (0.8)	0.53 ± 0.11	42.6 ± 10.9
E	Sham-immune (0.8)	0.54 ± 0.09	47.0 ± 9.4

[a] $P < 0.05$ (Student t test) versus group A.
[b] $P < 0.01$ versus group A.

TABLE 5. Inhibitory effect of purified yIgG to CA-GTase on plaque deposition and caries development in specific-pathogen-free rats infected with *S. mutans* MT8148R

Rat group	Concn (%) of yIgG to CA GTase	Plaque index (mean ± SD)	Total caries score (mean ± SD)	Smooth surface caries score (mean ± SD)
A	0	0.69 ± 0.15	54.9 ± 15.8	8.8 ± 4.3
B	0.025	0.64 ± 0.17	50.4 ± 14.2	7.1 ± 4.2
C	0.05	0.59 ± 0.14	50.3 ± 12.7	8.0 ± 3.3
D	0.1	0.41 ± 0.10[a]	40.4 ± 8.2[b]	4.9 ± 2.5[b]
E	0.25	0.49 ± 0.09[a]	36.4 ± 6.3[c]	4.5 ± 1.3[c]
F	0.5	0.47 ± 0.11[a]	39.3 ± 11.5[b]	5.0 ± 2.5[b]
G	1.0	0.32 ± 0.10[a]	30.6 ± 4.8[a]	3.5 ± 1.5[a]

[a] $P < 0.001$ versus group A.
[b] $P < 0.05$ versus group A.
[c] $P < 0.01$ versus group A.

use this GTase protein as a vaccine for immunizing hens and found that the yolks of their eggs contained high-titered yIgG specific for CA-GTase. It is well known that antibodies generated against GTase proteins effectively inactivate GTase activities (11, 38). The yIgG purified from the egg yolks exhibited strong inhibitory activity against CA-GTase but not against CF-GTase. Conversely, the yIgG against CF-GTase was found to inactivate CF-GTase but not CA-GTase. These findings showed that the immunological specificities of CF-GTase and CA-GTase were completely different.

It is of interest that yIgG specific for CA-GTase but not CF-GTase significantly inhibited the development of dental caries in rats that had been infected with serotype c *S. mutans*. This finding was supported by the in vitro experiments showing that yIgG to CA-GTase strongly suppressed the cellular adherence of *S. mutans* MT8148R to the glass surface, while yIgG to CF-GTase caused only a slight inhibition in cell adherence. The yIgG to whole cells of *S. mutans* MT8148 resulted in some but not significant decreases in caries development in rats. The yIgG to whole cells was found to contain antibodies to CA-GTase, PAc, serotype carbohydrate antigen, and lipoteichoic acids; it strongly induced heavy aggregation of the whole-cell suspension, and it inhibited the enzymatic activity of CA-GTase but not that of CF-GTase. In this regard, Otake et al. (28) have recently reported that passive oral administration of their crude yAb to *S. mutans* whole cells exhibited protection against *S. mutans*-induced dental caries in rats. However, the immunological specificities of the whole-cell yAb were not given in their report. Their results are not coincident with ours in terms of the anticaries effect of the yIgG. This discrepancy may be due to the differences in the specificities of antibodies elaborated by immunization with whole cells. This means that immunization with complex antigens, such as whole bacterial cells, should give rise to variable antibodies, and it is difficult to control the repertoire of antibodies required. On the other hand, yIgG to CA-GTase showed more effective anticaries activity in rats than did yIgG to whole cells. Furthermore, our pilot experiment has indicated that yIgG prepared against PAc exhibited some caries-inhibitory activity, but the degree of caries inhibition was less than that with yIgG to CA-GTase (results not shown).

It has been reported that the sera of rabbits immunized with *S. mutans* or other streptococcal species exhibited positive immunofluorescent staining on human heart tissue, and several streptococcal antigens have been proposed to be

cross-reactive with heart antigens, including antigen I/II (PAc) and cell membrane proteins (7, 14, 33, 40). Thus, careful immunological scrutinies should be done in order to eliminate the possibility of cross-reaction of antibodies with mammalian tissues, especially of the heart. Preliminary experiments indicated that yIgG to CA-GTase did not stain sarcolemmal tissues by immunofluorescent microscopy (results not shown).

Livetins α, β, and γ had been termed the water-soluble proteins in egg yolks. Williams (41) then found that γ-livetin was identical with serum gamma globulin, i.e., IgG. It was further elucidated that hen egg yolk contained IgG almost exclusively, while IgA and IgM were selectively located in egg white (32). Since the content of yIgG recovered from egg yolk was usually about 100 mg, eggs from hyperimmunized hens may provide a convenient and economical source of antibodies for passive immunization (39, 42, 43). Another advantage of yIgG is that collecting eggs from laying hens does not require the bleeding of animals for antiserum production, which is especially suited to current regulations for experimental animal protection.

yIgG has been purified by several methods, with polyethylene glycol (29, 30), dextran sulfate (15), organic solvent (2), hydrophobic interaction (12), and carrageenan (13). In this study, we have devised a new method for large-scale production of yIgG by fractional ethanol precipitation. As shown in Fig. 1, the yIgG obtained gave a homogeneous 220-kDa protein in SDS-PAGE, which was dissociated into 76-kDa heavy and 28-kDa light chains after treatment with 2-mercaptoethanol, as was reported previously (37). It was clearly shown that the molecular mass of yIgG was larger than that of IgG from mammalian species, such as humans and rabbits.

In conclusion, yIgG specific for *S. mutans* CA-GTase specifically inhibited the enzymatic activity which produces insoluble glucan from sucrose, resulting in the inhibition of cellular adherence and decreased cariogenicity in *S. mutans*-infected rats. High-titered yIgG could be obtained conveniently and stably from eggs of hens hyperimmunized with purified CA-GTase and an adjuvant.

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