Local and Systemic Antibody Response to Oral Administration of Glucosyltransferase Antigen Complex

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The salivary and serum immune responses to orally administered glucosyltransferase antigen complex from *Streptococcus mutans* strain 6715 were investigated in hamsters. An enzyme-linked immunosorbent assay was used to measure the antibody quantity and isotype, and a [^{14}C]glucosyl-labeled sucrose incorporation assay was used to measure functional inhibition of the enzyme. A total of 21 to 27 daily doses of antigen administered in hamster oral cavities elicited salivary immunoglobulin G and immunoglobulin A antibody responses and functional inhibitory activity. The salivary response increased throughout the immunization procedure, and the amount of salivary antibody was dependent upon the dose of antigen given. The salivary response to a second oral administration of antigen for 4 days showed some features of anamnesis. The response after a second antigen administration was detected sooner than the primary response, and somewhat higher levels of antibody and inhibitory activity were observed. Serum antibody (immunoglobulin G and immunoglobulin M) and functional inhibitory responses were also elicited by oral administration of the soluble enzyme antigen. These responses were lower than responses induced by local injections of antigen in complete Freund adjuvant. The ability to evoke a salivary immune response to the glucosyltransferase antigen complex may increase the potential of using this antigen in an effective caries vaccine.

Enteric vaccines have been investigated recently for their ability to induce a protective response against a variety of bacterial infections of mucosal surfaces (18, 20, 29). Administration of antigen by this route has been considered a possible mechanism to stimulate an immune response to protect the teeth, which are bathed by mucosal secretions. Experiments in rodent models have shown that dental caries caused by *Streptococcus mutans* can be reduced significantly by feeding either whole cells (14, 15) or soluble antigens (27). Preliminary studies in humans suggest that a salivary immune response can be elicited by feeding killed *S. mutans* cells in enteric capsules (13). However, little is known of the characteristics of the salivary and serum immune responses to oral administration of isolated antigens derived from this organism. The glucosyltransferase (GTF) antigen complex (26) of *S. mutans* is particularly interesting because the products of this constitutively synthesized enzyme have been implicated in dental plaque formation by *S. mutans* (8). In addition, injection or feeding of these antigens has been shown to diminish the extent of disease caused by *S. mutans* in rodents (25, 27, 30). Since antibody directed to antigens of the GTF complex may be protective, an understanding of the kinetics of antibody appearance in the saliva, the effect of

dose of this antigen on the amount of antibody elicited, and the nature of the secondary response to orally administered GTF may contribute to the development of a dental caries vaccine. Therefore, we investigated several of the characteristics of the primary and secondary salivary and serum immune responses after oral administration of the GTF antigen complex.

MATERIALS AND METHODS

Animals. NIH white, acromelanic hamsters were bred and raised at the Forsyth Dental Center. LHC/Lak cream hamsters (Charles RiverLakeview) were also used in this study because of the limited availability of the NIH white hamsters for all experiments. These two hamster strains show similar levels of salivary and serum antibodies to GTF after local immunization with GTF in adjuvant (unpublished data). All animals were individually caged and maintained on a pelleted diet (Purina Mouse Chow; Ralston Purina Co.) unless otherwise indicated. No *S. mutans* was observed on mitis salivarius agar plates after swabbing the dental surfaces of these two strains of hamsters while these animals were housed at the Forsyth Dental Center.

Antigen preparation. GTF for immunization and antibody analyses was prepared as previously described (26). Briefly, *S. mutans* strain 6715 was grown anaerobically in 10% CO_2 -90% N_2 for 24 h at 37°C in 6 to 10 liters of chemically defined medium. A cell-free supernatant, which was obtained by centrifugation

(13,000 $\times$ g), was brought to pH 6.5 with 1 N NaOH. Water-insoluble polysaccharide was then synthesized by incubation of the GTF-containing supernatant with 10% sucrose for 48 h at 37°C. Bacterial growth was inhibited by the addition of 0.02% sodium azide. The water-insoluble polysaccharide which formed was collected by centrifugation at 13,000 $\times$ g and then washed extensively with cold distilled water and 0.01 M sodium phosphate (pH 6.8) to which 0.02% sodium azide had been added. GTF was then eluted from the washed, water-insoluble polysaccharide by 1 h of incubation at 4°C with an equal volume of 6 M guanidine hydrochloride. After elution, guanidine was removed by dialysis, and the enzyme was concentrated by ultrafiltration. Enzymatic activity was determined by the Somogyi (28) and Glucostat (Worthington Biochemicals Corp.) assays, and protein was measured by the assay of Lowry et al. (11). Approximately 40% of the enzymatic activity which was present in the culture supernatant was recoverable after guanidine elution and dialysis (26). Fructose was the principal sugar released (78%) after incubation of the enzyme preparation with sucrose for 2 h at 37°C. As previously described, the guanidine-eluted preparations contain GTF, glucan, nonenzyme protein bound to glucan, and low-molecular-weight material. This type of antigen was used in all experiments. Antigen for use in the assays for antibody or enzyme-inhibiting activity was prepared by gel filtration on a column of 8% agarose (Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.01 M sodium phosphate, pH 6.8. Column fractions were monitored for protein spectrophotometrically at 280 nm and for enzymatic activity by the chemical assays described above. GTF activity was eluted in the column void volume. Fractions containing GTF activity were pooled and concentrated for use as antigen in the assays for antibody and enzyme-inhibiting activity. The gel filtration procedure eliminates the low-molecular-weight material. No significant invertase activity was detected in the column fractions. Glucose repre-

sented more than 97% of the labeled carbohydrate in the ethanol-insoluble polysaccharide synthesized from sucrose by GTF eluted from water-insoluble polysaccharide of *S. mutans* 6715 when [U - 14 C]sucrose and [fructose-1- 3 H(N)]sucrose incorporation assays were used (26). All guanidine-eluted GTF preparations formed both water-insoluble and water-soluble polysaccharide when incubated with 0.125 M sucrose for 4 h at 37°C.

Antigen administration protocol. Figure 1 shows the immunization protocols for all of the experiments. In the first experiment (experiment A), 23-day-old LHC/Lak cream hamsters were placed into the following two groups: (i) animals sham immunized orally with 0.2 ml of 0.01 M sodium phosphate buffer ($n = 14$) and (ii) animals immunized orally with 0.2 ml of GTF in phosphate buffer ($n = 14$). Antigen or buffer was administered daily for 27 consecutive days into the oral cavity with an automatic 0.2-ml pipette. Each dose of antigen contained 0.9 IU of enzyme activity in 400 μ g of protein. A total of 24.3 IU of enzyme activity in 10.8 mg of protein was administered during the 27-day immunization period. Hamsters in this experiment were bled and salivated at 10, 16, 22, 28, and 70 days after oral immunization was begun. Hamsters in groups i and ii were infected 30 days after the initial oral administration with approximately 10^8 colony-forming units of a cariogenic streptomycin-resistant strain of *S. mutans* 6715. The purpose of this infection was a subsequent study on the effect of oral immunization on *S. mutans* infections. At this time all animals were placed on cariogenic diet 2000 (10). Infections were confirmed in all animals of groups i and ii by swabbing and plating on mitis salivarius agar containing 200 mg of streptomycin sulfate per ml.

In the second experiment (experiment B), 24-day-old LHC/Lak cream hamsters ($n = 28$) were placed into two equal groups as in experiment A. Antigen or buffer was administered orally daily for 26 consecutive days. Each dose of antigen contained 0.25 IU in 250

FIG. 1. Immunization protocols for oral administration or injection of GTF antigen complex in hamsters. Orally administered antigen or buffer was given via a 0.2-ml pipette once a day for the times indicated. Injected antigen was given subcutaneously in the salivary gland vicinity in complete Freund adjuvant. The enzymatic activity (in international units [IU]) and protein content (in micrograms) of each single dose of antigen are shown. The times (weeks or months) after initiation of immunization are shown on the abscissa. Hamsters in experiment A were also infected with *S. mutans* 6715 between weeks 4 and 10.

μ g of protein. A total of 6.5 mg of protein was administered to each hamster during the immunization regimen. Hamsters were bled and salivated as previously described (30) at 1 day after the final daily dose of antigen.

In a third experiment (experiment C), 24-day-old white hamsters ($n = 28$) were separated into two equal groups as in experiment A. Antigen or buffer was administered orally daily for 21 consecutive days. Each dose contained 0.2 IU in 200 μ g of protein. A total of 4.2 IU of enzyme activity in 4.2 mg of protein was administered to each hamster during the immunization regimen. Hamsters were bled and salivated at 1 day after the final daily dose of antigen.

In a fourth experiment (experiment D), 24-day-old white hamsters were divided into the following two groups: (i) animals sham immunized orally with 0.2 ml of phosphate buffer ($n = 6$) and (ii) animals orally immunized with 0.2 ml of GTF in buffer ($n = 11$). Antigen or buffer was administered orally for 21 consecutive days. Each dose of antigen contained 0.14 IU of enzyme activity in 200 μ g of protein. A total of 4.2 mg of protein was administered during the 21-day period. All hamsters were bled and salivated at 7, 14, 22, 28, 35, and 42 days after the start of the immunization protocol. Those animals showing an immune response ($n = 3$) to this minimal antigen dose during this time were periodically bled and salivated during an additional 2.5 months. These hamsters were then orally immunized again with 0.2 ml of the same GTF preparation daily for 4 days. Salivas and sera were then periodically collected during an additional 4 months. At the end of the 4 months, hamsters ($n = 7$) which had not shown a response to either the primary or secondary antigenic challenge were injected with 0.1 IU of GTF enzyme activity in 75 μ g of protein in complete Freund adjuvant once in the salivary gland vicinity. Five untreated hamsters of approximately the same age were also injected once. Hamsters were bled and salivated at 7 days after the injection.

In a fifth experiment (experiment E), 29-day-old LHC/Lak cream hamsters were injected with 0.1 ml containing 0.23 IU of enzyme activity in 47 μ g of protein in 0.1 ml of complete Freund adjuvant. Antigen was injected into the vicinity of the parotid and submandibular glands once weekly for 4 weeks. Hamsters were bled and salivated at 6 and 47 days after the final injection.

Saliva and serum. Salivas and sera were collected and treated before the antibody assay, as previously described (30). In addition, salivas to be used in the inhibition of [14 C]glucose incorporation assays were dialyzed, first against phosphate-buffered saline containing 0.001 M ethylenediaminetetraacetate and then against phosphate buffer. All samples were stored at -20°C until antibody analysis.

Assay for inhibition of GTF activity. The procedure for determining inhibition of GTF activity by serum and saliva is a modification (24) of the method of Evans and Genco (7). This assay reflects the total amount of glucan synthesized by GTF. GTF activity was determined by [14 C]glucose incorporation from glucosyl-labeled sucrose into ethanol-insoluble polysaccharide. Additional modifications were incorporated into the assay to facilitate pipetting, increase

reproducibility, and decrease variability resulting from low protein concentrations (manuscript in preparation). In our assay for the measurement of inhibiting activity in serum, 5 μ l of serum was preincubated with 0.80 mIU of GTF (guanidine eluted from *S. mutans* 6715) in 100 μ l of 0.02 M phosphate buffer (pH 6.8) for 1 h at 37°C in a shaking water bath. A total of 330 μ g of primer dextran T10 (Pharmacia Fine Chemicals) and 0.018 μ g of [14 C]glucose-labeled sucrose (specific activity, 275 mCi/mmol; approximately 13,000 cpm; New England Nuclear Corp.) in 0.2 ml of 0.01 M sodium phosphate (pH 6.8) were then added. This mixture was incubated at 37°C for 2 h, and the reaction was stopped by precipitation with ethanol (final concentration, 75% vol/vol). After centrifugation of the precipitate at $13,000 \times g$, the pellet was dissolved in distilled water and reprecipitated with ethanol. The centrifuged pellet was dissolved in 0.2 ml of distilled water and mixed with 4 ml of Ready-Solv Solution VI (Beckman Instruments Inc.) and counted in a liquid scintillation spectrometer (model LS-100C; Beckman). Under these conditions approximately 1,000 cpm of [14 C]glucose is incorporated into ethanol-insoluble polysaccharide in the presence of buffer. Salivary inhibiting activity was assayed by incubating 20 μ l of dialyzed, unconcentrated saliva with 20 μ l of enzyme (0.38 mIU of activity) and 10 μ l of 1% bovine serum albumin (23) (both in 0.01 M sodium phosphate, pH 6.8) for 1 h at 37°C in a shaking water bath. A total of 330 μ g of primer dextran and 0.018 μ g of [14 C]glucosyl-labeled sucrose were then added in 50 μ l of sodium phosphate buffer. Mixtures were incubated for 5 h, after which polysaccharide was precipitated, washed, and counted as described above for the serum assay.

The inhibiting ability of an immune serum or saliva sample was determined as follows. The counts per minute incorporated into precipitated polysaccharide by the enzyme in the presence of immune fluid was first expressed as a percentage of the counts per minute incorporated by the enzyme in the presence of the appropriate control fluid. This value subtracted from 100 gave the percent inhibition. This measure of the inhibitory activity of immune fluids was found to deviate from linearity at high antibody concentrations (unpublished data). Thus, to determine accurately the inhibitory capacity of a given serum or saliva over a range of antibody concentrations, standard curves were prepared, in which the percent inhibitions of serum and saliva standards were measured at several twofold dilutions. The percent inhibition versus the reciprocal dilution ($\log_{10}$) of the standards showed a linear relationship, with a correlation coefficient of >0.999 ; 50% inhibition in the saliva and serum assays was assigned a value of 100 inhibition units so that the inhibitory activities in the two fluids could be compared. The inhibitory capacities of experimental serum and saliva samples were determined from these standard curves by using the following equations: for serum, percent inhibition = $48.7(\log_{10} \text{inhibition units}) - 45.9$; and for saliva, percent inhibition = $38.9(\log_{10} \text{inhibition units}) - 27.9$. The values from these equations were multiplied by the appropriate dilution factor.

Experimental sera were tested at 10- to 200-fold dilutions, depending on the level of inhibition found in

these sera. At these dilutions enzyme in the presence of normal serum incorporated 0 to 10% more radioactivity into polysaccharide than did enzyme in the presence of buffer. Experimental salivas were tested undiluted. Enzyme in the presence of undiluted, dialyzed normal saliva incorporated approximately 35% fewer counts per minute into polysaccharide than did enzyme in the presence of buffer. Therefore, little or no stimulation of enzyme activity was seen in these assays.

Antisera. Antisera specific for hamster gamma chains were obtained by injecting rabbits with hamster immunoglobulin G (IgG) (the 0.005 M sodium phosphate, pH 8.0, eluate after diethylaminoethyl cellulose [DE52; Whatman] chromatography of hamster serum). Light chain activity was removed with an immunosorbent prepared by linkage of IgA-rich hamster intestinal perfusates (50% ammonium sulfate precipitates [1, 9]) to cyanogen bromide-activated Sepharose 4B. Rabbit antisera directed to hamster alpha or mu chains and prepared as described previously (1, 2) were provided by John Bienenstock, McMaster University, London, Ontario, Canada.

Antibody analysis. Antibody in saliva or serum was determined by a modification of the indirect enzyme-linked immunosorbent assay (ELISA) (5, 6). The GTF antigen used for this assay was the same as that used for oral immunization in the respective experiments. Antigen in 0.1 M sodium carbonate (pH 9.6) was added to wells of polystyrene microtiter plates (Linbro Scientific) and incubated for 3 h at 37°C. After subsequent incubation overnight at 4°C, plates were washed and then incubated with appropriate dilutions of hamster saliva or serum. The amount of bound antibody was determined by reaction with monospecific rabbit anti-hamster antisera directed against hamster gamma, alpha, or mu heavy chains. After 2 h of incubation and washing, plates were reacted with goat anti-rabbit IgG (Miles Laboratories) conjugated to alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.). After overnight incubation at room temperature, the substrate (*p*-nitrophenylphosphate; Sigma 104) was added, and the degree of reaction was determined spectrophotometrically at 400 nm. To determine relative differences in the concentrations of salivary IgG and IgA antibodies or serum IgM and IgG antibodies, the salivas or sera giving the highest absorbance at 400 nm (A_{400}) were serially diluted and retested in the ELISA assay by using the appropriate monospecific antisera. The resulting A_{400} values were plotted against the dilution ($\log_{10}$) to obtain standard curves for each immunoglobulin. Linear relationships were generally obtained with A_{400} and the reciprocal dilution ($\log_{10}$) between optical densities of 0.250 and 1.200 nm. The following A_{400} values from the most active fluids were designated as 100 ELISA units (EU): anti- γ chain (saliva), 0.475; anti- γ chain (serum), 1.054; anti- α chain (saliva), 1.117; and anti- μ chain (serum), 0.650. The relative amounts of immunoglobulin bound of the remaining sera or salivas were then compared with these standard curves by using the following equations: for anti- γ chain (saliva), $A_{400} = 0.423(\log_{10} \text{EU}) - 0.489$; for anti- γ chain (serum), $A_{400} = 0.749(\log_{10} \text{EU}) - 0.443$; for anti- α chain (saliva), $A_{400} = 1.04(\log_{10} \text{EU}) - 0.963$; and for anti- μ chain (serum), $A_{400} = 0.525(\log_{10} \text{EU}) - 0.400$.

RESULTS

Immune responses to oral administration of GTF-containing antigens. The development of salivary and serum immune responses was followed in hamsters given 27 daily doses of GTF from *S. mutans* 6715 perorally (experiment A). The salivary IgA antibody responses (Fig. 2A) in the hamsters given GTF were significantly higher than the responses of the control group (data not shown) at 16 days after immunization was begun and throughout the duration of the experimental period. The salivary IgA

FIG. 2. Relative amounts of antibody in salivas and sera of hamsters given 0.9 IU of GTF antigen complex perorally once a day for 27 days. Antibody activity is expressed as EU, which were obtained by using separate equations for each isotype (see text). Thus, identical EU for different isotypes do not necessarily represent equivalent amounts of antibody. (A) Open bars, Salivary IgG antibody from orally immunized hamsters (5 to 11 animals); cross-hatched bars, salivary IgG antibody from injected hamsters (3 animals); solid bars, salivary IgA antibody (5 to 11 animals). Brackets enclose two standard errors. The columns marked 70c indicate antibody levels of sham-immunized control hamsters. Salivas were tested at 1:10 dilutions. (B) Open bars, Serum IgG antibody from orally immunized hamsters (5 to 11 animals); broken bars, serum IgM antibody from orally immunized hamsters (5 to 11 animals); cross-hatched bars, serum responses of injected hamsters (4 animals). Brackets enclose two standard errors. The columns marked 70c indicate antibody levels of sham-immunized hamsters. Sera were tested at 1:400 dilutions.

antibody responses continued to increase throughout the immunization period. At 6 weeks after completion of the immunization protocol, the salivary IgG and IgA antibody responses were still detectable. Both the sham-immunized control group and the GTF-immunized group were infected with cariogenic *S. mutans* 6715 on day 30. However, salivary antibody activity in the infected control group was not significantly different than activity in saliva from noninfected, nonimmunized hamsters at either day 30 or 70 (less than 1 EU for either IgG or IgA). The salivary IgG responses of animals given four weekly local injections of GTF emulsified in complete Freund adjuvant (experiment E) were nearly eightfold lower (Fig. 2A) than the responses of perorally immunized hamsters immediately upon completion of the respective immunization protocols. The salivary IgG antibody levels decreased twofold by day 68 in injected animals. A comparison (oral versus injection) of the salivary IgA responses was prevented by insufficient amounts of material.

The serum responses to the oral administration of GTF antigens are shown in Fig. 2B. A low but significant antibody response was detected in the IgM and IgG isotypes of sera obtained from immunized hamsters 10 days after GTF antigen feeding was begun. The peak IgM antibody response occurred by day 16. IgG antibody continued to increase until day 22. The serum IgG response in the orally immunized hamsters then gradually declined during the 6-week infection period but remained elevated. No serum IgA antibody was detected at any sampling period. Serum IgG antibody responses in the control infected group that had received buffer orally and in the uninfected group (data not shown) did not differ significantly during the experiment. The sera of injected hamsters contained 5.7 times as much IgG antibody and 14.6 times as much IgM antibody as the sera of orally immunized hamsters immediately after the completion of the respective immunization protocols. Antibody of the IgG isotype showed a modest increase in the sera of injected hamsters on day 68, whereas the IgM antibody fell to 39% of the day 27 levels.

Salivary and serum responses of orally immunized and injected hamsters were also analyzed for their ability to inhibit GTF-mediated glucose incorporation into total polysaccharide (Table 1). Although insufficient volumes of hamster saliva were available to complete the assay on days 10 and 16, significant inhibitory activity was observed in the salivas of orally immunized hamsters by day 22. Inhibitory activity was highest in the day 28 sample and decreased 1.8-fold by day 70, which corresponds to the decrease in specific antibody determined by the ELISA (Fig.

TABLE 1. Comparison of GTF inhibitory activities in salivas and sera of hamsters administered GTF orally or by injection in the salivary gland vicinity in Freund complete adjuvant^a

Days after start of GTF administration	Salivary inhibitory activity (inhibition units) ^b		Serum inhibitory activity (inhibition units $\times 10^{-3}$) ^b	
	Oral	Salivary gland vicinity	Oral	Salivary gland vicinity
10			2.9 $\pm$ 0.6 ^c	ND ^d
16			15.5 $\pm$ 1.2	ND
22	22.9 $\pm$ 1.8	ND	18.5 $\pm$ 4.9	ND
28 (27) ^e	47.7 $\pm$ 3.7	14.0 $\pm$ 4.2	15.6 $\pm$ 5.1	43.2 $\pm$ 2.6
70 (68)	26.2 $\pm$ 4.4	7.8 $\pm$ 6.9	6.0 $\pm$ 0.7	49.5 $\pm$ 2.2
70 (sham)	1.7 $\pm$ 1.7		<1	

^a For oral administration each dose was 0.90 IU of enzyme activity in 400 μ g of protein (27 doses), and for salivary gland vicinity injection each dose was 0.23 IU of enzyme activity in 47 μ g of protein (4 doses).

^b Values for saliva and serum were independently derived (see text). However, the 100-inhibition unit level for salivas and sera represents 50% inhibition of enzyme activity. The values shown were adjusted for the differences in dilution required to achieve 50% inhibition.

^c Mean $\pm$ standard error.

^d ND, Not determined.

^e Numbers in parentheses refer to days after start of injection schedule.

2A). Also similar to the antibody data (Fig. 2A), the salivas of injected hamsters contained approximately 30% of the inhibitory activity observed in the salivas of orally immunized hamsters at either time period. Analyses of the sera from orally immunized hamsters for inhibitory activity supported the ELISA data. A serum response was detected on day 10 (Table 1). The increase and decrease in serum inhibition corresponded to the changes observed for antibody of the IgG isotype (Fig. 2B), as did the difference between serum inhibitory activities after oral versus subcutaneous antigen administration. Thus, oral administration of soluble GTF-containing antigens over a 4-week period was sufficient to give rise to salivary IgG and IgA antibodies, as well as to serum IgM and IgG antibodies, which were detectable by assays which measured either antibody or functional inhibition of enzyme activity.

Effect of dose on immune response to orally administered antigen. Table 2 shows the effect of antigen dose on the induction of salivary and serum immune responses by oral administration of GTF antigen. Three different doses of enzyme antigen were tested for ability to elicit a salivary and/or serum immune response measured by both ELISA and functional inhibition. The highest dose of orally administered antigen (experiment A) induced salivary IgG and IgA antibody responses in all animals (14 of 14). The salivas of these hamsters also

contained relatively high levels of inhibitory activity. When the antigen dosage was lowered to a total of 6.5 IU of enzyme (experiment B), an immune response could be detected in only 9 of 11 hamsters. Furthermore, the levels of salivary antibody or inhibitory activity in the hamsters which did respond to the 6.5 IU dose were significantly lower than the levels of response to the 24.3 IU dose. Less than one-half the hamsters (5 of 11) given the lowest antigen dosage (4.2 IU of enzyme) (experiment C) had demonstrable salivary inhibitory activity. However, the mean levels of antibody and inhibitory activities in hamsters responding to the lowest dose were not significantly different than the mean positive responses to the 6.5 IU dose.

Some of these dose-response relationships were also observed with the serum responses (Table 2). Increasing the amount of total enzyme antigen administered from 6.5 to 24.3 IU resulted in a 10-fold increase in serum IgG antibody and a 7-fold increase in serum inhibitory activity. No significant difference in the amount of antibody of the IgM isotype occurred over the range of antigen doses tested in those hamsters showing a response. However, few animals given the lowest amount of antigen (4.2 IU) showed a significant serum antibody or serum inhibitory response (3 of 11 animals). Thus, both serum and salivary immune responses were apparently influenced by the dosage of orally administered GTF antigen.

It was also of interest to determine whether

the animals (6 of 11) which did not respond to oral administration of the lowest antigen dose (4.2 IU, 21 days) (Table 2) had been rendered tolerant to this antigen. These animals did not show a serum or salivary inhibitory response either after the initial 21-day oral administration of antigen or after a subsequent 4-day administration of the same antigen concentration (Table 3). The ability of these hamsters to respond to an injection of the same antigen 4 months after the second oral administration was compared with the response in injected hamsters that had not been exposed to antigen. The serum and salivary inhibitory responses of the previously orally immunized hamsters were in fact slightly higher than the responses of the previously untreated controls (Table 3). This may indicate that, rather than making the hamsters tolerant, some priming had in fact occurred in the hamsters given GTF orally, despite the inability to detect an immune response previously.

Anamnestic responses to orally administered soluble GTF antigens. Salivary and serum responses to a second oral exposure of the soluble GTF antigen preparation were investigated in weanling white hamsters given 0.14 IU of GTF activity in 200 μ g of protein daily for 21 days (experiment D). Under this regimen 3 of 11 hamsters showed significant salivary and serum responses by both ELISA and functional inhibition techniques. The antibody responses of the three responding hamsters were followed for 4 months (Fig. 3A and B) after completion of oral

TABLE 2. *Effect of dose on immune responses to orally administered antigen in salivas and sera of hamsters*

Fluid ^a	Total amt of protein (mg)	Total amt of enzyme (IU)	Days of oral administration	EU ^b			Inhibition units	No. showing inhibition/total no.
				IgA	IgG	IgM		
Salivas	4.2	4.2	21	10.0 $\pm$ 1.0 ^c	2.0 $\pm$ 0.5		10.1 $\pm$ 1.3 ^c	5/11
	6.5	6.5	26	13.9 $\pm$ 2.1	4.8 $\pm$ 2.0		9.5 $\pm$ 1.3	9/11
	10.8	24.3	27	28.1 $\pm$ 6.8	42.6 $\pm$ 12.9		46.5 $\pm$ 4.1	14/14
Sera	4.2	4.2	21		1.3 $\pm$ 0.1	7.2 $\pm$ 1.6	0.6 $\times$ 10 ³ $\pm$ 0.1 $\times$ 10 ³	3/11
	6.5	6.5	26		5.2 $\pm$ 1.9	6.6 $\pm$ 1.1	2.1 $\times$ 10 ³ $\pm$ 0.1 $\times$ 10 ³	8/11
	10.8	24.3	27		54.8 $\pm$ 13.4	5.1 $\pm$ 0.8	15.6 $\times$ 10 ³ $\pm$ 5.1 $\times$ 10 ³	14/14

^a Hamsters were bled and salivated 1 to 2 days after the last day of oral administration of the GTF antigen complex.

^b EU values were derived independently, and the 100-EU level was based on different optical densities for salivas and sera (see text). The EU values are intended to reflect relative differences in antibody activities within the respective fluids. The EU values for salivas were determined from 1:10 dilutions, and the EU values for sera were determined from 1:400 dilutions.

^c Mean $\pm$ standard error of hamsters which showed a positive response. A response was considered positive if it was more than 2 standard deviations above the responses of buffer-immunized hamsters. Salivary responses for buffer-immunized animals were 1.5 $\pm$ 0.6 EU for IgA, 1.0 $\pm$ 0.1 EU for IgG, and 0.4 $\pm$ 0.3 inhibition units for functional inhibition. Serum responses for buffer-immunized animals were 0.5 $\pm$ 0.2 EU for IgM, 1.1 $\pm$ 0.1 for IgG, and <100 inhibition units for functional inhibition.

TABLE 3. *Response to a single injection of GTF in untreated hamsters and in hamsters unresponsive to orally administered GTF^a*

Group	Fluid	Inhibition units ($\times 10^{-3}$) at:		
		7 Days after 21-day oral administration of PBS or GTF ^b	7 Days after 4-day oral boost with PBS or GTF	7 Days after single injection with GTF
Untreated/injected ($n = 5$)	Sera	0	ND ^c	6.9 ± 2.9^d
	Salivas	0.4 ± 0.4^d	0	2.6 ± 1.2
Oral (nonresponder)/injected ($n = 6$)	Sera	0	0.2 ± 0.05^d	10.3 ± 2.9
	Salivas	0.3 ± 0.1	0.2 ± 0.1	6.1 ± 1.0

^a The single injection of GTF (0.1 IU of GTF enzyme activity in 75 μ g of protein) was incorporated into complete Freund adjuvant (injection into salivary gland region). The hamsters unresponsive to orally administered GTF received a total of 4.2 mg of GTF (0.14 IU of GTF enzyme activity in 200 μ g of protein per dose).

^b Approximately 4 months elapsed between antigen exposures. PBS, Phosphate-buffered saline.

^c ND, Not determined.

^d Mean $\pm$ standard error.

GTF administration. During the first 2.5 months the mean salivary IgA and IgG antibody levels remained at or near the maximum primary response level, which occurred within 1 month of the start of antigen administration (Fig. 3A). On the other hand, the serum IgG antibody response (ELISA) (Fig. 3B) fell during the first month and was at pre-immunization levels 4 months after completion of the primary immunization regimen. A second course of antigen administration (0.14 IU/dose; duration, 4 days) was initiated at this time in the animals that responded to the primary series of antigen administrations. The peak salivary antibody response in either the IgA or IgG isotype occurred between 8 and 13 days after the second immunization protocol was begun. This was in contrast to the 21 to 28 days before a peak response was attained after primary immunization in this experiment or in the first experiment (Fig. 2A). The peak salivary IgG response after boosting showed a threefold increase compared with the peak primary response, whereas the peak salivary IgA response increased by approximately 1.5-fold after boosting. However, the salivary antibody of each isotype fell to levels within the range of the primary response during the 2 months after the second antigenic challenge. The inhibitory response in the salivas of boosted hamsters (Fig. 4) was similar to the salivary antibody response in general (Fig. 3A). Peak salivary inhibitory activity increased 1.7-fold and remained in the range of the peak primary response more than 4 months after the second administration of antigen.

The serum antibody response elicited by the second oral administration of the GTF antigen also demonstrated aspects of anamnesis (Fig. 3B). The peak IgG antibody response was not achieved until approximately 1 month after oral boosting. This response was threefold greater

than the primary IgG response. The secondary IgG antibody response declined within 2 months. The increase and duration of the serum response were also reflected in the pattern of inhibitory activity observed in the sera of the three animals to which the second antigen regimen was administered (Fig. 4).

DISCUSSION

Enteric administration of soluble antigens, including bovine serum albumin (3, 22), dinitrophenylated bovine gamma globulin (16, 17), and cholera toxoid (19, 21, 29), has resulted in the appearance of antibody in secretions such as intestinal and respiratory fluid, saliva, and milk. In the present study, 21 to 27 daily doses of GTF antigen complex administered into hamster oral cavities were sufficient to elicit an immune response in the saliva. Salivary antibody could be detected from 6 weeks (Fig. 2) to 4 months (Fig. 3 and 4) after primary oral challenge with GTF antigens. In addition, peroral antigen administration evoked a higher salivary response than did subcutaneous injection of antigen at the times tested (Fig. 2A).

Both IgA and IgG isotypes have been reported to occur in hamster intestinal washings and milk (1, 9). IgA has also been regularly identified in hamster saliva (1, 9). On the other hand, although Haakenstad and Coe (9) identified the IgG2 subclass in saliva, Bienenstock (1) was only able to detect IgA in this secretion. Salivary antibody directed against antigens contained in the GTF preparation was detected in both the IgA and IgG isotypes (Fig. 2A). Although the site of IgG synthesis was not studied directly, it is unlikely that the salivary anti-GTF antibody of the IgG isotype observed in the present study was a serum transudate. Salivary IgG antibody could be detected in hamsters in the absence of a detectable serum IgG antibody response, for

FIG. 3. Duration and anamnesis of salivary and serum antibodies in hamsters responding to oral administration of 0.14 IU of GTF antigen complex for 21 days (primary immunization [1°]) and, 4 months later, for 4 days (secondary immunization [2°]). Antibody activity is expressed as EU, which were obtained by using separate equations for each isotype (see text). Thus, identical EU for different isotypes do not necessarily represent equivalent amounts of antibody. The abscissa shows the number of months after completion of the primary immunization regimen. (A) Salivary IgA (●) and IgG (○) responses of two to three animals. Brackets enclose two standard errors. The slashed bars indicate the durations of primary and secondary oral administrations of GTF antigen. Salivas were tested at 1:10 dilutions. (B) Serum IgG (●) responses of two to three animals. Brackets and bars have the same meaning as in (A). Sera were tested at 1:400 dilutions.

example, after the use of low antigen doses (Table 2) or during the late stages of the primary response in some hamsters (Fig. 3A). Second, the peak serum IgG antibody response occurred 21 days after enteric antigen administration was begun (Fig. 2B), whereas the salivary IgG response was still increasing by day 28 (unpublished data). The local nature of the salivary IgA antibody response is emphasized by the absence of a detectable serum IgA response to any oral dose of antigen employed.

Administration of soluble antigens by the oral, intragastric, or intraduodenal route has been reported to result in the formation of serum antibody in a variety of animal model systems (17, 19, 22, 29), although the oral route does not seem to favor a serum response to particulate streptococcal antigens (13, 16). In the present study oral administration of soluble GTF antigens for 26 or 27 days resulted in a demonstrable serum response, which was predominantly of the IgG isotype (Fig. 2B and Table 2). However, the serum response to prolonged feeding with the highest dose of GTF (a total of 24.3 IU in 27 days) was 3-fold (IgG) to 15-fold (IgM) less (Fig. 2B) than that observed after subcutaneous injection of GTF antigen. Also, the serum IgG antibody response could be selectively eliminated by lowering the antigen dosage (Table 2).

The measurement of salivary and serum activities by both ELISA and functional inhibition techniques allowed the amount of antibody activity to be compared with the ability of the respective fluids to inhibit enzyme activity. In general, the data obtained in the two assays correlated quite closely, despite the possibility that antibody specificities in addition to anti-GTF may be detected by the ELISA. For example, comparison of the serum IgG antibody (EU) (Fig. 2B) with the inhibition units from

FIG. 4. Duration and anamnesis of salivary (○) and serum (●) responses to oral administration of 0.14 IU of GTF antigen complex for 21 days (primary immunization [1°]) and, four months later, for 4 days (secondary immunization [2°]). Responses were measured by the functional inhibition assay and are expressed as inhibitory units (IU). Brackets enclose two standard errors. The slashed bars indicate durations of primary and secondary immunizations. The abscissa shows the number of months after completion of the primary immunization regimen.

hamsters to which 0.9 IU of GTF was orally administered (Table 1) gave a correlation coefficient of 0.98 ($P < 0.01$). Likewise, a similar comparison of the salivary IgA antibody (Fig. 2A) with inhibition in the same samples (Table 1) in this experiment showed a correlation coefficient of 0.98 ($P < 0.01$). Although a significant correlation was not observed with serum IgM antibody, this relationship was probably masked by the high serum IgG antibody concentration. Although the ELISA technique is 8- to 30-fold more sensitive than the functional inhibition assay (unpublished data), these results suggest that the functional inhibition assay can give a reliable estimate of the relative amount of anti-GTF antibody activity in serum or saliva.

The primary response in the intestine to orally administered soluble antigen has been reported to be rather short lived in mice (29) and hamsters (3). These findings are presumably related in part to the relatively short half-life (4.8 days) of intestinal plasma cells (12). Pierce (19) has reported that primary immune responses in rat intestines to enteric immunization can be markedly increased by altering the structural characteristics of the antigen. In the present experiments the salivary immune response to prolonged oral antigen administration appeared to be influenced by dose. However, the salivary antibody response or inhibitory response in hamsters that received a low dose (4.2 IU in 21 days) of antigen (Fig. 3A and 4) decreased relatively slowly during the subsequent 2 to 3 months, whereas the salivary response in hamsters given the highest dose of antigen (Fig. 2B and Table 1) dropped severalfold in 6 weeks. In the latter experiment hamsters were infected with *S. mutans* 6715 (the organism from which the antigen had been obtained) after the primary immunization regimen. This dramatic decrease in salivary response may be explained by the adsorption of salivary antibody by organisms colonizing the teeth.

Until recently, the secretory immune system had seldom been reported to exhibit aspects of immunological memory (31). However, the jejunal immune response increased 19- to 43-fold when rats that were locally sensitized to cholera antigens were boosted intraduodenally with cholera toxoid (19). Furthermore, the ability to boost this response lasted for up to 8 months (19). In the present study hamsters orally primed with 0.14 IU of enzyme antigen per day for 21 days showed additional aspects of a local secondary response after oral boosting for 4 days (Fig. 3A and 4). The secondary antibody response in both salivary isotypes was more rapid and attained somewhat higher levels of salivary IgG and IgA antibodies. Also, the levels of func-

tional inhibitory activity observed 3.5 months after boosting were similar to levels of inhibitory activity seen at the peak of the primary response (Fig. 4). Thus, the form of the antigen, the amount of the antigen, or the mode of enteric administration may influence the degree to which memory is exhibited by the secretory IgA system after local stimulation.

The ability to evoke a salivary immune response with GTF administered by the oral route is significant in that immune responses to this antigen complex can be protective against the pathogenic effects of cariogenic streptococci (25, 27, 30). Our studies have shown that groups of immune hamsters fed any of the three antigen doses of GTF reported in Table 2 had fewer dental caries than similarly infected control groups (27). The results of the present study suggest, therefore, that potentially protective levels of antibody can be expected to occur for many months, provided that occasional boosting doses of antigen are applied enterically. Further investigations of enhanced immunogenicity and increased concentration at the critical site(s) for triggering a secretory immune response will assure more effective use of GTF in oral vaccines.

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LITERATURE CITED

1. Bienenstock, J. 1970. Immunoglobulins of the hamster. II. Characterization of the γ A and other immunoglobulins in serum and secretions. *J. Immunol.* **104**:1228-1235.
2. Bienenstock, J., and K. J. Block. 1970. Immunoglobulins of the hamster. I. Antibody activity in four immunoglobulin classes. *J. Immunol.* **104**:1220-1227.
3. Dolezel, J., and J. Bienenstock. 1971. γ A and non- γ A immune response after oral and parenteral immunization of the hamster. *Cell. Immunol.* **2**:458-461.
4. Ebersole, J. L., and J. A. Molinari. 1978. The induction of salivary antibodies by topical sensitization with particulate and soluble bacterial immunogens. *Immunology* **34**:969-979.
5. Ebersole, J. L., M. A. Taubman, and D. J. Smith. 1979. Thymic control of secretory antibody responses. *J. Immunol.* **123**:19-24.
6. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**:129-135.
7. Evans, R. T., and R. J. Genco. 1973. Inhibition of glucosyltransferase activity by antisera to known serotypes of *Streptococcus mutans*. *Infect. Immun.* **7**:237-241.
8. Gibbons, R. J., and J. van Houte. 1975. Bacterial

- adherence in oral microbial ecology. *Annu. Rev. Microbiol.* **29**:19-44.
9. Haakenstad, A. O., and J. E. Coe. 1971. The immune response in the hamster. IV. Studies on IgA. *J. Immunol.* **106**:1026-1034.
 10. Keyes, P. H., and H. V. Jordan. 1964. Periodontal lesions in the syrian hamster. III. Findings related to an infectious and transmissible component. *Arch. Oral Biol.* **9**:377-400.
 11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
 12. Mattioli, C. A., and T. B. Tomasi. 1973. The life span of IgA plasma cells from the mouse intestine. *J. Exp. Med.* **138**:452-460.
 13. Mestecky, J., J. R. McGhee, R. R. Arnold, S. M. Michalek, S. J. Prince, and J. L. Babb. 1978. Selective induction of an immune response in human external secretions by ingestion of bacterial antigen. *J. Clin. Invest.* **61**:731-737.
 14. Michalek, S. M., J. R. McGhee, and J. L. Babb. 1978. Effective immunity to dental caries: dose-dependent studies of secretory immunity by oral administration of *Streptococcus mutans* to rats. *Infect. Immun.* **19**:217-224.
 15. Michalek, S. M., J. R. McGhee, J. M. Mestecky, R. R. Arnold, and L. Bozzo. 1976. Ingestion of *Streptococcus mutans* induces secretory immunoglobulin A and caries immunity. *Science* **192**:1238-1240.
 16. Montgomery, P. C., J. Cohn, and E. T. Lally. 1974. The induction and characterization of secretory IgA antibodies. *Adv. Exp. Biol. Med.* **45**:453-462.
 17. Montgomery, P. C., K. M. Connelly, J. Cohn, and C. A. Skandera. 1978. Remote-site stimulation of secretory IgA antibodies following bronchial and gastric stimulation. *Adv. Exp. Biol. Med.* **107**:113-122.
 18. Nichols, R. L., E. S. Murray, and P. E. Nisson. 1978. Use of enteric vaccines in protection against chlamydial infections of the genital tract and the eye of guinea pigs. *J. Infect. Dis.* **138**:742-746.
 19. Pierce, N. F. 1978. The role of antigen form and function in the primary and secondary intestinal immune responses to cholera toxin and toxoid in rats. *J. Exp. Med.* **148**:195-206.
 20. Pierce, N. F., W. C. Cray, Jr., and B. K. Sircar. 1978. Induction of a mucosal antitoxin response and its role in immunity to experimental canine cholera. *Infect. Immun.* **21**:185-193.
 21. Pierce, N. F., and J. L. Gowans. 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. *J. Exp. Med.* **142**:1550-1563.
 22. Rothberg, R. M., S. C. Kraft, and R. S. Farr. 1967. Similarities between rabbit antibodies produced following ingestion of bovine serum albumin and following parenteral immunization. *J. Immunol.* **98**:386-395.
 23. Russell, M. W., S. J. Challacombe, and T. Lehner. 1976. Serum glucosyltransferase-inhibiting antibodies and dental caries in rhesus monkeys immunized against *Streptococcus mutans*. *Immunology* **30**:619-627.
 24. Smith, D. J., and M. A. Taubman. 1977. Antigenic relatedness of glucosyltransferase enzymes from *Streptococcus mutans*. *Infect. Immun.* **15**:91-103.
 25. Smith, D. J., M. A. Taubman, and J. L. Ebersole. 1978. Effects of local immunization with glucosyltransferase fractions from *Streptococcus mutans* on dental caries in hamsters caused by homologous and heterologous serotypes of *Streptococcus mutans*. *Infect. Immun.* **21**:843-851.
 26. Smith, D. J., M. A. Taubman, and J. L. Ebersole. 1979. Preparation of glucosyltransferase from *Streptococcus mutans* by elution from water-insoluble polysaccharide with a dissociating solvent. *Infect. Immun.* **23**:446-452.
 27. Smith, D. J., M. A. Taubman, and J. L. Ebersole. 1979. Effect of oral administration of glucosyltransferase antigens on experimental dental caries. *Infect. Immun.* **26**:82-89.
 28. Somogyi, M. 1945. A new reagent for the determination of sugars. *J. Biol. Chem.* **160**:61-73.
 29. Svennerholm, A.-M., S. Lange, and J. Holmgren. 1978. Correlation between intestinal synthesis of specific immunoglobulin A and protection against experimental cholera in mice. *Infect. Immun.* **21**:1-6.
 30. Taubman, M. A., and D. J. Smith. 1977. Effects of local immunization with glucosyltransferase fractions from *Streptococcus mutans* on dental caries in rats and hamsters. *J. Immunol.* **118**:710-720.
 31. Tomasi, T. B. 1976. The immune system of secretions. Prentice-Hall, Inc., Englewood Cliffs, N.J.