INFECTION AND IMMUNITY, Nov. 1997, p. 4424–4430 Vol. 65, No. 11
0019-9567/97/$04.00+0

Immunogenicity and Protective Immunity Induced by Synthetic Peptides Associated with a Catalytic Subdomain of Mutans Group Streptococcal Glucosyltransferase

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Received 23 June 1997/Returned for modification 5 August 1997/Accepted 29 August 1997

We examined the immunogenicity and induction of protective immunity of two 19-mer sequences (GGY and AND) which overlapped a highly conserved region which has recently been implicated in the enzymatic activity of glucosyltransferases (GTFs) of the mutans group streptococci. These peptides were synthesized as eight-branched constructs on a lysine core. Serum immunoglobulin G (IgG) antibody, induced by subcutaneous (s.c. [salivary gland vicinity]) injection with these peptide constructs, reacted with the inciting antigen, with mutans streptococcal GTFs, and with a 21-mer peptide (CAT) containing an aspartate previously shown to covalently bind sucrose. Several of these antisera also inhibited the ability of *Streptococcus sobrinus* GTF to synthesize insoluble glucan. Significant levels of salivary IgA antibody were also induced by GGY and AND peptide constructs after s.c. injection. The effect of immunization with the GGY and AND peptide constructs on the cariogenicity of *Streptococcus mutans* was studied in three experiments by immunization of weanling Sprague-Dawley rats, twice at 7- to 14-day intervals with peptides, *S. sobrinus* GTF, or phosphate-buffered saline. All rats were then orally infected with *S. mutans* SJ. After 63-day infection periods, the GGY and AND-injected groups had significant dental caries reductions compared with sham-injected groups in most experiments. These studies support the existence of an additional catalytic subdomain within the sequence defined by the GGY and AND peptides. Furthermore, the epitopes defined in these sequences have significant immunogenicity, can induce immune responses which interfere with GTF-mediated glucan synthesis in vitro, and can protect rats from experimental dental caries.

Glucan synthesis, which is the consequence of the catalytic action of glucosyltransferases (GTFs) on sucrose, is an important component of the colonization and accumulation potential of mutans group streptococci and, thus, of the expression of the virulence of these organisms (9). GTFs from mutans streptococci can induce immune responses that inhibit GTF catalytic activity, protect rodents from experimental dental caries (24), and interfere with reaccumulation of indigenous mutans streptococci in humans (20). Although the exact basis for experimental protection with GTF-type vaccines is unclear, it is quite likely that protection involves functional inhibition of the catalytic and/or the glucan-binding activities of GTF. Epitopes associated with these functions would theoretically be primary targets for immunological attack, provided that the relevant sequences are located in molecular areas that can be accessible to antibody. Subunit vaccines provide a method of blocking functional domains without inducing immunity to irrelevant or unwanted epitopes. We have reported that synthetic peptide vaccines that were associated with catalytic or glucan-binding domains of GTF could protect rats from experimental dental caries (27). For example, one of the peptides that was successfully used as a vaccine (21, 27) contained a sequence that included an aspartic acid to which the glucosyl moiety of sucrose was covalently bound (15).

GTFs from mutans streptococci and dextransucrase from *Leuconostoc mesenteroides* bear significant similarity in sequence (6). A second catalytic site (catalytic subdomain 1 [CD1]) has been suggested in *L. mesenteroides* dextransucrase by Funane and coworkers (6), approximately 40 residues toward the N terminus of the site of the first catalytic aspartate described in *Streptococcus sobrinus* GTF (15) (catalytic subdomain 2 [CD2]). Very recently, a catalytically active aspartate residue has been identified in the putative CD1 of *Streptococcus mutans* GTF-I by site-directed mutagenesis (29). The amino acid sequences in all mutans streptococcal GTFs surrounding this second catalytically active aspartate in CD1 are highly conserved (Table 1). Monoclonal antibody directed to a peptide whose sequence was based on this conserved GTF region was found to inhibit *S. mutans* GTF activity (2). Furthermore, CD1 and CD2 share very similar sequence immediately surrounding the respective aspartates [i.e., CD1, D.VDN; and CD2, D(A)VDN]. Recently, MacGregor and coworkers (13) have suggested that mutans streptococcal GTFs and the α-amylase family share structurally similar $(\beta/\alpha)_8$-barrel domains which are important to the catalytic activities of each enzyme. Amino acid sequence alignments of GTFs and α-amylases reveal that both catalytically active aspartates in GTFs lie adjacent to or are contained within the putative catalytically active barrel elements predicted from the crystallographic studies of the α-amylase family. Taken together, these observations suggest that the sequence encompassing this newly described catalytically active aspartate, if immunogenic, could serve as an additional functional (sub)domain target for immunological interference with GTF activity, and thus, with mutans streptococcal virulence.

In the present investigation, we explored the immunogenicity of synthetic peptide constructs (GGY, AND, and SAND) derived from the putative CD1 sequence (Table 1). We investigated the ability of these peptide constructs to induce antibody that reacted with and inhibited mutans streptococcal

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TABLE 1. Amino acid sequences of *L. mesenteroides* dextransucrase (DS) and mutans streptococcal GTFs in regions of aspartates associated with catalytic activity as well as peptide GGY, AND, SAND, and CAT sequences used in the present studies

Source of sequence (reference)	Amino acid sequence containing putative catalytic aspartate (*D*)[a]	Catalytic subdomain
GGY	GGYEFLLANDVDNSNPVVQ	CD1
AND	ANDVDNSNPVVQAEQLNWL	CD1
SAND	ANDVDNSNPVVQ	CD1
L. mesenteroides DS (6)	LQEDNSNVVVEA	
S. mutans GTF-I (18)	402 GGYEFLLANDVDNSNPVVQAEQLNWL	CD1
S. mutans GTF-SI (10, 30)	428 GGYEFLLANDVDNSNPVVQAEQLNWL	CD1
S. mutans GTF-S (11)	416 GGYDFLLANDIDNSNPVVQAEQLNWL	CD1
S. sobrinus GTF-I (1)	398 GGYDFLLANDVDNSNPVVQAEQLNWL	CD1
S. downei GTF-I (5)	404 GGYEFLLANDVDNSNPIVQAEQLNWL	CD1
S. downei GTF-S (8)	388 AGYELLLANDVDNSNPVVQAEQLNHL	CD1
S. mutans GTF-I (18)	442 DANFDSIRVDAVDNVDADVVQIA	CD2
S. sobrinus GTF-I (1)	438 DANFDSIRVDAEDNVDADQLQIS	CD2
CAT	DANFDSIRVDAVDNVDADVVQIA	CD2

[a] Putative catalytic aspartates in the CD1 (29) and CD2 (15) subdomains are indicated by underlining.

GTF or that reacted with a synthetic peptide construct that contained the originally described (15) catalytically active aspartate (CAT). Finally, we explored the ability of these peptides to induce protective immune responses in an experimental rat model of dental caries.

MATERIALS AND METHODS

Peptide constructs. GGY and AND were 19-mer peptides whose sequences overlapped residues in *L. mesenteroides* dextransucrase (6) and *S. mutans* GTF (29), which have been suggested to be associated with catalytic activity and, therefore, can be considered to contain a putative catalytic subdomain (CD1). These sequences are highly conserved among GTFs of *S. mutans*, *S. sobrinus*, and *Streptococcus downei* (Table 1). SAND was a 12-mer with sequence shared by GGY and AND. CAT was a 21-mer peptide (21) containing an aspartate residue originally shown by Mooser and coworkers to be catalytically active (15) and, for the purposes of these experiments, identified as CD2. The CAT sequence has been shown to induce GTF-inhibitory antibody (12, 27) and protective immunity in an experimental rat model of dental caries (27). Peptides were synthesized (Applied Diagnostics, Foster City, Calif.) by the stepwise solid-phase method of Merrifield (14) on a core matrix of lysines to yield macromolecules with four (CAT) or eight (GGY, AND, and SAND) identical peptides per molecule, after the method of Tam (23). Purity (>90%) was assessed by high-performance liquid chromatography, amino acid analysis, and molecular weight determination by mass spectrometry.

GTFs. GTFs from *S. sobrinus* 6715 and *S. mutans* SJ were obtained as previously described (25). Briefly, after bacterial growth in glucose-containing defined media, enzymes were isolated from culture media by affinity chromatography on Sephadex G-100 (Pharmacia Fine Chemicals), with 3 M guanidine HCl as the eluting solvent. These GTF-rich pools were then subjected to fast-performance liquid chromatography on Superose 6 (Pharmacia), with 6 M guanidine for elution. The gel filtration step removes non-GTF and other glucan-binding proteins from GTF preparations of *S. mutans* and *S. sobrinus*, as evidenced by the fact that the protein bands observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis were all associated with enzymatic activity after incubation of duplicate gels in sucrose. The *S. mutans* GTF preparation taken to this level of enrichment synthesized both water-insoluble and water-soluble glucan in both tube and filter assays (25). This preparation was designated GTFsm and was used in enzyme-linked immunosorbent assay (ELISA) measurements of antibody activity.

The *S. sobrinus* GTF preparation obtained after gel filtration on Superose 6 contained a mixture of GTF isozymes, including GTF-I and GTF-S, and was essentially free of other proteins. Approximately 90% of the glucan synthesized by this preparation was water insoluble under the conditions of the assay described below. This preparation was designated GTFss and was used for injection, inhibition assays, and ELISA measurement of antibody activity.

Immunogenicity of peptides. Sprague-Dawley CD strain 43-day-old male rats (Charles River Laboratories, Wilmington, Mass.) were used for injection. Six groups of four to five rats each were injected subcutaneously (s.c.) in the vicinity of the salivary glands with 50 μg each of either the GGY, AND, SAND, or CAT peptide constructs or 10 μg of GTF or were sham immunized with buffer alone. The initial injection included complete Freund adjuvant (CFA; Difco Laboratories, Detroit, Mich.); two subsequent injections at 21-day intervals included incomplete FA. Animals were bled and salivated prior to injection and 14 to 21 days after each injection. Antibody activity was measured in sera and saliva samples taken prior to injection and at the end of the experiment (21 days after the third injection and 63 days after the first injection). Sera from coagulated and centrifuged blood and clarified saliva samples were stored frozen at −20°C.

ELISA. Serum immunoglobulin G (IgG) and salivary IgA antibodies were tested by ELISA. Polystyrene microtiter plates (Flow Laboratories) were coated with 2.5 μg of each peptide construct per ml or 0.5 μg of *S. sobrinus* or *S. mutans* GTF per ml. Antibody activity was then measured by incubation with 1:100 to $1:10^6$ dilutions of serum or 1:4 to 1:32 dilutions of saliva. Plates were then developed for IgG antibody with isotype-specific rabbit anti-rat IgG, followed in sequence by alkaline phosphatase goat anti-rabbit IgG (Biosource, Inc.) and *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.). A mouse monoclonal reagent to rat α chain (Zymed, South San Francisco, Calif.) was used with biotinylated goat anti-mouse IgG (TAGO, Inc., Burlingame, Calif.) and avidin-alkaline phosphatase (Cappel) to reveal levels of salivary IgA antibody to peptides. Reactivity was recorded as A_{405} in a microplate reader (Biotek Instruments, Winooski, Vt.). Data are reported as A_{405} or as ELISA units (EU), which were calculated relative to the levels of appropriate reference serum or saliva samples from Sprague-Dawley rats thrice immunized with the respective peptide construct. Dilutions of 1:51,200, 1:25,600, 1:12,800, and 1:6,400 (A_{405} of approximately 1.0) were considered 100 EU for serum IgG to *S. sobrinus* GTF and *S. mutans* GTF, GGY, and AND constructs, respectively. Dilutions of 1:32 were considered 100 EU for salivary IgA to both GGY and AND constructs.

Antibody inhibition of glucan synthesis. Selected rat sera were evaluated for their ability to inhibit glucan synthesis catalyzed by *S. sobrinus* GTF by a filter assay. This GTF preparation contains GTF-I, which has complete homology with the AND peptide and differs from the GGY peptide by only one residue, which is well removed from the putative catalytic aspartate (Table 1). Ten-microliter volumes of sera (1:10 dilutions in 0.02 M sodium phosphate-buffered saline [PBS] and 0.2% sodium azide [PBSA], pH 6.5) were preincubated with the GTF for 1 h at 37°C in a total volume of 0.04 ml of PBSA. Next, 1.7 mg of sucrose and 44 nCi of [^{14}C]glucose-labeled sucrose (approximately 100,000 cpm) were added to 0.2 ml of PBSA in the absence of primer (27). Incubation proceeded for 2 h at 37°C after which water-insoluble glucan was collected on Whatman GF/F glass fiber filters, washed with PBS, and radioactivity was determined as previously described (21). Synthesis of water-soluble glucan was not tested, since it was less than 5% of the total glucan synthesized under these conditions.

Experimental protocol for protection experiments. The animals used in the present experiments were derived from germfree Sprague-Dawley rats that had been reared in the isolator facility of Charles River Laboratories (Area 051). A population of these rats was found to be free of indigenous mutans streptococci. These rats served as the initial breeding stock for the dams used in these experiments and were regularly monitored for the absence of mutans streptococci. The progeny of the dams used for the present experiments were weaned at approximately 21 days and were subsequently fed high-sucrose diet 2000 (24).

Three experiments were performed. Groups (n = 11 to 12) of 23- to 26-day-old rats were subcutaneously (sc) injected in the salivary gland vicinity as follows: experiment 1, GGY (50 μg), AND (50 μg), or GTFss (10 μg) or phosphate-buffered saline (control animals); experiment 2, GGY (50 μg) or PBS; experiment 3, AND (50 μg) or PBS. Each antigen was incorporated with CFA. Eight (experiment 1), 14 (experiment 2), or 7 (experiment 3) days later, rats were

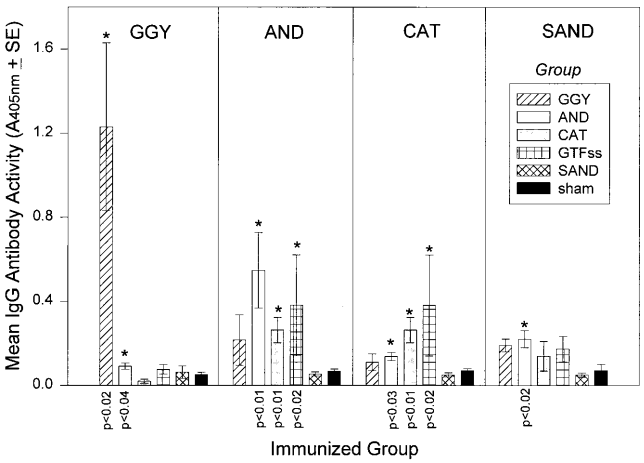

FIG. 1. IgG antibody activity in sera from peptide- and GTF-injected rats taken 63 days after initial injection (three injections total) evaluated by ELISA against all peptide constructs. The respective peptides used to coat ELISA plates are indicated at the top of each panel. Bars indicate the mean adsorbance for all rat sera of the indicated group (n = 4 to 5), tested at 1:200 dilutions, at least in duplicate. Brackets enclose 2 standard errors. Asterisks indicate group antibody levels that are significantly different from those of sham sera, tested against the same peptide in the same dilution. The levels of significance, determined by Kruskal-Wallace analysis of variance on ranks, are indicated below the bars.

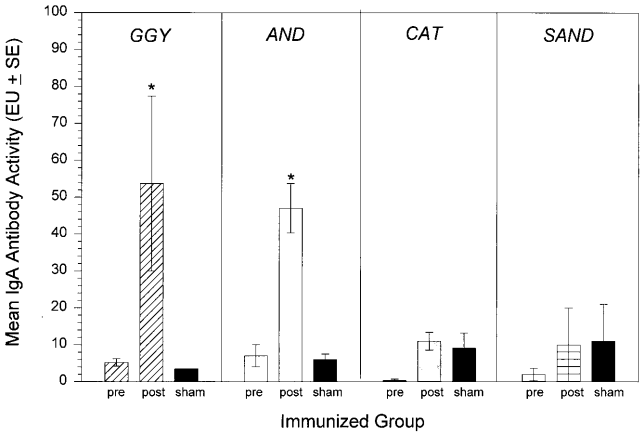

FIG. 2. IgA antibody activity in peptide-injected rat salivas evaluated by ELISA against the respective peptide construct used to induce the immune response. Preimmune saliva samples (pre) and saliva samples taken 63 days after initial injection (three injections total [post]) were tested (n = 4 to 5/group). Sham saliva samples taken at day 63 were also tested. Saliva samples were assayed in duplicate at 1:4 dilutions. The respective peptides used to coat ELISA plates are indicated at the top of each panel. Bars indicate the mean adsorbance for all rat salivas of the indicated group. Brackets enclose 2 standard errors. Asterisks indicate group antibody levels that were significantly different ($P < 0.05$; Kruskal-Wallace analysis of variance on ranks) from those of sham saliva samples tested against the same peptide in the same dilution.

reinjected with PBS or with the same antigen and dose in incomplete FA. Six (experiments 1 and 2) or 14 (experiment 3) days after the second injection, animals were bled from the tail vein and saliva was collected after injection of pilocarpine (1.0 mg/100 g of body weight). Fourteen (experiment 1), 10 (experiment 2), or 15 (experiment 3) days after the second injection, all rats were orally infected with approximately 10^8 *S. mutans* SJ32 cells for 3 consecutive days. Rats were singly caged after the infection protocol was completed. All three experiments were terminated 63 days after the initial infection.

Bacterial recoveries. The mutans streptococcal flora was assessed at termination as previously described (27). After systematic swabbing of teeth, sonication, and plating of appropriate dilutions on mitis salivarius (MS) agar (total streptococci) and MS agar with 0.006 mg of bacitracin (MSB) per ml (mutans streptococci), plates were incubated for 48 h at 37°C in 90% N_2–10% CO_2. Mutans streptococcal CFU were then enumerated microscopically on MSB agar.

Caries assessment. The extent and depth of carious lesions in all rat molar teeth (caries score) were microscopically evaluated by a modified Keyes method as previously described (24). Caries scores were determined separately on smooth and on occlusal dental surfaces.

RESULTS

Immunogenicity of peptides. Figure 1 presents the IgG antibody activities of all peptide-injected rat sera, taken at the end of the immunization period (day 63), assayed against each of the four peptide constructs (GGY, AND, CAT, and SAND). Both eight-branched 19-mer GGY and AND constructs, as well as the four-branched 21-mer CAT construct, induced serum IgG antibody to the inciting antigen on day 63. In fact serum IgG antibody to GGY and AND could be detected in the respectively injected rats 21 days after the first injection (not shown). In contrast, serum IgG antibody from rats injected with the 12-mer SAND peptide construct did not demonstrate a significant level of reaction with the SAND peptide. Immunogenicities of GGY and AND peptide constructs, whose sequences are based on putative CD1 (Table 1), were at least as great as those observed for the positive control CAT peptide construct whose sequence is based on CD2 (Table 1), both in terms of percentage of animals responding and reciprocal endpoint titer of the responding sera.

The antigenic similarity of the CD1 and CD2 subdomains was explored by reacting antisera to GGY, AND, SAND, or CAT with heterologous peptide constructs (Fig. 1). Antisera from rats immunized with AND (CD1) also recognized epitopes on GGY ($P < 0.04$ [CD1]) and CAT ($P < 0.03$ [CD2]). Although sera from one of four and two of four GGY-injected rats also recognized the AND (A_{405} of 0.572) and CAT (A_{405} of 0.187 and 0.154) peptide constructs, respectively, as a group they were not significantly different from sham sera. Interestingly the anti-AND serum IgG antibody also recognized epitopes on the 12-mer SAND peptide construct ($P < 0.02$) with which it shared sequence. Conversely, antisera from rats immunized with CAT (CD2) recognized epitopes on AND ($P < 0.01$ [CD1]). These data suggest that epitopes associated with CD2 (CAT) bear some antigenic similarity to epitopes associated with CD1 (GGY, AND, and SAND). However, differences in the length and sequence of these peptide constructs and in the genetic background of the injected rats result in significant differences in immunological responsiveness.

The IgA antibody activity in response to the inciting antigen in saliva samples of peptide construct-injected rats is shown in Fig. 2. The GGY and AND peptide constructs induced levels of IgA antibody activity that were significantly higher ($P < 0.05$) than those found in preimmune and sham-injected salivas by day 63. Salivary IgA antibody could be detected in three of four GGY- and four of five AND-injected rats 21 days after the first immunization. The level of IgA antibody in salivas of CAT peptide construct-injected rats (day 63) was significantly higher ($P < 0.01$) than those found in preimmune salivas, although antibody levels were not different from those of sham-injected rats. The 12-mer SAND peptide construct failed to induce a significant salivary IgA antibody to SAND, similar to the observed lack of serum IgG antibody induced by this antigen. However, the presence of salivary IgA antibody to GGY and AND peptides provides the possibility of immune intervention in the oral cavity by using these longer peptide constructs.

All antisera to peptide constructs were also assayed with the ELISA for IgG antibody reactive with intact *S. sobrinus* and *S. mutans* GTF preparations (Fig. 3). Sera from groups of rats injected with GGY and AND peptide constructs showed significant reactivity with *S. sobrinus*. Two of four of the rats

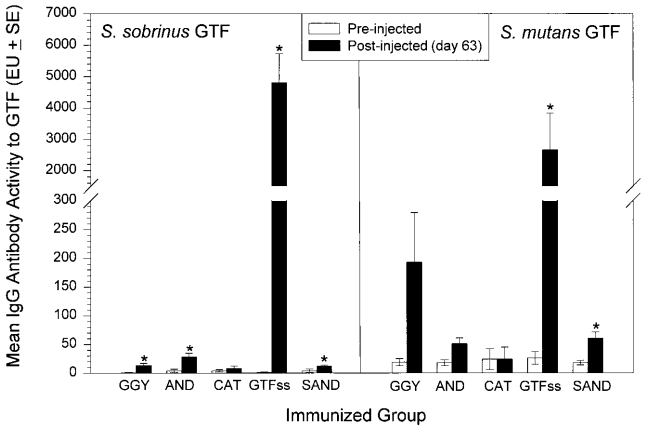

FIG. 3. Reactivity of peptide- and GTF-injected rat serum IgG (day 63) evaluated by ELISA against *S. sobrinus* and *S. mutans* GTF preparations. The source of the GTF preparation used to coat the ELISA plates is indicated at the top of each panel. Bars indicate the mean ELISA units for all rat sera of the indicated group (n = 4 to 5) taken prior to injection (open bars) or after three injections (day 63 [solid bars]). Sera were tested in duplicate. Brackets enclose 2 standard errors. Asterisks indicate group antibody levels that are significantly different ($P < 0.05$) from those of the respective preinjected sham sera (Kruskal-Wallace analysis of variance on ranks).

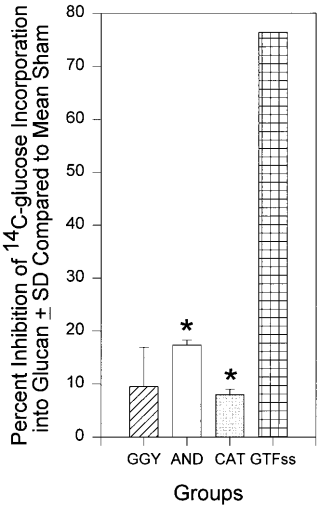

FIG. 4. Percentage inhibition of the *S. sobrinus* GTF-mediated incorporation of [^{14}C]glucose from labeled sucrose into water-insoluble glucan by sera from peptide- or GTF-injected rats. Day 63 rat sera to GGY (n = 4), AND (n = 4) and CAT (n = 2) peptide constructs were tested at 1:10 dilutions. A single day 63 serum sample from a rat injected with *S. sobrinus* GTF was tested at a dilution of 1:100. Data are expressed as the percentage [^{14}C]glucose incorporation of individual sera compared with the mean [^{14}C]glucose incorporation by three day 63 sera from sham-injected rats. Asterisks indicate peptide-immunized groups that significantly differed ($P < 0.02$) in level of inhibition.

injected with GGY also had elevated levels of serum antibody reactive with *S. mutans* GTF. Each of the peptide-injected groups had at least one rat serum sample with >100 EU of IgG activity to *S. mutans* GTF. Interestingly, sera from rats injected with the 12-mer SAND peptide showed significant levels of IgG antibody to both GTFs. This observation, coupled with the fact that IgG antibody from several serum samples from one of four *S. sobrinus* GTF-injected rats reacted positively with SAND (Fig. 1), might suggest that GTF contains an immunological epitope within the ANDVDNSNPVVQ sequence, which is shared by GGY, AND, and SAND. Furthermore, antibody to this putative epitope can be induced by peptides containing this sequence (e.g., Fig. 1, anti-AND IgG versus SAND peptide) and could be expected to modify GTF activity, since this sequence has been shown to include a catalytic aspartate (29).

Inhibition of GTF activity. To test this hypothesis, sera from rats injected with GGY, AND, or CAT peptide were evaluated for their ability to inhibit the formation of water-insoluble glucan from sucrose by *S. sobrinus* GTF (Fig. 4). Sera from all rats injected with AND peptide inhibited GTF activity ($P <$ 0.01). Inhibition with sera from rats injected with GGY peptide was more variable, although one rat serum sample gave considerable (35%) inhibition of GTF activity. The level of inhibitory activity of AND-injected rats exceeded that observed with sera from rats similarly immunized with CAT ($P < 0.02$), although the inhibitory levels of sera from all peptide-injected rats were far below those induced by the intact GTF protein.

Protection experiments. Three experiments were performed to measure the in vivo effect of immunization with the eight-branched GGY and AND peptide constructs on experimental dental caries in the Sprague-Dawley rat. In the first experiment, groups of rats were immunized with GGY or AND peptide constructs or *S. sobrinus* GTF (GTFss), or they were sham immunized with PBS. In the second and third experiments, groups of rats were immunized only with the GGY or AND peptide constructs, respectively, or sham immunized. Levels of antibody to the inciting antigen were measured before infection in all animals. In the GGY-immunized rat groups, levels of IgG antibody to GGY (experiment 1, 21.9 ± 17.8 EU; experiment 2, 108.0 ± 4.0 EU) were elevated above those of the sham-immunized groups (experiment 1, 1.7 ± 0.2 EU; experiment 2, 4.0 ± 3.0 EU) in both experiments and reached statistical significance ($P < 0.002$) in experiment 2. Salivary IgA antibody levels to GGY in the GGY-injected groups (experiment 1, 5.0 ± 2.0 EU; experiment 2, 11.7 ± 4.8 EU) were also elevated above those of the sham-immunized groups (experiment 1, 2.0 ± 1.0 EU; experiment 2, 2.0 ± 1.0 EU), although differences did not reach statistical significance. Levels of serum IgG and salivary IgA antibody to the AND construct were not significantly elevated in experiment 1, but they were significantly ($P < 0.02$) elevated for serum IgG (97.3 ± 15.9 EU) and salivary IgA (116.0 ± 22.9 EU), above those of sham sera (15.3 ± 2.3 EU) or salivas (4.2 ± 0.2 EU) in experiment 3. Levels of antibody to *S. sobrinus* GTF were significantly higher ($P < 0.01$) for serum IgG (250.0 ± 35.5 EU) and salivary IgA (72.5 ± 23.1 EU) in the GTF-immunized positive control group than the serum IgG (33.5 ± 1.5 EU) and salivary IgA antibody values (3.4 ± 2.4 EU) of the sham-immunized group.

Levels of *S. mutans* colonization were evaluated by swabbing of rats 59 days after initial infection. The median infection levels in the GGY- and AND-immunized groups were generally less than half of the median *S. mutans* infection level of the sham-immunized group in each of the three experiments. However, the differences did not reach significance because of the broad range of values.

Figure 5 presents the total molar and smooth surface caries scores of all groups in all experiments. The mean and median total, smooth surface, and occlusal (not shown) caries scores of AND- or GGY-immunized groups were lower than the respective scores of the sham-immunized groups in all experiments. Total molar caries scores of the groups injected with the GGY and AND constructs were significantly lower ($P < 0.05$) than those of the sham-injected groups in experiments 1 and 2 (GGY) and 3 (AND). Protection was significant on smooth (GGY, experiment 1, $P < 0.04$; experiment 2, $P < 0.03$) and

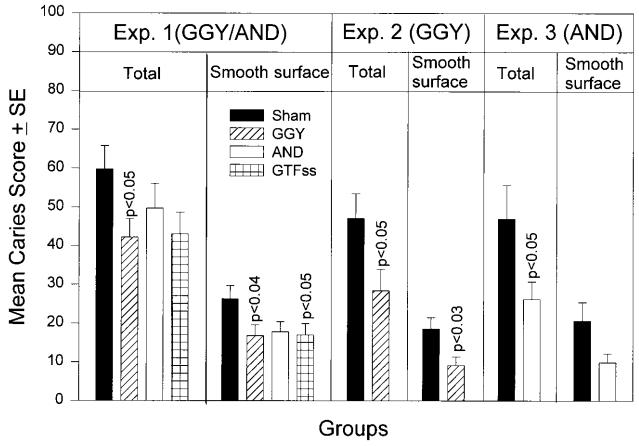

FIG. 5. Mean total and smooth surface caries scores of groups of rats (n = 11 to 12/group) injected with *S. sobrinus* GTF, AND, or GGY peptide constructs or sham injected after 63 days of infection with *S. mutans* SJ32. Results for all protection experiments are shown. The levels of statistical significance are indicated for immunized groups compared with those of sham-immunized groups.

occlusal surfaces (GGY, experiment 1, $P < 0.02$). Significant smooth surface ($P < 0.02$) and occlusal caries reductions ($P < 0.02$) were also observed in the group injected with *S. sobrinus* GTF (experiment 1). Thus, injection with peptide sequences containing the putative catalytic subdomain 1 can induce protective immunity from experimental dental caries in the rat.

DISCUSSION

Several lines of evidence suggest that GTFs of mutans streptococci may contain several subdomains within their sequence that participate in the catalytic activity of the enzyme. Mooser and coworkers (15) showed that the carboxyl side chain of aspartic acid residue 447 in *S. sobrinus* GTF-I bound an isotopically labeled transition state glucosyl moiety, indicating that this residue formed part of the active site. Subsequent studies indicated that active intermediates interacted with GTF at more than one subsite within the GTF molecule, although these subsites were not specified (4). Site-directed mutagenesis of *S. mutans* GTF identified several amino acid residues which influenced the nature of the glucan product (17). Funane and coworkers (6), working with *L. mesenteroides* dextransucrase, showed that a second site in this enzyme could be labeled with a fluorescent sucrose analog, even when the aspartate that corresponded to that identified by Mooser et al. (15) was blocked. This *L. mesenteroides* dextransucrase site was hypothesized to provide a second essential carboxyl group for catalytic activity (6). The identity of an additional catalytic subdomain in a corresponding sequence of *S. mutans* GTF-I was confirmed very recently by Tsumori et al. (29) by site-directed mutagenesis. These workers found that mutagenesis of aspartic acid residue 413 to threonine removed most enzymatic activity. The sequences within this region (CD1) are essentially identical among most mutans streptococcal GTFs (Table 1). Another indication that several subdomains may participate in catalytic activity arose from comparisons of GTF sequence with those of the α-amylase family of glucosidases whose structures have been visualized by X-ray crystallography. Characteristic of the catalytic motifs of these glucosidases are $(\beta/\alpha)_8$-barrel domains. Sequence comparisons revealed that mutans streptococcal GTFs and *L. mesenteroides* dextransucrase contain slightly permuted versions of these $(\beta/\alpha)_8$-barrel domains, and it has been suggested that this motif may form the catalytic domain of GTFs (13). Based on this hypothesis, several or all of the eight β strands may contain catalytically important residues. Interestingly, the peptides selected for this study lie adjacent to (GGY or AND) or are contained within (CAT) two of the β strands within this hypothetical motif.

The observed pattern and characteristics of immune responses to GGY and AND peptides support the hypothesis that a catalytic subdomain (CD1) exists at the location defined by the GGY and AND sequence. The sequence DVDNSN in CD1, which contains the catalytic aspartate identified by site-directed mutagenesis (29), is similar to the DAVDNVD sequence, which contains the catalytic aspartate in CD2, previously identified in *S. sobrinus* by Mooser et al. (15). Sequences from both regions are highly conserved. In fact, the two putative catalytic aspartates in CD1 and CD2 are 38 residues apart in all six mutans streptococcal GTFs in Table 1, suggesting a tightly controlled structure necessary for catalytic activity. Antibody raised to GGY or AND not only reacts with GTF (Fig. 3), but also inhibits its enzymatic activity (Fig. 4). Furthermore, our findings suggest that there is an immunological similarity between the epitopes on the CD1 (ANDVDNSNPVVQAEQ LNWL) and CD2 (DANFDSIRVDAVDNVDADVVQIA) catalytic subdomains, since antibodies to AND show significant reaction with epitopes within the CAT peptide (Fig. 1). Evidence from Chia and coworkers (2) has also suggested that a site in the putative CD1 could be important for GTF function. They observed that monoclonal antibody from mice immunized with an AND-bovine serum albumin conjugate could partially inhibit the ability of *S. mutans* GTFs to synthesize insoluble glucan. These monoclonal antibodies also inhibited the sucrose-dependent attachment of *S. mutans* to glass surfaces. Extending these observations, we demonstrated inhibition of insoluble glucan formation by GTF from *S. sobrinus* by a polyclonal antibody (Fig. 4). Since *S. mutans* GTF-I and GTF-SI, but not GTF-S, activities were inhibited, Chia et al. concluded that this GTF domain might participate in insoluble glucan synthesis (2). Taken together, these theoretical and laboratory observations indicate a catalytic role for the subdomain in the mutans streptococcal GTF sequence defined by GGY, AND, and SAND.

Epitopes expressed by the putative GGY-AND catalytic subdomain induced protective immunity after infection of rats with cariogenic *S. mutans* (Fig. 5). Protection was at a level similar in magnitude to that achieved by injection with the intact GTF from *S. sobrinus*, illustrating the effectiveness of polyclonal antibody raised to an important functional determinant. The extent of protection afforded by GGY immunization was somewhat more consistent than AND immunization (Fig. 5), but both peptides significantly diminished the cariogenicity of *S. mutans* in at least one experiment, and the mean levels of caries in each peptide-injected group were always lower than those observed in sham groups. The levels of protection were similar to those previously observed by immunization with CAT in a similarly executed protocol (27), further emphasizing the immunogenicity, and, perhaps, functional significance of these two sites. The functional basis for protection following GGY or AND peptide immunization may have emanated from antibody interaction with GTF epitopes of either CD1 or CD2, given the apparent immunological relationship of CD1 and CD2 (Fig. 1). The influence of antibody to CD1 could be even more broadly based if such immunological relationships also exist among the additional catalytic subdomains of GTF which have been predicted from comparison with the catalytic motifs of α-amylases (13).

Future experiments will reveal whether immunization with

the GGY and AND peptides also induces a protective immune response to infection with *S. sobrinus*. However, the sequence of these peptides is identical or nearly identical to the 26 residues containing the putative CD1 of all mutans streptococcal GTFs which have been sequenced. Thus, the resulting immune response could be expected to be domain specific, rather than strain or species specific. The ability of antibody to AND to inhibit *S. sobrinus* activity would support this notion. In this regard, immunization with the CAT peptide, whose sequence is based on CD2, induced protective immune responses to either *S. mutans* or *S. sobrinus* infection in the experimental rat model for dental caries (27).

The identification of multiple GTF epitopes which are capable of induction of significant protective immune responses would allow the design of subunit or recombinant vaccines which maximize the presentation of critical epitopes to the immune system. We have focused our selection of candidate sequences for subunit dental caries vaccines on those which are associated with functional domains of GTF. For example, we showed that the peptide construct GLU, whose sequence, TGAQTIKGQKLYFKANGQQVKG, was based on a putative glucan-binding domain (16), was immunogenic, contained both T- and B-cell epitopes, reacted with IgA antibody in human saliva samples (19), and could induce protective immunity in the experimental rat caries model (27). Other possibilities have included a 15-mer sequence, PQWNGESEKPYDDHL, in the N-terminal region (e.g., positions 342 to 356 of *S. sobrinus* GTF-I [30]). Dertzbaugh and Macrina (3) demonstrated that an equivalent *S. mutans* GTF-B peptide, when genetically fused to the B subunit of cholera toxin, induced GTF-inhibitory activity after s.c. immunization of rabbits. In contrast, we did not find that four-branched MAP constructs of this peptide were as immunogenic as GGY and AND, CAT, or GLU (22), nor did they induce protective immunity in our experimental rat model (data not shown). We also found that a consensus sequence, VTDRYGRISYYDGNSGDQIRN, containing the G...YY....G fundamental repeating unit in streptococcal GTF glucan-binding regions, as defined by Giffard and Jacques (7), induced little antibody, despite the fact that the immunogenic and protective GLU peptide construct also contains this fundamental repeating unit (26). The lack of immunogenicity of these peptide constructs may be attributed to the absence of appropriate T-cell epitopes. Further structural or functional studies should help to clarify these issues and define other potential sites for immune intervention.

Our preliminary studies (28) indicate that multiepitopic peptide constructs, consisting of both CAT and GLU peptide branches, induce enhanced immune responses, presumably because of the contribution of a potent T-cell epitope to the CAT construct. This strategy also could be used to increase the immune potential of the AND and GGY sequence described in the present study. Moreover, the combination of sequences from several catalytic and glucan binding sites into a synthetic or recombinant multiepitopic construct could increase the protective potential of subunit vaccines for dental caries. Finally, the subunit vaccine approach theoretically permits the combination of epitopes directed to multiple pathogens, together with epitopes derived from immunostimulatory molecules such as cholera toxin.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE-04733 and DE-06153 from the National Institute of Dental Research and by a student research grant from Harvard Medical School (B.S.).

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Editor: J. R. McGhee