Coimmunization with Complementary Glucosyltransferase Peptides Results in Enhanced Immunogenicity and Protection against Dental Caries

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Peptide constructs from the catalytic (CAT) and glucan-binding (GLU) regions of the mutans streptococcal glucosyltransferase enzymes (GTF) can provide immunity to dental caries infection. A strategy of coimmunization was tested to determine whether protection could be enhanced. Rats were immunized with one of the previously described peptide constructs from the CAT or GLU region of the GTF of mutans streptococci or coimmunized with a combination of these constructs (CAT-GLU). Coimmunized animals demonstrated significantly higher serum immunoglobulin G (IgG) and salivary IgA antibody levels to CAT or GTF than rats immunized with either construct alone. To assess the functional significance of coimmunization with these constructs, animals were immunized as above or with Streptococcus sobrinus GTF and then infected with S. sobrinus to explore the effects of immunization on immunological, microbiological, and disease (dental caries) parameters. Serum antibody from the coimmunized group inhibited S. sobrinus GTF-mediated insoluble glucan synthesis in vitro above that of the individual-construct-immunized groups. Immunization with CAT or GLU constructs resulted in significantly reduced dental caries after infection with S. sobrinus compared with sham-immunized animals. Coimmunization produced greater reductions in caries than after immunization with either CAT or GLU. Also, significant elevations in lymphocyte proliferative responses to CAT, GLU, and GTF were observed after coimmunization with CAT-GLU compared with the responses after immunization with the individual constructs. The results suggested that increased numbers of memory T cells, which could proliferate to CAT, were generated by coimmunization. The experiments support the functional significance of these GTF domains in dental caries pathogenesis and present coimmunization as a simple alternative to intact GTF to enhance protective immunity against cariogenic microorganisms.
significantly enhanced systemic and mucosal immune responses to CAT and GTF and resulted in significant reductions in dental caries.

MATERIALS AND METHODS

Synthetic peptides and antigens. (i) CAT peptide. The CAT peptide (DANF DSIRVADVDVNAALDDLQ) used in the present study contains a nonapeptide (DSIRVADVAD), located between residues 448 and 457 of GTF-I of Streptococcus downew (5), which contains an aspartic acid that has been shown to be involved in the catalytic reaction of GTF with sucrose (13, 14). An identical sequence was found in the catalytic region of Streptococcus mutans GTF-B (17), and the residues within the DSIRVADVAD peptide are either identical or conserved in S. sobrinus GTF-I (3). The peptide was synthesized as previously described (22, 26) (Applied Diagnostics, Foster City, Calif.) using the stepwise solid-phase method of Merrifield (11) on a core matrix of three lysines to yield a multiple antigenic peptide macromolecule with four identical 21-mer peptides per molecule, after the method of Tam and Lu (24). Purity (>90%) was assessed by high-pressure liquid chromatography, amino acid analysis, and molecular mass determination by mass spectrometry. This peptide multiple antigenic peptide construct, referred to as CAT, was used for immunization and antibody analyses.

(ii) GLU peptide. Repeating sequences within the C-terminal third of the GTF molecule have been associated with binding of glucan by these enzymes (23, 30, 33). One composition of a 22-mer GLU peptide, whose sequence (TGAQT IKQGQKLYF KANGQQVKG) was based on the derived sequence of one of the repeating regions of S. downew GTF-I (residues 1303 to 1324) which was 86% identical to an S. sobrinus GTF-I sequence (3) and 77% identical to an S. mutans GTF-B sequence (18), as described previously (26). The GLU peptide was synthesized (Applied Diagnostics) on a core matrix of three lysines to yield a macromolecule with four identical 21-mer peptides per molecule.

GTF inhibition assay. GTF from S. sobrinus strain 6715 was obtained as previously described (21). After bacterial growth in glucose-containing defined medium enzymes equally diluted in 6 M guanidine HCl were isolated by chromatography on Sephadex G100 (Pharmacia Biotech Inc., Piscataway, N.J.) in 3 M guanidine HCl as the eluting solvent. The GTF-rich pools were then subjected to fast protein liquid chromatography on Superose 6 (Pharmacia) in 6 M guanidine HCl, and gel filtration step separates non-GTF and other glucan-binding proteins from the catalytically active GTF, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). S. sobrinus GTF preparations obtained after gel filtration on Superose 6 contained a mixture of GTF-I (water-insoluble glucan [IG] product), GTF-U (primer-stimulated soluble glucan [SG] product), and GTF-S (primer-independent SG product) (1, 10, 28). This preparation was designated GTF and was used for injection and enzyme-linked immunosorbent assay (ELISA).

Animals. Gnotobiotic Sprague-Dawley rats CD (SD) originally reared in the isolator facility of Charles River Laboratories, Wilmington, Mass., were colonized and fed a flora found to be free of indigenous mutants streptococci. These rats were bred in our facility, weaned at approximately 20 days, raised on high-sucrose Diet 2000 (27), and used in the experiments described below. Females were used in the preliminary experiments, and males and females were used in all other experiments.

Protocol for animal experiments. (i) First experiment. An experiment was performed to determine if coimmunization with the two peptide constructs would enhance response to GTF or to the CAT construct. Groups of four female 23- to 29-week Sprague-Dawley rats (identical to the defined flora) were injected subcutaneously in the subcutaneous skin at the same time on day 0 and 3 weeks later with 0.2 ml of CAT-GTF mixture (50 

A second experiment was conducted to evaluate the effects of coimmunization on functional inhibition of GTF activities and on the levels of serum IgG antibody to CAT or GTF prior to infection (second experiment, 20 days after initial immunization); (Fig. 2A) and at the termination of the caries experiment after the animals had been infected for 62 days in a second experiment (Fig. 2B). Antibody levels to CAT were significantly elevated in the coimmunized group compared with either the CAT- or the GTF-immunized group. Additionally we investigated antibody levels to GTF compared with the CAT-immunized group. Antibody to GTF was significantly elevated compared with all groups after immunization with GTF (127 ± 5 ELISA units; not shown).

Serum IgG antibody to CAT and GTF after coimmunization. We also determined the levels of serum IgG antibody to CAT or GTF prior to infection (second experiment, 20 days after initial immunization); (Fig. 2A) and at the termination of the caries experiment after the animals had been infected for 62 days in a second experiment (Fig. 2B). Antibody levels to CAT were significantly elevated in the coimmunized CAT-GLU group compared with either the CAT- or the GTF-immunized group. Additionally we investigated antibody levels to GTF. Again the CAT-GLU-coimmunized group demonstrated significantly elevated IgG antibody to GTF compared with the CAT group.
CAT-immunized group when tested against CAT (Fig. 3). Furthermore, the level of IgA antibody to GTF determined after coimmunization with CAT-GLU was significantly elevated above the levels in the sham and GLU groups and also elevated above the level in the CAT-immunized group.

**Effects of coimmunization on GTF function.** Since we demonstrated that coimmunization gave rise to levels of antibody to CAT and GTF significantly higher than those of animals separately immunized with the peptide constructs, it was important to evaluate the effect of antibody initiated by coimmunization on GTF function. Water insoluble polyglucan formation by GTF is an important component in mutans streptococcal accumulation. Therefore, we studied the ability of sera from immunized rats to inhibit the formation of this glucan from radiolabeled sucrose (Fig. 4). Although inhibition of glucan formation was relatively low in the CAT- and GLU-immunized groups, this inhibition was significantly enhanced in the coimmunized group compared with the CAT (or the GLU) group. Serum from rats immunized with GTF inhibited glucan formation most effectively.

**Effects of coimmunization on dental caries.** To further assess the functional significance of enhanced levels of antibody to CAT and GTF, we evaluated the dental caries profile of the immunized and coimmunized animals in the second experiment, using a modified Keyes' method (Fig. 5). The sham-immunized animals demonstrated the highest level of dental caries, which was significantly higher than that in all other groups. The CAT-GLU-coimmunized group demonstrated significantly lower dental caries scores than either the GLU- or CAT-immunized group (Fig. 5). Thus, it appeared that coimmunization with CAT-GLU not only enhanced measurable serum antibody response to CAT and GTF and functional inhibition of IG formation, but also enhanced mucosal antibody to CAT and GTF and inhibited dental caries more effectively than immunization with the individual peptides.

**Cellular aspects of coimmunization.** Previous data indicating a feeble T-cell epitope on CAT may mean that only a small or insignificant number of T cells are reactive to CAT (27). The data presented herein imply that coimmunization expanded this small number of CAT-responsive T cells through the generation of increased numbers of memory T cells responsive to CAT following the generation of bystander help contributed by the strong GLU T-cell epitopes. This hypothesis was tested at the termination of the experiment by assessing the proliferation of lymph node T cells to GTF and to peptide antigens (Fig. 6). Highly significant elevations in proliferative responses

![Graph 1](image1.png)

**FIG. 1.** Serum IgG antibody to CAT and GTF (S. sobrinus 6715) in rats after immunization with the CAT construct or GLU construct and coimmunization with the CAT-GLU construct. Serum was collected 29 days after the first immunization. Bars indicate the mean antibody level in serum from four rats in each group, expressed as ELISA units. Error bars indicate the standard error of the mean. Differences are statistically significant at the following levels compared with the sham, CAT, and GLU groups by one-way analysis of variance followed by the Student-Newman-Keul multiple-comparisons test:

* p < 0.05 versus sham and CAT. Solid bars, antibody to the CAT construct; open bars, antibody to GTF. NT, not tested.

![Graph 2](image2.png)

**FIG. 2.** Serum IgG antibody in rats to CAT construct or to GTF in sera taken prior to infection (A; preinfection day 20) and at the termination of the experiment (B; after 62 days of infection). Bars indicate the mean level of serum IgG antibody from 9 to 10 rats in each of the designated groups (sham immunized, CAT construct immunized, GLU construct immunized, CAT-GLU immunized, and GTF immunized), expressed in ELISA units. Error bars indicate the standard error of the mean. Differences are statistically significant at the following levels compared with the sham, CAT, or GLU groups by one-way analysis of variance followed by the Student-Newman-Keul multiple-comparisons test:

* p < 0.05 versus sham and CAT; ** p < 0.001 versus sham, CAT, GLU, and CAT-GLU for panel A; and * p < 0.03 versus sham and CAT; ** p < 0.01 versus sham and P < 0.05 versus CAT for panel B. Solid bars, antibody to the CAT construct; open bars, antibody to GTF.
to CAT and significant responses to GTF were observed after coimmunization of animals with CAT-GLU. These results suggest that increased numbers of memory T lymphocytes which could proliferate to CAT were generated by coimmunization.

**FIG. 3.** Salivary IgA antibody levels in rats to the CAT construct and to GTF in saliva taken prior to infection. Bars indicate the mean antibody level of salivary IgA antibody from 9 to 10 rats in each of the designated groups, expressed in ELISA units. Error bars indicate the standard error of the mean. Differences are statistically significant at the following levels compared with the sham, CAT, or GLU group by one-way analysis of variance and the Student-Newman-Keul multiple-comparisons test: #, *P* less than at least 0.05 versus sham, CAT, and GLU; ***, *P* < 0.01 versus sham and CAT; **, *P* < 0.05 versus sham and CAT; *P* < 0.05 versus CAT. Solid bars, antibody to CAT; open bars antibody to GTF. NT, not tested.

**FIG. 4.** Effects of coimmunization on GTF function. Inhibition of IG synthesis by serum antibody to *S. sobrinus* GTF 20 days after initial immunization at preinfection. Bars indicate the mean percent inhibition ± standard error of incorporation of glucan from [14C]glucose-labeled sucrose into IG. The mean counts incorporated into IG in the presence of sham-immunized, uninfected sera were 1,908 ± 13 cpm. Differences are statistically significant at the following levels compared with sera from the immunized groups by one-way analysis of variance and the Student-Newman-Keul multiple-comparisons test: *, *P* less than at least 0.05 versus sham, CAT, and GLU; ***, *P* < 0.001 versus sham, CAT, GLU, and CAT-GLU.

**FIG. 5.** Dental caries scores of animals immunized or coimmunized and infected (62 days) with *S. sobrinus*. Bars show the mean total caries scores, including smooth and sulcal surfaces, and the standard errors. Differences are statistically significant at the following levels compared with the sham-immunized infected group by one-way analysis of variance and the Student-Newman-Keul multiple-comparisons test: ***, *P* < 0.001 compared with the sham group and for comparison with the CAT-GLU group; #, *P* less than at least 0.04 compared with the CAT or GLU group alone.

**FIG. 6.** Lymphocyte (cervical lymph nodes and axillary nodes) proliferation to CAT or GTF after immunization with CAT, GLU, or GTF or coimmunization with CAT-GLU and infection with *S. sobrinus*. Lymph node cell proliferation to the antigens above was assessed in animals sham immunized or immunized with CAT, GLU, or GTF. The bars indicate the mean counts per minute of tritiated thymidine incorporation by construct-stimulated cells from 5 to 10 animals per group. Statistical significance was evaluated by one-way analysis of variance and the Student-Newman-Keul multiple-comparisons test. Significant differences from sham, CAT, or GLU are indicated as: *, *P* less than at least 0.05 versus sham and CAT; ***, *P* < 0.001 versus sham, CAT, GLU, and CAT-GLU.

**DISCUSSION**

Coimmunization with CAT and GLU constructs resulted in enhanced serum IgG antibody response to CAT and to GTF compared with the response after immunization with single peptide constructs. Coimmunization also resulted in enhanced serum-mediated inhibition of GTF-mediated IG synthesis, a process critical to the pathogenic potential of mutans strepto-
coccii (35). The enhanced response produced by coimmunization was also able to give rise to enhanced caries reduction. It is clear that genetic control of responsiveness to synthetic peptides can be a factor in limiting the spectrum of host response to peptides which may be functionally highly significant. One approach to overcome this lack of response is to use the strategy of coimmunization (15, 16, 18). In the experiments described herein, we have used coimmunization with peptides from the functional domains of GTF to evaluate the possibility of enhancing responses to the CAT construct and to GTF from S. sobrinus. We also evaluated the ability of such a putative enhanced response to affect dental caries. We found that coimmunization significantly enhanced systemic and mucosal responses to CAT and resulted in significant reductions in dental caries compared with controls and with single peptide construct immunization. Dental caries was diminished in all immunized groups (as opposed to the sham-immunized group), and the coimmunized group dental caries scores were reduced even further compared with those for the individual peptide construct-immunized groups (Fig. 5). Lymphocyte proliferation in CAT-GLU-immunized rats was significantly enhanced above that in CAT-immunized animals in comparison to CAT or GTF, suggesting that memory T lymphocytes were generated to CAT (and also to GTF) by coimmunization (Fig. 6). Presumably the phenomenon relies on the provision of bystander help by the relatively large number of GLU-responsive T cells in the same anatomic location (27) to (i) the small number of CAT-specific T cells and to (ii) CAT-specific B cells resulting in generation of CAT memory T and B cells. The proximity of antigens to each other at the injection site is most significant, since simultaneous antigen deposition at two distant locations does not give rise to the coimmunization effect (15).

Enhanced salivary IgA antibody levels to CAT and GTF were also demonstrated after coimmunization with the CAT-GLU constructs. These findings indicate that the principles of coimmunization could also be extended to the mucosal immune system. While other studies have indicated that mucosal adjuvants can give rise to local (IgA) responses to coadministered protein (4, 31), to our best knowledge this report is the first demonstration of coimmunization with complementary immunogenic peptides resulting in enhanced mucosal immunity to the peptides and to the parent compound. This novel principle could be utilized further to promote mucosal immunity and caries-preventive measures.

The strategy of coimmunization can be used to enhance the immunogenicity of peptides derived from functionally significant regions of enzymes and will be of value in the utilization of subunit vaccines. It is clear from the work of Prieto et al. (16) with human immunodeficiency virus and Partidos and colleagues (15) with measles peptides that coimmunization is a valid procedure for increasing antibody levels to a B-cell epitope. Partidos et al. (15) demonstrated that coimmunization with nonimmunogenic B-cell epitopes combined with T-cell epitopes resulted in antibody to the B-cell epitope without requiring covalent linkage. The basis for the phenomenon is currently unclear. However, several hypotheses have been proposed. Partidos and coworkers (15, 18) believe the phenomenon can be attributed to bystander help from specific T cells to generate B memory cells. We have shown that there is minimal T-cell activity to CAT (27), which we think is enhanced by bystander activity (perhaps interleukin-2) from numerous GLU-specific T cells. Important in both hypotheses is the notion that the two peptides can be taken up by the same antigen-presenting cell. Our hypothesis suggests that uptake and processing of both peptides (CAT and GLU) by the same antigen-presenting cell results in presentation of both peptides or peptide segments. Recognition of these peptides by many GLU-responsive T cells which provide bystander help to a few CAT-responsive T cells can possibly give rise to memory T cells to CAT. Also, the T-cell bystander help from GLU-specific activated T cells can stimulate CAT-specific B cells, resulting in enhanced antibody production both systemically and in the mucosal (salivary) immune system. Thus, while bystander help could occur through the release of T-cell-derived factors that act nonspecifically on activated B cells, no direct link between the antigenic determinants recognized by the T cell and B cells is required. This would be in contrast to cognate help, which through direct interaction between Th and B cells results in transduction of a signal to the B cell in the form of locally released factors and/or cross-linking of small molecules. Despite the involvement of infection in modulating the serum response determined at experiment termination, the existence of a pronounced increase in anti-CAT IgG levels in serum at that time (Fig. 2) would support the generation of some memory T cells to CAT in addition to expansion of the demonstrated (27) CAT-specific B cells. Furthermore, these findings suggest that coimmunization may result in an anamnestic response to a peptide component with a minimal T-cell epitope.

The findings presented herein indicate that a combination of immunologically complementary functional peptides from separate domains of GTF can result in significant induction of antibody (systemic and mucosal) and cellular responses. Such antibodies appear to be significant in interference with the pathogenesis of dental caries. Although the combination of peptides used was not as immunogenic as the native GTF, the caries scores after immunization with either of these antigens were similar. We believe this can be attributed to inability to precisely measure dental caries. In any event, these data indicate that the infectious process leading to caries has been arrested in either case. An alternative explanation might suggest that antibody levels to GTF do not directly correlate with the ability to interfere with dental caries.

In addition to the CAT peptide (22) described herein and other peptides (GGY and AND) containing catalytically implicated aspartates of the GTF catalytic domain (20), we have also described potentially catalytic peptides (EAW and HDS), containing glutamate and tryptophan or aspartate and histidine residues, respectively, from additional subdomains (19). These peptides induce significant systemic and mucosal antibody responses which can inhibit GTF activity and dental caries caused by mutans streptococci (19, 20). The potential exists for further enhanced immunogenicity by coimmunization with a mucosal adjuvant (4, 31) and combinations containing more than two of these peptides. Combinations may be found which induce integrated immune responses that effectively target and block most sites participating in GTF function. A goal would be to provide functional blocking of GTF on a par with or superior to that induced by immunization with GTF itself. Such combinations would have significant potential as subunit vaccines for interference with dental caries.

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REFERENCES


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