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 communized 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.

The group of enzymes collectively called glucosyltransferases (GTF) have been implicated as important constituents in the active accumulation of mutans streptococci on teeth (9). The accumulation process involves glucans synthesized by GTF in the presence of sucrose (25). Several different isoforms of GTF exist within the various species of the mutans species group of streptococci, the predominant microorganisms implicated in the pathogenesis of human dental caries (32). The presence of sucrose is essential in this process in the rodent model. Glucan sucrases produced by oral streptococci all have three major domains, including an N-terminal highly variable region, a conserved core catalytic region, and a C-terminal glucan-binding domain (12). The catalytic domain, which exists primarily in the amino half of the molecule in a barrel configuration, contains at least one site with an aspartic acid residue which appears to function to stabilize glucosyl intermediates formed during the hydrolysis of sucrose (2, 13). Additional residues have also been implicated in the enzymatic activity of the catalytic domain (2, 29).

There are at least two further catalytic subdomains within this domain (20, 29). A second functionally important domain is found in the carboxyl half of the GTF molecule and is characterized as containing tandem repeats of certain sequences of aromatic amino acid motifs (7) which can bind carbohydrate (30, 33, 34). This second putative glucan-binding domain is immunogenic, contains both T and B epitopes (21, 27), and may function by binding and stabilizing the nascent glucan polymer during synthesis.

Synthetic peptides have been prepared from each of these regions (21, 22). When these peptides are presented in immunogenic fashion, the antibody produced can cause inhibition of some of the GTF functional properties (26). Thus, a monoclonal antibody to a catalytic-site peptide was shown to inhibit synthesis of glucan from sucrose by GTF-I from Streptococcus sobrinus (8, 22). Polyclonal antibody to a consensus sequence from the putative glucan-binding repeat region was also shown to inhibit GTF enzyme function (21). Immunization with either of these synthetic peptides utilized as tetramers on a lysine backbone has resulted in protection against infection with Streptococcus mutans or S. sobrinus and amelioration of dental caries caused by either of these organisms (26).

The peptide constructs designated CAT (from the catalytic site) and GLU (from the glucan-binding consensus sequence) have been shown to contain B-cell epitopes (21, 22). While the GLU peptide appears also to contain a major T-cell epitope (27), the CAT peptide construct contains only a feeble T-cell epitope (27). A simple strategy of coimmunization may enhance the host response to synthetic peptides lacking a major T-cell epitope or to which there is genetic unresponsiveness (15, 16, 18). In the experiments described herein, we have used the strategy of coimmunization with the peptides from the functional regions of GTF to evaluate the possibility of enhanced response to the CAT construct and to GTF from S. sobrinus. We also evaluated the ability of such enhanced response to affect dental caries. We found that coimmunization...
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 DSIRVDAVNVDAKDLLQ) used in the present study contains a nonapeptide (DSIRVDAVQ), located between residues 448 and 457 of GTF-I of Streptococcus downett (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 is found in a similar region of Streptococcus mutans GTF-B (17), and the residues within the DSIRVDAVQ 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 (22, 30). A 21-mer GLU peptide, whose sequence (SIGQKLYFKANGQQVKG) was based on the derived sequence of one of the repeating regions of S. downett GTF-I (residues 1303 to 1324) was considered 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 22-mer peptides per molecule.

GTF activity. 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 at the eluting solvent. The GTF-rich pools were then subjected to fast protein liquid chromatography on a Superoxel G100 Super guard column in 6 M guanidine HCl. 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 with defined flora found to be free of indigenous 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 for the carcinogenicity experiment.

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 25-g Sprague-Dawley rats (defined as female 24-day-old Sprague-Dawley rats [defined as female 24-day-old Sprague-Dawley rats]) were injected subcutaneously in the salivary gland vicinity (6) with CAT construct (50 μg/rat), GLU construct (50 μg/rat), or with the CAT and GLU constructs together (CAT-GLU; 50 μg of CAT and 50 μg of GLU) incorporated in complete Freund’s adjuvant (CFA) for immunization on day 0 and incomplete Freund’s adjuvant (IFA) 7 days later. A sham-immunized group receiving buffer with CFA and then IFA was also included. Animals were bled from the tail vein, and saliva was collected after injection of pilocarpine (1.0 mg/100 g of body weight; Sigma Chemical Co., St. Louis, Mo.) on day 29.

(ii) Caries experiment. A second experiment was conducted to evaluate the effects of coimmunization on functional inhibition of GTF activities and on dental caries. Five groups of 9 to 10 male and female 24-day-old Sprague-Dawley rats (defined as male 24-day-old Sprague-Dawley rats) were injected twice subcutaneously in the salivary gland vicinity at a 7-day interval. The initial injections were with the CAT construct (50 μg/rat), the GLU construct (50 μg/rat), or with a mixture of the CAT and the GLU constructs incorporated together in CFA (the coimmunized group, CAT/GLU; 50 μg of CAT plus 50 μg of GLU per rat) or immunized with GTF enzymes from S. sobrinus in CFA (25 μg/rat). The second injection, 1 week later, was in IFA. Prior to infection (day 21 after first immunization), animals were bled and saliva was collected. Oral infection with approximately 10^9 cells of S. sobrinus strain 6715 was performed in 24 consecutive days. The experiment was terminated after 62 days, at which time the experiment was terminated. Also, spleens, cervical lymph nodes, and axillary nodes were taken at this time for proliferation assays.

Antibody tumor assay. Serum immunoglobulin G (IgG) antibody levels were usually tested by ELISA to GTF, GLU, and CAT, and the levels were compared among the groups. Serum and saliva were tested for the presence of antibody by a previously described ELISA performed in microtiter plates (26). The antigens used on plates were as follows: 0.5 μg of CAT per well, 0.5 μg of GLU per well, or 0.15 μg of GTF per well. Isotype-specific rabbit anti-rat IgA or IgG (26) was used with goat anti-rabbit IgG conjugated to horseradish peroxidase (TAGO, Inc., Burlingame, Calif.). The plates were developed with 4-nitrophenyl-phosphate (Sigma Chemical Co.) and read on a photometric scanner (Dynatech, Vienna, Va.). at 405 nm. Antibody of each isotype (IgG and IgA) was expressed separately as ELISA units of a particular isotype (not comparable), which were calculated relative to the titration of appropriate reference sera from Sprague-Dawley rats hyperimmunized with each of the antigens mentioned above (23, 26). All test sera were diluted at least 1:100, and all saliva samples were diluted at least 1:4 for analysis.

GTF inhibition assay. Rat sera (preimmunization and termination) were evaluated for the ability to inhibit glucan synthesis by GTF in a modified filter assay described previously (26, 28). Briefly, the sera (1 μl) were combined with the respective GTF preparation in a final volume of 100 μl in 0.02 M sodium phosphate-buffered saline and 0.02% sodium azide (PBSA, pH 6.5) and incubated for 2 h at 37°C. To this was added 100 μl of PBSA containing 0.85 mg of sucrose and 22 ng of [14C]glucose-sucrose (approximately 50,000 cpm), and the mixture was incubated for 2 h at 37°C. IgG was detected on Whatman GF/F glass filters and washed with PBSA, and the radioactivity was determined as previously described (26).

Lymphocyte proliferation. T-cell proliferative responses to peptides were assessed at experiment termination. Axillary and cervical lymph node cells or spleen cells (5 × 10^9 viable cells/well) were cultured in triplicate in 0.2 ml in 96-well flat-bottomed tissue culture plates at 37°C in 5% CO_2 for 5 days in complete medium containing 5 × 10^5 cells/ml of RPMI 1640 (12.5% fetal calf serum, [H]thymidine (0.5 μCi/well) was added 18 to 24 h before harvest. Stimulation was done with 2 μg of GTF per well or 2.4 μg of CAT or GLU per well.

Bacterial recoveries. The mutants streptococcal flora was assessed at termination. Systematic swabbing of teeth, sonication, and plating appropriate dilutions on mitis salivarius agar (total streptococci; Difco Laboratories, Detroit, Mich.) were all performed as previously described (23, 26). Mutans streptococci were recovered from all animals 1 week after infection and at termination.

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 described previously (26). The caries scores were determined separately on smooth and sulcal dental surfaces and then combined.

RESULTS

Comimmunization compared with immunization. To test the efficacy of coimmunization, an experiment comparing coimmunization with CAT-GLU constructs and separate immunization with CAT or GLU constructs was performed. Significant elevation of serum IgG antibody to both CAT and GTF from S. sobrinus was found in the coimmunized group compared with the CAT-immunized rats (Fig. 1). Antibody to CAT (indicated by the solid bars) in the sera taken 29 days after initial immunization was significantly enhanced in the CAT-GLU-coimmunized group compared with the CAT-immunized group. The CAT-GLU-coimmunized group also demonstrated significantly enhanced antibody response 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 GLU-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.

Effects of coimmunization on salivary IgA antibody. In the second experiment, the levels of salivary IgA antibody to CAT and to GTF prior to infection were also determined by ELISA. The level of IgA antibody recovered from the CAT-GLU-coimmunized group was significantly elevated above that of the
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...
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.

**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 mutants strepto-
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).

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-processing/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 mutants 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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