Development of a DNA Vaccine Against Streptococcus mutans: a Novel Approach to Immunization Against Dental Caries

Thomas Han

University of South Florida

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Development of a DNA Vaccine Against *Streptococcus mutans*: a Novel Approach to Immunization Against Dental Caries

by

Thomas K. Han

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Biology
College of Arts and Sciences
University of South Florida

Major Professor: My Lien Dao, Ph.D.
Daniel V. Lim, Ph.D.
Kenneth E. Ugen, Ph.D.
Valerie J. Harwood, Ph.D.

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Development of a DNA Vaccine Against *Streptococcus mutans*: a Novel Approach to Immunization Against Dental Caries

Thomas K. Han

ABSTRACT

*Streptococcus mutans* is the main causative agent of dental caries, which is a widespread infectious disease. A number of surface molecules are involved in the pathogenicity of this organism, including adherence and aggregation factors. The wall-associated protein A (WapA) of *Streptococcus mutans* GS-5 was previously demonstrated to be a sucrose-dependent adherence and aggregation factor, and is a larger precursor to extracellular antigen A (AgA), a candidate antigen for a dental caries vaccine.

The full-length *wapA* gene and a C-terminal truncated version *agA* encoding the AgA were cloned into the mammalian expression vector pcDNA 3.1/V5/His-TOPO. The above constructs were mixed with a cationic lipid and used to transfect Chinese hamster ovary (CHO) cells. Transient expression of the *wapA* and *agA* genes was observed at 24 h post-transfection, as shown by Western immunoblot analysis. In CHO, cells WapA containing the membrane and wall-spanning region was found in apoptotic bodies, whereas the soluble AgA, which lacked the hydrophobic region, was found in extracellular medium.

A higher salivary IgA level was observed in mice immunized with the pcDNA-
wapA vaccine as compared to those immunized with the pcDNA-agA vaccine. Furthermore, the anti-WapA antibody inhibited *S. mutans* sucrrose-dependent adherence, suggesting potential protection of the tooth against *S. mutans* colonization, while anti-AgA had no significant effect. Indeed, prediction and analysis of protein epitopes showed that WapA contains highly promiscuous MHC-II binding motifs that are absent from AgA. Immunodot assay confirmed that WapA bound biotin-labeled dextran, whereas AgA did not. These data indicated that full-length wall-associated WapA is a better candidate vaccine antigen than the soluble AgA.

In co-immunization studies pcDNA-ctb was preferable to pcDNA-il-5 as genetic adjuvant. A comparable secondary response was obtained by priming with either pcDNA-wapA or WapA followed by a WapA boost, thus demonstrating the pcDNA-wapA as a valid contender primary vaccine. The successful utilization of the caries DNA-based vaccine protocol would represent a highly significant new approach to this important worldwide health problem.
INTRODUCTION

Background of Dental Caries

Dental caries is one of the most prevalent and costly chronic infectious diseases in humans. Despite improvement in dental hygiene and advances in caries research over the past decades, dental caries has been experienced by over 90 percent of all adults in the U.S. and over 80 percent of children by age 17, resulting in billion of dollars in health expenditures per year (53, 79, 140). In recent years, caries prevalence and distribution has shifted from being circulated evenly among the population, to disproportionately affecting individuals in economically-challenged populations with more severe forms of caries (20, 108). The incidence of dental caries is high in industrialized countries and on the rise in developing countries (4, 71). The risk of severe dental caries is increased in elderly people who are showing root-surface caries and in other specific groups such as those who suffer from hypo-salivation due to drug-induced side effects, systemic disease such as Sjogren’s Syndrome, and tumor patients receiving irradiation therapy (2, 3, 13, 128).

The etiologic role of mutans streptococci (MS) in dental caries was established in Keyes’ experiment in 1960, showing direct evidence that these organisms are the primary etiologic agents. MS has been divided into eight serotypes designated a-h based on differences of carbohydrates in the cell wall (9, 11, 97). DNA homology analysis further clarified MS into seven distinct species – known as Streptococcus mutans (serotypes c,e
and f), *S. rattus* (serotype b), *S. cricetus* (serotype a), *S. sobrinus* (serotypes d and g), *S.ferus* (serotype C), and *S. macacae* (serotype c) and *S. downei* (serotype H) (8, 17, 18, 138). These organisms have the ability to colonize the tooth surface, build up dental plaque by aggregating homologous and heterologous bacteria, and produce lactic acid which attacks the enamel. Of these species, *S. mutans* has been implicated as the major and most common of the two main etiological agents of human dental caries (37, 73, 135), the other being called *S. sobrinus*. *S. mutans* was first described by Clarke in 1924 after he isolated an organism that he felt to be from the initial carious lesions in humans (15). Clarke named *S. mutans* based on his observation that the organism formed different colony morphology with the change of growth media.

As with a large number of infectious diseases, dental caries is preventable by immunization. This was demonstrated in animal studies, but the prospect for a human dental caries vaccine using whole organisms or components of *S. mutans* has been hampered by the presence of antigens that are cross-reactive with proteins in human cardiac tissue (46). Consequently, much effort has been focused on developing recombinant vaccine antigens. Through this process, heart reactive antigens can be avoided or modified to eliminate the heart cross-reactive epitopes. With decades of research on *S. mutans* by dedicated researchers around the world, a number of proteins involved in colonization have been studied as important candidate dental vaccine antigens. One concern is how to make them affordable to large populations, especially since most of the populations at risk are from developing countries, or from low socioeconomic groups in industrialized nations.
Virulence Factors of *S. mutans* and Approaches for Development of Anti-caries Vaccine

A number of surface molecules contribute to the pathogenicity of the MS organism including adherence and aggregation factors (37). With recombinant DNA technology and advances in mucosal immunology, a number of virulence factors involved in *S. mutans*, a principal causative agent of dental caries, have been characterized, cloned and tested for the ability to induce a protective response through induction of mucosal immunity and production of specific salivary secretory IgA (sIgA). Today, much progress has been made in the field of dental caries vaccines based on the virulence factors involved in the *S. mutans* colonization of the tooth and build-up of dental plaque (Fig. 1). The factors tested to reduce dental caries in an animal model include: (1) A 190 kDa salivary adhesin called “AgI/II”; (2) Glucosyltransferase (GTF); (3) A glucan binding protein (GBP), (4) A 29 kDa extracellular antigen A (AgA), also known as antigen III; and (5) Wall-associated protein A (WapA), a precursor to the extracellular AgA (59, 64). These receptors could be blocked by vaccine-induced antibodies, thus preventing the *S. mutans* from colonizing or accumulating in the oral cavity. Recombinant DNA technology has been applied to clone the genes encoding the above virulence factors for the production of recombinant antigen and peptide vaccines, as well as avirulent recombinant organisms expressing the target antigen.
FIG. 1. Virulence factors of *Streptococcus mutans* during colonization and accumulation in dental biofilms.

**AgI/II**

A 190 kDa major cell-surface protein antigen of *S. mutans* has been variously known as AgI/II, P1 (33), Pac (93), and AgB (110) while designated as SpaA or PAg (55) in *S. sobrinus*. AgI/II is involved in the initial bacterial adherence to tooth surfaces (28, 109). The AgI/II contains two internal repeating amino acid regions: 1) A-region with an alanine-rich tandem repeat at N-terminal domain that can bind salivary components such as slgA, β2-microglobulin, histidine-rich polypeptides, a 60 kDa glycoprotein, high molecular mass glycoproteins, lysozyme, lactoferrine, and 2) a P-region with a proline-rich repeat in the center of the molecule where an adhesion epitope resides (19, 68, 87).
Antibody to intact AgI/II (38, 54, 69) or peptides containing salivary-binding domain epitopes (131) induced partial protection against dental caries in rodents, primates, and humans. Passive immunization with the anti AgI/II antibody have shown reduced tooth colonization with *S. mutans* (75, 76).

**Glucosyltransferases (GTF)**

Glucosyltransferases (GTF) in *S. mutans* are involved in the synthesis of extracellular glucans, and its role in sucrose-enhanced cariogenicity was obtained from the comparative insertional inactivation study of GTF genes (86, 144). Three gtf genes encoding GtfB, GtfC and GtfD are responsible for glucan synthesis in *S. mutans*: GtfB (118) synthesizes an α-1,3-linked insoluble glucan, while GtfD (44) is involved in the formation of a soluble α-1,6 linked glucan. GtfC (100) synthesizes both insoluble and soluble glucans. All GTF molecules contain a glucan-binding domain (GLU) on the C-terminal end and a sucrose-binding catalytic region (CAT) on the N-terminal part (84). Salivary and serum antibodies to GTF were detected and significant protection from dental caries was obtained by oral immunization with GTF in an animal model (126, 127).

Synthetic peptides from the catalytic or glucan-binding domains of GTF have been shown to induce protection against experimental dental caries (132). The newly formulated GTF peptide vaccine containing both the catalytic (CAT) and glucan-binding (GLU) regions induced significantly enhanced levels of antibody to GTF than either a CAT or GLU construct or coimmunization with CAT/GLU (133).

**Glucan-binding proteins (GBP)**

The *S. mutans* glucan-binding protein (GBP), which mediates binding of the
organisms to glucans synthesized by GTFs, is a virulence component in the development of dental plaque, and a candidate for a human caries vaccine (72). *S. mutans* synthesizes three glucan-binding proteins: GBP-A (110), GBP-B (123), and GBP-C (116). The predominant *S. mutans* GBP-A with a molecular mass of 74 kDa was purified (112), cloned, and sequenced (5). A *S. mutans* mutant insertionally inactivated in the *gbpA* was analyzed *in vitro* to demonstrate the role of *gbp* in colonization (6). Both the mutant and the parental type colonized smooth surfaces when grown in the presence of sucrose, suggesting that *S. mutans* may involve more than one protein in glucan binding.

GBP-B secreted by *S. mutans* having a molecular mass of 59 kDa has been purified (123) and compared structurally and antigenically to other GBP-A in *S. mutans* and to the glucan-binding region of *S. mutans* glucosyltransferases. The GBP-B was shown to be antigenically distinct from the GBP-A and induced significantly higher salivary immune response in humans (123). GBP-C has an estimated molecular mass of 64 kDa and has no sequence similarities to GBP-A or GTF, but shows significant sequence matching to the surface protein adhesion AgI/II (77, 125).

*Antigen A (AgA)*

One candidate vaccine antigen against dental caries is the *S. mutans* antigen A (47, 110, 111), a proteolytic cleavage product of the large precursor wall-associated protein A (WapA) (23, 113). Immunization with AgA-induced protection against caries has been performed on cynomolgus monkeys (111) and mice (47) and has been recognized as an anti-caries vaccine.

*Wall-associated protein A*

The gene encoding the wall-associated protein A (WapA), a precursor to the AgA,
was cloned and sequenced and proven to be involved in *S. mutans* sucrose-dependent adherence and aggregation (23, 30). Insertional inactivation of the *wapA* gene resulted in a significantly reduced sucrose-dependent adherence and aggregation, 40 and 52%, respectively, of the *wapA* mutant (101). These findings suggest that the antibody to the WapA or AgA may prevent dental caries by blocking both the colonization of the tooth and the build-up of dental plaque by *S. mutans*.

A genomic library of *S. mutans* strain GS5 was constructed in our laboratory via the shuttle vector *Streptoccus-E. coli* pSA3, and one clone was found to express the AgA and its precursor, the WapA, by Western immunoblot analysis using a monoclonal antibody against AgA (22). Subsequent sequence analysis of the gene showed that AgA was derived from WapA by proteolytic cleavage. AgA previously shown by Russell (110) was a good candidate dental vaccine antigen (47, 111). Immunization of rats and monkeys conferred protection in these animals, with a reduction in carious lesions upon infection of these animals with the *S. mutans* and feeding a cariogenic diet rich in sucrose content.

Since WapA has been observed to have binding affinity for dextran, this protein may be involved in glucan-mediated adherence and aggregation. Indeed, insertional inactivation of *wapA* in *S. mutans* caused a significant decrease in sucrose-dependent adherence and aggregation, by 40% and 50% respectively, as compared to the wild type tested in parallel (101). The role of WapA in sucrose-dependent adherence and aggregation was further demonstrated by cloning the *wapA* gene into *S. gordonii*, a heterologous organism known to produce GTF but devoid of WapA. Expression of WapA conferred to this organism the ability to attach to a culture dish and to aggregate
when grown in BHI containing 4% sucrose.

These observations provided an explanation of the protection obtained by immunization with AgA (111). Perhaps the antibody raised against AgA blocked \textit{S. mutans} adherence and aggregation through binding to WapA. Through comparative sequence analysis between the \textit{S. mutans} strain GS5 and the \textit{S. mutans} strain, Ingbritt showed that the \textit{wapA} gene was virtually identical in these strains, with the exception of a 24 base pair deletion in the GS-5 strain. This is in agreement with the immunological cross-reactivity of both strains with a rabbit anti-WapA antibody.

Isolation of WapA from the original \textit{E. coli} recombinant clone designated 4B2 was not efficient; therefore, production of vaccine using this clone is not practical for large scale immunization studies. To increase the expression of WapA and to facilitate its isolation twelve \textit{wapA} truncations were prepared by PCR amplification using primers designed to delete various regions of the \textit{wapA} gene. The truncated \textit{wapA} genes, designated \textit{wapA}-A to \textit{wapA}-L were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Then they were excised by digestion with EcoRI and cloned into the expression pGEX-6P-1 glutathione S-transferase (GST) fusion vector (Pharmacia, Pittacaway, NJ). Cloning of a series of truncations of the \textit{wapA} gene at the N-terminal and C-terminal ends into the expression fusion vector pGEX-6P-1 glutathione S-transferase (GST) demonstrated that the WapA and AgA reacted most strongly with a rabbit antibody to the \textit{S. mutans} WapA (146). The resulting plasmids were used separately to transform \textit{E. coli} BL21, a protease-deficient host strain for optimal expression of recombinant proteins (Pharmacia).

PGEX-6P-1 contains a tac promoter for inducible high-level intracellular
expression, an internal lac I\textsuperscript{q} repressor for induction with isopropyl beta-D-thiogalactoside (IPTG), a PreScission\textsuperscript{TM} protease cleavage site for removal of the GST by the PreScission protease enzyme, and an ampicillin resistance gene for selection of recombinants. Of the 12 truncated \textit{wap}A constructs, eight truncated \textit{wap}A were found to express proteins reacting with a rabbit anti-WapA. These included \textit{wap}A-D to \textit{wap}A-K.

The twelve \textit{wap}A constructs are presented in Fig 2.
FIG. 2. Construction of truncated wapA. Numbers on the top of the line represent amino acid positions. P is the promoter; SP is the signal peptide; PC is the protease cleavage site; W is the Cell wall domain; and M is the Membrane-spanning domain.

Two of the clones constructed are of interest to the present study since they expressed proteins corresponding to the wild type full-length WapA (clone WapA-E), and a proteolytic cleavage site corresponding to the wild type extracellular AgA (clone
Passive immunization

Passive immunization has also been considered as a safe way to administer a dental vaccine. Specific polyclonal and monoclonal antibodies prevent colonization of the teeth by *S. mutans*. Lehner et al. (67) obtained a decrease in colonization by repeated application of a monoclonal antibody to *S. mutans* AgI/II onto the teeth of Rhesus monkeys. Passive immunization by oral administration of antibody to GTF-generated protection was performed against *S. mutans* in the rat model (124). Other studies demonstrated a more convenient means of passive immunization with immunized cow’s milk. Application to mice showed a significant decrease in caries activity as compared to animals receiving milk from unimmunized cows (91). Hatta and Michalek (42) showed that a mouth rinse containing egg yolk IgY from chicken immunized with whole *S. mutans* prevented the *in vitro* attachment of the *S. mutans* to saliva-coated hydroxyapatite and reduced plaque formation in human volunteers. Ma et al. (76) ingeniously used a tobacco plant as a means to mass produce monoclonal sIgA. Application of the plant-derived antibody to human volunteers reduced plaque formation.

Advantages and disadvantages are associated with each type of vaccine. Long-term protection is obtained with active immunization using recombinant proteins or peptides, but the response is variable between individuals. Rapid and dependable protection is obtained with passive immunization; however, this protection is of limited duration and frequent booster applications of antibodies may be required.

**Advantages and Risks of DNA-based Immunization**

DNA vaccines possess many of the most attractive aspects of modern vaccination
strategies and, therefore, offer potential solutions for other diseases for which conventional vaccines have not yielded preventive measures. In comparison with traditional vaccines, DNA-based vaccines offer a major advantage in terms of the ease with which they can be constructed, modified, and purified. DNA vaccines are much more stable and can be manufactured inexpensively on a large scale at high levels of purity, which make them ideal prophylactic agents for use in developing countries (24, 129). Moreover, many different antigens from the same or different pathogens can be cloned into a single vector. Therefore, making multivalent vaccines against pathogens will decrease the number of vaccinations necessary, especially in children (27, 82).

Since DNA vaccines utilize the host cell’s transcriptional and translational machinery to produce conformationally-specific antigens, the native conformation of epitopes is conserved. An appropriate tertiary structure of protein conserved following administration of a DNA vaccine is essential for the induction of conformationally specific antibodies and protective immunity (36, 117). DNA vaccines can initiate both cellular and humoral immune responses that are long lasting (24, 61, 103).

To date, DNA vaccines have not shown any of the possible adverse effects that have been previously discussed as potential safety issues associated with DNA-based immunization, which include the integration of the plasmid DNA into the host genome (88, 141), leading to insertional mutation, induction of tumor formation by activation of oncogenes, or auto-immune disease due to the induction of anti-DNA antibodies (66, 143). The safety of DNA vaccines in humans has been demonstrated in clinical trials against several diseases and specific pathogens including cystic fibrosis, the hepatitis B virus, herpes simplex virus, HIV, malaria, and cancer (26, 78, 136).
Mechanism of DNA Vaccines

The cellular and molecular mechanisms by which DNA-based immunizations stimulate different types of T-cells have not been fully elucidated (64). The type of cells transfected may vary depending on the route and method of DNA delivery (61). After administration of antigen-encoding plasmid DNA, the vaccinee’s cells take up the DNA molecules and transport them to the nucleus for transcription, with eventual export of the transcribed messenger RNA to the cytoplasm for protein synthesis. In the nucleus, the plasmid DNA is retained in an extra-chromosomal location and thus an expression of the DNA is not influenced by surrounding chromosomal elements (39).

After translation at the ribosome, the antigens expressed intracellularly are processed and presented to T cells in the context of antigen presenting cell’s (APC) MHC class I or II molecules, depending on whether the antigen is processed through an exogenous or endogenous pathway (106). An antibody response is induced by secretion of expressed whole antigen or antigen peptides processed and presented to CD4+ T cells on the cell surface with host MHC class II by antigen-presenting cells. Transfected APC can also process the antigen to peptides which bind to an MHC class I molecule in the ER. This complex moves via the golgi apparatus to the cell surface where it can stimulate the cellular T cell response by activation of CD8+ cytotoxic T lymphocytes (CTL), which directly lyse infected target cells (104) (Fig. 3).
Mucosal Immune System and Salivary Immunity in the Oral Cavity

The mucosal surfaces provide the principal immune defense against most human pathogenic organisms. Mucosal immunity to a variety of antigens has been successfully induced by immunization with protein or DNA vaccine through mucosal routes including oral, nasal, and genito-rectal mucosa (134). The major antibody isotype found in external secretions is sIgA, which can traverse epithelial membranes and help provide specific immunity against pathogens, including cariogenic S. mutans (37). All the mucosal routes...
tested induced the production of secretory IgA in the immunized area, as well as at distant mucosal surfaces (84, 90).

One major function of the mucosal immune system is to develop salivary IgA antibodies in newborn infants (34). In mucosal immune systems, antigens or microorganisms are taken up by specialized epithelial cells termed M cells which overlie organized lymphoid follicles in tonsils and adenoids (Waldeyer’s ring) and on intestinal Peyer’s patches. After uptake and transcellular transport by the M cells, the antigenic materials are processed by underlying APCs, which present them to T helper cells. B cells are stimulated by both T-cell activation and specific cytokines. B cells then, differentiate into precursors of IgA-secreting plasma cells.

Following activation of B and T cells, the IgA-producing cells migrate to various mucosal effector sites in the mucosal tissues including the stroma of the salivary glands, where terminal differentiation of the B lymphoblasts into IgA-secreting plasma cells occurs under the regulation of cytokines secreted by the T cells and epithelial cells. The resulting polymeric IgA secreted in the salivary glands is taken up by a polymeric Ig receptor on the basolateral surface of glandular epithelial cells and released into the saliva with a bound secretory component to form salivary sIgA (81, 84, 90). Secreted salivary IgA, in turn, interferes with S. mutans binding to tooth surfaces via both sucrose-independent and sucrose-dependent mechanisms (122). Immunoglobulins derived from circulation pass through the gingival crevice into the oral cavity. These Igs, which include IgM, IgG, and IgA, are proportionally lower than sIgA in saliva compared to relative proportions in blood plasma (37).
Caries Vaccine Route to Mucosal Immune Response

Depending on the route of immunization and the type of adjuvants utilized, development of systemic and/or mucosal immunity can be favored. Induction of specific mucosal immunity with production of specific salivary IgA is the goal of a dental vaccine (37, 122). In mucosal system, the delivery of vaccine at any mucosal route results in the induction of secretory IgA antibody at the delivery site as well as in the distant mucosal sites (90). Oral immunization induces mucosal immune response in the gut-associated lymphoid tissues (GALT), one of the principal inductive sites of sIgA antibody response, which consist of the Peyer’s patches, the appendix, and solitary lymph nodes in the gastrointestinal tract (83).

One drawback of the oral route for immunization is the damaging effects of stomach acidity on the antigen. Thus, for oral immunization, the DNA vaccine has to be encapsulated in enteric-coated tablets, or delivered by intra-oral Jet in the cheek, which is also the case with protein vaccines (74). Rectal immunization is also an effective inductive method for a mucosal immune response. Inductive potential of the rectal route for salivary IgA responses was considered as an alternative route to mutans streptococcal antigens (65).

Of all the mucosal routes tested, intra-nasal application of protein or DNA vaccines has gained much popularity because it is not invasive (32, 38, 51, 92). This route is especially convenient for the immunization of young children, who cannot yet swallow enteric-coated tablets. Furthermore, unlike oral vaccine, a nasal vaccine does not need to be encapsulated into enteric-coated tablets and consequently costs less to prepare.
Intranasal immunization with a *S. mutans* glucan-binding region of glucosyltransferase (51) and a *S. mutans*-enriched fimbrial preparation (32) elicited specific salivary IgA production and protective immunity. Intranasal immunization with a DNA vaccine mixed with cationic lipids has been shown to be immunogenic. Immunization of animals with a luciferase gene-DNA construct complexed with cationic proteins resulted in the expression of luciferase in nasal tissue and an induction of a systemic and humoral response after a single dose of the vaccine (58).

A number of adjuvants have been identified as stimulating a mucosal response to protein or DNA vaccines. Adjuvants used in protein or peptide vaccines include cholera toxin CT, cholera toxin B subunit (CTB), and a detoxified heat labile *E. coli* toxin (LT R292G) (25, 142). Adjuvants used in DNA vaccines include liposome and various cationic lipids (57, 58, 89, 98). Further stimulation of the immune response may be obtained by co-immunization with genetic adjuvants consisting of plasmids encoding cytokines, for example IL-4 or IL-10, to induce a Th2 type response, or IL-12 and/or a granulocyte/macrophages colony stimulating factor to obtain a Th1 type of response (27, 92, 102, 139).
MATERIALS AND METHODS

Structural Analysis of the Wall-associated Protein A Gene (wapA)

Antigenicity prediction algorithms of the WapA protein

The identification of B cell epitopes within a WapA protein sequence was analyzed using four different prediction algorithms: Hopp-Woods, Parker, Welling, and Kolaskar and Tongaonkar. The hydropathic profile of the WapA was calculated using the Hopp-Woods algorithm (45), which assumes that antigenic sites are primarily hydrophilic at the surface of the protein. The Parker method (96) of prediction uses an experimentally-determined hydrophilic scale of polypeptides derived from high-performance liquid chromatography (HPLC) parameters for the antigenic determinants. Prediction of antigenic regions using the method of Welling (137) is based on the amino acid composition in known antigenic peptides. A semi-empirical algorithm of Kolaskar and Tongaonkar (60) uses data from both experimentally-determined epitopes and physicochemical properties of amino acids for the prediction of antigenic determinants.

Prediction and analysis of MHC Class II binding regions

Potential T cell epitopes within AgA or WapA were identified using a matrix-based quantitative algorithm, publicly available on line at http://www.imtech.res.in/raghava/propred/. The promiscuous binding regions were located and quantified by a virtual matrix for 51 known MHC class II alleles. Binding probabilities were set at a 3% threshold representing the 3% best scoring natural peptides.
**Analysis of wapA DNA sequence for glycosylation**

Inappropriate glycosylation of bacterial proteins that results in conformational change, thus causing alteration or loss of immunogenicity, has been of concern in DNA vaccine preparation (48, 50). Potential glycosylation sites of the WapA protein were evaluated. Prediction analysis of the N-glycosylation or O-glycosylation sites in WapA based on the amino acid sequence was performed using NetNGlyc (35) or a NetOGlyc prediction server (40, 41) available from URL: http://www.cbs.dtu.dk/services/.

**Kozak sequences on the 5′ untranslated region (UTR) of the mRNA transcript**

Initiation of translation is regulated by two fundamentally different mechanisms in prokaryotes versus eukaryotes. In prokaryotes, the ribosome binding site (RBS), also called the Shine-Dalgarno sequence, is engaged in its recognition by ribosome for efficient and accurate translation of mRNA. In eukaryotes, however, a 40S ribosomal subunit locates the initiator AUG codon by scanning the mRNA from the capped 5′ end for the first AUG codon. It has been proven that a specific translational initiating sequence called the Kozak consensus sequence is required for efficient translation initiation in higher eukaryotes (62). Prokaryotes and some eukaryotes do not have a Kozak sequence in their genes. To improve the translation efficiency of an expressed wapA gene in mammalian hosts, the Kozak sequence ANNATGG was incorporated into the wapA or agA forward primer in the process of caries DNA vaccine construction.

**Codon usage**

Codon usage was another topic of concern in bacterial DNA vaccine development. There is a significant variation of codon usage bias in all organisms and even between different genes. The difference in the codon usage can seriously limit the
gene expression efficiency in a heterologous system. Codon optimization may be considered in case the expression of the cloned gene is hampered due to the codon bias. In such case, the alteration of rare codons of the foreign gene to make codon usage match the available tRNA pool within the host cell may benefit by improving the expression rate of heterologous genes.

Commonly used codons for *S. mutans* serotype c or wapA were determined from the Codon Usage database located at www.kazusa.org.jp/codon/ and analyzed against that of *Homo sapiens* or *Mus musculus* using the graphical codon usage analyzer available at www.gcua.de. To locate cumulative low codon usage from wapA, each codon position of wapA sequence was analyzed against the codon usage table of *Homo sapiens* or *Mus musculus* using the codon usage analyzer. Codons were optimized according to the codon usage tabulated from GenBank.

**Identification and Characterization of Collagen Binding Properties by the WapA**

**Bacterial strains and growth conditions**

The *S. mutans* strain used for cloning and expression was GS-5 (serotype c) (our laboratory stock). *S. mutans* were grown in brain heart infusion (BHI) (Fisher Chemical Co., Cincinnati, OH) broth at 37 °C without agitation, or on BHI agar containing 1.5% agar at 37 °C in the presence of 10% CO₂.

**Specificity and localization of collagen binding domain**

The nucleotide sequence and the deduced amino acid sequence of wapA indicated that it contained a sequence specific collagen binding domain. Further analysis on the specificity and localization of the collagen binding domain of the WapA was performed with different functional domain algorithms including the NCBI Conserved Domain
Database (CDD) (80) and Protein Families (Pfam) Applications (7) of these programs.

*Structure prediction of collagen binding domain in WapA*

In order to investigate the biochemical function of the putative collagen binding domain, secondary structure analysis was carried out with the 3D-PSSM program (31, 56) using the amino acid sequence of the putative collagen binding domain in the WapA. The predicted secondary structure was matched against the 3D-PSSM fold library (http://www.sbg.bio.ic.ac.uk/~3dpssm/), which contains solved crystal structures. The protein sequence was also scanned against the SCOP (Structural Classification of Proteins) database, where protein domains are classified into four levels: family, superfamily, fold, and class (72).

*Cloning, expression and purification of recombinant collagen binding domain (rCBD) of WapA*

A C-terminal truncated version of *wapA* (*wapA*-G) encoding the AgA was used as a clone for a putative recombinant collagen binding domain (rCBD). A 885 bp DNA fragment of *wapA* lacking the promoter, the signal sequence, and the hydrophobic C-terminal region was cloned into the pGEX-6P-1 glutathione S-transferase (GST) fusion vector and hosted in *E. coli* BL21 (DE3). rCBD was overexpressed by inoculating 500 mL of 2x YTA medium with 5 ml of overnight culture. The culture was continuously grown at 37 °C until the *A*<sub>600</sub> reached around 1. IPTG was added to a final concentration of 1 mM to induce the expression of the cloned gene.

At 6 h post incubation, the cells were harvested by centrifugation at 7,700 x g for 10 min at 4 °C, and washed with 250 ml cold PBS containing 1 mM Phenylmethylsulphonylfluoride (PMSF), followed by 1 min sonication on ice and
centrifugation at 12,000 x g for 30 min. Solubilization of the fusion protein was aided by mixing 20% Triton X-100 for 30 min. After centrifugation at 12,000 x g for 10 min at 4 °C, the supernatant was incubated on a rocker for 30 min at room temperature with 2 ml of the 50% slurry of Glutathione Sepharose 4B (Pharmacia).

The mixture was transferred onto the Sepharose column, and the column was washed with 15 ml of PBS. After the column was washed with 2.5 ml of PreScission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0), the PreScission Protease mix containing 20 µl of PreScission Protease with 230 µl of PreScission cleavage buffer was loaded onto the column and incubated on a rocker at 5 °C for 4 h to cleave off the GST tag. The unbound rCBD protein was eluted with 800 µl of PreScission cleavage buffer. Eluted fractions containing rCBD protein were analyzed by SDS-PAGE.

*Biotin Labeling*

Biotin labeling of the purified rCBD was performed with Sulfo-NHS-LC-Biotin reagents (Pierce, Rockford, IL) as follows. rCBD in an amine-free elution buffer (pH 7.2) was mixed with a 10-fold molar excess of 10 mM sulfo-NHS-biotin reagent solution with the protein, and the mixture was incubated at room temperature for 1 h. To remove free biotin, the reaction solution was dialyzed against 3 changes (4 L each) of PBS (pH 7.3) overnight using dialysis tubing with a nominal Molecular Weight Cut Off’s (MWCO) 12,000 – 14,000.

*Dot Blot Collagen binding assay*

To test the ability of WapA to bind to collagen, a soluble native type I collagen (Sigma, St. Louis, MO) was diluted in PBS to 5 µg/ml and was dotted (10 µl per dot) onto a piece
of nitrocellulose membrane. The membrane was blocked by incubation with 5% skim milk in PBS to which biotin-labeled rCBD was added, and incubated for 1 h at room temperature. After extensive washes with PBST (PBS with 0.05% Tween 20), the bound protein was detected by alkaline phosphatase-conjugated streptavidin (AP-streptavidin) (Sigma) diluted 1:1,000 which was incubated in PBS for 1 h at room temperature. After a final wash in PBS-T, the membrane was stained with a chromogenic substrate (25 mg o-dianisidine tetrazotized, 25 mg Naphthyl acid phosphate in sodium borate buffer, pH 9.7 containing MgSO$_4$-7H$_2$O added at 1.2 mg per ml). Bovine Serum Albumin (BSA) on the dot was tested in parallel as a negative control.

**Solid Phase Binding Assay**

To further evaluate the binding abilities of WapA to an immobilized native type I collagen, an ELISA assay was carried out as a function of collagen concentration. Native type I collagen (Sigma, St. Louis, MO) was diluted in PBS at a concentration of 5 µg/ml, and coated onto 96 well microtiter plates (Immobilon IV from Dynex, Chantilly, VA) overnight at 4 °C. After rinsing of the plate with PBS-T, the wells were blocked with 200 µl of 10% skim milk in PBS containing 0.02% sodium azide, for 1 h at room temperature. 100 µl of various dilutions (1 µg to 12 µg) of biotin-labeled, purified rCBD without a GST tag was added separately to the wells and the plate was incubated at 37 °C for 1 h.

The wells were washed with PBS-T and incubated with AP-streptavidin diluted 1:20,000 in PBS for 1 h at room temperature. The absorbance at 405 nm of each well was recorded using an ELISA reader against a PBS-coated well used as a blank. Wells coated with BSA served as a control for nonspecific binding. Binding properties were calculated and plotted as a function of the dose of protein rCBD.
Construction of DNA vaccines

Cloning of the wapA and agA genes into the eukaryotic expression vector

pcDNA3.1/V5/His-TOPO

The wapA and agA were obtained by PCR amplification of a previously constructed (145) plasmid pGEX-6P-GST containing wapA-E and wapA-G gene encoding WapA and AgA, respectively, using the following two sets of primers:

wapA:
Forward: 5’-ACC ATG GAC CAA GTC ACA AAT TAT ACA-3’
Reverse: 5’-TTA TTA GCA TTA TTA TCA ATG TTA-3’

agA:
Forward: 5’-ACC ATG GAC CAA GTC ACA AAT TAT ACA-3’
Reverse: 5’-TTA GTA GCC TGT TTG ATT GGA-3’

The initiation codon ATG was included in each forward primer to ensure expression in eukaryotic cells. The PCR was performed as described below. The Kozak consensus sequence ANNATGG was also inserted into wapA or agA forward primer to increase the expression level of the genes. The plasmid used for cloning was engineered to contain the pUC origin for replication and an ampicillin resistance gene for propagation in the E. coli cells.

To obtain the expression of the cloned gene in mammalian cells the plasmid was constructed so that the cloned gene was under the control of a strong viral promoter, while the stability of the mRNA was provided by sequences downstream from the polylinker consisting of a polyadenylation signal and transcription termination sequences from the bovine growth hormone gene. The PCR products (Fig. 4) were ligated
separately to pcDNA3.1/V5/His-TOPO employing the human cytomegalovirus (CMV) immediate-early promoter/enhancer (Fig. 5).

The resulting DNA constructs were transferred into *E. coli* Top 10 cells by the heat shock method as described by the manufacturer (Invitrogen). The transformed cells were plated on LB agar containing ampicillin, and the resultant colonies were cultured and screened by plasmid DNA isolation, followed by digestion with BstXI and analysis by agarose gel electrophoresis. The PCR amplification, using vector sequence as a forward or a reverse primer (specific to the T7 sequence or BGH Reverse primer site, respectively), and the insert sequence (forward or reverse primer listed above), was performed to test the correct orientation of the cloned gene in relation to the vector.
FIGURE 4. PCR-generated *wap*A-E and *wap*A-G genes. The *wap*A was amplified by PCR. The location of the terminal amino acid positions and the letter designation of the corresponding constructs are indicated.
FIGURE 5. Map of pcDNA3.1/V5-His-TOPO with wapA insert. The wapA-E and wapA-G were inserted into this vector at the TA cloning site and transformed into E. coli Top10.

PCR

PCR was performed for each sample with a 50-µl reaction mixture containing 1.5 mM MgCl₂, 0.125 mM dNTP mixture, 1 U of Taq DNA polymerase, 50 pmol of each primer, and 100 ng of template DNA. The PCR reaction was carried out in a gene cycler (Bio-Rad, Hercules, CA) programmed for an initial denaturation step of 3 min at 95°C and 30 cycles each of 30 s at 95°C, 45 s at 54°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were visualized on 1% low melting
point agarose gel containing ethidium bromide.

**DNA sequencing**

After construction, the plasmids were subjected to sequencing. Plasmids were purified to sequencing grade using the alkaline lysis method described below and sequences were determined by DNA sequence analysis conducted at the DNA sequencing core facility of the H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL). The primer sets for sequencing were T7 forward primer: 5'–TAA TAC GAC TCA CTA TAG GG-3', and BGH Reverse primer: 5'-TAG AAG GCA CAG TCG AGG-3'.

**Optimization of wapA and agA DNA vaccine production and quality control**

**Chloramphenicol Amplification of Plasmid DNA containing agA**

Observation of overnight cultures of the recombinant *E. coli* indicated a striking difference in plasmid yield between clones harboring the *wapA* and *agA* DNA construct, *agA* clones being much less productive for plasmid isolation. Clones with the *agA* plasmid construct had the tendency to lyse, therefore decreasing overall plasmid production. To increase the yield of GST-AgA plasmid, chloramphenicol amplification of *E. coli* derived plasmids was used. 250 ml culture of LB medium containing 100 µg/ml ampicillin was grown and when the culture reached an O.D. of 0.4 at 600 nm, chloramphenicol was added to a final concentration of 170 mg/L and incubated further for 12 to 16 h at 37 °C with vigorous shaking. Plasmid production from a 250 ml culture without chloramphenicol treatment was performed in parallel with the above condition for comparison.
**Purification of DNA vaccine**

Plasmid DNA was isolated from *E. coli* TOP-10 containing pcDNA-wapA or pcDNA-agA by a modified alkaline lysis method using the Qiagen HiSpeed™ Plasmid Maxi Kit (Valencia, CA) and following the manufacturer’s instruction. Briefly, transformed *E. coli* TOP-10 was grown in batches of a 250 mL LB medium containing 100 µg/ml ampicillin. Following overnight (16 h) incubation at 37 °C, cells were collected by centrifugation at 6,000 x g for 15 min at 4 °C. The cells were resuspended with the resuspension buffer containing 50 mM Tris Cl (pH 8.0), 10 mM EDTA, and 100 ug/ml RNase A and were followed by lysis with the cell lysis buffer containing of 200 mM NaOH and 1% SDS (w/v) for 5 min at room temperature.

After neutralization using the neutralization buffer (3.0 M potassium acetate, pH 5.5), the lysate was filtered and washed with wash buffer containing 1.0 M NaCl, 50 mM MOPS, 15% isopropanol (v/v). Plasmid DNA from the filtered lysate was then purified using a pre-equilibrated HiSpeed Tip and eluted in the elution buffer containing 1.0 M NaCl, 50 mM MOPS, 15% isopropanol (v/v). Plasmid DNA was concentrated and desalted by isopropanol precipitation and eluted in TE buffer. Purity and concentration of DNA was determined by optical density (OD) reading in UV spectrophotometer at 260 and 280 nm. DNA measured larger than 1.7 in the ratio of OD260/OD280 was used in this study. 1 OD is equal to 50 µg/mL of DNA.

**Plasmid DNA preparation for immunization study**

Plasmid in TE solution was then concentrated by precipitation with 0.7 volume of isopropanol in the presence of 1:10 volume of 3 M Na Acetate (pH 3.0). The DNA was collected by centrifugation at 17,210 g in SS-34 rotor for 10 min at 4 °C, washed with
70% ethanol, and centrifuged again for 10 min. The pellet was dried and resuspended in sterile PBS to reach a concentration of 2 mg/ml. DNA purity was assessed spectrophotometrically and the ratio of OD$_{260nm}$/OD$_{280nm}$ was calculated. DNA preparation with a minimum absorbance ratio of 1.7 was used in the present study. For quality control, DNA vaccine constructs were confirmed by BstXI digestions and agarose gel electrophoresis, and by DNA sequence analysis conducted at the DNA sequencing Core Facility of the H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL).

**Expression of *S. mutans* Wall-associated Protein A Gene in Chinese Hamster Ovary Cells**

**Cell culture**

Chinese hamster ovary (CHO) cells were obtained from M. Kimble (Department of Biology, University of South Florida, Tampa, FL). The CHO cells were cultured in DMEM supplemented with 10% FBS, 200 mM L-glutamine, 100 mM sodium pyruvate, and antibiotics/antimycotic (penicillin 0.5 mg/ml, streptomycin 1 mg/ml, and 0.25 mg of amphotericin B/ml of 0.85% saline) at 37°C in 5% CO2. The *E. coli* Top 10 cells were cultured in Luria-Bertani (LB) broth or on an LB plate containing 1.5% agar. Ampicillin was added at 10 µg/ml for the culture of recombinant *E. coli*.

**Plasmids**

A gene encoding WapA or AgA was isolated from the plasmid pGEX-6P-GST containing *wapA*-E or *wapA*-G, respectively, and cloned into the mammalian expression vector pcDNA3.1/V5/His-TOPO as described previously. For isolation and purification of plasmid DNA, Qiagen HiSpeed™ Plasmid Maxi Kit (Valencia, CA) was used.
Propagation and isolation of Plasmid DNA vector pcDNA3.1/V5-His TOPO

Propagation of the vector pcDNA3.1/V5-His-TOPO was carried out by transformation of *E. coli* TOP10 using the heat shock method. When the 50 µl frozen competent cells were just beginning to thaw, 100 ng plasmid DNA was added and incubated on ice for 30 min. The cells were heatshock treated by placing them in a 37 °C water bath for 1 min and then returned to ice. After 2 to 3 min on ice, 650 µl LB medium was added and the cells were incubated at 37 °C for 1 h in a shaker at 15 rpm. Then, 25 to 100 µl of the suspension were aliquoted into the middle of an LB agar plate containing 100 µg/ml ampicillin and evenly spread over the plate using a spreader. The plates were incubated at 37 °C overnight. The inserted plasmid DNA vector was isolated by a modified alkaline lysis method as described above.

Transfection of CHO cells

Transient transfection was performed using the cationic lipid pfx-8 according to the manufacturer (Invitrogen). Briefly, CHO cells were seeded in a 12-well microtiter plate at a density of 5 X 10⁴/well and incubated overnight to approximately 50% confluency. The cells were washed by aspiration with sterile PBS, and a transfection solution (1 µg of DNA mixed with lipid solution at a 1:6 ratio [v:v]) was added (1.0 ml per well). After 4 h of incubation, the transfection solution was aspirated and replaced with an equal volume of complete medium, and the incubation was continued for another 24 h. On the following day, the cells were detached by scraping, and the cell suspension was collected.

Adsorption of antiserum

To avoid nonspecific binding of the antiserum to CHO cells, the antiserum was
incubated for 1 h at room temperature and then overnight at 4°C with a piece of nitrocellulose filter on which CHO cells were immobilized, and the free surface was blocked with 10% skim milk in PBS. The nonbinding fraction was tested by a dot immunobinding assay.

*Dot immunobinding assay*

To investigate the expression of *wapA* and *agA* in transfected CHO cells, a sonic extract (prepared as described below) of each sample was dotted (10 µl per dot) onto a piece of nitrocellulose membrane, which was next blocked by incubation with 10% heat-inactivated horse serum in PBS. The filter was incubated for 1 h with the adsorbed rabbit anti-*S. mutans* cell wall (1:200 dilution in PBS), followed by extensive washing in PBS containing Tween 20 added at 0.05% (PBS-T) and a 1-h incubation with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin diluted 1:1000 in PBS. Following an extensive wash in PBS-T, the peroxidase activity was identified using a chemiluminescent substrate (ECL for HRP from Pharmacia). A piece of X-ray film (Kodak) was next exposed to the filter for 1 min and developed in a Kodak M35A X-OMAT processor.

*Western immunoblot analysis of transfected CHO cells*

Expression of WapA and AgA in transfected CHO cells was investigated by Western blot analysis as follows. A sonic extract of the transfected CHO cells was prepared using two pulses of 30 sec each in a Vibra Cell™ (Sonics & Materials, Inc., Danbury, CT) set at 50 Hz. As controls, untransfected CHO cells and cells treated with the lipid alone were similarly processed. The sonic extract was incubated with the cracking buffer (0.019 M Tris, 0.5% SDS, 0.35 M 2-mercaptoethanol, 7.5% glycerol,
0.05% bromophenol blue) for 3 min in a boiling water bath. The particulate was sedimented by a 15-sec centrifugation at 6000 rpm, and the supernatant liquid from each sample was separated by electrophoresis in a 10% SDS-polyacrylamide gel. The protein bands were electrophoretically transferred to a piece of nitrocellulose membrane, which was then blocked by incubation with 10% horse serum in PBS for 1 h at room temperature. The membrane was processed for immunochemical staining as described above for dot immunobinding assay.

**Immunochemical staining of transfected CHO cells**

A 100-µl sample of each cell suspension was transferred to a microscope slide, let dry, and fixed with ice-cold acetone. The slide was next blocked with 10% nonfat dry milk for 1 h at room temperature with gentle rocking. The cells were incubated at room temperature for 1 h with a rabbit anti-*S. mutans* cell wall diluted 1:200 in PBS. The slide was next incubated with PBS containing 10 mM EDTA to inactivate endogenous alkaline phosphatase, as described by Dao (21), gently washed three times with PBS-T, then immediately incubated for 1 h with an alkaline phosphate-conjugated goat anti-rabbit immunoglobulin diluted 1:30,000 in PBS. After incubation, the slide was washed three times with PBS-T and stained with a Fast Red RC solution prepared as described by Sigma. After color development, the slide was rinsed three times with deionized water, observed on the light microscope, and photographed using an Olympus digital camera Model 3030Z (Olympus America Inc, Melville, NY).

**Differential immunogenicity of a DNA vaccine containing the *S. mutans* wall-associated protein A gene versus that containing a truncated derivative antigen A lacking in the hydrophobic carboxyterminal terminal region**
Animals

Six-to-eight-week-old female Balb/c mice were purchased from the National Cancer Institute (Frederick, MD), and maintained in the controlled animal facilities of the University of South Florida Medical School. All protocols using mice were approved by the Institutional Animal Care and Use Committee at the University of South Florida. The University program and facilities for animal care and use are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Bacterial strains and cell line

*E. coli* TOP10 cell (Invitrogen, Carlsbad, CA) clones harboring pcDNA-wapA or pcDNA-agA were prepared in a previous study as described above. The recombinant *E. coli* strain BL21 (DE3) transformed with the expression plasmid pGEX 6.1 Glutathione S-Transferase (GST), containing wapA or agA gene and expressing WapA or AgA as a GST fusion protein, were prepared previously and used in the present study as a source of recombinant antigens. HeLa cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) for use in *in vitro* transfection experiments.

DNA Vaccine

The eukaryotic expression vector pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA) containing the full length wapA gene (previously wapA-E) or truncated agA gene (previously wapA-G), encoding WapA and AgA, respectively, were cloned into the *E.coli* TOP 10 in a previous study and are referred to in the present report as pcDNA-wapA and pcDNA-agA vaccine, respectively.
Transfection of pcDNA-wapA in HeLa cells

To determine the optimal transfection conditions, HeLa cells were transfected with pcDNA3.1/V5-His-TOPO containing the β-galactosidase gene (pcDNA-β-gal) mixed with the cationic lipid DMRIE-C at various ratios. Briefly, HeLa cell cultures were seeded with 5 x 10⁴ cells in a 24-well microtiter plate and grown overnight at 37 °C in a CO₂ incubator until approximately 80% confluence. The cells were washed in a serum-free medium and incubated with a transfection solution consisting of 2 µg of pcDNA-β-gal mixed with 2-24 µg of DMRIE-C in a serum-free medium. After 4 h of incubation, a growth medium containing 20% fetal bovine serum (FBS) was added, and the incubation was continued for another 24 h. On the following day, the cells were solubilized with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL), and centrifuged, and the cell suspension was collected for β-galactosidase assay. 50 µl of cell lysate was added into the 96-well plate, mixed with 50 µl of 2X Assay Buffer (Promega, Madison, WI) that contained the substrate ONPG (O-nitrophenyl-β-D-galactopyranoside). β-galactosidase activity was measured at 420 nm with a spectrophotometer.

Immunization of mice by the intranasal inhalation technique

To investigate the diffusion of 100 µl of vaccine solution when administered by the intra-nasal drop technique, 100µl of sterile PBS containing 1/10 volume India ink were delivered slowly as droplets at the opening of the nostrils of the non-anaesthetized mouse held tightly by hand. Hand pressure was applied to the lower mandible of the mouse in order to reduce swallowing of the instilled material during administration. This procedure was performed on four mice, which were then sacrificed immediately and 30
min later. The mice tissues including nasal and oral cavities, lung tissue, esophagus, and trachea were inspected for any trace of the India ink as an indicator of vaccine diffusion.

**Immunization of mice**

Female BALB/c mice were distributed into five groups of three each and were immunized intra-nasally with one of the following: 50 µg pcDNA-\textit{wap} (Group 1) or pcDNA-\textit{ag} (Group 2) combined with 150 µg of the cationic liposome carrier DMRIE-C (1:3, w/w). The negative control mice were not immunized (Group 3) or administered with empty plasmid vector mixed with DMRIE-C at the same ratio (Group 4), or with PBS mixed with DMRIE-C (Group 5). Two doses were administered at three-week intervals.

**Collection of saliva and blood samples**

Saliva and blood samples were collected prior to immunization, and 3 and 6 weeks after boost immunization. Briefly, saliva samples were collected after intra-peritoneal injection of 100 µl of 1 mg/ml pilocarpine (Sigma, St. Louis, MO) in PBS to stimulate saliva secretion. Saliva was immediately centrifuged at 735 g for 30 min. The supernatant was collected, the protease inhibitor Phenylmethyl sulfonyl fluoride (PMSF, 100 mM) was added at 1% (v/v), and the sample was stored at –20 °C until use. Blood was collected from the tail vein, allowed to clot at room temperature for 1 h and stored 30 min at 4 °C. Serum was then separated from the clot by centrifugation at 3,210 g for 30 min and stored at –20 °C. Saliva samples from the mice in each group were pooled for analysis. Serum samples were collected and tested individually.

**Dot immunobinding assay**

To investigate the presence of specific IgA in the saliva of the immunized mice, a
piece of 96 well-embossed nitrocellulose membrane was dotted with 10 µl per dot of 5 µg/ml of purified GST-AgA fusion protein and allowed to dry at room temperature. After 10 min of incubation in the cold, the filter was blocked by incubation with PBS containing 2% Tween 20, at room temperature for 1 h. Next, small NTC pieces containing antigen dots were cut out and incubated individually overnight at 4 °C with the diluted (1:2) saliva samples to be tested. The pieces of NTC were washed six times with in PBS-T, and then incubated for 1 h with a HRP-conjugated goat anti-mouse IgA diluted to 1:10,000. After a final wash in PBS-T, the membrane was incubated with a HRP chemiluminescent substrate (obtained from Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min and exposed to an X-ray film (Kodak) for development.

**Enzyme linked immunosorbent assay (ELISA)**

Saliva and serum samples were assayed for anti-WapA salivary sIgA and serum IgG using enzyme-linked immunosorbent assay (ELISA). The purified GST-AgA fusion protein was used as the coating antigen (146). Immunolon IV 96-well microtiter plates (Dynex, Chantilly, VA) were coated overnight at 4 °C with 100 µl per well of 5 µg/ml solution of antigen in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). The wells were blocked with 200 µl of 10% skim milk in PBS for 1 h and washed four times with PBS containing 0.05% Tween 20 (PBS-T). Saliva was diluted 1:2 and serum at 1:16 in PBS, each sample was added in serial 2-fold dilution to the wells, and the plate was incubated at 4 °C overnight. After three washes with PBS-T, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA diluted to 1:1,000 in PBS-T was used. The plates were incubated for 1 h at room temperature. Following extensive washes in PBS-T, 100 µl of QuantaBlue Fluorogenic peroxidase substrate (Pierce, Rockford, IL)
was added to each well and incubated for 30 minutes at room temperature. Peroxidase activity was detected on the fluorometer by reading the excitation and emission at 325 nm and 420 nm, respectively. The end point titers antigen-specific IgG and IgA were determined in triplicate and expressed as the reciprocal of the highest dilution giving an OD corresponding to 3 X the standard deviation above the mean of the background control sample.

**Assay of antibodies to recombinant AgA**

Pooled saliva from each group of mice was extensively absorbed with *E. coli* BL21 homogenate immobilized on nitrocellulose in order to remove unwanted antibodies. The *E. coli* recombinant clone expressing AgA and the negative control *E. coli* transformed with the vector pGEX-6P-1 alone were sonicated and centrifuged at 12,000 x g for 30 min. The soluble fraction was loaded in triplicate lanes (2 µg/line) and separated by SDS-PAGE followed by electro-transfer of the protein bands onto a piece of nitrocellulose. The protein bands were incubated with purified anti-WapA or –AgA antibodies and subsequent steps were as described previously for the dot immunobinding assay.

**Assay of antibodies to S. mutans WapA**

Cultures of *S. mutans* were grown in BHI supplemented with 2% sucrose for 16 h at 37 °C. Cultures were sonicated and an equal volume of sonic extract was loaded in 4 sets of duplicate lanes (2 µg/lane). SDS-PAGE separated protein bands were transferred to 0.2 µm nitrocellulose membrane at 100V for 1 h. Differential immunoreactivity of anti-WapA or –AgA antibodies to WapA was tested by incubating the membranes with each antiserum and following steps for the Western blot analysis as described above.
In vitro Adherence inhibition assay

The influence of salivary IgA on the sucrose-dependent adherence of *S. mutans* GS-5 was assayed *in vitro* and quantified as described by Olson *et al.* (94) with some modifications. At 6 weeks post-booster immunization, saliva samples collected from immunized or control mice, and the antigen-specific sIgA level was measured using ELISA. Saliva samples with equivalent levels of specific sIgA were sterilized by mixing with 1/10 volume of chloroform and the aqueous layer, separated by centrifugation for 2 min at 16,000 g, was diluted to 1:2 in PBS. 200 µl of saliva diluted in PBS was added into 24 well cell culture plates containing 200 µl of BHI medium prepared at two times the standard concentration and supplemented with 2% filter-sterilized (Millipore Corp.; 0.45 µm pore size) sucrose.

A saturated overnight culture of *S. mutans* was diluted to 1:100 in BHI medium and 10 µl of the diluted suspension was added into each well. Binding assay was carried out by incubation of the plate at 37 °C for 20 h. Saliva samples from pre-immunized or non-immunized mice were used as controls, and assayed as described above for the test samples. After incubation, non-adherent *S. mutans* were removed by aspiration of the medium, and the wells were washed three times with 0.5 ml of 0.9% saline. The washes were pooled.

For quantitative analysis, the bacteria were detached from the wells by washing four times with 0.5 ml volumes of 0.5 N NaOH, and the washes were pooled. *S. mutans* were collected from the washes by centrifugation at 6,000 g for 1 min, the supernatant was discarded, and the cell pellet was resuspended with 400 µl of 0.5 N sodium hydroxide. The optical density (OD) of the suspensions was measured at a wavelength of
540 nm and the mean adherence level was determined and expressed as percent OD of the total cells (non-adherent cells in the culture medium plus adherent cells detached from the well by NaOH).

**Dextran binding Assay**

Dextran-binding properties were investigated for GST-WapA or GST-AgA fusion proteins using a Biotin-conjugated dextran (Sigma, St. Louis, MO) by dot immunobinding assay as follows. GST-WapA and GST-AgA fusion proteins were obtained from the corresponding recombinant *E. coli* BL 21 after induction with IPTG and the recombinant proteins purified by chromatography on a glutathione-conjugated Sepharose column following the manufacturer protocol (Amersham, Piscataway, NJ). Next, 10 µl of 5 µg/ml of the purified protein was dotted onto a piece of 96 well-embossed nitrocellulose membrane and processed as described above for the dot immunobinding assay. The membrane was incubated with biotin-conjugated dextran for 1 h at room temperature, and the dextran-binding protein was identified by incubation with AP-Streptavidin solution at room temperature for one hour, followed by *in situ* staining of AP activity (21).

**Statistical analysis**

Data for serum or saliva antibody titers were logarithmically transformed and statistical differences were determined by using the nonparametric Mann–Whitney U-test. Data obtained from the adherence inhibition assay were also analyzed with the nonparametric Mann–Whitney U-test for the difference in the median values among the groups.
Efficacy of DNA versus Protein Vaccine: Relative induction of mucosal IgA response to \textit{S. mutans} Wall Associated Protein A

\textit{Experimental animals}

Balb/c female mice, 6-8 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and were maintained in the animal facilities of the USF medical school.

\textit{Cell lines}

HeLa cells were purchased from ATCC (Manassas, VA) and maintained in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum at 37 °C in 5% CO2.

\textit{Bacterial strains, plasmids, and media}

The three clones needed for this study were obtained previously and included the following: Clone 1 consisted of recombinant \textit{E. coli} TOP10 clone containing the \textit{wap}A gene cloned into the mammalian expression vector pcDNA3.1/V5/His-TOPO. The clones were used to propagate the plasmid containing \textit{wap}A-pDNA. The DNA plasmid, namely pcDNA-\textit{wap}A which encodes the \textit{S. mutans} WapA, was described previously. Clones 2 and 3 consisted of recombinant \textit{E. coli} BL21 containing the \textit{wap}A gene or \textit{ag}A gene cloned into the prokaryotic high expression fusion vector pGEX 6-1 GST. These clones, designated BL21-WapA and BL21-AgA, expressed the WapA and AgA, respectively, as a fusion protein with GST. The WapA or AgA expression plasmid, designated GST-WapA or –AgA, respectively, was also described previously.

\textit{Cloning of murine IL-5 cytokine gene into the mammalian expression vector}

To investigate the effect of IL-5 on salivary IgA production in the immunized
mice, recombinant *E. coli* HB101 containing IL-5 cDNA cloned into vector pBR322 was obtained from ATCC. PCR amplification was performed using primers for IL-5, which was designed based on the IL-5 DNA sequence found in Genbank (Accession No. NM_010558). The primers used were as follows: Forward, 5’-ACC ATG AGA AGG ATG CTT CTG CAC-3’ and Reverse, 5’-TCA GCC TTC CAT TGC CCA-3’. The resulting fragment of 402 bp was cloned downstream of the CMV promoter into pcDNA3.1 vector. PCR and cloning protocols used were described previously.

**Cloning of the cholera toxin B-subunit (ctb) gene into the mammalian expression vector**

The *ctb* plasmid construct was prepared by cloning a ctb gene into the mammalian expression vector pcDNA3.1/V5/His-TOPO according to the protocol above. Genomic DNA of Vibrio cholera was obtained from ATCC. Since the *ctb* gene sequence was known (GenBank accession no. D30053), the genomic DNA obtained was used as a template for the amplification of the CTB gene by PCR using specific primers. The primers used were: Forward, 5’-ACC ATG ACA CCT CAA AAT ATT ACT GAT T-3’ and Reverse, 5’-TTA ATT TGC CAT ACT AAT TGC GG-3’.

**Cloning of the ctb gene into a pGEX expression vector**

The *ctb* gene was cloned into prokaryotic high expression pGEX-6P-1 glutathione S-transferase (GST) fusion vector using the manufacturer protocol (Pharmacia). PCR primers derived from the *ctb* gene sequence in GenBank (accession no. D30053) was used to amplify the ctb gene without signal peptide and was subcloned into the TA cloning vector pCR2.1-TOPO (Fig. 6) The ctb gene digested with EcoRI was ligated in frame with GST-tag into the pGEX GST fusion vector (Fig. 7). The *E. coli* BL21 competent cells were transformed with the ligation mixtures, the transformant containing
pGEX-ctb was grown at 37 °C, and expression was induced with 1mM IPTG as described previously. After induction, cells were lysed by sonication and were purified by the Glutathione Sepharose 4B protocol. The purity and identity of the protein was confirmed by 10% SDS-PAGE followed by Western blot analysis.

FIG. 6. Map of pCR2.1-TOPO plasmid vector. ctb gene was cloned into pCR2.1-TOPO.
FIG. 7. Map of prokaryotic expression vector pGEX-6p-1. The pCR2.1-TOPO clone containing \(ctb\) gene was excised and subsequently cloned via the EcoRI restriction enzyme site into pGEX-6p-1 plasmid from which proteins can be expressed as fusion proteins with glutathione S-transferase (GST).

**DNA sequencing**

After construction, the plasmids were sequenced to confirm the orientation and sequence. Plasmids were purified to sequencing grade using the alkaline lysis method described below and Sequences were determined by DNA sequence analysis conducted at the DNA sequencing core facility of the H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL). The primer sets for sequencing were T7 forward primer: 5′-TAA TAC GAC TCA CTA TAG GG-3′ and BGH Reverse primer: 5′-TAG AAG GCA CAG TCG AGG-3′.
Expression and Purification of WapA and AgA from E. coli recombinant clones

To obtain enough proteins for the immunization study and for the preparation of rabbit antibodies, isolation of these proteins was scaled up. Both WapA and AgA were obtained from the corresponding recombinant E. coli BL21 (DE3) clones. The clones expressing GST-WapA and GST-AgA were cultured at 37 °C in an incubator shaker overnight in batches of 500 ml 2x YTA medium containing 100 µg/ml ampicillin. The following day, the overnight culture was diluted 1:100 into fresh pre-warmed 2x YTA medium, and incubation was continued at 37 °C until the A$_{600}$ reached about 1. Induction of protein expression and preparation of the protein lysate was followed in the same manner as described above.

The protein lysate was incubated on a rocker for 30 min at room temperature, after 0.5 ml of glutathione-linked Sepharose 4B (Pharmacia) in a 50% slurry was added. The mixture was transferred onto a chromatographic column, and washed by adding 50 ml of PBS/1 mM PMSF. The fusion protein was eluted by 10 mM reduced glutathione in five aliquots of 300 µl each. Cleavage of GST tag was carried out by incubation with PreScission protease as described previously. The quantity of the AgA was determined by OD reading at 280 nm. 1 OD$_{280}$ equals 0.5 mg/ml, the formula based on the extinction coefficient of free GST. The purity and solubility of the AgA was analyzed by SDS-PAGE.

The amount of recombinant proteins without GST tags was determined by the bincinchonic acid method using reagents and protocols obtained from Sigma. Briefly, 0.1 ml of the sample was mixed with 2 ml of protein determination reagent (1 part Copper sulfate pentahydrate 4% solution added to 50 parts of Bicinchonic acid solution).
The mixture was mixed by vortex followed by incubation at 37 °C for 30 min. After cooling of the tube to room temperature, the absorbance was recorded at 562 nm, and the concentration of protein determined from a standard curve established with solutions of bovine serum albumin (20, 40, 60, 80, 100 µg/ml) assayed in parallel.

Measurement of GST Activity by CDNB Assay

Rapid enzymatic detection assay using the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) was used to optimize conditions for expression or to quantify the level of expression of GST fusion protein. High affinity between the GST and 1-chloro-2,4-dinitrobenzene (CDNB) resulted in a CDNB-glutathione with a strong absorbance at 340 nm. When a sample containing a GST fusion protein was incubated in a CDNB assay solution containing CDNB and glutathione, the relative or absolute amount of GST fusion protein in the sample could be calculated by comparing a standard curve of \( A_{340}/\text{min} \) versus fusion protein amount. Briefly, the total volume of 1000 µl CDNB assay solution (100 mM CDNB, 100 mM reduced glutathione, 10X reaction buffer and distilled water) was mixed and transferred into two UV-transparent cuvettes with 500 ml volume each.

The soluble (post-sonicate) fraction of the fusion protein or the GST-WapA purified soluble protein was added into the sample cuvette to be assayed. To the other cuvette (blank cuvette), a volume of 1X reaction buffer in an amount equal to the sample was added. Measurements were conducted at 340 nm in a UV spectrophotometer at one-minute intervals for 5 minutes. Blanking the spectrophotometer with the blank cuvette was done before each reading of the sample cuvette. Recorded absorbance readings were used to calculate \( A_{340}/\text{min/ml} \) values which could be used as a relative comparison of
GST fusion protein content between samples.

*Production of protein vaccines and DNA vaccines against S. mutans WapA and AgA*

The plasmid DNA containing *wapA* or *agA* gene and the corresponding recombinant WapA and AgA were isolated and an average yield was calculated as a function of culture volume for comparison. Plasmid DNA containing *wapA* or *agA* was prepared from *E. coli* TOP-10 by a modified alkaline lysis method using a Qiagen HiSpeed™ Plasmid Maxi Kit (Valencia, CA), as described previously. The goal was to optimize the isolation of these antigens as recombinant proteins or as genes separately inserted into a eukaryotic expression plasmid vector, and to compare the yield between the two target antigens. The purified recombinant protein and DNA vaccines were used in the immunization study.

*Immunogenicity of the fusion proteins*

Considering that fusion proteins can elicit a strong and specific antibody response to the target antigens, it is hypothesized that the presence of GST did not interfere with the immunogenicity of the target protein. Indeed, the antigenicity of WapA-GST and AgA-GST was demonstrated by immunodot analysis using a rabbit antibody to the wild type WapA and AgA. Considering the advantage of skipping the cleavage of GST from the fusion proteins in term of time and money, this step was omitted from our production of protein vaccines.

*Preparation of rabbit polyclonal antibody against WapA*

Rabbit polyclonal antibody against WapA was prepared for the identification and immunoochemical analysis of WapA and AgA in the present study. Briefly, six 4 kg female albino rabbits from the New Zealand strains were immunized intramuscularly
with 100 µl of 1mg/ml of WapA mixed with TiterMax Gold (Sigma) adjuvant Freund complete adjuvant (v:v). Booster injections were administered with WapA or AgA mixed with the TiterMax Gold adjuvant (v:v) at three-week intervals. Blood was collected before immunization and one week after each booster injection.

The serum was separated by centrifugation and decomplexed by heating at 55 °C in a water bath for 30 min. The serum was sterilized by adding 1/10 vol of chloroform, mixing, and centrifugation to obtain the aqueous upper layer containing the antibody. Dot immunobinding assay and ELISA were performed to test the serum for the presence of a specific IgG antibody, as described previously. Larger volumes of blood were obtained when a dilution of the antibody at 1:500 in PBS gave a positive reaction in immunodot analysis.

Absorption of anti-E. coli and anti-GST antibody from rabbit serum

Nonspecific binding of the antiserum to WapA was removed by extensive absorption of serum against E. coli and GST. Anti-E. coli absorption was completed as described previously. For the absorption of anti-GST antibodies, Glutathione Sepharose 4B attached with GST was packed onto a gravity-flow column. Serum was loaded on a column and incubated for 1 h at room temperature. Incubation was continued for 1 h at 4 C, and the absorbed serum flow-through was collected. Fractions were tested for antibody activity using dot immunobinding assay (Fig. 8).
FIG. 8. Absorption of immune serum. To remove non-specific binding of antiserum to WapA, anti-*E. coli* and −GST activity was absorbed from serum against sonicated *E. coli* fraction and purified GST dotted on a nitrocellular membrane. A1 (anti-WapA), A2 (anti-GST) or A3 (anti-*E. coli*) shows its antibody activity reacting on purified GST-WapA, GST, or *E. coli* fraction, respectively, before absorption. B1, B2, and B3 are corresponding antibody activity after absorption.

**Purification of IgG antibody against WapA**

WapA or AgA specific antibodies from post-immune rabbit serum were purified using the Melon™ Gel IgG Purification Kit, by the procedure specified by the manufacturer. Serum was diluted by 1:10 with melon gel purification buffer and loaded on a gravity-flow column. The specific antibodies were eluted with Melon Gel Purification Buffer, and the absorbance of the antibody fractions were measured at 280 nm.

**Induction of WapA in presence of sucrose**

 Cultures of *S. mutans* were grown in BHI or BHI supplemented with 2% sucrose for 16 h at 37 °C. Cultures were sonicated and an equal volume of sonic extract was loaded in 4 sets of duplicate lanes (2 μg/lane). SDS-PAGE separated protein bands were
transferred to 0.2 µm nitrocellulose membrane at 100V for 1 h. Expression of WapA in the presence of sucrose was evaluated by probing membranes with anti-WapA antibodies.

**Immunization protocols**

The mice were divided into 3 groups as follows: Group 1 mice were intra-nasally immunized with pcDNA-wapA alone, or with pcDNA-il-5 encoding IL-5 or with pcDNA-ctb encoding the cholera toxin B subunit (CTB). Group 2 mice were immunized with WapA and CTB. Group 3 mice were primed with pcDNA-wapA and boosted with WapA.

**Collection of saliva**

Mice were injected by i.p. 100 µl pilocarpin (1 mg/ml) to induce salivary flow. Saliva was collected by aspiration from the cheek pouch. Phenyl methyl sulfonyl fluoride (PMSF) was added at 1mM as a protease inhibitor and the saliva was stored at -70 °C until use, at which time the sample was centrifuged and the supernatant used in immunochemical assays.

**Immunodot analysis**

The presence of specific sIgA expressed in the saliva of immunized animals was detected by immunodot analysis of the *in situ* staining for alkaline phosphatase. A purple coloration was indicative of the presence of salivary sIgA against WapA. Saliva from unimmunized mice served as negative controls.

**Enzyme-linked immunosorbent assay**

To follow the production of specific salivary IgA over time, ELISA was used to determine the salivary IgA titre. Production of salivary IgA (mean titer from 6 animals and standard deviation) was calculated and plotted as a function of time post-immunization.
RESULTS

B cell epitope prediction of the WapA protein

Identification of B cell epitopes on the WapA was made using different parameters for prediction of antigenic determinants, including hydrophilicity, accessibility and flexibility, experimental antigenic determinant data, and physicochemical properties of amino acids (Fig. 9). Consensus epitopes were identified based on predicted epitopes and their frequency within different algorithms. Table 1 shows the consensus antigenic determinants in the WapA sequence, indicating 11 strongly antigenic sites within AgA and 4 sites around the membrane-spanning domain.
FIG. 9. B cell epitope prediction by four algorithms; Hopp and Woods (A), Parker (B), Welling (C), and Kolaskar and Tongaonkar algorithm (D). Values greater than 0 are predicted antigenic sites and are likely to be exposed on the surface of a folded protein (A, B, and C). Kolaskar and Tongaonkar algorithm (D) gives sites that are potentially antigenic a value above 1.0.
TABLE 1. Consensus antigenic determinants within the WapA sequence.

<table>
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<tr>
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<th>Start Position</th>
<th>Sequence</th>
<th>End Position</th>
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<td>440</td>
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</table>

Detection of MHC class II binding regions

The amino acid sequences of AgA and its larger precursor WapA were analyzed using virtual matrices algorithms by which binding values were compared with 51 sets of known MHC class II alleles. MHC Class II binding specificity was shown as the
percentage of motif matches within the sets of 51 DRB alleles that cover more than 90% of MHC Class II molecules expressed on antigen presenting cells. Analysis at a 3% threshold identified 6 more promiscuous binding regions in WapA, but not in AgA (Fig. 10). One of these regions, beginning at residue 400 (Fig.10), showed the highest promiscuous binding regions with over 90% promiscuousness for the predicted region of peptide against 51 known MHC II alleles.
FIG. 10. Detection of MHC-II promiscuous binding motifs in WapA. Motifs associated with 51 MHC class II alleles (listed under the graph) were identified in the sequence of WapA using a MHC class II binding motif-matching algorithm. Each peak represents percent of promiscuousness for the predicted region of peptide against 51 known MHC II alleles that cover more than 90% of MHC Class II molecules expressed on antigen presenting cells. Six distinct regions beginning at residue 294, 300, 382, 388, 394, and 400 were identified which were within the WapA and not AgA.
Identification of N- or O-glycosylation sites in WapA sequence

Asn-Xaa-Ser/Thr sequences were identified on WapA, and asparagines residues predicted to be N-glycosylated were determined based on the potential value calculated using the NetNGlyc server. Six sites were predicted to be N-glycosylated with high specificity across the WapA sequence (Fig. 11). The O-glycosylated sites were predominantly predicted to be in coil regions (Fig. 12).

**FIG. 11.** Prediction of N-glycosylation sites in WapA sequence. The X-axis represents protein length from N- to C-terminal and the Y-axis the predicted N-glycosylation potential at that position. Values above the threshold (horizontal line at 0.5) are predicted to be N-glycosylated.
FIG. 12. Predicted O-glycosylation sites in WapA sequence. The X-axis represents the WapA sequence position in the multiple alignments and the Y-axis the predicted O-glycosylation potential at that position. A position with a potential (vertical lines) crossing the threshold (dotted line) is regarded as O-glycosylated.

Glycosylation of the anti-WapA DNA vaccine candidates

The possibility of WapA or AgA glycosylation was considered in the process of selecting the anti-S. mutans DNA vaccine candidates. Cloning of a series of truncations of the wapA gene at the N-terminal and C-terminal ends into the expression vector pGEX-6P-1 glutathione S-transferase (GST) fusion vector demonstrated that only constructs GST-WapA-D, -E, -F, and –G, showed a strong cross-reaction with the polyclonal WapA antibody raised in rabbit (146). Detailed descriptions of those inserts are as follows:

wapA-D: 1335 b.p. fragment of wapA missing the promoter
**wapA-E**: 1251 b.p. fragment of **wapA** missing the promoter and signal peptide

**wapA-F**: 968 b.p. fragment of **wapA** missing the promoter and wall spanning region.

**wapA-G**: 885 b.p. fragment of **wapA** missing promoter, signal peptide, and wall-spanning region

The truncated **wapA-E** and **wapA-G** were selected to prevent target proteins from being glycosylated in mammalian host cells for the source of DNA vaccine construction. The **wapA-E** and **wapA-G** were truncated versions of **wapA**, both lacking in signal peptide sequence, therefore preventing target proteins from being glycosylated in mammalian host cells. In eukaryotic cells, proteins are synthesized in the cytoplasm or in the endoplasmic reticulum. Protein glycosylation occurs in the RER, and RER-synthesized proteins are distinguished from those synthesized in the cytoplasm by the presence of signal peptides (13-36 residues containing 7-13-residue hydrophobic core flanked by several relatively hydrophilic residues usually with one or more basic residues near the N-terminus). Similarly, bacterial membrane proteins are also preceded by signal peptides such as in the case of WapA.

To avoid glycosylation of WapA, **wapA-E**, and **wapA-G** were selected, which were truncated versions of the **wapA** gene that did not contain sequences encoding the **S. mutans** signal peptide. Taken with the lack of a eukaryotic signal sequence in the cloning vector, this meant that it was not likely that nascent protein synthesized in the cytoplasm could be translocated to the RER for further elongation and glycosylation. Despite a number of potential glycosylation sites with Thr and Ser residues in the **wapA** gene, glycosylation was successfully prevented and antigenicity was conserved.
Codon optimization

The ability of the vaccinee to produce the protein encoded by the DNA vaccine is often correlated with comparative codon usage between foreign and host genes. Analysis of the *S. mutans* or WapA gene sequence showed the difference of the codon usage frequencies compared to those prevalent in the human genome (Fig. 13 and 14). The pattern of usage difference in the *wapA* position revealed 19 low-usage codons for expression in mammalian systems (Fig. 15). In order to improve the heterologous expression of *wapA* sequence in the mammalian system, the frequency of wapA and human codon usage was determined, and optimized codons were suggested in Table 2.
FIG. 13. Comparison of codon usage for each amino acid used by *S. mutans* and *H. sapiens* genes. Mean difference of 26.7% was calculated between two species. *S. mutans* codon was colored red. The black bar was used for *H. sapiens* codon usage.
FIG. 14. Codon usage of *wapA* gene compared with the *H. sapiens* genes for each amino acid. Mean difference of 24.89% was calculated between two species. Codon fraction colored in red is *wapA*. *H. sapiens* was shown as the black bar.
FIG. 15. Analysis of codon usage pattern of \textit{wapA} to the common codon usage of \textit{H. sapiens} at each position of the gene. Red bar indicates low usage codon with less than 10\% codon match to the codon usage of \textit{H. sapiens}. Grey bar shows less than 20\% of codon usage match.
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</tr>
<tr>
<td>Gly</td>
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<td>GCC</td>
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<tr>
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<td>CTA</td>
<td>7</td>
<td>CAG</td>
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<tr>
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<td>AAA</td>
<td>42</td>
<td>AAG</td>
<td>58</td>
<td>58</td>
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<tr>
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<td>ATG</td>
<td>100</td>
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<td>100</td>
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<td>TTC</td>
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<td>TTC</td>
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<tr>
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<td>GTC</td>
<td>24</td>
<td>GTT</td>
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<td>18</td>
</tr>
</tbody>
</table>

Table 2. Codon optimization of wapA gene. The codons and their frequency of
occurrence in the WapA gene are compared with the codon frequencies of the most common codon usage of the human gene. The codon-optimized WapA gene represents the optimal codon for human codon usage.

*Comparative sequence analysis of the putative collagen binding domain in WapA*

Blasting the deduced amino acid sequence of WapA against the NCBI Conserved Domain Database (CDD) revealed a significant hit (E = 9e-20) for the collagen binding domain (Fig. 16A). Amino acids at position 150 to 286 of WapA were aligned 100% with pfam05737 consensus residues of the collagen binding domain (Fig. 16B).
FIG. 16. Alignment of deduced amino acid sequences of WapA with conserved domain databases. (A) Domain organization of *S. mutans* WapA. Amino acids 150-286 (putative CBD of WapA) were aligned 100% with 134 consensus residues of collagen binding domain in NCBI Conserved Domain Database (CDD). WapA signal peptide (S), proteolytic cleavage site (PC), cell wall domain containing LPSTG motif (W), and membrane-spanning domain (M) are indicated. The shaded region denotes the putative collagen binding domain (150-286) that was represented by CBD. The region of the expressed recombinant protein (rCBD) used in this study is also shown. (B) Sequence identity (E = 9e-20) to the collagen binding domain family was identified within the pfam database (pfam05737), where 33.8% identity in 134 amino acids overlap.
Structure analysis of putative collagen binding domain in WapA

Results of a secondary structure prediction algorithm using the 3D-PSSM program suggested that the submitted portion of WapA was a collagen binding domain. Prediction of secondary structure and its probable function as a collagen binding domain was matched significantly with the polypeptide chain of the collagen binding domain of the Cna protein from *S. aureus* with over a 95% confident hit (E = 1.68e-08) (Fig. 17). The putative collagen binding domain also has a significant hit (E = 5.3e-22) with the collagen-binding domain of *S. aureus*, from the result of sequence searches against SCOPE, suggesting a common function and implying a probable common evolutionary origin.
FIG. 17. The predicted secondary structure for the putative collagen binding domain in WapA was generated using the 3D-PSSM program, and was aligned to the significant structural match (E = 1.68e-08) of the fold library. The predicted secondary structure of the putative collagen binding domain in WapA shares structure motifs showing similar secondary structure topology with the assigned collagen binding domain of the Cna protein from *S. aureus*. E is extended beta strand; C is coil; and H is alpha helix.

Expression of recombinant collagen binding domain

A WapA gene fragment encoding the predicted collagen-binding domain was expressed in *E. coli* BL21 clone, and denoted rCBD. rCBD was purified and cleaved from glutathione S-transferase with the PreScission protease. The purified protein
representing the collagen binding domain of \textit{wapA} was shown as a single band of approximately 29 kDa in SDS-PAGE (Fig. 18).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure18.png}
\caption{Expression and purification of recombinant collagen binding domain. C-terminal truncated \textit{wapA-G} cloned into pGEX-6P-1 was expressed in \textit{E. coli} BL21. The resulting fusion proteins were purified on glutathione-Sepharose column followed by cleavage of the GST tag with PreScission protease. The purity of rCBD was evaluated on 10\% SDS-PAGE gels stained with Coomassie blue. All lanes showed column pool and subsequent column passages.}
\end{figure}

\textit{Collagen Binding Properties of WapA}

Immunodot analysis was used to detect the collagen binding properties of WapA, which contained the putative collagen binding domain. As shown in Fig. 19B, biotin-labeled rCBD was bound to native type I collagen, indicating the putative collagen binding domain. BSA, serving as the negative control (19A), did not bind native type I collagen.
collagen, confirming specificity.

FIG. 19. Collagen binding Assay. The collagen binding property was identified for the putative collagen binding domain in WapA by an adaptation of the dot immunobinding method, but using Biotin conjugated recombinant CBD protein in place of the primary antibody. A: No binding was detected with BSA used as a negative control. B: Binding of soluble native type I collagen to rCBD protein was observed.

Quantitative analysis of collagen binding property of WapA

The WapA protein was evaluated for its ability to bind soluble collagen immobilized onto a microtiter plate in a concentration-dependent manner. The putative rCBD in WapA bound quantitatively to the immobilized collagen type I, whereas no significant concentration-dependent binding was observed to the BSA (Fig. 20). Relative binding was measured by monitoring absorbance at 405 nm following the alkaline phosphatase reaction. Data points represent the means of OD405 nm values ± standard deviation from 3 independent experiments.
FIG. 20. Binding of recombinant putative CBD of WapA protein to native type I collagen used at different concentrations. Wells coated with BSA were used as the negative control. Serially diluted, biotin-labeled rCBD protein was added to the wells in a volume of 100 µl, and the bound protein was detected by AP-streptavidin by reading the absorbance of the reaction mixture at 405 nm. Data are plotted as the mean values of three experiments.

Cloning of truncated wapA genes into pcDNA3.1/V5/His-TOPO

After transformation of *E. coli* Top 10 with the eukaryotic expression vector pcDNA3.1/V5/His-TOPO containing a truncated wapA gene, wapA-E or wapA-G, plasmid DNA was isolated from the recombinant clones and amplified by PCR using a forward primer specific to the T7 sequence of the vector upstream of the multicloning site.
and a reverse primer specific to the *S. mutans* gene insert. Analysis of the PCR products by agarose gel electrophoresis showed that both constructs contained a DNA insert of the correct size and in the correct orientation (Fig. 21). The complete nucleotide sequence of these fragments was confirmed (Fig. 22 and Fig. 23).

FIG. 21. Insertion of *wapA-E* and *wapA-G* fragments containing an initiation codon into the pcDNA3.1 eukaryotic expression vector. Confirmation of the correct orientation of the inserts. The PCR amplification of *wapA-E* and *wapA-G* DNA with different combination of primers. Lane 1, Molecular mass markers, Lanes 2 and 3, a forward T7 primer specific to the sequence of the vector upstream of the cloning site with BGH reverse for downstream of the cloning site for each truncation; Lane 4, Forward *wapA-E* specific primer with BGH reverse primer; Lane 5, Forward *wapA-G* primer with BGH reverse primer; Lane 6, a forward T7 primer with *wapA-E* specific reverse primer; Lane 7, a forward T7 primer with *wapA-G* reverse primer; Lane 8, forward *wapA-E* primer with reverse *wapA-E*; Lane 9, forward *wapA-G* primer with reverse *wapA-G*.
FIG. 22. Nucleotide sequence of the recombinant wapA (wapA-E) cloned into a
eukaryotic expression vector, pcDNA3.1. The underlines indicate the regions used as
primers for the amplification of the wapA. The Kozak sequence, initial Met, and
terminal codon are highlighted in yellow.
FIG. 23. Nucleotide sequence of the recombinant *agA* (*wapA-G*) cloned into a eukaryotic expression vector, pcDNA3.1. The underlines indicate the regions used as primers for the amplification of the *agA*. Yellow highlight shows the Kozak sequence, initial Met, and terminal codon.

**Quality control of plasmid production for immunization**

The plasmid DNA production must include a good quality control process in order to assure the plasmid DNA, especially when used as a therapeutic agent including vaccination, is free of any contaminants, which include proteins, RNA, endotoxins, genomic DNA of the host cell, or any components used in the purification process. Purification of supercoiled plasmid without containing other isofoms such as nicked, linear, dimers or concatemers plasmids is an essential factor for quality control of
plasmid production, as well as function as a reproducible, scalable and economical purification process. Plasmid isolation was performed using the Qiagen procedure approved to produce plasmid DNA for human clinical Phase I studies in the U.K. (14) and other European countries, as well as in the United States by the FDA (49).

Supercoiled plasmid and any other isoforms were analyzed by running total plasmid DNA in 1% agarose gel electrophoresis (Fig. 24A). Plasmid DNA identity and quality were accessed through restriction analysis by BstXI digest of the parental vector and \( \text{wapA} \) or \( \text{agA} \) DNA vaccine constructs (Fig. 24B). DNA purity was assessed by using spectrophotometric analysis and calculation from its absorbance ratio of 260nm/280nm.

A.  

B.  

FIG. 24. Agarose gel electrophoresis and restriction enzyme digestion of pcDNA3.1-\( \text{wapA} \) or pcDNA3.1-\( \text{agA} \) for assuring the quality of DNA vaccines. A. Lane 1, \( \lambda \) DNA-\( \text{Hind III Digest} \). Lane 2, pcDNA3.1-\( \text{wapA} \). Lane 3, pcDNA3.1-\( \text{agA} \). Lane 4, pcDNA 3.1 parental vector. B. Lane 1, 100 base pair ladder. Lane 2, pcDNA3.1-\( \text{agA-BstXI digest} \). Lane 3, pcDNA3.1-\( \text{wapA-BstXI digest} \). Lane 4, pcDNA3.1-BstXI digest.
Expression of WapA and AgA by dot immunobinding assay

The expression of wapA and agA genes was investigated by immunodot analysis of the sonic extract of transfected CHO cells against a rabbit antiserum to the S. mutans cell wall. Chemiluminescence detection showed a high level of expression of both wapA and agA. No reaction was obtained with the negative control CHO cells (Fig. 25).

Western immunoblot analysis of WapA and AgA expressed in transfected CHO cells

To determine the molecular size of the proteins expressed in transfected CHO cells, Western immunoblot analysis was performed on aliquots of the cell sonic extract. Proteins with the anticipated molecular weights of 52,000 and 29,000 were observed with the transfected CHO cell samples, but not with the negative control untransfected CHO cells (Fig. 26).
FIG. 26. Western immunoblot analysis of wapA-E and wapA-G gene expression using rabbit antiserum to S. mutans cell wall antigens in CHO cells at 24 h post-transfection.

Lane 1, WapA-E sonic extract. Two bands of 52 and 29 kDa were obtained corresponding to WapA and AgA, respectively. Lane 2, WapA-G sonic extract. The anticipated 29-kDa band was obtained (smaller bands are probably attributable to proteolytic degradation). Lane 3, CHO cell treated with the lipid alone as a negative control. Lane 4, Untransfected CHO cells as a negative control.

In situ immunochemical staining of transfected CHO cells

To localize the WapA and AgA protein in transfectants, immunochemical staining was performed using a rabbit antiserum to S. mutans cell wall antigens followed by an alkaline phosphatase-conjugated goat antibody to rabbit immunoglobulins. Detection
was performed using the chromogenic substrate fast red stain. The presence of antigen was identified by a red coloration. The WapA protein was found mainly associated with the CHO cell membrane and budding vesicles, whereas AgA was found in transfected cells and extracellular surroundings (Fig. 27). Thus, the \textit{wap}A and \textit{ag}A gene constructs were efficiently taken up by CHO cells through lipid-mediated transfection and expressed in these eukaryotic cells.
FIG. 27. *In situ* expression of WapA and AgA at 24 hours post-transfection. Transfected CHO cells were fixed on a slide with cold acetone, blocked with skim milk, and immunochemically stained with antibody to *S. mutans* cell wall. Fast Red RC was used as a substrate for alkaline phosphatase. (A) A CHO cell treated with the lipid alone as a negative control. (B) Expression of AgA in transfected cells and extracellular medium. (C) Expression of WapA in transfected cells and budding vesicles. (D) Expression of WapA in large vesicles. (A, B and C were identified on the light microscope at X400 and photographed at X1000).
**Optimization of pcDNA-lipid complex**

HeLa cells were transiently transfected to determine the optimum ratio of pcDNA-lipid complex. The highest transfection efficiency was obtained with 2 µg pcDNA and 10 µg DMRIE-C, the ratio corresponding approximately to the ratio in molarity of 1:2 of pcDNA and DMRIE-C. Decreased β-galactosidase activity was indicative of cell death (Fig. 28).

![Diagram](image)

**FIG. 28.** Optimization of DMRIE-C Reagent and pcDNA ratio for transfection. In order to determine the optimal DMRIE-C formulation, different pcDNA3.1-βgal DNA:DMRIE-C lipid ratios were tested in Hela cells. At 24 h post-transfection, cells were solubilized and assayed for the β-galactosidase activity at 415 nm with a spectrophotometer. Data are means and standard deviation of duplicates.

**Dot immunobinding assay**

Four weeks after the booster immunization, mice immunized with plasmid containing *wap*A or *ag*A complexed with DMRIE-C showed the presence of anti-*Ag*A salivary sIgA by immunodot analysis using a HRP-conjugated anti-mouse IgA and a
chemiluminescence substrate for HRP (Fig. 29). No reaction was detected in the saliva obtained from the control mice.

FIG. 29. Immunodot analysis of anti-WapA and AgA salivary IgA production. Purified GST-AgA fusion protein was dotted onto a piece of nitrocellulose filter in line 2. Purified GST only was used as a control in line 1. The filter was incubated overnight with the saliva (1:2 dilution in PBS) of mice that were immunized with (A) PBS, (B) DMRIE-C lipid, (C) pcDNA3.1 vector with DMRIE-C, (D) WapA DNA vaccine construct with DMRIE-C, and (E) AgA DNA vaccine construct with DMRIE-C. Chemiluminescence detection showed the presence of anti-WapA and AgA salivary IgA in 2D and 2E. No reaction was obtained with the saliva of control mice.

Humoral immune responses induced by DNA vaccines

To access the level of humoral immune response after DNA immunization, serum or saliva was collected from the control mice and the mice immunized with pcDNA-wapA or pcDNA–agA at various time points after the second immunization. ELISA of the saliva obtained showed a significantly higher level (P < 0.05) of antigen-specific IgA in the immunized mice as compared to the background level in the control group (Fig.
30A). Similarly, the levels of antigen-specific IgG were observed in the serum of the immunized mice as compared to that of the control animals (P < 0.05) (Fig. 30B). Both salivary IgA and serum IgG titers were higher in the samples collected from the pcDNA-\textit{wapA} immunized mice as compared to those immunized with pcDNA-\textit{agA} vaccine (P < 0.05) (Fig. 30).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure30.png}
\caption{Salivary IgA and serum IgG responses in mice. Following intra nasal immunization with pcDNA-\textit{wapA} or pcDNA-\textit{agA}, pooled saliva (A) and serum (B) samples were analyzed by ELISA using plates coated with recombinant AgA. Mice immunized with either pcDNA-\textit{wapA} or pcDNA-\textit{agA} showed a significantly higher signal than the background level (control mice), both in saliva and serum (P < 0.05).}
\end{figure}

\textit{Western immunoblot analysis of antibody response against AgA}

Analysis of the saliva of the mice immunized with pcDNA-\textit{agA} or with pcDNA-\textit{wapA} by Western immunoblot showed the presence of sIgA binding to the GST-WapA antigen band of approximately 58 kDa (Fig. 31).
FIG. 31. Western immunoblot analysis of salivary sIgA Abs to \textit{wapA} and \textit{agA} DNA vaccines. The results showed the anticipated antigen bands (GST-AgA fusion protein of approximately 58 kDa) with the saliva of mice immunized with \textit{agA-pDNA} (Lane 3) or with \textit{wapA-pDNA} (Lane 5). No band was obtained with either mouse saliva against the extract of the control \textit{E. coli} harboring empty vector (lanes 4 and 6). As anticipated, no band was obtained with the saliva of the control mice (Lane 1 and 2).

\textit{Induction of \textit{S. mutans} WapA}

The WapA is involved in sucrose-dependent adherence and aggregation (101). In order to determine whether sucrose induces further the expression of WapA, \textit{S. mutans}, Western blot analysis was performed on sonic extracts of each culture grown with or without sucrose presence. Expression of \textit{S. mutans} WapA was strongly enhanced in presence of 2% sucrose, suggesting WapA expression is regulated by sucrose (Fig. 32).

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FIG. 32. Expression of WapA in presence of sucrose. Enhanced WapA expression was shown in the *S. mutans* culture grown in presence of 2% sucrose.

*Western immunoblot analysis of antibody response against WapA*

Reactivity against WapA was higher with the anti-WapA antibody than with the reactivity of the anti-AgA, suggesting a stronger reaction is attributable to the additional epitopes seen in WapA but not in AgA (Fig. 33).

FIG. 33. Western blot analysis of *S. mutans* WapA using polyclonal antiserum raised against AgA or WapA. Stronger immunoreactivity was shown with anti-WapA serum that that of anti-AgA.
**Sucrose-dependent adherence inhibition assay**

Saliva of the immunized mice, with pcDNA-wapA or pcDNA-agA, was evaluated for the ability to inhibit the *S. mutans in vitro* sucrose-dependent adherence. A significantly higher inhibition of adherence (P<0.05) was obtained with the saliva of the mice immunized with pcDNA-wapA (21% inhibition) as compared to the Saliva of the mice immunized with pcDNA-agA (7% inhibition) shown in Table 3.

**TABLE 3. EFFECT OF SALIVARY IgA PRODUCED IN RESPONSE TO IMMUNIZATION ON BACTERIAL ADHERENCE**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization Treatment a</th>
<th>Mean % adherent cells/total cells ± SD b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pcDNA Vector only</td>
<td>61.11 ± 1.75</td>
</tr>
<tr>
<td>B</td>
<td>pcDNA-wapA</td>
<td>48.53 ± 1.36</td>
</tr>
<tr>
<td>C</td>
<td>pcDNA-agA</td>
<td>57.00 ± 1.44</td>
</tr>
</tbody>
</table>

a Saliva collected was diluted 1:2 in PBS and added to triplicate *S. mutans* culture wells.

b Significant inhibition (P<0.05) of *S. mutans in vitro* sucrose-dependent adherence was observed with the saliva of mice immunized with pcDNA-wapA (B), and to a lesser extent with the saliva of mice immunized with pcDNA-agA (C) or with pcDNA vector alone (A).

**Dextran-binding properties**

Analysis of recombinant WapA or AgA for the ability to bind biotin-labeled dextran showed that WapA bound dextran, but AgA did not. As anticipated, no dextran-binding activity was observed with GST used as a negative control (Fig. 34).
FIG. 34. Dextran-binding assay. The dextran-binding property was identified on GST fusion proteins containing WapA or AgA against the Biotin-dextran conjugate using the Dot immunobinding method. The WapA showed a capacity to bind dextran (1), but the AgA did not (2). GST only was dotted as a control (3).

Cloning and sequencing of *il-5* and *ctb* recombinant genes

The orientation and identity of the *il-5* cDNA and *ctb* gene clones were confirmed by the nucleotide sequencing (Fig. 35). The cloned *il-5* cDNA was 402 bp of ORF without signal peptide, and it encoded 133 amino acid residues. The sequencing of the *ctb* clone showed correct identity of the *ctb* gene insert including 312 nucleotides encoding 103 amino acids.
FIG. 35. Nucleotide sequence of il-5 or ctb gene insert. The il-5 cDNA or ctb gene subunit was cloned into the pcDNA3.1 mammalian expression vector and sequenced. The coding regions, including the ATG start codon, are in red, and the Kozak sequence is underlined. The primers used are indicated in blue.

Western immunoblot analysis of the purified GST-CTB fusion protein

The purified GST-CTB fusion protein was confirmed by the Western immunoblot with monoclonal antibody to Ctb. The 40-kDa band was identified on the nitrocellulose lines containing purified GST-CTB fusion protein or lysate of E. coli containing the construct. No band was shown on the line with purified GST (Fig. 36).
FIG. 36. Western immunoblot analysis of purified GST-CTB fusion protein. Specific binding of the anti-CTB monoclonal antibody to the cloned and purified GST-CTB protein (Line 2). No immunoreactivity was shown to the purified GST protein (Line 3). The molecular masses standards (lane 1) are indicated (in kilodaltons) on the left of the blot.

Expression of recombinant WapA or AgA

WapA and AgA are expressed as GST-fusion proteins in the E. coli recombinant clones WapAE and WapA-G, respectively. Expression of both antigens was observed (Fig. 37 and Fig. 38).
FIG. 37. SDS-PAGE analysis of the GST-*wapA* fusion protein expression. A highly expressed truncated *wapA*-G gene as GST fusion was used as the coating antigen in an ELISA assay. Lane 1, *E. coli* BL21 lysate. Lane 2, a soluble sonic extract containing GST-*wapA*-G. Lane 3, GST-*wapA*-G protein purified from the soluble fraction. Lane 4, M.W. marker.

Fig. 38. Expression of *wapA* and *agA* genes as GST fusions. SDS-PAGE of purified 29 kDa GST (lane 1), 81 kDa GST-*WapA* (lane 2), and 58 kDa GST-*AgA* (lane 3) are shown on coomassie blue stained gel. Molecular weight standards were run in lane 1.
Production of DNA vaccine

The recombinant \textit{E. coli} TOP10 containing the target DNA was cultured in an LB medium containing ampicillin added at 50 micrograms per milliliter, and plasmid DNA was isolated by the modified alkaline lysis method using a kit from Qiagen (HiSpeed Maxi Kit), Valencia, CA. The average yield of plasmid DNA containing the \textit{wapA} gene (pcDNA-\textit{wapA}) was 2 mg/L \textit{E. coli} culture, whereas that of the plasmid containing the \textit{agA} gene (pcDNA-\textit{agA}) was only 0.92 mg/L. One reason for the low yield of pcDNA-\textit{agA} is the tendency of the recombinant \textit{E. coli} to lyse. In order to improve the yield of pcDNA-\textit{agA}, the antibiotic chloramphenicol was added to the culture. As a result, the yield of plasmid copies was improved by approximately 2.5 fold (2.3 mg/L \textit{E. coli} culture). Hence, we have established the conditions for the obtaining of comparable levels of production of pcDNA-\textit{wapA} and pcDNA-\textit{agA}.

Production of Protein vaccine

The proteins WapA and AgA were obtained separately, each as a fusion protein with the enzyme glutathione S-transferase (GST), respectively designated as WapA-GST and AgA-GST. The yield of these proteins was determined by optical density readings at 280nm, and calculated based on the extinction coefficient of GST ($1 \text{ OD}_{280} = 0.5 \text{ mg protein/ml}$). The fusion proteins were purified by affinity column chromatography on GST-Sepharose (Promega, Madison WI) and purity was demonstrated by SDS-PAGE, where a single protein band was obtained for each GST-fusion protein. Originally, the yield of AgA-GST and WapA-GST was 2.5 mg/L and 1.2 mg/L, respectively. One reason for the lower yield of WapA-GST was due to the insolubility of WapA, since this protein contains an extra hydrophobic membrane-binding region at the C-terminal end as
compared to AgA. Sarkosyl was used to solubilize the *E. coli* membrane and improve the recovery of WapA-GST. The results showed a two-fold increase up to 2 mg/L.

**Immunodot analysis**

The presence of anti-WapA salivary IgA was detected by Immunodot analysis. Weak staining was shown in the saliva from the DNA vaccine immunized mice while a much stronger reaction was obtained with the protein vaccinated mice (Fig. 39). No reaction was detected from the saliva of the negative control or that of the DNA vaccine alone without cationic lipid.

![Immunodot analysis](image)

**FIG. 39.** Immunodot analysis. Saliva from immunized mice were tested for immunogenicity of specific S-IgA against WapA. Group 1 mice immunized with pcDNA-wapA alone (2), or with pcDNA-*il*-*5* (3) or pcDNA-*ctb* (4) showed weak immunogenicity to WapA. Group 2 mice immunized with AgA (6) or WapA (7) showed strong reaction to WapA. Group 3 mice that is primed with pcDNA-wapA and boosted with WapA protein (5) revealed strong immunogenicity to WapA. No immunogenicity was shown for non-immunized (1) or WapA DNA vaccine only without adjuvant (8).

**Immunomodulatory effect on humoral immunity**

To investigate the influence of *il*-*5* or *ctb* co-immunization on the level and duration of *salivary IgA* response, saliva from the control and immunized mice with or
without the adjuvant was analyzed using an ELISA assay. Co-immunization with pcDNA-*il-5* resulted in a significant (*P* < 0.05) but temporal sIgA response after boosting with a third injection of DNA as compared to that of the control mice group (Fig. 40A). This was followed by a drop in the level of sIgA with the additional booster, indicating that the booster effect was aborted and, therefore, no memory immune response was elicited. The level of sIgA antibody induced by pcDNA-*ctb* as an adjuvant was improved more rapidly and consistently as compared to that of the control or pcDNA-*il-5* co-immunization (Fig. 40A).

Mice primed with pcDNA-*wap* followed by the WapA boost produced sIgA levels comparable to mice receiving 2 WapA doses (Fig. 40B). The antigen-specific antibody was not affected in the groups administered with DNA only without adjuvant and the non-immunized group (Fig. 40).
A. FIG. 40. WapA-specific sIgA antibody response in the saliva of mice immunized with DNA or protein vaccine. Levels of the antigen-specific S-IgA was determined in the saliva of immunized mice given pcDNA-wapA alone, or with pcDNA-il-5 or pcDNA-ctb (A) or primed with pcDNA-wapA and boosted with WapA or WapA and CTB (B) by the i.n route. Each vaccine was injected biweekly, as shown by black arrows, for a total of four inoculations. Each point is the mean of triplicate values.
DISCUSSION

Dental caries is a significant health problem in humans and treatment is costly due to the high prevalence in the population. Despite longstanding effort at the development of a caries vaccine, an efficacious vaccine protocol has not yet been developed. The establishment of an effective mucosal vaccine would be an exceptionally significant accomplishment that would greatly enhance the ability to provide preventive vaccination for this disease. This is especially true for the groups at risk such as underserved communities which do not have access to water fluoridation and periodical preventive dental care, and children and adults with medical problems or receiving medical treatments that reduce salivary flow. Once developed, the dental vaccine should also serve as a model for the preparation of similar vaccines against other infectious agents, particularly those infecting the oral cavity.

The overall objective of this study was to develop a novel mucosal vaccine that is safe, cost-effective and efficacious at inducing protective immunity against dental caries. A number of virulence factors have been identified as candidate vaccine antigens, most of which have been cloned and sequenced, and some have been tested with much promise. Since salivary immunoglobulin A (IgA) to various S. mutans adherence and aggregation factors has been showed to confer protection against S. mutans infection, induction of mucosal immunity has become the main target for dental caries vaccine development (37,
The target antigens used in this study were the *S. mutans* antigen A (AgA), a recognized candidate vaccine antigen, and its precursor the wall-associated protein A (WapA), a factor involved in colonization and build up of dental plaque. Previously, WapA gene and the truncated agA gene had been cloned into a high expression vector for expression as Glutathione-S-Transferase fusion proteins to improve yield and facilitate purification (146). However, considering that the costs associated with the extraction, purification and storage of recombinant antigens and antibodies can be an obstacle to mass immunization, it is necessary to explore alternative approach.

With recent advances made in the construction of DNA vaccines comes the hope for a more economical way to immunize against infectious disease. In the present study, genetic immunization using naked DNA vaccine was used as an alternative method of inducing mucosal immunity characterized by the induction of secretory IgA against *S. mutans*. DNA vaccine is both potentially safer and more economical than conventional vaccines when considering the ease of isolation of plasmid DNA, low cost of mass-production, stability at extreme temperatures, ease of administration, and potential for induction of long-lasting immunity without causing any severe side effects (1, 39). Therefore, the prospect of a DNA vaccine against dental caries is particularly attractive due to the high incidence of this disease in the world, with populations of low socioeconomic status being at the highest risk.

DNA vaccines induce both specific antibody and cell-mediated immune responses (27). However, their use has been mainly focused on viral infections (81). DNA vaccines against bacterial infections have been limited to intracellular bacterial pathogens such as *Mycobacteria spp.* or *Salmonella spp.* where cytotoxic or a Th1 type T cells
confer protection (130). Moreover, induction of mucosal immunity against extracellular bacterial infection where secretory IgA mediates resistance has been rarely studied.

The feasibility of DNA vaccine efficacy was demonstrated in terms of inducing specific mucosal immunity against *S. mutans*, an extracellular bacterium, and relative immunogenicity of full-length and C-terminal truncated *wapA* was compared and evaluated.

Further exploration was made to test the efficacy of two forms of vaccine; Gene versus Protein in inducing specific sIgA response. The efficacy of co-immunization with genetic adjuvants at enhancing salivary IgA response was also investigated. A number of cytokines have been identified as enhancing the mucosal IgA response to DNA vaccines, notably IL-5 in mice (102, 139). Intranasal administration of recombinant adenovirus vectors expressing IL-5 has been shown to enhance secretory IgA response to the adenovirus in the lung (102). Increased IgA response to *Salmonella* LPS has also been observed in mice immunized with the bacterial strain engineered to express murine IL-5 (138). IL-5 was chosen over other cytokines as it has been found to be the primary cytokine inducing the generation of IgA producing B cells in mice.

Effects of co-immunization with either gene or protein of a CTB subunit were studied. CTB is a proven adjuvant for the induction of a mucosal response to a protein-based vaccine in mice (142). Unlike CT and CTA subunits, CTB is not toxic, and hence has been considered as a good candidate adjuvant for human use (145). Bergquist *et al.* (10), using recombinant CTB in intranasal immunization of humans, did not observe any systemic adverse reactions, but only local irritations of short duration at high doses (1,000 µg per dose) in all volunteers. At a lower dose (100 µg), the local reaction was
mild and tolerable, and occurred only in approximately one third of the subjects.

In a study conducted by Johansson et al. (52), 21 female volunteers were administered nasally 250 µg of recombinant CTB using an atomizer, and no apparent side effect was reported. CTB was also found to be safe in the immunization of Israeli young adults against enterotoxigenic E. coli (16). By using pcDNA-ctb as an adjuvant in DNA immunization, it was possible to not only compare the efficacy of protein versus DNA vaccine, but also that of protein versus DNA adjuvant. The enhancement of mucosal immunity to protein conjugated with CTB was due to two functions of CTB: (1) transmucosal carrier, and (2) immuno-stimulatory molecule. In immunization with pcDNA, delivery of pcDNA across the mucosal barrier is mediated by DMRIE-C, thus any enhancement obtained by co-immunization with pcDNA-ctb will be attributable to the strong immunogenic properties of CTB.

Cloning of the truncated wapA genes, wapA-E and wapA-G, into the eukaryotic expression vector pcDNA3.1/V5/His-TOPO was completed, and the genes were shown to be inserted in the correct orientation. To test the expression of the cloned genes, CHO cells were used as hosts in transfection studies. The cationic lipid pfx8 was used, as it has been reported by the manufacturer to work best for CHO cells. Indeed, transfection of CHO cells was successful, and the cloned genes were expressed as anticipated. It is noteworthy that the wapA-E gene product (WapA-E), corresponding to the S. mutans WapA, was expressed in the transfected cells and budding vesicles, probably from cells undergoing apoptosis, whereas the wapA-G gene product (WapA-G), corresponding to the S. mutans AgA, was found in both the cells and the surrounding medium.

The differential expression of WapA and AgA may be attributable to the presence
of the hydrophobic C-terminal end of WapA, allowing this molecule to interact with hydrophobic components in the host cell membrane. The AgA protein is truncated in this hydrophobic region and hence is more soluble and may be released from leaky or lysed cells.

Whether the genes are expressed as soluble or cell-and vesicle-associated proteins, it is reasonable to anticipate an antibody response, as previously obtained by immunization with the WapA and AgA proteins. In fact, both cellular and humoral immune responses were obtained by immunization with DNA vaccines, indicating that in the host cells, the foreign proteins were processed both through the endogenous pathway of antigen processing, and presented associated with MHC-I, or through release and uptake by antigen-presenting cells for processing by the exogenous pathway and presentation in association with MHC-II (61). The present study illustrated two ways by which foreign proteins could be released from the host cells. The immunization study showed that it was feasible to induce a mucosal response to S. mutans antigen by intranasal administration of naked DNA vaccine containing the wapA gene or its truncated derivative agA gene. Both DNA constructs induced antigen specific sIgA antibodies in the saliva of the immunized animals, with a higher antibody titre observed with pcDNA-wapA as compared to pcDNA-agA.

It was hypothesized that due to its larger size and hydrophobicity, due to the presence of a membrane and wall-spanning region, WapA may be more immunogenic than its hydrophilic truncated derivative AgA. Data from a previous in vitro transfection study showed that WapA was found expressed in budding vesicles (presumably apoptotic bodies), whereas AgA was diffused in the extracellular medium. Such an occurrence in
vivo should facilitate the phagocytosis and processing of apoptotic bodies and the presentation of antigen peptides to T helper cells by macrophages. This hypothesis was further supported by the sequence analysis of WapA and AgA using virtual matrices algorithms. The results obtained revealed putative binding sequences within the hydrophobic C-terminal end of WapA, along with the rest of the sequences, based on MHC binding motifs for known major histocompatibility complex class II binding alleles. Those predicted MHC class II ligands corresponded with promiscuous binding motifs associated with 51 MHC class II alleles; one of the most prominent promiscuous binders was located at residues 400 to 409, within the C-terminal end. Those promiscuous binding motifs allow for a large degree of compatibility between the antigen peptide sequence and dimeric MHC II molecule (121). The resulting peptide-MHC II complexes are delivered to the membrane for detection by CD4+ T cells and induce humoral immune responses (99).

Most of the peptides presented by MHC class II molecules are derived from the endocytosis and processing of extracellular proteins (99, 122). Previously, it was demonstrated by others that endogenous proteins produced by a DNA vaccine could be released from transfected cells and uptaken by antigen-presenting cells for processing by the exogenous pathway and presentation in association with MHC class II. Moreover, in the host cells, endogenous antigens enter the MHC Class II processing and presentation pathways much more efficiently than the endocytosis and processing of the same protein from the extracellular compartment (12, 95, 115). Another major factor affecting the antibody response is transfection efficiency (29, 43, 114). The present study also demonstrated that transfection efficiency of HeLa cells was dependent on the ratio of
pcDNA:DMRIE-C starting at 1:1 (w/w) and reaching an optimal level at 1:5, then decreasing thereafter. The decrease in transfection efficacy and expression was associated with cell death due to the toxicity of DMRIE-C at high dose.

In addition to the above, the inhibition of *S. mutans* adherence to the culture vessel when grown in the presence of sucrose by a salivary antibody of mice immunized with pcDNA-*wapA*, and not by the salivary antibody of the mice immunized with pcDNA-*agA*, further demonstrated that WapA was a better candidate vaccine antigen than AgA. Dot immunobinding assay where the WapA in its larger precursor form showed a glucan-binding property, but not the 29-kDa C-terminal truncated version (*wapA*-G) encoding the AgA, supports this view. These findings are in agreement with the previous data in an experiment where WapA, but not the AgA, showed a binding affinity to various dextran matrices (23). The work presented here explores the feasibility of genetic immunization against *S. mutans*. DNA vaccine containing the *S. mutans* wall-associated protein A gene (*wapA*) were proven to be better immunogens than others which contained the truncated derivative corresponding to the antigen A gene (*agA*) for the reasons described above.

In the present study, we further evaluated if co-immunization with genetic adjuvant would enhance the levels of sIgA response elicited by the caries DNA vaccine containing the WapA gene. Attempts were also made to compare the efficacy of DNA versus protein in inducing the WapA-specific sIgA response. Our data shows that a DNA vaccine encoding WapA protein is less effective than a corresponding protein vaccine in inducing mucosal antibody response. Furthermore, subsequent boosts with DNA vaccine were not capable of amplifying Ab memory generated by the DNA vaccine prime,
suggesting homologous DNA vaccine is not an efficient booster of immune memory.

Specific salivary IgA response was transiently enhanced by co-immunization with IL-5 expressing plasmid, while comparatively steady increase was observed by a co-immunization with plasmid containing the CTB gene. This observation indicates that expressed CTB may have continuing adjuvant activity by enhancing presentation of expressed antigen released from transfected cells and by stimulating mucosal Th2-type cytokine responses, such as IL-5, leading to increased mucosal IgA production. Antigen-specific IgA response was improved by the action of pcDNA-il-5 expression, indicating immunomodulatory effect of the cytokine in improving isotype differentiation of B cells to mucosal IgA formation. However, the IgA antibody response failed to be sustained, probably due to the lack of booster effect in generating continuous IL-5 as seen with pcDNA-wapA gene immunization.

Prime-boost immunization regimen of pcDNA-wapA priming, followed by boosting with a WapA protein, showed considerable enhancement on the level that sIgA produced, almost as high as protein-protein vaccination. Thus, pcDNA-wapA was shown to be a valid candidate primary vaccine and that subsequent natural exposure to S. mutans may provide the necessary boost to induce higher sIgA response.

It has been reported that plasmid DNA immunization predominantly induces a Th1-type cellular immune response (104) over a Th2 type immune response that produces humoral antibodies. The lower salivary IgA immune response generated from a DNA vaccine might be due to the Th1-biased characteristic of the vaccine. Insufficient memory response was consistently observed in other studies indicating that boosting may not efficiently stimulate the memory B cells with the DNA vaccine (70, 107, 119).
Indeed, the data demonstrated that intranasal immunization with a DNA vaccine followed by a boost with the corresponding protein vaccine showes a significant enhancement of the antigen-specific salivary sIgA response, a key factor for protective mucosal immunity.

With recent advances in the understanding of B and T-cell memory, prime-boost vaccination strategies appear to create a synergistic effect from the two vaccines, and the quality and longevity of T-cell memory generated from DNA prime can be significantly enhanced by subsequent protein boosting. From the experiments performed herein, we have been able to establish an alternative, novel DNA vaccine strategy of inducing mucosal immunity against *S. mutans*. Dental caries induction involves multiple *S. mutans* proteins, and this study should serve as a model for the preparation of other gene constructs to include in a combination dental DNA vaccine. The results obtained in this study will have an impact on future vaccine development, not only against *S. mutans*, but also against other pathogens utilizing the mucosal lining of the respiratory, gastro-intestinal, and genito-urinary tract as a route of entry into the host.
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About the Author

Thomas Han received his bachelor’s degree in genetics from Iowa State University, and a master’s degree in immunology from Louisiana Tech University.

During his doctoral studies at the University of South Florida, he was awarded The THARP Fellowships for 3 consecutive years. He was also selected to receive The Outstanding Poster Presentation Award at the First Annual USF Graduate Student Research conference. He was a recipient of the 2005 Corporate Activities ASM Student Travel Grant Award. He was a primary author of four published articles in various scientific journals. He made several research presentations at national and regional scientific meetings. He is one of inventor on pending patent vaccine application. His research data was used to secure funding of Innovative Grants from NIH.