A specific salivary IgA (sgIgA) response was obtained in mice by intranasal immunization with a naked DNA vaccine consisting of the *Streptococcus mutans* wall-associated protein A gene (*wapA*) inserted into the mammalian expression vector pcDNA3.1/V5/His-TOPO. In the present study, the vaccine, referred to as pcDNA-*wapA*, was administered with or without the cationic lipid DMRIE-C. No mucosal response was observed in mice immunized with the vaccine alone, whereas a weak and temporal sgIgA response was obtained when the vaccine was mixed with DMRIE-C. To investigate the use of pcDNA containing the interleukin 5 (IL-5) gene (pcDNA-il-5) or the choler toxin B gene (pcDNA-crb) as genetic adjuvants, these constructs were used in coinmunization studies. The enhancement effect was transient with pcDNA-il-5, but longer lasting with pcDNA-crb, thus supporting the use of the latter as a genetic adjuvant to DNA vaccine.

DNA-based vaccines. The adjuvants used in protein or peptide vaccines include choler toxin, choler toxin B subunit (CTB) and a detoxified heat labile *Escherichia coli* toxin (LT R292G) (De Magistris, et al., 1998; Wu & Russell, 1993), and DNA vaccine adjuvants include liposome and various cationic lipids (Klavinskas et al., 1997, 1999; Ochiya et al., 1999, Perrie et al., 2001). Cytokines were found to further provide immunopotentiating effects, for instance interleukin (IL)-4 or IL-10 induced a Th2 type response, whereas IL-12 and/or a granulocyte/macrophage colony stimulating factor favoured a Th1 type of response (Donnelly et al., 1997; Okada et al., 1997; Ramsay & Kohonen-Corish, 1993; Whittle et al., 1997). Intranasal administration of recombinant adenovirus vectors expressing IL-5 has been shown to enhance mucosal IgA response to co-expressed heterologous antigen in the lung of immunized mice (Ramsay & Kohonen-Corish, 1993). Increased IgA response to *Salmonella* lipopolysaccharide has also been observed in mice immunized with a bacterial strain engineered to express murine IL-5 (Whiley et al., 1988).

The goal of the present study was to explore the ability of plasmids encoding CTB or cytokine IL-5 as a genetic adjuvant to boost the mucosal antibody response to a DNA vaccine administered intranasally. The target antigen used in this study was the *S. mutans* wall-associated protein A (*WapA*), a factor involved in tooth colonization and build-up of dental plaque (Qian & Dao, 1993). Three different plasmids were constructed by separately cloning *wapA*, *il-5* and *ctb* into the mammalian expression vector pcDNA3.1/
V5-His-TOPO. The constructs, designated pcDNA-wapA, pcDNA-il-5 and pcDNA-ctb, were mixed with the cationic lipid transfection adjuvant DMRIE-C, used to transfect HeLa cells in order to check for the expression of the corresponding proteins in these cells, and then administered intranasally to the BALB/C mice. Specific sIgA was determined for comparison between pcDNA-wapA without DMRIE-C, with DMRIE-C, with DMRIE-C plus pcDNA-il-5 or with DMRIE-C plus pcDNA-ctb.

**METHODS**

**Experimental animals.** BALB/c female mice of 6–8 weeks old were purchased from Charles River Laboratories and were maintained in the animal facilities of the University of South Florida, College of Medicine. All protocols using mice were approved by the Institutional Animal Care and Use Committee at the University of South Florida. The University programme and facilities for animal care and use are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

**DNA vaccine construction.** The three clones needed for this study consisted of a recombinant *E. coli* TOP 10 clone containing the *wapA*, *il-5* or *ctb* gene inserted separately into the mammalian expression vector pcDNA3.1/V5/His-TOPO (Invitrogen) (Fig. 1a). In order to improve the translation efficiency of the expressed genes in mammalian hosts, the Kozak sequence ACCATG was incorporated into all the inserted genes. The final constructs obtained were designated pcDNA-wapA, pcDNA-il-5 and pcDNA-ctb. The recombinant expression plasmid pcDNA-wapA was constructed as described previously (Han et al., 2001), whereas, pcDNA-il-5 and pcDNA-ctb were cloned in the present study as follows: recombinant *E. coli* HB101 containing IL-5 cDNA cloned into the vector pBR322 was obtained from the American Type Culture Collection (ATCC) and the plasmid was used as a template for PCR amplification of *il-5* using primers that were designed based on the IL-5 DNA sequence available in GenBank (accession no. NM_010558). The primers used included the forward primer, 5'-ACC ATG AGA AGG ATG CTT CTG GAC-3', and the reverse primer, 5'-TCA GCC TTC CAT TGC CCA-3'. The resulting PCR product was cloned downstream of the CMV promoter into the pcDNA3.1/V5/His-TOPO vector. Construction of a plasmid containing the *ctb* gene was performed similarly using genomic DNA of *Vibrio cholerae* (ATCC) as a template for the PCR amplification of the *ctb* gene. Primers for the amplification of *ctb* were designed based on the gene sequence available in GenBank (accession no. D300553), and included the forward primer, 5'-ACC ATG ACA CCT CAA AAT ATT ACT GAT T-3' and the T7 forward primer, 5'-TAA TAC GAC TCA CTA TAG GG-3'.

**DNA sequencing.** The plasmids constructed as described above were purified to sequencing grade by a modified alkaline lysis method using the HiSpeed plasmid maxi kit (Qiagen) and following the manufacturer’s instruction. Sequences were determined at the DNA sequencing core facility of the H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA, in order to confirm the sequence and orientation of the target genes. The primer sets for sequencing were the T7 forward primer, 5'-TAA TAC GAC TCA CTA TAG GG-3', and the BGH reverse primer, 5'-TAG AAG GCA CAG TCG AGG-3'.

**Expression and purification of WapA from *E. coli* recombinant clones.** To obtain enough protein for the preparation of rabbit antibodies and immunochromatographic analysis of WapA in the present study, purification of WapA protein was optimized and scaled up. WapA was obtained from the corresponding recombinant *E. coli* BL21 (DE3) clone as a fusion protein with glutathione S-transferase (GST) (Yoder et al., 2000). The clone was cultured at 37 °C in an incubator shaker overnight in batches of 500 ml 2 × YT medium (prepared by

![Fig. 1. Cloning of ctb, il-5 and wapA into pcDNA3.1/V5/His-TOPO and in vitro expression of the cloned genes. (a) The diagram shows the site at which the target genes were inserted separately into the vector. Copyright 2007 Invitrogen Corporation. Used with permission. (b) Western immunoblotting of transfected HeLa cells shows the expression of antigens with the anticipated molecular size: from left to right, strip 1/lane 1, CTB; strip 2/lane 1, IL-5; strip 3/lane 1, WapA with a smaller band of approximately 29 kDa corresponding to the antigen A (AgA) protein, which was derived from WapA by proteolytic cleavage. In all three strips, lane 2 containing HeLa cells transfected with empty vector was negative.](image-url)
dissolving 16 g trypetone, 10 g yeast extract and 5 g NaCl in 900 ml distilled H2O, pH 7.0) supplemented with 100 μg ampicillin ml⁻¹ (2 × YTA). The following day, the overnight culture was diluted 1:100 into fresh pre-warmed 2 × YTA medium, and incubation was continued at 37 °C until the culture reached an OD₆₀₀ of 1. Induction of protein expression and preparation of the protein lysate were performed as described previously (Yoder et al., 2000). Purification of GST-WapA was carried out by adding 50% (v/v) slurry of glutathione-linked Sepharose 4B (Pharmacia) to the protein lysate, and the mixture was incubated on a rocker for 30 min at room temperature. The mixture was transferred onto a chromatography column, followed by washing with 50 ml PBS containing 1 mM PMSF. The fusion protein was eluted by 10 mM reduced glutathione in five aliquots of 300 μl each. Protein concentration was determined by optical density reading at 280 nm. One OD₂₈₀ equals 0.5 mg protein ml⁻¹ using a formula based on the absorption coefficient of free GST. Protein purity was analysed by SDS-PAGE using 12% (w/v) acrylamide gel.

**Immunogenicity of GST-WapA fusion protein.** Considering the advantage of skipping the cleavage of GST from the fusion protein in term of time and money, this step was omitted from the production of WapA protein for immunization. The validity of this approach was tested by demonstrating the reactivity of GST-WapA with a previously prepared rabbit anti-WapA antibody.

**Preparation of rabbit polyclonal antibody against WapA.** Rabbit polyclonal antibody was prepared as follows: briefly, two 4 kg female albino rabbits from the New Zealand strains (Harlan) were immunized intramuscularly with 100 μg purified GST-WapA fusion protein mixed in a 1:1 (v/v) ratio with TiterMax Gold (Sigma) adjuvant. Two booster injections of the same vaccine construct were administered at 3 week intervals. Blood was collected before the immunization and 1 week after each booster injection. Serum was separated by centrifugation and decompomelled by heating at 55 °C in a water bath for 30 min. Serum was sterilized by adding 1/10 (v/v) chloroform, mixed and centrifuged to obtain the aqueous upper layer containing the antibody.

**Absorption of antiserum.** Non-specific binding of antiserum to WapA was removed by extensive absorption of serum against E. coli, GST and HeLa cells as described previously (Han et al., 2001). For the absorption of anti-GST antibodies, glutathione Sepharose 4B attached with GST was packed onto a gravity-flow column. Serum was loaded on the 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 a dot immunobinding assay.

**Purification of IgG antibody against WapA.** WapA specific antibodies from post-immune rabbit serum were purified using the Melon gel IgG purification kit (Pierce), according to the procedure specified by the manufacturer. Serum was diluted by 1:10 (v/v) with Melon gel purification buffer and loaded onto a gravity-flow column. The specific antibody was eluted with the purification buffer, and absorbance of the antibody fractions was measured at 280 nm.

**Transfection.** HeLa cells were purchased from ATCC and transfected separately with the plasmid constructs pcDNA-wapA without DMRIE-C, pcDNA-wapA with DMRIE-C, pcDNA-wapA with DMRIE-C and pcDNA-il-5 or pcDNA-ctb. DMRIE-C (Invitrogen) was added at 1:2 molar ratio of pcDNA construct/DMRIE-C, providing the optimal condition determined in previous studies (Han & Dao, 2005). Briefly, HeLa cells were seeded with 2 × 10⁵ cells in 2 ml culture medium, which consisted of Dulbecco’s modified Eagle’s medium supplemented with 1 mM sodium pyruvate, 5 mM l-glutamine, 100 IU penicillin, 100 μg streptomycin ml⁻¹, 0.25 μg amphotericin ml⁻¹ and 10% fetal bovine serum (FBS), onto a 6-well tissue culture plate and grown overnight at 37 °C in a CO₂ incubator until approximately 70% confluence. The cells were washed once with 2 ml serum-free medium, and then overlaid with the lipid-DNA complex solution. After 4 h of incubation, growth medium containing 20% FBS was added to each well, and the incubation was continued for another 24 h. At 24 h post-transfection, the cells were solubilized with M-PER mammalian protein extraction reagent (Pierce), and the cell suspension was collected.

**Western immunoblotting of transfected HeLa cells.** Expression of the transfected genes was assayed by Western blotting, as previously described (Han et al., 2001). Briefly, a sonic extract of the transfected HeLa cells was separated by SDS-PAGE on a 12% gel and transferred by electrophoresis onto a piece of nitrocellulose membrane, which was then blocked by incubation with PBS containing 5% skimmed-milk powder and then probed with 1:500 dilution of monoclonal anti-IL-5 (Imgenex) or anti-CTB (Biodesign) antibody, or absorbed and purified polyclonal anti-WapA antibody raised as described previously. The blot was incubated with a horseradish peroxidase (HRP)-conjugated corresponding secondary antibody diluted to 1:2,000, and specific bands were developed on an X-ray film (Kodak) using an HRP chemiluminescent substrate (Amersham).

**Immunization protocols.** For DNA vaccination, 50 μg each DNA vaccine constructed was intranasally administered in the presence of DMRIE-C at 1:2 molar ratio of DNA/DMRIE-C unless otherwise indicated. The mice were immunized, six mice per group, as follows: group 1 with pcDNA-wapA alone; group 2 with pcDNA-wapA plus pcDNA-il-5; group 3 with pcDNA-wapA plus pcDNA-ctb; group 4 with pcDNA-wapA without DMRIE-C. All mice were immunized four times biweekly.

**Collection of saliva.** Mice were injected intraperitoneally with 100 μl pilocarpin (1 mg ml⁻¹) to induce salivary flow. Saliva was collected by aspiration from the cheek pouch using a plastic pipette tip. PMSF was added at 1 mM 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 immunonochemical assays.

**Dot immunobinding assay.** The presence of specific IgA expressed in the saliva of the immunized mice was detected by immunodot analysis as follows. A piece of 96-well-embossed nitrocellulose membrane was dotted with 10 μl per dot of 5 μg purified WapA protein ml⁻¹. The membrane was blocked with 5% skimmed milk for 1 h and small membrane pieces containing the antigen dots were cut out and incubated separately overnight at 4 °C with diluted (1:2) saliva samples. After extensive washing, the membrane was incubated with an alkaline-phosphatase-conjugated anti-mouse IgA diluted to 1:10,000 for 1 h, followed by antibody detection by staining with a chromogenic substrate solution containing O-dianisidine tetrazotized (0.25 mg ml⁻¹) and β-naphtyl acid phosphate (0.25 mg ml⁻¹) in 0.06 M sodium borate buffer at pH 9.7 (Dao, 1985). A purple colouration was indicative of the presence of sIgA against WapA. Saliva from pre-immunized mice served as negative controls.

**ELISA.** To follow the production of specific sIgA over time, ELISA was used to determine the sIgA titres as described previously (Han & Dao, 2005). Serial twofold dilutions of saliva were made and then incubated at 4 °C for overnight in the purified WapA-coated plate. The level of specifically bound sIgA antibody was quantified by using an HRP-conjugated goat anti-mouse IgA diluted to 1:1000 and then read on a fluorometer with 325 nm excitation and 420 nm emission. Production of sIgA (mean titre from six animals and SD) was calculated and plotted as a function of time post-immunization. The end-point titres were expressed as the highest saliva dilution that was
resulted in an absorbance value three times the sd above the mean of the pre-immune saliva.

**Statistical analysis.** Data for saliva antibody titres were exponentially transformed and statistical differences were determined by using Kruskal–Wallis nonparametric one-way analysis of variance (ANOVA) with Dunn’s multiple comparison post test.

**RESULTS AND DISCUSSION**

**Expression of DNA vaccine plasmids containing the target gene in eukaryotic cells**

The expression of the genes inserted into the plasmid vector was verified by in vitro transfection of HeLa cells. Western blotting confirmed that these cells expressed the corresponding antigens, based on the anticipated size for CTB, IL-5 or WapA (Fig. 1b). In contrast, no immunoreactive protein was expressed in the HeLa cells that were transfected either with the empty pcDNA3.1/V5/HisTOPO vector or with pcDNA-wapA without the cationic adjuvant DMRIE-C. Thus, cationic lipid was proven to be a necessary adjuvant.

**Antigenicity and serospecificity to GST-WapA fusion protein**

Antisera obtained from rabbits immunized with GST-WapA fusion protein were adsorbed with E. coli antigens and GST, then tested against S. mutans GS5 wild-type and E. coli recombinant clones by immunodot analysis. The results showed the presence of specific antibody (Fig. 2). The GST-WapA fusion protein elicited a specific antibody response to the WapA in rabbit, suggesting that the presence of GST did not interfere with the immunogenicity of the target protein. Thus, the cleavage of GST was not necessary, at least in studies involving immunization of murine models of experimental caries.
lack of WapA expression by HeLa cells transfected with pcDNA-wapA without DMRIE-C.

**Immunomodulatory effect of genetic adjuvants on humoral immunity**

To investigate the influence of co-immunization with pcDNA-il-5 or pcDNA-ctb on the level and duration of sIgA response, saliva from the different groups of mice was collected and analyzed at various time intervals. Group 1 mice that were immunized with pcDNA-wapA were shown to produce a significant specific sIgA antibody response ($P < 0.05$) after a primary immunization and one booster dose ($P < 0.05$), but there was no apparent memory enhancement by subsequent booster doses (Fig. 4). Group 2 and group 3 mice showed a significantly higher sIgA response after one primary and two booster doses of the corresponding DNA constructs ($P < 0.001$). However, this was followed by a drop in the sIgA level in group 2 mice that received pcDNA-wapA and pcDNA-il-5 as an adjuvant despite an additional booster dose, although the level was still significantly higher than in group 1 mice that did not receive any DNA adjuvant. Data from group 3 mice indicated that, unlike with pcDNA-il-5, the use of pcDNA-ctb was associated with a sustained production of sIgA levels for an additional 4 weeks ($P < 0.001$) as shown in Fig. 4. In summary, the antigen-specific sIgA response to the DNA vaccine pcDNA-wapA was generally enhanced by co-immunization with pcDNA-il-5 or pcDNA-ctb, suggesting an immunomodulatory effect of IL-5 or CTB expression. Additionally, the enhancement obtained by co-immunization with pcDNA-ctb could be attributed to the carrier function of CTB that is associated with the high affinity of CTB to GM1 ganglioside receptors present at the surface of epithelial cells and leukocytes, thus promoting the uptake and presentation of co-expressed WapA to the mucosal immune system. George-Chandy et al. (2001) reported a similar observation that the coupling of antigen to CTB augmented the antigen-presenting capacity to dendritic cells and B cells, as well as macrophages. Insufficient memory response was consistent with data from other studies with DNA vaccine (Letvin et al., 1997; Robinson et al., 1999; Shiver et al., 1996). Jia et al. (2006) showed an immunomodulatory effect of anti-caries DNA vaccine by fusion of cytoxic T lymphocyte antigen-4 (CTLA4) to S. mutans antigens, suggesting that the fusion vaccine may improve the protective efficacy of DNA vaccine against dental caries.

Our work is to the best of our knowledge the first to test the usefulness of genetic adjuvants in immunization with anti-caries DNA vaccine, and the results indicate that co-immunization with pcDNA-ctb induces steadier production of the sIgA response than with pcDNA-il-5, supporting the use of this construct as an adjuvant in DNA vaccine regimens. This observation is in agreement with the recognized use of CTB as an adjuvant for the induction of a mucosal response to a protein-based vaccine in mice (Wu & Russell, 1993). Furthermore, unlike choleratoxin and cholera toxin A subunits, CTB is not toxic, and hence is considered as a good candidate adjuvant for human use (Yasuda et al., 1998).

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**REFERENCES**


