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Research paper

Enhanced immune response to a dual-promoter anti-caries DNA vaccine orally delivered by attenuated *Salmonella typhimurium*

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ABSTRACT

The strength of immune responses induced by DNA vaccine is closely associated with the expression level of cloned antigens available to the antigen presenting cells (APCs). To acquire a larger and more persistent amount of antigen, a dual-promoter, which could double the target antigen output through its expression both in prokaryotic and eukaryotic cells, was employed in the constructed anti-caries DNA vaccine with attenuated *Salmonella* as mucosal delivery vector in this study. Here, both *CMV* and *nirB* promoters were included in the plasmid that harbors the genes encoding the functional epitopes of two virulence factors of *S. mutans*, i.e. the saliva-binding region (SBR) of PAc and the glucan-binding region (GBR) of glucosyltransferase-I (GTF-I). Delivered by attenuated *Salmonella Typhimurium* strain SL3261, the anti-caries vaccine was administered intragastrointestinally to BALB/c mice for evaluation of the effectiveness of this immune regime. Specific anti-SBR and anti-GBR antibodies were detected in the serum and saliva of experimental animals by week 3 after immunization. These immune responses were further enhanced after a booster vaccination at week 16. However, in mice receiving *Salmonella* expressing SBR and GBR under the control of *nirB* alone these antibody responses were significantly ($P < 0.01$) lower. The serum IgG subclass profiles suggested a Th1/Th2-mixed but Th2 biased immune response to the cloned antigens, which was further confirmed by a significant increase in the Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines in splenocytes of immunized mice upon stimulation with SBR or GBR. To further determine the protective efficacy of these responses, a challenge test with *S. mutans* strain UA159 was performed in mice after the second immunization. Following challenge, mice immunized with *Salmonella* expressing SBR and GBR under the control of the *CMV-nirB* promoter showed a significant ($P < 0.01$) reduction in the number of *S. mutans* in the dental plaque compared to the empty vector-immunized or unimmunized mice, and the reduction was also significant at weeks 3–8 ($P < 0.05$) post-challenge when compared with those receiving *Salmonella* clones with *nirB* promoter alone. These results provide evidence for the effectiveness of a dual-promoter strategy in the anti-caries DNA vaccine when employing attenuated *Salmonella* as delivering vehicle for mucosal immunization.

1. Introduction

Dental caries is a worldwide disease, caused mainly by mutans streptococci (MS), especially *S. mutans* (Taubman and Nash, 2006). It affects 60–90% of school children and nearly all adults, often leading to pain, tooth loss and expensive cost (Petersen, 2003). Thus, it is necessary that some public-health measures (such as vaccination) be taken to combat this disease (Taubman and Nash, 2006). The molecular pathogenesis of MS is closely associated with several cell-surface proteins of *S. mutans,* which are found involved in the mutans streptococcal colonization on the tooth surface. For initial colonization, MS need to adhere to saliva-coated tooth surface, which is mediated by an adhesion molecule known as PAc (also designated as Ag I/II or P1) via the saliva-binding region (Koga et al., 2002). This process is followed by the accumulation of MS on tooth surface, which depends on extracellular water-insoluble glucans synthesized by glucosyltransferases (GTFs) (Koga et al., 1986; Koga et al., 2002). *S. mutans* expresses three forms of GTF (GTF-S, GTF-SI, and GTF-I) for producing water-soluble and water-insoluble glucans; each having a distinct role in the pathogenesis of dental caries (Koga et al., 2002; Taubman and Nash, 2006). The C-terminal glucan-binding region (GBR) of GTF is an functional domain that is important to the activity of GTFs (Smith and Mattos-Graner, 2008).

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Experiments have showed that antibodies targeting the functional regions of these virulence factors can significantly inhibit the colonization of MS on tooth surface and reduce the dental caries (Smith and Mattos-Graner, 2008; Sun et al., 2009).

Secretory IgA (sIgA) is the principal immunoglobulin isotype in saliva and acts as an important agent in immune defense against dental caries by blocking MS colonization on tooth surface (Hajishengallis et al., 1992). Such IgA-mediated mucosal immunity are generally most efficiently induced via mucosal route rather than by systemic ways (Neutra and Kozlowski, 2006). Attenuated *Salmonella typhimurium* as a bactofection vector has been used in many ways and shown obvious advantages for mucosal immunization. The orally administered vector can mimic the natural process of *Salmonella* infection and target the gut-associated lymphoid tissues, delivering the foreign antigens or DNA vaccines directly to APCs (Castillo Alvarez et al., 2013). This feature provides the *Salmonella*-carried vaccine a favorable condition in inducing a mucosal immune response with a gastrointestinal stage (Rescigno et al., 2001). In addition, the *Salmonella* carrier can also serve as a natural adjuvant by stimulating the secretion of proinflammatory mediators that trigger an early innate immune response and create a local environment conducive to antigen presentation (Salazar-Gonzalez et al., 2006). Thus, the *Salmonella*–based vaccine system provides an efficient platform technology for mucosal immunization and novel vaccination strategies (Spreng et al., 2006).

Research studies exploring immunological intervention in dental caries have demonstrated that immunization via mucosal route can efficiently induce salivary MS-specific IgA antibody responses and suppress the MS colonization in dental plaques (Huang et al., 2001; Smith and Mattos-Graner, 2008). However, some concern still remains that previous mucosal-based vaccines may not induce host responses strong enough or of sufficient duration to block the colonization of MS on a long-term basis (Smith, 2010; Taubman and Nash, 2006). This dilemma is caused by multiple factors, and an important one may be that previous vaccines could not yield sufficient amount of antigens to the APCs. Therefore, in this study we designed a dual promoter (*CMV-nirB*) expression system hoping to provide a large and persistent amount of antigen protein. In this regard, the in vivo-inducible *nirB* promoter should be activated immediately after *Salmonella* infecting the host (Huang et al., 2001; Salam et al., 2006), whereas the *CMV* promoter should be active when the dual-promoter plasmid was released and transferred into the cytosol and nucleus of the infected host cells (Darji et al., 1997). Thus, a high amount of antigen protein along with different ways of presentation could be available in this vaccination formula. The main purpose of this study was to determine if this dual-promoter immune regime was superior to *nirB* promoter used alone in inducing a protective immunity against *S. mutans* colonization. The results showed this immune strategy was promising.

2. Materials and methods

2.1. Bacterial strains, cell line, and culture conditions

Salmonella enterica serovar Typhimurium SL3261 (*aroA* r^+ m^+) and *S.* Typhimurium LB5010 (*galE* r^- m^+) were kindly provided by Dr. Dunstan SJ (Imperial College London, UK). Bacterial strains were routinely grown at 37 °C in Luria-Bertani (LB) broth or agar, supplemented with 100 μg/ml ampicillin (LB+amp) as needed. For anaerobic growth, recombinant clones of SL3261 was grown in LB+amp broth overlaid with sterile mineral oil (Sigma) at 30 °C overnight in a candle jar without shaking. *S. mutans* UA159 was cultured in brain heart infusion broth (BHI; Becton Dickinson, Sparks, MD) at 37 °C in the presence of 5% CO_2.

Chinese hamster ovary (CHO) cells were propagated in DMEM/F-12 (1:1, v/v) medium supplemented with L-proline (150 μg/ml), penicillin (100U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. For transient transfection, CHO cells were seeded in 6-well plate at a density of 3.3×10^5 cells per ml. After cultured overnight, CHO cells were transfected with plasmids using FuGENE 6 transfection reagent (Roche Diagnostics Corp., USA) according to the manufacturer's instructions, and afterwards incubated in serum-free medium at 37 °C in a 5% CO2 atmosphere.

2.2. Genetic construction

Plasmid pCN-SS/SG (Wang et al., 2007) was constructed by cloning DNA segments encoding SBR and GBR into the dual-promoter vector pCMVnir (Bian et al., 2005), as previously described. Plasmid pCMVnir (or pCN) contains prokaryotic promoter *nirB* and eukaryotic promoter *CMV*. In plasmid pCN-SS/SG, *sbr* and *gbr* segments were under the control of the *CMV-nirB* promoter and separated by internal ribosome entry site (IRES) to prevent fusion expression (Fig. 1A). Furthermore, signal sequences of the tissue-type plasminogen activator (tPA-sp) were designed with the help of SignalP 3.0 to form an in-frame fusion with the 5′ end of *sbr* and *gbr* to facilitate exocytosis of the expressed SBR and GBR. To confirm that target genes were properly expressed under the control of *CMV-nirB* promoter in both prokaryotic and eukaryotic cells, EGFP gene encoding the enhanced green fluorescent protein was cloned in place of the *sp-gbr* segment to construct another plasmid pCN-SSIE (Liu et al., 2005) (Fig. 1B). In addition, fragment *SS/SG* with sticky ends *NcoI* and *XhoI,* was PCR amplified with plasmid pCN-SS/SG and cloned into plasmid pNir-16L1E7 (Bian et al., 2005) digested with *NcoI* and *XhoI* to construct pNir-SS/SG as a positive control.

2.3. Preparation of SBR and GBR and mouse polyclonal anti- SBR and GBR antibodies

Recombinant SBR and GBR were expressed in the JM109 (DE3) cells using plasmids pTriEx-4-SBR (Lu et al., 2004) and pTriEx-4-GBR (Wang et al., 2006) respectively, and purified with HisTrap™ kit (Amersham). The molecular weights of SBR and GBR were 43.5 kDa and 42.2 kDa respectively, including 8 × His·Tag.

Six-week-old BALB/c mice were immunized subcutaneously in the back with 40 μg of SBR or GBR, incorporated in complete Freund's adjuvant on day 0 and in incomplete Freund's adjuvant on days 14, 28 and 42. Seven weeks after the first inoculation, blood was collected. Sera were retrieved from the coagulated specimens and centrifuged at 5000 × *g*, 4 °C for 10 min, then frozen at −80 °C for later use.

2.4. Gene expression under the control of CMV-nirB promoter in vitro

To assess the efficacy of the *CMV-nirB* promoter in controlling the target genes expression in both prokaryotic and eukaryotic cells, plasmids pCN-SSIE and pCN-SS/SG were used to transform *S. typhimurium* strain SL3261 and CHO cells, as previously described (Liu et al., 2005; Oyston et al., 1995). Briefly, pCN-SSIE and pCN-SS/SG were first passaged through *S. typhimurium* strain LB5010 for DNA-methylation, and then electroporated into *S. typhimurium* SL3261. The transformants were anaerobically cultured in LB+amp broth overnight. CHO cells were transfected with pCN-SSIE or pCN-SS/SG plasmid using FuGENE 6 transfection reagent (Roche Diagnostics Corp., USA). Culture supernatant as well as CHO cells was retrieved after 48 h of incubation. SBR and GBR expressions