Therapeutic vaccination with *Salmonella*-delivered codon-optimized outer inflammatory protein DNA vaccine enhances protection in *Helicobacter pylori* infected mice

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**A B S T R A C T**

Vaccination had demonstrated as an alternative way to combat *Helicobacter pylori* challenge. In the present study, codon-optimized outer inflammatory protein gene (oipA) for *Mus* species codon usage, the inclusion of optimal Kozak sequence, and modified of GC content was applied to construct a novel DNA construct. The *Salmonella*-delivered wild type oipA construct (SL7207/poipA) and the *Salmonella*-delivered codon-optimized oipA construct (SL7207/poipA-opt) were prepared and their therapeutic efficacy was evaluated in *H. pylori*-infected mice. The codon-optimized oipA construct (poipA-opt) expressed almost six-fold higher protein than that of wild type construct (poipA) as normalized to the β-actin expression in AGS cells. Oral therapeutic immunization with SL7207/poipA-opt significantly eliminated *H. pylori* colonization in the stomach; and protection was related to a robust Th1/Th2 immune response. Therefore, our results suggested that fine therapeutic efficacy was related to sufficient expression of the antigen. It is supposed that codon-optimized oipA gene improves protein expression and consequently enhances the immunogenicity of DNA vaccine, which resulted in a significant reduction of bacterial loads in *H. pylori* infected mice. The *Salmonella*-delivered codon-optimized DNA construct could be a candidate vaccine against *H. pylori* for the clinical application.

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1. Introduction

*Helicobacter pylori* (*H. pylori*) is a spiral bacterium that colonizes the gastric mucosa of more than half the world’s population. *H. pylori* infection can lead to gastritis, peptic ulcer disease, and a risk factor for adenocarcinoma and mucosa associated lymphoid-tissue lymphoma [1]. The current antibiotic therapy to *H. pylori* infection has many drawbacks including therapy failure due to the emergence of bacterial resistance, lack of patient compliance, high cost of treatment, and failure to prevent re-infection, which calls for a reliable, inexpensive and highly immunogenic vaccine that can eradicate *H. pylori* infection and prevent re-colonization [2].

Therefore, vaccination has been demonstrated as an effective way to combat *H. pylori* infection.

Either prophylactic vaccination to prevent *H. pylori* infection or therapeutic vaccination had been studied in variety of antigens and adjuvant combinations in many animal models with promising results [3–8]. Among numerous vaccination strategies, DNA vaccination poses as an attractive approach for protecting and immunotherapy against *H. pylori*. Compared to live viral or bacterial vectors, naked DNA plasmid vaccines are flexible to design and easy to prepare on a large scale with high purity and high stability (for reviews, see [9]). Furthermore, DNA vaccines are relatively safe and can be easily administered.

Extensive experiments have shown DNA vaccines’ ability to elicit systematic immune responses in several model systems. However, the naked DNA vaccine is limited by its insufficient immunogenicity. One promising approach for improving the response of naked DNA vaccine is to maximize its expression in mammalian cells [10–12]. The other approach for enhancing DNA vaccine efficacy is required an appropriated delivery system, which can play a key role in the magnitude and quality of the triggered immune response. Attenuated *Salmonella typhimurium* strains have
been evaluated for using as live vaccines for delivery of a variety of bacterial, viral, and parasitic antigens to mucosal lymphoid tissue. Vaccines administered via mucosal routes can induce mucosal, cell-mediated, as well as systemic immune responses [13–16]. The auxotrophic mutants of *S. typhimurium* r–m+ strain SL7207 had been applied as vaccine strains for delivering many antigens, which conferred protection against lethal challenge in mice [17,18].

In the current study, we attempted to investigate whether murine codon optimization of the oipA gene could enhance the antigen expression and its immunogenicity. We next tried to explore whether attenuated *Salmonella* SL7207 carrying codon-optimized oipA gene could confer more elimination of bacterial colonization in *H. pylori* infected mice. To this end, we generated a codon optimization of the oipA DNA construct which could highly express protein in eukaryotic cell. *Salmonella* carrying codon-optimized oipA construct could elicit a stronger immune response and confer more protective efficacy against *H. pylori* challenge in mice, suggesting that therapeutic efficacy of DNA vaccine against *H. pylori* can be significantly improved by genetic modification.

2. Materials and methods

2.1. Bacterial strains and cell culture

Human gastric epithelial cells (AGS) were obtained from the American Type Culture Collection and cultured in DMEM containing 10% fetal bovine serum, and an antibiotic mixture with 100 units of penicillin and 100 μg of streptomycin (Invitrogen, Carlsbad, CA). Mouse-adapted *H. pylori* strain SS1 was maintained on Columbia agar (Oxoid, Cambridge, UK) supplemented with 5% defibrillated rabbit blood and 1% Dent’s selective supplement (Oxoid, Cambridge, UK). To prepare for infection experiments, bacteria inoculated in brain–heart infusion were harvested at 48 h. Live attenuated *S. typhimurium* strain SL7207, which has a deletion in the aroA gene in the aromatic amino acid biosynthetic pathway, was kindly provided by Prof. Zhongtian Qi (The Second Military Medical University, China). This mutant strain of bacteria is not pathogenic to mice via oral delivery [17].

2.2. Codon-optimization of *H. pylori* oipA gene

To generate the codon-optimized gene, the oipA gene of *H. pylori* J99 strain (GenBank accession number: NC_000921) was modified to include six nucleotides of Kozak sequence (GCCACC) upstream of the start codon, codon usage was optimized based on frequencies of transfer RNA in *Mus* species and GC content was increased to promote RNA stability. Modification of 27.3% nucleosides optimized oipA sequence and resulted in G+C content being increased from 38.6% to 48.5% (see Supplementary Figure S1). Despite such DNA sequence changes, the final codon-optimized sequence should still produce the identical oipA amino acid sequence as in the original bacterium. The codon-optimized sequence was chemically synthesized by Sangon Biological Engineering Company (Shanghai, China) with added restriction enzyme sites of Pst I and Xho I for subcloning purpose immediately upstream of Kozak sequence and downstream of the stop codon, respectively.

2.3. Construction of *H. pylori* oipA DNA vaccine plasmids

The wild type of oipA DNA plasmid (poipA) was constructed as previously described [19]. The codon-optimized oipA sequence was cloned into the same eukaryotic expression vector pVAX1 (Invitrogen, Carlsbad, CA) at Pst I and Xho I cloning sites to produce the novel DNA vaccine construct, which was designated as poipA-opt. Both of the DNA vaccine plasmids were verified by double-restriction enzymes analysis and DNA sequencing (Takara, Dalian, China). The plasmids were prepared using Qiagen Plasmid Mega Kit according to the manufacturer’s instruction.

2.4. In vitro transfection studies of DNA vaccine

For transfection experiments, five hundred thousand of AGS cells were cultured in a plate with ~90% confluent. Then cells were transfected with the recombinant plasmids by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) transiently, cells were harvested at 48 h post-transfection. Total cell lysates were prepared by using RIPA lysis buffer (Beyotime, Jiangsu, China). Equal amounts of cell lysates were separated by a 12% SDS-PAGE and then transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were blocked for 1 h at room temperature in PBS containing 3% BSA and 0.1% Tween 20 under gentle shaking. Membranes were incubated overnight with rabbit anti-oipA serum and actin antibody (Santa Cruz, CA, USA), respectively. Followed with a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Zhongzhan Golden Bridge, Beijing, China). Blots were developed by enhanced chemiluminescence (Amersham Biosciences, UK) and exposed to Kodak-XO-mat-AR film (Kodak, Rochester, NY) for 1 min. The intensity of each band was read by using a Chemilager 4400 densitometer (Alpha Innotech, San Leandro, CA) and the relative intensity of oipA were normalized by β-actin expression.

2.5. Attenuated *S. typhimurium* carrying DNA plasmids construction

To make competent cells, SL7207 were grown in LB broth at 37 °C overnight to an optical density of 0.6–0.7 at 600 nm. Following centrifugation, the bacterial pellet was washed three times in sterile ice-cold water, resuspended finally in sterile water supplied with 10% glycerol. The plasmids were subsequently electroporated into the competent cells in a 2-mm cuvette under the following conditions: 2.0 kV, 25 μF and 200 Ω (Gene Pulser Xcell, Bio-rad, Hercules, CA) for 5 s. The positive transformants were selected on LB agar containing 50 μg/mL kanamycin and identified by PCR amplification and restriction enzyme digestion. The PCR products were sequenced to further confirm the introduction of plasmids in SL7207. These produced the strains of SL7207/poipA, SL7207/poipA-opt and SL7207/pVAX1, respectively.

2.6. Mice and immunization

Six-week old female C57BL/6 mice were obtained from Shanghai Laboratory Animal Center (Shanghai, China). All animals were maintained under specific pathogen-free conditions at the animal center of Fujian Medical University (Fuzhou, China). Animals were housed under standard day–night cycle and provided with sterile food and water ad libitum. The Animal Care and Ethics Committee of Fujian Medical University (Fuzhou, China) approved all procedures. Fifty mice were challenged with three doses of 5.0 × 10⁸ CFU of *H. pylori* SS11 in brain heart infusion broth intragastrically at 2-day intervals. Quantitative cultures of *H. pylori* and histopathological evaluation of gastric tissues demonstrated that these *H. pylori* challenge doses resulted in nearly 100% infection rates in C57BL/6 mice. Mice were orally immunized with 5.0 × 10⁸ CFU/200 μL *S. typhimurium* stains as follows: (1) SL7207/poipA-opt strain; (2) SL7207/poipA strain; (3) SL7207/pVAX1 strain; (4) SL7207 strain and (5) sham-immunized with 200 μL of PBS. Water and food were available to the mice 2 h after immunization. The same way, booster immunization was carried out 2 weeks after the prime immunization.

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2.7. Detection of serum antigen-specific antibody

For H. pylori-specific antibody assay, blood was withdrawn from an orbital vein of each mouse 4-week post-immunization. The sera total IgG, IgG2a and IgG1 were determined by indirect ELISA as described previously [19].

2.8. Determination of H. pylori colonization

Four weeks post-immunization, the mice were sacrificed for the determination of H. pylori colonization in the stomach. The colonization of H. pylori was quantified by quantitative culture as described previously [19].

2.9. Antigen specific cytokines assay

Mice spleen was grounded through a screen mesh. Red blood cells were lysed with ACK lysis buffer (Beyotime, Jiangsu, China) from spleen samples. Two million splenocytes were added to a plate and incubated with 30 μg of H. pylori sonicate in complete RPMI 1640 containing 5% fetal bovine serum (Invitrogen, Grand Island, NY) at 37 °C. Following incubation, supernatants were collected at 72 h and concentrations of IFN-γ and IL-4 were determined using OptEIA™ mouse cytokines detection system (BD Biosciences, San Jose, CA). The optical density of each well was read and data were analyzed by BIOTEK software (Bio-Tek, Winooski, VT) using five parameter logistics.

2.10. Statistical analysis

One-way ANOVA and Student–Newman–Keuls (SNK) test were performed by the SigmaPlot software (Systat Software, Inc., San Jose, CA) to compare statistically significant differences among groups. Differences were considered statistically significant at p < 0.05 between groups.

3. Results

3.1. Expression of oipA protein in AGS cell

To characterize DNA vaccine that incorporates codon-optimization, we constructed a codon-optimized DNA vaccine that could be a higher expression of the H. pylori oipA protein in mammalian system. Following construction, proteins expression was confirmed by Western blot. The presence of ~34 kDa protein was detected in the cell lysates of poipA-opt and poipA transfected AGS cells, while control pVAX1 empty vector-transfected lysates were negative for oipA expression (Fig. 1A). The poipA-opt transfected AGS cells produced approximately six-fold higher oipA protein than that of poipA transfected AGS cells, as normalized to β-actin expression, respectively (as shown in Fig. 1B).

3.2. Stability of S. typhimurium carrying recombinant DNA vaccines

To construct the attenuated S. typhimurium carrying recombinant DNA vector vaccines, poipA, poipA-opt and pVAX1 vectors were transformed into SL7207 by electroporation. The stability of the plasmid maintained in SL7207 was assessed in culture without antibiotic selection. After growth for fifty passages without kanamycin selection, above 90% of bacteria still harbored the plasmids (as shown in Fig. 2). Enzyme digestion with Pst I and Xho I revealed that all plasmids extracted from ten randomly picked SL7207/poipA-opt and SL7207/poipA colonies grown on kanamycin-free plates contained an insert of about 924 bps, the same size of oipA gene (data not shown).

3.3. Serum specific antibody responses in mice

The capacity of attenuated S. typhimurium vaccine to induce oipA-specific serum antibodies was evaluated by ELISA. In the induction of sera anti-oipA specific IgG, IgG1 and IgG2a,
SL7207/poipA and the SL7207/poipA-opt immunized mice primed significantly higher levels of sera antibodies than that of the control groups (p < 0.001). Furthermore, the SL7207/poipA-opt elicited higher levels of sera antibodies than that of the SL7207/poipA (p < 0.001) (Fig. 3).

3.4. Therapeutic immunization induced antigen specific cytokine responses

To assess the polarization of the immune response, ELISA was utilized to determine the production of IFN-γ and IL-4 cytokines in the supernatants of spleen cell cultures. In immunized mice, higher concentrations of IFN-γ and IL-4 were observed in the SL7207/poipA-opt groups and the SL7207/poipA group, as compared to those of the control groups with triple independent experiments (p < 0.001). Interestingly, in the SL7207/poipA-opt immunized mice, the concentrations of IFN-γ and IL-4 were nearly two-fold increase as compared to those of the SL7207/poipA immunized mice (as shown in Fig. 4A and B). Thus, oral inoculation with attenuated *Salmonella* carrying recombinant *oipA* DNA vaccine facilitated a mixed Th1 and Th2 immunized response, concurrently codon-optimized *oipA* gene DNA vaccine improved both humoral and cellular immune responses in *H. pylori* infected mice.

3.5. Effect of vaccination against *H. pylori* infection

To determine whether oral immunization of SL7207/poipA-opt or SL7207/poipA could reduce the bacterial load in the stomach of *H. pylori* infected mice, quantitative culture of *H. pylori* was performed four weeks post-immunization. A significantly decreased bacterial load was achieved in mice immunized with SL7207/poipA, with almost 3-log decrease in bacteria load as compared to that of the control groups (p < 0.001). Furthermore, SL7207/poipA-opt group had conferred nearly 2-log decrease in bacterial load than that of SL7207/poipA group; three mice had no detectable *H. pylori* with both the quantitative culture method and the PCR-based detection of *H. pylori* urease C and 16S rRNA genes. These data demonstrated that SL7207/poipA-opt improved therapeutic efficacy in infected mice, which could obtain sterile immunity in some of the animals (Fig. 5).
4. Discussion

In the current study, we constructed live-attenuated *S. typhimurium*-delivered codon-optimized oipA vaccine (SL7207/poipA-opt) and evaluated the therapeutic efficacy in the *H. pylori* infected mice. Mice oral administered with either SL7207/poipA or SL7207/poipA-opt had significantly eliminated *H. pylori* colonization. However, mice administered with SL7207/poipA-opt elicited stronger immune responses and reduced more bacterial load in the stomach, indicating that *Salmonella*-delivered DNA vaccine could effectively eliminate *H. pylori* colonization in mice, which was associated with robust Th1/Th2 immune responses.

DNA vaccine has posed an attractive approach for protecting against bacterial, viral and parasitic pathogens. However, the naked DNA vaccine is limited by its insufficient immunogenicity. Various attempts have been made to improve DNA vaccines, including the use of molecular or genetic adjuvant, modification of subcellular localization of expressed antigen [20,21], the targeting of antigen to the major histocompatibility complex class I or II processing pathway [22], and the use of self-destructing attenuated intracellular bacteria as carriers for the DNA vaccines [23]. Recently, several reports showed that codon optimization or codon usage modification of genes toward human consensus codon usage significantly increased both the protein expression and the immunogenicity of DNA vaccines. In this study, oral immunization with SL7207/poipA-opt induced significantly higher *H. pylori*-specific antibody response and profile of cellular response, indicating that the SL7207/poipA-opt expressed much protein in vivo. These results were consistent with other researches, demonstrating that the codon-optimization of antigen encoding construct delivered by *S. typhimurium* could improve the immunogenicity of DNA vaccine.

The oipA sequence was optimized in many ways to improve protein expression efficiency. Firstly, the mRNA translation efficiency can be improved by codon usage optimization. Codon-optimized of the sequence for species could result in significantly higher levels of viral protein expression for genetic vaccination purpose [24,25]. Secondly, an up-stream Kozak sequence flanking the AUG start codon has been shown to be essential and necessary for the optimal translation genes in mammalian cells [26,27]. In this study, the oipA gene was optimized for the most prevalent codons used in *Mus* species, and modified to include a consensus Kozak sequence (GCCACC). As a result led to approximately 27.3% of codons being modified in oipA-opt construct (Supplementary Figure S1). Thirdly, in order to promote mRNA stability, GC content was dramatically increased from 38.64% to 48.5%. We had demonstrated that by optimizing the sequence of oipA gene for expression in mammalian cells, the expression of oipA protein can be significantly increased three times approximately. This is in accordance with the findings of recent researches [24,28,29]. However, understanding the relative contribution of each molecular determinant (i.e. promoter, Kozak sequence, codon optimization, etc.) individually may help design future optimized other sub-unit vaccines; the work needs to be explored in the future.

The ability to deliver the DNA vaccine efficiently to host cells to produce robust levels of protein expression is another key to successful vaccination. *Salmonella* vector vaccine has been proposed as a novel strategy for developing an effective vaccine against some infectious diseases [30–32]. The use of live-attenuated *Salmonella* to deliver recombinant antigens decreases potential pathogenicity while taking advantage of the ability to invade intestinal cells and deliver expressed antigen directly to the immune system [33,34]. Studies had shown that attenuated *Salmonella* has been used as a live vector to deliver heterologous antigens that can stimulate mucosal, humoral and cellular immune responses after immunizing animals via mucosal surfaces [30–32]. In the current study, oral immunization with *S. typhimurium*-delivered oipA construct dramatically eliminated *H. pylori* colonization in C57BL/6 infected mice. Interestingly, SL7207/poipA-opt vaccination obtained sterile immunity in some of the experimental animals. We presume that the protection could be relevant to the type and extent of immune responses. However, sterile immunity could be achieved only when immune responses modulate and increase to a certain level. Therefore, it is possible that codon optimization gene improved the recombinant antigen expression in mice, and the latter resulted in modulating the immune response to certain level. Hence, an attenuated *Salmonella*-delivered DNA construct could be a promising candidate for the development of *H. pylori* vaccination.

Whether Th1 or Th2 cell play a key role in vaccine-induced protection against *H. pylori* is still debated. Data obtained with transgenic mice supposed that a Th2 response be owing to a protective mechanism [35]. However, recent studies have demonstrated the importance of the Th1 response in an effective protection following vaccination against *H. pylori* [36,37]. In addition, Blanchard et al. [38] also proposed a role of regulatory T cell in *H. pylori* vaccination and suggested that the site of T cell activation influences protection. Therefore, the definite protective mechanism against *H. pylori* challenge is still need to be explored. In this study, significantly elevated IFN-γ and IL-4 expression and anti-H. pylori balanced IgG2a/IgG1 antibody production were evident, indicating that the mixed Th1/Th2 type response could be more important in the clearance of *H. pylori*.

In summary, our results suggested that codon optimization of the oipA DNA construct could improve protein expression and consequently enhance therapeutic efficacy against *H. pylori*. Furthermore, the strategy of combining DNA vaccine of codon-optimized *H. pylori* antigen with other promising approaches could facilitate the design of improved anti-*H. pylori* vaccine for clinical application. In the future, we attempt to construct a *S. typhimurium* ghost vector carrying codon-optimized DNA vaccine for preclinical study in mice and in nonhuman primates. In addition, trials of using therapeutic *Salmonella*-delivered DNA vaccines on humans are expected.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.06.052.

References


