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Intragastric administration of attenuated *Salmonella typhimurium* harbouring transmissible gastroenteritis virus (TGEV) DNA vaccine induced specific antibody production

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**Article info**

**Abstract**

Attenuated *Salmonella typhimurium* was selected as a transgenic vehicle for the development of live mucosal vaccines against transmissible gastroenteritis virus (TGEV). A 2.2 kb DNA fragment, encoding for N-terminal domain glycoprotein S of TGEV, was amplified by RT-PCR and cloned into eukaryotic expression vector pVAX1. The recombinant plasmid pVAX-S was transformed by electroporation into attenuated *S. typhimurium* SL7207, the expression and translation of the pVAX-S delivered by recombinant *S. typhimurium* SL7207 (pVAX-S) was detected in vitro and in vivo respectively. BALB/c mice were inoculated orally with SL7207 (pVAX-S) at different dosages, the bacterium was safe to mice at dosage of 2 × 10⁹ CFU and eventually eliminated from the spleen and liver at week 4 post-immunization. Mice immunized with different dosages of SL7207 (pVAX-S) elicited specific anti-TGEV local mucosal and humoral responses as measured by indirect ELISA assay. Moreover, the immunogenicity of the DNA vaccine was highly dependent on the dosage of the attenuated bacteria used for oral administration, 10⁹ CFU dosage group showed higher antibody response than 10⁸ CFU and 10⁷ CFU dosages groups during week 4–8 post-immunization. The results indicated that attenuated *S. typhimurium* could be used as a delivery vector for oral immunization of TGEV DNA vaccine.

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administration of DNA vaccines via mucosal surfaces as well as delivery of the plasmid DNA directly to professional antigen presenting cells (APCs), which can elicit strong humoral and cellular responses against the pathogens [20–22]. In particular, attenuated Salmonella typhimurium (S. typhimurium) has been used to deliver DNA vaccines encoding immunogens of pathogenic microorganisms including HIV, HBV, HSV, NDV, IBDV, PRRSV and Toxoplasma gondii [23–29]. However, the use of S. typhimurium as a DNA vaccine vector in TGEV has almost not been reported. In this study, we used the attenuated intracellular bacteria, attenuated S. typhimurium SL7207, as a carrier for delivery of DNA vaccines encoding the N-terminal half of TGEV glycoprotein S. Our data indicated that orogastric intubation of the recombinant S. typhimurium could induce a specific immune response against TGEV.

2. Materials and methods

2.1. Bacterial strains, plasmid, virus and cell lines

The attenuated S. typhimurium aroA– strain SL7207 (S. typhimurium 2337–65 derivative hisG46, DEL407 [aroA::Tn10(16°)]) was kindly provided by Professor Kai Schulze of Helmholtz Centre for Infection Research, Germany. Eukaryotic expression vector, pVAX1 contains cytomegalovirus (CMV) immediate-early promoter for efficient expression and bovine growth hormone (BGH) poly A signal for mRNA stability, purchased from Invitrogen. The virulent TGEV strain SC-H was isolated from Sichuan, P.R. China in 2005, ST cells and COS-7 cells were purchased from China Center for Type Culture Collection (Wuhan, China).

2.2. Experiment mice

Female BALB/c mice (six weeks old, 17–22 g), purchased from Chengdu Institute of Biological Products (Chengdu, China), were maintained in animal holding laboratory under controlled conditions with temperature of 25 ± 1 °C, humidity of 40 ± 10% and had free access to standard mouse diet and water.

2.3. Construction of expression plasmid

Viral RNA was extracted from TGEV-infected ST cells using Mini BEST Viral RNA/DNA Extraction Kit (TaKaRa). A 2.1 kb DNA fragment, encoding for the N-terminal of glycoprotein S was ampliﬁed from virus genomic RNA by RT-PCR, using the primers S1: 5′-CCGAAGCTTACATGGAAAATCTATTGTG-3′ (forward) and S2: 5′-TCGAGCTCTAGGCATAGCGCTCT-3′ (reverse), which respectively introduce the HindIII and BamHI restriction sites at the 5′ ends of the primers. The ampliﬁed DNA fragment of TGEV S gene was cloned into a pMD19-T vector (TaKaRa) and sequenced. Then, the S gene fragment was released from the pMD19-T plasmid by HindIII and BamHI digestion and ligated into the pVAX1 vector cut by the same enzymes, the resulting plasmid was named pVAX-S.

2.4. Transient expression of the recombinant plasmid

Six-week tissue culture plates (Costar) were seeded with COS-7 cells. Monolayer of 70–80% confluent cells were transiently transfected with the plasmid pVAX-S and empty plasmid pVAX1 using Lipofectamine 2000 (Invitrogen). Thirty-two hours after transfection, cells were washed with phosphate-buffered-saline (PBS), then ﬁxed with ice-cold methanol/acetone (1:1) at 4 °C for 30 min and washed once again with PBS. Diluted primary and secondary antibodies were incubated at 37 °C for 1 h, respectively. Primary antibodies were anti-serum of pig to TGEV, which were kindly provided by researcher Li Feng (Harbin Veterinary Research Institute, China) and secondary antibodies were FITC-conjugated-rabbit-anti-pig IgG (Sigma).

2.5. Transformation of attenuated S. typhimurium

The puriﬁed plasmid pVAX-S or control vector pVAX1 was transformed into S. typhimurium competent cells by electroporation at 2.5 kV, 25 μF and 200–400 Ω. The positive transformants were selected on LB agar containing 50 μg/mL kanamycin, and then were veriﬁed by PCR ampliﬁcation and digestion with restriction enzymes. The Salmonella strains containing plasmid pVAX-S or pVAX1 were named strain SL7207 (pVAX-S) and strain SL7207 (pVAX) respectively.

2.6. Plasmid transfer from attenuated S. typhimurium to mammalian host cells in vitro

Twelve-week-old BALB/c mice were sacriﬁced by cervical dislocation, and their peritoneal cavities were injected with 6 mL of RPMI 1640 medium. After gentle abdominal massage, the maximum amount of ﬂuid was collected. The peritoneal exudates cells were separated by centrifugation and resuspended into 10 mL of RPMI 1640 medium. The isolated peritoneal macrophages were allowed to adhere for 3 h in a six-well tissue culture plates (Costar) in antibiotic-free medium, at which time the no-adherent cells were removed by gently washing plates two times with antibiotic-free medium. The adherent cells were infected with recombinant S. typhimurium strains SL7207 (pVAX) or SL7207 (pVAX-S) at a multiplicity of infection (MOI) of 50:1. After 20–30 min incubation at 37 °C, the infected cells were washed with PBS and incubated in fresh RPMI 1640 containing 10% fetal bovine serum (FBS) and 100 μg/mL gentamicin for 2 h. The medium was then removed and replaced with fresh RPMI 1640 containing 10% FBS and 10 μg/mL tetracycline. 42–60 h after infection, the expression of S gene was detected by indirect immunofluorescence assay (IFA).

2.7. RT-PCR detection the transcription of S gene in vivo

Six-week-old mice were inoculated intragastrically with 1 × 109 CFU SL7207 (pVAX-S), control mice were given with the same dosage of SL7207 (pVAX). Three days after the immunization, Payer’s patches were removed from three mice and pooled. Cellular RNA was isolated from homogenized Payer’s patches with Trizol (TaKaRa) according to the manufacturer’s instructions. The transcripts of TGEV S gene in Payer’s patches was analyzed by RT-PCR using speciﬁc primers, S3: 5′-AATTTTCCTGGTCTTCAATTGAC-3′ (forward) and S4: 5′-TCCTTTCTAAA ACTATACGTGCTCT-3′ (reverse), which contain the B, C site of S gene (549 bp in length). Mice β-actin speciﬁc primers, β1: 5′-CATGTGCCCATCTACGA-3′ (forward) and β2: 5′-ACAG GATGATCCACCAACG-3′ (reverse), with its ampliﬁed fragment length 334 bp, was used as a quality control.

2.8. Safety and bacterial colonization in organs

S. typhimurium strains SL7207 (pVAX-S) were cultured in condition as described [30] previously. The bacterial cells were collected by centrifugation at 5000 × g for 10 min and resuspended in PBS containing 5% sodium bicarbonate (m/v) to the expected cell populations, as determined by plating serial dilution on LB agar plates. Three groups of six-week-old BALB/c mice, with eight mice in each group, were inoculated intragastrically using a gavage needle with SL7207 (pVAX-S) at dosage of 5 × 108, 1 × 109, 2 × 109 CFU and boosted with the same dosage two weeks later. The immunized mice were monitored daily for clinical changes. Two mice of each group were sacriﬁced every week post-immunization, spleens and livers were collected and homogenized in 2 mL PBS containing...
0.1% Triton X-100 (v/v). The bacterial colonies were determined by plating 100 µL of the homogenized spleens and livers samples on LB agar plates containing 50 µg/mL kanamycin. Bacteria colonies were picked randomly for PCR identification of TGEV S gene and digestion by restriction enzymes.

2.9. Mice immunization and sample collection

Six-week-old BALB/c mice were randomly divided into five groups, each of them consisting of twenty mice, and immunized three times with two weeks intervals. All mice were deprived of food and water for 4 h before oral immunization. Mice in groups A, B and C were inoculated intra-gastrically with SL7207 (pVAX-S) with different dosages of 10^7, 10^8 and 10^9 CFU per mouse, respectively. Mice in group D were inoculated intra-gastrically with the control strain SL7207 (pVAX) at 10^8 CFU per mouse. Group E mice received PBS as a negative control.

Three mice from each group were sacrificed for sera and intestinal lavage collection at weeks 0, 2, 4, 6 and 8 after the primary immunization. Blood were obtained from the submaxillary sinus of the mice, sera were prepared and stored at −20 °C prior to analysis. To prepare intestinal fluid, a 15 cm section of the small intestine was removed from mouse, cut into pieces and washed in 5 mL of PBS containing 1 mmol/L phenylmethylsulfonyl fluoride and 50 mmol/L EDTA (PBS-PE). Samples were centrifuged at 12,000 × g for 20 min to remove cellular debris. The supernatant were freeze-dried using freeze dryer and dissolved in 0.5 mL PBS-PE. After centrifuged at 12,000 × g for 10 min, the supernatant were collected as resultant intestinal lavage and stored at −20 °C prior to analysis.

2.10. Measurement of antibody levels

All measurements of antibody levels in individual animal were determined in duplicate. For enzyme linked immunosorbent assays (ELISA) to determine serum IgG and intestinal IgA level of immunized mice, 96-well polystyrene microtitre plates (Costar) were coated overnight at 4 °C with 50 µL 5 µg/mL purified TGEV-antigen, in carbonate buffer (pH 9.6), and blocked for 1 h at 37 °C with PBS containing 1% w/v BSA. The coating antigen was prepared through sucrose density gradient centrifugation as described previously [4]. Plates were then washed three times with PBS containing 0.02% Tween 20 (PBST) and incubated with 100 µL of mouse serum (1:40 diluted) or intestinal lavage samples for 1 h at 37 °C. After washed three times with PBST, HRP-conjugated goat anti-mouse IgG or IgA (Sigma), diluted 1:2000 were used as the secondary antibody and incubated for 1 h at 37 °C. After incubation, the reaction was stopped by the addition of 50 µL of H2SO4 (2 mol/L), and the optical density at 450 nm was measured in an ELISA micro-plate reader. Total serum IgG and intestinal lavage IgA specific for TGEV were represented as the optical density. Data were analyzed using the one-sided Student’s t test. Differences were considered statistically significant with P < 0.05.

3. Results

3.1. Construction and transient expression of pVAX-S in COS-7 cells

A 2.1 kb DNA fragment was amplified by RT-PCR from TGEV SC-H strain. Sequence analysis showed that the amplified TGEV SC-H strain S gene fragment was 2124 bp in length and encoded 707 amino acids. The result of sequence alignment showed that the predicted amino acids sequence of TGEV SC-H strain S gene fragment shared homology of 97.7%, 94.1%, 98.0%, 97.9%, 99.6%, 99.7%, 97.1%, 99.0% and 98.3% with the sequence of TS, 96–1933, HN2002, Miller,
3.4. Safety and colonization of recombinant S. typhimurium in organs

BALB/c mice were inoculated orally with SL7207 (pVAX-S) at different dosages for safety analysis. No clinical aberrations and visible lesions in liver and spleen were observed during the four weeks observation period. The kinetics of colonization and persistence of the bacteria in vivo was investigated. Bacteria could be isolated from liver and spleen of different dosage group during weeks 1–3 post-inoculation and were eventually eliminated from liver and spleen at four weeks post-inoculation (Fig. 5). PCR and enzyme digestion revealed the constant presence of S gene in recovered bacterial isolates.

3.5. Mucosal antibody responses induced by attenuated S. typhimurium harbouring TGEV DNA vaccine

The ability of the recombinant S. typhimurium to induce a mucosal immune response was determined by measuring the level of IgA antibody to TGEV in intestinal lavage sample. As shown in Fig. 6, recombinant S. typhimurium induced detectable IgA antibodies to TGEV Ag in mice as rapidly as week 2 post-vaccination. Considerably enhanced antibodies titers could be observed in groups B and C, which received SL7207 (pVAX-S) at dosages of 10^8 and 10^9 CFU respectively, at week 4–6 post-immunization. In addition, the antibody level of group C was significantly higher (P < 0.05) than that of group B, at week 6 post-vaccination. However, only low-level antibody response could be detected from mice in group A, which received a low dose (10^7 CFU) of strain SL7207 (pVAX-S), throughout the whole experiment. No specific anti-TGEV antibodies were detected in intestinal lavage sample of the SL7207 (pVAX) group (group D) and PBS group (group E) during the experiment.

3.6. Humoral immune responses induced by attenuated S. typhimurium harbouring TGEV DNA vaccine

The ability of the recombinant S. typhimurium to induce a humoral immune response was determined by measuring the level...
of IgG antibody to TGEV in serum sample. As shown in Fig. 7, none of the groups had any detectable antibody response at week 2 post-vaccination. Mice in groups A, B and C, which received SL7207 (pVAX-S) at dosages of $10^7$, $10^8$ and $10^9$ CFU respectively, showed different levels of antibody response during the experiment. Mice in groups B and C showed higher ($P<0.05$) anti-TGEV ELISA antibody level than group A during week 4–8 post-immunization. At week 6 post-immunization, the antibodies titers generated in groups B and C reached their peak, meanwhile the antibodies level of group C was higher ($P<0.05$) than group B. However, all through the experiment, mice in group A only induced a negligible antibody response at week 6 post-vaccination. There were no detectable specific anti-TGEV antibodies in the SL7207 (pVAX) group (group D) and PBS group (group E) during experiment.

4. Discussion

Most pathogenic microorganisms are either restricted to the mucosal membranes or in need of transit across the mucosal barrier during the early steps of infection. Thus, the elicitation of mucosal immune responses after vaccination is highly desired. A potential approach to achieve this aim is the use of attenuated Salmonella as carrier for heterologous antigens. In this report, we demonstrated that a DNA vaccine encoding N-terminal of TGEV glycoprotein S, delivered by attenuated S. typhimurium is a simple and potent vaccine that elicits both serum IgG and mucosal IgA antibody response against TGEV. We have also demonstrated that the dosage of $10^9$ CFU elicited a higher antibody response than that of $10^7$ and $10^8$ CFU.

It is generally assumed that after crossing the intestinal mucosal barrier (mainly via M cells) a large amount of attenuated S. typhimurium carrying the eukaryotic expression plasmid are taken up by APCs in local lymphoid tissues like Payer’s patches. In these phagocytes, the bacteria will start to replicate and die possibly due to their metabolic attenuation. This should result in the release of their plasmid and the in vivo transfection of the infected cells which in turn will produce the antigen [31]. In this study, when using attenuated Salmonella as a vehicle for TGEV S gene eukaryotic expression plasmid, we detected the expression of S gene in mouse peritoneal macrophages by IFA in vitro and the transcription of S gene in Payer’s patches by RT-PCR in vivo. The results indicated, when immunized orally, attenuated S. typhimurium SL7207 has the ability to deliver TGEV DNA vaccine for antigen expression by APCs.

Safety is a prerequisite when using live bacterial as vaccine carrier. Attenuated S. typhimurium SL7207 with deletion mutations in aroA gene has impaired ability to grow in cultured macrophages and in mammalian tissues. In safety study, neither deaths nor side effects were found in mice post-inoculation with attenuated S. typhimurium SL7207 (pVAX-S) at different dosage ($5 \times 10^6$ CFU, $1 \times 10^7$ CFU, $2 \times 10^7$ CFU). No bacterial were recovered in liver and
spleen in different dosage group after two weeks of boosting, indicated that attenuated S. typhimurium could be eliminated by immune system after completing the plasmid delivery. At the same time we noticed one week after boosting, bacterial separated from liver and spleen in different dosage groups were significantly lower than one week after first inoculation, which indicated the elimination of immune system to bacterial enhanced with the increasing of inoculation frequency. All the results above indicate that the use of attenuated S. typhimurium is an ideal choice of delivery vector for DNA vaccine orally, concerning safety for livestock.

Live bacterial vectors offer many potential clinical advantages. They are stable, easy and relatively inexpensive for mass-production. They are also able to deliver multiple antigens. Oral delivery increases the safety and ease of administration. In addition, bacteria naturally possess immunostimulatory molecules such as lipopolysaccharide (LPS) that can function as adjuvant [33]. When comparing the three different dosages of DNA vaccine delivered by the attenuated S. typhimurium SL7207 (pVAX-S), we found that immunogenicity of the TGEV DNA vaccine was highly dependent on the dosage of the attenuated bacteria used for oral administration. Delivery of the DNA vaccine at a low dosage (10⁸ CFU) only elicits weak anti-TGEV IgG and IgA antibodies through out the experiment. When the vaccination dosage increased to 10⁹ CFU, the IgG and IgA antibody level was enhanced, meanwhile the highest antibody response was detected at week 6 post-vaccination when the vaccination dosage was increased to 10¹⁰ CFU.

In summary, this study provided preliminary evidence that attenuated S. typhimurium strain SL7207 could be utilized as the oral delivery vector for TGEV DNA vaccines. The target gene could be expressed not only in vitro but also in vivo to develop a specific humoral and mucosal immune response against TGEV. Although these data are preliminary and an ultimate TGEV vaccine delivery vector needs to be developed in further experiments with DNA vaccine of TGEV to obtain a maximum immune response.

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In summary, this study provided preliminary evidence that attenuated S. typhimurium strain SL7207 could be utilized as the oral delivery vector for TGEV DNA vaccines. The target gene could be expressed not only in vitro but also in vivo to develop a specific humoral and mucosal immune response against TGEV. Although these data are preliminary and an ultimate TGEV vaccine may require incorporation of other TGEV antigens [34], or together with co-stimulatory molecules or immunomodulatory cytokines [35], the attenuated S. typhimurium may be an adequate delivery system to be tested in further experiments with the DNA vaccine of TGEV to obtain a maximum immune response.