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Immune response induced by oral DNA vaccination against FMDV delivered by attenuated *Salmonella choleraesuis* C500

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Abstract A recombinant strain of *Salmonella choleraesuis* C500, containing a eukaryotic expression plasmid pBO1 with the immune-dominant epitope of foot-and-mouth disease virus, was constructed. Specific immune response to this recombinant strain was evaluated by oral administration of the recombinant live bacteria pBO1/S. cho in rabbits. Results showed that T cell response and specific antibody production were elicited. This approach may present a general strategy for eliciting immune responses with DNA vaccine delivered by live bacterial vectors. The stimulated indexes of T lymphoproliferation by specific antigens of FMDV in rabbits, can reach up to 11.0 and an antibody titer of 1/32 as detected in the serum with liquid block ELISA.

Keywords *Salmonella choleraesuis*, foot-and-mouth disease virus, recombinant live vaccine

1 Introduction

Since 1970s, many new vaccination strategies have been developed, especially on the use of safe and effective recombinant live vaccines. *Bacille Calmette-Guerin* (BCG), *Listeria monocytogenes*, *Salmonella* and *Shigella* were shown to be promising vaccine vectors in experimental models. Bacterial vectors possess three main advantageous

attributes: (1) they are relatively inexpensive to manufacture and are well suited for large-scale administration; (2) they can induce strong humoral, cell-mediated immune responses to passenger antigens; (3) the tropism of enteric bacterial vectors for dendritic cells and macrophages in the lymphoid tissue of the intestinal mucosa naturally targets them to these sites, allowing the development of mucosal vaccines.

Salmonella, a member of enteric bacilli, is one of the most severe etiological agents of food-borne diseases. It is widely accepted that recombinant live vaccines using attenuated *Salmonella* as vector to deliver passenger antigen induce immune responses not only against salmonella infection but also against passenger pathogenic infection (Curtiss et al., 1994). Many researches focusing on using attenuated *Salmonella* as a vaccine vector have been done abroad (Susan and Stocker 1981; Fagan et al., 1997; Neutra 1999). Furthermore, gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium* 7270 (aroA-) resulted in protective immunity against a murine fibrosarcoma (Raglia et al., 1998). Protective immune response was induced by oral DNA vaccination against pseudorabies delivered by *Salmonella choleraesuis* in mice (Shiau et al., 2001). Early in 1970s, Fang et al. and Huang et al. screened out an attenuated *Salmonella choleraesuis* C500 which was an effective live strain against swine paratyphoid (Fang et al., 1981; Huang et al., 1981). However, the study using this strain as a vector of genetically engineered live vaccine has not been explored.

Foot-and-mouth disease virus (FMDV) is a picornavirus that is the cause of a highly contagious disease of cloven-hoofed farm animals. FMDV shows a high genetic and antigenic variability, which is reflected in the seven serotypes and the numerous variants described to date (Bachrach 1968). For centuries, outbreaks of FMDV have caused heavy economic losses worldwide. It is difficult to prevent FMD, though there are two main approaches at present. One is inactivating the virus by physical and chemical methods, combined with quarantine and slaughter

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of the infected and impressionable animals around the outbreak areas. The other is broad vaccination. Our group has been researching on genetically engineering a vaccine against FMDV type O since 1983. We have been able to produce both peptide vaccine and DNA vaccine (Huang et al., 1999). FMDV's spread is mainly through the mucosa system, but the vaccines are mainly inoculated by injection. If oral inoculation was feasible, it could build a first line of defense against the disease and highly reduce labor costs. In this paper, we do a preliminary study on the feasibility of a recombinant live vaccine against FMDV type O delivered by attenuated *Salmonella choleraesuis* C500 in rabbits.

2 Material and methods

2.1 Animal, bacteria and plasmid

Rabbits, weighing 1.5–2.0 kg and without intestinal disease, were used in this study. Attenuated vaccine strain *Salmonella choleraesuis* C500 was obtained from Nanjing Biological Pharmaceutical Co. Attenuated *Salmonella typhimurium* LB5010 (genotype: MetA22 metE551 trpC2 ilv-452 H1-b H2-e, n, x(cured of Fels2) fla-66 rpsL120 xyl-404 hsdL6 hsdSA29 hsdSB; Phenotype: Met(-); Try(-); ilv(-); r(-)LT m(+)LT; r-(SA) m(+)SA; r-(SB) m(+)SB; Str^R; Xyl(-); Leu(-)) was kindly provided by Prof. Aoquan Wang (Institute of Microbiology, Chinese Academy of Sciences). Eukaryotic expression plasmid pBO1 was constructed and conserved in our laboratory.

2.2 Main reagents, culture medium and PCR primers

The PCR kit was bought from the Fuhua Company, and the ELISA kit was bought from the Lanzhou Veterinary Institute of China Academy of Agriculture Science. The kit for *Salmonella* identification was bought from the Shanghai Institute of Biological Products. Solid and liquid LB culture medium was used for culturing *E. coli* and *S. cho*. The primers used for detecting recombinant bacteria pBO1/ *S. cho* are as follows:

Forward (3M): TAAGTAGCGGCCGCAGGTCGACTCTAG
Reverse (5P): GGAAACACATATGACCATGATTACGG

2.3 Construction and identification of recombinant pBO1/*S. cho*

In order to prepare the recombinant pBO1/*S. cho*, the attenuated *Salmonella typhimurium* LB5010, an intermediate host cell was first transformed with plasmid pBO1 by a standard CaCl₂ method (Joseph and David 1998). Then the Plasmid pBO1 was extracted and transferred to a terminal host cell-attenuated *Salmonella choleraesuis* C500 by electroporation. The preparation of cells for electroporation was as follows. C500 cells were cultured in 3 mL LB

medium at 200 r / min overnight at 37 °C, and then were inoculated into 25 mL liquid LB medium by 1% (v/v) at 37 °C, shaking at 200 r/min. Bacteria were grown to OD = 0.6~0.7 phase, chilled and then collected by centrifugation at 4 000 r / min for 10 min at 4 °C. Cells were then washed extensively with 10% glycerol to reduce the ionic strength of the cell suspension by three times. The cells were re-suspended in 10% glycerol at a concentration of 3×10^{10} cells / mL, frozen in dry ice, and stored at -70 °C. A 200 µL aliquot of the cells was thawed and used for transformation. One µg DNA (the control setup with no DNA) was mixed with cold cell suspension and then transferred to a chilled cuvette. Transformation was carried out with Bio-Rad Gene Pulser at 2.0 kV, 25 µF, 200 Ω at 0~4 °C. Transformed competent cells were transferred to a tube, and 400 µL of media was added prior to incubating the cultures for 1.5 hours at 50 r / min at 37 °C. 80 µL of the cultures were transferred onto solid LB medium containing 100 µL / mL penicillin. Recombinant bacteria were identified via a PCR method. Recombinant plasmid was confirmed by digestion with Hind III. Serological characteristics of recombinant *Salmonella choleraesuis* C500 were tested by congregation reaction on a glass plate to confirm its immunogenicity.

2.4 Vaccination, serum collection and assay of T cell proliferation

Rabbits were orally immunized with 1×10^9 of recombinant live pBO1/*S. cho* and *Salmonella choleraesuis* C500, respectively. The control group was injected with 800 µg of plasmid DNA pBO1. Live bacteria were prepared by culturing cells in 25 mL liquid LB medium at 37 °C for 18 hours followed by inoculating 2.5 mL cultures into 25 mL liquid LB medium till up to $2 \times 10^9 \sim 5 \times 10^9$ CFU / mL. Counts of live bacteria were performed by serial dilution of cultures followed by spread plating. DNA was prepared according to the reference protocol (Joseph and David 1998).

Two or eight weeks after vaccination, the blood was extracted from an ear vein and allowed to clot for 30 min at 37°C. The blood clots were allowed to contract overnight at 4 °C. The sera was then harvested and clarified by centrifugation at 3000 r / min and stored at -20 °C.

The T-cells were isolated using 1.077 g / mL Percoll solution (Pharmacia) from the spleens of immunized rabbits 8 weeks after inoculation. They were then resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 6×10^{-5} mol / L β-mercaptoethanol, 60 µg / mL penicillin and 60 µg / mL streptomycin to 1×10^5 cells / mL. A 100 µL aliquot of the T cell suspension was added to each well of a 96-well round-bottomed plate. A total of 50 µL of the diluted pure FMDV serotype O antigens were added to the wells and these set-ups were done in triplicate. The cells were incubated at 37 °C for 60 hrs followed by inoculation with 1 µCi per 30 µL of [3H.] thymidine for 12hrs. The cells were harvested and were measured using a liquid scintillation

counter (Beckman LS6500). Results were obtained as mean counts per minute (cpm) and expressed as stimulation index (SI, mean cpm of cultures with antigen divided by mean cpm of cultures without antigen). The response was considered significant only when the SI was 2.0 or higher.

2.5 Detection of sera antibody by liquid-phase blocking ELISA

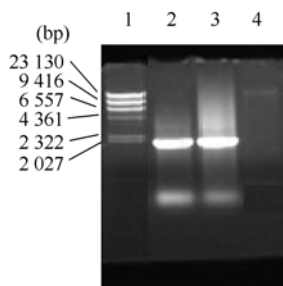
The antigen used was standard FMDV type O antigen. Coating antibody was 146S rabbit antiserum against FMDV type O. Testing antibody was 146S guinea pig antisera against FMDV type O. Enzyme was conjugated to a guinea-pig reactive rabbit antibody.

ELISA plates were coated with coating antibody and left overnight in a humid chamber at 4 °C. Block response was performed in U-bottomed 96 wells plates overnight at 4 °C by mixing standard FMDV type O antigen with different dilutions of serum taken from immunized rabbits. After the ELISA plate was washed 5 times with PBS, serum / antigen mixtures from the carrier plates were transferred to the coated ELISA plate and incubated at 37 °C for 1 hour on a rotary shaker. The ELISA plate was washed 3 times, and the testing antibody was added into each well and incubated at 37 °C for 1 hour on a rotary shaker. The conjugated enzyme solution was added and incubated for 40 min. Orthophenylene diamine containing 0.05% H₂O₂ was added to each well, then the color reaction was allowed to develop in a dark room. The reaction was stopped after 15 minutes with 1.25 M sulphuric acid. The results were read and PI was calculated.

3 Results

3.1 Identification of recombinant plasmid in *Salmonella choleraesuis*

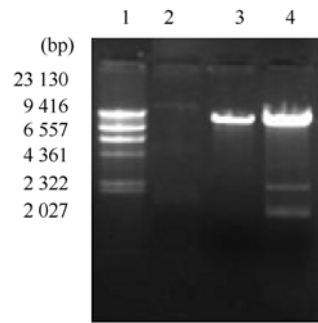
Plasmids isolated from recombinant *Salmonella choleraesuis* C500 were identified by PCR with primers of 3M and 5P. Results showed that a 2.2kb specific band was amplified as expected (Fig.1). A confirmatory test was performed by subjecting the recombinant plasmids to HindIII digestion.



1: DNA Marker; 2: PCR result of pBO1/JM101
3: PCR result of pBO1/S.cho; 4: PCR result of S.cho.

Fig.1 PCR test of transformed pBO1 plasmid in *S.cho*

The result of showed an expected 12.7kb specific band (Fig.2). This demonstrated the plasmid pBO1 was transferred into *S.cho* via electroporation.



1: DNA Marker; 2: *S.cho*
3: pBO1/JM101; 4: pBO1/*S.cho*

Fig.2 Restriction Enzyme test of pBO1 plasmid in *S.cho*

3.2 Serum congregation test of transformed bacteria

Serum congregation test was done using the identification kit for *Salmonella choleraesuis* in order to confirm whether the selected recombinant *Salmonella choleraesuis* C500 still retain the serum characteristics of *Salmonella choleraesuis*. Results showed that the recombinant *Salmonella choleraesuis* C500 still had the O7 factor characteristics of immunogenesis.

3.3 T cell proliferation test

Eight weeks after vaccination, spleen T-cells were separated to test their proliferation response. Figure 3 showed that the T-cells of rabbits immunized with pBO1/*S.cho*, stimulated by 1/40 specific antigen of FMDV type O, had significant proliferation response, with the stimulation indices reaching up to 11.0. The stimulation index of rabbits immunized with pBO1 was 4.4 with 1/20 dilution, while the control group had no proliferation reaction with any dilutions.

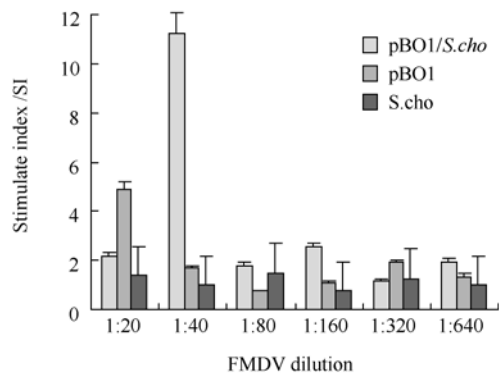


Fig.3 The response of T cell of rabbit immunized by oral recombinant DNA vaccine pBO1/*S.cho*

3.4 Detection of specific antibody in sera

Rabbits were separated into three groups for single dose vaccination. Two to eight weeks later, venous blood was taken from the ears for liquid-phase blocking ELISA to detect specific antibodies in sera against FMDV type O. A serial dilution was performed, and the sera at different dilutions were neutralized with excessive antigens. The mixtures were transferred to the ELISA plate coated with the coating antibody. Block value was calculated by measuring OD492 to determine titer. The results summarized in table 1 show that the antibody titer of the sera in groups of rabbits immunized with pBO1/S.cho and pBO1 were both 1/32, while *S.cho* was negative.

Table 1 Specific Antibody response in rabbit receiving oral inoculation of recombinant DNA vaccine

Groups	Number of animals	Titer of antibody after inoculation	
		2 weeks	8 weeks
pBO1/ <i>S.cho</i>	5	1:32	1:32
pBO1	5	1:32	1:32
<i>S.cho</i>	5	negative	negative

4 Discussion

Recent studies show that DNA vaccines, which can move into the APC of the lymphatic node after muscle injection or being inoculated into dermis tissue by gene gun and DNA vaccine, may play an important role in eliciting immune response through direct transfer of DNA and/or a cross allergic reaction (Dietrich et al., 1999). *Salmonella*, an enteric bacillus, is an intracellular infective etiological agent. Orally-inoculated *Salmonella* has the ability to infect and settle in intestine-associated lymphoid tissue, or Peyer's Patches, which makes it possible to be a proper carrier of DNA vaccines. There are several advantages for bacteria as a vector of DNA vaccines. First, compared with muscle injection or gene gun inoculation, a proper bacterial vector can present the plasmid directly to the APC of specific organs to generate immune responses. Second, some components of bacteria can enhance the immunogenicity of DNA vaccines as an immune adjuvant, such as the LPS of Gram-negative bacteria and the non-methylated DNA sequence of the bacterial genome. Third, bacterial vectors can be used to deliver the DNA vaccine orally. In our work, attenuated *Salmonella choleraesuis* C500 was used as a vector to deliver an FMDV DNA vaccine. A T-cell proliferation test showed that immune memory cells had a strong response to the stimulator of antigen (1 / 40 dilution) as compared with those of rabbits inoculated via muscle injection (Fig.3).

Although there are many different theories about the mechanism of *Salmonella* DNA delivery (Fless et al., 1996; Dietrich et al., 1999; Darji et al., 2000; Shata et al., 2000), some researches have given strong support for *Salmonella* as a delivery system for DNA vaccine. Darji et al.

demonstrated that highly specific antibodies against β -galactosase, T cell proliferation and CTL response were produced from a eukaryotic β -gal-expressing plasmid under the control of a CMV promoter orally delivered by an attenuated *aroA*- *Salmonella* (Darji et al., 1997). Furthermore, a DNA vaccine containing pseudo-rabies virus glycoprotein D gene, delivered by an attenuated *Salmonella choleraesuis*, elicited a good protective immunoresponse in mice (Shiau et al., 2001). These works aroused our strong interest in research.

In this work, we constructed a recombinant live vaccine pBO1/*S.cho* by transforming the eukaryotic expression plasmid pBO1 into attenuated *Salmonella choleraesuis* C500. After being inoculated into rabbits, pBO1/*S.cho* elicited significant T-cell response and specific antibodies against FMDV type O. It provides some insights for further research on oral vaccines against FMDV using attenuated *Salmonella choleraesuis* as the vector.

Among many ELISA methods, there are several reasons why we chose liquid-phase blocking ELISA to test the specific antibody in sera. In liquid phase, the main neutralizing epitopes of virus antigens are in their natural conformation, and can react with neutralizing antibodies in sera omni-directionally, which was the same as the assay of virus neutralization. Liquid-phase blocking ELISA also has the advantage of using inactivated antigens, as compared with virus neutralization. Thus, there is no need to worry about the virus diffusion or the need to do cell culture and animal experiments. This can make the diagnosis of FMD and the test of vaccine effect a universal application. Furthermore, in T-cell proliferation tests, the antigen can induce significant cell proliferation reaction only at 1 / 40 dilution. It may have something to do with the competitive combination of antigens and cells, or some other unknown factors, which need to be studied further.

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