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Immunogenicity of a Tuberculosis *Salmonella typhimurium* Vaccine Expressing a Fusion Protein HspX-ESAT6 in Mice

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Abstract: Tuberculosis remains a major infectious disease worldwide due to the low efficacy of available vaccine of the *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). There is a need to develop protective vaccines against Tuberculosis (TB) that elicit full immune responses including mucosal immunity. Here, a live attenuated *salmonella typhimurium* aroA SL7207 vector TB vaccine, namely SL (E6-HspX), harboring the *Mycobacterium tuberculosis* (Mtb) H37Rv *ESAT6-HspX* fusion gene was developed. Its immunogenicity and protective efficacy were assessed in a mouse model of tuberculosis. Vaccination with the SL (E6-HspX) significantly increased the frequency of peripheral blood CD4+ and CD8+ T cells and induced significantly higher levels of cell-mediated immune response compared with vaccination with PBS or the pVAX1 vector. Vaccination with the SL (E6-HspX) also induced the strongest TB Ag-specific mucosal, humoral responses and exerted high protective efficacy in mice against virulent Mtb H37Rv challenge compared to the other vaccinated groups (mice immunized with SL (HspX) or BCG only). This strategy may represent a novel promising mucosal vaccine candidate for the prevention of TB and may be used for the prevention and therapeutic intervention of Mtb infection.

Key words: Tuberculosis, *Mycobacterium tuberculosis*, HspX, ESAT6, mucosal immunity, salmonella vector

INTRODUCTION

Tuberculosis (TB) is a highly infectious disease infecting one-third of the world population (World Health Organization, 2006). In 1993, The World Health Organization has declared TB a global health emergency. Emergence of multi drug resistance in *M. tuberculosis* strains has lead to a general consensus that the disease should be best controlled by vaccination. This high rate of mortality has persisted despite the availability of an attenuated strain vaccine of *Mycobacterium bovis*, termed Bacillus Calmette-Guerin (BCG) for >50 years. BCG only decreases childhood TB and gives little protection against adult lung tuberculosis; the protection in adults varies from 0-80% (Andersen and Doherty, 2005; Franco-Paredes *et al*., 2006). These data indicate that the BCG vaccine lacks the immunogenicity required to generate protective immunity in the immunized adult populations. The major reasons behind the failure of BCG have been ascribed to lack of protective antigens in BCG (Agger and Andersen, 2002) and sensitization of infants with environmental mycobacteria (Brandt *et al*., 2002). Therefore, the development of safe, efficacious and mucosal TB vaccines that can confer potent protection in the respiratory tract mucosa remains a major challenge to TB prevention.

Comparative genomic analyses have identified >100 coding sequences that are missing from BCG but are present in *Mycobacterium tuberculosis* (Mtb) (Behr *et al*., 1999) including the 6 kDa early secreted antigenic target antigen (Ag) (ESAT6) which has been shown to be a protective Ag in several animal studies (Brandt *et al*., 1996; Pym *et al*., 2003). An earlier study has shown that vaccination with Esat6 alone has a moderate protection against Mtb infection (Zabolotnykh *et al*., 2008). An earlier study has found that the HspX, a 16 kDa protein has strong immunogenicity and can induce a potent Th1 immunity in Mtb exposed subjects but a week 1 in BCG-vaccinated individuals (Geluk *et al*., 2007). Accordingly, vaccination with multiple antigens should result in high protective effects.

Attenuated derivatives of Salmonella enterica have been proposed as vehicles for the mucosal delivery of heterologous antigens and as a basis for multivalent vaccines. In fact, strains of *S. typhi* and *S. typhimurium* were among the first bacterial recombinant vaccine vectors used (Curtiss, 2002; Formal *et al*., 1981). Recombinant salmonella vaccines are quite easy to produce and store on a large scale, making these vaccines more economically feasible and accessible. They have been used as oral vaccines and as live vectors to deliver

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heterologous Ags such as gp120, HIV Gag and Ag85A which can stimulate mucosal, humoral and cellular immune responses after animals are immunized via mucosal surfaces (Feng *et al*., 2008; Parida *et al*., 2005). However, attenuated salmonella vector TB vaccine that carrying ESAT6 and Ag85B of virulent Mtb H37Rv strain has not been reported so far. Thus, in the present study, two recombinant, orally delivered and attenuated *S. typhimurium* vaccine strains, SL (HspX) and SL (E6-HspX) that harbor the *Mtb H37Rv HspX* gene and the *ESAT6-HspX* fusion gene carried by a plasmid pVAX1, respectively were constructed. Researchers compare and analysis of the immune responses and protective efficacy of the SL (85B) and SL (E6-85B) vaccines, the DNA vaccine the BCG vaccine against Mtb.

MATERIALS AND METHODS

Animal: Man BALB/c mice at 6-8 weeks of age were purchased from Liaoning University Medicine (Liaoning, China) and housed in a special pathogen free facility in the Institute of Laboratory Animal Science of Liaoning University Medicine (Jinzhou, China). For the MTb H37Rv challenge experiment, the mice were maintained in a ABSL-2 lab in the Institute of Laboratory Animal Science of Liaoning University Medicine. Animal experiments were performed in accordance with the guidelines of the Chinese Council on Animal Care. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

Bacterial culture and *M. tuberculosis* antigen preparations: MTb H37Rv strain was obtained from the Jilin University (Jilin, China) and was passed through the mice twice to recover virulence. *Mycobacterium bovis* BCG strain was purchased from the Changchun Institute of Biological products (Changchun, China). The number of bacteria was examined by serially diluting with 7H9 medium and inoculated in 7H10 medium with OADC at 37°C for 3-4 weeks, followed by counting CFUs. The HspX and ESAT6 antigens were kindly provided by Professor Peng Zhang (Jilin University). Those antigen proteins with a purity of >88% were used for following experiments.

DNA vaccine construction and preparation: The entire *HspX* gene sequence from the genomic DNA of the Mtb H37Rv strain was implified by PCR and subcloned into the prokaryotic expression vector pET-32 (Invitrogen USA) and the eukaryotic expression vector pAVX13.1 (Invitrogen, USA). The primer sequences were (forward) 5'-AGAATTCGCCACCATGGCCACCACCCTTCCCGTTC-3' and (reverse) 5'-AAAGCTTTCAGTTGGTGGACCGGA TCTG-3' where the underlined oligonucleotides represent EcoRI and HindIII sites, respectively, to facilitate cloning. The amplified products were purified with the Quiaex II gel extraction kit (QIAGEN, Valencia, CA), digested with EcoRI and HindIII and ligated into the prokaryotic expression vector pET32 that was digested by EcoRI and HindIII. The plasmid pET-32 was cleaved with BamHI and EcoRI to generate an HspX fragment that was subcloned into BamHI and EcoRI-digested pVAX1 to yield the recombinant plasmid pVAX1-Ag85B. The entire *ESAT6* gene sequence of Mtb H37Rv was also successfully amplified by PCR. The primer sequences were (forward) 5'-GCAAGCTTATGACAGAGCAGCAGTGGAA-3 and (reverse) 5'-TTCCTAGGTGCGAACATCCCAGTGACGT-3'. ESAT6 was digested with HindIII and BamHI and ligated to the eukaryotic expression plasmid pVAX1-HspX which was also digested with HindIII and BamHI to yield the eukaryotic expression plasmid pVAX1-ESAT6-Ag85B. The plasmids pGEX-HspX, pVAX1-HspX (also called pV-HspX) and pVAX1-ESAT6-Ag85B (also called pV-E6-HspX) were confirmed by DNA sequence analysis.

Construction of attenuated *S. typhimurium* recombinant vaccine strains: The eukaryotic expression plasmids pVAX1-HspX and pVAX1-ESAT6-HspX were electrotransformed into the attenuated *S. typhimurium* strain aroA SL7207 via the *S. typhimurium*-modifying strain LB5000 (r-m+) with Ap^R selection according to previous reports (Liao *et al*., 2007). Then, this two attenuated salmonella recombinant vaccine strains were generated and confirmed by PCR and plasmid DNA restriction enzyme digestion analysis.

Immunization animal: One hundred sixety, 7-8 weeks old female BALB/C mice were divided into 8 groups (20 mice per group). Three groups of mice were orogastrically (o.g.) immunized with the attenuated salmonella vaccine strains SL (HspX) or SL (E6-HspX) or the parental bacterium strain SL7207 by placing 100 μL of vaccine suspension containing 10^7 CFU into the lower esophagus using a gavage needle on days 0, 15 and 30. Another two DNA vaccination groups of mice that were preinjected with 10-12% lidocaine were intramuscularly immunized with 100 μg plasmid DNA pV-85B or the empty vector control pVAX1 per mouse on days 0, 15 and 30 by gene gun with an Electric Square Porator (Scientz Biotechnology), basically according to earlier method (Li *et al*., 2007). The BCG group of mice were injected subcutaneously with a

single dose of BCG (5×10^5 CFU/mouse) and the a negative control group received equal volumes of subcutaneously administered phosphate-buffered PBS.

Measurement of antibody levels by Enzyme-Linked Immunosorbent Assay (ELISA): The anti-HspX IgG or IgA antibody was detected 2 weeks after each immunization using an ELISA Method. Briefly, 5 μ mL^{-1} of the purified HspX were coated on 96 well microtiter plates overnight at 4°C. After being blocked with 0.5% Bovine Serum Albumin (BSA) in PBS at 37°C for 2 h, individual serum samples at 1:100-500 dilutions were added in triplicate and incubated at 37°C for 1 h. Subsequently, the bound antibodies were detected with 1:500 diluted HRP-conjugated goat anti-mouse IgA (DAKO) or 1:1000 diluted HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, USA) at 37°C for 1 h, respectively. After being washed, individual wells were added with Tetramethyl Benzidine (TMB) substrate (200 μL/well) and the reaction was stopped by adding 50 μL/well of 1M H_2SO_4. The Optical Density (OD) was measured at 450 nm.Titers are shown as the sample dilution resulting in an OD 450 equal to twice the mean background of the assay.

Analysis of peripheral CD4+ and CD8+: The 2 weeks after the final immunization, peripheral blood samples were obtained from individual mice and the frequency of CD4+ and CD8+ cells was characterized by flow cytometry analysis using a FACS calibur instrument (BD Biosciences). Briefly, peripheral blood samples were stained with a combination of FITC-anti-CD3, PE-anti-CD4, PE/Cy5-anti-CD8 or PE-anti-CD3 (Biolegend, San Diego, USA), respectively.

Experimental infections: The 2 weeks after the final immunization, mice were divided into eight groups (6 mice in each group) and were infected i.v., via the lateral tail vein or intransally (i.n.) with an inoculum of 1×10^6 CFU of Mtb H37Rv suspended in 0.1 mL PBS (Chen *et al*., 2007).

Statistical analysis: The data were analyzed using one-way ANOVA and the Tukey's post hoc test was used for selected pairwise comparisons. The p-values of <0.05 were considered significant. Prism 4.0 Software (GraphPad, CA, USA) was used for statistical analyses of mouse data.

RESULTS

Construction of recombinant salmonella vaccine strains harboring the *HspX-ESAT6* fusion gene: To construct the recombinant plasmids, the DNA fragment of the entire *HspX* gene sequence from Mtb H37Rv was amplified and cloned into the eukaryotic expression vector pVAX-1. The DNA fragment of the entire *EAST6* gene sequence was amplified from Mtb H37Rv and then inserted into pVAX1-HspX to yield the recombinant plasmid pV-E6-HspX. Recombinant plasmid pV-E6-HspX, pV-HspX were transformed into the attenuated salmonella strain (SL7207) after DNA sequence analysis, respectively (Table 1). Recombinant salmonella vaccine strain was generated and confirmed by plasmid DNA restriction enzyme digestion analysis. Restriction enzyme digestion analysis (Fig. 1) showed that the relative molecular mass (Mr) of each inserted DNA fragment was identical to the value predicted (Fig. 1).

Table 1: The frequency of different subsets of T cells. Data are expressed as mean%±SD of each group of mice (n = 6 per group) from three separate experiments. [a]$p<0.05$ vs. the PBS group; [b]$p<0.05$ vs. the HspX group

Groups	CD4 (%)	CD8 (%)
SL (HspX)	25.76±2.18[a]	6.78±1.38
SL (E6-HspX)	28.98±3.96[ab]	9.12±1.74[a]
BCG	26.17±3.45[ab]	8.92±1.66[a]
PV-HspX	23.75±3.10	6.46±1.34
pVAX 1	21.08±2.07	6.25±1.18
PBS	20.89±2.00	5.89±1.09
SL7027	21.42±2.21	6.14±1.15

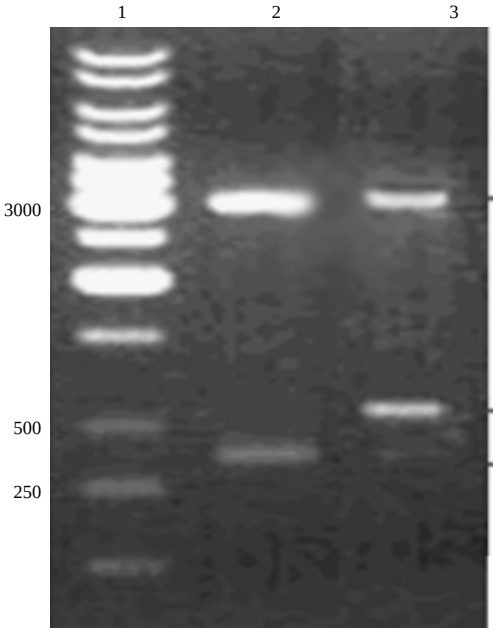
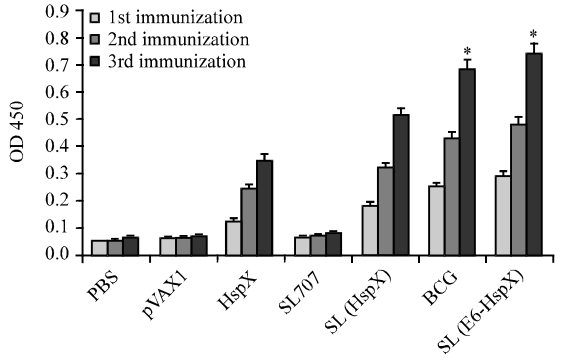


Fig. 1: Analysis of plasmid constructs in salmonella by DNA restriction enzyme digestion analysis. Restriction enzyme digestion analysis of recombinant plasmids pVAX1-HspX (BamHI and EcoRI digestion, Lane 2) and pV-E6-HspX (HindIII and BamHI digestion, Lane 3); Lanes 1 DNA marker

Vaccination with recombinant salmonella vaccine induces potent humoral responses and mucosal immune responses in mice: To determine the antibody responses against the recombinant salmonella strains, BALB/c mice were immunized orogastrically with the recombinant vaccine strains. Every 2 weeks after the immunization, levels of HspX-specific IgG in the sera of immunized mice were determined by ELISA. Titers of HspX-specific IgG increased in each immunized group as the number of immunizations increased (Fig. 2). After the third immunization, all vaccine groups that were immunized with the DNA vaccine, recombinant salmonella vaccine strains or BCG developed higher levels of IgG antibody production compared to the parental strain (SL7207), the empty vector group (pVAX-1) and PBS controls) (Fig. 2, *$p<0.05$). In addition, the antibody titers were similar in the SL (E6-HspX) group, the BCG group which is higher than other group. These data showed that these two groups induced the strongest IgG antibody responses among all groups.

To observe the mucosal immune responses against recombinant salmonella vaccine strains, titers of HspX specific mucosal secretory IgA (SIgA) antibodies from the lung tissues of immunized mice were determined by ELISA. Mice immunized with SL (E6-85B) elicited much higher levels of lung SIgA and serum IgA than other groups (Table 2).

Vaccination with salmonella vaccine induces cell immune responses in mice: Next, researchers examined the impact of vaccination with salmonella vaccine on peripheral T cell population and antigen-specific T cell response in mice. The 2 weeks after the final vaccination, the frequency of peripheral blood CD4+ and CD8+ were analyzed. As shown in Table 2, the frequency of peripheral blood CD4+ and CD8+ T cells in mice that had been vaccinated with SL (E6-HspX) group were similar to that of mice vaccinated with BCG and they were significantly higher than that of the parental strain (SL7207), the empty vector group (pVAX-1), the DNA vaccine group (pVAX-HspX) and the non-immunized mice (PBS controls). Hence, vaccination with salmonella vaccine for the fusion protein expression increased the frequency of CD4+ and CD8+ T cells and stimulated antigen-specific T cell responses *in vivo*.



Fg. 2: Otal serum HspX-specific IgG responses of immunized mice. Mice were injected i.m. with DNA vaccine pV-HspX or o.g. with SL (HspX) or SL (E6-HspX). The immunization was performed on days 0, 15 and 30 and HspX-specific IgG titers were assayed by ELISA 2 weeks after each immunization. Six mice were used in each group. Data points represent means±SEM. *$p<0.05$ vs. parental vector control groups

SL (E6-85B) induced protective immunity against i.v. Mtb H37Rv infection: To evaluate protective responses after Mtb challenge, immunized mice were infected i.v. with Mtb H37Rv. The recombinant salmonella vaccine strain groups SL (E6-HspX) and the BCG group had longer 50% death times (T50) and lower death rates mice compared to the DNA vaccine pV-HspX group, the parental strain group and the non-immunized PBS group (Table 3). These data indicated that the bacterial vaccines can induce stronger protective immunity against Mtb than the DNA vaccine. In addition, the mice immunized with SL (E6-85B) survived nearly as long as the mice immunized with BCG which was the most effective because all mice in this group were alive at 50 days post-infection while 16-80% of the mice in the other groups were dead (Table 3).

Table 2: Titers of IgA in serum and lung tissues. [a]$p<0.05$ vs. the DNA vaccine and the parental vector control groups. [b]$p<0.05$ vs. BCG, DNA vaccine and the parental vector control groups

Groups	Serum	Lung
SL (HspX)	39±5.8	16±2.1
SL (E6-HspX)	94±7.4[ab]	33±3.8[a]
BCG	50±4.5[a]	32±3.4[a]
pV-HspX	23±3.1	18±2.9
pVAX 1	17±4.0	16±2.5
PBS	15±3.5	15±2.0
SL7027	18±4.2	17±2.3

Table 3: Comparisons of various vaccines-induced protection efficacy in mice infected with H37Rv. T50: Time when 50% mortality

Groups	T_{50} (days)	Mortality (%) (50 days)	Sample
SL (HspX)	>60	14.00	20
SL (E6-HspX)	>60	0.00	20
BCG	>60	19.00	20
PV-HspX	38	33.00	20
pVAX 1	31	75.25	20
PBS	26	70.18	20
SL7027	30	80.00	20

DISCUSSION

Currently, great progresses have been made in the development of novel TB vaccines such as attenuated live or inactive whole bacterium, recombinant BCG, subunit vaccine and DNA vaccine (Hoft, 2008). In comparison with conventional vaccines, recombinant salmonella vaccines are quite easy to produce and store on a large scale, making these vaccines more economically feasible and accessible. Moreover, the bacterial vector may mimic natural infection and interact with the mucosal, humoral and cellular compartments of the immune systemn (Kotton and Hohmann, 2004; Roland *et al*., 2005; Boyle *et al*., 2007). Therefore, in this study, researchers have constructed recombinant salmonella vaccine strains and evaluated their efficacy.

In the present study, researchers generated two recombinant salmonella vaccine strains, SL (HspX) and SL (E6-HspX) which harbor the *Mtb HspX* gene and the *ESAT6-HspX* fusion gene, respectively. The results strongly suggest that a novel recombinant salmonella vaccine strain, SL (E6-85B) that harbors an *ESAT6-HspX* polyprotein gene can protect against Mtb infection. Mice vaccinated with SL (E6-HspX) contained high titers of Ag85B-specific IgG in their serum and SIgA in their lung indicating that vaccination with SL (E6-85B) induced potent humoral responses and mucosal immune responses. Moreover, the SL (E6-85B) salmonella vaccine strains also enhanced T cells proliferations which demonstrated that SL (E6-85B) triggered a strong cellular immune response. Both HspX and ESAT6 proteins have been identified as immunogenic for human leukocyte Ag class I-restricted CD8+ T cells (Flynn *et al*., 1992; Caruso *et al*., 1999). Therefore, the SL (E6-HspX) vaccine strain could induce high levels of IFN-γ and granzyme expression and improved Th1-type cellular responses which play an important role in the prevention of TB. Importantly, challenge with Mtb by SL (E6-HspX) survival studies also indicated that immunization with the SL (E6-85B) vaccine strain induced significant protective immunity.

CD4+ and CD8+ T cells are crucial for defending intracellular pathogens. In addition, CD8+ T cells can also produce perforin and granulysin which can directly kill the Mtb-infected cells and attack Mtb (Flynn *et al*., 1992; Caruso *et al*., 1999). Researchers found that the frequency of peripheral blood CD4+ and CD8+ T cells increased in the SL (E6-HspX) and BCG-treated mice and that there was a higher frequency of other groups, further supporting that vaccination with the SL (E6-85B) induced Th1 response in mice. The data are consistent with previous findings that vaccination with a salmonella vaccine strains encoding a fusion protein of Ag85B and human ESAT6 significantly increases the frequency of peripheral blood CD4+ and CD8+ T cells and inhibits Mtb replication in mice (Hoiseth and Stocker, 1981). Indeed, several DNA vaccines can elicit protective responses, similar to that of BCG in primary infection models. Researchers found that the protective effect of SL (E6-HspX) vaccination was similar to that of BCG but significantly higher than that of vaccination with HspX in mice. The data are in agreement with e zarlier findings and further support the notion that vaccination with a salmonella vaccine strains that expresses multiple antigens improves the protective effect against Mtb infection (Lindsay *et al*., 2009; Wang *et al*., 2009). Therefore, vaccination with a salmonella vaccine strains for multiple antigens is better to induce protective responses in mice.

CONCLUSION

The data demonstrate that the most effective recombinant salmonella vaccine strain, SL (E6-HspX) would be a novel vaccine candidate for the prevention of TB. Furthermore, treatments combining vaccination with SL (E6-HspX) and BCG against TB seem to be promising.

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