Salmonella expressing a T-cell epitope from Sendai virus are able to induce anti-infection immunity

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Bacterial fimbriae can accept foreign peptides and display them on the cell surface. A highly efficient gene replacement method was used to generate peptide vaccines based on *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2. DNA encoding an epitope from Sendai virus, SV9 (Sendai virus nucleoprotein peptide 324–332, FAPGNYPAL), which is known to induce cytotoxic T lymphocytes, was incorporated into the gene encoding AgfA (the major subunit protein of thin aggregative fimbriae of *Salmonella*) by replacing an equal length DNA segment. To improve cytotoxic T lymphocyte recognition, both termini of the peptide were flanked by double alanine (AA) or arginine (RR) residues. Western blotting and immunofluorescence microscopy using AgfA-specific antiserum verified the expression of chimeric AgfA; expression was also proved by a Congo red binding assay. Oral immunizations of C57BL/6 mice with the four strains induced an epitope-specific T-cell response (detected by enzyme-linked immunosorbent spot assay). When the mice were challenged with the Sendai virus, the magnitude of the infection was significantly reduced in the immunized groups compared with the controls. The *Salmonella* fimbrial display system efficiently induces a cellular immune response and anti-infection immunity *in vivo*, providing a new strategy for the development of efficient peptide vaccination.

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INTRODUCTION

Vaccination is one of the most important achievements in medicine and immunology, and is an effective means to prevent infectious diseases. Different approaches have been successfully developed to deliver vaccines against pathogenic bacteria and viruses, including inactivated whole cell vaccines, subunit vaccines and live attenuated vaccines (Spreng *et al.*, 2006). Generally, live vaccines can proliferate *in vivo* and are able to provoke strong, long-lasting adaptive immune responses. Therefore, numerous strategies have been tried that use attenuated microbes as carriers of heterologous antigens.

Attenuated *Salmonella* have been shown to be excellent carriers for foreign antigens derived from bacteria, viruses, parasites or tumour cells, as vaccines developed with such carriers can stimulate strong systemic and local immune

responses against the corresponding antigens in animal models (Echchannaoui *et al.*, 2008; Kulkarni *et al.*, 2008; Qu *et al.*, 2008; Spreng *et al.*, 2006; Wang *et al.*, 2008). Plasmid-based expression systems have been widely used in presenting foreign antigens in attenuated *Salmonella*, but plasmids tend to be unstable and require selection (Garmory *et al.*, 2003; Haga *et al.*, 2006; Isoda *et al.*, 2007; Xu *et al.*, 2007). Furthermore, plasmids may have negative effects on the colonization ability of the carrier strain *in vivo* (Coulson *et al.*, 1994). Although chromosomal integration of DNA encoding the foreign antigens may solve the problem, expression of the antigens from a single locus is usually below desirable levels (Spreng *et al.*, 2006). Therefore, DNA encoding an abundantly expressed protein would be a desirable site for chromosomal integration of DNA encoding foreign antigens. Fimbriae, for example, are long and thread-like protein structures on the surface of bacterial cells, and a *Salmonella* cell usually has several hundred fimbrial fibres per cell (Klemm & Schembri, 2000).

Fimbriae are adhesive organelles, which enable bacteria to target and colonize specific host tissues, and are well

Abbreviations: CR, Congo red; ELISPOT, enzyme-linked immunosorbent spot; IFN- γ , gamma interferon; IL, interleukin; SFC, spot-forming cell; TCID₅₀, median tissue culture infective dose.

A table of primer sequences is available as supplementary data with the online version of this paper.

known to elicit strong humoral and cell-mediated immune responses (Ochoa-Reparaz *et al.*, 2004; Scavone *et al.*, 2007; Tiels *et al.*, 2008). They are polymers composed of large numbers of identical protein subunits called fimbrins. Thin aggregative fimbriae (called Tafi or curli in *Escherichia coli*) and the major subunit, AgfA (CsgA or curlin in *E. coli*), have been found in a wide range of *Salmonella enterica* subsp. *enterica* serovars, such as Typhimurium (*S. Typhimurium*) and Enteritidis (*S. Enteritidis*) (Doran *et al.*, 1993). Tafi are highly flexible for displaying a 16 aa epitope (PT3) from *Leishmania major* gp63 protein, and the chimeric AgfA could be detected by anti-PT3 antibody, but it was not known whether this could induce an epitope-specific immune response (White *et al.*, 1999, 2000).

In this study, we established an antigen expression model to evaluate the immune effects of the Tafi presentation system. We engineered the *agfA* gene of *S. Typhimurium* LT2 by replacing a 27 bp segment with a DNA segment encoding a T-cell epitope, i.e. Sendai virus nucleoprotein 324–332 (also called SV9) (Kast *et al.*, 1991; Sandberg *et al.*, 1998; Schumacher *et al.*, 1991). Oral immunization of C57BL/6 mice with the engineered bacterial strains induced an epitope-specific T-cell response. When the mice were challenged with the Sendai virus, the magnitude of the infection was significantly reduced in the immunized groups compared with the control groups.

METHODS

Bacterial strains, media and growth conditions. *S. Typhimurium* LT2 (LT2) was grown routinely in Luria–Bertani (LB) broth or on LB agar for 14–18 h at 37 °C. LT2 or *E. coli* containing recombinant plasmid were grown in LB broth or on LB agar supplied with kanamycin (50 µg ml⁻¹) for 20–24 h at 28 °C as described by White *et al.* (1999). For analysing AgfA fimbrin, LT2 strains were grown on T agar for 48–60 h at 28 °C as described by Romling *et al.* (1998). For Congo red (CR) binding experiments, strains were grown on T agar supplemented with 100 µg CR ml⁻¹ (TCR).

Construction of LT2 strains containing *agfA::SV9* in the chromosome and a LT2 Δ *agfA* strain. The chimeric *agfA::SV9* genes were generated by two-step overlap extension PCR using primers A, B-SVAA, C-SVAA and D or A, B-SVRR, C-SVRR and D (Supplementary Table S1 available with the online journal) and were cloned into pHSG415 (Fig. 1a). The chimeric genes were recombined into the chromosome of LT2, replacing the wild-type *agfA* gene [Fig. 1(a), following procedures outlined by White *et al.* (1999, 2007)]. Both sides of the SV9 epitope were flanked by two alanine residues (LT2-SVAA strain) or arginine residues (LT2-SVRR strain). Using the same procedure, we replaced a DNA segment in *agfA* gene with several termination codons to construct an AgfA-deficient Δ *agfA* strain.

To facilitate the introduction of the SV9 epitope into AgfA with flanking residues, we changed two bases in the *agfA* sequence, i.e. GATGCC→GACGTC, and generated an *Aat*II restriction site, leading to an Ala to Val change in residue 43 of the mature AgfA protein. The changes were immediately upstream to the site chosen for SV9 insertion, which was site A5 (Fig. 1) as described by White *et al.* (2000). The mutated *agfA* gene was cloned into pHSG415 to generate pHSGAAT (Fig. 1b). Instead of two-step overlap extension

PCR, one-step PCR could create a chimeric *agfA* gene (Fig. 1b) using primers E-SVAA or E-SVRR and D (see Supplementary Table S1 available with the online journal), with pHSGAAT as the template. The resultant recombinant plasmids, pHSGSVAA and pHSGSVRA, were used to construct two bacterial strains in which SV9 was flanked by AA (LT2-SVAA strain) or RR (LT2-SVRR strain). PCR using primers SV9 and D was performed to certify the insertion of SV9 DNA into *agfA* in the chromosome of the LT2 strains. For final confirmation of gene replacement, fragments containing *agfA* and the surrounding DNA region were amplified with primers *agf1* and *agf2*, and sequenced by BGI LifeTech. Restriction enzymes and the high-fidelity *Taq* enzyme were supplied by New England Biolabs, and ligase was purchased from Promega.

CR binding assay. The CR binding assay was performed as described by White *et al.* (2000). LT2 and LT2-SV9 mutant strains were grown on TCR plates; cells were scraped off the plates and suspended in 10 mM Tris buffer (pH 7.0) at an A_{650} value of 1. The cell suspension was equilibrated for 1 h and 1 ml aliquots were transferred into Eppendorf tubes containing 1 drop (50 µl) of 30 % PEG8000 in 100 mM Tris buffer (pH 7.0). Cells were removed from the suspension by centrifugation (15 000 g for 5 min) before determining the amount of CR released into the supernatant by measuring the absorbance at 480 nm using a spectrophotometer.

SDS-PAGE and Western blotting. Bacterial cells were harvested from T agar plates, suspended in Tris buffer (10 mM Tris, pH 7.5) and mixed with an equal volume of 2×SDS-PAGE sample buffer containing 0.2M glycine (pH 2). The mixture was boiled for 10 min and cell debris was sedimented by centrifugation (15 000 g for 5 min). The insoluble pellet was washed with distilled water and treated with 90 % formic acid, then lyophilized (Collinson *et al.*, 1991). Samples were dissolved in 1×SDS-PAGE loading buffer and loaded immediately on SDS-PAGE gels. SDS-PAGE was performed with a 5 % acrylamide stacking gel and 15 % acrylamide resolving gel. Proteins were electrophoretically transferred to nitrocellulose. Western blotting was performed using rabbit anti-AgfA antiserum (Collinson *et al.*, 1991).

Immunofluorescence. Bacterial cells were heat-fixed on glass slides prior to incubation with AgfA-specific rabbit immune serum. The secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG(H+L), was purchased from Invitrogen. Cells were examined with a fluorescence microscope BX60-32FB3-E01 (Olympus) using a 100× magnification oil-immersion lens and photographed.

Animal immunization. Female C57BL/6 mice, 6–8 weeks of age, were obtained from the Department of Experimental Animal of Peking University Health Science Center (Beijing, PR China) and maintained in a pathogen-free facility. Six mice per group were immunized with wild-type LT2, LT2-SVAA, LT2-SVAAa, LT2-SVRR and LT2-SVRRa strains. Each mouse was administered a dose of 10⁷ c.f.u. bacteria orally in 100 µl saline (0.9% NaCl) by pipette. Oral immunization was repeated twice, at intervals of 14 days, for a total of three immunizations.

Enzyme-linked immunosorbent spot (ELISPOT) assay. The SV9 peptide, FAPGNYPAL, was synthesized by BGI Life Tech (Beijing, China). The mouse gamma interferon (IFN- γ) ELISPOT kit was purchased from U-CyTech Biosciences. Mice were euthanized 1 week after the last immunization and their spleens were removed. Mono-splenocytes were prepared and cultured for 7 days in RPMI 1640 (Invitrogen) supplemented by 10 % calf serum (Hyclone), 100 µg streptomycin ml⁻¹, 100 U penicillin ml⁻¹ and 10 U murine interleukin (IL)-2 ml⁻¹ (Cytolab); the culture medium was refreshed every 48 h after incubation. The day before ELISPOT testing, 10 µg ml⁻¹ of SV9 peptide was added into the cell culture clusters. Cells

Fig. 1. Schematic of the method of generating *S. Typhimurium* strains carrying a foreign epitope in fimbrin by a two-step PCR method (a) and a modified (simplified) one-step method (b). (a) *S. Typhimurium* LT2 genomic DNA was used for amplifying chimeric *agfA* genes by two-step overlap PCR. The chimeric genes were constructed in pHS415, an unstable and temperature-sensitive plasmid, to generate pHS415A and pHS415B. Then, the recombinant plasmids were used to transform LT2. Finally, we isolated mutant strains through gene replacement by temperature control and biotic pressure as described by White *et al.* (1999). (b) A new restriction site *Aat*II was introduced into the plasmid pHS415 with the *agfA* gene by the same method as shown in (a). Then, with primers D and E, and taking pHS415A as the template, one-step PCR was performed to generate chimeric *agfA* gene. The subsequent steps were the same as for (a).

were collected and counted before performing the ELISPOT assay, using conditions specified by the manufacturer. Four replicates of 5×10^5 and 1×10^5 cells per well were added to 96-well mixed PVDF plates pre-coated with anti-mouse IFN- γ antibody and each well was supplemented with $10 \mu\text{g}$ synthesized peptides ml^{-1} and 10 U murine IL-2 ml^{-1} . No peptides were applied to media in the negative control group. After incubation at 37°C for 24 h without agitation, plates were washed and incubated first with a biotinylated secondary antibody and then with alkaline phosphatase-conjugated streptavidin, followed by addition of freshly prepared AEC substrate buffer (supplied with the kit). The plates were photographed and analysed with an ImmunoSpot Analyser (Cellular Technology).

Virus infection. Parainfluenza 1 (Sendai) virus was purchased from Wuhan Institute of Virology, Chinese Academy of Science (Wuhan, PR China), and was propagated in 9-day-old chicken embryos. The allantoic fluid was harvested after 72 h of inoculation and titrated on LLC-MK2 cells (Institute of Biochemistry and Cell Biology, Chinese Academy of Science) to calculate the median tissue culture infective dose (TCID_{50}). Groups of mice ($n=20$) vaccinated with one of the five LT2 strains, along with a control group immunized with saline, were inoculated intranasally with $2 \times 10^3 \text{ TCID}_{50}$ ($10^{6.3}$ copies) Sendai virus in a total volume of 0.02 ml 10 days after the last immunization.

Viral outgrowth determination. Detection of viral load in mice was performed as described by van der Sluijs *et al.* (2003). On days 1, 4

and 8 after inoculation, 6 mice from each group of 20 were anaesthetized and euthanized by bleeding out from the vena cava inferior. Lungs were harvested and homogenized on ice in four volumes of DEPC-treated saline. Samples ($100 \mu\text{l}$) of lung homogenates were treated with 1 ml TRIzol reagent (Invitrogen) to extract RNA. The final RNA was suspended in $40 \mu\text{l}$ nuclease-free water. cDNA synthesis was performed using $10 \mu\text{l}$ RNA suspension and a RevertAid first strand cDNA synthesis kit (Fermentas International). A total of $5 \mu\text{l}$ of each cDNA suspension was used in a quantitative real-time PCR, using the following primers: 5'-GGCTAACC-ACCCCTCTACAG-3' (forward), 5'-TCAGAGCCTCATTGCATGTGT-3' (reverse) and 5'-FAM-CCTGTTGGCATCCTTG-3' [5'-FAM (6-carboxyfluorescein)-labelled probe]. The standard curve was determined using virus dilutions of chicken embryo allantoic fluid.

The presence of infectious particles was also evaluated. After centrifugation at $12\,000 \text{ g}$, the suspension of the lung homogenate was tested by haemagglutination of chicken red blood cells (CRBCs). Viruses at 1 : 2 dilutions in 0.5 % CRBCs were incubated for 30 min at room temperature.

Statistical analyses. Experimental data were analysed with one-way analysis of variance followed by Tukey's multiple range test for significant differences. In all cases, the criterion for statistical significance was $P < 0.05$.

RESULTS AND DISCUSSION

Generation of mutants and CR binding

Four individual strains of *S. Typhimurium* LT2 were engineered to express chimeric AgfA proteins containing a Sendai viral T-cell epitope, SV9 (FAPGNYPAL) using different strategies (Fig. 1a, b). Using the method described by White *et al.* (1999) (Fig. 1a), two strains, LT2-SVAA (SV9 flanked by two alanine residues) and LT2-SVRR (SV9 flanked by two arginine residues), were constructed; the other two strains, LT2-SVAAa and LT2-SVRRa, were constructed by the simplified method (Fig. 1b). The final strains generated by the original method and the simplified method differ by the presence of an alanine residue (SVAA and SVRR) versus a valine residue (SVAAa and SVRRa) at position 43 in mature AgfA. An AgfA-deficient strain of LT2 ($\Delta agfA$) was also generated.

The five LT2 mutant strains, along with the LT2 parent, were analysed for their ability to bind the hydrophobic dye CR. The binding of CR to *Salmonella* strains is proportional to the relative amounts of thin aggregative fimbriae (Tafi, curli) produced (White *et al.*, 2000). The mutated strains had a similar morphology to the LT2 parent strain on TCR plates, indicating that the AgfA::SV9 proteins were expressed and assembled into functional Tafi. The binding of CR by the four mutated strains with chimeric AgfA was a little lower than or equal to the parent strain, but significantly higher than $\Delta agfA$ strain ($P < 0.05$) (Fig. 2a). This verified the expression of AgfA in the constructed strains and demonstrated that insertion of SV9 did not cause significant reduction in AgfA expression.

To simplify the gene replacement procedure, we made a single amino acid mutation (AgfA protein 63-A $\rightarrow$ V) upstream of the replaced AgfA fragment. Using the modified method, we constructed the chimeric *agfA* fragment used in gene replacement by one-step PCR using plasmid pHSGAAT as the template, which is more convenient than two-step overlap extension PCR, especially when a large quantity of construction is needed. Our results are indicative that the amino acid mutation caused by the modified method did not affect the expression of the chimeric fimbriae. Therefore, this modified method can be used for future vaccine developments.

Expression of AgfA in LT2 strains

The expression of AgfA in LT2 strains grown on TCR plates was further examined by Western blotting and immunofluorescence using AgfA-specific immune serum. Western blotting analysis showed that the AgfA protein was expressed by all the LT2 and mutant strains except the $\Delta agfA$ strain, and that the expressed protein products had different molecular sizes (Fig. 2b). The immunofluorescence showed expression of thin aggregative fimbriae on the cell surface of LT2 parent strain [Fig. 2c(i)] and SV9 mutant strains [Fig. 2c(ii, iv, v, vi)], but not the $\Delta agfA$

Fig. 2. Expression of chimeric fimbriae. (a) CR binding assay. The amount of CR bound by *Salmonella* cells at an A_{650} value of 1 from TCR plates was measured. CR bound by LT2- $\Delta AgfA$ was lower than other LT2 strains ($P < 0.05$). (b) Western blotting of fimbriin of *S. Typhimurium* LT2 strains. AgfA samples from LT2 (lane 2), LT2-SVAA (lane 3), LT2-SVAAa (lane 4), LT2-SVRR (lane 5) and LT2-SVRRa (lane 6) reacted with rabbit anti-AgfA serum. An AgfA sample from LT2- $\Delta AgfA$ (lane 1) did not. Molecular mass marker sizes are indicated on the left. (c) Immunofluorescence of *S. Typhimurium* LT2 strains using rabbit anti-AgfA serum. (i) LT2 strain, (ii) LT2- $\Delta AgfA$ strain, (iii) LT2-SVAA strain, (iv) LT2-SVAAa strain, (v) LT2-SVRR strain, (vi) LT2-SVRRa strain. Bars, 5 μ m.

strain [Fig. 2c(ii)]. The differences shown by immunofluorescence may be due to the change of fimbrial structure caused by the insertion of foreign peptides. With these data we confirmed the expression of the various chimeric AgfA::SV9 proteins in mutated LT2 strains.

Epitope-specific T-cell immune response in mice immunized with LT2 vaccine strains

C57BL/6 mice were immunized with the four AgfA::SV9 LT2 strains and the LT2 parent strain, and the epitope-specific T-cell immune response was analysed using ELISPOT. In mice, the four bacterial mutants all induced epitope-specific T-cell responses and there was no difference between these four groups (Fig. 3). In mice immunized with the parental LT2 strain, there were no SV9-specific spot-forming cells (SFCs). This is believed to be the first report of the use of the ELISPOT assay in relation to this epitope. We concluded from these results that the LT2 mutant strains expressing chimeric AgfA::SV9 were able to induce an epitope-specific T-cell immune response.

Infection in immunized mice after viral challenge

As a test of vaccine efficacy, groups of mice immunized with the different LT2 strains were challenged with live parainfluenza 1 (Sendai) virus. The level of viral infection was monitored by real-time PCR (see Methods). The TCID₅₀ of chicken embryo allantoic fluid was 1×10^4 per 100 µl. The viral load in the lungs of the mice was calculated according to a standard curve established with dilutions of chicken embryo allantoic fluid.

On day 1, the viral load in each group was similar, with no statistical difference between groups (Fig. 4). On day 4,

Fig. 3. ELISPOT assay of IFN- γ of mice spleen cells. Mice were euthanized 1 week after the last immunization and their spleens were removed. Single cell suspensions of spleen cells were prepared and cultured in RPMI 1640 for 7 days with IL-2. The day before ELISPOT testing, $10 \mu\text{g}$ SV9 peptide ml^{-1} was added into the cell culture clusters. ELISPOT was performed using a mouse IFN- γ ELISPOT kit. The y-axis values indicate the SV9-specific SFCs (10^5 splenocytes)⁻¹ in each group. The error bars indicate significant differences for each group. SV9-specific SFCs were observed in the SV9 groups but not in the parental LT2 group.

Fig. 4. Viral load in mice lungs after challenge with Sendai virus. Groups of mice ($n=20$) vaccinated with one of the five LT2 strains, along with a negative control group immunized with saline, were inoculated intranasally with Sendai virus after the last immunization. The number of viral copies in chicken embryos allantoic fluid was used to determine a standard curve and the viral load expressed as RNA copies per lung (mean $\pm$ SE) as determined by real-time PCR. The viral loads in mice were detected on day 1, 4 and 8 after viral challenge. Asterisks indicate that the viral load was lower than in the parental LT2 group and the saline group. ●, Negative control; ○, LT2; ▼, LT2-SVAA; △, LT2-SVAAa; ■, LT2-SVRR; □, LT2-SVRRa.

viral loads increased in most of the mouse groups, with the greatest increase observed in control mice immunized with saline (negative control) (Fig. 4). The parental LT2 group had a similar viral level to the LT2 mutant groups and this was much lower than the saline group, which may be explained by the fact that many kinds of cytokines and chemokines can be produced, such as tumour necrosis factor alpha, IL-1, IL-6, IL-12, IL-18 and IFN- γ (reviewed by Mittrucker & Kaufmann, 2000), during the initial stages of *Salmonella* infection, and some cytokines, such as IL-1, IFN- γ , granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor, are effective in stimulating non-specific protection against Sendai virus infection (Azuma *et al.*, 1992; Iida *et al.*, 1989). However, on day 8, the viral loads in all groups with SV9 mutant strains dropped to levels lower ($P<0.05$) than the parental LT2 group, and the parental LT2 group was similar to the saline group, because specific cellular immune response plays an important role in recovery from infection in normal mice after day 4 (Anderson *et al.*, 1979) and parental LT2 cannot induce a virus-specific T-cell response in mice. In contrast, the depressed viral load in mice immunized with the SV9 vaccine strains was accounted for by specific cellular immunity induced by SV9 epitope on the bacterial surface. We were unable to determine any statistical differences between the four AgfA::SV9 strains. But the result on day 8 did not correspond with the result

on day 4, in which SVAA and SVRR strains did not show any protection effect, which may be explained by the fact mentioned above that specific cellular immune response is more important in recovery after day 4 (Anderson *et al.*, 1979). The protection was not significant on day 4, but was on day 8 in these two groups. These results demonstrated that each mutant strain expressing chimeric AgfA::SV9 was effective in protecting the animals against viral challenge.

In conclusion, using *Salmonella* thin aggregative fimbriae as vectors for foreign epitopes from Sendai virus succeeded in inducing a specific cellular immune response and anti-infection immunity. This vaccine strategy has the following advantages. First, *Salmonella* replicates directly in lymphoid organs and antigens are presented more efficiently; second, fimbriae are strong immunogens that can serve as an effective natural adjuvant; third, epitopes have a stable and high expression in this system, which is very advantageous, since recombinant proteins introduced by conventional methods (plasmid expression and chromosomal integration of foreign DNA at a single locus) are either unstable or poorly expressed; and fourth, oral immunization is very convenient and economic. The display system in our study provides a good choice for peptide vaccination in the future to combat tumours, as well as infectious diseases.

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