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Evaluation of a novel Vi conjugate vaccine in a murine model of salmonellosis

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Abstract

Immunisation of BALB/c mice with a vaccine containing Vi polysaccharide conjugated to the *Klebsiella pneumoniae* outer membrane 40 kDa protein (rP40), in combination with *Escherichia coli* heat-labile toxin adjuvant (LT), elicited anti-Vi IgG antibodies after administration using different routes. Testing of the immune serum in opsonisation assays demonstrated the specific enhancement of Vi-positive bacterial uptake by cultured murine bone marrow derived macrophages. Intra-peritoneal challenge of mice immunised with the Vi-based vaccine elicited a degree of protection against virulent Vi⁺ *Salmonella enterica* serovar typhimurium (*S. typhimurium*). In contrast, Vi vaccination did not confer protection against oral challenge with virulent Vi-positive *S. typhimurium* or *S. dublin*.

Keywords

Vi polysaccharides; *Salmonella*; Typhoid; Vaccination; Antibody

1 Introduction

Salmonella enterica subspecies *enterica* serovar typhi (*S. typhi*) is the causative agent for human typhoid with over 22 million cases reported annually resulting in an estimated 200,000 deaths. *S. typhi* expresses the surface-associated polysaccharide antigen Vi composed of repeating acetylated α 1–4 galacturonic acid moieties [1]. Vi antigen expression reduces the efficiency of uptake of *S. typhi* into phagocytic cells [2–4] and has been reported to have immunomodulatory activities [5]. Antigenically identical Vi antigen is expressed on the surface of *S. typhi* [6–8], some isolates of *S. dublin* [9], as well as strains of

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Citrobacter freundii [10,11]. The key genes for *Salmonella* Vi antigen synthesis and surface expression in *S. typhi* are located on *Salmonella* pathogenicity island 7 (SPI-7) [12–16].

Typhoid vaccines based on purified Vi antigen have been licensed for use in many countries [17–19] and they have consistently shown an efficacy of over 60% in adults in typhoid endemic areas [20–25]. Protection may be relatively short-term, possibly due to the fact that Vi is a polysaccharide and therefore is a T cell independent antigen. Thus, vaccinees may need boosting every 3–5 years [26,27].

Many polysaccharide-based vaccines have additional drawbacks in that they do not normally induce good immune responses in infants under the age of two [28,29] and may fail to induce isotype switching and affinity maturation of antibody responses. Vi-polysaccharide-protein conjugate vaccines have the potential to elicit superior protection, which is of a longer lasting nature in both adults and children. To date, several candidate proteins such as diphtheria, tetanus and cholera toxins [30–32] and the B subunit of the heat-labile toxin (LT-B) of *Escherichia coli*, have been considered as conjugate partners for Vi and other polysaccharides [33]. One Vi conjugate based on a recombinant exotoxin A (rEPA) of *Pseudomonas aeruginosa* [34] has been tested in the field with extremely encouraging efficacy [35,36].

We have evaluated a novel Vi conjugate vaccine, Vi-rP40, based on the *C. freundii* Vi linked to the 40 kDa recombinant outer membrane protein (rP40) of *Klebsiella pneumoniae* [37–40]. One of the difficulties in evaluating Vi-based typhoid vaccines is that *S. typhi* is host restricted to humans [41–43]. *S. typhimurium* infection of mice is frequently used as an animal model for typhoid but *S. typhimurium*, unlike *S. typhi*, normally lack Vi expression. To overcome this problem we have used a novel mouse virulent *S. typhimurium* modified to express the *S. typhi* Vi antigen by transfer of SPI-7 from *S. typhi*. In addition, we have also exploited a mouse virulent *S. dublin* strain naturally expressing Vi. These Vi⁺ strains have enabled us to establish in vivo and in vitro models for exploring the immunogenicity and protective efficacy of Vi-based conjugate vaccines.

2 Materials and methods

2.1 Mice and immunisation strategy

Groups of 10–15 BALB/c female mice, 6–8 weeks old, were used in immunisation experiments. In pilot experiments small groups of mice were immunised, sub-cutaneously, with different doses of Vi-rP40 with or without *E. coli* heat labile toxin (LT) as adjuvant. Doses of 1 µg LT, or 1 µg LT in combination with 10 µg Vi-rP40, were selected as optimal vaccine dose for subcutaneous (sc) or intranasal (in) immunisation. Different immunisation schedules involving sc, in or combinations of these routes were investigated. Immunisations were normally performed on days 0, 7, and 21. Intranasal immunisation was routinely in a volume of 25 µl of PBS with or without 1 µg of LT. For sc immunisation, groups of mice normally received 100 µl doses of vaccines. If required, blood samples were collected 1 week after the last immunisation and thereafter for a period of 6–13 weeks. In some experiments sub-groups of mice received further sc. boosts or were selected for challenged with either 2×10^8 *Salmonella* orally or 10^4 intra-peritoneally. Mice were ex-sanguinated

between one to seven days after challenge depending on when and if they developed clinical signs of severe infection, At the time of ex-sanguination sera was collected and spleens and livers were removed for viable bacterial counts.

2.2 Bacterial strains, preparation of inocula for the infection of mice and enumeration of internalised organisms

S. typhimurium C5.507 is a Vi-positive derivative of *S. typhimurium* C5 that has been modified to express the Vi polysaccharide following selection of Vi-positive recipients in conjugation experiments with *S. typhi* (Popoff, Institut Pasteur, Paris, France, unpublished results). *S. typhimurium* C5.507 harbours the entire SPI-7 pathogenicity island of *S. typhi*, including the *via* genes responsible for Vi expression (D. Pickard, unpublished results). *S. dublin* is a natural V-positive strain that also harbours SPI-7 and the *via* genes. Both strains express Vi on their surface in a form detectable by agglutination and colony blotting. *S. typhimurium* C5, *S. dublin* or *S. typhimurium* C5.507 was normally grown overnight at 37 °C in 10 ml of Luria Bertani (LB) broth (Difco) in an orbital shaker. The following morning, a volume of 100 µl was transferred to 10 ml of LB-broth and cultured to a stationary phase state at 37 °C overnight. Bacterial numbers were adjusted by OD at 600 nm and then the culture was centrifuged and resuspended in PBS ready for infection. Before use, bacterial cultures were checked for the expression of Vi using commercially available rabbit anti-Vi polyclonal antisera (Remel Europe, Dartford, UK). This was performed both by slide agglutination and fluorescence microscopy. To enumerate the numbers of bacterial counts in organs, mice were killed by cervical dislocation. Spleens and livers were aseptically removed and homogenized in 5 ml of sterile distilled water in a Colworth Stomacher. Viable bacterial counts were determined using spotted or pour plates of LB agar.

2.3 Antigens and reagents

Recombinant outer membrane protein A of *K. pneumoniae*, called rP40, was expressed in *E. coli* and purified as described previously [37]. Vi polysaccharide was purified from *C. freundii* (strain 5362, Institut Pasteur Collection, Paris, France) as previously described [30]. Briefly, *C. freundii* was grown on a chemically defined medium containing 60 g/l glucose as the carbon source. Culture was stopped after complete consumption of glucose, heated to 60 °C for 1 h and centrifuged. Vi was precipitated from the supernatant with 2% cetyltrimethyl ammonium bromide (CETAB). CETAB was eliminated with NaCl treatment and ethanol precipitation. Precipitate was solubilised in sodium acetate, and purified with cold phenol as described previously [44]. Purified Vi was dialysed against water and freeze-dried. Vi content was measured by acridine orange binding assay [45]. *O*-acetyl was measured with acetyl choline as a standard [46]. Protein was determined by the bicinchoninic acid assay with bovine serum albumin as a standard. Nucleic acids content was determined by measuring absorbance at 260 nm [47]. Endotoxin determination was performed with *Limulus* amoebocyte assay (LAL).

Vi-rP40 was synthesised with adipic acid dihydrazide (ADH) as the linker [48]. Firstly, protein was derivatised with ADH. ADH (3.5 mg/mg of protein) was added to rP40, 2.5–3 mg/ml in 0.2 M NaCl containing 0.1% (w/v) Zwittergent 3–14. After addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDAC) at 0.4 mg/mg of

protein, pH was adjusted to 5 with 0.2 M HCl. The reaction was carried out for 1 h at room temperature with gentle stirring. The pH was maintained at 4.9–5.1 with 0.2 M HCl. The reaction mixture was dialysed overnight at 4 °C against 0.2 M NaCl containing 0.1% (w/v) Zwittergent 3–14. The extent of derivatisation with ADH was determined using the 1,3,5-trinitrobenzenesulfonic acid assay with ADH as a reference [49]. Fifty mM EDAC was added to Vi, 5 mg/ml in 0.2 M NaCl, in order to obtain a final concentration of 5 mM, and mixed for 2 min at room temperature. ADH-rP40 was added dropwise, and the conjugation was carried out for 3 h at room temperature and at pH of 5.6–5.9 maintained with 0.2 M HCl. The reaction mixture was dialysed overnight at 4 °C against 0.2 M NaCl. Conjugate was stored at 4 °C after addition of thiomersal at the final concentration of 100 µg/ml. It was analysed for protein by the bicinchoninic assay, and for polysaccharide by acridine orange dye binding method [45] and a ratio of 1:1 polysaccharide: protein established for all batches. It was also further characterised by SDS-PAGE and gel-filtration.

2.4 Enzyme-linked immunosorbent assay (ELISA) for murine antibodies

Flat bottom 96 well micro-titre plates (Corning-Costar) were coated with 50 µl of 1 µg/ml tyraminated Vi, diluted in PBS, at 4 °C overnight. The wells were washed three times with PBS/0.05% Tween 20 (PBS/T) prior to blocking with 100 µl PBS/1% bovine serum albumin (BSA)/0.05% Tween 20 (PBS/BSA/T) for 1 h at 37 °C. Dilutions of mouse serum in PBS/BSA/T were added to the plates (50 µl) and incubated at 4 °C for 24 h prior to washing three times with PBS/T. Negative and positive controls of normal mouse serum (NMS) and murine anti-Vi IgG1 sera respectively, were included in every assay.

To detect total immunoglobulin (Ig), 50 µl of horseradish peroxidase (HRP) labelled rabbit anti-mouse Ig (1:1000) (Dako) was added to each well and the plates were incubated 1 h at 37 °C. After three washes in PBS/T, the reaction was developed with 50 µl of *O*-phenylenediamine (OPD, Sigma) for 15 min and stopped with 50 µl 3 M H₂SO₄. Optical density (OD) was measured at 490 nm.

To detect IgG subclasses, 50 µl biotin-conjugated anti-mouse IgG1 or IgG2a (Pharmingen), diluted 1 in 2000 in PBS/T were added to each well and incubated at 37 °C for 2 h. After three washes in PBS/T, 50 µl of Streptavidin-HRP (Sigma) diluted 1 in 1000 in PBS/T was added and incubated at 37 °C for 1 h. Detection of bound antibody was with OPD as described above.

2.5 Opsonisation and macrophage uptake

S. typhimurium strains C5 and C5.507 were grown overnight at 37 °C as a stationary phase culture in LB broth in the presence of 50 µg/ml kanamycin for the growth of C5.507. The overnight culture was then washed once in PBS and aliquots of 500 µl were transferred to Eppendorf tubes. Bacteria were opsonised with sub-agglutinating concentrations of either NMS (1:50) or sera containing antibodies against LT (1:50), LT + Vi-rP40 (1:50) or *Salmonella* (1:100). Excess sera were removed by washing opsonised bacteria twice in PBS. Effective opsonisation was confirmed by fluorescence microscopy.

Bone marrow-derived macrophages were prepared from BALB/c mice as described previously [50]. Briefly, bone marrow cells were extracted from tibias and femurs and

cultured for 6 days at 3×10^5 cells/ml in bacterial-grade plastic Petri dishes containing RPMI 1640 supplemented with 10% foetal bovine serum, 5% horse serum, 2 mM; L-glutamine, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate (all from Sigma) and 20% conditioned medium from L929 cells. The adherent cell population was washed with PBS and detached from the plastic Petri dishes using cell scrapers. Bone marrow-derived macrophages (BMM) were used from days 6 to 21 and were found uniformly CD11b⁺CD11c⁻CD19⁻TCR $\alpha\beta$ ⁻ as confirmed by flow cytometric analysis.

Bone marrow-derived macrophages (2×10^5 cells/well) were plated in 24-well plates (Nunc) in RPMI 1640 and infected with opsonised *S. typhimurium* C5 or C5.507 at various multiplicity of infection (MOI) for 1 h at 37 °C. BMMs were then washed three times in RPMI 1640 to remove extracellular bacteria and incubated in RPMI 1640 containing 100 µg/ml gentamicin for 1 h at 37 °C. Cells were finally washed three times in RPMI 1640, incubated in RPMI 1640 containing 10 µg/ml gentamicin for 4 h at 37 °C before being lysed in 0.5% Triton X-100 for 10 min at 37 °C. Numbers of intracellular bacteria were estimated by plating serial dilutions of the lysates on LB plates overnight at 37 °C.

2.6 Statistical analysis

Statistical significance was calculated using the Student's *t*-test, two tailed assuming unequal variance between populations and with a confidence level of 95% ($p < 0.05$).

3 Results

3.1 Mice immunised with the Vi-rP40 conjugate vaccine develop anti-Vi immunoglobulin responses

Pilot experiments were conducted in small groups of mice (5 per group) to determine the dose range for the immunogenicity of the Vi-rP40 vaccine conjugate in BALB/c mice. These established that the Vi-rP40 vaccine, in combination with LT, was reproducibly immunogenic with doses as low as 10 µg (data not shown). Subsequently, larger groups of BALB/c mice (15 per group) were immunised intranasally on days 0 and 7 followed by two sub-cutaneous immunisations on days 21 and 117. Mice were sample bled on day 42 (15/15 mice) and 133 (3–5/15 mice) and sera tested for the presence of anti-Vi total immunoglobulin (Ig) (Fig. 1a). By day 42, mice immunised intranasally with vaccine harbouring Vi-rP40 plus LT had developed anti-Vi antibody responses (Fig. 1a A) and this was still present at day 133 (Fig. 1a B). As expected, the control mice did not produce detectable anti-Vi antibody responses. Further groups of mice (15 per group) were immunised using three subcutaneous immunisations (days 7, 21 and 117) and although anti-Vi antibodies were not detected in the serum of these mice by day 42 (15/15 mice) they had seroconverted by day 133 (3–5/15 mice) (Fig. 1a A and B). Analysis of serum harbouring anti-Vi immunoglobulin (Fig. 1b A) in repeated intranasal immunisation experiments indicated that the majority of total immunoglobulin present was IgG1 (Fig. 1b B) whilst all mice exhibited variable low IgG2a titres (Fig. 1b C). Thus, the Vi-rP40 vaccine was immunogenic in BALB/c mice using different routes of immunisation.

3.2 In vitro opsonisation into bone marrow-derived macrophages

Sera from mice immunised with vaccines containing Vi-rP40 plus LT, LT or PBS were used to opsonise *S. typhimurium* C5 or C5.507 Vi⁺ bacteria in vitro. Sera from mice immunised with a live vaccine based on a distinct Vi-negative *S. typhimurium*, SL3261 [51] were also included as an additional control. The sera were used at sub-agglutinating concentrations and specific antibody binding to the bacterial surface was confirmed by immunofluorescence (data not shown). Data presented in Fig. 2 show that the intracellular numbers of *S. typhimurium* C5 were higher when the bacteria were opsonised with anti-*Salmonella* serum as compared to bacteria opsonised with anti-LT or anti-LT + Vi-rP40 serum. The intracellular bacterial numbers of *S. typhimurium* C5.507 Vi⁺ were lower than the numbers of intracellular C5, consistent with the established anti-phagocytic role of the Vi polysaccharide antigen. Opsonisation of strain C5.507 with naive or anti-LT serum did not have any detectable effect of intracellular numbers of viable bacteria. Conversely, opsonisation of *S. typhimurium* C5.507 Vi⁺ bacteria with anti-*Salmonella* serum or anti-LT + Vi-rP40 serum significantly increased the intracellular bacterial load. Thus, immunisation with LT + Vi-rP40 induces anti-Vi antibodies that effectively bind to the surface of Vi⁺ bacteria and enhance their uptake by cultured bone marrow-derived macrophages.

3.3 Influence of immunisation with Vi-rP40 on bacterial challenge with Vi⁺ *Salmonella* strains

To further explore the humoral component of the immune response, immunised mice were subsequently challenged with virulent Vi-positive *Salmonella*. Complete protection against *Salmonella* in *Salmonella*-susceptible BALB/c mice is known to be dependent on a combination of humoral and cellular immunity. However, the contribution of antibody to this protection can be monitored using the intraperitoneal (ip) route of challenge with low doses of *Salmonella* bacteria [52]. Hence, the challenge dose of *S. typhimurium* was reduced to 10⁴ organisms per mouse. Both *S. typhimurium* C5 (Vi-negative) and C5.507 (Vi-positive) challenge were utilised separately to demonstrate the specificity of immunisation. In Fig. 3a we show data for the numbers of *Salmonella* isolated from spleens (A) and livers (B). The data indicates that only mice immunised with vaccines containing Vi-rP40 (10 per group) were able to control the growth of *S. typhimurium* C5.507 Vi-positive bacteria (5 of the 10 immunised) with respect to control mice (the remaining five immunised mice). *S. typhimurium* C5 Vi-negative bacteria infect normally, regardless of the recipients immune status. Interestingly, mice given only LT intra-nasally exhibited slightly higher bacterial loads than control animals, perhaps reflecting the immunomodulatory activity of LT.

To see if we could detect any evidence for a protective effect of Vi-rP40 immunisation against oral challenge with virulent Vi-positive *Salmonella*, BALB/c mice that had been vaccinated with LT + Vi-rP40 along with control vaccinated groups were initially challenged orally on day 133 with 10⁸ Vi-positive *S. dublin* (five mice per group). The data illustrated in Fig. 3b highlight the variation in bacterial load seen within groups, for both spleen (A) and liver (B). Even though the Vi-positive *S. dublin* has a relatively low murine virulence, bacterial burden in LT + Vi-rP40 or LT vaccinated groups and controls were not reproducibly different. However, there was a slight trend towards lower bacterial counts in the mice immunised with the vaccine containing Vi-rP40 + LT. This was not entirely

unexpected as non-living vaccines based on *Salmonella* LPS or whole dead bacteria are known to be poorly protective against virulent oral *Salmonella* challenge in BALB/c mice [53]. To further establish this observation, experimental oral challenges were performed utilising 10^8 Vi-positive *S. typhimurium* C5.507. In these experiments, C5.507 was as virulent as the parent C5 strain and the infected mice were culled over a period of 2–5 days, post-challenge, as and when they appeared ill. Results were similar to the previous experiment and showed little protection (data not shown).

Prior to the inoculation of immunised and control animals, the bacterial inoculum was routinely checked for Vi expression by agglutination with anti-Vi polyclonal antibody. To confirm that bacterial colonies recovered from immunised mice were still able to express Vi antigen, the bacterial colonies isolated from those animals were screened for the presence of Vi by anti-Vi colony blotting. All *Salmonella* recovered from mouse tissues were still able to express Vi antigen (results not shown).

4 Discussion

Here we report the evaluation of a murine model in which immune responses were assessed using both in vitro and in vivo assays following immunisation with a novel Vi-polysaccharide conjugate vaccine, Vi-rP40. *S. typhi* infections are still a major problem in the developing world and are likely to be an increasing threat if the acquisition of bacterial antibiotic resistance is not halted [54,55]. Currently available vaccines against typhoid are restricted to those based on heat- and phenol-inactivated whole bacteria, live attenuated *galE* mutant bacteria (Ty21a) or purified Vi polysaccharide. These vaccines have significant efficacy but the level is not ideal and their administration is generally limited to adults and infants over the age of five. As a consequence of their T-independent nature, polysaccharide vaccines are known to generally generate a relatively short-lived response and requiring routine boosting. However, it is hoped that the new generation of polysaccharide conjugate vaccines will improve efficacy and reduce the need to boost. The Vi-rP40 vaccine described in this text is one of several new conjugate Vi vaccines that will hopefully improve the efficacy of typhoid vaccines, particularly in the young. Thus, the preclinical testing of Vi-conjugate vaccines is likely to be of increasing importance in the future. Vi is a surface exposed capsule associated with most clinical isolates of *S. typhi*. Vi-based vaccines have efficacy, which correlates with the levels of IgG antibodies in the serum of vaccinees. Thus, significant protection in humans against typhoid can be elicited by generating the production of serum antibodies. However, the level of protection may be enhanced if other arms of the immune system were stimulated, for example using strong adjuvants [56], optimal conjugates or appropriate live vaccines. It is well established that optimal immunity to salmonellosis in mice relies on the combined effects of both the cellular and humoral arms of the immune response [57].

Mice cannot be readily infected with *S. typhi* and most murine studies of salmonellosis employ murine virulent isolates of promiscuous *S. enterica* serovars such as *S. typhimurium* or *S. Enteritidis* that unfortunately do not express Vi antigen. In this study, we have employed natural Vi-positive *S. dublin* and recombinant *S. typhimurium* to investigate the influence of Vi antibody on bacterial infectivity using both in vitro and in vivo assays.

Interestingly the *S. typhimurium* Vi positive isolate, which was generated by conjugation, encodes the whole of SPI-7 (our unpublished observations). BALB/c mice are known to be deficient in full macrophage function as demonstrated by Nramp1 status and as such are utilised as a susceptible murine strain for vaccine studies. Our data indicate that these hyper-susceptible mice could be partially but not completely protected against virulent *Salmonella* challenge. Considering that Vi-antigen can be regulated in response to environmental signals such as osmolarity and ionic concentration it is probably not surprising that complete protection against challenge was not achieved. Nevertheless, serum from Vi-immunised mice was shown to be active in opsonisation assays and could transfer partial protection to naïve animals. This offers a potential method for monitoring the activity of Vi-based vaccines in terms of a measurable protective effect in mice. We believe this model could be further developed, possibly by the use of naturally *Salmonella* resistant Nramp1-positive inbred mice and we will explore this possibility in the future.

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Fig. 1.

Anti-Vi antibody titres after immunisation with various vaccines. (a) Shows anti-Vi total immunoglobulin titres of sera collected on (A) day 42 (15/15 mice), (B) day 133 (3–5/15 mice). (b) Is of anti-Vi titres compiled from day 29 sera (9 naïve, 11 LT and 14 LT + Vi-rP40 mice per group) and illustrates (A) total immunoglobulin, (B) IgG1 and (C) IgG2a. Data is represented as individual (♦) and mean (–) titres. LT represents *E. coli* heat-labile toxin, Vi-rP40 represents the Vi conjugate vaccine, in and sc represent the intranasal or subcutaneous route respectively.

Fig. 2.

Ability of mice sera to opsonise Vi-positive and Vi-negative *Salmonella*. Bone marrow-derived macrophages (BMMs) (2×10^5 per well) were infected with *S. typhimurium* C5 or C5.507 previously opsonised with either normal mouse sera (1:50), sera from 20 mice immunised with LT (1:50), sera from 20 mice immunised with LT + Vi-rP40 (1:50) or sera from 20 mice immunised with a live vaccine based on *S. typhimurium* SL3261 (1:100) for 1 h at 37 °C. Extracellular bacteria were removed by three consecutive washes in PBS and the addition of 100 µg/ml gentamicin for 1 h at 37 °C. Data represents the total cfu of *S. typhimurium* C5 or C5.507 recovered in 10^6 macrophages after opsonisation with different antibodies. Data are expressed as mean $\pm$ S.D. for $n = 3$. * for $P < 0.05$ when comparing to opsonisation with anti-LT antibodies and as determined using a Student's t-test.

Fig. 3.

Bacterial counts in the livers and spleens of mice following challenge with virulent *Salmonella*. (a) represents the viable cfu recovered from immunised mice challenged with either 10^4 *S. typhimurium* C5 (5 mice per group) or C5.507 (5 mice per group) intra-peritoneally and (b) from mice challenged orally with 2×10^8 Vi-positive *S. dublin* (5 mice per group), A represents spleen and B represents liver cfu, respectively. Data is represented as individual (♦) and mean (—) cfu per organ. * for $P < 0.05$ between experimental groups as determined using a Student's t-test.