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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 *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-

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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 Vi-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