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Vi-CRM₁₉₇ as a new conjugate vaccine against *Salmonella* Typhi

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Abstract

An efficacious, low cost vaccine against typhoid fever, especially for young children, would make a major impact on disease burden in developing countries. The virulence capsular polysaccharide of *Salmonella* Typhi (Vi) coupled to recombinant mutant *Pseudomonas aeruginosa* exoprotein A (Vi-rEPA) has been shown to be highly efficacious. We investigated the use of carrier proteins included in infant vaccines, standardized the conjugation process and developed key assays required for routine lot release at production scale. Vi from a BSL1 organism, *Citrobacter freundii*, strain WR7011, was used as an alternative to Vi from *S. Typhi*. We showed that Vi conjugated to CRM₁₉₇, a non-toxic mutant of diphtheria toxin, widely used in commercial vaccines, was produced at high yield. Vi-CRM₁₉₇ proved immunogenic in animal studies, even without adjuvant. Thus, Vi-CRM₁₉₇ appears to be a suitable candidate for the development of a commercially viable, effective typhoid vaccine for developing countries.

Keywords

Vi; Conjugate vaccine; *Salmonella* Typhi

1. Introduction

Typhoid fever is a common serious disease in many parts of the world and remains a major health problem in developing countries with an estimated 21 million cases and 200,000 deaths in 2000 [1,2]. The highest incidence is in South Asia (India, Pakistan and Bangladesh) but cases occur throughout Asia, Africa and the Americas. Travellers visiting these areas are at risk of contracting typhoid fever and approximately 400 cases are recorded annually in the USA and in the UK, mostly with a travel history to India [3,4]. In highly endemic areas, children are at particular risk with the peak age inversely proportional to the incidence in the community [5,6]. Although commonly quoted as a disease of school age children [7,8], one study from Bangladesh showed that the most common age of infection in

hospitalised children was 1–2 years [5]. As *Salmonella enterica* serovar Typhi only infects humans, vaccines targeting young children would give protection and also reduce transmission of typhoid fever in nonvaccinated members of the community, as was seen in a recent vaccine trial in Kolkata, India [9].

The capsular polysaccharide of *Salmonella* Typhi (Vi) is a linear homopolymer of α 1,4-N-acetylgalactosaminouronic acid, 60–90% O-acetylated at the C-3 position [10].

Unconjugated Vi polysaccharide is one of the two widely available licensed vaccines together with an oral live attenuated vaccine (Ty21a). The Ty21a vaccine is distributed as enteric coated capsules, licensed only for people 6 years and older [11]. Several manufacturers produce unconjugated Vi vaccine, licensed for adults and children 2 years and older [12]. There is no typhoid vaccine that is licensed for use in infants.

A recent meta-analysis of both Ty21a (oral) and Vi polysaccharide (parenteral) vaccines estimated that the cumulative efficacy is 51% (95% CI 36–62%) for Ty21a and 55% (95% CI 30–70%) for Vi [13,14]. The duration of protection is not well determined, with estimates of five to seven years for the Ty21a vaccine and three years for Vi vaccination [13,14]. Despite these limitations, several studies have illustrated the importance of vaccination against typhoid fever for populations at risk [11]. The World Health Organization and GAVI have recommended, but not yet funded, introduction of the existing Vi vaccine, and support the development of more effective vaccines [15]. A vaccine that could be administered to infants would be highly beneficial.

As observed with other polysaccharides [16,17], conjugation of Vi to a carrier protein substantially increases the antibody response. A conjugated vaccine of Vi coupled to recombinant mutant of *Pseudomonas aeruginosa* exoprotein A (Vi-rEPA) was shown to be safe in all ages including infants [18]. Vaccination provided excellent and long lasting immunity with 92% protection over two years post vaccination in a randomized, two-dose placebo controlled trial in 2–5-year-old children in Vietnam, and with 89% protection over 46 months [19,20].

Diphtheria toxoid (DT), tetanus toxoid (TT), and CRM₁₉₇ (a non-toxic variant of diphtheria toxin), a outer-membrane protein complex (OMPC) from *Neisseria meningitides* and *Haemophilus influenzae* outer membrane protein D (OMPD) are used as protein carriers in licensed glycoconjugate vaccines [17,21]. This study reports use of CRM₁₉₇ for the preparation of a Vi conjugate, its characterization and immunogenicity in mice as part of a program to develop a consistent and affordable conjugate vaccine for use in all ages in developing countries. Unlike DT or TT, CRM₁₉₇ does not require detoxification with formaldehyde and homogeneous preparations of purified antigen can be readily obtained. CRM₁₉₇ is a precisely defined protein, consistent from batch to batch. Unlike rEPA, CRM₁₉₇ is licensed for human use in several efficacious conjugate vaccines already used in hundreds of millions of children [22–24]. Its use as carrier protein should facilitate the manufacturability of a Vi conjugate vaccine and simplify its pathway to licensure.

In order to study the influence of a different carrier protein on the efficacy of a Vi conjugate vaccine, the same chemistry has been used for the synthesis of Vi-CRM₁₉₇ and Vi-TT vaccines and their immunogenicity compared in mice.

Citrobacter freundii WR7011 has been chosen as source of Vi instead of *Salmonella enterica* serotype Typhi (Ty-2). Vi from *Citrobacter* is structurally similar and immunologically indistinguishable to Vi from *S. Typhi* [25,26]. Vi from *Citrobacter freundii* WR7011 has been successfully used as the Vi source in studies assessing immunogenicity of Vi-vaccine conjugates [10,27,28]. As a low risk organism and a high Vi yield strain, *C. freundii* constitutes a safer and more economic source for Vi production than BSL3 *S. Typhi*.

2. Materials and methods

Polysaccharide

Vi polysaccharide from *C. freundii* WR7011 was from the Program in Developmental and Molecular Immunity, the National Institute of Child Health and Human Development, National Institutes of Health. Characterization of the polysaccharide was done at Novartis Vaccines Institute for Global Health (NVGH) by A₂₆₀ for nucleic acid content, micro BCA for protein estimation, ¹H NMR for Vi identity and O-acetylation level. O-acetyl groups were also estimated by the Hestrin method [29]. Thermogravimetric analysis and Karl Fisher were used for dried weight and residual moisture measure, respectively. Na⁺ content was evaluated by atomic absorption spectroscopy [30].

Proteins

CRM₁₉₇ and tetanus toxoid were from Novartis Vaccines and Diagnostics (NV&D). Tetanus toxoid was further purified by gel filtration through Sephacryl S-300 (GE Healthcare) equilibrated in 0.15 M NaCl, 10 mM NaH₂PO₄, pH 7.2. The fractions, corresponding to the monomeric molecular weight of tetanus toxoid, as verified by MALDI-TOF (average molecular mass of 155.3 kDa), were pooled.

Reagents and materials

The following materials were used in this study: adipic acid dihydrazide (ADH), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), formic acid, 4-morpholine ethanesulfonic acid (MES) and trifluoroacetic acid (TFA) [Sigma]; sodium chloride (NaCl) [Merck]; sodium hydroxide (NaOH, 50% solution) [J. T. Baker]; sodium phosphate monobasic monohydrate (NaH₂PO₄·H₂O) [Carlo Erba]; acetonitrile [LC-MS Chromasolv]; sodium nitrate (NaNO₃) [Fluka]; Spectra/Por dialysis membrane MWCO 6000–8000 Da, 10 mm diameter [Invitrogen]; and Sephacryl S-1000 [GE Healthcare].

2.1. Analytical methods

Protein analysis—Protein concentration was measured by micro BCA, using bovine serum albumin (BSA) as a reference following the manufacturer's instructions [Thermo Scientific].

Derivatized proteins and conjugates were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7% Tris-acetate gels (NuPAGE, from Invitrogen). The samples (5–20 μL with a protein content of 5 μg) were added to 0.5 M dithiothreitol (1/5, v/v) and NuPAGE LDS sample buffer (1/5, v/v). The mixtures were heated at 100 $^{\circ}\text{C}$ for 1 min and the samples loaded into the wells of the gel. The gel was subjected to electrophoresis at 45 mA in NuPAGE Tris-acetate SDS running buffer (20 $\times$, Invitrogen) and stained with Simply Blue Safe Stain (Invitrogen).

Nucleic acid analysis—Nucleic acid content was determined by UV spectroscopy at a wavelength of 260 nm. Nucleic acid concentration of 50 $\mu\text{g/mL}$ produces an $\text{OD}_{260} = 1$.

Thermogravimetric analysis—Vi sample was analysed in duplicate for volatile content estimation using a Perkin-Elmer TGA-6. Keeping the sample in inert atmosphere, temperature was increased from 25 $^{\circ}\text{C}$ to 150 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C/min}$ and then left at 150 $^{\circ}\text{C}$ for 15 min.

Karl Fisher analysis—Residual moisture was determined by the Karl Fisher coulometric method with Metrohm 756.

Mass spectrometry—Protein samples were analysed by MS Q-TOF and MALDI-TOF. For Q-TOF analysis, sample of protein at 2 mg/mL was ultrafiltered using a Microcon 3 (Millipore) for 15 min at 10 $^{\circ}\text{C}$ and $10,000 \times g$. The treatment was repeated three times, using 5 mM ammonium acetate buffer pH 6.8 to reconstitute the retentate. The sample was then diluted 1:4 with 50% acetonitrile/ H_2O containing 0.1% formic acid and injected by direct infusion in a Q-TOF micro (Micromass) at a flow rate of 10 $\mu\text{L/min}$. The mass spectrometer was tuned as follows: ESI positive mode, capillary 3000 V, sample cone 30 V, desolvation temperature 250 $^{\circ}\text{C}$, source temperature 100 $^{\circ}\text{C}$, scan range from 500 to 2500 Da.

For MALDI-TOF analysis, the protein was diafiltrated, using a Centricon-10 (Millipore), against 10% acetonitrile containing 0.1% TFA. Two microliters of protein (about 80 pmoles) were mixed with 2 μL of a saturated solution of sinapinic acid in 30% acetonitrile solution containing 0.1% TFA. One microliter of the mix was spotted on a MTP 384 stainless steel target (Bruker Daltonics GmbH, Bremen, Germany) and allowed to air-dry. Measurements were recorded on an Ultraflex (Bruker Daltonics) MALDI-TOF/TOF MS in linear mode. External calibration was performed by spotting 0.5 μL of protein calibration standard II (Bruker Daltonics) containing the following proteins: trypsinogen (23,982 Da), protein A (44,613 Da) and bovine serum albumin (66,431 Da). Data were collected using an Accelerating Voltage of 25 kV, Ion Source1 20 kV, Ion Source2 17 kV and Lens 9.60 kV. The laser power was adjusted between 30 and 50% intensity, using a 337 nm nitrogen laser with a frequency of 50 Hz. All mass spectra were recorded by summing up to 400 laser shots. The Flex Analysis version 2.4 software packages provided by the manufacturer were used for data processing.

^1H NMR spectroscopy—Nuclear Magnetic Resonance (NMR) experiments were recorded at 25 $^{\circ}\text{C}$ on Bruker DRX 500 MHz spectrometer, equipped with a Bruker 5 mm

dual $^1\text{H}/^{13}\text{C}$ Z gradient probe. For data acquisition and processing XWINNMR 2.6 software package (Bruker Daltonics) was used. 1D proton NMR spectra were collected using a standard one-pulse experiment. Chemical shifts were referenced to HDO at 4.79 ppm. 32k points were collected on 4000 Hz spectral window. ^1H NMR spectra were obtained in quantitative manner using a total recycle time to ensure a full recovery of each signal ($5 \times$ Longitudinal Relaxation Time T1). FID (free induction decay) were Fourier transformed applying 0.1 Hz of line-broadening.

Dried Vi sample was solubilized in deuterated water (Aldrich) and transferred to 5 mm NMR tube (Wilmad, 535-PP-7). Two spectra were collected: the first one in deuterated water and the second one after addition of sodium deuterioxide to a final concentration of 200 mM. The first spectrum was recorded in order to assure that other impurities did not fall at the same chemical shift of the acetate anion (released after de-O-acetylation of the sample), with implications for the quantification of O-acetyl content.

Addition of sodium deuterioxide to the sample caused rapid de-O-acetylation (the sample was heated at 37 °C for 20 min to have complete de-O-acetylation) and consequently sharper peaks appeared in the proton spectrum. The spectrum of the de-O-acetylated polysaccharide in alkaline medium was better suited for use as an identification test than that of the untreated sample, based on the presence of five typical signals (from about 5 to about 4 ppm) corresponding to the protons of the carbohydrate ring. Well resolved N-acetyl and acetate resonances were observed so that the degree of O-acetylation of Vi was determined by comparison of the integrals of these two peaks [31].

HPLC-SEC—Analytical size exclusion high pressure liquid chromatography (HPLC-SEC) analysis was used for proteins and conjugates characterization. The HPLC-SEC system consisted of an Ultimate 3000 Dionex equipped with a G6000PW (30 cm $\times$ 7.5 mm) column (particle size 17 μm ; Sigma 8-05765) with a TSK gel PWH guard column (7.5 mm ID $\times$ 7.5 cm L; particle size 13 μm ; Sigma 8-06732) (TosohBioscience). The effluent was monitored with a Photodiode Array Detector and a RF2000 Fluorescence Detector (Dionex). CRM₁₉₇ and its derivative CRM_{ADH} were also analysed using a G3000PW (30 cm $\times$ 7.5 mm) column (particle size 10 μm ; Sigma 8-05762). Separation was performed with a flow rate of 0.5 mL/min using isocratic elution of 0.1 M NaCl, 0.1 M NaH₂PO₄, 5% CH₃CN at pH 7.2. Column void and bed volumes were measured with λ -DNA (λ -DNA Molecular Weight Marker III, 0.12–21.2 kbp, Roche) and sodium azide (NaN₃, Merck), respectively. Eluted polysaccharides and proteins were detected setting UV detector at 214nm (polysaccharide and protein) and at 280 nm (protein). Protein peaks were also detected using tryptophan fluorescence (emission spectrum at 336 nm, with excitation wavelength at 280 nm).

HPAEC-PAD—After evaluating different conditions of hydrolysis, Vi samples were treated with NaOH at a final concentration of 2 M. Samples were heated at 110 °C for 4 h in a closed screw-cap test tube, then chilled at 2–8 °C for about 30 min, filtered with 0.45 μm Acrodisc (PALL) filter and analysed. Vi polysaccharide was used for building the calibration curve, set up with standards in the range of 15–200 $\mu\text{g/mL}$ (Vi was considered as acid form fully acetylated in C-3 position). High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was performed with

a Dionex ICS3000 equipped with a CarboPac PA1 column (4 mm × 250 mm) coupled with PA1 guard column (4 mm × 50 mm). Samples were run with a flow rate of 1 mL/min, using a gradient from 40 mM to 150 mM NaNO₃ in 100 mM NaOH. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8.

Acridine orange method—Vi content in free and conjugated samples was measured also by acridine orange binding according to the procedure reported in the literature [32]. Vi samples were analysed in water, while conjugates were dialysed against 2 mM phosphate buffer before the analysis.

2.2. Immunogenicity in mice

Immunization—Fourteen groups of 8 BALB/c female mice 5–6 weeks of age (Table 1) were immunized with Vi, Vi-conjugates (Vi-CRM₁₉₇, Vi-TT) or a physical mixture of Vi and carrier protein (CRM₁₉₇ or TT). Three subcutaneous injections of 200 µL each containing 2.5 or 10 µg of Vi were given at two weeks intervals and blood was drawn prior to and two weeks after each immunization. Mice received Vi conjugates formulated either without adjuvant, or adjuvanted with 400 µg aluminum hydroxide per injection (Alum, manufactured by Novartis Vaccines and Diagnostics) or Freund's adjuvant (complete Freund's adjuvant (CFA), on the first injection; and incomplete Freund's adjuvant (IFA), on the second and third injections).

Analysis by ELISA—The wells of 96-well ELISA plates (Maxisorp, Nunc) were coated overnight with 100 µL of 1 µg/mL Vi or 2 µg/mL CRM₁₉₇ in 0.05 M carbonate buffer, pH 9.6 at 4 °C. The plates were blocked with 200 µL/well of 5% fat-free milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 1 h at room temperature (RT). After washing with PBST, 100 µL/well of mouse sera (diluted 1:200 in PBST containing 0.1% BSA) were incubated for 2h at RT. After three more washes, 100 µL/well of alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma A3438, diluted 1:10,000 in PBST containing 0.1% BSA) was incubated for 1 h at RT. After another wash, alkaline phosphatase substrate (*p*-nitrophenol phosphate tablets, Sigma N2765), dissolved in 1 M diethanolamine buffer, pH 9.8, was added to the plates and incubated for 1 h at RT. Absorbance at 490 nm and 405 nm were obtained using an ELISA reader (ELx800, BioTek) and OD₄₀₅ minus OD₄₉₀ values were used for subsequent analysis. Antibody units were expressed relative to a mouse anti-Vi or anti-CRM₁₉₇ standard serum curve, with best 4 parameter fit determined by modified Hill Plot. One ELISA unit was defined as the reciprocal of the dilution of the standard serum that gave an absorbance value equal to 1 in this assay. Each mouse serum was run in triplicate. Data are presented as scatter plots of individual mice ELISA units, and bars represent the geometric mean of each group.

Statistical methods—Kruskal–Wallis ANOVA was used to compare the anti-Vi ELISA units elicited by the various Vi-conjugates.

2.3. Protein derivatization with ADH

CRM₁₉₇ and TT were derivatized by treatment with ADH and EDAC [27]. Derivatization was performed at pH 6.0–6.2 rather than at the usual pH of 4.9–5.1 used for this kind of reaction in order to avoid protein precipitation. MES buffer was used in order to maintain constant pH during the entire reaction time. In the standard reaction, ADH (ADH/protein = 3.5, w/w) was added to either CRM₁₉₇ or TT (10–12 mg/mL in 60–80 mM MES buffer pH 6.0–6.2) and mixed. When the solution became clear, 1.5–1.8 mg/mL EDAC (EDAC/protein = 0.15, w/w) was added. The reaction was carried out for 1h at RT, then dialyzed at 2–8 °C first against 0.2 M NaCl, 10 mM MES buffer pH 7.2 and then against 5 mM MES buffer pH 7.2. Protein recovery was estimated using micro BCA while the level of derivatization was measured by mass spectrometry.

2.4. Vi conjugates

Conjugates were synthesized as described by Kossaczka et al. [27], with slight modifications.

Vi conjugation to derivatized CRM₁₉₇—Multiple Vi-CRM₁₉₇ lots were synthesized. For all lots, EDAC was added to Vi (molar ratio EDAC/Vi of 0.9–1.4, referred to Vi repeating unit) in 100 mM MES buffer pH 6.0 and mixed for approximately 5 min at RT. The individual lots also varied in the ratio of Vi to derivatized CRM₁₉₇ used. The details of the various synthesis are given in Table 2.

Derivatized CRM₁₉₇ in 5 mM MES buffer pH 7.2 was added to the solution under mixing and allowed to react with activated Vi for 3h at RT. Final conjugation mixture contained >40 mM MES buffer pH 6, in order to maintain a constant pH during the reaction. The reaction mixture was then dialyzed against 10 mM NaH₂PO₄, 0.2 M NaCl, pH 7.0 at 4 °C and purified by size exclusion chromatography on a 1.6 cm × 90 cm Sephacryl S-1000 column eluted at 0.2 mL/min in either 10 mM NaH₂PO₄, 0.2 M NaCl, pH 7.0 (conjugation lots 1–6) or 10 mM NaH₂PO₄, 5 mM NaCl pH 7.0 (conjugate lots 7–11). Fractions were analysed by SDS-PAGE and those showing presence of conjugate without free protein were collected. Two different pools were collected according to polysaccharide profile on the same column in the same eluting conditions: the first of higher molecular weight did not containing free Vi, while the second of lower molecular weight overlapped with unconjugated Vi.

Vi conjugation to derivatized tetanus toxoid—A similar procedure was used to synthesize Vi-TT. EDAC was added to Vi in 100 mM MES buffer pH 6.0 and allowed to react for approximately 5 min at RT. Derivatized TT (Vi/TT, w/w, ratio of 0.5:1) in 5 mM MES buffer pH 7.2 was added over few seconds to activated Vi solution and mixed slowly at RT for 3h. The conjugation reaction mixture contained 3.15 mg/mL TT_{ADH}, 1.53 mg/mL Vi and 1.47 mg/mL EDAC. The reaction was dialyzed against 0.2 M NaCl, 10 mM NaH₂PO₄ pH 7.0 at 4 °C and purified by Sephacryl S-1000 (1.6 cm × 90 cm column) in 10 mM NaH₂PO₄, 0.2 M NaCl pH 7.0 eluted at 0.2 mL/min. Fractions were analysed by SDS-PAGE and those not containing free protein were collected. As was done for Vi-CRM₁₉₇, two different pools were collected according to polysaccharide profile on the same column

in the same eluting conditions: the first of higher molecular weight did not contain free Vi, while the second of lower molecular weight overlapped with unconjugated Vi.

3. Results

3.1. Characterization of Vi polysaccharide

Characterization of Vi showed that this preparation contained 1% of nucleic acids, 0.3% of proteins, an O-acetylation level of 68% (according to ^1H NMR) and of 63.4% (according to Hestrin method). Spectral assignments of ^1H NMR of de-O-acetylated Vi saccharide (5.10ppm H1, 4.70ppm H5, 4.44ppm H4, 4.2ppm H2, 4.13 ppm H3, 2.06 ppm N-acetyl in non-O-acetylated residues (3H), 1.91 ppm acetate anion arising from de-O-acetylation (3H)) were in good agreement with published results [31]. Na^+ content was <0.08%. Thermogravimetric and Karl Fisher analysis gave 76% of dried weight and 21.6% of residual moisture, respectively. This Vi with a purity of 76% by weight was used as standard for High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) analysis.

3.2. Assay for unconjugated and conjugated Vi

To measure the concentration of Vi in solution or in glycoconjugate samples, we developed an assay based on the measurement of the product released following hydrolysis of the Vi polysaccharide based on HPAEC-PAD. During the development the following aspects were investigated:

Hydrolysis conditions—Hydrolysis of Vi in 4 M TFA at 120 °C for periods up to 2 h resulted in a mixture of oligosaccharides that were separated on HPAEC and detected by PAD (Fig. 1A). Hydrolysis of Vi in 4 M TFA at 120 °C after de-O-acetylation or reduction of COOH groups [33,10] also resulted in a mixture of oligosaccharides. Hydrolysis with TFA after treatment for complete de-acetylation in 2 M NaOH at 110 °C for 6 h [33] resulted in a single peak on HPAEC-PAD. Only the step of hydrolysis with 2 M NaOH at 110 °C resulted in this single peak (Fig. 1B). Kinetic of hydrolysis was studied (data not shown) and, as a result of this investigation, 4 h at 110 °C in 2 M NaOH were selected as conditions of hydrolysis.

Range of detection—Calibration curves generated from hydrolysis of purified Vi and HPAEC-PAD analysis showed excellent linearity over the range of 1–200 $\mu\text{g/mL}$ when 25 μL samples were analysed (0.025–5 μg per sample) ($R^2 > 0.999$).

Under the conditions described above, the HPAEC-PAD method gave a close correlation with the acridine orange method [32,34] for unconjugated Vi samples and for some Vi-CRM₁₉₇ conjugates tested (Table 3). In contrast to other reported procedures for Vi quantification, our new method was more sensitive. For example, the intensities of the Vi absorbance peaks observed by FT-IR spectroscopy were proportional to Vi in the range of 0.25–2.0 mg [32], and using acridine orange method Vi in the range of 20–700 $\mu\text{g/mL}$ can be assayed. Furthermore, the HPAEC-PAD method can be applied to samples in different and complex matrixes unlike FT-IR that requires salt-free solids or the acridine orange assay

that is sensitive to ionic strengths greater than 3 mM. In the latter assay and in the presence of higher salt concentrations or impurities, the binding of the cation dye acridine orange to Vi is inhibited. Attempts to quantify Vi in culture supernatants or in intermediate steps of purification using acridine orange method failed, as did the quantification of Vi in conjugates without including a step of dialysis. We also had difficulty obtaining quantitative results with some Vi conjugates when using spectrophotometric titration with acridine orange, resulting in underestimations of Vi concentration. The reason for this has not been determined.

3.3. Protein derivatization with ADH

CRM₁₉₇ derivatized with ADH (CRM_{ADH}) resulted in a similar mobility on SDS-PAGE and similar retention time on HPLC-SEC as the underivatized CRM₁₉₇ (Fig. 2). Yields were >85%. There was no significant formation of protein–protein dimers or higher oligomers, a theoretical side reaction with the chemistry chosen (Figs. 2 and 3). The level of CRM₁₉₇ derivatization with ADH was measured by mass spectrometry. Q-TOF analysis showed the formation of several products characterized by the presence of a different number of ADH linkers ranging from 3 to 10 with the major peak containing 6 bound linkers (Fig. 3A). Analysis by MALDI-TOF showed a broad peak shifted by a mass of 830 Da compared with un-derivatized CRM₁₉₇. As shown by Kossaczka et al. [27], the level of derivatization depended on the concentration of EDAC in the reaction mixture. Using half the concentration of EDAC, the CRM_{ADH} was less derivatized containing 2–3 linkers per mole of protein (Fig. 3B).

TT was derivatized using the same conditions described for CRM₁₉₇, with a recovery of 86%. The TT_{ADH} SDS-PAGE pattern and SEC profile were similar to native TT. Molar ratio of ADH to TT was measured by MALDI-TOF mass spectrometry and had an average of 11 linkers per TT (data not shown).

3.4. Vi-CRM₁₉₇ and Vi-TT

Sephacryl S-1000 gel filtration profiles of Vi-CRM₁₉₇ and Vi-TT mixtures showed peaks containing protein (as judged by A₂₁₄ and A₂₈₀) at higher MW than either unconjugated Vi or the unconjugated carrier protein, CRM₁₉₇ or TT (Fig. 4). Analysis of the conjugates showed that they contained both protein and Vi (Table 4). Consistent with the large size of the conjugate, close to the void volume of the Sephacryl S-1000 column, the conjugate was unable to migrate into the SDS-PAGE gel and protein stained band was observed in the well [27].

Conjugates were easily separated from unconjugated CRM₁₉₇ or TT but, due to the polydisperse nature of Vi and Vi conjugates and to the polysaccharide large size compared with the protein, the elution of conjugate and free Vi overlapped (Fig.4). Fractions pooled from the first half of the conjugate peak (pool 1) were characterized by a Vi to protein ratio lower than that in fractions from the second half (pool 2). As pool 1 was collected at elution times that did not overlap with free Vi, we assume that this material did not contain unconjugated Vi, while the presence of unconjugated Vi could not be ruled out for pool 2. Similar results were obtained with analysis of the Vi-TT conjugates.

Separation on Sephacryl S-1000 was subsequently optimized and elution with 10 mM NaH_2PO_4 pH 7.0 with 5 mM NaCl gave a better separation of conjugate and free Vi than with 200 mM NaCl. Vi-CRM₁₉₇ Lots 7–11 were purified in this way. A better separation between conjugate and free Vi was obtained and enabled also the purification of lower molecular weight conjugates prepared by using a higher ratio of Vi to CRM₁₉₇ (Fig. 5).

Reproducibility of conjugate formation—Three lots of Vi-CRM₁₉₇ using the same batch of CRM_{ADH} were synthesized to test the reproducibility of the process on small scale. Conjugations were performed on 4.6 mg of Vi (1.53 mg/mL) and a ratio Vi/CRM₁₉₇ of 0.5:1 (w/w). EDAC was used at 1.47 mg/mL. As shown in Table 5, the three independent conjugate lots gave similar recoveries.

Varying the ratio of Vi to CRM₁₉₇—Additional lots of Vi-CRM₁₉₇ were conjugated varying the initial ratio of Vi to CRM₁₉₇ (Table 2, Lots 7–11). In each case as judged by profile on gel filtration, conjugates were successfully formed. As the initial ratio of Vi to CRM_{ADH} was increased, the peak elution time increased, consistent with fewer CRM₁₉₇ molecules attached to polysaccharide. As expected the Vi to protein ratio also increased (Table 6).

3.5. Immunogenicity of Vi conjugates in mice

The immunogenicity of unconjugated Vi, Vi conjugates (with or without adjuvant) and mixtures of Vi with CRM_{ADH} or TT_{ADH} was tested in mice (Table 1). Tested conjugates differed by the ratio of Vi to protein. Furthermore, Vi-CRM₁₉₇ Lot 1 and Vi-TT Lot 1 pool 1 did not contain free polysaccharide, while Vi-CRM₁₉₇ Lot 3 pool 2 and Vi-TT Lot 1 pool 2 likely contained unconjugated Vi after purification by Sephacryl S-1000. Anti-Vi IgG were measured by ELISA (Fig. 6A) and compared with anti Vi levels in mice serum obtained after Vi-rEPA immunization (serum provided by NIH). The anti-CRM₁₉₇ responses for the groups of mice receiving free CRM_{ADH} or Vi-CRM₁₉₇ conjugates are shown in Fig. 6B. Unconjugated Vi, either alone (Fig. 6A, group 2) or mixed with CRM_{ADH} or TT_{ADH} (Fig. 6A, groups 3 and 4, respectively), failed to generate a detectable antibody response, even after three injections. By contrast all mice receiving Vi conjugates, with either CRM₁₉₇ or TT as the carrier protein, showed a significant increase in Vi antibody levels even after the first injection and in the absence of adjuvant (Fig. 6A, groups 5–8). The anti-Vi response peaked after the second injection, while the anti-CRM₁₉₇ antibody levels increased also after the third injection (Fig. 6B).

No significant difference in anti-Vi antibody levels was detected in sera of mice immunized with Vi-conjugates (Fig. 6A, groups 5–14) when compared on a given bleed day (Kruskal–Wallis ANOVA, $p > 0.05$). Additionally, no differences in anti-Vi antibody levels were observed between 2.5 μg and 10 μg doses (Fig. 6A, group 5 in comparison with 13 and 14), or between the unadjuvanted or adjuvanted formulations (Fig. 6A, group 5 in comparison to 9, 13 and 14; group 6 in comparison to 10; groups 7 and 8 in comparison to 11 and 12, respectively). Finally, animals immunized with Vi-CRM₁₉₇ or Vi-TT produced anti-Vi antibody levels similar to that detected in NIH mice serum (mice immunized with Vi-rEPA conjugate). Differences in Vi/CRM₁₉₇ ratios (Fig. 6A, groups 5 and 6) or possible presence

of free Vi (Fig. 6A, groups 6 and 8 vaccinated with pool 2) did not produce significant variations of anti-Vi antibody responses.

4. Discussion

Surface polysaccharides from bacteria have been widely used in vaccines, being both essential virulence factor and protective antigens. As saccharides are T-independent antigens, they are poorly immunogenic, do not induce immunological memory and are not effective in infants. Re-vaccination is required at regular intervals as antibody levels decline, but reinjection at any age does not elicit a booster effect [17,35,36]. It is well known that conjugation to a carrier protein converts T-independent antigens into T-dependent ones, thereby providing a long lasting protection and enhancing memory responses. Antibody response is boosted by repeated immunization and glycoconjugate vaccines confer protection in children younger than 2 years of age [17,35,37].

The first conjugate vaccine was developed against *Haemophilus influenzae* type b (Hib) [38,39]. With the success of the Hib conjugate vaccine, glycoconjugate vaccines against *Neisseria meningitidis* and *Streptococcus pneumoniae* have been developed and licensed [22–24,35].

In addition to native purified polysaccharides, oligosaccharides obtained by polysaccharide degradation or through organic synthesis can be used for the preparation of glycoconjugate vaccines [37]. In general, native polysaccharides are widely polydisperse in molecular weight as are their corresponding conjugates. Whereas, the use of oligosaccharides allows for well defined vaccines in a more reproducible way [37]. Saccharide chain length as well as saccharide to protein ratio are important parameters that can influence the immunogenicity of a glycoconjugate.

As shown for other polysaccharides, the introduction of Vi vaccine into routine immunization programs has been limited by its age-related immunogenicity and T-cell-independent properties. Conjugation to a carrier protein conferred T-cell dependence and increased immunogenicity [28,40,41]. Several proteins, such as diphtheria toxoid (DT), tetanus toxoid (TT), cholera toxins (CT), the B subunit of the heat-labile toxin (LT-B) of *Escherichia coli*, recombinant OMP of *Klebsiella pneumoniae* (rP40) and iron-regulated OMP of *S. Typhi* have been tested as carriers for Vi polysaccharide [42]. In particular, clinical trials of Vi-rEPA conjugate conferred 89% protection over 46 months in Vietnamese children 2–5 years old [19,20]. However, the lack of regulatory precedent for rEPA in licensed vaccine hinders its use in areas where a vaccine is more needed. Recently a Vi-TT conjugate has been licensed for local distribution in India [43] and the International Vaccine Institute (IVI) is planning to perform clinical evaluation of the safety and immunogenicity of a Vi-DT vaccine [42]. In the present study, we described the preparation, characterization and immunological properties of an anti-typhoid fever conjugate vaccine composed of the *C.freundii* capsular polysaccharide covalently coupled to the non toxic mutant of diphtheria toxin CRM₁₉₇.

We decided to use the high molecular weight native polysaccharide since it has been reported that conjugates obtained after reduction of Vi molecular size elicited lower levels of antibodies than those prepared with native Vi [41]. Furthermore, Vi polysaccharide is resistant to acid hydrolysis and its depolymerisation is unlikely in conditions that would maintain its structure substantially unchanged.

CRM₁₉₇ was derivatized with adipic acid dihydrazide and conjugated to Vi polysaccharide by carbodiimide chemistry. As there are multiple activation points within each Vi chain and multiple linkage points on each protein, crosslinked network of very high molecular weight was formed. The Vi-CRM₁₉₇ conjugates were fully characterized, including a newly developed method for total sugar quantification based on HPAEC-PAD.

Manufacture of vaccines requires good characterization and quality control of all its components. The standardization of vaccines composed of conjugated or unconjugated Vi requires a method for quantifying Vi [44]. Colorimetric methods for measuring amino sugar residues or uronic acids do not work for Vi because of its resistance to acid hydrolysis. This also prevented the application of traditional HPAEC-PAD methods [45–48] for Vi quantification. Our new method is based on alkaline hydrolysis with NaOH, in conditions that cause both complete de-acetylation and hydrolysis of the Vi. In this way quantification of the released species by HPAEC-PAD becomes possible. The same procedure can be used to quantify Vi in conjugates. The optimized procedure is reproducible, simple and precise (CV < 2% both for Vi and Vi conjugate samples) and more sensitive than other methods previously employed [29,32] (detection of 1 µg/mL Vi concentrations). Furthermore, it is suitable for quantifying Vi in complex matrixes and for analysis of formulated conjugates.

By modifying the reaction conditions described in published Vi conjugation methods, we have generated conjugates with carbohydrate/protein ratio ranging from 0.5:1 to 10:1 (Table 2). Vi-CRM₁₉₇ conjugates with a ratio 1.15 and 0.71 were tested in this preliminary study and their immunogenicity was compared to those induced by Vi-TT conjugates containing a similar ratio of 0.41 and 0.67 (Table 1). Similar levels of serum IgG Vi antibodies were elicited in mice by all Vi conjugates tested and were significantly higher than those elicited either by Vi alone or its physical mixture with carrier proteins (Fig. 6A). Conjugation ratio or dose within range tested had little impact on immunogenicity. It would be interesting to test other conjugates with higher Vi to protein ratios. Furthermore, anti-Vi IgG levels were similarly elicited by Vi-CRM₁₉₇ and Vi-TT conjugates and were also comparable to those elicited by Vi-rEPA at NIH. The nature of the carrier protein used (i.e., CRM₁₉₇, TT or rEPA) did not impact on immunogenicity, and confirms the data of Cui et al. [42] where Vi-DT was compared with Vi-rEPA produced at NIH. Importantly, a powerful adjuvant (Freunds complete followed by incomplete) did not increase antibody responses.

Based on these preliminary results, additional immunogenicity studies of Vi-CRM₁₉₇ have been conducted (Rondini et al., manuscript submitted). Vi-CRM₁₉₇ appears to be a suitable candidate for the development of a commercially viable, effective typhoid vaccine, especially for young children in low income countries.

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Fig. 1.

TFA and NaOH hydrolysis of Vi detected by HPAEC-PAD. HPAEC-PAD profiles were obtained with CarboPac PA1 column using PA1 guard-column and a flow rate of 1 mL/min. Vi treatment with (A) 4 M TFA at 120 °C for 2h resulting in a mixture of oligosaccharides, analysed by elution with 18 mM NaOH for 15 min and then reaching 40 mM NaOH, 10 mM NaNO₃ in 5 min and then 100 mM NaOH, 80 mM NaNO₃ in 10 min and finally 100 mM NaOH, 200 mM NaNO₃ in 30 min. (B) 2 M NaOH at 110 °C for 4h resulting in a single peak, analysed by elution in gradient from 40 mM to 150 mM NaNO₃ in 100 mM NaOH in 22 min.

Fig. 2.
HPLC-SEC analysis of underivatized CRM₁₉₇ and CRM_{ADH}. Samples were run on TSK gel 3000 PW column and eluted with 100 mM NaH₂PO₄, 100 mM NaCl, 5% CH₃CN pH 7.2 with UV detection at 214 nm. Derivatized CRM₁₉₇ (CRM_{AHD}) showed similar retention time on HPLC-SEC as the underivatized protein.

Fig. 3. Impact of EDAC/CRM₁₉₇ ratio on derivitization of CRM_{ADH} as detected by MS Q-TOF. Derivatized CRM_{ADH} was prepared using (A) EDAC/CRM₁₉₇ ratio of 0.15 (w/w) or (B) half amount of EDAC. The result is the formation of several products characterized by the presence of a different number of linkers bound to the protein: (A) 3–10 linkers per mol protein with the principle product containing 6 linkers and (B) 2–3 linkers per mole of protein.

Fig. 4.

Sephacryl S-1000 profile of Vi, CRM_{ADH} and Vi-CRM₁₉₇. Vi-CRM₁₉₇ Lot 3 was purified on Sephacryl S-1000 (1.6 cm × 90 cm) eluting with 10 mM NaH₂PO₄, 200 mM NaCl pH7.0 at a flow rate of 0.2 mL/min. Conjugate reaction mixture profile is in comparison with unconjugated Vi run on the column in the same conditions; black arrow indicates the peak corresponding to unconjugated protein.

Fig. 5.

Sephacryl S-1000 purification eluted with 5 mM NaCl instead of 200 mM NaCl gives better separation of Vi and Vi-CRM₁₉₇. Vi-CRM₁₉₇ Lot 11 purification on Sephadex S-1000 in comparison to free Vi. Sephadex S-1000 (1.6 cm × 90 cm column); eluent: 10 mM NaH₂PO₄, 5 mM NaCl, pH 7.0; flow: 0.2 mL/min. Separation conditions on Sephadex S-1000 were optimized, finding that elution with this buffer containing lower NaCl gave a better separation between conjugate and free Vi, also for lots synthesized using a higher ratio of Vito CRM₁₉₇ and so producing a conjugate shifted at lower MW.

Fig. 6.

Conjugation of Vi to carrier protein is required to generate anti-Vi antibodies. Fourteen groups of BALB/c female mice ($N = 8$ per group) were subcutaneously immunized on days 0, 14 and 28 as detailed in Table 1. Sera were assessed by ELISA for (A) anti-Vi IgG antibodies and (B) anti-CRM₁₉₇ IgG antibodies (measured just in those groups receiving CRM₁₉₇). Bars represent the geometric mean ELISA units of the group, individual animals are represented by the scatter plots. Comparisons are made to serum from mice vaccinated

with Vi-*r*EPA (serum provided by PDML, NIH, obtained from mice immunized with 2.5 µg of the Vi-*r*EPA conjugate used in human trials in Vietnam).

Table 1

Immunogenicity study.

Group number	Vaccine	Vi/protein ratio in conjugate (w/w)
1	PBS	
2	Vi	
3	Vi+CRM _{ADH}	
4	Vi+TT _{ADH}	
5	Vi-CRM ₁₉₇ Lot 1	1.15
6	Vi-CRM ₁₉₇ Lot 3 pool 2	0.71
7	Vi-TT Lot 1 pool 1	0.41
8	Vi-TT Lot 1 pool 2	0.67
9	Alum formulated Vi-CRM ₁₉₇ Lot 1	1.15
10	Alum formulated Vi-CRM ₁₉₇ Lot 3 pool 2	0.71
11	Alum formulated Vi-TT Lot 1 pool 1	0.41
12	Alum formulated Vi-TT Lot 1 pool 2	0.67
13	CFA/IFA formulated Vi-CRM ₁₉₇ Lot 1	1.15
14	CFA/IFA formulated Vi-CRM ₁₉₇ Lot 1	1.15

Vi dose per vaccination was 2.5 µg for all the groups with the exception of groups 13 and 14 that received 10 µg (both groups were identical).

Table 2

Conjugation conditions used for generation of different Vi-CRM₁₉₇ lots.

Conjugate	Vi concentration (mg/mL)	Vi/CRM ₁₉₇ ratio (w/w)	EDAC/Vi ratio (w/w)
Lot 1	2.1	1:1	0.88
Lot 3	2.1	0.5:1	0.88
Lots 4–6	1.5	0.5:1	0.96
Lot 7	2.2	0.7:1	0.69
Lot 8	2.2	1:1	0.69
Lot 9	2.2	2:1	0.69
Lot 10	2.2	5:1	0.67
Lot 11	2.2	10:1	0.67

Table 3

Vi concentrations in unconjugated Vi and Vi-CRM₁₉₇ samples determined by HPAEC-PAD after alkaline hydrolysis and by acridine orange method.

Sample	<u>Vi concentration (µg/mL) determined by</u>	
	HPAEC-PAD	acridine orange
Unconjugated	30.0	34.6
Vi	50.0	55.8
	100.0	122.0
Vi-	23.2	26.0
CRM ₁₉₇	99.6	97.5
	116.0	115.2

Table 4Vi-CRM₁₉₇ and Vi-TT conjugate characterization for protein and saccharide content.

Conjugate	Vi content ^a µg/mL (HPAEC-PAD)	Vi content ^b µg/mL(acridine orange)	Protein content µg/mL (micro BCA)	Vi/protein ratio (w/w)
Vi-CRM ₁₉₇	70.0	60.9	53.0	1.32 ^a
Lot 1				1.15 ^b
Vi-CRM ₁₉₇	23.2	25.9	41.9	0.55 ^a
Lot 3 pool 1				0.62 ^b
Vi-CRM ₁₉₇	99.6	97.6	137.6	0.72 ^a
Lot 3 pool 2				0.71 ^b
Vi-TT	nd	26.8	64.64	0.41 ^b
Lot 1 pool 1				
Vi-TT	84.7	82.8	123.68	0.68 ^a
Lot 1 pool 2				0.67 ^b

^aVi quantified by HPAEC-PAD analysis.^bVi quantified by acridine orange colorimetric assay.

CV < 2% for HPAEC-PAD and < 3% for micro BCA on analysed conjugates. Acridine orange measurements were more variable (CV from 3 to 12%).

Table 5

Reproducibility of Vi-CRM₁₉₇ conjugation and purification.

Conjugate	Vi conc. µg/mL (HPAEC-PAD)	CRM ₁₉₇ conc. µg/mL (micro BCA)	Vi/CRM ₁₉₇ ratio (w/w)	% yield	
				Vi	CRM ₁₉₇
Lot 4 pool 1	24.37	52.06	0.47	84.0	64.9
Lot 4 pool 2	78.36	106.1	0.74		
Lot 5 pool 1	34.51	63.17	0.55	86.8	63.9
Lot 5 pool 2	71.39	93.42	0.76		
Lot 6 pool 1	39.12	59.1	0.66	86.2	56.9
Lot 6 pool 2	67.73	82.41	0.82		

Table 6Characterization of Vi-CRM₁₉₇ produced by varying the ratio of Vi to CRM₁₉₇

Conjugate	Nominal Vi/CRM ₁₉₇ ratio in reaction	Vi conc. µg/mL (HPAEC-PAD)	CRM ₁₉₇ conc. µg/mL (micro BCA)	Actual Vi/CRM ₁₉₇ ratio (w/w) in purified conjugate	Retention time of the major peak on Sephacryl S-1000 (mL)
Lot 7	0.7:1	18.6	27.4	0.68	100.33
Lot 8	1:1	14.3	16.6	0.86	102.57
Lot 9	2:1	22.4	11.9	1.88	106.79
Lot 10	5:1	49.5	8.4	5.90	107.14
Lot 11	10:1	32.9	5.0	6.58	107.47