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Preparation and evaluation of immunogenic conjugates of *Salmonella enterica* serovar Typhi O-specific polysaccharides with diphtheria toxoid

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Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), is a major health problem particularly in developing countries. The available vaccines have certain limitations regarding their efficacy, and inability to induce an immune response especially in individuals under 2 years of age. Conjugate vaccines which consist of a bacteria-specific polysaccharide chemically bound to a carrier protein overcome these problems by inducing a T-cell dependent immune response characterized by enhanced immunogenicity in all ages. In this study, O-specific polysaccharides (OSP) of *S.* Typhi were conjugated to diphtheria toxoid (DT) using adipic acid dihydrazide (ADH) as a linker. These conjugates (OSP-AH-DT) were then evaluated for their immunogenicity using mice as a model and showed significantly higher levels of IgG ELISA titers ($P = 0.0241$ and 0.0245) than lipopolysaccharides alone. Different immunization schedules were compared and it was found that schedule-B (three injections with 4-weeks interval) induced higher immune responses than schedule-A (three injections with 2-weeks interval). We showed that diphtheria toxoid can be successfully employed as a carrier protein for conjugation with *Salmonella* OSP and play an important role in facilitating adequate immune response.

Surface polysaccharides are essential virulence factors and protective antigens in many bacterial pathogens. These may be capsular polysaccharides such as found on *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Hemophilus influenzae* or the O-specific polysaccharides (OSP) domain of lipopolysaccharides (LPS) of some Gram-negative bacteria.[1] Polysaccharide antigens are large molecules consisting of repeat units that are not processed by antigen presenting cells (APC) and interact directly with B lymphocytes. These antigens induce antibody synthesis without the assistance of T-helper cells (thus, designated as T-cell independent antigens). Most of the OSP do not elicit serum antibodies at any age, probably because of their relatively low molecular weight, and are mostly considered as haptens.[2] Vaccines based on polysaccharide alone fail to induce protective levels of antibodies in young children less than 2 y old and even re-immunization of most polysaccharides does not induce a booster response.[3]

Production of conjugate vaccines involves the conjugation of polysaccharide antigen of a particular pathogen with a carrier protein. This conjugation converts the T-cell independent polysaccharide antigen into a T-cell dependent antigen, which results in better immunological response.[4] Polysaccharide-protein conjugate vaccines that are currently licensed include *Hemophilus influenzae* type b, *Neisseria meningitidis*, and *Streptococcus pneumoniae* while conjugate vaccines against *Salmonella, Shigella, Pseudomonas* and many other bacteria are at different stages of clinical evaluation.[5,6] Recent reports from endemic countries indicated a significant typhoid burden in children <2 y.[7,8] These reports highlighted the need to develop an effective vaccine that targets this age group. Vi-rEPA conjugate vaccine, prepared by conjugation of Vi capsular polysaccharide of *S.* Typhi to a nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA), has shown an enhanced immunogenicity in adults and in children aged 5 to 14 y.[9,10] The Vi-rEPA conjugate vaccine was found to be safe and immunogenic and has more than 90 percent efficacy in children age 2 to 5 y.[11] This Vi-rEPA conjugate vaccine is now undergoing clinical evaluation in younger age groups. In addition, efforts are underway to develop and evaluate improved live, attenuated, oral vaccines with the goal of maintaining safety while improving efficacy and reducing the number of doses required.[12]

Vi conjugate vaccines are being developed by many investigators.[7,13,14] In the current study, we investigated an alternative vaccine development strategy by conjugating OSP of *S.* Typhi to diphtheria toxoid (DT) followed by immunogenicity evaluation of the resultant conjugates in mice. The OSP of Gram-negative bacteria are their important shielding antigens and the serum immunoglobulins (IgG) to OSP confer protective immunity to these enteric pathogens.[1,15] The OSP of different human enteric

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pathogens have already been used in developing conjugate vaccine candidates against these bacteria.[16-18] To the best of our knowledge this is the first report of conjugation of *S.* Typhi OSP with carrier protein DT.

Results

***S.* Typhi OSP-AH preparation.** A yield of 54 mg of LPS per 100 g of wet cell-pellet and a subsequent yield of 33.5 mg of OSP per 100 mg of LPS was obtained. Results of silver staining of *S.* Typhi LPS are shown in **Figure 1**. Nucleic acids and protein contaminations in purified LPS and OSP were found to be less than 1%. The LPS was found to have > 3000 endotoxin units (EU) per mg and the OSP less than 3 EU per mg which is acceptable according to WHO standards.[19] After derivatization of OSP, the ADH content was found to be 2.07%. Antigenicity of the purified LPS and OSP samples was determined against the standard hyper immune sera by immuno-diffusion assay and the precipitation bands confirmed their antigenicity.

Conjugation of *S.* Typhi OSP-AH with DT. On the basis of size exclusion chromatography profile, proteins and polysaccharides assays, and immuno-diffusion patterns, the Sepharose CL-6B column fractions were pooled as *S.* Typhi OSP-AH-DT conjugates (**Fig. 2**). The OSP yield in the final *S.* Typhi OSP-AH-DT conjugate-1 and 2 was 45.6% and 41.6%, respectively while the DT yield in these conjugates was 27.3% and 30.6%, respectively (relative to the total amount of OSP and DT added to the conjugation reaction). The OSP/DT ratio was found as 1.65 and 1.36 in *S.* Typhi OSP-AH-DT conjugate-1 and 2, respectively. The results of immuno-diffusion assays for both conjugate pools are shown in **Figure 3**.

Immunogenicity evaluation of the conjugates in mice. *S.* Typhi OSP-AH-DT conjugates-1 and 2 induced significantly higher geometric mean antibody titers ($p = 0.0241$ and 0.0245 respectively) than *S.* Typhi LPS alone when injected with dosing schedule-B. No significant difference was observed between antibody titers induced by *S.* Typhi OSP-AH-DT conjugates-1 and 2 ($p = 0.7847$). *S.* Typhi OSP-AH-DT conjugate-1 using schedule-A did not induce a significantly higher antibody titer ($p = 0.3262$) as compared with LPS alone. These results are summarized in **Table 1**.

Discussion

Typhoid fever is an important health problem especially in the developing countries, with approximately 22 million new cases each year of which 5% are fatal.[20,21] The main cause of enteric fever is *S.* Typhi but *S.* Paratyphi A is emerging as an important secondary cause displaying similar symptoms to those seen in *S.* Typhi infections.[22] Vi-negative variants of *S.* Typhi have been isolated from patients

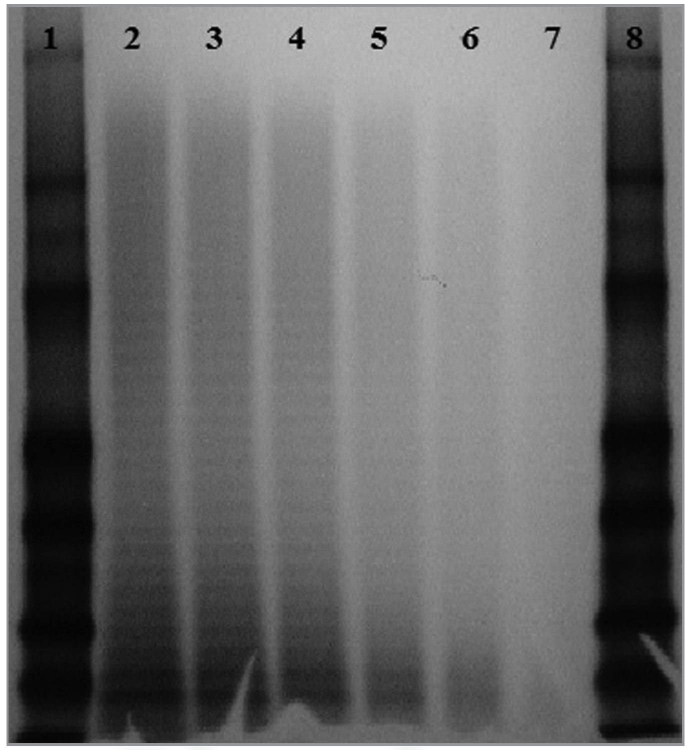

Figure 1. Silver staining of *S.* Typhi LPS on SDS PAGE. Lanes 1 and 8: BanchMark prestained ladder (Invitrogen Cat# 10748–010). Lane 2–7: Different amounts of *S.* Typhi LPS in μg 20, 10, 5, 2.5, 1 and 0.5 respectively.

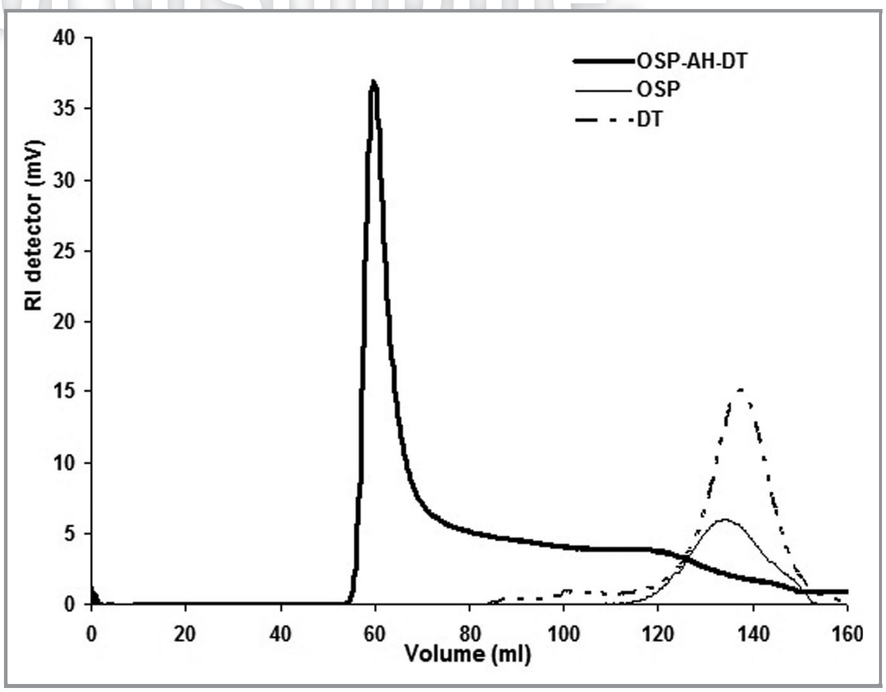

Figure 2. Gel filtration profiles of DT, *S.* Typhi OSP and *S.* Typhi OSP-AH-DT. Samples were passed through Sepharose CL-6B (1.6 X 84 cm) column against saline.

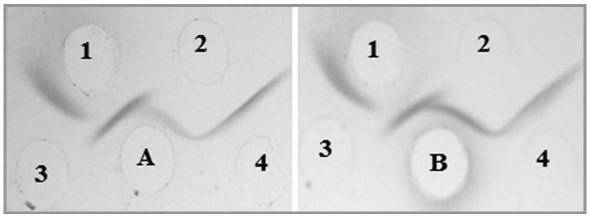

Figure 3. Double Immunodiffusion of conjugates against anti-OSP and anti-DT sera. Wells A and B: *S.* Typhi OSP-AH-DT conjugate-1 and conjugate-2 (100 μg each) respectively. Wells 1 and 2: Anti-OSP and anti-DT (15 μl each) respectively. Wells 3 and 4: *S.* Typhi OSP and DT (100 μg each) respectively.

with typhoid fever[23,24] justifying the need for development of an OSP conjugate vaccine in parallel to Vi conjugate vaccines. The long-term solution for prevention of enteric fever is providing improved sanitation and food hygiene. However, this solution is expensive and a drain on already scarce resources in developing countries. The immediate solution to reduce the burden of typhoid fever is vaccination of the people at greater risk living in endemic areas.

Currently there are three licensed vaccines against typhoid which include parenteral inactivated whole-cell vaccine, oral live attenuated Ty21a vaccine and parenteral Vi capsular polysaccharide vaccine. The use of inactivated whole cell vaccine was suspended due to the unacceptably high incidence of adverse effects, 25–40% of the people immunized by this vaccine developed systemic reactions[25] and 10% of vaccinees missed school or work after vaccination.[26] The Ty21a vaccine was found to have cumulative 3 y efficacy as 51% and it has also not been widely adopted as a routine public health tool in any country with endemic typhoid fever due to its high cost.[27] The Vi polysaccharide vaccine of *S.* Typhi is approved for the persons more than 2 y old with a three-year efficacy of more than 65%.[13] None of the currently licensed typhoid vaccines is widely adopted as a routine public health tool in any country with endemic typhoid fever.[27] This fact highlights the need for the preparation of a vaccine with enhanced efficacy and effectiveness even in infants which can be achieved by conjugation with a protein.

The first glycoconjugate vaccine licensed in 1987 for use in humans was *Hemophilus influenzae* type b (Hib) conjugate vaccine and shortly thereafter it was introduced into the US infant immunization schedule.[28] This vaccine is now recommended for the worldwide use. Other licensed conjugate vaccines with high efficacy include meningococcal and pneumococcal conjugate vaccines.[29] Conjugate vaccines against organisms causing enteric fever are being developed by many groups and the Vi-rEPA conjugate vaccine has proven to be safe and immunogenic and is now undergoing evaluation in younger age groups.[6,11]

In addition to capsular polysaccharide, different other vaccines are being developed where the OSP is conjugated to a variety of carrier proteins. Reports of OSP based conjugate vaccines include *Shigella dysenteriae* type 1 OSP with tetanus toxoid,[16] *Escherichia coli* O157 OSP with bovine serum albumin, rEPA, exotoxin C of *Clostridium welchii*,[30] and with nontoxic B subunit of Stx1.[17] The OSP of *S.* Typhi has been conjugated to tetanus toxoid, bovine serum albumin,[18] and also with human serum albumin.[31]

Conjugation of *S.* Typhi OSP with carrier protein, diphtheria toxoid (DT) has not been reported. We have conjugated OSP of *S.* Typhi (common in all strains of *S.* Typhi, both Vi-positive and Vi-negative) with DT followed by the evaluation of immunogenicity in mice. Two conjugates of *S.* Typhi OSP were prepared and both *S.* Typhi OSP-AH-DT conjugates induced significantly higher anti-LPS IgG antibody response ($p = 0.0241$ and 0.0245 respectively) after three injections compared with LPS alone (**Table 1**). Difference between antibody titers raised by two *S.* Typhi OSP-AH-DT conjugates was found non-significant ($p = 0.7847$). Injection schedule-B (three injections with 4-weeks interval) induced significantly better response than injection schedule-A (three injections with 2-weeks interval). The possible reason could be that four weeks interval in each of three doses gave proper time for establishing immunological memory for better immune response with the booster doses.

The results presented here demonstrated that OSP purified from *S.* Typhi can be conjugated to DT carrier protein. The antibody responses generated following three doses of conjugates were superior to those generated by LPS alone using the same dosing regimen. The superior immune response and the increase in size of the conjugate as compared with polysaccharide alone are indicative of the successful preparation of the conjugate. The use of OSP is also advantageous to broaden the scope of typhoid conjugate vaccine as it will be effective against different variants of *S.* Typhi. Being a conjugate, this vaccine could also be used for infants.

Table 1. Immunogenicity of *S.* Typhi LPS alone and conjugates after three injections as compared with standard hyperimmune sera

Immunogen	Geometric mean of Antibody titers (n = 10)	95% confident limit		P value when compared with LPS alone
		Lower	Upper	
[a]*S.* **Typhi LPS alone**	13.89	1.079	26.701	N/A
[a]*S.* **Typhi OSP-AH-DT Conjugate-1**	18.24	1.051	35.429	0.3262
[b]*S.* **Typhi LPS alone**	13.07	4.605	30.745	N/A
[b]*S.* **Typhi OSP-AH-DT Conjugate-1**	76.19	15.210	137.17	0.0241
[b]*S.* **Typhi OSP-AH-DT Conjugate-2**	66.39	14.802	117.98	0.0245

[a]Injection Schedule A (days 1–14–28); [b]Injection Schedule B (days 1–28–56)

Materials and Methods

Purification of polysaccharide antigens. A field isolate from China, *S.* Typhi (O9, O12, Vi), reported earlier,[32] was taken from International Vaccine Institute (IVI) collection. The inoculum was added at the rate of 6% (v/v) to each of the two 10 L fermentors containing 8 L TSB (Tryptic soy broth, Merck, Cat. # 105459) and fermentation conditions controlled at 32°C with 30% dissolved oxygen at pH 7 for 10 h. Optical density of the culture was measured every hour at 600 nm. After 10 h fermentation, bacterial cells were killed by adding 1% formalin and kept at room temperature overnight with continuous stirring at 200 rpm. Harvesting of bacteria was done by centrifugation at 7,000 g at 4°C for one hour. LPS were extracted and purified from the harvested wet cell-pellet by hot-phenol method,[16] and freeze-dried. Purified *S.* Typhi LPS were electrophoresed on SDS-PAGE and checked by silver staining.[33] Purification of OSP of *S.* Typhi was done by acid hydrolysis as previously described.[16] Briefly, the LPS of *S.* Typhi was dissolved as 10 mg/ml in 1% glacial acetic acid and kept in boiling water for 90 min. The pH was neutralized by using 1 N sodium hydroxide and the contents were ultracentrifuged at 96,000 g for 5 h at 4°C with a drop of 1 M calcium chloride. The supernatant was applied to the chromatography column (1.5 X 34 cm) using Sephadex G-25 media against deionized water. The void volume fractions were pooled and freeze-dried as *S.* Typhi OSP. GE Healthcare's AKTA Basic FPLC chromatographic system was used during all chromatography experiments with automated fractionation and attached UV and refractive index [RI] detection systems.

Nucleic acids contaminations in *S.* Typhi LPS and OSP were measured spectrophotometrically at A_{260}. Protein estimation was done by Coomassie assay reagent (Pierce, Cat. # 23200) as instructed by the manufacturer. *Limulus* Ameobocyte Lysate (LAL) test,[34] was used to determine endotoxin levels in *S.* Typhi LPS and OSP.

Derivatization of *S.* Typhi OSP. *S.* Typhi OSP was derivatized with adipic acid dihydrazide (ADH) as described earlier.[35] Briefly, *S.* Typhi OSP was dissolved in saline (20 mg/ml) and pH of the solution was adjusted as 5.0 to 6.0 with 0.1 N NaOH. It was followed by addition of 1-cyano-4-dimethylamino-pyridiniom tetrafluoroborate (CDAP, Sigma, Cat. # C2776) to the OSP solution slowly to make final ratio CDAP/OSP = 0.5/1.0 (wt/wt). Tri-ethylamine (TEA) solution was added drop wise to bring the pH 7.5 to 8.5 followed by addition of equal volume of ADH (0.8 M) solution. The reaction mixture was kept at room temperature for 2 h with pH maintained at 8.0 to 8.5. The reaction mixture was then dialyzed against pyrogen free saline overnight at 4°C and passed through chromatography column (Sephadex G-25, 1.5 X 34 cm) against deionized water. Void volume fractions were pooled and freeze-dried as derivatized *S.* Typhi OSP (*S.* Typhi OSP-AH). The extent of derivatization of OSP with ADH was measured by trinitrobenzene sulfonic acid (TNBS) assay with ADH as standard.[36]

Conjugation of *S.* Typhi OSP-AH with DT. *S.* Typhi OSP-AH was dissolved as 20 mg/ml in saline and DT solution was slowly added to make the final *S.* Typhi OSP-AH /DT ratio = 1/1 (wt/wt). The mixture was then put on ice bath and EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydro-chloride] powder was added slowly to make its final concentration as 15mM. The reaction mixture was stirred on ice bath for 4 h with pH maintained at 5.0 to 5.5 and then dialyzed against saline overnight at 4°C. The contents of dialysis tube were applied to chromatography column Sepharose CL-6B (1.6 X 84 cm) and passed against saline. Polysaccharides assay,[37] protein assay (Coomassie assay reagent, Pierce, Cat. # 23200) and immuno-diffusion assay,[38] were performed on collected fractions. The void volume fractions, which showed precipitation lines with anti-LPS and anti-DT serum samples, were pooled as *S.* Typhi OSP-AH-DT conjugates.

Preparation of standard hyper immune sera. The overnight culture of *S.* Typhi was killed by adding 2% formalin in TSB flasks and diluted with saline to get the final OD as 1.0 at 600 nm. These cells were then used for injecting the 8 weeks old female Balb/C mice (20 mice). The mice were injected for three consecutive weeks scheduled as during first week three injections (0.1 ml each) subcutaneously, second week three injections (0.15 ml each) intra peritoneally and third week three injections (0.2 ml each) intra peritoneally. The blood was drawn after one week of the last injection and serum was analyzed for the presence of antibodies against *S.* Typhi LPS using double immuno-diffusion assay.[38] The serum was aliquoted into small tubes (50 µl each) and stored at -70°C as standard sera for immunoassays.

Immunogenicity evaluation in mice. Groups of 10 Balb/C mice (female, 6 week old) were inoculated subcutaneously as follows: Group 1 was injected with saline only, group 2 and 3 with LPS only, group 4 and 5 with *S.* Typhi OSP-AH-DT conjugate-1 and group 6 with *S.* Typhi OSP-AH-DT conjugate-2. The injection schedule for groups 2 and 4 was: Schedule A (dosed on day-1, day-14 and day-28) and for all other groups was Schedule B (dosed on day-1, day-28 and day-56). The dose of polysaccharide in all groups was 2.5 µg of polysaccharides/injection. Heart bleeding was done 2 weeks after the third injections and the serum was stored at -70°C.

Immunoassay and data analysis. Serum IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) using Nunc Maxisorp plates (coated with purified LPS of *S.* Typhi) as described previously.[39,40] ELISA data were processed with Program ELISA for Windows, Centers for Disease Control and Prevention,[41] for calculations of antibody titers. Titers of the test sera were determined by interpolation of OD values from the standard curve and by averaging the calculated concentrations obtained in a defined region of curve (only values of OD lying within the range 0.15 and 2.20 were used).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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