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Synthesis and immunogenicity evaluation of *Salmonella enterica* serovar Paratyphi A O-specific polysaccharide conjugated to diphtheria toxoid

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Salmonella enterica serovar Paratyphi A (*S.* Paratyphi A) is a human restricted pathogen that can cause systemic infection (paratyphoid fever) with recently increased incidence particularly in developing countries. Currently there is no licensed vaccine for prevention of infection from *S.* Paratyphi A. In this study the O-specific polysaccharide (OSP) of *S.* Paratyphi A was conjugated to diphtheria toxoid (DT) with and without adipic acid dihydrazide (ADH) as a linker. Binding of the OSP to a carrier protein was intended to convert a T-cell independent OSP response to a T-cell dependent response inducing higher levels of anti-OSP antibodies and immunological memory. These conjugates (OSP-AH-DT and OSP-DT) were evaluated for their immunogenicity in mice. The *S.* Paratyphi A OSP-DT conjugate induced a poor anti-OSP response less than that observed with LPS while the OSP-AH-DT conjugate induced a significantly higher antibody titer compared with LPS alone. The study also demonstrated diphtheria toxoid as a potential carrier protein for conjugate vaccine candidates using *S.* Paratyphi A OSP.

Introduction

Paratyphoid fever caused by *Salmonella enterica* serovar Paratyphi A (*S.* Paratyphi A) is a human enteric infection which can lead to bacteremia and enteric fever. Generally *S.* Typhi is believed to be the major cause of most episodes of the enteric fever, but several recent reports have shown an increasing prevalence of *S.* Paratyphi A infections resulting in a disease of major public health concern particularly in Asia.[1] Vaccination against *S.* Typhi has resulted in sharp decline of enteric fever in many areas of the world exposing an increase in paratyphoid episodes.[2] In spite of the increase in the number of enteric fever episodes caused by *S.* Paratyphi A, the paratyphoid vaccine development is lagging behind[3] and currently no vaccine against *S.* Paratyphi A is available.[4]

Surface polysaccharides of many bacteria whether they are capsular polysaccharides or associated with the LPS in the case of gram-negative enteric bacteria are usually protective antigens.[5] The OSP purified from the LPS is poorly immunogenic and behaves as a T-cell independent antigen.[6] T-cell independent responses are characterized by low levels of anti-OSP antibodies and a lack of induction of memory cells and a failure to boost on subsequent doses.[6]

Conjugation of a polysaccharide antigen to a carrier protein results in a T-cell dependent immune response characterized by higher titers of anti-polysaccharide antibodies having bactericidal activity and induction of memory cells.[7] This conjugation can be achieved by direct linkage of OSP with carrier protein or using an intermediate linker molecule such as adipic acid dihydrazide (ADH). *S.* Paratyphi A OSP-protein conjugates were previously shown to be safe and to elicit anti-OSP IgG antibodies in all age groups.[8] The currently licensed polysaccharide-protein conjugate vaccines include *Hemophilus influenzae* type b, *Neisseria meningitidis*, and *Streptococcus pneumoniae* while various conjugate vaccines candidates against *E. coli*, *Shigella, Salmonella, Pseudomonas,* and many other bacteria are at different stages of clinical trials.[9,10]

In this study we used direct and a linker molecule to conjugate OSP of *S.* Paratyphi A to diphtheria toxoid (DT) followed by an evaluation of the immunogenicity of the conjugates in mice. The use of DT as carrier protein in developing conjugate vaccine candidates against *S.* Typhi has been reported earlier[11] but to the best of our knowledge this is the first report of conjugating DT with *S.* Paratyphi A OSP.

Results

S. Paratyphi A OSP antigen preparation

Growth of *S.* Paratyphi A and LPS extraction resulted in LPS yield as 29 mg per liter of culture. Results of silver staining of

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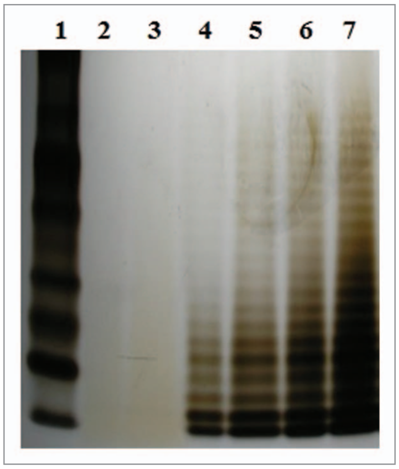

Figure 1. Silver staining of *S.* Paratyphi A LPS on SDS PAGE. Lane 1: BanchMark prestained ladder (Invitrogen Cat# 10748–010). Lanes 2 and 3: *S.* Paratyphi A OSP 20 µg and 10 µg respectively. Lanes 4–7: Different amounts of *S.* Paratyphi A LPS in µg 0.5, 1, 2.5, and 5 respectively.

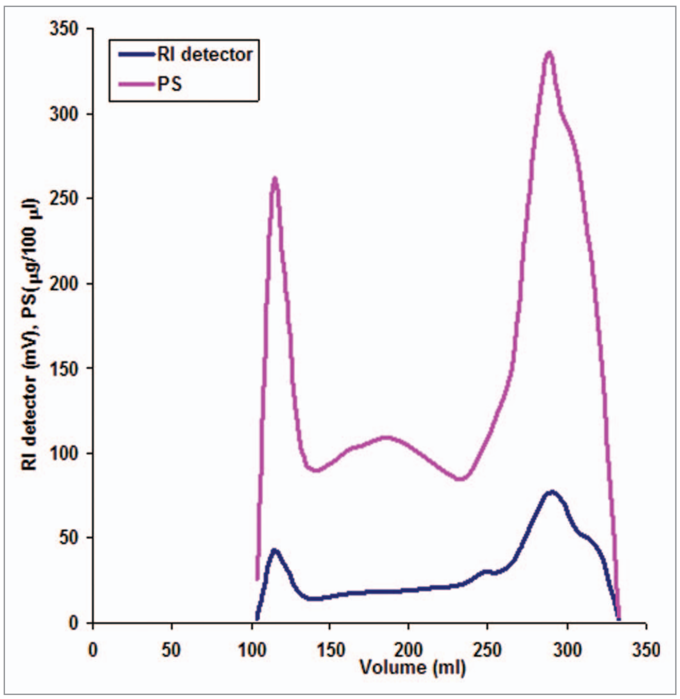

Figure 2. Sephadex G-75 profiles of *S.* Paratyphi A OSP. Elution volume was plotted against refractive index (RI) detector readings and polysaccharides (PS) concentrations as measured by Anthrone assay. The fractions from 101 ml to 257 ml were pooled together as higher molecular weight antigenic part of *S.* Paratyphi A OSP.

S. Paratyphi A LPS are shown in **Figure 1**. After acid hydrolysis, Sephadex G-75 column fractions of the crude *S.* Paratyphi A OSP resulted in high (101 ml to 257 ml) and low (258 to 350 ml) molecular weight fractions based on Anthrone assay and refractive index (RI) detector (**Fig. 2**). Immunodiffusion assay of fractions showed that the high molecular weight portion of *S.* Paratyphi A OSP reacted with the anti LPS serum and was selected for preparation of conjugates. Nucleic acid and protein contamination in purified LPS and OSP were found to be less than 4% and 1% respectively. The LPS had >3000 endotoxin units (EU) per mg and the OSP less than 3 EU per mg which is acceptable according to WHO standards.[12] Derivatization of the OSP resulted in 0.87% ADH content in *S.* Paratyphi A OSP-AH. The O-acetyl content as measured by Hestrin assay was found to be 70 nmol/ml in the OSP and 16 nmol/ml in the OSP-AH.

***S.* Paratyphi A OSP-AH-DT conjugate**

The OSP and DT yields in the final OSP-AH-DT conjugate (compared with the starting OSP and DT added to the conjugation reaction) were 43.8% and 27.3% respectively and the OSP/DT ratio was found to be 1.6.

***S.* Paratyphi A OSP-DT conjugate**

The OSP conjugated directly to DT without linker yielded higher recoveries of both OSP and DT compared with the conjugate generated with the linker. The OSP and DT yields in the final OSP-DT conjugate were 80.2% and 41.4% for polysaccharide and protein respectively and the OSP/DT ratio was 1.94. The results of immuno-diffusion assays for both conjugates are shown in **Figure 3**.

Immunogenicity evaluation of the conjugates

The immuno-diffusion result confirmed the presence of OSP antigen in the conjugate prepared without a linker, however, the anti-OSP response was poor and less than that induced by LPS alone (**Table1**). In contrast the conjugate with the ADH linker induced a significantly higher (P = 0.0446) anti-OSP response compared with the response to LPS alone.

Discussion

Bacteria of the genus *Salmonella* cause a variety of diseases in humans as well as animals commonly referred as salmonellosis. In humans, severe disease and bacteremia are associated primarily with *S.* Typhi and *S.* Paratyphi A serotypes. Symptoms of paratyphoid fever in most cases cannot be distinguished from those of typhoid fever (caused by *S.* Typhi), but it seems to follow a distinct route of transmission: whereas typhoid fever is spread predominantly within the household, paratyphoid fever is mainly transmitted outside the patient's home.[13] The emerging incidence of *S.* Paratyphi A infections has gained great concern, particularly because of the fact that in the areas where paratyphoid incidence is high there is a risk that even a highly effective *S.* Typhi vaccine may appear as poorly efficacious.[4] The old whole-cell bacterial vaccine (TAB) against *S.* Typhi and *S.* Paratyphi A and B was de-licensed because of multiple factors including high incidence of reactogenicity, gradual hydrolysis of essential O-acetyl group of LPS[7] and inability of parenterally injected inactivated gram-negative bacteria to induce high levels of IgG LPS antibodies.[14,15] Since no vaccine against paratyphoid is available, a combination vaccine covering both serotypes seems needed, particularly in Asia.[16]

Salmonella LPS consists of the 3-deoxy-D-manno-octulosonic acid (KDO) terminus of a conserved core region linked to lipid A on one side and to a variable OSP chain on the other side. The

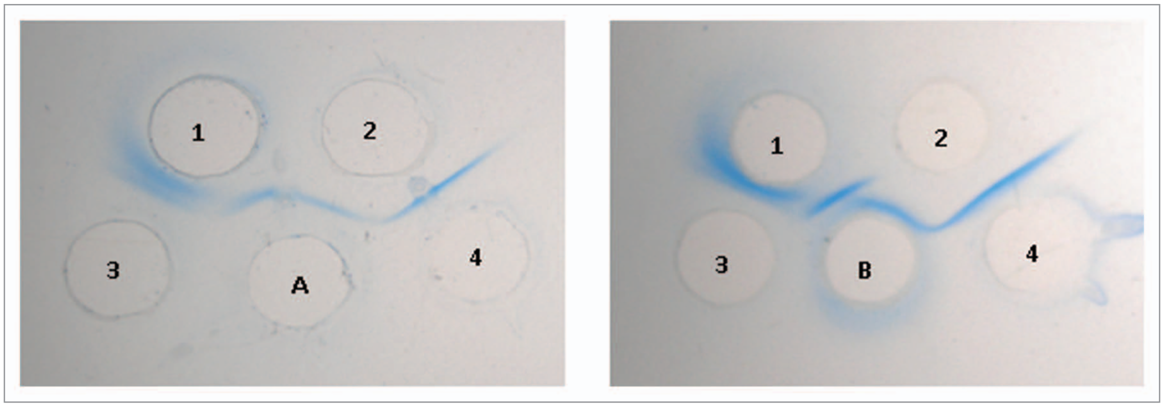

Figure 3. Double Immunodiffusion of conjugates against anti-OSP and anti-DT sera. Wells A and B: *S.* Paratyphi A OSP-AH-DT conjugate and *S.* Paratyphi A OSP-DT conjugate (100 μg each) respectively. Wells 1 and 2: Anti-OSP and anti-DT (15 μl each) respectively. Wells 3 and 4: *S.* Paratyphi A OSP and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

serovar specific OSP of *S.* Paratyphi A is the immunodominant portion of the molecule that extends as a repeating polymer[17] and consists of a trisaccharide backbone (rhamnose, mannose, and galactose), with a branch of paratose from the C-3 mannose (which confers serogroup specificity: factor 2) and glucose from the C-6 of galactose.[7,8] The OSP-DT conjugation most likely occurred via activated hydroxyl groups on the sugar repeat units of the OSP, in contrast, in the binding in the OSP-AH-DT conjugate the ADH most likely was bound to carboxyl groups on the KDO groups on the core polysaccharide region and subsequently the DT was bound to the ADH. The differences in the position of the conjugation on the OSP may explain the differences observed in the anti-OSP response, or it may be due to the distance between the OSP and the DT permitting better access to the antigen sites on the OSP in the conjugate with the spacer. Another possible reason could be a higher free polysaccharide content in the conjugate without the linker (OSP-DT) compared with the one with the linker (OSP-AH-DT). Although free polysaccharide was not directly measured the conjugate OSP-AH-DT was a pool of fractions 59–83 ml from the CL-6B column, free OSP eluted at 85–145 ml, and OSP-DT was a collection of fractions 53–101 ml. The OSP-AH-DT was likely to contain very little free OSP whereas OSP-DT likely contained some free OSP.

The OSP-protein linkage in conjugate vaccines converts T-cell independent polysaccharide antigen into a T-cell dependent antigen, eliciting better immune response with immunological memory. There is experimental evidence that serum antibodies to the OSP of group B and D salmonellae, whether actively induced or passively administered, confer protection to mice.[18-20]

Various conjugation strategies have been reported including recent conjugation chemistry to link the *S.* Paratyphi A O:2 and the carrier protein CRM197, using the terminus 3-deoxy-D-manno-octulosonic acid (KDO), thus leaving the O:2 chain unmodified. These new conjugates were found to have bactericidal activity against *S.* Paratyphi A.[4] *S.* Paratyphi A OSP was also bound to tetanus toxoid (TT) with ADH linker (SPA-TT1) and without ADH (SPA-TT2), the conjugate with the linker was more immunogenic (not statistically significant) than the conjugate without the linker. Only SPA-TT2 was injected into younger children and it did not elicit a booster response in the 2 to 4 y-old group.[8] This is in contrast to the booster response elicited in this age group by pneumococcus type 6B-TT, Vi-rEPA, and *Shigella* ñexneri 2a-rEPA conjugates.[21,22]

Conjugation of *S.* Paratyphi A OSP with diphtheria toxoid (DT) has not been previously reported. We have conjugated OSP of *S.* Paratyphi A with DT and evaluated immunogenicity in mice. Two conjugates of *S.* Paratyphi A OSP were prepared with DT: one using an ADH linker molecule and the other directly linking OSP to DT. The conjugate with the linker showed a significantly higher anti-OSP response compared with LPS alone whereas the conjugate prepared by directly linking OSP to DT was poorly immunogenic. The results presented here demonstrated that OSP purified from *S.* Paratyphi A can be conjugated to DT carrier protein. The enhanced immune response seen with the OSP-AH-DT conjugate is indicative of a successful conjugation and warrants further development as a vaccine candidate.

Materials and Methods

Purification of polysaccharide antigens

An isolate of *S.* Paratyphi A from Guangxi Province, China was used for fermentation and was obtained from the collection held at the International Vaccine Institute (IVI). The seed inoculum was added as 6% (volume/volume) to each of the two 10 L fermentors containing 8 L of TSB (tryptic soya broth Merck, Cat. # 105459). The fermentation was controlled at a temperature of 32 °C with 30% dissolved oxygen and pH 7. The fermentation process was of 10 h duration. Optical density of the culture was measured every hour at 600 nm. The bacterial cells were killed by addition of formalin to a final concentration of 1% and kept stirring at 200 rpm overnight. Bacteria were harvested by centrifugation at 7000 g at 4 °C for one hour. The wet cell-pallet was processed further for extraction and purification of LPS by hot-phenol method[23] and freeze-dried. Purified *S.* Paratyphi A LPS were electrophoresed on SDS-PAGE and checked by silver

Table 1. Immunogenicity evaluation of the prepared *S.* Paratyphi A conjugates with DT

Immunogen	Geometric mean of Antibody titers (n = 10)	Standard deviation (SD)	Standard error of means (SEM)	Lower 95% confident limit	Upper 95% confident limit	*P* value when compared with LPS alone
***S.* Paratyphi A LPS alone**	27.70	29.78	9.417	6.398	49.002	N/A
***S.* Paratyphi A OSP-AH-DT Conjugate**	84.11	89.98	28.454	19.747	148.47	0.0446
***S.* Paratyphi A OSP-DT Conjugate**	9.58	11.70	3.700	1.211	17.949	0.1008

Groups of ten mice were used per immunogen and the table shows the comparison of the anti-LPS IgG ELISA titers produced against the *S.* Paratyphi A LPS 2 wk after 3 injections and statistical parameters.

staining.[24] Purification of *S.* Paratyphi A OSP was done by acid hydrolysis as previously described.[23] Briefly, the LPS of *S.* Paratyphi A at 10 mg/ml was made up to 1% glacial acetic acid and incubated in boiling water for 90 min. The pH was bought up to 7.0 using 1 N sodium hydroxide and the mixture was then ultracentrifuged at 96000 g for 5 h at 4 °C after addition of a drop of 1 M calcium chloride. The supernatant containing the OSP was dialyzed against purified water and freeze-dried. The OSP was then made up to 10 mg/ml in purified water and applied to chromatography column (2.6 × 70 cm) using Sephadex G-75 media against purified water. Fractions were collected and checked for the presence of polysaccharides (by Anthrone assay[25]) and antigenicity (by immuno-diffusion assay[26]). The high molecular weight fractions of OSP were pooled and freeze-dried for later preparation of *S.* Paratyphi A OSP conjugates.[7] The AKTA Basic FPLC chromatographic system (GE Healthcare) was used during all chromatography experiments with automated fractionation and attached UV and refractive index (RI) detection systems. Nucleic acid contamination was 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,[27] was used to determine endotoxin levels in *S.* Paratyphi A LPS and OSP.

Derivatization of *S.* Paratyphi A OSP

The OSP was dissolved in MES buffer as 10 mg/ml. The ADH and EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydro-chloride) were added as powder to the OSP solution to make final concentrations of 500 mM and 100 mM respectively. After 30 min reaction time, an additional amount of EDC was added to make a final concentration of 200 mM. Reaction was allowed to continue at room temperature for two hours with pH maintained at 5.0 to 6.0. The reaction mixture was then dialyzed against saline overnight. The dialysate was applied to the chromatography column Sephardex G-25 (1.6 × 50 cm) for desalting and the void volume fractions were harvested and freeze-dried and designated OSP-AH. The trinitrobenzene sulfonic acid (TNBS) assay[28] was performed to measure the hydrazide group and Hestrin assay[29] was performed on OSP and OSP-AH to determine the O-acetyl concentration (C-3 of rhamnose in OSP is partially O-acetylated[30]) as it is essential for the immunogenicity of *S.* Paratyphi A OSP.[7]

Conjugation of *S.* Paratyphi A OSP-AH to DT

Derivatized OSP was conjugated to DT as previously reported.[7] Briefly the OSP-AH was dissolved in saline at 20 mg/ml. The solution was put in an ice bath and EDC powder added slowly to a final concentration of 15mM. The DT solution was added into the reaction mixture slowly to make the final ratio OSP-AH /DT = 1/1 (wt/wt). The reaction mixture was stirred on ice for 4 h with pH maintained between 5.0 and 5.5, followed by then dialysis against saline overnight at 4 °C using 6~8 K_d cut off membrane. The dialyzed conjugate mix was run through a chromatography column containing Sepharose CL-6B (1.6 × 84 cm) and eluted with saline. Anthrone assay, Coomassie protein assay, and immuno-diffusion assay were performed on collected fractions. The void volume fractions, which showed precipitation lines with anti-LPS and anti-DT serum were pooled and designated conjugate OSP-AH-DT.

Conjugation of *S.* Paratyphi A OSP to DT without linker

The OSP was conjugated directly to DT using 1-cyano-4-dimethylaminopyridiniom tetrafluoroborate (CDAP, Sigma, Cat. # C2776) activation.[7] The *S.* Paratyphi A OSP was dissolved in saline at 20 mg/ml and the pH adjusted between 5.0 to 6.0. The CDAP was added slowly to make the final ratio OSP/ CDAP = 1/1 (wt/wt). After addition the pH was adjusted to 7.5–8.5 using 0.2 M solution of TEA (Tri-ethylamine). After two minutes of reaction, DT was slowly added to make the final ratio OSP/DT = 1/1 (wt/wt). The reaction mixture was stirred at room temperature and the pH maintained at 8.0–8.5 for two hours followed by dialysis against saline overnight at 4 °C. The dialyzed conjugate mixture was then loaded onto the chromatography column Sepharose CL-6B (1.6 × 84 cm) and eluted with saline. Anthrone protein and immuno-diffusion assays were performed on collected fractions. The void volume fractions, which showed precipitation lines with anti-LPS and anti-DT serum, were pooled and designated OSP-DT conjugate.

Preparation of standard hyper immune sera

Formalin inactivated *S.* Paratyphi A cells were diluted in saline to a final OD_{600} of 1.0. These cells were then used to inject 8 wk old female ICR mice[11]; the sera were analyzed for the presence of antibodies against *S.* Paratyphi A LPS using double immuno-diffusion assay.[26]

Mice immunization

Six-week-old female ICR mice (Groups of 10) were injected subcutaneously on day-1, day-28 and day-56 as follows: Group 1 was injected with saline only, group 2 with LPS only, group 3 and 4 with OSP-AH-DT conjugate and OSP-DT conjugate respectively. The dose of polysaccharide in all groups was 2.5 μg per injection and heart bleeding was done after 2 wk of the last injection.

Immunoassay and data analysis

The anti-OSP IgG levels in serum were determined by enzyme-linked immunosorbent assay (ELISA) using Nunc

Maxisorp plates (coated with purified LPS of *S*. Paratyphi A as described previously.[31,32] For calculations of antibody titers, ELISA data were processed with Program ELISA for Windows, Centers for Disease Control and Prevention.[33] Titers of the test sera were determined by interpolation of OD values.[11]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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