

# Protection in a mouse peritonitis model mediated by iron-regulated outer-membrane protein of *Salmonella typhi* coupled to its Vi antigen

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Vi polysaccharide and iron-regulated outer-membrane proteins (IROMPs) were extracted and purified from the standard strain of *Salmonella typhi*, Ty2. These were then conjugated by chemical coupling using the carbodimide method. Vi–IROMP conjugate was tested for its ability to protect against colonization by *S. typhi* in different organs. Mice immunized with 2.5 µg Vi–IROMP conjugate showed the most protection, as the least bacterial colonization of spleen, liver and Peyer's patches was observed. Peritoneal macrophages obtained from conjugate-treated mice phagocytosed bacteria efficiently. Circulating antibodies and the delayed type hypersensitivity response elucidated by mouse foot-pad swelling was significantly higher in conjugate-treated animals. These results clearly demonstrate that an IROMP and polysaccharide conjugate of *S. typhi* prepared from the same strain has the potential to protect animals against challenge.

## INTRODUCTION

Vaccination has long been considered as a cost-effective means of preventing typhoid fever, which is caused by *Salmonella typhi*. In clinical trials, Vi polysaccharide-containing vaccines have been found to provide 60–70% protection (Acharya *et al.*, 1987; Szu *et al.*, 1994; Yang *et al.*, 2001). The usefulness of Vi vaccine is limited by its age-related immunogenicity and its T-cell-dependent properties. Thus, it does not elicit protective levels of antibodies in infants and young children and re-injection at any age does not induce a booster effect (Robbins & Schneerson, 1990; Cadoz, 1998). To improve its immunological properties, Vi polysaccharide has been covalently bound to carrier proteins such as tetanus toxoid (TT) (Szu *et al.*, 1994), B-subunit of heat-labile cholera-like enterotoxin (LT-B) of *Escherichia coli* and the recombinant exoprotein A (r-EPA) of *Pseudomonas aeruginosa* (Kossaczka *et al.*, 1999). The conjugate converted Vi polysaccharide to a T-cell-dependent antigen, providing a better vaccine candidate which was more effective than Vi polysaccharide alone in the above-mentioned studies.

The bacterial envelope of the micro-organism is always exposed to a particular environment. There is now much evidence that the composition and related biological properties of the bacterial surface are largely determined by the growth environment. Besides many factors, iron is one of the essential nutrients for all bacteria. In most enteric organisms,

including *S. typhi*, expression of the iron-uptake system is characterized by the secretion of siderophores and expression of a number of new proteins on the cell surface (Guerinot, 1994; Reissbrodt *et al.*, 1997). These proteins are referred to as iron-regulated outer-membrane proteins (IROMPs). The potential of IROMPs as candidate vaccine components has been reported, as these have been found to be immunogenic and protective against typhoid fever as well as other Gram-negative bacterial infections (Chibber & Bajaj, 1995).

To develop a more effective antigen for protection against *S. typhi*-mediated infections, we attempted to couple the Vi antigen to purified fractions of IROMPs from the same strain.

## METHODS

**Bacterial strain.** *S. typhi* strain Ty2 was used in this study. The strain was obtained from the Central Research Institute, Kasauli, India, and maintained on nutrient agar at 4 °C until the period of study.

**Animals.** BALB/c male mice weighing 20–25 g were obtained from the central animal house, Panjab University, Chandigarh. All the animals were given an antibiotic-free diet (Hindustan Lever, Mumbai) and water *ad libitum*.

**Extraction of Vi polysaccharide.** Vi polysaccharide from *S. typhi* Ty2 was prepared by the cold ethanol extraction method of Gotschlich (1975) (Fig. 1). The production/seed medium was used and prepared according to the modified method of Frantz (1942). Seed culture was prepared by inoculating a single colony of *S. typhi* Ty2 into 100 ml nutrient broth.

**Extraction of outer-membrane proteins (OMPs) and IROMPs.** OMPs and IROMPs were isolated using the method of Filip *et al.* (1973)

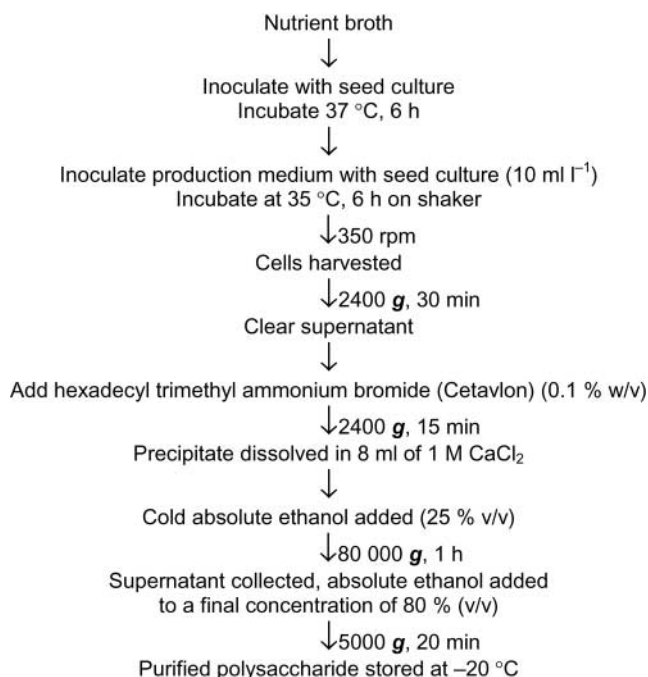
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Abbreviations: DTH, delayed type hypersensitivity; IROMP, iron-regulated outer-membrane protein.

as modified by Williams *et al.* (1983). To create iron-deficient medium, 150  $\mu\text{M}$  2,2'-dipyridyl was used. *S. typhi* Ty2 was grown at 37 °C for 18 h with shaking. The cells were harvested by centrifugation (3500 g, 20 min, 4 °C), washed twice in PBS (pH 7.2) and suspended in 10 ml of 50 mM Tris/HCl (pH 7.8). Cells were broken by sonication ( $3 \times 60$  s pulses) at 4 °C, centrifuged (900 g, 15 min, 4 °C) and the OMPs pelleted by ultracentrifugation (30 000 g, 60 min, 4 °C); OMPs were then suspended in 1 ml of 2 mM Tris/HCl (pH 7.2). The same procedure was followed for isolating IROMPs. The OMPs/IROMPs were further purified on a Sephadex G-150 column and eluted with 0.1 M Tris/HCl (pH 8.0) buffer containing 0.05 % sodium azide. The 3.0 ml fractions were collected and monitored for absorption at 280 nm on a spectrophotometer. The first two peaks obtained were collected separately, pooled and dialysed extensively against distilled water, and the purity and identity were checked by SDS-PAGE.

**Preparation of conjugate.** Chemical coupling of IROMPs to Vi polysaccharide was carried out using the method of Beuvery *et al.* (1982) using [1-ethyl-3-(dimethyl-aminopropyl) carbodiimide] (EDAC). Gel filtration of Vi-IROMP conjugate on a Sephadex G-150 column was performed using 0.1 M Tris/HCl (pH 8.0). The fractions were monitored at 280 nm, pooled and concentrated by acetone precipitation. Precipitates of conjugate were then removed, dissolved in 2 mM Tris/HCl (pH 7.2) and stored at -20 °C.

**Protection studies.** For active immunization studies, mice were divided into three different groups of 12 animals each. The animals in different groups were immunized with 5  $\mu\text{g}$  Vi ml<sup>-1</sup>, 50  $\mu\text{g}$  IROMP ml<sup>-1</sup> or 2.5  $\mu\text{g}$  Vi-IROMP ml<sup>-1</sup> antigens in isotonic saline on day 0. The animals were given a second dose of antigen after 14 days. These immunized animals were challenged intraperitoneally with 10<sup>9</sup> organisms ml<sup>-1</sup> of *S. typhi* Ty2 with 5 % hog gastrin mucin. The surviving mice were sacrificed 7 days post-challenge. In each group an equal number of untreated mice were used as controls. These were given 0.1 ml of untreated saline.



**Fig. 1.** Flow-chart for extraction of Vi polysaccharide.

The number of viable bacteria in the organs was assessed. Organs (Peyer's patches, spleen, liver) were excised aseptically from each animal in different groups, homogenized and serial tenfold dilutions were made in PBS (pH 7.2) under aseptic conditions. These were plated on MacConkey agar, viable counts were determined and results expressed as c.f.u. (g tissue)<sup>-1</sup>.

**Toxicity studies.** Lethal toxicity of each antigen preparation was assessed. The mice were sensitized by injecting 18  $\mu\text{g}$  D-galactosamine intraperitoneally followed within 30 min by intravenous injection of the preparation to be checked (5  $\mu\text{g}$  Vi antigen, 50  $\mu\text{g}$  IROMP, 5  $\mu\text{g}$  Vi-IROMP conjugate).

**Immune response studies.** Phagocytosis studies were performed according to the method of Allen *et al.* (1987). Mice were sacrificed and peritoneal macrophages collected ( $10^7$  cells ml<sup>-1</sup>). A bacterial suspension of the overnight culture was made in saline (pH 7.2) and the count was adjusted to  $1 \times 10^9$  c.f.u. ml<sup>-1</sup>. To measure uptake and killing of bacteria by macrophages, a mixture of 0.5 ml macrophages ( $10^7$  cells ml<sup>-1</sup>), 0.4 ml diluted serum and 0.1 ml bacterial suspension ( $1 \times 10^9$  c.f.u. ml<sup>-1</sup>) was prepared. The mixture was gently vortexed and incubated at 37 °C in a 5 % CO<sub>2</sub> atmosphere. Samples (20  $\mu\text{l}$ ) were taken after 0, 30 and 60 min and suspended in cold RPMI 1640 (2 ml) for the uptake assay. The number of viable bacteria in the supernatant was determined by plating serial dilutions in triplicate on MacConkey agar plates.

Delayed type hypersensitivity (DTH) was checked using the method of Desiderio & Campbell (1985). The mice were divided into different groups: control (untreated saline); immunized (days 0 and 14) with 50  $\mu\text{g}$  IROMP, with 2.5  $\mu\text{g}$  Vi-IROMP conjugate or with 5  $\mu\text{g}$  Vi polysaccharide; immunized-infected, immunized as above and then infected with *S. typhi* Ty2, 7 days after immunization.

Mice were examined for DTH response to *S. typhi* Ty2 with the antigens as the test inoculum on days 7, 20 and 60 after the second dose of immunization. Each animal received 0.025 ml antigen intradermally to the left hind foot pad and the same volume of PBS to the right hind foot pad. Foot-pad thickness was measured with vernier callipers calibrated to 0.01 mm.

The level of IgG antibodies was measured in the serum of different groups of animals by ELISA (Kussi *et al.*, 1979). One hundred microlitres of antigen (2  $\mu\text{l}$ /100  $\mu\text{l}$ ) in bicarbonate buffer was added to each well of a 96-well propylene plate. Plates were incubated at 4 °C overnight and then washed with PBS-I (washing buffer) three times, treated with PBS-II (washing buffer containing 3 % BSA and 0.05 % Tween 20) and incubated at 37 °C for 2 h before washing with PBS-I. Antiserum (100  $\mu\text{l}$ ) was added to each well and the plate incubated at 37 °C for 2 h before being washed three times with PBS-I. Conjugate (100  $\mu\text{l}$ ) was added and the plate was incubated at 37 °C for 1 h, and then washed with PBS-I thrice. Finally, 100  $\mu\text{l}$  substrate mixture (80 mg ortho-phenylene diamine; 0.1 M citric acid phosphate buffer, pH 5.0; 5  $\mu\text{l}$  30 % hydrogen peroxide) was added. The reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> (0.6 %). Absorbance was measured at 490 nm on an ELISA reader (LX-800; Wiprotech). SDS-PAGE was performed according to the standard method of Laemmli (1970).

**Statistical analysis.** Student's *t*-test was applied for statistical evaluation of the results.

## RESULTS

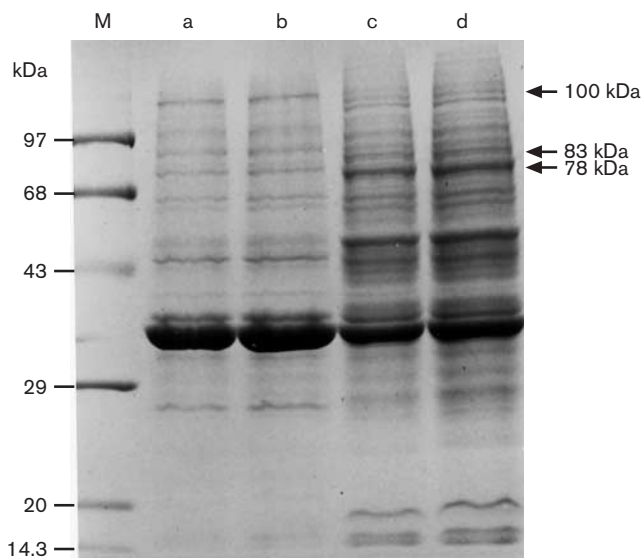
Total OMPs of *S. typhi* Ty2 grown in iron-replete and -depleted media were resolved on a 10 % polyacrylamide SDS gel and stained with Coomassie blue. Three additional proteins were observed in the OMP profile of *S. typhi* Ty2

grown under iron-limiting conditions. The apparent molecular masses of these proteins were 78, 83 and 100 kDa (Fig. 2). Vi polysaccharide was extracted and purified. The chemical composition of the pure preparations was determined. The carbohydrate and protein contents, respectively, of Vi polysaccharide were 46.0 and 0.93  $\mu\text{g ml}^{-1}$ , IROMP 20 and 92  $\mu\text{g ml}^{-1}$  and Vi-IROMP 24 and 29.5  $\mu\text{g ml}^{-1}$ . The protein/polysaccharide ratios (w/w) were 0.002 (Vi polysaccharide), 4.6 (IROMP) and 1.23 (Vi-IROMP).

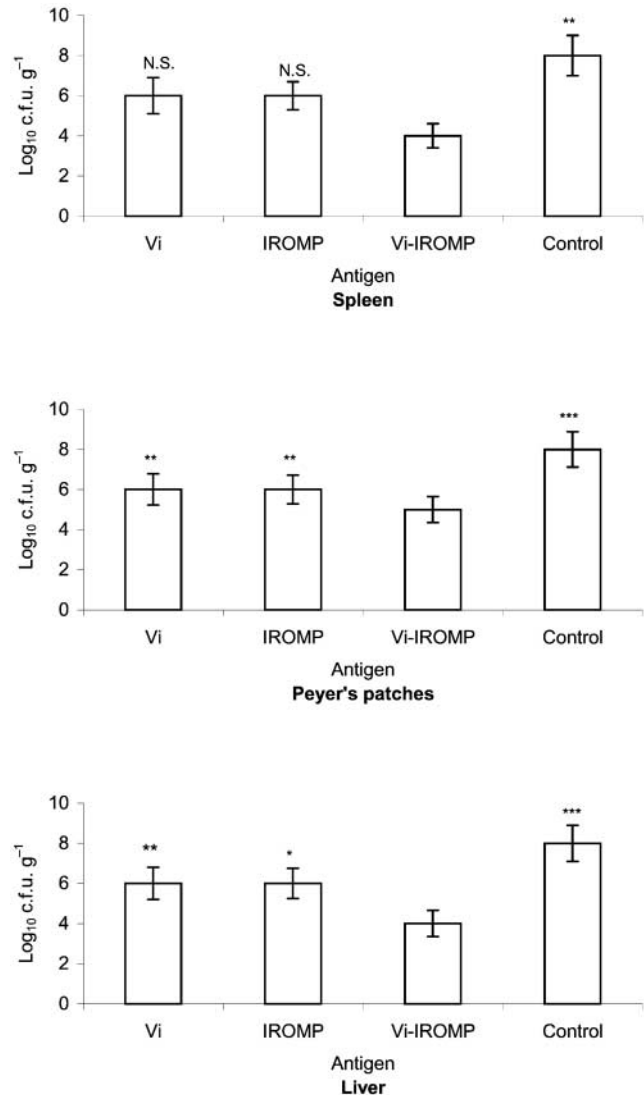
A comparative analysis of the lethal toxicity of Vi, IROMP and Vi-IROMP conjugate showed that Vi-polysaccharide (5  $\mu\text{g}$ ), IROMP (50  $\mu\text{g}$ ), Vi-IROMP (5  $\mu\text{g}$ ) when injected intravenously into mice were non-toxic in control as well as sensitized mice. The quantitative bacterial counts determined in the spleen, Peyer's patches and liver from mice immunized with Vi-IROMP showed a fourfold decrease in the bacterial counts compared with controls. The decrease in bacterial number was almost two log units in conjugate-immunized animals compared with Vi- and IROMP-treated animals (Fig. 3).

The underlying mechanism of protection to *S. typhi* Ty2 infection in terms of phagocytic uptake and killing ability showed that peritoneal macrophages from the Vi-IROMP conjugate-treated animals were remarkably more efficient in engulfing the bacteria compared with those obtained from Vi polysaccharide or IROMP-treated animals at 30 and 60 min (Fig. 4).

Pooled antisera from a group of four animals each treated with Vi, IROMP or Vi-IROMP were screened for the presence of antibodies by ELISA. The highest titre was found for the Vi-IROMP group with a mean ELISA value of



**Fig. 2.** Electrophoretic pattern of OMPs (a, b) and IROMPs (c, d). M, Molecular mass markers in kDa.



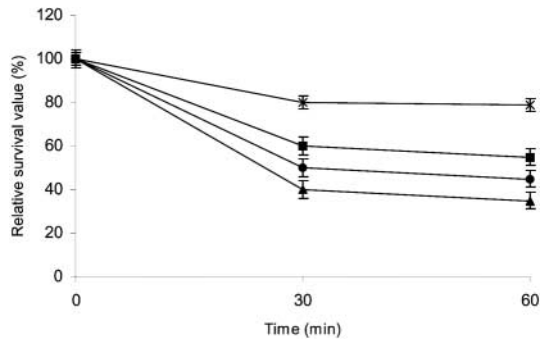
**Fig. 3.** Bacterial count of *S. typhi* in the spleen, Peyer's patches and liver of Vi- (5  $\mu\text{g ml}^{-1}$ ), IROMP- (50  $\mu\text{g ml}^{-1}$ ) and Vi-IROMP- (2.5  $\mu\text{g ml}^{-1}$ ) immunized mice. Significance is compared with the Vi-IROMP group at levels of  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*).

$0.586 \pm 0.14$  (not significant, NS), IROMP  $0.410 \pm 0.15$  (NS) and Vi  $0.39 \pm 0.08$  (NS) compared with Vi group (each ELISA value represents the mean  $\pm$  SD of triplicate samples).

The DTH response indicated by mouse foot-pad swelling was significantly higher in the immunized-infected group of mice at 20 days post-infection and no significant increase in the response was observed at 60 days (Table 1). Vi-IROMP-immunized mice showed the highest DTH response.

## DISCUSSION

Attempts have been made in the past to join the polysaccharide (Vi) moiety of *S. typhi* covalently to different proteins so



**Fig. 4.** Kinetics of uptake of *S. typhi* by peritoneal macrophages in the presence of serum from mice immunized with saline (X), Vi (●), IROMP (■) or Vi-IROMP (▲).

as to increase its immunogenicity and to confer the property of T cell dependence on the antigen (Szu *et al.*, 1994). In recent years, conjugates have been made by chemical coupling of Vi antigen with *P. aeruginosa* recombinant exoprotein A (rEPA) as the carrier (Kossaczka *et al.*, 1999; Lin *et al.*, 2001). Another approach has been to conjugate Vi to porins isolated from the same organism (Paniagua *et al.*, 1992; Singh *et al.*, 1999). In the present study we have conjugated the Vi antigen to IROMPs, as the latter have been shown to be protective in Gram-negative infections (Banerjee-Bhatnagar & Frascch, 1990; Chibber & Bajaj, 1995). These high molecular mass OMPs, in conjunction with Vi polysaccharide, provided protection in the mouse peritonitis model as was evident from bacterial counts obtained in different organs of conjugate-treated animals. This protection was higher than when Vi antigen was used alone. Such an approach would be expected to elicit a better immune response, as these proteins (IROMPs), which are expressed *in vivo* under prevailing iron-limiting conditions,

may account in part for the improved immunogenicity over the polysaccharide alone. Paniagua *et al.* (1992) considered that, with a more relevant antigen as a carrier, the conjugate would elicit antibody responses towards two protective antigens. Since both the antigens in the conjugate are from the same organism, this approach is more meaningful, as is evident from the results of this study.

In an earlier study, a conjugate of Vi bound to porin was shown to induce both systemic and mucosal immune responses, which were protective against challenge with *S. typhi* (Singh *et al.*, 1999). A higher level of anti-Vi IgG on coupling of Vi to rEPA was noticed in 5- to 14-year-old children (Kossaczka *et al.*, 1999). In our study as well, the conjugate was found to elicit both humoral and cell-mediated immune response. The higher antibody levels in mice immunized with conjugate, coupled with efficient phagocytic uptake by peritoneal macrophages and a higher DTH response, might have contributed towards protection against challenge. Antibodies against IROMPs have been shown to interfere with iron uptake, leading to protection of mice against infections (Banerjee-Bhatnagar & Frascch, 1990; Lucier *et al.*, 1996).

The results of this study clearly indicate that Vi antigen bound to IROMPs, both isolated from the same organism, was able to elicit protection in mice. This observation favours coupling of Vi antigen to the high molecular mass IROMP, as both types of antigens are expressed on the cell surface, which is likely to come into contact with the immune system in an infection process.

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**Table 1.** DTH response to antigens in different groups of mice

Significance is compared with the Vi group at levels of significance of  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*), NS, not significant. Each value in the table represents mean  $\pm$  SD of triplicate samples.

Antigen	Time post-infection (days)	Foot-pad swelling (mm)		
		Control	Immunized	Immunized-infected
Vi	7	0.85 $\pm$ 0.25 <sup>NS</sup>	0.89 $\pm$ 0.035 <sup>NS</sup>	0.97 $\pm$ 0.05***
	20	0.91 $\pm$ 0.061 <sup>NS</sup>	1.03 $\pm$ 0.21*	1.12 $\pm$ 0.021***
	60	0.90 $\pm$ 0.145 <sup>NS</sup>	1.09 $\pm$ 0.15**	1.13 $\pm$ 0.020***
IROMP	7	0.80 $\pm$ 0.15 <sup>NS</sup>	0.90 $\pm$ 0.150 <sup>NS</sup>	0.01 $\pm$ 0.20***
	20	0.95 $\pm$ 0.045 <sup>NS</sup>	1.67 $\pm$ 0.25 <sup>NS</sup>	2.75 $\pm$ 0.035***
	60	0.96 $\pm$ 0.01 <sup>NS</sup>	1.59 $\pm$ 0.01***	1.95 $\pm$ 0.07***
Vi-IROMP	7	1.01 $\pm$ 0.025 <sup>NS</sup>	1.01 $\pm$ 0.13 <sup>NS</sup>	1.25 $\pm$ 0.12**
	20	1.15 $\pm$ 0.50 <sup>NS</sup>	1.95 $\pm$ 0.26**	3.55 $\pm$ 0.01***
	60	1.13 $\pm$ 0.015*	1.25 $\pm$ 0.05 <sup>NS</sup>	3.20 $\pm$ 0.02***

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