A challenge study to assess the protective efficacy of typhoid Vi-Polysaccharide-protein conjugate vaccine in laboratory animals

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Abstract

Salmonella typhi, an enteric pathogen causing typhoid fever, is still extremely common in developing parts of the world. Research on developing polysaccharide conjugate vaccines gained interest for its specificity in producing efficient immune responses when compared to native polysaccharide vaccines. A novel Typhoid Vi-capsular-polysaccharide-Tetanus toxoid conjugate vaccine was tested for its protective efficacy when challenged with 20LD50 of live S. typhi cultures (222x10^5.3) to the pre-immunized mice with test and reference vaccines. The ED50 values for test and reference vaccines were calculated as 10.09 and 8.99 respectively. This study has proved that the immunization with the experimental ViPs-TT conjugate vaccine was able to protect mice even at the lowest concentration tested.

Key words: Salmonella typhi, Vi-capsular-polysaccharide, ViPs-TT conjugate vaccine

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Introduction

Vaccines presently available for immunization for typhoid fever are satisfactory, but resulted in eliciting poor immune responses with lack of secondary memory responses in children and particularly in infants below 2 years of age. In contrast, carbohydrate-protein antigens were reported to be effective for the conjugates' capability to recruit T-cells for immune mechanism with cell mediated immune responses. This aspect facilitated in producing high affinity antibodies with secondary memory responses (Lindberg, 1999). Typhoid fever vaccines are assessed in field trials to determine the ability of the vaccine components to induce protection when challenged with virulent S. typhi strains. Many experiments were carried out with S. typhosa strain; approximately 1000 LD50 dose in the presence of gastric mucin was challenged and relative potency of alcohol-killed, alcohol-preserved, heat-killed, phenol-preserved vaccines were studied (Geoffrey et al., 1959). When mice were administered challenged with live bacterium some mice were susceptible, and a group of mice population were resistant against the infection due to the host immune differentiation between the species. Generally, mice challenged with virulent Salmonella cultures are resistant for few days after the challenge administration, due to the innate immune mechanism of the mice. An investigation was carried out to determine the activity of the macrophages during the infection of S. typhimurium. The prime scope of the study was to investigate whether killing of activated macrophages was faster than the resident macrophages (Langermans et al., 1990). The present study was carried to determine the protective efficacy of a newly designed Vi-capsular polysaccharide-Tetanus toxoid conjugate vaccine, including at lowest concentrations of antigen, when challenged with live S. typhi Ty2 culture in mice models. The LD50 for S. typhi cultures were determined by Reed and Muench method and the mice immunized with experimental test vaccine were challenged with live cultures at 20 LD50 concentrations.
Materials and methods

Bacterial strain

The strain S. typhi Ty2 was obtained from National Institute of Child Health and Human development (NICHD), USA. The purity of the strain was confirmed on different selective media such as Xylose Lysine Deoxycholate agar (XLD agar), Bismuth Sulphite Agar (BSA) and Triple Sugar Iron (TSI) agar (Hossain, 2006).

Virulence Testing of S. typhi: Animals

Female mice weighing 17-22 g were divided into four groups, each group containing 10 mice. 10 mice were used as control animals.

Vaccination and challenge protocol

Two-fold dilutions of test (Vi-TT/BB/01) and reference (TYPBAR™) vaccines were injected into pre-labelled groups of mice intra-peritoneally on 0th and 7th days. All the mice were monitored to check the number survived or dead during 15-day period, the mortality ratio and percentage of mortality were calculated.

Statistical analysis

Determination of mean lethal dose (LD₅₀) was calculated (Reed and Muench, 1938).

Table 1. Determination of mean Lethal dose LD₅₀ for S. typhi Ty2 by intra-peritoneal route of administration (NS-Normal saline)

<table>
<thead>
<tr>
<th>Dilutions of S. typhi culture (10⁴-10⁸)</th>
<th>No. of mice per group</th>
<th>Injected Volume (Intraperitoneal)</th>
<th>Number of live and death during 15 days period of observation</th>
<th>Cumulative live mice</th>
<th>Cumulative death in Mice</th>
<th>Mortality ratio</th>
<th>% of mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁴</td>
<td>10</td>
<td>0.25 mL</td>
<td>Live 2  Dead 8</td>
<td>2</td>
<td>18</td>
<td>18/20</td>
<td>90</td>
</tr>
<tr>
<td>10⁵</td>
<td>10</td>
<td>0.25 mL</td>
<td>Live 4  Dead 6</td>
<td>6</td>
<td>10</td>
<td>10/16</td>
<td>62.5</td>
</tr>
<tr>
<td>10⁶</td>
<td>10</td>
<td>0.25 mL</td>
<td>Live 6  Dead 4</td>
<td>12</td>
<td>4</td>
<td>4/16</td>
<td>25</td>
</tr>
<tr>
<td>10⁷</td>
<td>10</td>
<td>0.25 mL</td>
<td>Live 8  Dead 2</td>
<td>20</td>
<td>2</td>
<td>2/20</td>
<td>10</td>
</tr>
<tr>
<td>Control (NS)</td>
<td>10</td>
<td>0.25 mL</td>
<td>Live 10  Dead -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Determination of active mice protection upon immunization of test Vi-capsular polysaccharide – Tetanus toxoid conjugate and reference Vi-capsular polysaccharide vaccines after challenging with suitable dose of S. typhi cultures

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Dilutions</th>
<th>Survivals</th>
<th>Deaths</th>
<th>Cumulative Survivals</th>
<th>Cumulative Deaths</th>
<th>Mortality ratio</th>
<th>% Mortality</th>
<th>ED₅₀</th>
<th>%Proportionate distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Vaccine (Vi-TT)</td>
<td>1/4</td>
<td>8</td>
<td>2</td>
<td>18</td>
<td>2</td>
<td>2/20</td>
<td>10</td>
<td>0.2</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>6</td>
<td>6/16</td>
<td>37.5</td>
<td>0.25</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>12</td>
<td>12/16</td>
<td>75.0</td>
<td>0.25</td>
<td>8.9</td>
</tr>
<tr>
<td>Reference vaccine TYPBAR™</td>
<td>1/4</td>
<td>7</td>
<td>3</td>
<td>16</td>
<td>3</td>
<td>3/19</td>
<td>15.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>7</td>
<td>7/16</td>
<td>43.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>14</td>
<td>14/17</td>
<td>82.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Challenge Culture Preparation

Working organisms of Salmonella typhi Ty2 were grown in soya bean casein digest medium (SCDM) and incubated at 37±1°C for 12 hours. The culture suspension was serially diluted as 10⁴, 10⁵, 10⁶ and 10⁷, using sterile normal saline and 5% of gastric mucin (Sigma). From each dilution 0.25 mL of culture suspension was injected intraperitoneally to all the pre-labelled mice groups. Simultaneously, the number of viable organisms in each dilution was determined by plating the culture on SCDA agar plates. The mice were challenged on 21st day with 20LD₅₀ of S. typhi cultures using the intra-peritoneal route (0.25 mL/mice). All the mice were monitored to check the number survived or dead during 15-day period, the mortality ratio and percentage of mortality were calculated.
Results

The bacterium *S. typhi* was identified based on the following characteristics: glucose positive without gas formation, H2S positive on a XLD Agar, and positive serology with antiserum of Vi capsular polysaccharide. Virulence of *S. typhi* challenge preparations were determined in four mice groups, each containing 10 mice per group, by injecting 0.25mL of *S. typhi* Ty2 bacterium mixed with 5% of mucin (Sigma) intra-peritoneally (Table 1). The dilution 10\(^{-5} \text{ to } 10^{-3}\) (220-250 organisms / 0.25 mL) was found to be lethal were 50% of mouse killed resulting in mortality of 62.5%. All the mice injected with saline as control were found to be alive.

*Calculation of LD\(_{50}\) by Reed and Muench method*

\[
\text{PD} = \text{Proportionate Distance} = \left(\% \text{ of Mortality above } 50\% \right) - 50 \times \log \text{Dilution factor)} \left/ \% \text{ of Mortality above } 50\% \right. - \% \text{ of Mortality below } 50\%
\]

Using the above formula the Proportionate Distance was calculated as below.

\[
\text{PD} = (62.5-50) / (62.5-25) \times 1 = (12.5 / 37.5) = 0.30,
\]

Accordingly 1 LD\(_{50}\) for *S. typhi* Ty2 was calculated as 10\(^{-3}\)/0.25mL.

For assessing protective efficacy, six mice groups, each containing 10 mice per group, were segregated separately for test vaccine (Vi-capsular polysaccharide-Tetanus toxoid conjugate vaccine) and reference vaccine (Vi-capsular-polysaccharide vaccine, TYPBAR\(^\text{TM}\)). Both vaccines were serially diluted in three two-fold dilutions (1/4, 1/8, 1/16) and injected into intra-peritoneally into mice weighing 17–22 g. on days 0 and 7 (Table-2). The lethal dose dilution of *Salmonella* strain in the present study was determined as 10\(^{-3}\) and 20LD\(_{50}\) of the same challenge (4449x10\(^3\)) was injected intra-peritoneally to the pre-immunized mice at 21\(^{st}\) day of the study (0.25 mL). All the mice groups were monitored for and all abnormalities and deaths were recorded. 1/4\(^{th}\) vaccine dilution protected mice up to 5 days after challenging with the bacterium and on days 6 and 7 two mouse each was found to be dead out of 10 resulting in 10% mortality. For the test vaccine dilutions 1/8\(^{th}\) and 1/16\(^{th}\) the percentages of mortality were 37.5% and 75%, respectively. Similarly, for reference vaccine groups TYPBAR\(^\text{TM}\) the deaths at the initial 1/4\(^{th}\) dilution were recorded as three deaths out of 10 resulting in 15.7% of mortality. When compared to the test vaccine the protection levels of the reference was not more significant but minimum difference in protection level was established. The relative potency and ED\(_{50}\) of test and reference vaccines were calculated as 10.09 and 8.99 (Spaun, 1964).

The ED\(_{50}\) values in terms of percentage were approximated by Miller and Tainter (Batson 1949). After challenging to the pre-vaccinated mice the survival rates using proportionate distance (PD) and percentages of ED\(_{50}\) values were calculated separately for test and reference vaccines as mentioned above.

*Calculation of test Vaccine ED\(_{50}\)*

It is interpreted as ‘log reciprocal of higher dilution of vaccine-proportionate distance’.

\[
\text{Antilog of 1.004} = \text{The ED}_{50} \text{ of test Vi-capsular-polysaccharide-Tetanus toxoid conjugate vaccine} = 10.09.
\]

Reference Vaccine ED\(_{50}\)=1.204-0.25 = 0.94.

\[
\text{Antilog of 0.94} = \text{The ED}_{50} \text{ of reference Vi-capsular-polysaccharide vaccine} = 8.99.
\]

In contrast, we found that there was no remarkable difference in protection levels of test and reference vaccines when tested mice models. Both vaccines could offer maximum protection and these results suggested that the test Vi-capsular-polysaccharide-Tetanus toxoid conjugate vaccine was protective and hence the vaccine clearing the way for further testing in humans for immunogenicity assessment.

Discussion

The Vi-capsular polysaccharide of *S. typhi* is both a virulent factor and a protective immunogen against *S. typhi* when used as vaccine component. (Daniels et al., 1989). The Vi capsular polysaccharide of *S. typhi* is a linear homopolymer of poly-a (1-4) GalNAcp variably O-acetylated at the C-3 position (Szu et al., 1991). Capsular polysaccharide of *S. typhi* is potent and highly virulent factor and escapes phagocytosis easily; capsule based antibodies mediated with complement can cause lysis and elimination of the bacterium. As existing vaccines’ failure to induce protective immune responses in children below 2 years of age has contributed the development of polysaccharide-protein
conjugate vaccine (Szu et al., 1994). Many field trials of *Salmonella* vaccines witnessed its efficacy when challenged with different LD$_{50}$ doses. A mouse protection study of with a lyophilized monovalent typhoid vaccine prepared from *S. typhosa* strain 58 was conducted by Department of biological products, Army Medical Centre, Washington, D.C. The scope of the study was to determine the relative effect of graded immunizing and challenge doses in determining the protection of typhoid vaccines (Batson, 1949). Findlay and Bensted measured the lethal dose of *S. typhi* bacilli in response to presence of its Vi antigen. They found 80x10$^6$ as lower lethal LD$_{50}$ dose and for typhoid and para typhoid bacilli is about 8000x10$^6$ organisms (Archer et al., 1957). Several potency tests on acetone killed vaccines of *S. typhi* Ty2 were assessed using the intra-peritoneal route of challenge.

In regard with this, a laboratory test was performed by University of Maryland to assess the protection of *S. typhi* components when vaccinated in B6D2 mice. Acetone killed *S. typhi* Ty2 vaccines (2x10$^8$) with the Vi antigen-free variant O-901, or with *Yersinia* enterocolitica and *Serratia marcescens*, *S. typhi* and *S. marcescens* endotoxin and corresponding lipid A components were investigated. Results of the study suggested that nonspecific inactivation of the intra-peritoneal challenge (i.p) elicited immune response seen in mice vaccinated with other specific typhoid antigens (Carter and Collins, 1997). Laboratory centre for Disease control conducted a study to assess the potency of *Salmonella typhosa* Ty2 vaccines prepared by acetone, alcohol treatment or followed by heat killing. These immunized mice were challenged with *Salmonella typhimurium* hybrids constructed by genetic crosses of *S. typhosa* antigens 9, d, and Vi and *S.typhosa* Hfr donor. Results of the study observed both acetone and alcohol treated typhoid vaccines were more protective when compared to the heat killed and phenol-preserved vaccine (Diena et al., 1973). Cell wall components of *Salmonella typhi* were investigated and revealed the ability of purified porins to induce systemic and mucosal immune responses. To study porin-induced cellular immune responses an experiment was carried out at Departments of Paediatrics and Experimental Medicine and Biotechnology, India. Porin immunized mice were challenged with a lethal dose of 300xLD$_{50}$ of *S.typhi* cultures and induction of systemic and mucosal immune responses assessed from the isolated spleen, lamina propria, macrophages and lymphocytes of infected and control mice. It was found that the porin immunized mice found to elicit high T-cell counts with increased CD4+/CD8+ ratio .Induction of secretory (sIgA) was also found to be increased. The study was evident in inducing cellular and humoral immune responses both at systemic and mucosal levels.

In Contrast, immunogenicities differing in different species of mice were studied with available typhoid vaccines. Protective responses (ED$_{50}$) of acetone-inactivated typhoid vaccine and soluble Vi antigen were tested in (CFW, NIH, and Balb/cAnN) mice and later seven days the mice were challenged with 1,000 organisms of the Ty 2 strain of *S. typhosa* by intraperitoneal and subcutaneous route of challenge. It was found that intraperitoneal immunized mice were better protected when compared to the subcutaneous route immunized mice (Esposito et al., 1969). In designing new generation typhoid conjugate vaccines similar studies could be done to assess the relative potency of the vaccine components. A recent mouse immunogenicity experiment proved the in vivo immune responses of Vi-capsular Polysaccharide-Tetanus conjugate vaccine in reference with an native polysaccharide vaccine (TYPBAR™). Vaccinated sera were tested for IgG antibody titres using indirect ELISA method. Immunogenicity profile proved 100% seroconversion which resulted in complete protection in mice (Venkatesan and Srinivas, 2010). The results of the present study proved that experimental typhoid conjugate vaccine was able to protect even at highest dilution tested (lowest concentrations of antigen) and found to be safe and efficacious. This experimental study helped to decide on testing the vaccine at human clinical trial for its safety and immunogenicity in young children and adults.

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