공학박사 학위논문

Development of Efficient Vi Polysaccharide Conjugate Vaccine and Its Vaccination Strategy for Prevention of Typhoid Fever

장티푸스 예방을 위한 효과적인 Vi 다당체 단백결합 백신의 개발과 접종 방법의 연구

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안소정의 공학박사 학위논문을 인준함

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ABSTRACT

Development of Efficient Vi Polysaccharide Conjugate Vaccine and Its Vaccination Strategy for Prevention of Typhoid Fever

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The incidence of typhoid fever still remains high in infants in many developing countries. Vi capsular polysaccharide vaccine was licensed in 1995 and has provided protection against typhoid fever, however this vaccine is not ideal as it does not induce immunological memory and does not induce an antibody response in infants less than 2 years of age.

In this study, an improved vaccine, where the Vi polysaccharide was conjugated to a diphtheria toxoid (DT) carrier protein, was developed and evaluated for immunogenicity.

One important factor in the Vi-DT conjugate vaccine is the ratio of Vi polysaccharide to DT in the final product. The physical and chemical characteristics of several conjugates were assessed using size exclusive chromatography and the results showed that the size and cross-linking formation of the conjugate increased when more DT was bound to the Vi. The Vi-DT conjugates were also evaluated for immunogenicity in mice and the results showed that highly cross-linked conjugate with the Vi/DT ratio of 0.7 induced a very strong primary anti-Vi response. This

study establishes a correlation between the physico-chemical characteristics of the conjugate and the magnitude of the anti-Vi response.

In some instances with other polysaccharide conjugates vaccine un-conjugated polysaccharide can inhibit the response induced by the conjugate. However, in this study, the presence of un-conjugated Vi polysaccharide, up to 50%, in Vi-DT conjugate vaccine did not negatively affect the anti-Vi response. Conversely, the presence of increasing amounts of un-conjugated Vi, up to 50%, administered with the Vi-DT conjugate resulted in increasingly higher levels of both anti-Vi and anti-DT response.

Pre-exposure to Vi polysaccharide vaccine suppressed the response to subsequent doses of either Vi or Vi-DT conjugate vaccine. This immune suppression by pre-exposure to Vi could be overcome by two doses of Vi-DT conjugate vaccine. Following one dose vaccination of Vi-DT conjugate vaccine the anti-DT IgG response was strong and protracted and continued to rise for 12 weeks.

Therefore the Vi-DT conjugate vaccine developed during this study could be used to vaccinate infants in developing countries. Due to the efficient and high yield of the process developed, this vaccine should be available at affordable prices for public health use in developing countries.

Keyword: Vi polysaccharide, Conjugation, Conjugate vaccine,
Typhoid vaccine, *Salmonella typhi,* Diphtheria toxoid

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Chapter 1

Research Background and Objective

Chapter 1. Research Background and Objective

Typhoid fever continues to be a public health problem in many developing countries and WHO conservatively estimates the global incidence of typhoid fever to be 21 million cases, of which 1-4% end in fatality [1]. To combat the problem there are currently many vaccine manufacturers producing Vi capsular polysaccharide vaccines and one company producing a live oral Ty21a attenuated vaccine for protection against typhoid fever [2-3]. Unfortunately those most at risk from infection, the people living in areas where the disease is endemic, remain unvaccinated and at risk of contracting typhoid fever. Recent studies on disease incidence indicate that typhoid fever has a higher incidence in children less than two years of age than previously thought [4]. To protect infants, it is essential to have a vaccine that is licensed in children less than two years of age currently, none of the internationally licensed typhoid vaccines are registered for use in this age group [5].

Polysaccharide only vaccines are poorly immunogenic in children under two years of age [6]. In order to induce a satisfactory response in this age group it is necessary to convert the response from a T cell independent to T cell dependent response and this can be achieved by conjugating the polysaccharide to a carrier protein [7-9]. Several carrier proteins such as tetanus toxoid, cholera toxin and *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA) have been tried as carrier proteins to produce a Vi conjugate [9-11]. However, most trials of Vi conjugate were in laboratory studies, which could not be adapted to vaccine

manufacture. Therefore, more an efficient carrier protein was required for massive production of Vi conjugate. It is also important to study the physical and chemical properties of a carrier protein for efficient Vi conjugate vaccine and for the further development of other polysaccharide vaccines. Diphtheria toxoid (DT) was chosen for this conjugate development because it is readily available at low cost and it is stable in the range of pH encountered during the conjugation process. As the primary target for this vaccine is people living in typhoid endemic areas, it is therefore critical that the vaccine is affordable given the limited financial resources available to purchase vaccine for use in these impoverished communities. At the same time these people are entitled to receive a high quality, safe and efficacious vaccine.

In order to ensure an affordable high quality vaccine, it is essential to develop a production process that is reliable, reproducible, high yielding and scalable, and the Vi-DT conjugate vaccine product complies with the quality requirements.

In summary, the aims of this research purposes are:

1. To evaluate various factors in the Vi-DT conjugate reaction that correlate with immunogenicity of Vi-DT conjugate vaccine.

2. To establish a correlation between physico-chemical properties of the Vi-DT conjugate and its immunogenicity.

3. To demonstrate an immunogenic influence of un-conjugated Vi in the Vi-DT conjugate vaccine.

4. To evaluate the influence of pre-exposure to Vi polysaccharide vaccine to the subsequent immune response induced by Vi-DT conjugate vaccine.

Chapter 2

Literature Review

Chapter 2. Literature review

2.1. Typhoid fever and typhoid vaccine

Typhoid fever is a life-threatening disease caused by infection with *Salmonella enterica* serovar Typhi (*Salmonella typhi*). This pathogen is human specific and transmitted through food or water contaminated with feces of an infected person. The main symptoms are typically characterized by high fever, diarrhea and abdominal symptoms. Typhoid fever was endemic in Europe and United State in the late 19th and early 20th centuries during the industrial revolution when public water systems were inadequate to control infection. The introduction of sanitation in the form of water purification systems in the early 20th century has led to a steady declined in the incidence of typhoid fever since early 20th. In developed countries the risk of typhoid fever is limited to travel in areas where typhoid is endemic [12-13]. According to a report of the World Health Organization (WHO) [14], in many developing countries, typhoid fever still remains a serious public health problem. It is estimated that 21 million cases and 216,000 deaths occurred in 2000. South-central Asia and south-east Asia were the highest incidence regions of typhoid fever where it is estimated that there are more than 100 deaths per 100,000 cases per year. Moreover, the high incidence of age distribution had a high proportion of cases in the 0-4 year old age group. There are two effective licensed typhoid vaccines to prevent typhoid fever. A live attenuated *Salmonella typhi* Ty21a vaccine and a purified Vi polysaccharide

vaccine are now used worldwide. The live attenuated vaccine is delivered by the oral route. The Ty21a strain, used in this vaccine is non pathogenic, a mutant strain of *Salmonella* Typhi Ty2 and Vivotif® (Berna Biotech) is currently the only licensed live oral typhoid vaccine. Through the oral vaccination route the Ty21a vaccine induced mucosal IgA as well as serum IgG [15]. The Vi polysaccharide vaccine induces an anti-Vi IgG antibody but does not induce a boost response after additional vaccination using Vi vaccine. So the Vi vaccine is a single dose vaccine delivered by the parenteral route. Therefore both licensed vaccines have a poor response in children younger than 2 years of age which is the highest risk group for typhoid fever.

2.2 Development of a conjugate vaccine for infants

It is necessary to develop a new vaccine to overcome the poor immunogenicity of existing typhoid vaccines in children. This limitation can overcome by conjugation of Vi polysaccharide to carrier protein. Covalently binding molecules to a protein carrier to produce a better response to the bound molecule was first established by Landsteiner [16]. In early 1930, this idea was developed further by Avery and Goebel who bound bacterial polysaccharide to a protein to generate an immunogenic response to the polysacchride [17]. Following this early work, *Haemophilus influenza* type b (Hib) capsular polysaccharide (polyribosyl ribitol phosphate, PRP) was covalently bound to diphtheria toxoid and was the first licensed polysaccharide – protein conjugate vaccine back in 1987 and is used to prevent

meningitis and pneumonia in infants [18-20]. At present, four Hib conjugate vaccines are licensed and all use with different carrier proteins bound to Hib polysaccharide (Tetanus toxoid, CRM197 mutant *C. diphtheria* toxin protein, outer membrane protein of N. meningitis and diphtheria toxoid). These Hib conjugate vaccines are included in vaccination programs of many developed and developing countries [21]. *Streptococcus pneumonia* is recognized as having 91 different capsular polysaccharide serotypes up to now [22]. A 14-valent pneumococcal polysaccharide vaccine was licensed in 1977 for use in adults, which was followed by the development of 7-valent pneumococcal conjugate vaccine then subsequently a 13-valent pneumococcal conjugate vaccine for children, less than 2 years of age. Several meningococcal conjugate vaccines were developed and licensed for group C serotype, and tetravalent conjugate vaccines (A, C, Y, W-135) were subsequently developed and licensed up to now [23].

2.3. Conjugation techniques for conjugate vaccines

Basically in the conjugation technique for polysaccharide conjugate vaccine, a critical element is defining the significant attributes of a polysaccharide to generate a conjugate product with a carrier protein. If a polysaccharide or a protein needs to be chemically modified to prepare the materials for a conjugation with detectable compound for conjugation, it is called activation or derivatization. Two methods of derivatization for a polysaccharide antigen, periodate oxidation and cyanylation, are

broadly used [24]. The periodate oxidation method involves breaking C-C bonds on a ring structure of sugars modified to reactive aldehyde groups (C=O). These aldehyde groups conjugate with amino groups on a protein by sodium cyanoborohydride. The cyanylation method creates reactive cyanoester groups on a polysaccharide using CNBr: the cyanoester groups on a polysaccharide have high activity to act with amines to generate an O-alkyl-isourea linkage. This method was used for the development of the first conjugate vaccine, Hib conjugate vaccine [18, 20]. The CNBr was replaced with other cyanylating reagent, CDAP (1-cyano-4-dimethylaminopyridinium tetraflourobarate), which has a higher efficacy than CNBr [25].

In other case, if a polysaccharide contained carboxylates, a carrier protein can be activated with bis-hydrazide-containing reagent. Carboxylate groups on a protein are covalently modified with bis-hydrazide compound creating terminal hydrazide groups (hydrazide derivative) in the presence of a carbodiimide. Finally this derivatized protein is conjugated with a polysaccharide containing carboxyl groups. Adipic acid hydrazide (ADH) is used as bis-hydrazide reagent for this conjugation reaction and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) is widely used as a water soluble carbodiimide in a conjugating a carboxylate and an amine to produce a polysaccharide conjugate vaccine [26-27].

This conjugate method based on ADH an activated protein and EDC mediated conjugation with polysaccharide was used in this study for Vi polysaccharide conjugate vaccine development.

2.4. Vi conjugate vaccine

The concept of the Vi conjugate vaccine is covalently bonding Vi polysaccharide antigen to diphtheria toxoid carrier protein (Figure 2.4.1).

Antigens of *Salmonella typhi* consist of lipopolysaccharide in the cell wall (O antigen), Virulence factor (Vi) capsular polysaccharide surrounding the bacterial cell (Vi antigen) and flagella (H antigen) [28]. Vi polysaccharide consists of a linear homopolymer, ([alpha] 1-4), 2-deoxy-2-N-acetyl galacturonic acid (Fig. 2.4.1), *O*-acetylated at the C-3 position and *N*-acetylated at C-2 position. The *O*-acetylation Vi is associated with the immunogenicity of Vi [29-31]. The carboxyl group on each monomer has the potential to bind to ADH spacer molecules, thus the native Vi has abundant binding sites for chemical conjugation.

In the beginning of the development of the Vi-DT conjugate the Vi purification method yielded Vi with a broad range of molecular weight [18]. The new method developed at International Vaccine Institute (IVI, Korea) produced Vi with a more uniform, narrower range of molecular weight [12], which permitted easy visualization of molecular size changes that occurred after conjugation of the DT to the Vi.

The choice of carrier protein can be influenced by a number of factors including availability, price, chemical characteristics such as stability at certain pH, adjuvant effect and so on. Diphtheria toxoid (DT) was chosen for the Vi-DT conjugate development because it is: an existing vaccine material with defined quality

specifications; readily available at low cost; stable in the range of pH encountered during the conjugation process.

Several other Vi conjugate vaccines are being developed using different carrier proteins including *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA), CRM_{197} the mutant diphtheria toxin and tetanus toxoid [9, 13, 19, 20, 21]. The Vi-rEPA conjugate was tested in 2 to 5 year old Vietnamese children and was shown to be safe and 89% efficacious against typhoid fever for 46 months [22] and safe and immunogenic in infants [23].

Vi-DT Conjugate is prepared in two steps, based on the EDC mediated method as described previously: firstly binding adipic acid dihydrazide (ADH) spacer molecules to diphtheria toxoid (DT) carrier protein then secondly binding varying amounts of this derivatized DT to a Vi polysaccharide purified from *Salmonella enteric* Serovar Typhi.

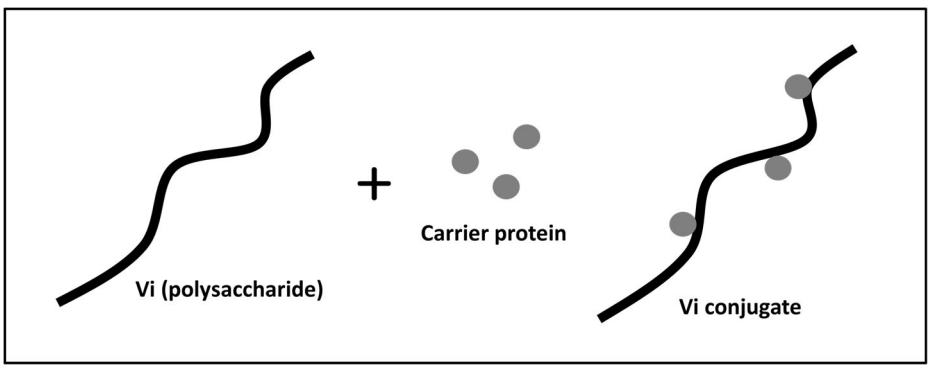


Figure 2.4.1. The concept of a Vi-DT conjugate vaccine

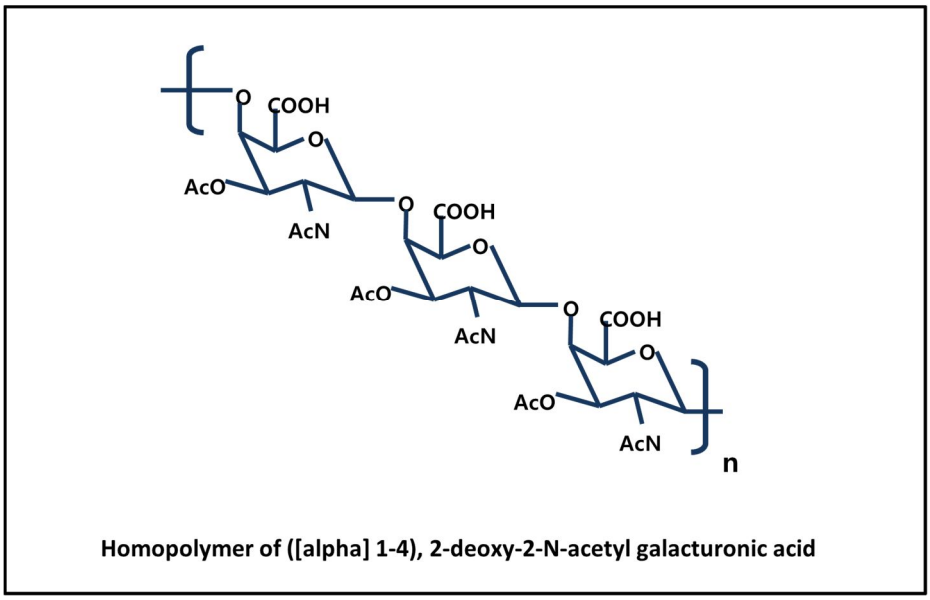


Figure 2.4.2. Structure of Vi polysaccharide

2.5. Immunological properties of the conjugate vaccine

The poor immunogenicity of polysaccharides in infants can be explained by the T-cell independent properties of polysaccharide antigens (Fig. 2.3.1). Immune responses to polysaccharides in children less than 2 years of age are generally poor as they lack immunological material to develop a T cell independent response. Basically polysaccharides cannot be recognized with major histocompatibility complex class II (MHC-II) molecules and thus do not recruit CD4+ T-cell help. Immunological memory is not induced by polysaccharide antigens therefore no boosting effect of the antibody response is generated by revaccination with polysaccharide. Immunoglobulin responses to purified polysaccharides are largely of the IgM and IgG2 isotypes in humans [32-34].

Conjugate vaccines in which polysaccharide is bound to carrier protein induce a T-cell dependent response and immunological memory by recruiting CD4+ T-cell help. The memory cells induced by a conjugate vaccine allow a boosting response following a second vaccination and elicit more anti-polysaccharide IgG than IgM. A conjugate vaccine elicits high anti-polysaccharide IgG, mainly IgG1 which represents switch from IgM and IgG3 which are induced by polysaccharide only vaccines. Most importantly, conjugate vaccines induced strong anti-polysaccharide responses in infants [18, 35-37].

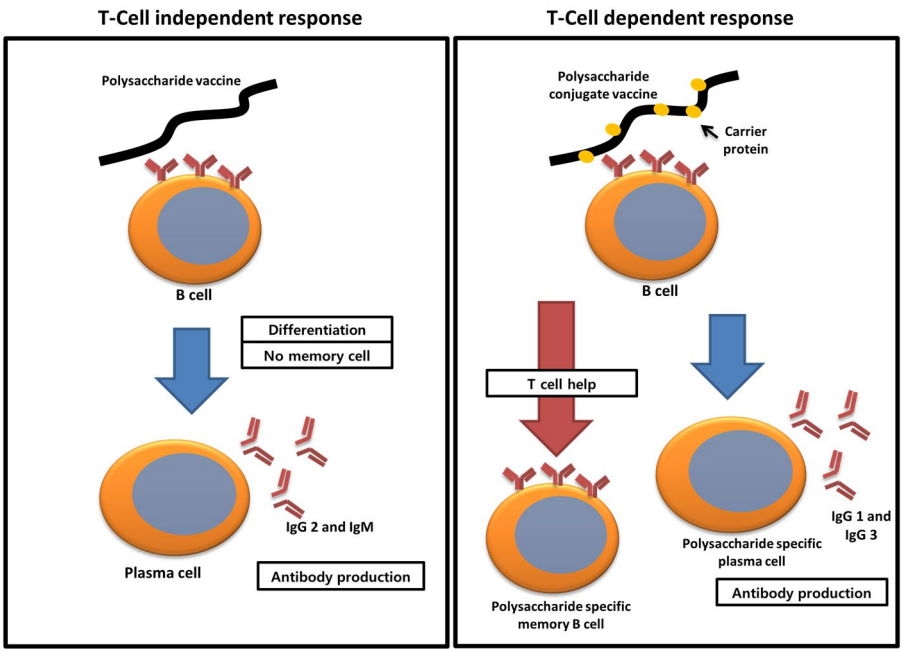


Figure 2.5.1. The acquired immune respone to polysaccharide vaccine and conjugate vaccines.

Chapter 3

Experimental Procedures

Chapter 3. Experimental Procedures

3.1. Purification of Vi polysaccharide

Vi polysaccharide (Vi) was purified from *Salmonella enterica* Typhi isolate number C6524 strain obtained from a patient in Kolkata India by the National Institute of Cholera and Enteric Diseases (NICED). Isolate C6524 was cultivated in a bioreactor to maximize Vi production and inactivated with formalin [38]. Vi was released from the cell to the culture supernatant. Vi was clarified from the culture supernatant using a 0.45μm Hydrosart (Sartorius) cross flow cassette and concentrated and diafiltered against 1M NaCl and concentrated and diafiltrated to change buffer to pure water using a 30 kDa Hydrosart (Sartorius) cassette. Vi is strongly negative charged. Therefore, it could be precipitate by mixing with cetyltrimethlammonium bromide (CTAB, or cetavlon) [38] at a final concentration of 0.5% for 2 hours. This cetavlon treated 1st precipitate was washed with 20% ethanol and increased ethanol concentration to 60% to dissolve the Vi precipitate in the 60% ethanol. 5M NaCl added to make 1M NaCl to the dissolved Vi in 60% ethanol and increase the ethanol concentration to 75%, then the dissolved Vi was precipitated again and settled for overnight. The 2nd precipitate in 75% ethanol was washed with absolute ethanol and dissolved in water. Remaining impurities were removed by ammonium sulfate precipitation then filtered through a 0.2μm filter. Finally remaining ammonium sulfate was removed by 100 kDa hydrosart (Sartorius)

diafiltration and sterilized using 0.2μm Sartopore 2 (Sartorius) [39]. The fermentation of *Salmonella typhi* and the purification of the Vi polysaccharide were performed by Vaccine Development Department of IVI.

3.2. Quality control of Vi polysaccharide

Vi polysaccharide quality was matched to the WHO requirement [40] for Vi polysaccharide typhoid vaccine. WHO requires that purified Vi shall contain not less than 2.0 mmol *O*-acetyl per gram of Vi and shall contain less than 10mg of protein by Lowry assay and less than 20mg of nucleic acid per gram of Vi. The molecular size of purified Vi shall be such that at least 50% of it shall elute from Sepharose CL-4B gel column (GE Healthcare) before a distribution constant (K_D) of 0.25 is reached. Comparison of the elution profiles on Sephacryl S-1000 (GE Healthcare) of the Vi with Dextran 2000 (GE Healthcare) indicated that the molecular size of the Vi was mainly greater than 2000 kDa.

3.3. Preparation of Vi-DT conjugates

Two steps are involved in the conjugation of the DT carrier protein to the Vi polysaccharide: 1). derivatization of the DT and 2). conjugation of the derivatized DT to the Vi polysaccharide (Fig. 3.4.1). The method used was based on that reported by Kossaczka et al [41] and modified as follows.

3.3.1. Derivatization of the diphtheria toxoid as carrier protein

DT was produced by Shantha Biotechnics Hyderabad India. The DT was concentrated to 30 mg/ml and diafiltered against five volume changes of 80 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma) pH 5.6 buffer using a 30kD Hydrosart (Sartorius-Stedim) ultrafiltration membrane. The protein concentration of the diafiltered concentrate was measured by Lowry assay [42]. The DT was derivatized by adding adipic acid dihydrazide (ADH) (Sigma-Aldrich) followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich) so that the final concentration of DT, ADH and EDC were 10 mg/ml, 35 mg/ml and 0.05-4.0 mg/ml respectively. The reaction was allowed to proceed for 60 minutes maintaining pH at 5.6 by addition of 1M HCl. At the end of 60 minutes the reaction was stopped by addition of 1M NaOH to bring the pH to greater than 7.0. Unbound ADH and residual EDC were removed by diafiltration against 15 volume changes of 80 mM MES buffer using a 30 kD Hydrosart ultrafiltration membrane. The ADH concentration measured in the TNBS assay was corrected by subtracting the value obtained for DT only (at the same protein concentration as the derivatized DT) from the value obtained for the derivatized DT.

3.3.2. Conjugation of the diphtheria toxoid to the Vi polysaccharide

The Vi used in this study contained less than 0.02% protein, less than 0.5% nucleic acid, 2.2 mmol *O*-acetyl per g of Vi and was of large molecular weight with

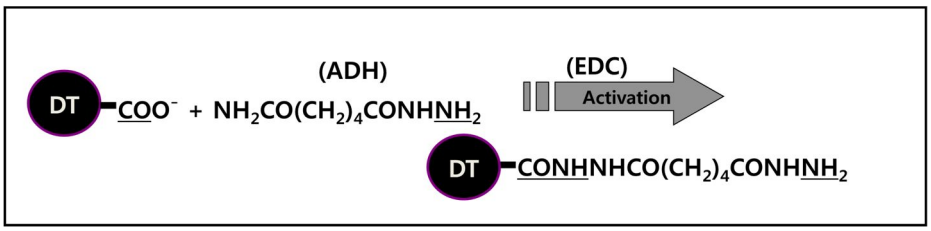
65% of the Vi eluting before K_D of 0.25 on a Sepharose CL-4B column. Thus the Vi was highly pure and met all the WHO specifications for Vi polysaccharide vaccine.

The Vi polysaccharide was concentrated to more than 3 mg/ml and the diafiltration performed against 5 volume changes of 80mM MES buffer. Conjugation of the derivatized DT to the Vi polysaccharide was performed by adding EDC, then derivatized DT to the Vi polysaccharide such that the final concentrations of Vi and EDC in the reaction mixtures were 1.0 mg/ml and 2.0 mg/ml respectively and the final concentration of DT varied depending on the design of the reaction. The reaction was allowed to proceed for 180 minutes and the pH maintained between 5.6 and 5.8 during the reaction.

3.3.3. Diafiltration of Vi-DT conjugates using 300 kDa diafiltration

Unbound DT and residual EDC was removed by diafiltration against 10 volume changes of phosphate buffered saline pH 7.2 using a 300 kDa Polyethersulfone membrane (Sartorius-Stedim). The membrane size, 300 kDa is bigger than the DT molecular size, 64 kDa or dimer size of the DT. Remaining unbound DT in the Vi-DT conjugate product after the diafiltrarion was evaluated by SDS-PAGE and size exclusive chromatography (Sephacryl S-1000).

1) Step 1: derivatization of DT



2) Step 2: conjugation with Vi

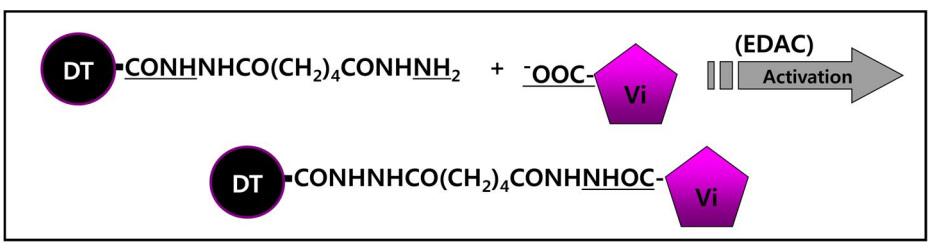


Figure 3.3.1. Two steps of Vi-DT conjugation procedure

3.4. Reagents and assay methods for the Vi-DT conjugation

3.4.1 Reagents for the Vi-DT conjugation

Carboxylate groups on protein were modified with adipic acid dihydrazide (ADH) in the presence of carbodiimide to produce hydrazide derivatives.

Carboxylate groups on Vi polysaccharide were conjugated to the terminal hydrazide of the ADH spacer molecules bound to the DT. To form an amide between carboxylate and amine using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). EDC is a zero-length cross-linking agent capable of activating a carboxylate group for coupling with an amine-containing compound [22].

3.4.2. TNBS assay

The trinitrobenzene sulfonic acid (TNBS) assay was used to measure the hydrazide group or primary amine on the derivatized DT (DT_{AH}). TNBS reacts with an amine containing molecule to form a chromogenic derivative which has a maximum absorbance at 500nm. To compare the DT_{AH} to a standard curve, the value should be multiplied by 2 because the standard of ADH is a divalent hydrazide and the ADH bound to DT has only one hydrazide group [22][43][44].

3.4.3. Hestrin assay

To check Vi concentration of Vi-DT conjugate and Vi in a solution, the Hestrin assay was used. The Hestrin assay is basically colorimetric method in which *O*-acetyl groups mix with hydroxylamine in alkali to show a purple-brown color when add Fe^{3+} is added in acidic conditions. The Vi concentration was calculated by *O*-acetyl content on Vi polysaccharide [11].

3.5. Chemical and physical analysis of Vi-DT conjugates

Conjugates were assayed for *O*-acetyl content by Hestrin assay and converted to mg/ml using a Vi standard of known dry weight, protein content by Lowry assay, size by size exclusion chromatography using Sephacryl S-1000 (GE Healthcare). The morphology analysis of the conjugates was performed using filed-emission scanning electronic microscopy (Carl Zeiss) in National Instrumentation Center for Environmental Management (NICEM, Korea). S.G. Baek provided technical support for the images.

3.6. Immunization of mice

Groups of 10, 6 week old female, ICR mice were injected subcutaneously 2 or 3 times at 4week intervals with 2.5 μg of Vi polysaccharide in each of the conjugates in 0.1ml of PBS, Control groups received Vi or DT alone and negative

controls received 0.1 ml of PBS. A hyper-immune mouse serum against *Salmonella typhi* used a standard was prepared with formalin-killed whole *Salmonella typhi* cells. The killed bacteria cells were prepared at OD 1.0 and injected intra peritoneally to 8 weeks old BALB/c mice. The injection plan was 3 times injection a week every other day for 3 weeks with 0.1 ml of the killed cell during the 1st week, 0.15 ml during the 2nd week and 0.2 ml each for the 3rd week.

3.7. Immunogenicity study

3.7.1. Anti-Vi and anti-DT IgG antibody responses to Vi-DT conjugates

Mice were bled by retro-orbital puncture, the blood spun down at 5000 rpm for 30 minutes, and the serum collected for antibody quantification. Anti-Vi and DT antibody levels in mouse sera were assayed by Vi and DT enzyme-linked immunosorbent assays (ELISA). The Vi and DT ELISA methods were adopted and developed based on the method from the laboratory of Dr. John B. Robbins [10, 45]. The ELISA antigen coating condition was validated using Vi produced and purified in IVI. 2 μg/ml of Vi was selected for the Vi ELISA and 5 μg/ml of DT was used for the DT ELISA. The antibody titers were expressed as the geometric mean (GM) of ELISA units. A titer lower than the detectable level of the ELISA was assigned a value of 0.02 EU and the hyper-immune mouse serum pool was assigned a value of 100 EU and used as a standard.

3.7.2. Immunoglobulin subclass analysis

Equal volumes of serum from each individual mouse in each group were pooled and assayed for IgG subclass using a kit "Mouse Monoclonal Antibody Isotyping Reagents" (Sigma-Aldrich). The IgG subclass titers were expressed as the serum dilution giving an OD of 0.5 and a titer lower than the ELISA detectable level was assigned a value of 25. Pooled sera were used due to the limited amount of individual serum from each mouse and the relatively large quantity required in each test.

3.8. Statistical analysis

Comparison of immune responses between the different conjugates and control groups were performed. The Student's t-test, Welch's t-test or Wilcoxon rank sum test was used whether the variance was equal or not or depending on the distribution. The booster effect following repeat dosing of conjugates was analyzed by paired t-test or Wilcoxon matched-pairs signed ranks test depending on the distribution. The threshold of significance was $P < 0.05$ and 95% confidence interval was calculated. Statistical analysis was done with Stata software (version 11.0). The method design for the statistical analysis and the statistical analysis were performed by D.R. Kim, statistical analyst of IVI.

Chapter 4

Evaluation of Various Vi-DT Conjugates for Immunogenicity

Chapter 4. Evaluation of Various Vi-DT Conjugates for Immunogenicity

4.1. Evaluation of DT derivatization

4.1.1. Time dependent derivatization of DT and the effect of EDC

During the derivatization reaction using EDC and ADH, hydrazide functional groups are bound to DT to form derivatizated DT. These hydrazide group on the derivatised DT are reacted with carboxylic group on the Vi polysaccharide during the conjugation reaction. So the level of derivatization on DT is a key element of the conjugation reaction. Therefore it is necessary to evaluate conditions for the derivatizarion. First of all the effect of EDC concentration on the derivatization was performed using varying EDC concentrations under a fixed 3.5 mg of ADH amount per mg of DT and monitored the derivatizarion reaction for 120 minutes to check that a steady-state of the reaction of derivatization was achieved (Fig. 4.1.1).

The EDC concentrations in the derivatization reactions were 0.05 mg, 0.1 mg, 0.2 mg and 0.4 mg of EDC per mg of DT. All of the reactions showed that the derivatization was essentially complete after 60 minutes with no or very little ADH being bound after this. The result showed the level of derivatization was dependent on EDC concentration: as the EDC concentration increased so did the amount of ADH bound to the DT. Therefore an EDC concentration of 0.4 mg of EDC per mg of

DT was selected for the derivatization reaction for all subsequent Vi-DT conjugations. Based on molar weight of the DT derivatization, the maximum of derivatization, 6.4 % of derivatization it was assumed that 22 ADH molecules were bound to one molecule of DT, and 1.8 % of derivatization meant that 6 ADH molecules bound to each DT molecule.

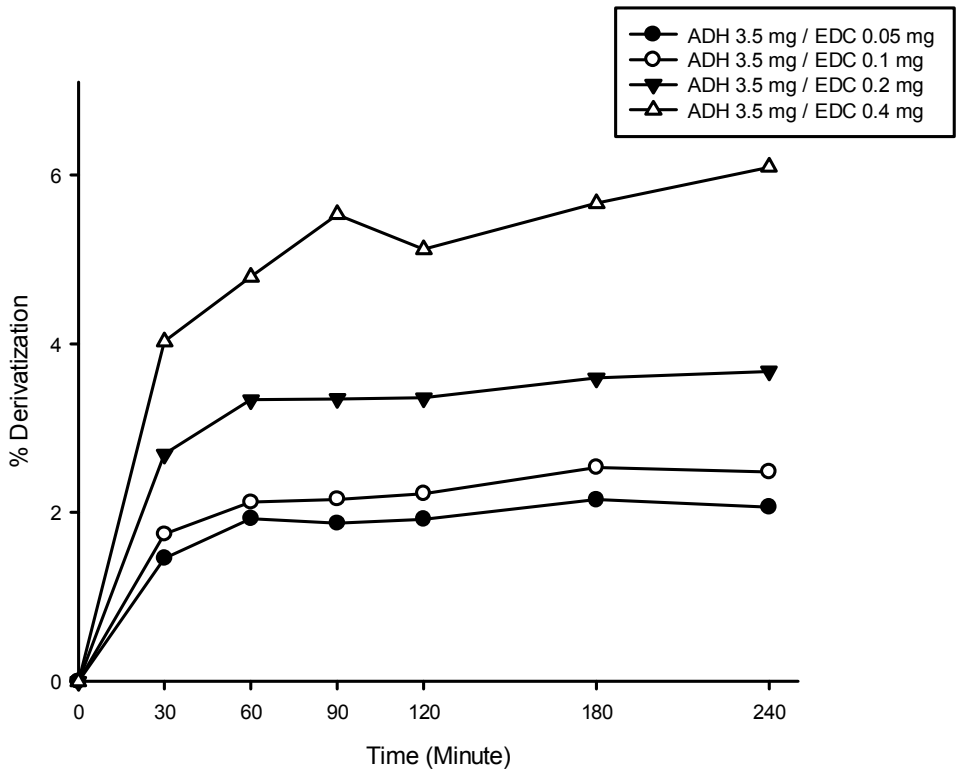


Figure 4.1.1. Degree of binding of ADH to DT (w/w) at varying EDC concentrations.

4.1.2. Influence of pH on the derivatization of DT

EDC has an optimal pH range for reaction from pH 4.7 to 7.5. To investigate the pH dependence of the EDC reaction with DT, derivatization reactions using varying conditions of pH from 5 to 7 were performed. The derivatization levels were decreased with increasing pH.

The size of derivatized DT using different pH conditions was analyzed using Superdex^{TM} 200 10/300GL (GE healthcare) (Fig. 4.1.3). The derivatized DT at pH 5 and 5.5 showed molecular weights larger than 64 kDa. As the concentration of ADH is in large access, all available carboxyl groups on the DT are saturated. The likely cause of the increase in molecular weight at pH 5.5 and lower is therefore precipitation of the DT as opposed to binding of DT to DT. To avoid precipitation of DT, further derivatization reactions were performed at pH higher than 5.5.

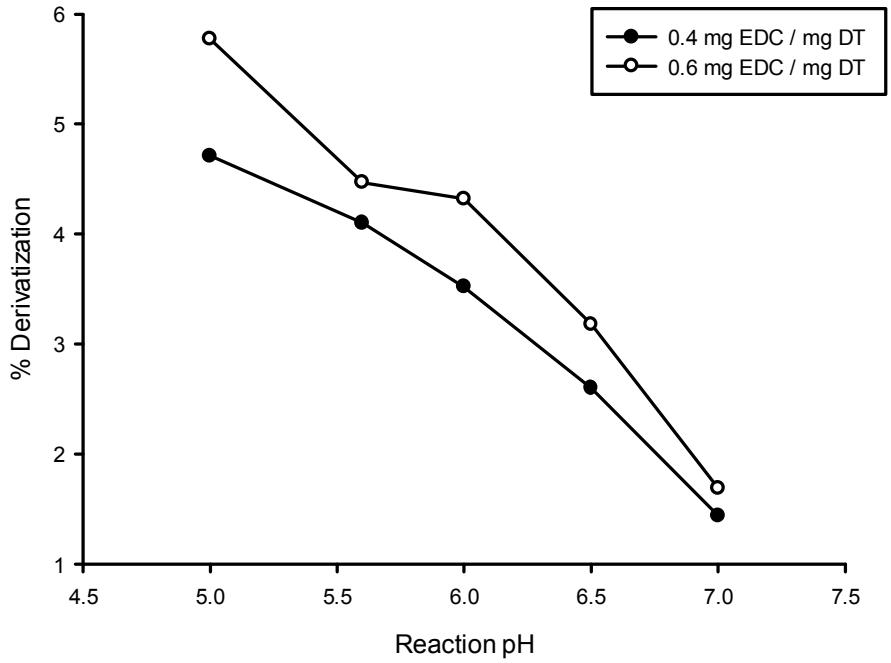
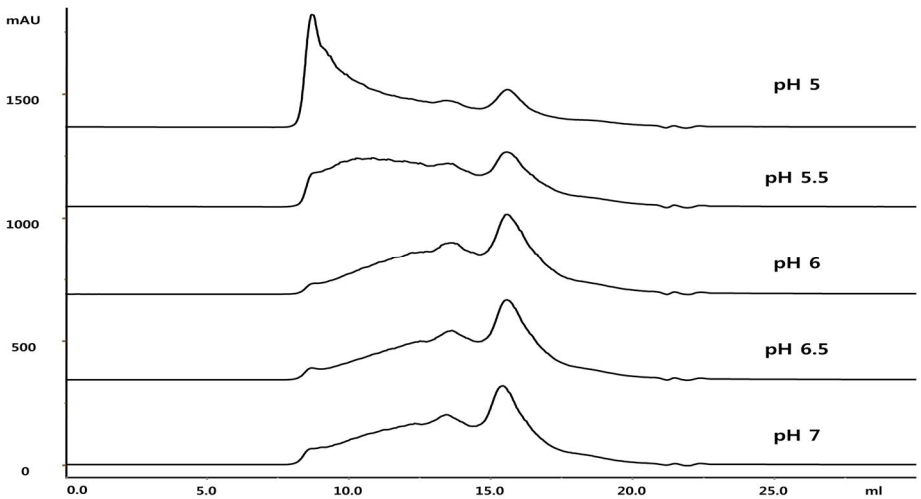


Figure 4.1.2. Derivatization of DT under varying pH conditions.

a) EDC 0.4mg / mg DT



b) EDC 0.6mg / mg DT

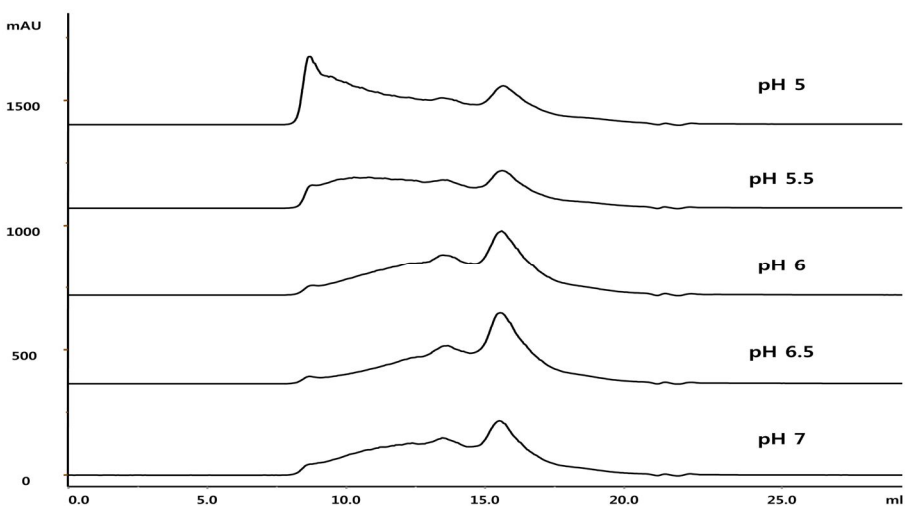


Figure 4.1.3. Size exclusive gel chromatography profiles (Superdex^{TM} 200 10/300GL) of derivatized DT under varying pH condition during derivatization

4.2. Preparation of Vi-DT conjugates using various derivatization levels of derivatized DT and EDC concentrations

Six batches of Vi-DT conjugates were prepared in order to examine an effective level of derivatization on DT and an effective EDC concentration to form the Vi-DT conjugate (Table 4.2.1). After the conjugation, the unbound derivatized DT level in the Vi-DT conjugates was confirmed by size exclusive chromatography column (Sephacryl S1000) (Fig. 4.2.1). The conjugates loaded onto the column contained the same amount of DT. The chromatography profiles and chemical analysis of conjugates indicated that the Vi-DT conjugate made from DT with the low derivation level yielded the least amount of conjugated Vi. In order to remove free Vi and free DT, conjugates were diafiltered on 300 kDa ultrafiltration membrane.

The removal of unbound DT in conjugates was checked by SDS-PAGE (Fig. 4.2.2). The conjugated DT did not enter the PAGE gel, and no unbound DT was present after 300 kDa diafiltration. The result of the conjugated Vi and DT yield showed that both the Vi and DT were higher of a yield of conjugation when the EDC concentration was 10 mM compared with 3.3 mM EDC. A higher amount of DT bound to Vi at higher EDC concentration. The results indicate that the more derivatized the DT was the greater the yield of both Vi and DT in the conjugation. The highest yield of Vi was obtained with the conjugate prepared with 4.8% derivatized DT and the higher concentration of EDC. This derivatization condition

contained 17 units of ADH on one DT molecule. The one ADH unit on the DT was bound to 23 units of Vi. The increased ADH bound on the DT resulted in more tight conjugation with Vi polysaccharide. The results showed that the derivatization level of DT affects the conjugation yield (Table. 4.2.2).

The immunogenicity testing of the Vi-DT conjugates was performed in mice. The anti-Vi IgG titer showed that in this experiment conjugates prepared with the more highly derivatized DT were more immunogenic (Fig. 4.2.3). The highest anti-Vi IgG antibody titer was obtained with the Vi-DT conjugate A1. After the 2^{nd} dose the titer of A1 was higher than B1 and C1: the *P* values were 0.003 and 0.04. Therefore the highest immunogenicity was obtained with a conjugate made with low EDC and the highest derivatization of DT.

Table 4.2.1. Vi-DT conjugates, prepared with various derivatization levels of derivatized DT and EDC

Vi-DT Conjugate Batch	DT Derivatization (%, wt/wt)	Vi:DT Reaction Mixture (mg/ml)	EDC (mM)
A1	4.8	1:1	3.3
A2		1:1	10
B1	3.3	1:1	3.3
B2		1:1	10
C1	2.1	1:1	3.3
C2		1:1	10

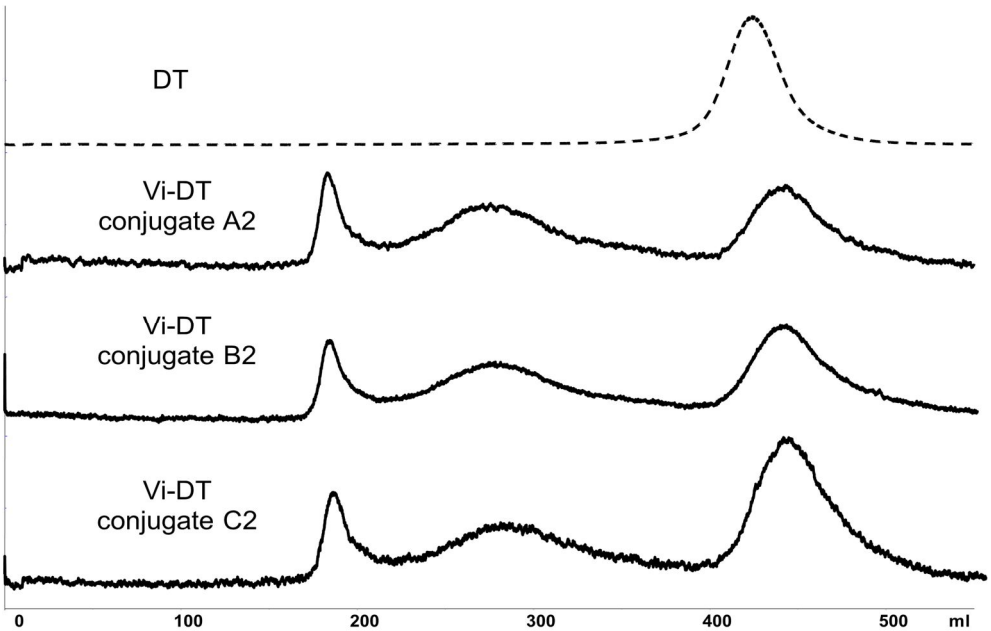


Figure 4.2.1. Size exclusive chromatography of Vi-DT conjugates, for each Vi-DT conjugate the same amount of Vi was loaded onto the column and the results detected at UV wavelength 280nm.

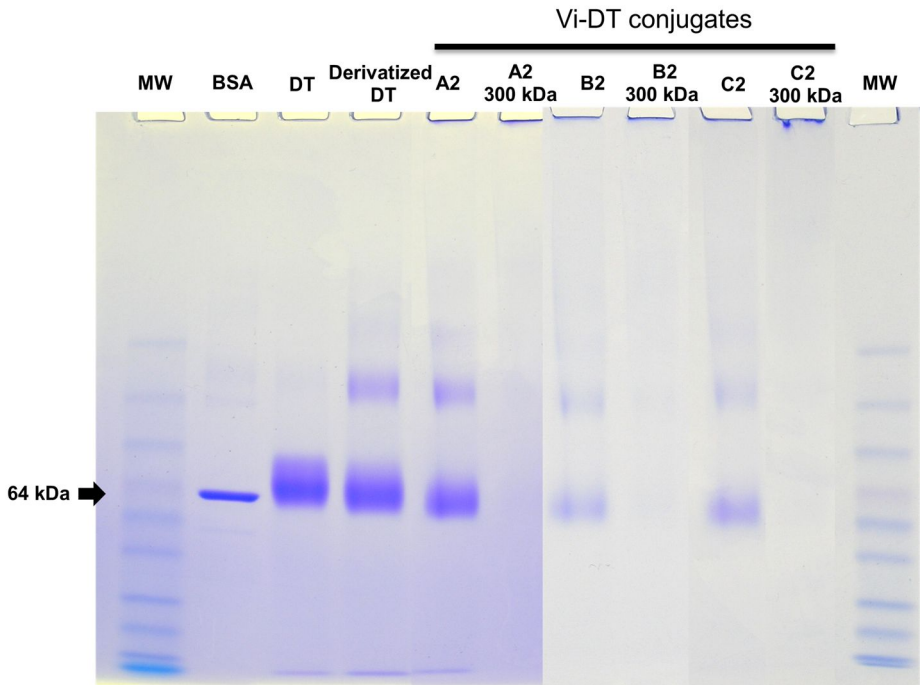


Figure 4.2.2. SDS-PAGE analysis of Vi-DT conjugates, DT and derivatized DT. It has shown the removal of unbound DT. The arrow indicates the DT size before derivatization. MW, Molecular weight.

A2, B2 and C2: Vi-DT conjugate batches before 300 kDa diafiltration

A2 300 kDa, B2 300 kDa and C2 300 kDa : Vi-DT conjugate batches after 300 kDa diafiltration

Table 4.2.2. Yield of conjugated Vi and DT in conjugates after 300 kDa diafiltration.

Conjugate batch	Vi Yield	DT Yield	Vi Monomers* per DT
A1	51%	24%	527
A2	70%	45%	386
B1	49%	16%	760
B2	69%	34%	503
C1	41%	13%	782
C2	62%	38%	405

* Calculation based on the following: DT, *Mr* = 62,000; Vi monomer, *Mr* = 250.

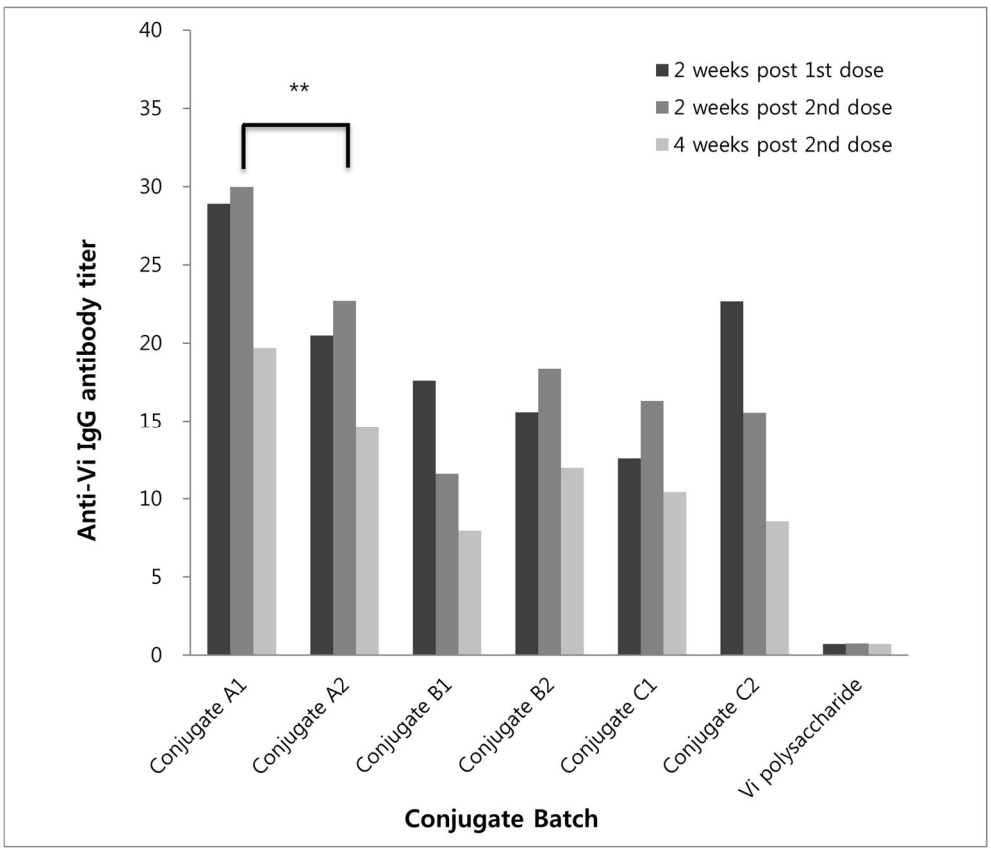


Figure 4.2.3. Anti-Vi IgG titer of conjugates prepared with various level of derivatized DT. Bleeds were taken at 2 (1st) and 6 (2nd) weeks and sera assayed for anti-Vi antibodies. Conjugate A1 versus conjugate B1 after 2nd dose, ** $P < 0.01$.

4.3. Effect of the Vi and DT ratio on the conjugation

To evaluate the importance of the Vi and DT ratio in the conjugation and test the correlation with immunogenicity, six conjugates were made as Table 4.3.1. The EDC concentration used for the conjugates was 3.3 mM which produced more immunogenic conjugates in the previous experiments. The ratios of Vi:DT in conjugation reaction were 0.3, 0.7 and 1.0 mg/ml of derivatized DT with 1 mg/ml of Vi. The Vi/DT ratios in the conjugates after removal of unbound DT were not dependent on the level of derivatization of DT. The Vi/DT ratios of the conjugates were related to the ratio of the Vi and DT in the conjugate reactions.

The immunogenicity of the different conjugates showed a correlation with the ratio and anti-Vi IgG level, the lowest Vi/DT ratio of conjugate induced significantly higher anti-Vi IgG level than higher level of Vi/DT ratios of conjugates after the first dose (Fig. 4.3.1). The anti-Vi levels after the second dose were similar, indicating that the boosting response is less dependent on the Vi:DT ratio.

Two conjugates with different Vi:DT ratios were made using 10 mM of EDC concentration to reconfirm of the correlation between the Vi/DT ratio and the immunogenicity of the Vi-DT conjugates (Table 4.3.2).

The data of size exclusion chromatography showed that the lower ratio of Vi/DT conjugate produced a conjugate of larger size eluting near the void volume of the column (Sephacryl S1000) compared with the higher ratio conjugate. In addition both Vi-DT conjugates contained similar amounts of unbound Vi polysaccharide in

the region from 310 ml to 410 ml which is the Vi polysaccharide standard region. Therefore, the effect of unbound Vi in these conjugates was excluded in the immunogenicity result (Fig. 4.3.2).

The immunogenicity results for the conjugates showed that the lower Vi/DT ratio conjugate and higher amount of DT bound to lower units of repeating Vi induced higher levels of anti-Vi IgG titer than the higher Vi/DT ratio conjugate. In conclusion, the Vi-DT conjugate size affects to the immunogenicity of the conjugates (Fig. 4.3.3), and the larger the conjugate the higher the anti-Vi levels induced.

Table 4.3.1. Different ratios of Vi-DT conjugate

Vi-DT Conjugate Batch	Derivatization of DT (%, wt/wt)	Vi:DT Reaction Mixture (mg/ml)	EDC (mM)	Vi/DT (wt/wt) in Conjugate	Vi Monomers* per DT in Conjugate
D1	4.0	1:0.3	3.3	2.5	620
D2		1:0.7		1.6	397
D3		1:1		1.1	273
E1	3.1	1:0.3	3.3	2.6	645
E2		1:0.7		1.6	397
E3		1:1		1.2	298

* Calculation based on the following: DT, *Mr* = 62,000; Vi monomer, *Mr* = 250.

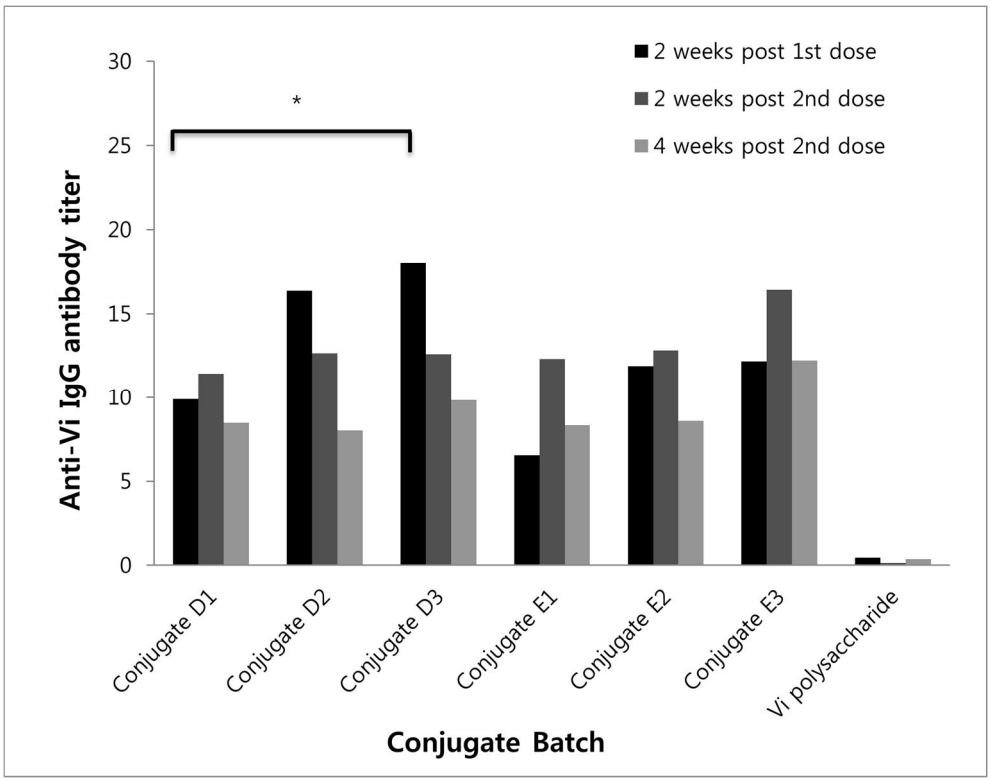


Figure 4.3.1. Anti-Vi IgG titer of Vi-DT conjugates prepared with different ratios of Vi and DT. Bleeds were taken at 2 (1st) and 6 (2nd) weeks and sera assayed for anti-Vi antibodies. Conjugate D1 versus conjugate D3 after 1st dose, * $P < 0.05$.

Table 4.3.2. Vi-DT conjugates prepared with different ratios of Vi and DT

Vi-DT Conjugate Batch	Derivatization of DT (%, wt/wt)	Vi:DT Reaction Mixture (mg/ml)	EDC (mM)	Vi/DT (wt/wt) in Conjugate	Vi Monomers* per DT in Conjugate
F1	**3.8**	**1:0.3**	**10**	**2.9**	**719**
F2		**1:1**		**1.5**	**372**

* Calculation based on the following: DT, *Mr* = 62,000; Vi monomer, *Mr* = 250.

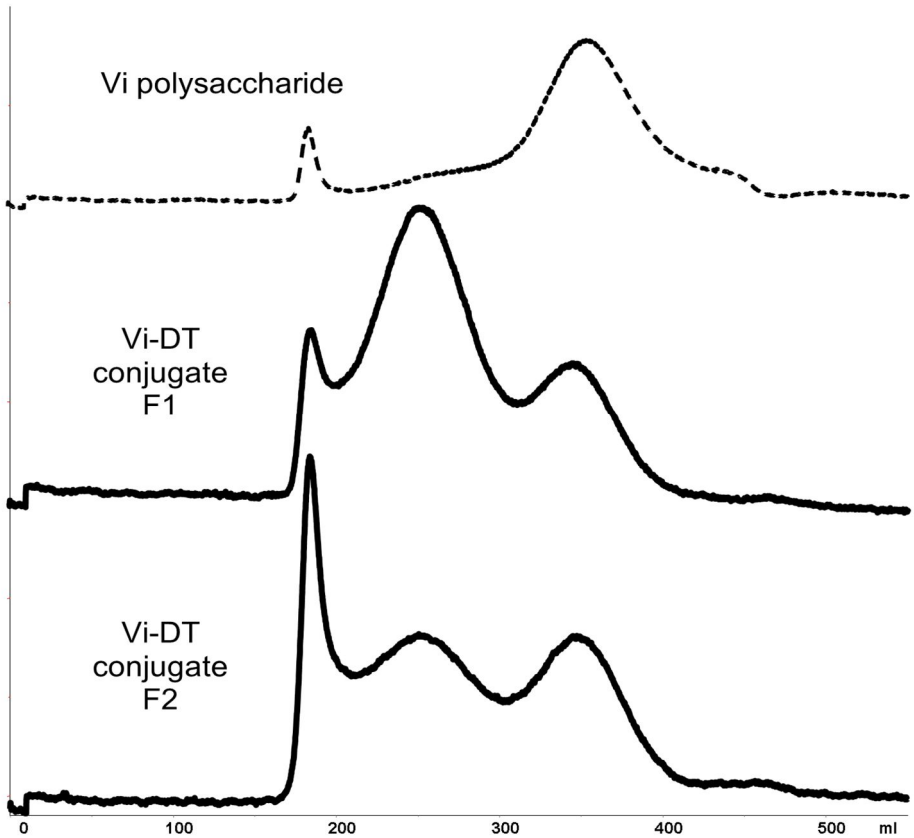


Figure 4.3.2. Size exclusive chromatography profile (Sephacryl S1000) of Vi-DT conjugates batches F1 and F2, the results detected at UV wavelength 206nm.

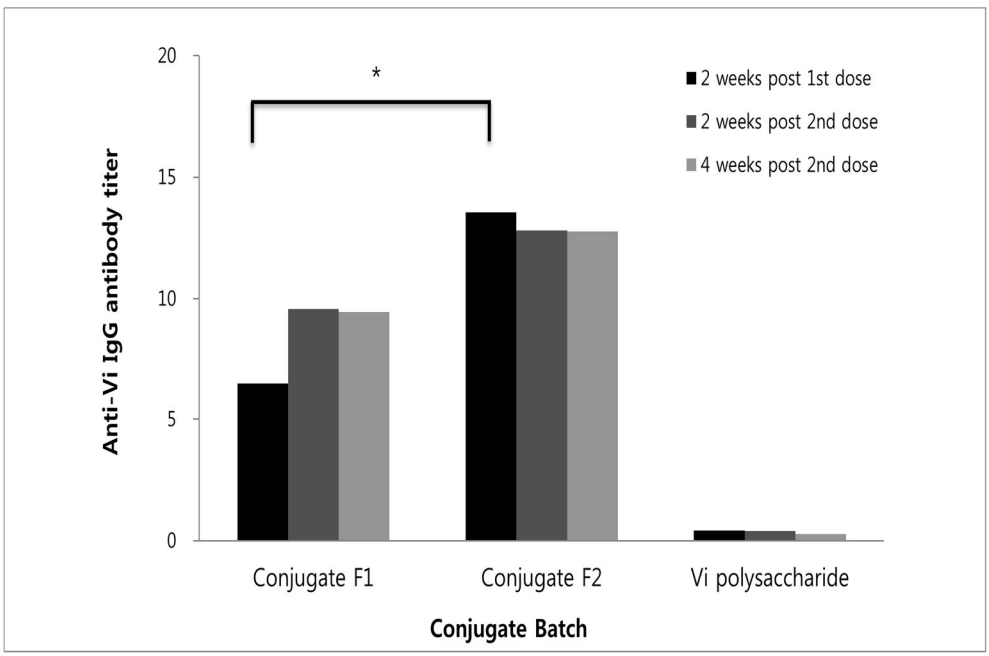


Figure 4.3.3. Anti-Vi IgG titer of Vi-DT conjugates batches F1 and F2

Bleeds were taken at 2 (1st) and 6 (2nd) weeks and sera assayed for anti-Vi antibodies. Conjugate F1 versus conjugate F2 after 1st dose, * $P < 0.05$.

Chapter 5

Physico-Chemical Properties and Immunogenicity of Vi-DT Conjugate Vaccines

Chapter 5. Physico-Chemical Properties and Immunogenicity of Vi-DT Conjugate Vaccines

5.1. Introduction

The results presented previously demonstrate a relationship between the Vi-DT conjugate product size and its immunogenicity. In order to ensure the Vi-DT conjugate vaccine quality, it is important to define the physico-chemical properties of the conjugate. There are two important factors. Firstly an effect of more DT bound to Vi in the Vi-DT conjugate and the immunogenicity of the Vi-DT conjugate. Secondly the increased immunogenicity may be due to increased conjugate size. It may also be due to a critical amount of DT required to stimulate enough T cells to induce an optimal T cell dependent response. The second factor is the size of the Vi-DT conjugate and that is related to the degree of cross-linking. The influence of conjugate size on immunogenicity has not been extensively studied but recently it was shown that larger conjugates of group B Streptococcal Type III were more immunogenic than smaller ones [46]. No attempt to size-reduce the Vi was made in this study but previously it has been shown that the immunogenicity of the Vi whether alone or as a component of a conjugate is related to its molecular size: size-reduced Vi is less immunogenic than native Vi [10]. Therefore this study needs to show that a larger Vi-DT conjugate is more immunogenic or not.

5.2. Preparation of the derivatized DT carrier protein

DT was activated with EDC and derivatized with ADH. The lowry protein assay and the TNBS assay were used to determine the protein content and the hydrazide (AH) content of the derivatized DT respectively. The derivatized DT was found to have a corrected AH : Protein ratio of 2.9% which corresponds to approximately 10 AH molecules per DT molecule. The amount of ADH in the reaction mixture is in gross excess to prevent cross-linking of the DT molecules, only 1% of the starting ADH bound to the DT.

5.3. Conjugation of the derivatized DT to the Vi polysaccharide

A series of conjugates with varying amounts of the derivatized DT (DT_{AH}) bound to a fixed amount of Vi capsular polysaccharide purified from *Salmonella typhi* were prepared. The Vi and EDC concentrations were fixed at 1.0 mg/ml and 2.0 mg/ml respectively and the DT_{AH} concentration ranged from 0.125 mg/ml to 4.0 mg/ml in the reaction mixtures (Table 5.3.1). In all reactions during the 3 hour incubation the pH was stable and there was no need to make adjustments. The reaction mixture containing 4.0 mg/ml DT_{AH} formed an insoluble gel, all other reaction mixtures remained as solutions. The amount of DT_{AH} bound to the Vi was proportional to the amount of DT_{AH} (over the range tested) added to the starting reaction mixture.

The ratio of Vi to DT_{AH} after conjugation ranged from 0.7 to 7.1 and the recovery of Vi in the bulk conjugate preparations ranged from 70 to 80%.

Table 5.2.1. Comparison of Vi-DT conjugates

Conjugate	DT_{AH} conc. in reaction[a] (mg/ml)	Vi recovery %	Ratio Vi/DT (wt/wt)	Vi monomers[b] per DT	ADH %[c]	Vi dose[d] µg	DT dose[d] µg
1	4.0	Insoluble gel	-	-	-	-	-
2	1.5	80	0.7	170	2.7	2.5	3.5
3	1.0	78	0.9	220	2.1	2.5	2.7
4	0.5	72	1.7	420	2.3	2.5	1.5
5	0.25	70	3.0	740	2.2	2.5	0.8
6	0.125	71	7.1	1760	2.4	2.5	0.4

[a] DT_{AH} concentration used in conjugation reaction mixture, the concentration of Vi was 1.0 mg/ml in all reaction mixtures.

[b] Calculation based on DT *M*r =62,000 and Vi monomer *M*r = 250.

[c] Calculation based on DT *M*r =62,000 and ADH monomer *M*r = 174.

[d] The Vi dose and DT dose refer to the doses used in the immunogenicity studies.

5.4. Chemical and physical analysis of Vi-DT conjugate vaccines

Elution profiles of the Vi-DT conjugates on Sephacryl S-1000 gel chromatography (Fig. 5.4.1) showed three regions in the conjugate profiles, region 1 beginning at the void volume (160 ml) to 210 ml, region 2 from approximately 210 ml to 310 ml and region 3 from 310 to 410 ml. The distribution coefficient (*K*av) of region 1, region 2 and region 3 were 0.15, 0.44 and 0.77, respectively. The Vi-only profile shows that the free polysaccharide appears in region 3. The fractions eluted before the *K*av = 0.44 were of higher molecular weight than the Vi and the DT molecules.

Conjugates made with lower DT_{AH} concentrations (0.125 and 0.25 mg/ml) exhibited a profile where most of the absorbing material (UV 206 nm) appeared in region 2. When the DT_{AH} concentration was increased to 0.5 mg/ml there was a substantial increase in the amount of material appearing in region 1. Further increases in the DT_{AH} concentration progressively increased the amount of material in region 1 with consequent decreases in the amount of material in regions 2 and 3. It was not possible to run conjugate 1 on the Sephacryl S-1000 column as it had formed an insoluble gel. The Vi only peak in region 3 shows a uniform distribution of molecular weight and the width of this peak was similar to the peak widths seen in the conjugates made with lower DT_{AH} concentrations (i.e. conjugates 5 and 6). The shift of the Vi peak to region 2 without a change of shape indicates that the DT_{AH}

was evenly distributed on all the Vi molecules. Elution fractions were collected for each of the conjugates and assayed for Vi and protein content, the ratio of Vi to protein was the same in regions 1 and 2 for each conjugate but varied in magnitude from conjugate to conjugate (results not shown). The same Vi to protein ratio in regions 1 and 2, but the larger size of material in region 1, indicates that the material in region 1 was cross-linked. Vi polysaccharide is conjugated to hydrazide group (ADH) on a derivatized DT in the presence of EDC. By calculating of the remaining ADH molecules on a single DT of the conjugates, the cross-linking formations of the conjugates were indicated indirectly. Except for conjugate 2, higher amount of ADH groups were used for conjugate 3 than conjugate 4, 5 and 6. The original ADH on the derivatized DT was 2.9%. 0.8 % of ADH molecules bound to Vi polysaccharide means that three of ten original ADH molecules on the single DT were used for the conjugate 3. In conjugate 6, 0.5 % of ADH was used for the conjugation. Two of ten ADH molecules were used for conjugate 4, 5 and 6. More ADH molecules on a single DT conjugated with Vi represents more Vi bound to a single DT unit which causes cross-linking. In case of conjugate 2, excessive DT loading caused the decreasing usage of ADH groups on the single DT for the conjugation. The cross-linked formulation of the conjugate was evaluated by Scanning Electron Microscope (SEM) (Fig. 5.4.2). The SEM images show that after conjugation the linear structure of Vi polysaccharide was changed to a more mesh-like structure in the conjugate, where conjugate unit are bound to other conjugate units. By varying the concentration of DT used in the conjugation reaction a series of conjugates with

increased size and increased cross-linking was generated.

The derivatized DT used in this study had 10 ADH spacer molecules bound so it too contained multiple binding sites thereby facilitating cross-linking under certain conditions. The Vi/DT ratios in fractions eluted from Sephacryl S-1000 were the same in regions 1 and 2 indicating that the density of DT bound to the Vi was the same in both regions. The molecular size of the material in region 1, however, was greater than that in region 2 indicating that region 1 contained predominantly cross-linked conjugate. Adding increasing amounts of DT to the conjugation reaction increases the likelihood of cross-linking. Minor cross-linking occurred when the Vi/DT ratio was 3.0 or greater but as the ratio dropped to 1.7 the tendency towards cross-linking became more pronounced and at ratios less than 1.0 virtually all of the Vi and DT were presented as cross-linked conjugate.

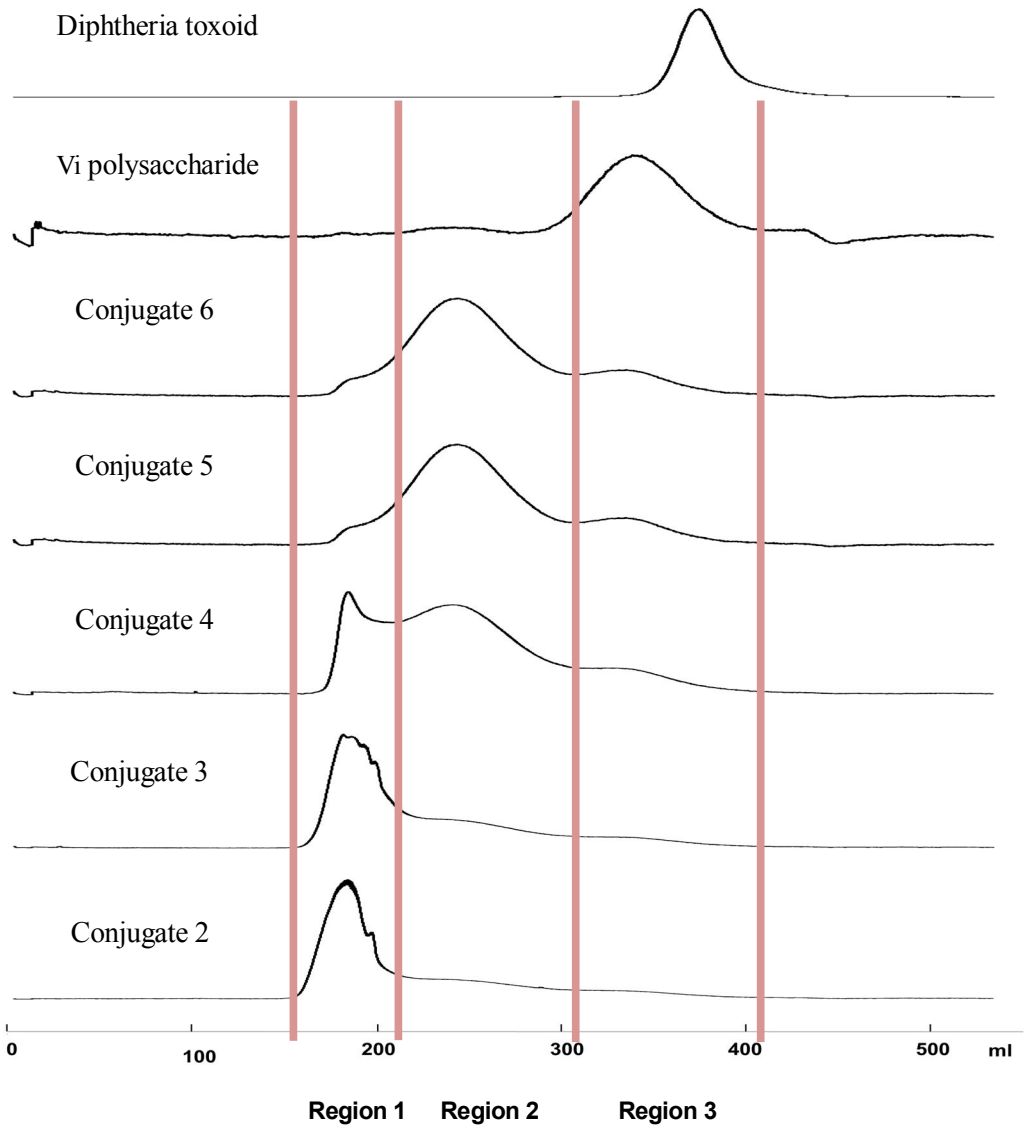


Figure 5.4.1. Sephacryl S-1000 profiles of diphtheria toxoid (Abs at 280 nm), Vi and Vi-DT conjugates (Abs at 206 nm). The void volume was 160 ml and the column volume 460 ml. The profiles of Vi and the conjugates show three distinct regions, region 3 being free Vi (or Vi with low levels of DT bound), region 2 conjugate with low levels or no cross-linking and region 1 cross-linked conjugate.

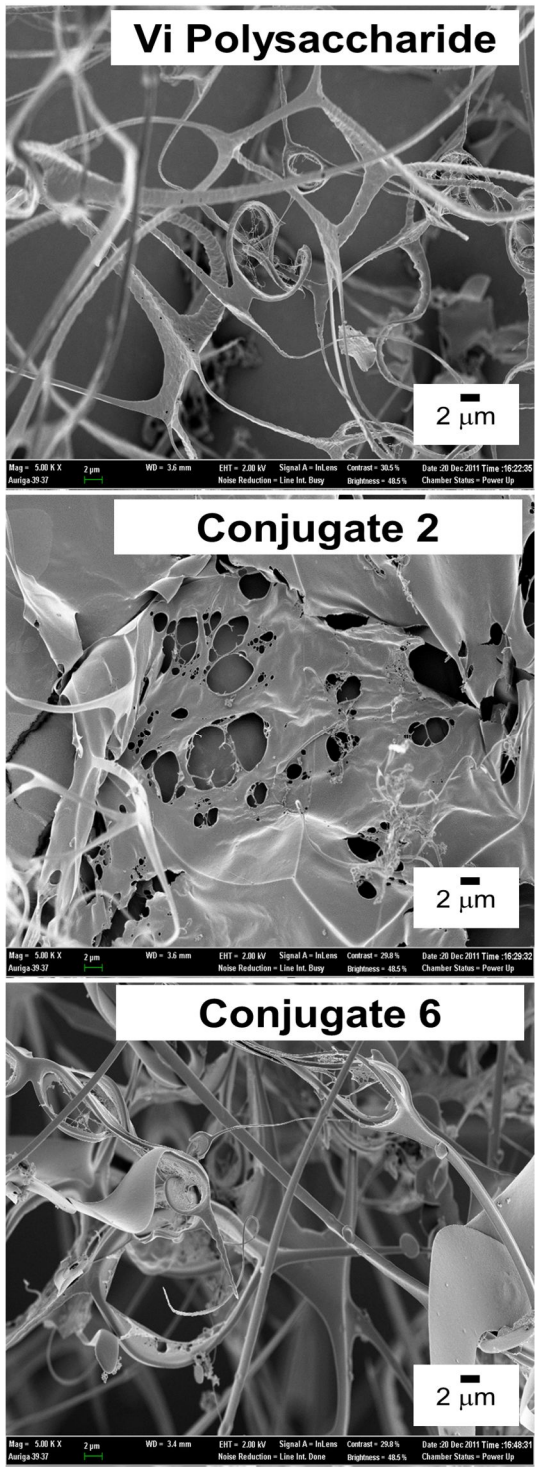


Figure 5.4.2. Morphology of the Vi polysaccharide and Vi-DT conjugates, SEM image.

5.5. Immunogenicity of Vi-DT conjugate vaccines

5.5.1. Antibody response to Vi polysaccharide in the Vi-DT conjugate

Groups of 10 mice were injected with a 2.5 μg dose (based on Vi content) of each of the conjugates or a Vi-only 2.5 μg dose control. Three doses were given at 0, 4, and 20 weeks. The anti-Vi response following each of three doses was measured by ELISA and monitored for a period of 22 weeks and the results are presented in Table 5.5.1. No significant boosting of the antibody response was observed with any of the conjugates after the second dose at week 4, however, all conjugates induced a significant rise after the third dose at week 20. The response to Vi-only was poor and showed no boosting on re-dosing, all conjugates induced a significantly higher response after one and two doses than Vi-only ($P < 0.001$) and one dose of conjugate 2 induced a greater than 50 fold higher anti-Vi titer than Vi-only. Comparing the different conjugates the anti-Vi responses after one and two doses progressively increased in magnitude as the conjugate contained more DT and statistically after 1 dose conjugate 2 induced a significantly higher response than conjugates 4, 5 and 6 (P values 0.008, 0.0005 and 0.0002 respectively) but not significantly higher than conjugate 3 ($P = 0.165$). After three doses the magnitude of the anti-Vi response to conjugate 2 was not significantly different from conjugates 3 and 4 (P values 0.073 and 0.192 respectively) but was significantly higher than conjugates 5 and 6 (P values 0.025 and 0.002 respectively). Hence these anti-Vi response results show that

the conjugates with a Vi/DT ratio of 0.7 and 0.9 induced strong anti-Vi responses after one dose, whereas it took three doses of the conjugates with ratios of 1.7 and 3.0 to achieve a similar level of anti-Vi antibodies.

Table 5.5.1. Anti-Vi IgG immune responses in mice following dosing with Vi and Vi-DT conjugates[a].

Conjugate	Geometric Mean anti-Vi IgG (95% confidence interval)		
	1st injection	2nd injection	3rd injection
2	31.93 (21.57 - 47.26)	26.55 (18.90 – 37.30)	44.51 (30.68 – 64.57)
3	26.75 (22.31 – 32.08)	16.79 (11.16 – 25.25)	31.24 (22.80 – 42.82)
4	14.24 (9.89 – 20.49)	15.00 (10.12 – 22.23)	32.06 (20.26 – 50.76)
5	7.59 (4.86 – 11.86)	10.22 (7.73 – 13.51)	29.06 (22.36 – 37.77)
6	2.05 (1.30 – 3.21)	3.24 (2.05 – 5.12)	12.24 (7.88 – 19.02)
Vi only	0.62 (0.34 – 1.15)	0.42 (0.16 – 1.07)	N.D.
PBS	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.02)

[a] The mice were injected subcutaneously with three doses at 0, 4 and 20 weeks. The results presented above are assays of sera taken 2 weeks after each injection.

5.5.2. Antibody response to DT carrier protein

The dose of DT given with each of the conjugates varied and was dependent on the amount of DT bound to the Vi in each conjugate. Poor responses to the DT carrier protein even after three doses were observed with conjugates 4, 5 and 6 (Table 5.5.2) and this may have been due to the lower dose of the DT in these groups. The difference in DT dose between conjugates 2 and 3 was not large (3.5 μg versus 2.7 μg) and the physical and chemical differences between the two conjugates was not that striking as assessed by Sephacryl S-1000 chromatography and Vi/DT ratio, but the response following all three doses of conjugate 2 was significantly greater than that to conjugate 3. Conjugate 2 induced significantly higher anti-DT titers than conjugates 3, 4, 5 and 6 after all doses. After dose 1 the *P* values were 0.0032, 0.0002, 0.0001 and 0.0001, after dose 2 the *P* values were 0.0015, 0.0004, 0.0001 and 0.0001 and after dose 3 the *P* values were 0.0052, 0.0019, 0.0001 and 0.0001. Boosting of the anti-DT response occurred after each dose of conjugate 2, after 2 doses the anti-DT titer was significantly higher than after 1 dose (*P* value 0.0125) although the statistical analysis showed no significant difference between the titers after the second and third dose (*P* value 0.115).

The highly cross-linked conjugate with the Vi/DT ratio of 0.7 induced a primary anti-DT response which was strongly boosted by a second dose then further boosted by a third dose. The other conjugates produced weak anti-DT responses and this may have been due to the lower dose of DT, however, the difference in DT dose between

conjugate 2 and 3 was not large 3.5 μg compared to 2.7 μg so the greater responses may have also been due to a more favorable presentation of the DT in the more cross-linked conjugate.

The main purpose of the carrier protein in a conjugate vaccine is to induce a T cell dependent response to the polysaccharide but it may be desirable to also induce a response to the carrier protein. The strong anti-DT responses to Vi-DT conjugate 2 may indicate that this conjugate would not require carrier protein priming in order to generate a robust anti-Vi response in humans.

Table 5.5.2. Anti-DT immune responses in mice following dosing with Vi-DT conjugates[a].

Conjugate	Geometric Mean anti-DT IgG (95% confidence interval)		
	1st injection	2nd injection	3rd injection
2	2.35 (0.96 – 5.75)	17.61 (8.47 – 36.61)	38.45 (22.03 – 67.12)
3	0.16 (0.05 – 0.56)	1.25 (0.32 – 4.91)	4.56 (0.91 – 22.92)
4	0.03 (0.02 – 0.07)	0.32 (0.05 – 1.99)	1.79 (0.22 – 14.59)
5	0.02 (0.02 – 0.02)	0.03 (0.02 – 0.06)	0.11 (0.02 – 0.49)
6	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.02)	0.04 (0.01 – 0.14)
PBS	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.02)

[a] Dosing of conjugate was at 0, 4 and 20 weeks. Bleeds from weeks 4, 12 and 22 were assayed and analyzed for DT antibody levels by ELISA.

5.6. Summary

In this study it was demonstrated that the immunogenicity of Vi polysaccharide-diphtheria toxoid conjugates was related to the physical and chemical structure of the conjugate. Conjugates were prepared in two steps, firstly binding adipic acid dihydrazide (ADH) spacer molecules to diphtheria toxoid (DT) carrier protein then secondly binding varying amounts of this derivatized DT to a fixed amount of Vi capsular polysaccharide purified from *Salmonella enteric* Serovar Typhi. As the amount of DT bound to the Vi increased naturally the size of the conjugate increased but also the degree of cross-linking increased. The SEM image showed the cross-linking stricture which bound conjugates with conjugates to produce a larger structure. The immunogenicity of the conjugates was tested in mice and measured by ELISA for anti-Vi and anti-DT IgG responses, and the results revealed a trend that as the amount of DT bound to the Vi increased the anti-Vi responses increased. Testing these conjugates for immunogenicity established a correlation between conjugate size and cross-linking and the magnitude of the anti-Vi and anti-DT responses induced in mice.

The conjugation conditions resulted in a yield of 80% of the Vi antigen in the conjugate. Moreover, there was no un-bound Vi polysaccharide in this conjugate.

Therefore, this study showed a possibility to evaluate an immunogenicity of a Vi-DT conjugate vaccine by its physico-chemical properties. And the conjugation yield can be controlled by the DT concentration in the reaction. The conjugate with the

greatest degree of cross-linking without forming an insoluble gel was chosen as the candidate for the following studies.

Chapter 6

Adjuvant-like Effect of Unbound Vi on Immunogenicity of Vi-DT Conjugate Vaccine

Chapter 6. Adjuvant-like Effect of Unbound Vi on Immunogenicity of Vi-DT Conjugate Vaccines

6.1. Introduction

The presence of un-conjugated polysaccharide in a conjugate vaccine is generally considered to have no benefit to the immune response and in some instances free polysaccharide can inhibit the response induced by the conjugate [47] . The presence of un-conjugated polysaccharide in a conjugate vaccine may affect the anti-polysaccharide response. Un-conjugated polysaccharide in *Streptococcus pneumoniae* polysaccharide-tetanus toxoid conjugates was shown to have a negative influence on the anti-polysaccharide response. The presence of relatively large amounts of un-conjugated type 6B [48] and type 4 [47] polysaccharides in their respective conjugate vaccines decreased the anti-polysaccharide response and in the case of type 4 the presence of low doses of un-conjugated polysaccharide up to 10% did not influence the anti-polysaccharide response. In one study it was concluded that the presence of un-conjugated Vi was not likely to be a problem in a Vi-CRM_{197} conjugate vaccine [48].

The Vi-DT conjugate used in this study was characterized by its high molecular weight and did not contain un-conjugated Vi. To ensure that un-conjugated Vi does not negatively impact on the responses induced by Vi conjugate, the anti-Vi and anti-DT responses to Vi-DT conjugate were evaluated after

immunization of the Vi-DT conjugate mixed with varying quantities of un-conjugated Vi.

6.2. Vi-DT conjugate vaccines containing various amounts of un-conjugated Vi polysaccharide

The preparation of Vi polysaccharide and Vi-DT conjugate vaccines used in this study was described previous chapter and the Vi-DT conjugate used in this study was conjugate 2 from the earlier study.

Various amount of un-conjugated Vi polysaccharide ranging from 10% to 75% of the total Vi content were mixed with the Vi-DT conjugate in PBS. In all preparations the final dose of conjugated Vi was 2.5 μg and the dose of un-conjugated Vi is presented in Table 6.2.1.

Table 6.2.1. Dosing with in mixtures of un-conjugated Vi and conjugate Vi

% un-conjugated Vi	[a]Dose of Vi in mixture (μg)		
	Un-conjugated Vi	Vi in conjugate	Total
10	0.28	2.5	2.78
25	0.83	2.5	3.33
50	2.5	2.5	5.00
75	7.5	2.5	10.0

[a] Mice immunized with conjugate mixed with un-conjugated polysaccharide all received 2.5 μg of conjugated Vi and varying amounts of un-conjugated Vi as defined above.

6.3. Influence of un-conjugated Vi in Vi-DT conjugate vaccine on immunogenicity

The influence of un-conjugated Vi in the conjugate preparation on the immune response to Vi (Table 6.3.1) and to DT (Table 6.3.2) was examined. The anti-Vi responses to the priming doses were similar for the conjugate-only and the conjugate plus 10%, 25%, and 50% un-conjugated polysaccharide groups but significantly lower for the conjugate plus 75% un-conjugated polysaccharide group ($P = 0.004$). The anti-Vi levels induced after the second dose were increasingly higher with increasing un-conjugated Vi up to 50%, however, only the 50% un-conjugated Vi group being significantly higher than the conjugate ($P = 0.013$), at 75% un-conjugated Vi the anti-Vi response was less but not significantly, compared to the conjugate-only group. After the third dose groups receiving conjugate containing 25% and 50% un-conjugated Vi had significantly higher anti-Vi levels than the conjugate-only group ($P = 0.019$ and 0.008, respectively), the 75% un-conjugated Vi group continued to show an inhibited response with a significantly lower anti-Vi titer compared to the conjugate only group ($P = 0.028$). The anti-DT responses displayed similar patterns to the anti-Vi response, the presence of increasing amounts of un-conjugated Vi progressively increased the response to the DT. The anti-DT response to the 50% un-conjugated Vi group was significantly higher than to the conjugate only group for all three doses ($P = 0.007$, 0.008 and 0.005, respectively). The anti-DT response after three doses of the conjugate plus 50% un-conjugated Vi was

higher but not significantly higher than the group receiving three doses of DT. Mice receiving conjugate plus 75% un-conjugated Vi responded with similar anti-DT levels to that of the conjugate only group, so did not enhance nor suppress the response to DT (results not shown).

Table 6.3.1. Anti-Vi IgG immune responses in mice to different % of un-conjugated Vi in the Vi-DT conjugate[a]

% un-conjugated Vi[c]	Geometric Mean anti-Vi IgG (95% Confidence Interval)		
	1st[b]	2nd	3rd
0	27.07 (22.07 – 33.20)	24.14 (19.17 – 30.40)	35.24 (27.03 – 45.94)
10	23.28 (15.22 – 35.59)	28.52 (19.35 – 42.02)	49.42 (30.10 – 81.15)
25	22.99 (15.90 – 33.24)	32.40 (23.15 – 45.34)	53.11 (40.50 – 69.82)
50	29.11 (18.26 – 46.43)	44.14 (28.16 – 69.20)	66.42 (47.06 – 93.73)
75	6.68 (3.57 – 12.51)	12.42 (6.89 – 22.38)	11.69 (5.12 – 26 68)

[a] Six week old female mice were injected subcutaneously and doses were given at 0, 4 and 8 weeks.

[b] Bleeds were taken at 2 (1st), 6 (2nd) and 10 (3rd) weeks and sera assayed for anti-Vi antibodies.

[c] Mice in the four groups below received three doses of Vi-DT conjugate spiked with additional amounts of un-conjugated Vi ranging from 10 to 75%.

Table 6.3.2. Anti-DT IgG immune responses in mice to different % of un-conjugated Vi in the Vi-DT conjugate[a]

% of un-conjugated Vi[c]	Geometric Mean anti-DT IgG (95% Confidence Interval)		
	1st[b]	2nd	3rd
0	1.44 (0.73 – 2.85)	10.20 (5.49 – 18.94)	19.26 (11.33 – 32.75)
10	2.14 (1.07 – 4.28)	16.17 (7.49 – 34.93)	29.55 (15.21 – 57.40)
25	3.87 (1.85 – 8.08)	20.17 (9.80 – 41.50)	32.22 (16.22 – 62.45)
50	6.58 (3.73 – 11.62)	33.16 (18.57 – 59.19)	63.28 (34.80 – 115.07)

[a] Six week old female mice were injected subcutaneously and doses were given at 0, 4 and 8 weeks.

[b] Bleeds were taken at 4 (1st), 8 (2nd) and 12 (3rd) weeks and sera assayed for anti-DT antibodies.

[c] Mice in the four groups below received three doses of Vi-DT conjugate spiked with additional amounts of un-conjugated Vi ranging from 10 to 50%.

6.4. IgG subclass distribution following dosing with of Vi-DT conjugate plus with un-conjugated Vi

Comparing conjugate only with conjugate containing an additional 50% un-conjugated polysaccharide (Table 6.4.1), the IgG subclass distribution was similar following all three doses. The magnitude of the response to all subclasses, however, particularly after the second and third doses was greater in the group receiving conjugate containing 50% un-conjugated Vi. The IgG2a portions of the both groups, the conjugate and the conjugate plus 50% unbound, were similar but the level of IgG2a induced by the conjugate plus 50% was higher than the group of conjugate only. It was comparatively an adjuvant effect by the un-bound Vi in the conjugate vaccine.

Consequently, the presence of up 50% un-bound Vi in the conjugate vaccine showed a adjuvant-like effect and was not inhibitory of the T cell dependent immune response to Vi-DT conjugate.

Table 6.4.1. Subclass composition of anti-Vi IgG antiboby

Dosing regimen[a]	Reciprocal of serum dilution giving an OD of 0.5 [c] (% of sum of subclasses in brackets)				
	Dose [b]	IgG1	IgG2a	IgG2b	IgG3
Vi/Vi/Vi	1st	135 (22)	25 (4)	163 (26)	295 (48)
	2nd	174 (23)	68 (9)	200 (27)	310 (41)
	3rd	163 (23)	79 (11)	220 (30)	260 (36)
C/C/C	1st	16,000 (56)	1,880 (7)	6,200 (18)	4600 (16)
	2nd	24,000 (77)	1,750 (6)	3,100 (10)	2500 (8)
	3rd	30,000 (78)	1,970 (5)	3,850 (10)	2690 (7)
Conjugate plus 50% un-conjugated Vi (3 doses)	1st	25,000 (64)	2,200 (6)	4,700 (12)	7,200 (18)
	2nd	42,000 (76)	2,900 (5)	5,100 (9)	5,000 (9)
	3rd	64,000 (79)	3,800 (5)	7,200 (9)	6,300 (8)

[a] Six week old female mice were injected subcutaneously with three doses of combinations of either Vi-DT conjugate (C) or Vi according to the regimen above, doses were given at 0, 4 and 8 weeks.

[b] The 1st, 2nd and 3rd dose refer to bleeds taken 2 weeks after each dose.

[c] A pool of sera from each group was assayed for levels of IgG subclass by ELISA.

6.5. Summary

In this study the presence of un-conjugated Vi, up to 50%, in the Vi-DT conjugate vaccine enhanced the anti-Vi IgG response and was non-specific in that both the anti Vi and anti DT responses were elevated. Enhanced responses were only obvious after the second and third dose. The response was dose dependent and the more un-conjugated Vi, up to 50%, the greater the anti Vi and DT responses. The anti Vi but not the anti DT response was inhibited in the presence of 75% un-conjugated Vi.

These findings have implications for vaccine quality and a limit for un-conjugated polysaccharide should not exceed 50% and from a vaccine program perspective if the results presented here translate to humans then a Vi conjugate, once it becomes available, should replace Vi polysaccharide vaccines.

Chapter 7

An Efficient Vaccination Strategy for Using Vi-DT Conjugate Vaccines

Chapter 7. An Efficient Vaccination Strategy for Using Vi-DT Conjugate Vaccines

7.1. Introduction

Vaccination with Vi polysaccharide has been shown to protect individuals from typhoid fever but Vi vaccine has a number of limitations. Vi is poorly immunogenic and revaccination does not elicit a booster response [6, 49]. The response to Vi polysaccharide in children under two years of age is poor and consequently Vi vaccines are not licensed for use in this at risk age group. However, interest in preventing typhoid fever by vaccination is increasing and a number of trials investigating the programmatic use of Vi in endemic settings are currently underway and it is anticipated that these initiatives will have significant impact on reduction of typhoid fever in these communities. It is not known, whether pre-exposure to Vi will induce hyporesponsiveness to subsequent doses of either Vi or Vi conjugate vaccine.

Hyporesponsiveness has been demonstrated in humans with other polysaccharides notably meningococcal serogroups A and C [50]. The available limited data suggests that all four meningococcal vaccine serogroup polysaccharides (A, C, W-135 and Y) have the potential to induce hyporesponsiveness at high doses, including in older age groups [51]. Recently it was shown that prior receipt of a 23 valent pneumococcal polysaccharide vaccine caused immune hyporesponsiveness illustrated by a lack of response to all 23 serotypes following re-challenge with the

polysaccharide vaccine [52]. The mechanism of induction of antigen specific hyporesponsiveness is not clear although a number of possibilities have been suggested. One explanation for serotype-specific hyporesponsiveness resulting from pneumococcal carriage is B cell exhaustion due to continuous exposure of high polysaccharide antigen loads [53]. This is not a plausible explanation for the results observed here as mice received low doses of antigen and it is highly unlikely that they were exposed to *S. typhi* infection and therefore chronic exposure to Vi. Another possible explanation for hyporesponsiveness is the induction of regulatory T cells. The antibody response to pneumococcal polysaccharide type III was shown to be controlled by the activity of regulatory suppressor and amplifier T cells [54]. The magnitude of the response to *Helicobacter pylori* was shown to be linked to *H. pylori*-specific regulatory T cells that actively suppress the responses [55]. Hyporesponsiveness following chronic antigen exposure to bacterial, fungal, parasitic and viral infections has been linked to $CD4^+CD25^+$ Treg cells and IL-10 and TGF-β secretion [56][57]. Suppression of the magnitude of the anti-Vi response following exposure to Vi is likely to also be controlled by induction of Treg cells. Vi also suppresses other aspects of the immune response, it was shown that the scarcity of neutrophils in intestinal infiltrates of typhoid fever patients is due to Vi mediated reduction of TLR-dependent IL-8 production in the intestinal mucosa [58].

Human studies have shown that hyporesponsiveness to meningococcal polysaccharide vaccines can also be partially overcome with one dose of conjugate vaccine [59-60].

Previous studies investigating multiple dosing of Vi have produced conflicting results, in one study pre-exposure to Vi vaccine did not induce hyporesponsiveness to a second dose of Vi given 27 to 34 months later [49] whereas later studies with a combined hepatitis A and typhoid fever (Vi) vaccine did generate Vi specific hyporesponsiveness [61].

In the this study in addition to looking at the overall IgG response following each of three doses of various dosing combinations, the IgG subclass composition generated in response to Vi and Vi-DT conjugate was also examined. It is the first to examine hyporesponsiveness to Vi-DT conjugate induced by pre-exposure to Vi polysaccharide.

7.2. Immunization plan

Groups of 10, six week old female ICR mice, were injected subcutaneously using varying dosing regimens as defined in Table 7.3.1. Mice received a total of 3 doses at 0, 4 and 8 weeks, and were bled by retro-orbital puncture at 2, 4, 6, 8, 10 and 12 weeks and serum collected for antibody quantification.

7.3. Anti-Vi responses following different vaccination strategies

The anti-Vi responses following vaccination with various dosing regimens of

conjugate and Vi are presented in Table 7.3.1. Mice receiving a single dose of conjugate developed a strong anti-Vi response peaking two weeks after dosing then declining steadily over the 10 week observation period. Mice receiving three doses of conjugate induced a significant (P = 0.042) rise in anti-Vi levels following the third dose and the titer surpassed that achieved after the priming dose but was not significantly higher (P = 0.148). Conjugate primed mice receiving a second dose of either conjugate or Vi maintained anti-Vi titers at slightly lower levels than achieved after the initial dose of conjugate. Conjugate primed mice that received Vi as the third dose, regardless of whether the second dose was conjugate or Vi, induced a slight but not significant (P > 0.5) rise in anti-Vi levels that did not exceed the levels achieved after the conjugate prime.

Mice receiving a priming dose of conjugate induced a T-cell dependent response and as such stimulated immunological memory. A second and third dose of Vi was able to induce higher anti-Vi levels but to lower levels than that induced by the primary conjugate dose suggesting that Vi is capable of stimulating the existing pool of memory B cells to differentiate into antibody secreting plasma cells but was incapable of stimulating production of new memory cells to replenish the memory B cell pool. This mechanism of memory B cell stimulation without replenishment has been suggested for meningococcal polysaccharide vaccine [62].

Mice receiving a primary dose of Vi responded poorly with geometric mean anti-Vi titers less than 0.4, in comparison, those receiving conjugate responded with GM titers ranging from 27.07 to 32.45 (Table 7.3.1). A second and third dose of Vi

following a first dose of Vi failed to induce an increase in the anti-Vi titers. the poor T-cell independent response seen after vaccination with Vi failed to boost IgG levels beyond the primary dose and the geometric mean titers dropped after each subsequent inoculation of Vi, albeit not statistically significant, suggesting that the Vi may have induced hyporesponsiveness. A single dose of conjugate following either one or two doses of Vi induced a significant booster response (P = 0.004 and 0.009 respectively), however, the magnitude of the response was significantly less than the response obtained after a single dose of conjugate (P = 0.002). Two doses of conjugate following an initial dose of Vi progressively boosted the anti-Vi titer so that after the second dose the titer had risen to levels achieved after a single dose of conjugate. Mice receiving Vi as the first dose, followed by conjugate, then finally Vi, responded to the final Vi dose with anti-Vi titers similar to those achieved after the conjugate (second) dose but was not able to boost the response to levels achieved with a single dose of conjugate.

A second dose of conjugate overcame the suppression induced by Vi resulting in a further boost of anti-Vi levels up to that achieved after a single dose of conjugate in naïve mice.

Table 7.3.1. Anti-Vi IgG immune responses in mice following different dosing regimens with Vi-DT conjugate and Vi.

Dosing regimen[a]	Geometric Mean anti-Vi IgG		
	1st[c]	2nd	3rd
C[b]	32.13	15.23	10.62
C/C/C	27.07	24.14	35.24
C/C/Vi	32.45	22.72	25.98
C/Vi/Vi	28.95	21.92	27.53
Vi/C/Vi	0.39	12.26	15.41
Vi/C/C	0.32	5.97	27.09
Vi/Vi/C	0.27	0.22	4.46
Vi/Vi/Vi	0.39	0.18	0.12
PBS	0.02	0.02	0.02

[a] Six week old female mice were injected subcutaneously with three doses of combinations of either Vi-DT conjugate (C) or Vi according to the regimen above, doses were given at 0, 4 and 8 weeks.

[b] This group received one dose only of conjugate.

[c] Bleeds were taken at 2 (1st), 6 (2nd) and 10 (3rd) weeks and sera assayed for anti-Vi antibodies.

7.4. Anti-DT responses following vaccination with the Vi-DT conjugate

The anti-DT response following one dose of Vi-DT conjugate was characterized by a prolonged rise in titer up to 12 weeks post vaccination after which the titer stabilized. No significant difference was observed in the magnitude of the anti-DT response, at any time point, in mice receiving three doses of conjugate compared with mice receiving a single dose (Table 7.4.1). The primary response to DT of one dose of Vi-DT conjugate was characterized by induction of low anti-DT titers which peaked at four weeks. The anti-DT response following one dose of Vi-DT conjugate was in contrast characterized by a prolonged rise in titer for up to 12 weeks post vaccination after which the titer stabilized. This prolonged duration of stimulation of the immune system may be explained by the slow release of DT as the conjugate cross-linked structure is slowly degraded within the mouse.

Mice inoculated with a first dose of DT responded with a small rise in anti-DT titer, a second dose at four weeks induced an increase in anti-DT titer which peaked two weeks later then declined slightly by four weeks post second dose (two weeks post second injection data not shown). A third dose of DT at eight weeks induced a strong boost peaking four weeks later which then began to decline. The anti-DT response to the conjugate was less than that achieved after DT but the difference was not significant.

Table 7.4.1. Anti-DT IgG immune responses in mice following different dosing regimens with Vi-DT conjugate and DT.

Dosing regimen[a]	Geometric Mean anti-DT IgG		
	1st[c]	2nd	3rd
C[b]	2.88	12.10	16.83
C/C/C	1.44	10.20	19.26
DT/DT/DT	0.30	7.22	40.74
PBS	0.02	0.02	0.02

[a] Six week old female mice were injected subcutaneously with three doses of either Vi-DT conjugate (C), or diphtheria toxoid (DT) as per the regimen above, doses were given at 0, 4 and 8 weeks.

[b] This group received one dose only of conjugate.

[c] Bleeds were taken at 4 (1st), 8 (2nd) and 12 (3rd) weeks and sera assayed for anti-DT antibodies.

7.5. IgG subclass distribution following conjugate or Vi inoculation

Pooled sera from each of the groups of mice were assayed for IgG subclasses distribution and the results are presented in Table 7.5.1. Mice receiving a first dose of conjugate responded with high anti-Vi titers represented by all IgG subclasses with the dominant subclass being IgG1 accounting for 56-78% of the sum total of all the subclasses. Conjugation of Vi to DT results in change in the way the immune system responds to the Vi antigen and one aspect of that change is a subclass switch from a predominantly IgG3 response with Vi to a predominantly IgG1 response with conjugate. In these mice, regardless of whether the second or third dose was conjugate or Vi, the proportion of IgG1 increased and the proportion of IgG2b and IgG3 decreased with subsequent dosing. Mice receiving Vi as the first dose responded with low anti-Vi titers with all four IgG subclasses represented and the dominant subclass being IgG3 accounting for 36-48% of the total in previous test (Table 6.4.1). The proportion of IgG1 and IgG2b were similar and accounted for between 22-30% of the sum total.

Subsequent dosing with conjugate resulted in an increase in the magnitude of all IgG subclasses with a distribution switch similar to that seen after dosing with conjugate and dominance of the IgG1 subclass.

Table 7.5.1. Subclass composition of anti-Vi IgG antibody

Dosing regimen[a]	Reciprocal of serum dilution giving an OD of 0.5[c] (% of sum of subclasses in brackets)				
	Dose[b]	IgG1	IgG2a	IgG2b	IgG3
C/C/C	1st	16,000 (56)	1,880 (7)	6,200 (18)	4,600 (16)
	2nd	24,000 (77)	1,750 (6)	3,100 (10)	2,500 (8)
	3rd	30,000 (78)	1,970 (5)	3,850 (10)	2,690 (7)
Vi/C/C	1st	188 (29)	25 (4)	145 (22)	290 (45)
	2nd	4,000 (56)	520 (7)	1,100 (15)	1,500 (21)
	3rd	29,000 (77)	2,200 (6)	2,750 (7)	3,860 (10)

[a] Six week old female mice were injected subcutaneously with three doses of combinations of either Vi-DT conjugate (C) or Vi according to the regimen above, doses were given at 0, 4 and 8 weeks.

[b] The 1st, 2nd and 3rd dose refer to bleeds taken 2 weeks after each dose.

[c] A pool of sera from each group was assayed for levels of IgG subclass by ELISA.

7.6. Summary

The influence pre-exposure of mice to Vi capsular polysaccharide, purified from *Salmonella enterica* Serovar Typhi, on the subsequent immune response induced by a Vi-DT conjugate was evaluated. Vi induced low anti-Vi IgG titers with the dominant subclass being IgG3. The Vi-DT conjugate induced high titers of anti-Vi IgG with the dominant subclass being IgG1 but with considerable quantities of IgG2a, IgG2b and IgG3. Priming of mice with Vi suppressed the response to a subsequent dose of conjugate and the suppression was overcome by a second dose of conjugate. Priming with conjugate prevented suppression of the anti-Vi response and subsequent dosing with Vi raised titers back to previous levels but did not boost to new higher levels. In this study hyporesponsiveness to Vi was partially overcome with one dose of conjugate and fully overcome with two doses of conjugate. The anti-DT IgG response to one dose of conjugate was relatively strong and protracted and continued to increase for 12 weeks, compared to the response to one dose of DT which was poor and peaked at two weeks. The prolonged anti-DT response was most likely due to the slow release of DT from the conjugate lattice as it degrades within the mouse resulting in a continuous stimulation of the immune response.

Chapter 8

Overall Discussion and Further Suggestions

Chapter 8. Overall Discussion, Conclusions and Further Suggestions

8.1. Overall discussion

In order to develop a safe and effective Vi conjugate vaccine for infant in developing countries, in this study, DT was chosen for the protein carrier for the conjugate. DT is stable in the range of pH used in the derivatization and Vi-DT conjugation. By adjusting parameters of derivatization, it was shown that the derivatization of DT was time, EDC concentration and pH dependent. It was demonstrated that the derivatization level of DT could be controlled by the EDC concentration. The derivatization reaction had high activity at a pH lower than 6.0 but a pH lower than 5.5 caused DT to precipitate during derivatization. Therefore, the pH range 5.5 to 6.0 was selected for the derivatization. In the conjugation reaction with Vi, higher than 3% derivatized DT resulted in higher than 70% yield of conjugated Vi in Vi-DT. More DT was bound to Vi at higher EDC concentrations. The conjugates produced during the optimization experiment were tested for immunogenicity in mice. The highest anti-Vi response was obtained with conjugate made with low EDC and the highest derivatization of DT. The conjugate size was also shown to affect immunogenicity. The larger conjugates were more immunogenic.

The relationship of the physical and chemical structure of the conjugate with immunogenicity was evaluated. As the amount of DT bound to the Vi increased, the

size of the conjugate increased and so did the degree of linking. Results of this varying size of the Vi-DT conjugates on immunogenicity established a correlation between conjugate size and cross-linking and the anti-Vi and anti-DT responses in mice. The anti-Vi response showed significantly higher immunogenicity even after the first dose of Vi-DT conjugate. Therefore, the rapid onset of a high level of anti-Vi antibodies may offer an advantage in that highly cross-linked conjugate might provide protection after a single injection similar to that seen with the Hib PRP polysaccharide-outer membrane protein complex (OMPC) of *Neisseria meningitides* conjugate and, unlike other Hib conjugates induces a strong primary response to the PRP polysaccharide [63]. Furthermore, group B streptococcal type III polysaccharide conjugate vaccine increased anti-polysaccharide response as the extent of cross-linked conjugate, it was assumed that the high immunogenicity is due to high stability of an epitope on the cross-linked polysaccharide to carrier protein [46]. A study suggested that the polysaccharide of a glycoconjugate entered an endosome in the antigen presenting cell with carrier protein and the peptides which derived from the carrier protein with polysaccharide component co-localized with MHC II on the antigen presenting cell surface and these epitopes recruit T helper cells for the adaptive immune response [64]. It means the stability of the conjugate vaccine can induce a slow releasing immunogenicity in vivo. Thus this hypothesis can explain slow releasing of the DT antigen and long lasting anti-DT response of Vi-DT conjugate without boost immunization.

Moreover, there was no free polysaccharide in the conjugate containing high

cross-linking, thus enabling simplification of the purification method and elimination of size selection by chromatography. The simplified process and high yields obtained are a positive step towards a high quality affordable vaccine targeting vaccination of low income people living in areas where typhoid is endemic.

The un-conjugated Vi, up to 50%, in the Vi-DT conjugate vaccine increased the anti-Vi and anti-DT IgG responses compared with Vi-DT conjugate-only dosing. As with other conjugate vaccines [47, 65], the negative effect of un-conjugated polysaccharide in a conjugate vaccine was confirmed in the presence of 75% un-conjugated Vi. These findings suggest a key element for the conjugate vaccine quality control for a limit for un-conjugated polysaccharide that should not exceed 50% for the Vi-DT conjugate.

The anti-DT IgG response to one dose of conjugate was relatively strong and protracted and continued to increase for 12 weeks. The prolonged anti-DT response was possibly due to slow releasing of DT antigen from the conjugate from the cross-linked structure. The highly cross-linked Vi-DT conjugate vaccine appears to be the best candidate as it generates strong antibody response to Vi and DT after only one dose. A single dose in mice of Vi polysaccharide induced low anti-Vi IgG titers with the dominant subclass being IgG3. The Vi-DT conjugate induced high titers of anti-Vi IgG with the dominant subclass being IgG1. The suppressed response of Vi polysaccharide was overcome by a second dose of conjugate. From an immunological point of view, conjugation of Vi to DT results in a change in the way the immune system responds to the Vi antigen and one aspect of that change is a

subclass switch from an IgG3 response with Vi to an IgG1 response with conjugate. A similar subclass switch from predominantly IgG3 with polysaccharide to IgG1 with conjugate has also been observed with pneumococcal polysaccharides [29].

Additionally, Vi-DT conjugates prepared at IVI induced comparable anti-Vi IgG levels to Vi-rEPA conjugate vaccine which was used in clinical trials by the National Institutes of Health (NIH) [66].

With these results, the Vi-DT conjugate vaccine is a more effective and affordable vaccine for a typhoid vaccine candidate and easily to adaptable to scale-up for vaccine manufacture.

8.2. Conclusions and Further Suggestions

The Vi-DT conjugate candidate with high degree cross-linking induced a very high anti-Vi response in mice. There was no un-bound Vi polysaccharide in this Vi-DT conjugate. The correlation of immunogenicity with molecular size and the easy visualization of the molecular size using size exclusive chromatography of Vi-DT conjugates is of significance in Vi-DT vaccine process development for scale-up for manufacture and to control the quality if the vaccine. The methods developed in this study means that the vaccine process could be simplified and eliminate the need for size selection using chromatography. Un-conjugated Vi, up to 50%, in Vi-DT conjugate showed an adjuvant-like effect in immunogenicity in mice. This result supports the simplification of the Vi-DT conjugate manufacturing process and

moreover provides an idea for further investigation that free Vi polysaccharide could be used as an adjuvant for other vaccines as well as a need to evaluate other adjuvant effects in the Vi-DT conjugates.

Pre-exposure to Vi may have induced hyporesponsiveness to subsequent doses of Vi, and clearly caused suppression of the response to a subsequent dose of conjugate, this suppression was overcome with a second dose of conjugate. The clinical significance of Vi induced suppression is not known, however, if Vi induced suppression also occurs in humans then Vi vaccines should be replaced by Vi conjugate vaccines when they are licensed especially in areas where children are at increased risk of contracting typhoid fever.

A remarkable result was the strong induction of an anti-DT IgG response which continued to increase for 12 weeks post one dose Vi-DT conjugate vaccination. This result suggests an idea about a slow releasing vaccine. The slow releasing vaccine could generate protective immunity from a one dose vaccination. Most slow release vaccines are based on microspheres composed of biodegradable polymers, as the polymer degrades antigen is slowly released providing continuous stimulation of the immune system and it is hoped that delivery of vaccine in this manner will reduce the need for booster doses [67] [68]. It is intended to further investigate the capacity of Vi-DT conjugate to behave as slow release delivery systems for other carrier proteins. In conclusion, the results obtained in mice in this study offer a high quality affordable vaccine candidate for use in preventing typhoid fever in typhoid endemic settings.

초 록

저개발국가에서 장티푸스는 여전히 높은 발병률을 보이는 중요 질병으로 남아있다. 1995년 이후 승인된 Vi 다당체 백신이 20년 가까이 상용되고 있지만 이 다당체 백신은 2차 면역에 의한 memory B세포가 형성되지 않는 T 세포 비 의존성의 특성과 2세 이하의 소아에서의 면역성이 현저히 떨어지는 단점들을 가지고 있기에 본 연구에서는 Vi 다당체와 면역을 유도하는 단백질로서의 디프테리아 톡소이드를 결합한 Vi 단백결합 백신의 개발을 통해 소아에서 효과적인 장티푸스백신의 개발을 목적으로 하였다.

Vi 단백결합 백신의 생산에 있어서 결합되는 Vi 다당체와 디프테리아 톡소이드 단백질의 비율은 결합의 수율을 결정하는 중요한 요소이다. 본 연구에서는 결합되는 단백질의 양을 조절하는 시도를 통해 여러 가지 크기의 Vi 다당체 단백결합 백신을 유도하였다. 결합되는 단백질이 증가할수록 단백결합 백신의 분자량의 크기가 커짐과 동시에 단백질이 결합된 Vi 다당체 사이의 교차 결합이 생성됨을 보였다. 이렇게 생성된 각각의 다른 크기의 단백결합 백신을 생쥐에서의 접종실험을 통해 단백결합 백신의 교차 결합 비율이 증가할수록 유도된 항 Vi 항체와 항 디프테리아 톡소이드의 항체의 생성이 증가함을 확인할 수 있었다. 특히 디프테리아 톡소이드와 결합된 Vi 다당체의 비율이 0.7이하에서 많은 수의 단백질이 부착된 교차결합의 비율이 증가하였고 그로 인한 강한 항 Vi 항체가 생성되었다. 이로써 Vi 단백결합 백신의 물리 화학적 특성과

그것을 통해 유도된 면역성의 연관성을 증명하였다.

현재 양산되는 다른 다당체 단백결합 백신에서 단백질과 결합되지 않고 남은 다당체가 백신 생산공정에서 제거되지 못할 경우 단백결합 백신의 면역반응을 억제한다는 일련의 결과들이 있었다. 그런 결과가 Vi 다당체 단백결합 백신도 적용되는지 확인해 보고자 하였다. Vi 단백 결합백신에 여러 비율의 Vi 다당체를 혼합하여 생쥐에 접종하여 면역성을 확인한 결과 면역억제의 반응을 보이는 다른 다당체 단백결합 백신의 결과와는 달리 Vi 단백결합 백신에 50%까지 혼입된 Vi 다당체는 Vi 단백 결합 백신에서 유도되는 항 Vi 항체보다 높은 수치를 보여 혼입된 Vi 다당체는 면역 증강제와 같은 효과를 가짐을 확인 하였다.

현재 접종되고 있는 Vi 다당체 백신을 접종했을 시 2차접종에서 보이는 면역억제반응은 잘 알려져 있는 결과이다. Vi 다당체 백신과 Vi 단백 결합백신을 여러 가지 백신접종의 순서로 생쥐에 접종해 보았을 때 1차 접종된 Vi 다당체 백신을 통해 억제된 면역성은 2차와 3차로 이어지는 Vi 단백결합 백신의 접종을 통해 면역성이 회복됨을 보였고 재 접종 없이 1차로 접종된 Vi 단백결합 백신으로 유도된 항 Vi 항체가 점진적으로 감소됨을 보였으나 항 디프테리아 항체는 12주까지 재 접종 없이도 항체의 양이 증가하였고 지속됨을 보이고 있었다.

그리하여 본 연구를 통해 확인한 결과인 Vi 단백결합 백신의 물리화학적 특성과 면역성과의 연관관계와 Vi 다당체가 가지는 단백결합 백신에의 긍정적은 효과는 백신의 대량생산에 활용하여 더 안전하며 항체생성 능력이 높은 단백결합 백신의 생산에 활용될 수 있겠다.

그리고 기존의 Vi 다당체 백신의 면역억제의 효과를 Vi 단백결합 백신으로 극복 가능하다는 결과를 통해 이 Vi 단백결합 백신이 저개발국가의 소아의 장티푸스 예방에 크게 도움이 될 것이라 생각된다.

주요어: 장티푸스, 장티푸스백신, Vi 다당체, 단백결합백신

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