Vaccine 29 (2011) 7618–7623

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Physico-chemical properties of *Salmonella typhi* Vi polysaccharide–diphtheria toxoid conjugate vaccines affect immunogenicity

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ARTICLE INFO

Article history:
Received 24 June 2011
Received in revised form 1 August 2011
Accepted 2 August 2011
Available online 16 August 2011

Keywords:
Vi polysaccharide
Diphtheria toxoid
Conjugation

ABSTRACT

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 enterica* Serovar Typhi. As the amount of DT bound to the Vi increased the size of the conjugate increased but also the degree of cross-linking increased. 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. This study establishes a correlation between physico-chemical characteristics of the conjugate and the magnitude of the anti Vi and anti DT responses.

1. Introduction

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. With the exception of the Vietnamese Vi vaccine the primary target for these vaccines is for travelers to endemic regions or to the private sector in developing countries [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 younger children, and to target the Extended Program of Immunization (EPI) to deliver the vaccine, 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. This can be achieved by conjugating the polysaccharide to a carrier protein [7–9]. 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 IVI conjugate development program because it is readily available at low cost and it is stable in the range of pH encountered during the conjugation process. 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 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 final product complies with the quality requirements defined in the appropriate regulatory documents. In order to ensure vaccine quality it was considered important to define the physico-chemical properties of the conjugate and, if possible, to establish a relationship between these properties and immunogenicity.

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doi:10.1016/j.vaccine.2011.08.019

The conjugation chemistry used in these studies was previously described [10] and modified in our laboratories to ensure consistency of manufacture. By varying the concentration of DT used in the conjugation reaction a series of conjugates with increased size and increased cross-linking was generated. 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.

2. Materials and methods

2.1. Bacterial strain

Salmonella enterica Serovar Typhi isolate number C6524 isolated from a patient in Kolkata India by the National Institute of Cholera and Enteric Diseases (NICED) was used as the source of Vi capsular polysaccharide.

2.2. Purification of Vi polysaccharide

Isolate C6524 was cultivated in a bioreactor to maximize Vi capsular polysaccharide production as previously described [11] and the Vi purified as previously described [12]. Comparison of the elution profiles on Sephacryl S-1000 (GE Healthcare) of the Vi with Dextran 2000 (GE Healthcare) indicated that the relative molecular size of the Vi was mainly greater than 2000 kDa. The Vi used in this study was prepared using the method developed at IVI and 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 [14].

2.3. 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. The method used was based on that reported by Kossaczka et al. [10] and modified as follows.

2.3.1. Derivatization of DT 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 30 kD Hydrosart (Sartorius-Stedim) ultrafiltration membrane. The protein concentration of the diafiltered concentrate was measured by Lowry assay [13]. The DT was derivatized by adding adipic acid dihydrazide (ADH) (Sigma) followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma) so that the final concentration of DT, ADH and EDC were 10 mg/ml (0.16 mM), 35 mg/ml (200 mM) and 4.0 mg/ml (21 mM) respectively. The reaction was allowed to proceed at room temperature for 60 min maintaining pH at 5.6 by addition of 1 M HCl. At the end of 60 min the reaction was stopped by addition of 1 M 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 TNBS assay [9,15] 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.

2.3.2. Conjugation

The Vi polysaccharide was concentrated to 3.3 mg/ml and diafiltered against 5 volume changes of 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 concentration of Vi and EDC in the reaction mixtures were 1.0 mg/ml (4.0 mM of Vi monomer units) and 2.0 mg/ml (10.5 mM) respectively and the final concentration of DT was 4.0, 1.5, 1.0, 0.5, 0.25, and 0.125 mg/ml (0.065, 0.024, 0.016, 0.008, 0.004 and 0.002 mM, respectively) depending on the reaction. The reaction was allowed to proceed at room temperature for 180 min and the pH remained stable between 5.6 and 5.8 during the reaction. Free DT and residual EDC were removed by diafiltration against 10 volume changes of phosphate buffered saline pH 7.2 using a 300 kD polyethersulfone membrane (Sartorius-Stedim).

2.4. Chemical and physical analysis of conjugates

Conjugates were assayed for *O*-acetyl content by Hestrin assay [16] 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).

2.5. Immunization and antibody responses to conjugates

Groups of 10 mice were injected subcutaneously with 2.5 μg dose (based on Vi) in each of the conjugates, plus Vi alone controls. Mice received a total of 3 doses at 0, 4 and 20 weeks, and were bled by retro-orbital puncture at 2, 4, 6, 12 and 22 weeks and serum collected for antibody quantification. Vi and DT antibody levels in mouse sera were evaluated by enzyme-linked immunosorbent assay (ELISA) [17]. The antibody titers were expressed as the geometric mean of ELISA unit. A titer lower than detectable level of the ELISA was assigned a value of 0.02 EU and a hyper-immune mouse serum pool was assigned a value of 100 EU and used as standard.

2.6. Statistical analysis

Comparison of immune responses between the different conjugates and control groups were performed. The Student *t*-test, Welch *t*-test or Wilcoxon rank sum test was used whether the variance was equal or not or depending on the distribution. 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).

3. Results

3.1. Derivatization of the DT carrier protein

DT was activated with EDC and derivatized with ADH. Lowry protein assay and TNBS assay were used to determine the protein content and the ADH content of the derivatized DT respectively. The derivatized DT was found to have a corrected ADH: Protein ratio of 2.9% which corresponds to approximately 10 ADH 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. The derivatized DT migrated to a similar position to that of the native DT when analyzed by polyacrylamide gel electrophoresis and Superdex 200 (GE healthcare) size exclusion chromatography (data no shown) indicating no cross-linking of the DT molecules had occurred. The effect of varying the EDC concentration was tested at fixed ADH and DT concentrations, as the EDC concentration increased so did the amount of ADH bound