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Comparison of CRM_{197}, diphtheria toxoid and tetanus toxoid as protein carriers for meningococcal glycoconjugate vaccines

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ABSTRACT

Glycoconjugate vaccines are among the most effective and safest vaccines ever developed. Diphtheria toxoid (DT), tetanus toxoid (TT) and CRM_{197} have been mostly used as protein carriers in licensed vaccines. We evaluated the immunogenicity of serogroup A, C, W-135 and Y meningococcal oligosaccharides conjugated to CRM_{197}, DT and TT in naïve mice. The three carriers were equally efficient in inducing an immune response against the carbohydrate moiety in immunologically naïve mice. The effect of previous exposure to different dosages of the carrier protein on the anti-carbohydrate response was studied using serogroup A meningococcal (MenA) saccharide conjugates as a model. CRM_{197} showed a strong propensity to positively prime the anti-carbohydrate response elicited by its conjugates or those with the antigenically related carrier DT. Conversely in any of the tested conditions TT priming did not result in enhancement of the anti-carbohydrate response elicited by the corresponding conjugates. Repeated exposure of mice to TT or to CRM_{197} before immunization with the respective MenA conjugates resulted in a drastic suppression of the anti-carbohydrate response in the case of TT conjugate and only in a slight reduction in the case of CRM_{197}. The effect of carrier priming on the anti-MenA response of DT-based conjugates varied depending on their carbohydrate to protein ratio. These data may have implications for human vaccination since conjugate vaccines are widely used in individuals previously immunized with DT and TT carrier proteins.

1. Introduction

Meningococcal disease epidemiology features unpredictable epidemics, and variation in endemic serogroup distributions [1], highlighting the importance of developing multivalent vaccine formulations to confer protection against the most relevant serogroups [2]. Five serogroups, A, B, C, W-135 and Y, cause the majority of meningococcal meningitis [3]. Purified capsular polysaccharides, one of the major virulence factors of encapsulated bacterial pathogens, are not immunogenic in infants and poorly immunogenic in adolescents and adults. This limitation, due to their inability to engage T cells, has been completely overcome by conjugation to carrier proteins that provide T cell epitopes [4–7]. Currently, three conjugate vaccines against *Neisseria meningitidis* serogroup C and three quadrivalent meningococcal conjugate vaccines against serogroups A, C, Y and W-135, are commercially available and use DT, CRM_{197} and TT protein carriers respectively [8–10]. In addition to using different carrier proteins, the existing conjugate vaccines contain other variables like conjugation approach, (Fig. 1A), and formulation technology. Here, we report a comparison of the three most-commonly used carrier proteins, CRM_{197}, TT and DT, conjugated with the same chemistry [7,11] to meningococcal oligosaccharides from *N. meningitidis* serogroups A, C, W-135 and Y (MenA, MenC, MenW, MenY). The resulting glycoconjugates were compared in terms of their physicochemical and immunogenic properties in mice with focus on the effect of carrier priming on the anti-saccharide response.

2. Materials and methods

2.1. Starting materials

Meningococcal oligosaccharides, activated at their reducing termini [11], CRM_{197}, TT and DT were obtained from Novartis Vaccines & Diagnostics Manufacturing Facilities. Prior to conjugation, TT was further purified by gel filtration on Sephacryl S300 equilibrated in

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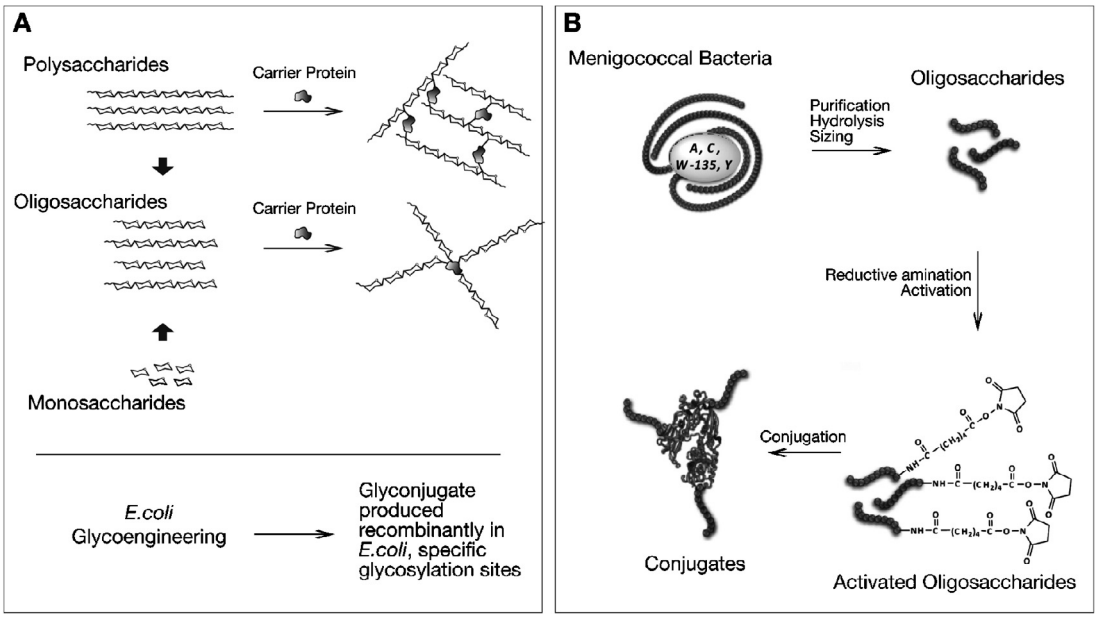

Fig. 1. (A) Different ways to make conjugate vaccines. Upper panel: coupling of carrier proteins to randomly activated polysaccharides, to end groups activated oligosaccharides derived from native polysaccharides or by chemical synthesis. Lower panel: glyco-engineering the *E. coli* N-glycosylation pathway [41]. (B) Scheme of conjugates preparation. Starting from bacterial polysaccharides hydrolysis and sizing is performed to obtain the oligosaccharides. Oligosaccharides are activated introducing the N-hydroxysuccinimide ester moiety and conjugated to the proteins CRM_{197}, TT and DT.

0.15 M NaCl, 0.01 M sodium phosphate buffer at pH 7.2 (Suppl. Fig. 1s).

2.2. Meningococcal A, C, W-135 and Y oligosaccharide-protein conjugates

Activated MenA, MenC, MenW and MenY oligosaccharides were independently conjugated to CRM_{197}, DT and TT with an active ester to protein molar ratio varying from of 12:1 to 30:1, in 0.1 M sodium phosphate buffer pH 7.0. The reactions proceeded with gentle stirring overnight at room temperature (r.t.). Purification of the conjugates from the unreacted oligosaccharides was performed either by repeated ammonium sulphate (500 g/L) precipitation/solubilization cycles, or by hydrophobic chromatography on phenyl sepharose.

2.3. Characterization of glycoconjugates

SDS-PAGE followed by Coomassie staining was performed on glycoconjugates and carrier proteins using NuPage 3–8% Tris-acetate gradient gels (Invitrogen). Protein content was determined by MicroBCA assay (Pierce) [12]. Total saccharide content of MenA, MenW and MenY conjugates was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) essentially as described [13,14]. The total saccharide content of MenC conjugates was also determined by HPAEC-PAD: briefly, samples were hydrolyzed in 1 M trifluoroacetic acid at 90 °C for 2 h, neutralized with 2 M NaOH and loaded on a CarboPac PA1 column connected to a Dionex ICS3000 system. A NaOH/sodium acetate gradient was applied for elution. All chromatographic data were integrated and processed using Chromeleon™ 6.8 software.

Unconjugated (free) saccharide was separated from the conjugate by analytical ultrafiltration and quantified on filtrates applying the HPAEC-PAD methods for total saccharide. HPLC-size exclusion chromatography was performed with a TSK 4000 Gel SW (Tosoh Bioscience) analytical column connected to an Ultimate™ 3000 HPLC system (Dionex -Thermo Fisher Scientific) equipped with a PDA Detector. Chromatography was performed in 0.1 M sodium sulphate 0.1 M sodium phosphate 5% CH_3CN pH 7.2 at flow rate of 0.5 mL/min and data was processed using Chromeleon™ 6.7 software.

2.4. Vaccines formulation and animal immunization

Experimental guidelines set forth by the Novartis Animal Care Department were followed in all animal studies. Conjugated antigens were formulated in 12 mM phosphate buffered saline pH 7.2 (PBS) with aluminum phosphate ($AlPO_4$) as adjuvant (0.12 mg/dose expressed as Al^{3+}). Non-conjugated protein carriers were also formulated in PBS with $AlPO_4$ to obtain dosages of 0.5, 5 or 50 μg. The adsorption of the antigens to $AlPO_4$ was determined by checking the protein content in the supernatant after 1 h incubation at r.t. and centrifugation at 13,000 × *g*, 4 °C, 40 min. The percentage of adsorption was calculated relative to a formulation without adjuvant. In all cases more than 75% of conjugate was found not adsorbed.

2.4.1. Immunogenicity in naïve mice

All vaccines were injected subcutaneously in a volume of 200 μL. Each dose contained 1 μg of conjugated oligosaccharide in the case of MenC, MenW and MenY antigens, or 2 μg of conjugated oligosaccharide in the case of MenA antigen. Quadrivalent formulations (MenACWY) contained 1 μg of MenC, MenW and MenY antigens, 2 μg of MenA antigen and $AlPO_4$. The immunogenicity of the individual glycoconjugates or the quadrivalent formulations was tested in groups of 8 BALB/c mice immunized on days 1, 14 and 28. Bleedings were performed on day 0 (pre immune), day 27 (post 2) and day 42 (post 3). Control groups received PBS/$AlPO_4$.

2.4.2. Immunogenicity in mice primed with carrier proteins

According to different schemes mice were immunized, at day 1 with 0.5, 5 or 50 μg of CRM_{197}, or DT or TT, and at day 14 with 2 μg of serogroup A oligosaccharide conjugated to CRM_{197} or DT or TT. In one scheme, mice were primed with carrier at day 1 or at day