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Preclinical studies on new proteins as carrier for glycoconjugate vaccines

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ABSTRACT

Glycoconjugate vaccines are made of carbohydrate antigens covalently bound to a carrier protein to enhance their immunogenicity. Among the different carrier proteins tested in preclinical and clinical studies, five have been used so far for licensed vaccines: Diphtheria and Tetanus toxoids, the non-toxic mutant of diphtheria toxin CRM_{197}, the outer membrane protein complex of *Neisseria meningitidis* serogroup B and the Protein D derived from non-typeable *Haemophilus influenzae*. Availability of novel carriers might help to overcome immune interference in multi-valent vaccines containing several polysaccharide-conjugate antigens, and also to develop vaccines which target both protein as well saccharide epitopes of the same pathogen. Accordingly we have conducted a study to identify new potential carrier proteins. Twenty-eight proteins, derived from different bacteria, were conjugated to the model polysaccharide Laminarin and tested in mice for their ability in inducing antibodies against the carbohydrate antigen and eight of them were subsequently tested as carrier for serogroup meningococcal C oligosaccharides. Four out of these eight were able to elicit in mice satisfactory anti meningococcal serogroup C titers. Based on immunological evaluation, the *Streptococcus pneumoniae* protein spr96/2021 was successfully evaluated as carrier for serogroups A, C, W, Y and X meningococcal capsular saccharides.

1. Introduction

Glycoconjugate vaccines are safe and efficacious tools to prevent infection and mortality against different encapsulated bacterial pathogens [1–3].

In these type of vaccines carbohydrate antigens are covalently bound to a carrier protein which provides T-cell epitopes that confer a T dependent character to the saccharide moiety.

Five carrier proteins are currently included in licensed glycoconjugate vaccines [4–7]. Diphtheria (DT) and Tetanus toxoids (TT), derived from the respective toxins after chemical detoxification with formaldehyde, were initially selected as carriers because of the safety track record accumulated with tetanus and diphtheria vaccination [8]. CRM_{197}, a non-toxic mutant of diphtheria toxin [9], has been extensively used as carrier for licensed conjugate vaccines against *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, *Neisseria meningitidis*) and for other vaccines in clinical development. The outer membrane protein complex (OMPC) of *N. meningitidis* serogroup B has been used as carrier for the Hib conjugate vaccine developed by Merck [10]. Protein D, derived from non-typeable *H. influenzae* (NTHi), has been used as carrier for most of the polysaccharides included by GSK into the multivalent pneumococcal conjugate vaccine recently licensed [11,12].

Multivalent conjugates administration and vaccines co-administration can be associated with immune interference such as carrier-specific enhancement of T-cell help, carrier-induced-epitopic suppression (CIES) or bystander interference. Carrier priming and suppression effect were investigated in both animal and human studies and different behaviors between the commonly used carriers were reported [13–15]. Considering these aspects, novel carriers might help in avoiding or reducing the impact of the above mentioned issues and enable the further development of pediatric schedules and combinations [16].

Other proteins are increasingly used as carrier and some of them have also been tested in clinical trials. The recombinant non-toxic form of *Pseudomonas aeruginosa* exotoxin A (*r*EPA) has been used as carrier for *Shigella* O-antigens [17] and *Staphylococcus aureus* type 5 and 8 capsular polysaccahrides as well as for *Salmonella Typhi* Vi antigen [18–20]. *r*EPA is also used as a carrier for glycoconjugate vaccines directly synthesized in *Escherichia coli* against *Shigella dysenteriae* type 1 [21]. A rationally designed recombinant protein containing strings of promiscuous human CD4+ T-cell epitopes derived from various pathogens including

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tetanus, influenza virus, *Plasmodium falciparum* and hepatitis B virus, proved to be a very good carrier for Hib and meningococcal oligosaccharides [22–24]. Endeavors to develop carrier proteins which might also provide protection against a target pathogen, have recently broadened to include candidates from diverse vaccine categories [25–30].

Taking advantage from the availability of several recombinant proteins originated from a variety of reverse vaccinology projects undertaken in our Research Center, we have conducted a study to identify new carrier proteins, derived from different bacteria like extraintestinal pathogenic *E. coli* (ExPEC), *N. meningitidis* serogroup B (Men B), group A *Streptococcus* (GAS), group B *Streptococcus* (GBS) and *S. pneumoniae* (sp).

The strategy was based on two steps: (a) immunogenicity screening in mice of several recombinant proteins conjugated to a model saccharide in comparison to CRM_{197} as benchmark carrier protein; (b) testing selected carriers conjugated to meningococcal carbohydrate antigens. Here we describe the results of this study. Laminarin (Lam) was the model saccharide used for the first immunogenicity screening, a neutral sugar antigen studied as vaccine candidate against infections induced by *Candida albicans* [31].

2. Materials and methods

2.1. Reagents

Laminarin was obtained from Sigma Aldrich (L9634).

Serogroup A, C, W, Y meningococcal oligosaccharides were provided by the Manufacturing Department (GSK, Siena, Italy).

Men X polysaccharide was produced internally by Research Center (GSK, Siena, Italy).

CRM_{197} was provided by the Manufacturing Department (GSK, Siena, Italy).

Proteins tested as new carrier candidates were produced internally by Research Center (GSK, Siena, Italy).

Men B 961c (NadA or GNA1994), GNA2132-1030 (NHBA-GNA1030) and GNA2091-fHbp (GNA2091–GNA1870) proteins were provided by the Manufacturing Department (GSK, Siena, Italy).

2.2. Preparation of protein carriers

The twenty eight recombinant proteins tested as potential new carrier candidates were expressed in *E. coli*, twenty five of them were expressed as His-tag fusion proteins and purified by immobilized metal affinity chromatography. Details of cloning, expression and purification strategy are reported in SI. All the proteins tested are reported in Table 1 SI with bacteria origin, protein name and alternative name reported in the bracket. Sp proteins were identified as spr when derived from R6 genome or D39 (spr1418 and spr1712), non spr are derived from TIGR4 genome or INV104 genome (PitB).

2.3. Preparation of conjugates

Lam has been treated by reductive amination using the following conditions: 2 mg/ml of polysaccharides were incubated for 5 days at 50 °C in 300 mg/ml ammonium acetate (Sigma–Aldrich) and 0.2 M sodium cyanoborohydride ($NaBH_3CN$) (Sigma–Aldrich) pH 7.5. Aminated Lam was purified by diafiltration on regenerated cellulose membrane (cut-off 1 kDa; Millipore) and the number of primary amino groups introduced were determined by colorimetric assay [32].

The amino-oligosaccharides were vacuum dried, solubilized in 1:9 H_2O:DMSO solution to a final amino group concentration of 40 μmol/ml, and reacted with a 12-fold molar excess of adipic acid bis(N-hydroxysuccinimmide) (SIDEA), in presence of 5-fold molar excess triethylamine as compared with amino groups [33]. The reaction was kept under gentle stirring at room temperature for 2 h. The activated oligosaccharides were obtained by precipitation with 4 volumes of acetone and dried under vacuum. The content of N-hydroxysuccinimide ester groups introduced was determined [34].

Lam conjugates have been prepared in 10–100 mM NaH_2PO_4 pH 7 or in phosphate buffer saline pH 7 (PBS, 150 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4) depending on the protein buffer, using an active ester (AE):protein molar ratio of 30:1, and a protein concentration in the range of 0.4–6 mg/ml. Reactions were carried out overnight at room temperature with gentle stirring.

The conjugates have been purified by affinity chromatography with His MultiTrap 96-Well Filter Plates (GE Healthcare) using the His tag portion on the recombinant protein to bind the conjugate to the resin. Conjugates have been loaded on the resin (250 μg of protein per well in a volume of 200 μl in the same buffer of conjugation) and incubated for 30 min after gently mixing on the well. After, the flow through have been taken by using the vacuum air and two washes of the resin have been done with 200 μl of PBS buffer pH 7.2. Conjugates have been eluted from the resin by adding 150 μl of elution buffer 0.5 M NaCl, 0.5 M NaH_2PO_4, 0.5 M Imidazole pH 6.3, incubating for 15 min and collecting the flow through; the elution has been repeated two times. Eluted conjugates have been dialyzed with 6–8 kDa membrane (Spectra/Por 1, diameter 6.4 mm) against PBS buffer pH 7.2 to eliminate the elution buffer, dialysis have been carried out one day at 4 °C changing the buffer for four times. Non His-tag protein conjugates were purified by Ultrafiltration with Vivaspin 10 K (Sartorius).

SIDEA-activated Men A, C, W, and Y oligosaccharides and their CRM_{197} conjugates were obtained from GSK Vaccine Manufacturing, produced as previously described [35]. Spr96/2021–Men A, C, W, Y conjugations were carried out in 10–100 mM NaH_2PO_4 pH 7 or in PBS pH 7 depending on the protein buffer, at a protein concentration of 3–3.5 mg/ml and a saccharide to protein molar ratio of 12:1. Reactions were let to proceed overnight at room temperature with gentle stirring. Purification has been done by gel filtration or by ultrafiltration as reported above. The same conditions were applied to obtain the conjugates between Men C activated oligosaccharides and the proteins Upec-5211, Orf3526, GNA2091-fHbp, RrgB I-II-III, spr907 and spr1418.

Men X polysaccharide was size reduced and activated for the conjugation as reported before [36]. Conjugation of spr96/2021 was performed at a protein concentration of 10 mg/ml with an oxidized polysaccharide to protein ratio 4:1 (w:w) and a saccharide to $NaBH_3CN$ ratio of 1:1 (w:w), at 37 °C for 48 h. The conjugate was purified by ammonium sulfate precipitation (500 g/l) and the pellet was dissolved in 10 mM NaH_2PO_4 at pH 7.2. CRM_{197}–Men X conjugate was prepared as previously published [36].

Conjugates were characterized by micro BCA for total protein content [37] and by HPAEC-PAD analysis (SI) for total saccharide content.

2.4. Mice immunization

Groups of 6–8 mice (BALB/c or CD1) were immunized subcutaneously on days 1, 14 and 28; bleedings were performed on day 0 (pre immune), day 27 (post 2) and day 42 (post 3).

All vaccines were administered on saccharide base in a volume of 200 μl. Lam conjugates were tested at 5 μg dose, meningococcal conjugates at 2 μg for Men A and 1 μg for Men C, W, Y, X.

Lam and Men A, C, W, Y conjugates were tested without any adjuvant while Men X conjugates were formulated with $AlPO_4$ at 0.12 mg Al^{3+}/dose.

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