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Impact of conjugation chemistry on the immunogenicity of *S.* Typhimurium conjugate vaccines

G. Stefanetti, S. Rondini, L. Lanzilao, A. Saul, C.A. MacLennan, F. Micoli*

Novartis Vaccines Institute for Global Health, Via Fiorentina 1, 53100 Siena, Italy

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ABSTRACT

Salmonella Typhimurium is major cause of invasive nontyphoidal *Salmonella* disease in Africa. Conjugation of *S.* Typhimurium O-antigen to an appropriate carrier protein constitutes a possible strategy for the development of a vaccine against this disease, for which no vaccines are currently available. The conjugation chemistry used is one of the parameters that can affect the immunogenicity of glycoconjugate vaccines. Herein different glycoconjugates were synthesized to investigate the impact of this variable on the immunogenicity of *S.* Typhimurium conjugate vaccines in mice, all with CRM_{197} as carrier protein. Random derivatization along the O-antigen chain was compared with site-directed activation of the terminal KDO sugar residue of the core oligosaccharide. In particular, two different random approaches were used, based on the oxidation of the polysaccharide, which differently impact the structure and conformation of the O-antigen chain. For the selective conjugation methods, linkers of two different lengths were compared.

When tested in mice, all conjugates induced anti-O-antigen IgG antibodies with serum bactericidal activity. Similar anti-O-antigen antibody levels were elicited independent of the chemistry used and a higher degree of saccharide derivatization did not impact negatively on the anti-O-antigen IgG response. Bactericidal activity of serum antibodies induced by selective conjugates was similar independent of the length of the spacer used. Random conjugates elicited antibodies with greater bactericidal activity than selective ones, and an inverse correlation was found between degree of O-antigen modification and antibody functional activity.

1. Introduction

In many parts of Africa, nontyphoidal *Salmonellae* (NTS) are the leading cause of bacteremia. Incidence of disease caused by different serovars varies depending upon the country, but *S.* Typhimurium is the overall major cause of invasive NTS (iNTS) disease [1,2]. iNTS disease was recently estimated at 2.58 million cases per year with a 20% case-fatality rate leading to 517,000 deaths [3]. Young children [4,5], children with HIV infection [6], malaria [7], anemia and malnutrition [8], and HIV infected adults [9,10] are particularly affected. Antibiotics are widely used to treat iNTS disease, but the increasing frequency of multidrug-resistant clinical isolates is concerning and hampers the effectiveness of this treatment in man [11]. Until improved sanitary conditions and widespread provision of clean drinking water can be guaranteed, vaccination constitutes the most promising strategy for the control of iNTS disease in developing countries. No vaccines are currently available to prevent iNTS disease in man.

Surface polysaccharides from bacteria have been used for many years in vaccine applications, being both essential virulence factors and targets for protective antibodies. Covalent conjugation to an appropriate carrier protein is an important mean of increasing the immunogenicity of polysaccharides [12–15]. Glycoconjugate vaccines elicit T cell-dependent immunogenicity against the saccharide. With the involvement of T cells, immunological memory is induced, and affinity maturation and isotype switching from IgM to IgG occur. Unlike pure polysaccharides, glycoconjugate vaccines are effective in young infants.

Antibodies directed against the O-antigen (OAg) of NTS mediate killing [16–18] and confer protection against infection in animal models [19,20]. Therefore, OAg glycoconjugates have been proposed as a vaccine strategy against *Salmonella* for use in man [21].

The synthesis of glycoconjugate vaccines requires a covalent linkage between the saccharide and the carrier protein. Many conjugation methods have been proposed, all following two main approaches: random chemical activation along the polysaccharide

* Corresponding author. Tel.: +39 0577 539087; fax: +39 0577 243540.
E-mail address: francesca.micoli@novartis.com (F. Micoli).

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chain, followed by conjugation to the carrier protein, and coupling to the protein through selective activation of the terminal reducing unit of the saccharide chain [14,15,22,23]. The choice of conjugation strategy can affect the efficiency of conjugation, saccharide to protein ratio and glycoconjugate structure and size, with consequent impact on immunogenicity [15]. Spacer molecules are often introduced between the saccharide and protein to reduce steric hindrance and facilitate conjugation.

Here we investigate different conjugation strategies for linking *S.* Typhimurium OAg to CRM_{197} [23] and compare the impact of these chemistries on the immunogenicity of the resulting conjugates in mice.

2. Materials and methods

SI Materials and Methods feature additional information.

2.1. OAg purification and characterization

S. Typhimurium OAg was purified as previously described [24], following fermentation of the animal-derived isolate, 2192, obtained from the University of Calgary, or of the laboratory strain LT2, obtained from the Novartis Master Culture Collection. OAg preparations were characterized by protein content <1% (by micro BCA), nucleic acid content <0.5% (by A_{260}) and endotoxin level <0.1 UI/μg (by LAL). Full characterization of the OAg chains from these two strains have been previously reported [25]. In particular, 2192 OAg, used for the synthesis of the conjugates tested in mice, was 24% glucosylated and 100% O-acetylated on C-2 abequose (Abe). It showed an average molecular weight (MW) distribution of 20.5 kDa, determined from the molar ratio of rhamnose (Rha; sugar of the OAg chain) to N-acetyl glucosamine (GlcNAc; core sugar), sugar composition analysis by HPAEC-PAD and considering the level of O-acetylation by NMR analysis. OAg chains showed the presence of NH_2 groups (NH_2 to GlcNAc molar ratio % of 37.6), as detected by TNBS colorimetric method [26,27], probably as pyrophosphoethanolamine residues in the core region (Fig. S1).

2.2. Synthesis of OAg-CRM_{197} glycoconjugates

OAg-oxNaIO$_4$-CRM$_{197}$: random activation of the OAg chain with NaIO$_4$ and conjugation to CRM$_{197}$. OAg (10 mg/mL in AcONa 100 mM pH 5) was stirred for 2 h in the dark with 3.75 mM $NaIO_4$. The mixture was desalted using a HiPrep™ 26/10 desalting column 53 mL, prepacked with Sephadex™ G-25 Superfine [GE Healthcare], and the pool, eluted at the void volume of the column, was dried. The activated OAg was designated OAg-oxNaIO$_4$. For conjugation to CRM_{197}, OAg-oxNaIO$_4$ was added to CRM_{197} in NaH_2PO_4 100 mM pH 7.2 to give a final concentration of 10 and 5 mg/mL, respectively. $NaBH_3CN$ was added immediately after (OAg-oxNaIO$_4$:$NaBH_3CN$ = 1:1 w/w), and the reaction mixture stirred overnight at 37 °C. After this time, $NaBH_4$ (OAg-oxNaIO$_4$:$NaBH_4$ = 1:1 w/w) was added and the mixture was stirred at 37 °C for 2 h. The conjugate was designated OAg-oxNaIO$_4$-CRM_{197}.

OAg-oxTEMPO-CRM$_{197}$: random activation of the OAg chain with TEMPO and conjugation to CRM$_{197}$. OAg (3 mg/mL, corresponding to [CH_2OH] of 7.69 mM) and $NaHCO_3$ (molar ratio $NaHCO_3/CH_2OH$ = 30), were added to a stirred solution of TEMPO (molar ratio TEMPO/CH_2OH = 0.05) in DMF. The reaction was cooled to 0 °C and TCC (molar ratio TCC/CH_2OH = 1.6) was added. The activated sugar was recovered from the reaction mixture by precipitation with EtOH (85 v/v% in the final mixture) after 2 h of stirring at 0 °C. The pellet was washed twice with 100% EtOH (1.5 volumes with respect to the reaction mixture volume) and lyophilized. The activated OAg was designated OAg-oxTEMPO2h. The same procedure was used for the synthesis of OAg-oxTEMPO12h, increasing the reaction time to 12 h. OAg-oxTEMPO2 h and OAg-oxTEMPO12h were conjugated to CRM_{197}, using the same conditions for OAg-oxNaIO$_4$. The two corresponding conjugates were designated OAg-oxTEMPO2h-CRM_{197} and OAg-oxTEMPO12h-CRM_{197}, respectively.

OAg-ADH-SIDEA-CRM$_{197}$: selective activation of the terminal KDO with ADH, followed by reaction with SIDEA and conjugation to CRM$_{197}$. The synthesis of this conjugate was performed as previously described [28] and detailed in SI.

OAg-NH$_2$-SIDEA-CRM$_{197}$: selective activation of the terminal KDO with NH$_4$OAc, followed by reaction with SIDEA and conjugation to CRM$_{197}$. OAg was solubilized in 500 mM NH_4OAc pH 7.0 at a concentration of 40 mg/mL. $NaBH_3CN$ was added immediately ($NaBH_3CN$:OAg = 2:5 w/w). The solution was mixed at 30 °C for 5 days. The reaction mixture was desalted on a G-25 column and the OAg-NH_2 was dried. The following steps of conjugation were performed as for OAg-ADH-SIDEA-CRM_{197} and the resulting conjugate was designed OAg-NH_2-SIDEA-CRM_{197}.

All conjugates were purified by hydrophobic interaction chromatography (HIC) on a Phenyl HP column [GE Healthcare], loading 500 μg of protein for mL of resin in 50 mM NaH_2PO_4 3 M NaCl pH 7.2. The purified conjugate was eluted in water and the collected fractions were dialyzed against 10 mM NaH_2PO_4 pH 7.2.

2.3. Conjugates characterization

Total saccharide was quantified by phenol sulfuric assay [29], protein content by micro BCA (using BSA as standard and following manufacturer's instructions [Thermo Scientifics]) and the ratio of saccharide to protein calculated. OAg-CRM_{197} conjugates profiles were compared with free CRM_{197} by HPLC-SEC and SDS-PAGE (see SI).

Methods used for the characterization of derivatized OAg intermediates are described in SI.

2.4. Immunogenicity study in mice and serological analysis

Seven groups of eight 5-week old female C57BL/6 mice were purchased from Charles River Laboratory and maintained at Novartis Vaccines Animal Care. Mice received three subcutaneous immunizations at 14 days-interval with 200 μL/dose of 1 μg of conjugated OAg. Mice were bled before the first immunization (day 0) and two weeks after each immunization. All animal protocols were approved by the local animal ethical committee (approval N. AEC201018) and by the Italian Minister of Health in accordance with Italian law.

Serum IgG, IgM and IgA levels against both OAg and CRM_{197} were measured by ELISA (see SI) [28,30]; day 42 sera were additionally assessed for serum bactericidal activity (SBA) and binding capacity (flow cytometry) of two invasive clinical isolates (see SI). Statistical analysis of ELISA results was conducted using Kruskal–Wallis test, with Dunn's post hoc analysis ($\alpha = 0.05$).

3. Results

3.1. OAg oxidation with NaIO$_4$ and reductive amination with CRM$_{197}$

$NaIO_4$-based oxidation affects vicinal diols to generate two aldehyde groups, opening the sugar ring. In the case of *S.* Typhimurium OAg, this reactivity can involve Rha and glucose (Glc) residues (Fig. 1a). The resulting aldehyde groups can then react with the amine group on lysine residues of the carrier protein to form a covalent C=N linkage, which is subsequently reduced to a stable