Vaccine 33 (2015) 1345–1352

Contents lists available at ScienceDirect

Vaccine

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

Structural correlates of carrier protein recognition in tetanus toxoid-conjugated bacterial polysaccharide vaccines

Kay Lockyer[a], Fang Gao[a], Jeremy P. Derrick[b], Barbara Bolgiano[a,*]

[a] Division of Bacteriology, National Institute of Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, South Mimms, Potters Bar EN6 3QG, UK
[b] Faculty of Life Sciences, The University of Manchester, Manchester, UK

ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form
23 December 2014
Accepted 12 January 2015
Available online 29 January 2015

Keywords:
Conjugate vaccine
Tetanus toxoid
Chromatography
Fluorescence quenching

ABSTRACT

An analysis of structure-antibody recognition relationships in nine licenced polysaccharide-tetanus toxoid (TT) conjugate vaccines was performed. The panel of conjugates used included vaccine components to protect against disease caused by *Haemophilus influenzae* type b, *Neisseria meningitidis* groups A, C, W and Y and *Streptococcus pneumoniae* serotype 18C. Conformation and structural analysis included size exclusion chromatography with multi-angle light scattering to determine size, and intrinsic fluorescence spectroscopy and fluorescence quenching to evaluate the protein folding and exposure of Trp residues. A capture ELISA measured the recognition of TT epitopes in the conjugates, using four rat monoclonal antibodies: 2 localised to the H_C domain, and 2 of which were holotoxoid conformation-dependent. The conjugates had a wide range of average molecular masses ranging from 1.8×10^6 g/mol to larger than 20×10^6 g/mol. The panel of conjugates were found to be well folded, and did not have spectral features typical of aggregated TT. A partial correlation was found between molecular mass and epitope recognition. Recognition of the epitopes either on the H_C domain or the whole toxoid was not necessarily hampered by the size of the molecule. Correlation was also found between the accessibility of Trp side chains and polysaccharide loading, suggesting also that a higher level of conjugated PS does not necessarily interfere with toxoid accessibility. There were different levels of carrier protein Trp side-chain and epitope accessibility that were localised to the H_C domain; these were related to the saccharide type, despite the conjugates being independently manufactured. These findings extend our understanding of the molecular basis for carrier protein recognition in TT conjugate vaccines.

1. Introduction

The global burden of bacterial meningitis is primarily due to invasive infection by *Neisseria meningitidis* and *Streptococcus pneumonia*. *Haemophilus influenzae* serogroup B (Hib) accounted for many cases of bacterial meningitis in the developed world prior to the introduction of the Hib conjugate vaccine in 1987. Hib vaccines have reduced incidence of disease attributed to Hib by 80% or more, dependent on vaccine uptake [1,2]. Monovalent meningococcal group C (MenC) vaccines, licenced in 1999–2000, have reduced the incidence of invasive meningococcal disease caused by MenC by over 90% in the UK [3,4]. There are currently three licensed tetravalent meningococcal conjugate vaccines, which also offer protection from serotypes A, W and Y [5], and pneumococcal conjugate vaccines can protect against up to 13 disease-causing serotypes [6,7]. The significant mortality rates and long-term sequelae following infection by encapsulated bacteria have made such vaccination strategies highly sought after worldwide.

Conjugate vaccines have purified oligo- or polysaccharide (PS) covalently linked to a carrier protein, e.g. tetanus toxoid (TT), in a process known as conjugation. A conjugate vaccine elicits a T-cell dependent antibody response, leading to high-avidity, circulating antibodies and the establishment of immune memory in infants and other at-risk groups, which are not evoked by plain PS vaccines [4]. The failure of plain PS vaccines to elicit IgG memory in mice has led to the belief that elicitation of T-cell help by glycoconjugates was attributable to MHC Class II presentation of peptides to the T-cell receptor. Carbohydrates fail to directly bind MHC Class II receptor molecules and are not presented to T-cells, and are, therefore, truly 'T-cell independent' [8]. The 2011 study carried out by Avci et al. [8] has demonstrated that MHC Class II-presented glycopeptides elicit T-cell help; glycoconjugated carbohydrates are processed into smaller glycans which are presented to the T-cell

* Corresponding author. Tel.: +44 1707 641 209; fax: +44 1707 641 054.
E-mail address: barbara.bolgiano@nibsc.org (B. Bolgiano).

http://dx.doi.org/10.1016/j.vaccine.2015.01.046

receptor on the APC surface. Carbohydrate epitope presentation to CD4+ cells plays a vital role in inducing polysaccharide-specific adaptive immune responses. A separate study suggested that the carbohydrate component of a pneumococcal glycoconjugate is presented to the APC surface and co-localises with the MHC class II protein [9].

Glycoconjugate vaccines vary immensely due to biological variations such as polysaccharide type and chemical variations such as conjugation chemistry. These factors as well as the choice of carrier protein can provide glycoconjugate vaccines varying in terms of both size and structure. The size of the conjugate can depend on the oligomeric state (and monomeric size) of the carrier protein, the chain-length of the PS, the saccharide-to-protein loading and the conjugation chemistry used [2,10]. Previous studies have suggested that the immunogenicity of conjugate vaccines is partly dependent on their PS chain length and structural properties [11–13], as well as the intrinsic properties of the carrier protein, but studies have not been done to survey the protein epitope accessibility. In this study, a comparison of the protein structural and antibody recognition features of a panel of polysaccharide-tetanus toxoid conjugate vaccines has been undertaken to determine if the accessibility of the exposed TT epitopes is affected by high PS loading in polysaccharide-TT conjugates.

2. Materials and methods

2.1. Vaccines

A panel of nine glycoconjugates manufactured with TT as carrier protein by a variety of manufacturers was obtained. The panel included Hib-TT-A and Hib-TT-B (coded as described by Ho et al. [14]; two MenC-TT conjugates and two MenA-TT conjugates (arbitrary codes were assigned); and, one of each of the following conjugates; MenW-TT, MenY-TT and Pneumo 18C-TT. The bulk purified carrier protein conjugated to MenC-TT (2), MenW-TT and MenY-TT was also included in the panel.

Prior to analysis, samples of bulk intermediate polysaccharide-protein conjugates supplied by vaccine manufacturers were dialysed at 4 °C with three changes of phosphate buffered saline (PBS 'A') (10.1 mM Na_2HPO_4, 1.84 mM KH_2PO_4, 171 mM NaCl, 3 mM KCl pH 7.3–7.5) for 24–26 h using dialysis membranes with a 10 kDa-molecular-mass cut-off pore size (Spectra/Por® 7 Dialysis Membrane, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA).

Polysaccharide loading ratios of mol polysaccharide repeating unit: mol tetanus toxoid monomeric unit were converted from the g PS:g ratios determined by the manufacturers.

2.2. Monoclonal antibodies

Four rat IgG monoclonal antibodies, TT04, TT07, TT08 and TT10, against tetanus toxoid were produced from rat hybridoma cell lines, provided by Wellcome Research Laboratories (Beckenham, U.K.). The hybridomas were prepared [15] and the antibodies characterised [15,16] as described previously.

2.3. Determination of protein content

For TT conjugates, protein concentration was determined using a molar absorption coefficient, $A_{278\ nm,\ 0.1\%}$ of 1.229 cm^{-1} mg^{-1} ml for TT [17]. Samples were analysed on a Perkin–Elmer Lambda 800 UV-VIS spectrophotometer. A data increment of 0.50 nm was used, scan speed was 125 nm/min, slit width was 1.00 nm and integration time was 0.24 s. A cell volume of 1.0 ml with 10 mm path-length was used. Following determination of protein concentration, the samples were diluted in PBS '*A*' to 500 μg protein/ml, where possible, for SEC-MALS and 6–25 μg protein/ml for fluorescence spectroscopy.

2.4. Determination of dn/dc

The specific refractive increment, *dn*/*dc*, was measured using an interferometric refractometer (Optilab rEX; Wyatt Technology Corp. Santa Barbara, CA, U.S.A.) which had been calibrated with five different concentrations of BSA in PBS 'A', ranging from 0.25 to 2.0 mg/ml. A series of five different sample concentrations ranging from 0.05 to 1.0 total mg/ml [(mg of saccharide + mg of protein)/ml, for conjugates], was prepared from dialysed sample and syringed through the refractometer, which had been equilibrated with filtered dialysis buffer, starting with the lowest concentration. For conjugate samples, the saccharide and protein concentrations were added together to give the sample concentration, *c* (mg/ml). *dn*/*dc* values (ml/g) were calculated using the software supplied by the manufacturer (DNDC™; Wyatt Technology Corp.). Percentage errors were determined by the Astra software as a combination of noise for each detector combined with the quality of the line projected [18].

2.5. Molecular sizing by SEC/MALS

The HPLC-SEC/MALS system consisted of a gradient pump (Thermo Fisher (Dionex) UK Ltd., Hemel Hempstead, UK) and autosampler fitted with a 200 μl injection loop. A Tosoh Bioscience TSK PWXL guard column and TSK gel $G6000_{PWXL}$ + $G5000_{PWXL}$ analytical columns were connected in series. Chromatographic signals were collected by an ICS series multi-wavelength UV detector (Thermo Fisher UK Ltd.) an interferometric refractometer (Wyatt Technology Corp., Santa Barbara, USA) and a DAWN-EOS 18-angle angle light scattering detector (Wyatt Technology Corp.). PBS 'A' ultra-filtered through 0.10 μm Millipore filter was used as eluent at a flow rate of 0.25 ml/min. Fifty μg protein (500 μg/ml where possible) was injected per sample and total mg protein + mg saccharide (by calculation) were used for subsequent calculations.

Data from 11 detectors between the angles of 57.0° and 141.0° were used and weight-average molar mass and % recoveries were determined using Astra for Windows 5.3.4™ software from Wyatt Technology Corp. The *dn*/*dc* values determined for each conjugate/carrier protein (Table 1) were used in calculations, with the exception of two different MenA-TT conjugates which gave unusually low *dn*/*dc* values. For these a *dn*/*dc* value of 0.191 ml/g was used [19]. The weight-average molecular mass (Mw) and polydispersity (Mw/Mn) were determined by the Zimm method and were obtained for high molar mass (peak 1), lower molar mass (peak 2) and total combined peak data (peak 3), also used for the purpose of determining the % recovery. Errors are calculated by the Astra software in the same way as described for the refractive index calculation.

2.6. Fluorescence spectroscopy

Intrinsic fluorescence spectra were obtained on a Horiba Jobin Yvon FluoroMax-4 spectrofluorometer in 1 ml, 1 cm path-length quartz cells. Excitation wavelengths (λ_{ex}) of both 280 and 295 nm were used, with a band pass of 4.25 nm for excitation and emission monochromators and data was collected at increments of 0.5 nm. Emission spectra were collected using a wavelength range from 260 to 550 nm (280 nm excitation) or from 275 to 550 nm (295 nm excitation). Fluorescence spectra were corrected by subtracting the corresponding base-line spectra of buffer alone (PBS 'A'). Samples of 6–25 μg protein/ml in PBS 'A' were used. Fluorescence