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Research paper

Development of four sandwich ELISAs for quantitation of capsular polysaccharides from *Neisseria meningitidis* serogroups A, C, W and Y in multivalent vaccines

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ABSTRACT

Neisseria meningitidis is a Gram negative bacterium that has been classified in 13 serogroups according to the biochemical composition of the capsular polysaccharide (CP). However, invasive infections are most frequently caused by six of these serogroups: A, B, C, W, X and Y (MenA, MenB, MenC, MenW, MenX, MenY). Individual CP quantitation in multivalent meningococcal CP-based vaccines is required for quality control testing of these products. In this regard, four sandwich enzyme-linked immunosorbent assays (ELISAs) were developed for the quantitation of CP. The quantitation and detection limits of the four ELISAs were below 1 ng/mL. The assays showed good reproducibility and repeatability as calculated for each point of the standard curve (CV < 15%). In addition, five multivalent meningococcal CP-based vaccines were evaluated and the proposed ELISAs showed that these vaccines were found into the accepted range (±30%) of CP content. These assays are suitable for screening multiple plain or conjugated meningococcal CP-based vaccines and could be useful for monitoring lot-to-lot consistency and stability analysis.

1. Introduction

Meningococcal disease, caused by *Neisseria meningitidis*, occurs mainly as either septicemia or meningitis, and is a worldwide health problem (Chang et al., 2012). *N. meningitidis* strains have been classified into at least 13 serogroups on the basis of the structure of the capsular polysaccharide (CP) (Harrison et al., 2009). However, invasive infections are most frequently caused by six of these serogroups: A, B, C, W, X and Y (MenA, MenB, MenC, MenW, MenX, MenY) (Stephens et al., 2007). Most meningococcal vaccines are monovalent or multivalent mixtures of either polysaccharides or polysaccharides conjugated to carrier proteins (Tan et al., 2010). Quantitative determination of the individual polysaccharide components in multivalent meningococcal vaccines is an important step in manufacturing and regulatory control (Cook et al., 2013a).

Several methods for individual quantitation of meningococcal polysaccharides have been described. Determination of total phosphorus content (Chen et al., 1956) has been employed for measurement of MenA and MenX, while sialic acid content (Svennerholm, 1957) can be used for MenC, MenW, and MenY. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been used for the quantitation of polysaccharides in multivalent meningococcal CP-based vaccines (Cook et al., 2013b). These methods can provide accurate and sensitive measurement of meningococcal

Abbreviations: CP, capsular polysaccharide; MenA, *N. meningitidis* serogroup A; MenC, *N. meningitidis* serogroup C; MenW, *N. meningitidis* serogroup W; MenY, *N. meningitidis* serogroup Y; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; MAbs, monoclonal antibodies; TT, tetanus toxoid; HRP, horseradish peroxidase; IC, internal controls; OD, optical density; 4PL, four-parameter logistic equation; QCs, quality control; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; CZE, capillary zone electrophoresis.

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polysaccharides but require prior conversion to monosaccharides by acid digestion. The use of strong acids, often at elevated temperature, represents a potential safety concern for laboratory staff and has a cost associated with material disposal (Lamb et al., 2005).

Simple and affordable techniques to quantitate polysaccharides in meningococcal vaccines are essential for the evaluation of antigen content and lot-to-lot consistency of manufacture. Immunological assays such as rate nephelometry or inhibition enzyme-linked immunosorbent assay (ELISA) may be used (WHO Expert Committee on Biological Standardization, 2006). In addition, sandwich ELISA is a common tool for antigen quantitation (Trad et al., 2011; Suzaki et al., 2006; Ma et al., 2013). Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. However, monoclonal antibodies (MAbs) recognize a single epitope that allows fine detection and quantitation of small differences in antigen. In a previous study, our group obtained MAbs for MenA, MenC, MenW and MenY CPs. The selectivity of these MAbs was confirmed for each CP, with no cross-reactivities to heterologous CPs, or carrier proteins like tetanus toxoid (TT) and CRM_{197} (Reyes et al., 2013). The aim of the present work was to develop four sandwich ELISAs using these MAbs to quantitate CPs from MenA, MenC, MenW and MenY in meningococcal CP-based vaccines.

2. Materials and methods

2.1. Reagents and buffers

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (USA). The following buffers were used: Coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), blocking buffer (PBS, 3% non-fat dried milk), washing buffer (PBS, 0.05% (v/v) Tween 20, pH 7.4), and substrate buffer (35 mM citric acid, 67 mM Na_2HPO_4, 0.012% (w/v) H_2O_2, pH 5.0).

2.2. MAbs

Murine MAbs against MenA, MenC, MenW and MenY CPs were produced and characterized as described previously (Reyes et al., 2013). Briefly, hybridomas were produced by fusion of SP2/O myeloma cell line with splenocytes of BALB/c mice that were immunized with subcutaneous injections of meningococcal polysaccharide group A conjugated to TT (MenAfriVac®, Serum Institute of India, Ltd.) and meningococcal polysaccharide groups A, C, W135 and Y conjugated to CRM_{197} (MENVEO®, Novartis, Switzerland) vaccines. Four hybridomas, coded as 7E1F7 (anti MenA CP), 7E12B3 (anti MenC CP), 5C11F1 (anti MenW CP) and 5H10D9 (anti MenY), were selected and grown as mouse ascites in the peritoneal cavity of pristane-primed BALB/c mice. The MAbs were purified from the ascites fluid by affinity chromatography using HiTrap Protein G (GE Healthcare, Germany) following manufacturer's instructions.

2.3. Peroxidase labeling of MAbs

Purified MAbs were conjugated in-house to horseradish peroxidase type VI (HRP) as described by Wilson (1978). Briefly, 8 mg of each MAb was coupled to 4 mg of HRP and was purified by gel filtration chromatography. Conjugates containing 1% BSA, 0.05% thiomersal and 50% glycerol were stored in small aliquots at −20 °C.

2.4. Calibrators, quality controls and calibration curve construction

In the absence of International Standards, calibrators consisted of purified CPs from MenA, MenC, MenW and MenY produced at Finlay Institute (Havana, Cuba) and used as internal controls (IC). MenC, MenW and MenY ICs were quantitated using resorcinol colorimetric assay (Chen et al., 1956) with N-acetylneuraminic as the standard while MenA IC was quantitated using Chen colorimetric assay (Svennerholm, 1957) using KH_2PO_4 as the standard. These ICs are supplied as lyophilized powders with 100 μg of each CP. A two-fold serial dilution of each IC was used to generate a six-point calibration curve with a range from 0.3125 to 10 ng/mL. GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for plotting the optical density (OD_{492}) against the CP concentrations and four-parameter logistic equation (4PL) was applied to obtain the function describing a sigmoid model. The quality controls (QCs) consisted of meningococcal CP powders (Finlay Institute, Havana, Cuba) which were weighted, dissolved in PBS and adjusted to a final concentration of 1.25 ng/mL (w/v). Calibrators and QCs were stored as single ready-to-use aliquots at −20 °C.

2.5. Description and preparation of vaccines

Three registered vaccines and two lots of two experimental vaccines were analyzed.

- MENVEO® vaccine (Novartis, Switzerland): Meningococcal polysaccharide groups A, C, W_{135} and Y conjugated to CRM_{197} (10 μg/dose of MenA CP and 5 μg/dose of each MenC, MenW and MenY CPs).
- MENCEVAX®ACWY vaccine (GlaxoSmithKline): Meningococcal polysaccharide groups A, C, W and Y (50 μg/dose of each CP).
- vax-MEN-ACW_{135}® (Finlay Institute, Cuba): Meningococcal polysaccharide groups A, C and W (50 μg/dose of each CP).
- ACW_{135}Y (lots 105, 106) and ACW_{135}XY (lots 101, 102) vaccines: Experimental vaccines (Finlay Institute, Cuba), meningococcal polysaccharide groups A, C, W, Y or A, C, W, X and Y (50 μg/dose of each CP).

All the vaccines were reconstituted as described by the manufacturers and were prepared to a final concentration of 1.25 ng/mL taking into account the standard curve range.

2.6. ELISA sandwich for serogroup quantitation

Polystyrene microwell plates (Maxisorp, Nunc, USA) were coated with 10 μg/mL of MAb 7E1F7, 7E12B3, 5C11F1 or 5H10D9 in coating buffer (100 μL/well). After overnight incubation at 4 °C, the coated wells were washed three times and blocked with blocking buffer for 1 h at 37 °C. The calibrators, QCs and samples were added (100 μL/well) and incubated 1 h at 37 °C. Subsequently, the wells were washed three times and peroxidase conjugated MAbs diluted to 1:15000 in washing buffer containing 1% non-fat dried milk were added to the wells

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