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Identification of the common antigenic determinant shared by *Streptococcus pneumoniae* serotypes 33A, 35A, and 20 capsular polysaccharides

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ABSTRACT

In order to better understand cross-reactions of serogroup 33 polysaccharides and the typing sera, the structure of pneumococcal capsular polysaccharide serotype 33A was elucidated. Serotype 33A has been shown to have an identical polysaccharide backbone as that of serotype 33F, with two additional sites of O-acetylation at C5, and C6 of the 3-β-Gal*f* residue in serotype 33A. This finding is consistent with the presence of an additional functional acetyltransferase gene (wcjE) in the cps biosynthetic locus of serotype 33A compared to 33F. The identical polysaccharide backbone with at least one common O-acetylation site (C2 of 5-β-Gal*f*) shared by serotype 33A and 33F polysaccharides is proposed to be the epitope recognized by typing serum 33b. In addition, a 5,6-di-O-acetylated →3)-β-D-Gal*f*5,6Ac-(1→3)-β-D-Glc*p*-(1→ disaccharide unit, a common structural motif present in serotypes 33A, 20, and 35A polysaccharides, is proposed to be the antigenic determinant recognized by typing serum 20b.

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1. Introduction

Streptococcus pneumoniae is a major human pathogen responsible for causing a wide spectrum of diseases ranging from relatively mild sinusitis and otitis media to more severe invasive infections including meningitis, septicaemia, and pneumonia. It is widely accepted that the expression of capsular polysaccharides (capsules) by *S. pneumoniae* is key for the pathogen's evasion of the host immune system, enabling the bacteria to survive within the human nasopharynx and the subsequent spread to and colonization of other organs. More than 90 pneumococcal capsule types (commonly referred to as serotypes) differing in the structures of their respective polysaccharide capsules have been identified based on their reactivity with reference sera.[1–3] Current pneumococcal vaccines elicit immune responses to the polysaccharide capsules. The use of polysaccharides and especially polysaccharide-conjugate vaccines has led to an almost complete reduction of vaccine serotype-specific invasive pneumococcal diseases worldwide.[4–6]

Capsular polysaccharide serotype 33F is included in the adult polysaccharide vaccine PNEUMOVAX 23®, and in a 15-valent polysaccharide-conjugate vaccine currently undergoing clinical development.[7] Serotype 33F belongs to serogroup 33, in which five distinct serotypes (33A, 33B, 33C, 33D, and 33F) have been identified based on reactivities with typing sera. Typing serum 33a reacts with all serogroup 33 polysaccharides,[8,9] however serotype 33A and 33F polysaccharides are the only members that also show reactivity with typing serum 33b.[8,9] Cross-reactivity of 33A and 33F polysaccharides with rabbit immune antisera was also observed in a multiplex-serotyping assay,[10] suggesting common structural motifs uniquely shared by 33A and 33F polysaccharides.

Whereas the chemical structure of serotype 33F polysaccharide is known,[11,12] the structure of serotype 33A has not been determined.[13] Genetic analysis of the *cps* biosynthetic loci for serotypes 33A and 33F has revealed high sequence relatedness between them.[1,9] Herein we present our findings on the structure of capsular polysaccharide 33A, and confirmation that the previously suggested →3)-β-D-Gal*f*5,6Ac-(1→3)-β-D-Glc*p*-(1→ disaccharide unit is the structural epitope shared by the serotype 20, 33A, and 35A polysaccharides, and that this disaccharide is recognized by typing serum 20b.

2. Experimental

Purified serotype 33A and 33F polysaccharides were purchased from Statens Serum Institut (SSI). Serotype 33F polysaccharide was also obtained from the American Type Culture Collection (ATCC).

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To generate de-O-acetylated polysaccharide samples, approximately 5–10 mg of polysaccharides were dissolved in 1 mL of 0.2 N NaOH solution and incubated at room temperature for two hours, followed by repeated dialysis using 3000 MWCO regenerated cellulose dialysis tubing (Spectrum Laboratories) against distilled water to remove residuals.

2.1. Monosaccharide composition analysis by high performance anion exchange chromatography with pulsed-amperometric detection (HPAEC-PAD)

Serotype 33A and 33F polysaccharide samples (20–50 μg) and a 1:1 Glc/Gal monosaccharide standard (50 μg of each) were subjected to acid hydrolysis with 2 N TFA at 115 °C for 4 h. The hydrolysates were concentrated by heating at 40 °C for 3 days under a stream of air until samples were completely dried, and then redissolved in 500 μL of deionized water. The samples were then diluted 10-fold, and injected onto a Dionex HPAEC-PAD instrument for monosaccharide composition analysis. Monosaccharides were separated on a Dionex CarboPac PA1 analytical column and eluted with a linear gradient of 15 mM NaOH/0 mM NaOAc to 100 mM NaOH/150 mM NaOAc in 40 min. The relative peak responses of the untreated 1:1 Glc/Gal standard were compared to those of the TFA-treated 1:1 Glc/Gal standard. It was determined that, under the hydrolysis conditions used, the peak response of Gal is approximately 25% lower than that of Glc, and this was taken into account when determining the monosaccharide composition of 33A and 33F polysaccharides.

2.2. Linkage analysis by methylation/gas chromatography–mass spectrometry (GC–MS)

The serotype 33A and 33F polysaccharide samples from SSI were derivatized to the corresponding partially-methylated alditol acetates (PMAAs) based on the method of York.[14] Dry polysaccharide samples were resuspended in DMSO and stirred for 2 days to fully dissolve. The samples were then permethylated using iodomethane according to the method of Ciucanu and Kerek.[15] The permethylated material was hydrolyzed to partially methylated monosaccharides, reduced with sodium borodeuteride, and acetylated to PMAAs with acetic anhydride and trifluoroacetic acid. The PMAAs were analyzed on an Agilent 7890A GC interfaced to a 5975 mass selective detector (MSD, electron-impact ionization mode); separation was performed on a 30 m Rtx 2330 fused-silica capillary column using the following temperature gradient program: 80 °C for 3 min, 10 °C/min to 140 °C, 30 °C/min to 170 °C, 2.5 °C/min to 210 °C, then 5 °C/min to 240 °C and held for 10 min at 240 °C.

2.3. NMR spectroscopy

NMR experiments were carried out on a Varian INOVA 500 MHz (^{1}H) spectrometer with a 3 mm gradient probe at 25 °C with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C) using standard pulse sequences: DQCOSY, TOCSY (mixing time 120 ms), ROESY (mixing time 500 ms), gHSQC and gHMBC (100 ms long range transfer delay). AQ time was kept at 0.8–1 s for H–H correlations and 0.25 s for HSQC, 256 increments were acquired for t1.

2.4. Capillary electrophoresis–mass spectrometry (CE–MS)

CE–MS analysis[16] was performed using a 4000 Q-Trap mass spectrometer (Applied Biosystems/Sciex, Concord, Ontario, Canada) via a CE–MS interface with 90 cm length of bare fused-silica capillary using 15 mM ammonium acetate in deionized water, pH 7.0. A sheath solution (isopropanol–methanol, 2:1) was delivered at a flow rate of 1.5 uL/min. The orifice voltage was set at −110 V. For MS/MS analysis, fragment ions formed by collision activation of selected precursor ions with nitrogen in the RF-only quadrupole collision cell and were recorded using a time-of-fight mass analyzer. For pseudo MS/MS/MS analysis, the precursor ions were generated with an orifice voltage of +120 V and mass spectra were acquired with nitrogen in the RF-only quadrupole collision cell.

3. Results

3.1. Monosaccharide composition and linkage analysis of serotype 33A polysaccharide

Both HPAEC-PAD and GC–MS analyses demonstrated that 33A and 33F polysaccharides are composed of galactose and glucose in approximately a 5:1 ratio. This is consistent with the literature report for the structure of 33F polysaccharide.[11,12] Furthermore, linkage analysis of both polysaccharides by GC–MS revealed that linkages of 33A polysaccharide are identical to that of 33F.

3.2. NMR analysis

Full assignment of all ^{1}H and ^{13}C resonances in native 33A polysaccharide was accomplished using a combination of standard 1D and 2D NMR homo- and heteronuclear correlation experiments and the ^{1}H and ^{13}C chemical shift data are summarized in Table 1. Residues are labeled in the order A–F as depicted in Figure 1. In the ^{1}H spectrum of 33A polysaccharide, several resonances of unequal intensities in the anomeric region are observed, some of which are also observable in the ^{1}H spectrum of 33F polysaccharide when the two spectra are overlaid (Fig. 2). As in the case of 33F polysaccharide, this feature is indicative of heterogeneity in the levels and locations of O-acetylation in the polysaccharide chain. Monosaccharide compositions determined by HPAEC-PAD and GC–MS were also confirmed by COSY, TOCSY, and NOESY cross peak patterns and ^{13}C NMR chemical shifts. The low field position of the C1–C4 carbon resonances in residues C and E indicates a five-membered ring furanose configuration. In addition, data from the GC–MS analysis indicate that these are galactofuranoses and the chemical shifts of their C-1 resonances (>108 ppm) are consistent with these residues having the β-anomeric configuration.[17] Among the four remaining pyranose residues, two of which (residues A and D) are assigned the β-anomeric configuration based on the large $J_{1\text{-}2}$ proton coupling constants (8 Hz), where residue A was assigned as β-galactopyranose and residue D was assigned as β-glucopyranose. The other two (residues B and F) were assigned α-anomeric configuration. These α-pyranose residues (B and F) have overlapping H-2 and H-3 signals, but their carbon chemical shifts suggest they are not furanosides,[18] consistent with GC–MS linkage analysis. They were assigned the *galacto*-configuration (instead of *gluco*-configuration) based on the singlet appearance of their H-4 signals.

Connections between monosaccharides were determined from transglycosidic NOE correlations. The following transglycosidic NOE correlations were observed: B1:C3; B1:F1,2,5; E1:A1,2,3,4; C1:D3; F1:B2; F1:C3,5; A1:B3; and D1:E5. Arrangement of the substituents at α-Gal*p* (residue B) was not obvious because of the overlapping H-2 and H-3 proton signals in residue B. The conclusion that the terminal α-Gal*p* (residue F) is linked to the 2 position of α-Gal*p* (residue B) was deduced from the observation of the B1:F1 NOE correlation. Moreover, the linkage between the β-Gal*p* (residue A) to the OH-3 of α-Gal*p* (residue B) was supported by the strong A1:B4 NOE cross peak, in addition to A1:B3. The deduced linkage pattern for the 33A polysaccharide repeating unit based on NMR data is consistent with the GC–MS data, which is identical to that of the previously-reported serotype 33F

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