Structural and Immunochemical Characterization of the Type VIII Group B Streptococcus Capsular Polysaccharide*

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The type VIII capsular polysaccharide has been isolated and purified from a newly described strain of group B Streptococcus which is a leading cause of sepsis and neonatal meningitis in Japan. The polysaccharide contains D-glucose, D-galactose, L-rhamnose, and sialic acid in the molar ratio 1:1:1:1. By means of high resolution 1H nuclear magnetic resonance (1H NMR), 13C NMR, and homo- and heterocorrelated NMR, the repeating unit structure of the type VIII polysaccharide was delineated as the following:

\[
\begin{align*}
\rightarrow 4\beta-D-GlcP(1 \rightarrow 4\beta-D-GalP(1 \rightarrow 4\beta-L-Rhap(1 \rightarrow \\
\rightarrow 2\alpha-NeuAc
\end{align*}
\]

** Structure 1

Enzymatic studies established this polysaccharide as the first from which sialic acid, linked to a branched \(\beta-D\)-galactopyranosyl residue, is known to be removed by bacterial neuraminidase.

The group B Streptococcus (GBS)1 has long been recognized as a major cause of neonatal sepsis and meningitis (1, 2). GBS strains are classified into serotypes on the basis of their type-specific capsular polysaccharides. The strains isolated from clinical cases usually belong to one of the major capsular types (Ia, Ib, II, and III) (2), but five new serotypes have recently been described: IV, V (3), VI (4), VII, and VIII. Type VIII (originally designated type M9 (5)), while not isolated in North America, has been identified with increasing frequency over the last six years among disease-causing isolates in Japan, where it is now a prevalent strain (5).

The structures of the capsular polysaccharides of GBS types Ia, Ib, II, III (6), IV (7), V (8), VI (9), and VII (10) have been elucidated. Despite their structural relatedness they are largely distinct immunologically. We report the isolation, structural analysis, and immunochemical characterization of the type VIII GBS capsular polysaccharide which like all the other type-specific GBS polysaccharides contains terminal sialic acid.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Isolation of the Type VIII Capsular Polysaccharide—Type VIII GBS strains M9 Prague No. 130013 and M9 Prague No. 130672 were kindly provided by Dr. J. J. jčinková, Institute of Hygiene and Epidemiology, Prague, Czech Republic. Strain 130013 was used in the production and purification of the type VIII capsular polysaccharide. GBS strains used in adsorption studies, which had been maintained at -70 °C in cultures at the Channing Laboratory, included: 909 (type Ia), H36B (type Ib), 18RS21 (type II), M781 (type III), M15 (capsular type III), 3139 (type IV), 1169 (type V), and SS1214 (type VI). GBS strain 130013 was grown in a 16-liter fermenter and the type VIII polysaccharide was isolated and purified by methods utilized previously for other GBS polysaccharides (11).

Analytical Methods—The relative molecular weight of the purified type VIII polysaccharide was determined by gel filtration chromatography on a Superose 6 column (Pharmacia) calibrated with dextran standards. The identity and the immunospecificity of the polysaccharide were tested in double diffusion (Ouchterlony) assay and the competition ELISA, respectively, with use of GBS type-specific antisera.

The glycose composition of purified polysaccharide was assessed by gas-liquid chromatography of component alditol acetates prepared by acid hydrolysis of capsular polysaccharide, as described below in the section dealing with the determination of the absolute configuration of the component monosaccharides. Total carbohydrate content was determined by the phenol-sulfuric acid assay (12) with galactose used as the standard. Purified capsular polysaccharides were analyzed for protein by the method of Larson et al. (13) and were studied spectrophotometrically at \(A_{280}\) for nucleic acids. The presence and quantity of sialic acid were determined by the method of Warren (14) after hydrolysis of the purified polysaccharide with 3% acetic acid (v/v) at 80 °C for 1 h.

The absolute configurations of the monosaccharides were determined by a modification of the method described by Gooi et al. (15). The polysaccharide sample (1 mg) was hydrolyzed with 0.25 M sulfuric acid at 100 °C for 20 h, and the hydrolyzate was neutralized with barium carbonate. The sediment was removed by centrifugation and the supernatant was freeze-dried. The lyophilized residue was dissolved in 0.5 ml of (−)-2-butan, 1 drop of trifluoroacetic acid was added, and the mixture was heated at 80 °C for 16 h. The solvents were removed by evaporation in vacuum and the dried sample was trimethylsilylated using 0.5 ml of N-O-bis(trimethylsilyl)acetamide (Pierce) and 0.5 ml of pyridine. The trimethylsilylated (−)-2-butyglycosides of the constituent monosaccharides were analyzed by capillary gas-liquid chromatography using a Varian Saturn II GC-MS instrument equipped with a DB-17 capillary column (0.25 mm x 30 m, film thickness 0.25 μm) in the temperature program 150 to 210 °C at 2°C/min.
Preparation of the Chemically Modified Type VIII Polysaccharide—

Desialylated type VII polysaccharide was prepared by hydrolysis of the native polysaccharide with 1% acetic acid at 80 °C for 1 h. After dialysis, the contents of the dialysis bag were freeze-dried. Carboximidation reactions and periodate oxidation of the terminal sialic acid residues in the native type VII polysaccharide were conducted as described by Jennings et al. (16). If required, the modification procedure was repeated several times until a completely derivatized product was obtained, as confirmed by NMR spectroscopy and mass spectrometry.

Enzymatic Methods—The relative susceptibilities of the GBS type II, type III, and type VIII polysaccharides to the action of neuraminidase (type II from Vibrio cholerae, EC 3.2.1.18, Sigma) were determined in a following manner: 1 ml of a solution of each polysaccharide (1 mg/ml) was treated with 5 milliunits of neuraminidase at 37 °C for 48 h. A 250-μl volume of each sample was then removed and, after the addition of 36 μl of glacial acetic acid (final concentration, 6%) was hydrolyzed for 1 h at 80 °C. The amount of sialic acid released by each treatment was quantified in the thiorbituric acid assay, as described by Skoza and Mohas (17). In other experiments, 1-ml volumes of solutions of the polysaccharides (1 mg/ml) were treated with 5 or 10 milliunits of neuraminidase at 37 °C; 200-μl aliquots were collected after 8, 24, 32, 48, and 120 h; and the amount of liberated sialic acid was determined in the thiorbituric acid assay.

Instrumental Methods—All NMR experiments were performed on a Bruker AMX 600 spectrometer using a 5-mm broad band probe with 1H coil nearest to the sample. 1H and 13C NMR spectra were recorded at 330 K in D2O at pH 7.0. Acetone was used as internal standard, with the CH3 resonance at 31.07 ppm for 13C spectra and 2.225 ppm for 1H spectra. The experiments were conducted without sample spinning. Two-dimensional homo- and heterocorrelated experiments (COSY, TOCSY, NOESY, HMQC) were carried out as described previously (18). HMQC-TOCSY experiment was performed according to Lerner and Bax (19) and 1H-detected multiple-bond correlation experiment was carried out by the method of Bax and Summers (20). Spin-echo Fourier transformation experiment was conducted using an interleaved decoupling during the spin-echo period (21).

Antiserum—Type-specific antisera to polysaccharides of GBS types Ia, Ib, II, and III were obtained by the immunization of New Zealand White rabbits with GBS polysaccharide-tetanus toxoid conjugate vaccines (22–24). Formalin-killed GBS cells of serotypes IV, V, VI, and VIII (strain J M9 Prague No. 130013) were used as whole cell immunogens for the preparation of rabbit antiserum by the method of Lancefield et al. (25). Rabbit antiserum to Streptococcus pneumonia type 14 was prepared by the Staten Serum Institut (Copenhagen) and obtained through Dako Corp. (Santa Barbara, CA).

Serotype Specificity of Rabbit Antiserum to Strain 1300013—Whole GBS type VIII polysaccharide purified from a broth culture (10 mg/ml) were used to evaluate the specificity of type VIII antiserum. Log phase cells (1.0 ml; approximately 1010 colony-forming units/ml) were added to rabbit antiserum to type VIII strain 130013 (0.1 ml; diluted 1:10). After 60 min of incubation at 37 °C, serum was clarified by centrifugation, adjusted to a total volume of 2 ml with 0.9 ml of phosphate-buffered saline containing 0.01% NaN3, and sterilized by filtration (filter pore diameter, 0.45 μm). Adsorbed serum (initial dilution, 1:200) was added to a 96-well microtiter plate, each well of which was coated with 100 μg of purified type VIII capsular polysaccharide linked to poly-L-lysine (2); this preparation was processed in a standard ELISA, with alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:3,000) as the secondary antibody. Plates were developed for 60 min after addition of the substrate, and A405 was measured with an ELISA reader (Biotek, Winooki, VT). Values were expressed as the percentage of type VIII-specific antibodies adsorbed relative to quantities of antibody in an unadsorbed antiserum control.

Competition ELISA—The relative affinities of type VIII antiserum for native, desialylated, and chemically modified type VIII polysaccharides were evaluated in a competition ELISA. Polysaccharide inhibitors were subjected to serial 2-fold dilution, mixed with equal volume (100 μl) of type VIII antiserum (diluted 1:400), and added to type VIII polysaccharide-coated ELISA wells. The latter antiserum, kindly provided by Dr. Patricia Ferrieri of the University of Minnesota, was adsorbed to remove GBS C-protein activity. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as the secondary antibody at a dilution of 1:3,000, and the plate was incubated at 37 °C for 60 min. Results are expressed as follows: % inhibition = [(Asig with inhibitor – A200 with inhibitor)/Asig without inhibitor] × 100.

RESULTS

Structural Analysis of GBS Type VIII Polysaccharide—The type VIII capsular polysaccharide preparation was characterized for carbohydrate composition, molecular size, immunological identity, and impurities. Compositional analysis of the type VIII polysaccharide showed that it contained l-rhamnose, D-glucose, D-galactose, and N-acetylneuraminic acid (sialic acid) in 1:1:1:0.9 molar ratio. The sialic acid content was 30% (as determined in the thiorbituric acid assay) and the Mw was 200,000 (as determined by gel filtration chromatography). In a double diffusion immunoelectrophoresis, type VIII polysaccharide formed a single line of identity with rabbit antiserum to whole cells of GBS type VIII strain 130013. Type VIII antiserum did not cross-react with purified capsular polysaccharides of types Ia, Ib, and II through VI. The protein content of the purified polysaccharide was < 3% (w/w). Spectrophotometric analysis of purified type VIII capsular polysaccharide (1 mg/ml) showed a strong absorbance at 206 nm that is characteristic of carbohydrate; absorbances at 260 and 280 nm were indicative of low residual levels of nucleic acid and protein, respectively. That the type VIII GBS polysaccharide was composed of the tetrasccharide repeating units was deduced from its 1H NMR spectrum (600 MHz, 330 K) (Fig. 1) which contained three anomer signals at 4.857 (unresolved doublet), 4.825 (J1,2 = 7.3 Hz), and 4.740 ppm (J1,2 = 7.4 Hz) in the ratio 1:1:1, as well as the signals at 2.734 and 1.822 ppm of the equatorial and axial H-3 of α-linked N-acetyl-D-neuraminic acid, respectively, whose integration was consistent with the presence of one sialic acid residue in the repeating unit. The presence of a tetrasccharide repeating unit was also confirmed by analysis of the 13C NMR spectrum of the polysaccharide (150 MHz, 330 K) which exhibited only three signals in the anomeric region at
Type VIII Group B Streptococcal Capsular Polysaccharide

The structure of the native (top) and desialylated (bottom) type VIII polysaccharide antigens of GBS.

104.49 (1C), 103.41 (1C), and 101.38 ppm (2C), but the last signal was shown to contain two overlapping signals as they could be resolved by a spin-echo Fourier transformation experiment. The signal at 101.28 ppm was assigned to the anomeric carbon of the β-L-rhamnopyranosyl residue, while the other one was at 101.40 ppm originates from a quarternary carbon, and was thus assigned to C-2 of sialic acid. In addition, signals at 174.29, 52.63, and 39.37 ppm were observed that correspond to C-1, C-5, and C-3 of the sialic acid, as well as those at 175.83 and 22.87 ppm due to its N-acetyl group.

The assignment of the 1H and 13C NMR signals was performed using homocorrelated two-dimensional COSY, TOCSY, and NOESY techniques, as well as the heterocorrelated HMQC and HMQC-TOCSY methods. The hexose components of the native repeating unit shown in Fig. 2 were designated a, b, and c according to the sequence of their anomic signals in the 1H NMR spectrum. N-Acetylneuraminic acid was designated as unit d. The location of the H-2 signal of unit b at 3.31 ppm (doublet of doublets with large diaxial couplings 3 J = 7.3 Hz, 5 J = 9.6 Hz) unambiguously characterized unit b as being β-D-glucose. A small coupling constant (1,2 3 J = 3 Hz) and very low-field resonance for H-4, together with a large value of 1,2 5 J coupling for H-3c, characterized unit c as β-D-galactose. By elimination, the remaining unit a was assigned to be β-D-rhamnose. Because of the mannopyranosyl configuration of the rhamnose ring, it was impossible to determine its anomic configuration on the basis of 2,5 J value. However, NOESY experiments (Table I) showed that the anomeric protons of all the sugar units had cross-peaks with their respective H-3 and H-5 resonances. This finding indicated that the rhamnopyranosyl residue had the β-anomeric configuration as well. The complete assignment of the 1H and 13C NMR signals of the native and desialylated type VIII GBS polysaccharides are presented in Tables II and III, respectively.

The sequence of monosaccharides in the repeating unit of both the native and desialylated type VIII GBS polysaccharides was established from separate analyses of their two-dimensional-NOESY spectra (18), and the NOE data (Table I) are consistent with their structures shown in Fig. 2. Independent confirmation of the sequence of monosaccharides in the repeating unit of the desialylated polysaccharide was also obtained using an 1H-detected multiple-bond correlation experiment which showed cross-peaks between C-1a and H-4c, C-1b and H-4a, as well as between C-1c and H-4b. These long-range correlations are in agreement with the substitution pattern established by the NOESY experiment. Additional evidence for the position of sialylation (O-3) to galactose was also obtained from the downfield displacements exhibited by the H-3 (Table II) and C-3 (Table III) signals of galactose on desialylation of the native type VIII polysaccharide.

Immunological Properties—Rabbit antiserum to whole type VIII cells was determined by adsorption experiments to be specific for GBS type VIII strains. Type VIII strains 130013 and 130672 bound >90% of type VIII polysaccharide-specific antibodies, whereas <30% of type VIII polysaccharide-specific antibodies were adsorbed with strains of other GBS serotypes.

Competition ELISA revealed that rabbit antiserum to whole cells of type VIII strain 130013 had high affinity for the native type VIII polysaccharide. The concentration of native type VIII polysaccharide that resulted in a 50% inhibition of the binding of type VIII-specific antiserum was 2.8 μg (Fig. 3). Desialylated, periodate oxidized/borohydride reduced, or carbodiimide-reduced type VIII polysaccharide failed to inhibit binding of type VIII-specific antiserum even when used at a concentration of 0.5 mg (Fig. 3).

Susceptibility to Neuraminidase—The susceptibility of sialic acid to removal by neuraminidase or acid treatment varies among the known GBS type-specific polysaccharides. Unlike the others, type II polysaccharide is resistant to removal of sialic acid by neuraminidase. This resistance of the type II polysaccharide to neuraminidase cleavage has been believed to be a result of the unique position of sialic acid directly linked to galactose in the backbone. The sialic acid residue in the proposed type VIII polysaccharide structure is similarly situated. However, in contrast to the type II polysaccharide, the rate of release of free sialic acid from the type VIII and type III polysaccharides was similar over a 120-h period (Table IV). As previously shown, the type II polysaccharide remained resistant to neuraminidase digestion. The optimal specific activity of neuraminidase resulting in >80% release was 5 milliunits/mg of type III polysaccharide and 10 milliunits/mg of type VIII polysaccharide.

DISCUSSION

The structure of the type VIII polysaccharide represents a different motif from previously studied capsular polysaccharides of GBS. Capsular polysaccharides of types Ia, Ib, II, III, IV, V, and VII are all composed of α-D-galactose, α-D-glucose, N-acetyl-α-D-glucosamine, and N-acetylneuraminic acid and contain complex repeating units built from five to seven monosaccharides. The type VIII polysaccharide is not only the first GBS capsular polysaccharide composed of tetrasaccharide repeating units, but is also unique in that β-D-rhamnopyranosyl residues replace the 2-acetamido-2-deoxy-β-D-glucopyranosyl residues found in all other GBS type-specific polysaccharides except type VI (9). The overall structure of the type VIII polysaccharide most closely resembles that of the type II polysaccharide (26) in that the α-D-galactopyranosyl residues are situated in the backbone rather than in the side chains, the latter being the case in all other types (6-10). Like all the other GBS capsular polysaccharides, the type VIII polysaccharide has terminal sialic acid residues linked to O-3 of α-D-galactopyranosyl residues.

Although it shares structural features with other GBS polysaccharides, the type VIII polysaccharide is antigenically distinct. No significant cross-reactions were detected by type VIII-specific ELISA after adsorption of type VIII-specific rabbit antiserum with GBS organisms of heterologous serotypes. The lack of cross-reactions among the GBS polysaccharides (7, 8, 28) indicates that sialic acid is not, and by itself, an immu-
nodominant epitope. The failure of sialic acid to be immunodominant might be expected in light of its ubiquity in human and animal tissues (29). However, it is only "non-immunodominant" in the classical sense, that is, not being a direct epitope for antibody binding, but its presence is still crucial to the immunospecificity of antibody raised to the native type VIII polysaccharide. This was demonstrated by the inability of desialylated or otherwise chemically modified type VIII polysaccharide to bind to a significant extent to type VIII-specific antibodies. The involvement of sialic acid in the formation of an immunospecific epitope has also been reported for GBS serotypes Ia, II, III, and VI (4, 28), and hypothesized that the explanation for this specificity lies in the ability of sialic acid to exert conformational control over the epitopic expression of these polysaccharides.

![Fig. 3. ELISA inhibition of GBS type VIII rabbit antiserum with native (closed circles), carbodiimide-reduced (open circles), periodate-oxidized/borohydride-reduced (closed squares), and desialylated (open squares) type VIII polysaccharides. Values are the mean of duplicate determinations.](image)

The epitopic expression based on extended helical domains of polymers of α-(2→8)-polysialic acid has been well documented (28, 30, 31). A similar explanation could apply to the epitopic expression of some of the GBS polysaccharides; in the case of the type III polysaccharide, the chain-length dependence (number of repeating units) of the epitope, which is a requirement for extended helical epitopes (28, 32), has been established (33).

### Table II

| Sugar residue | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 |
|---------------|-----|-----|-----|-----|-----|-----|
| PS            | 4.857 | 4.10 | 3.82 | 3.62 | 3.48 | 1.371 |
| dsPS          | 4.854 | 4.08 | 3.82 | 3.62 | 3.50 | 1.374 |
| PS            | 4.825 | 3.31 | 3.68 | 3.60 | 3.52 | 3.92,3.80 |
| dsPS          | 4.656 | 3.39 | 3.66 | 3.61 | 3.50 | 3.95,3.81 |
| PS            | 4.740 | 3.67 | 4.20 | 4.15 | 3.71 | 3.78,3.71 |
| dsPS          | 4.669 | 3.61 | 3.77 | 4.16 | 3.71 | 3.83,3.77 |
| α-NeuAc-(2 → PS) | 1.822(ax) | 3.71 | 3.81 | 3.61 |

### Table III

| Sugar residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---------------|-----|-----|-----|-----|-----|-----|
| PS            | 101.28 | 71.39 | 73.59 | 82.13 | 71.54 | 17.71 |
| dsPS          | 101.32 | 71.50 | 73.62 | 82.12 | 71.65 | 17.78 |
| PS            | 103.41 | 74.31 | 76.44 | 74.88 | 75.10 | 61.92 |
| dsPS          | 104.42 | 74.57 | 76.43 | 77.75 | 75.47 | 61.90 |
| PS            | 104.94 | 71.07 | 76.22 | 75.67 | 75.20 | 61.46 |
| dsPS          | 104.69 | 72.95 | 74.14 | 77.99 | 75.34 | 61.51 |
| α-NeuAc-(2 → PS) | 174.29 | 101.40 | 39.37 | 69.20 | 52.63 | 73.78 |

### Table IV

| Time of incubation | Percentage of sialic acid release with GBS capsular polysaccharide |
|--------------------|---------------------------------------------------------------|
| h                  | Type II | Type III | Type VIII |
| 8                  | 0       | 66.2     | 71.4     |
| 24                 | 0       | 76.9     | 76.2     |
| 48                 | 0       | 79.4     | 100      |
| 120                | 0       | 100      | 100      |

*Neuraminidase (10 milliunits/mg of polysaccharide).*
displacements may also be attributable to deshielding of the anomeric carbon and proton of the β-D-glucopyranosyl residue by the carboxylate group of the terminal sialic acid.

GBS type II polysaccharide and G₉M₁ ganglioside (II³NeuAcGgOse₄Cer) are known to have in their structures terminal β-D-glucopyranosyl residues that are linked directly to O-3 of a branched β-D-galactopyranosyl residues that are resistant to treatment with neuraminidase (26, 34, 35). This resistance has been attributed to steric hindrance of the enzyme because sialic acid is attached directly to O-3 of the backbone β-D-galactopyranosyl residues that have vicinal substituents in position O-2 or O-4 (26). Although the type VIII polysaccharide also exhibits the above structural features, its terminal sialic acid is readily removed with neuraminidase. Obviously this effect is dependent on specific structural features surrounding β-D-galactopyranosyl residues of the type VIII polysaccharide. In this region type VIII polysaccharide differs in structure from both type II polysaccharide and G₉M₁ ganglioside; the most pronounced difference is that its backbone β-D-galactopyranosyl residue is linked glycosidically to a unique β-L-rhamnopyranosyl residue. It is interesting to speculate that the enzymatic source for the biosynthesis of the rhamnose substitution in the type VIII polysaccharide may have been from the same enzymes used by all GBS to synthesize the common group B-specific cell wall associated polysaccharide which consists of β-L-rhamnose, β-D-galactose, β-D-glucitol, and N-acetyl-β-D-glucosamine arranged in a complex multiantennary structure of four structurally distinct oligosaccharides (27).

All GBS polysaccharides whose structures have been elucidated possess side chains that are composed of or terminate with sialic acid. Sialic acid has been shown to be a critical virulence component (36) of these organisms by limiting the deposition on cells of C3b for opsonization in the absence of specific antibodies (37). That this distinguishing characteristic has been maintained on all capsular polysaccharides of GBS isolated from human sources, including type VIII, emphasizes the importance of this sugar in GBS pathogenesis.

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