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Isolation and Characterization of Type IV Group B *Streptococcus* Capsular Polysaccharide

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An antigenically distinct serotype, type IV, has recently been added to the recognized serotypes of group B streptococci (GBS). We isolated and purified the capsular polysaccharide antigen from a prototype type IV GBS strain. The type IV capsular polysaccharide formed a precipitin line with rabbit antiserum to type IV GBS organisms but not with antiserum to organisms of GBS serotype Ia, Ib, II, or III. Enzyme-linked immunosorbent assay inhibition experiments showed no cross-reaction between type IV antiserum and other GBS serotypes. Capsular polysaccharide released from the bacterial cells with mutanolysin and that isolated from the culture supernatant had similar elution profiles on Sepharose CL6B, with a K_{av} of 0.30 and an estimated M_r of 200,000. The purified type IV polysaccharide was found to contain galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) as exclusive sugars. The polysaccharide contained 23% (by weight) sialic acid and galactose, glucose, and *N*-acetylglucosamine in a relative ratio of (1):1.10:0.55. These results are compatible with a repeating structure of six monosaccharide residues containing galactose, glucose, *N*-acetylglucosamine, and sialic acid in a molar ratio of 2:2:1:1. Unlike type Ia, II, and III GBS polysaccharides, desialylation of the type IV polysaccharide produced an antigen which formed a line of identity with the native type IV antigen in double diffusion in agar against homologous antiserum. This result suggests that sialic acid is not as critical to the immunodeterminant structure of the type IV antigen as it is for other GBS capsular types.

Group B streptococci (GBS) are a leading cause of neonatal sepsis and meningitis. These organisms have been classified into serotypes based on antigenic differences in their capsular polysaccharides and the presence or absence of c protein antigen (8, 16, 17, 28). Most GBS isolates from human neonatal infections bear one of the four major capsular types Ia, Ib, II, and III. Recently, however, a number of previously nontypable GBS isolates have been identified as belonging to an antigenically distinct serogroup, type IV (9, 10, 21). Here we report the isolation, purification, and immunochemical characterization of the type IV GBS capsular polysaccharide.

The previously described GBS capsular polysaccharides have all been found to consist of repeating polymers containing galactose, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) (11, 12, 27). While the precise structure of each of the capsular polysaccharides is distinctive, they share the common feature of having a relatively simple oligosaccharide repeating structure of five monosaccharide residues for types Ia, Ib, and III and seven residues for type II. In addition, sialic acid is present as a terminal side-chain residue in the repeating structure of each of these antigens. In the type Ia, II, and III polysaccharides, the sialic acid residues have been shown to play an important role in the immunodeterminant structure of the polysaccharide, with the desialylated or core polysaccharides forming immunologically incomplete antigens, as demonstrated by agarose double-diffusion experiments and by quantitative precipitation studies using antiserum absorbed with native or core polysaccharide (12, 13, 24). In contrast, the immunodeterminant structure of the Ib polysaccharide does not appear to be affected by desialylation, with the core polysaccharide showing a line of identity with the native polysaccharide in double-diffusion experiments using rabbit antiserum (24, 25).

Our studies of the type IV GBS capsular polysaccharide show that this antigen is composed of the same monosaccharides as the other capsular types, although the ratio of component sugars is unique. Sialic acid is present in the type IV polysaccharide, but interestingly, it appears not to be critical to the immunodeterminant structure of the native antigen as recognized by immune rabbit serum. No cross-reactivity was detected between the type IV polysaccharide and the previously described GBS capsular antigens.

MATERIALS AND METHODS

Bacterial strains. Type IV GBS strain 3139 (Czechoslovak National Collection of Type Cultures strain 1/82) was kindly donated by J. Jelínková, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia, and was used for preparation of type IV capsular polysaccharide and production of type-specific antiserum. The other GBS strains used included 090 (type Ia), H36b (type Ib), 18RS21 (type II), and 090R (unencapsulated variant of strain 090), all originally from the collection of Rebecca Lancefield, The Rockefeller University, New York, N.Y., and strain M732 (type III), provided by Carol Baker, Baylor University School of Medicine, Houston, Tex.

Preparation of capsular polysaccharide. A 500-ml overnight culture was used to inoculate 16 liters of a dialysate (10,000-M_r membrane; Pellicon Cassette System; Millipore Corp., Bedford, Mass.) of Columbia broth (Difco Laboratories,

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Detroit, Mich.) containing 80 g of glucose per liter. Culture was performed in a 20-liter fermentor (Biolafitte, Poissy, France) for 22 h at 37°C with continuous stirring at 150 rpm, aeration at 10 liters/min, and pH titration to 7.1. The bacterial cells were recovered by centrifugation and washed twice with phosphate-buffered saline (PBS), pH 7.4. The pellet (133 g, wet weight) was suspended in 750 ml of 30 mM potassium phosphate buffer, pH 7.0, containing 40% (wt/vol) sucrose and 10 mM $MgCl_2$ and treated with 10,000 U of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) in a modification of previously described procedures (2, 29). After incubation at 37°C for 16 h, the supernatant was recovered following centrifugation and dialyzed extensively against water. The dialyzed supernatant was concentrated to 400 ml (PM 30 membrane; 30,000-M_r cutoff; Amicon Corp., Danvers, Mass.) and treated with 2 mg of DNase (Sigma) and 10 mg of RNase (Sigma) at 37°C for 2 h and again at 37°C overnight. Pronase (20 mg) was added for 2 h at 37°C and again at 37°C overnight. The solution was concentrated to 25 ml, dialyzed against water, and loaded onto a column (2.5 by 30 cm) containing DEAE-Sephacel equilibrated in 10 mM Tris, pH 8.3. After being washed with 500 ml of the same buffer, the material bound to the column was eluted with a linear gradient of 0 to 0.2 M NaCl in the same buffer. Ten-milliliter fractions were collected and tested for the presence of type IV capsular polysaccharide and group B antigen with specific antisera in capillary precipitin tests (18). Fractions reactive with type IV antiserum and not with group B antiserum were pooled, dialyzed against water, and lyophilized.

Capsular polysaccharide was also purified from the fermentor culture supernatant. After removal of the bacterial cells, the broth was filter sterilized and then concentrated to 500 ml (10,000-M_r membrane; Pellicon). The concentrate was brought to a concentration of 30% (vol/vol) ethanol. The precipitate formed was removed by centrifugation, and the supernatant was brought to a concentration of 80% ethanol and allowed to stand at 4°C overnight. The precipitate was recovered by centrifugation and dried. This material was dissolved in 100 ml of 10 mM Tris, pH 7.4; treated with DNase, RNase, and pronase as described above for the mutanolysin extract; dialyzed; concentrated; and run on the DEAE column as described above. Protein content, UV spectrophotometry, and molecular size estimates were made on both supernatant and mutanolysin-extracted polysaccharides at this stage of purification, as described below. The capsular polysaccharide preparation derived by mutanolysin extraction was subjected to alkaline hydrolysis to remove contaminating group B polysaccharide before component sugar analysis. The polysaccharide was treated with 0.5 M NaOH for 48 h at 50°C, neutralized, dialyzed against water, and lyophilized, as described previously (19). Since this procedure results in partial de-*N*-acetylation of the capsular polysaccharide, the polysaccharide was re-*N*-acetylated by treatment with acetic anhydride in 5% ammonium bicarbonate as described previously (24).

Preparation of antisera. Antiserum was raised in New Zealand White rabbits with Formalin-fixed whole organisms of type IV GBS strain 3139 grown at neutral pH as described previously (6). Sera from three animals were used. This type IV antiserum was absorbed with organisms of the unencapsulated type Ia variant GBS strain 090R. Antiserum against the common group B antigen was prepared by immunizing rabbits with Formalin-fixed organisms of strain 090R.

Immune electron microscopy. Type IV GBS organisms (strain 3139) were grown to early log phase in Todd-Hewitt broth, fixed in 2% glutaraldehyde in PBS containing 0.05% calcium chloride, and incubated with type IV GBS antiserum previously absorbed with cells of strain 090R to remove antibodies to the common group B antigen. After being washed four times with PBS, the cells were incubated with ferritin-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika, Malvern, Pa.), washed five times in PBS, and suspended in a fixation buffer of 0.267 g of sodium cacodylate, 1 ml of 25% glutaraldehyde, and 11.5 ml of water for processing for electron microscopy as described previously (15).

Chemical analysis of capsular polysaccharide. Protein content was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.) and following the manufacturer instructions. Sialic acid content was quantitated by the thiobarbituric acid method of Warren (26) after hydrolysis in 3% acetic acid at 80°C for 4 h or after incubation with 0.5 U of neuraminidase (from *C. perfringens*; Organon Teknika) per ml at 37°C for 4h. Analysis for glycose constituents was performed as follows. Following acid hydrolysis of the polysaccharide (0.5 M trifluoroacetic acid at 100°C for 16 h), individual glycoses were reduced and peracetylated (23) and identified by gas chromatography as described previously (19).

Sepharose CL6B chromatography. The molecular size of the capsular polysaccharide was estimated by gel filtration chromatography on a column 2.6 by 90 cm of Sepharose CL6B (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated in 40 mM phosphate buffer, pH 7.0, as previously described (15).

Immunologic assays. Capillary precipitin tests were performed by the method of Lancefield (18). Double diffusion in agar was performed as described by Ouchterlony (20). For enzyme-linked immunosorbent assays (ELISA), the type IV GBS capsular polysaccharide was coupled to poly-L-lysine (Sigma) and coated onto 96-well plates, as previously described (7, 22), at a concentration of 1 μg of polysaccharide per ml. After the plates were washed three times with 40 mM sodium phosphate, pH 7.0, containing 0.05% Tween 20 (Sigma), nonspecific protein-binding sites were blocked by incubating the wells with 200 μl of 5% skim milk powder (Difco) in the same buffer at 37°C for 1 h. After the plates were washed three times with the same buffer, type IV GBS antiserum was added at an appropriate dilution in the same buffer and incubated at 37°C for 1 h. The subsequent steps of addition of an alkaline phosphatase-conjugated secondary antibody, addition of a substrate, and measurement of color development were performed as described previously (22).

RESULTS

Immune electron microscopy. Incubation of type IV GBS organisms with homologous antiserum and ferritin-conjugated anti-rabbit immunoglobulin G demonstrated the presence of a layer of electron-dense material exterior to the bacterial cell wall (Fig. 1A). The distribution of this material in a layer around the outside of the cell and the localization of the ferritin-labeled antibody to this extracellular layer are compatible with the presence of a type-specific capsule recognized by type IV-specific antiserum. Incubation of type IV GBS organisms with nonimmune serum followed by ferritin-conjugated antirabbit immunoglobulin G showed negligible ferritin binding to the cells and no capsular layer (Fig. 1B). Completely analogous results have been obtained in immune electron microscopic studies of other GBS serotypes with homologous antisera (14, 22).

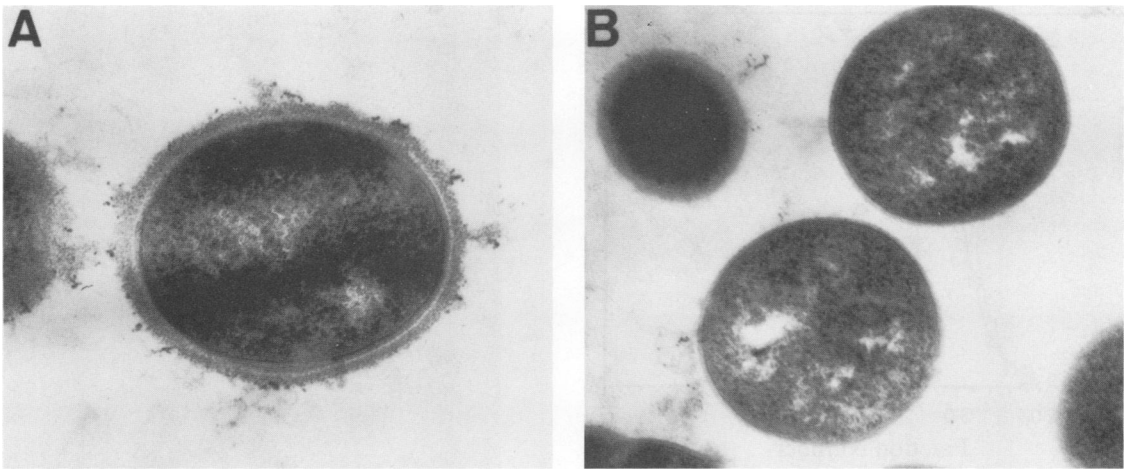

FIG. 1. Electron micrograph of type IV GBS treated with type IV antiserum (A) or nonimmune serum (B) and ferritin-conjugated anti-rabbit immunoglobulin G (Magnification, ×38,500).

Isolation of type IV GBS capsular polysaccharide. Similar results were obtained on DEAE passage of the crude capsular material extracted from the bacterial cells by mutanolysin treatment and that obtained from the culture supernatant. Group B antigenic activity was detected by capillary precipitins in fractions 64 to 120, while type IV antigenic activity was detected in fractions 114 to 137 (Fig. 2A). Fractions 121 to 137 were pooled, dialyzed against water, and lyophilized as purified capsular polysaccharide. In the purification of capsular polysaccharide from a second lot, fractions containing type IV GBS capsular polysaccharide were detected by ELISA inhibition (Fig. 2B). Samples (100 μl) of column fractions were added to microtiter wells coated with type IV capsular polysaccharide immediately before addition of 100 μl of type IV GBS antiserum diluted 1:5,000. Capsular polysaccharide in the fractions was detected as a decrement in optical density compared with control wells that received buffer instead of column fractions. The results obtained with the ELISA inhibition method agreed well with those of capillary precipitin testing, but the ELISA method was more sensitive, required less antiserum, and permitted a more precise estimate of the quantity of polysaccharide present in each fraction.

Chemical analysis of type IV capsular polysaccharide. UV spectrophotometry of a 1-mg/ml solution of the polysaccharide over a wavelength range of 190 to 300 nm showed a single major absorbance peak at 206 nm, with absorbance at 260 and 280 nm of less than 1% of the 206-nm detector response, compatible with a highly purified polysaccharide containing minimal amounts of contaminating nucleic acid or protein. Protein content as measured by the Bio-Rad Protein Assay was 1% or less. The polysaccharide was found to contain 23% (by weight) sialic acid, as detected by the thiobarbituric acid method by using acid hydrolysis to free the sialic acid from the polysaccharide. Incubation of the polysaccharide with neuraminidase yielded an amount of free sialic acid identical to that liberated by acid treatment. Glycose composition was assessed by gas chromatography of the component alditol acetates prepared from an acid hydrolysate of the polysaccharide. As in the other GBS capsular polysaccharides, in addition to sialic acid, glucose, galactose, and *N*-acetylglucosamine were detected as exclusive sugars. However, the relative amounts of component sugars in the type IV polysaccharide appeared to be different

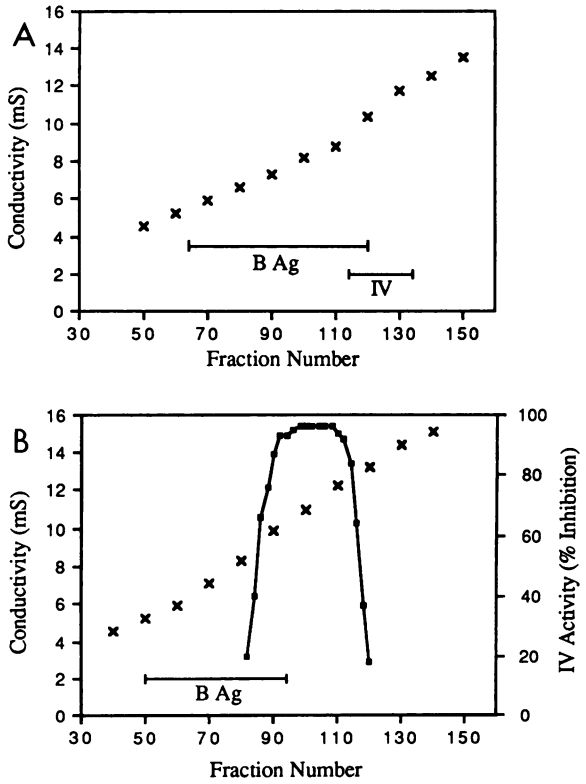

FIG. 2. Elution profile of mutanolysin-extracted type IV polysaccharide and group B antigen on a DEAE-Sephacel column. (A) Batch 1 purification. Column fractions reactive with group B antiserum are indicated by the bar labeled B Ag, and fractions reactive with type IV antiserum are indicated by the bar labeled IV. Fractions were assayed by capillary precipitin tests. Conductivity is plotted on the ordinate as an index of salt concentration. (B) Batch 2 purification. Column fractions reactive with group B antiserum by capillary precipitin tests are indicated by the bar labeled B Ag. The reactivity of fractions with type IV antiserum was assayed by ELISA inhibition and is plotted on the right ordinate. Conductivity is plotted on the left ordinate.

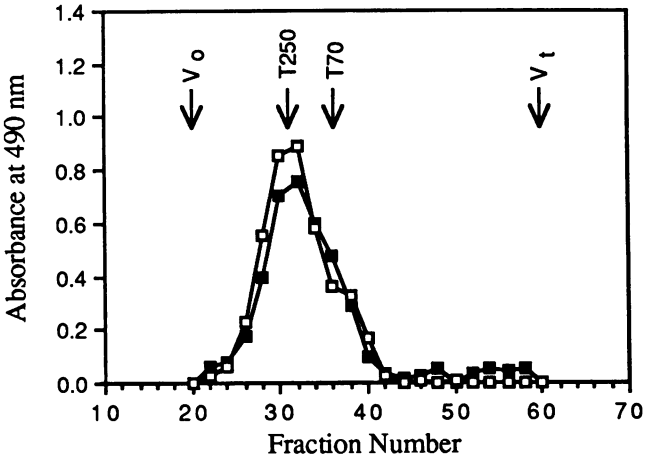

FIG. 3. Sepharose CL6B elution profile of type IV polysaccharide isolated from culture supernatant (open symbols) and that extracted from bacterial cells by mutanolysin treatment (closed symbols). Fractions were assayed for hexose content, indicated by the A_{490}, by the phenol-sulfuric acid method (5). The arrows indicate the void volume (V_0), the bed volume (V_t), and the elution volumes of dextran T250 (T250) and dextran T70 (T70).

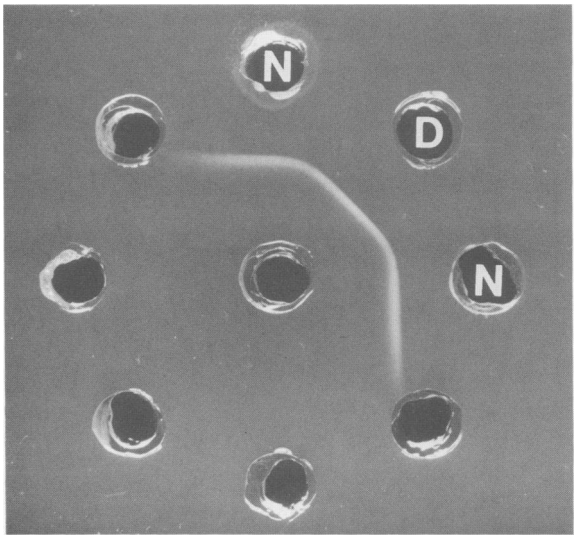

FIG. 4. Ouchterlony double diffusion in agar demonstrating a line of identity between the native (N) and desialylated (D) forms of type IV capsular polysaccharide, when tested against type IV antiserum (center well).

from those of the other capsular types, with an abundance ratio of galactose–glucose–*N*-acetylglucosamine of (1):1.10:0.55 (mean of three determinations with a range of experimental measurements of ±5% or less for each sugar).

Molecular size of type IV polysaccharide. The molecular size of the type IV polysaccharide was estimated by elution volume on a column of Sepharose CL6B. Samples of type IV polysaccharide isolated from the culture supernatant and by mutanolysin extraction of the bacterial cells gave nearly identical elution profiles (Fig. 3). The K_{av} of the polysaccharide was 0.30 on the basis of void volume and bed volume estimates based on the elution volumes of blue dextran 2000 and phenol red, respectively. The molecular weight of the polysaccharide was estimated to be 200,000 by comparison with dextran standards. Dextran standards seem more likely to give an accurate estimate of molecular weight than do globular proteins, although the elution of a linear dextran polymer may differ somewhat from that of a polysaccharide likely to have a branched structure.

Immunochemical characterization of type IV capsular polysaccharide. The purified type IV polysaccharide formed a single precipitin line with type IV antiserum in agarose double-diffusion experiments. No cross-reaction was observed between the type IV polysaccharide and rabbit antiserum to type Ia, Ib, or III GBS or burro antiserum to type II GBS (generously provided by John Robbins, National Institutes of Health, Bethesda, Md.). Interestingly, removal of the sialic acid residues from the type IV polysaccharide by treatment with 3% acetic acid at 80°C for 4 h did not appreciably alter the reaction of the polysaccharide with type IV antiserum. The desialylated type IV polysaccharide formed a line of identity with the sialylated native antigen (Fig. 4).

The absence of cross-reactivity of the type IV polysaccharide with other capsular types was confirmed by ELISA experiments using type IV antiserum previously absorbed with organisms of different GBS serotypes. Samples (100 μl each) of type IV antiserum diluted 1:10 in PBS were absorbed for 1 h at 37°C with 5×10^8 CFU of type Ia, Ib, II, III, or IV GBS. After absorption, the serum samples were diluted with 900 μl of PBS, and the bacterial cells were removed by centrifugation. The adsorbed serum samples were then assayed for reactivity with type IV polysaccharide coated onto ELISA plates. Only homologous type IV GBS organisms effectively absorbed type IV anti-capsular antibodies from the serum, while serum samples absorbed with organisms of the heterologous serotypes did not differ from the unabsorbed control (Fig. 5).

DISCUSSION

Type IV GBS has recently been established as a new serogroup of GBS which is antigenically distinct from the

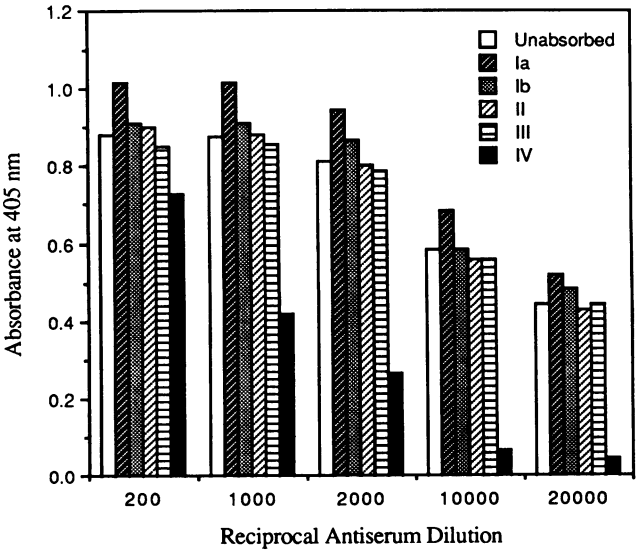

FIG. 5. ELISA using type IV GBS antiserum absorbed with organisms of GBS type Ia, Ib, II, III, or IV. Each group of bars represents ELISA readings (A_{405}) at the indicated antiserum dilution.

previously described capsular types (9, 10, 21). The type IV serotype-specific antigen was presumed to be a structurally and antigenically unique capsular polysaccharide. Isolation and purification of the capsular polysaccharide antigen from a type IV GBS strain has permitted immunologic and chemical characterization of this type specific antigen. Immune electron microscopy using type-specific antiserum demonstrated the presence of a layer of extracellular material surrounding the organisms which bound a type-specific antibody, as evidenced by the deposition of a ferritin-conjugated secondary antibody. This demonstrates that antiserum to type IV organisms contains antibodies which recognize the capsular polysaccharide antigen, and the appearance of the capsule is similar to that demonstrated with other GBS serotypes when homologous antisera were used (14, 22).

Like the other described GBS capsular polysaccharides, the type IV antigen could be recovered from broth culture fluid or extracted from bacterial cells with mutanolysin, an *N*-acetylmuramidase from *Streptomyces globisporus* shown previously to disrupt the cell wall of GBS, releasing the capsular polysaccharide antigen (3, 4, 29). As is typical of GBS capsular polysaccharides, negatively charged sialic acid residues permitted purification of the type IV polysaccharide on a column of DEAE-Sephacel. Elution with a shallow salt gradient resulted in separation of the common group B polysaccharide, which eluted at 5 to 10 mS, from the type IV polysaccharide, which eluted at 9 to 12 mS, with major portions of the peaks not overlapping.

The capsular polysaccharide purified by DEAE chromatography contained minimal protein or contaminating nucleic acid. Based on the elution volume on a column of Sepharose CL6B, the M_r of the polysaccharide was estimated to be 200,000 by comparison with dextran standards. We and others have found mutanolysin-extracted capsular antigen from type III GBS to be of higher M_r than that recovered from broth culture supernatants, perhaps because of cross-linking of multiple polysaccharide chains via a cell wall fragment (3, 4). Capsular polysaccharide extracted from type III GBS with mutanolysin is heterogeneous in size and elutes on Sepharose 4B from near the void volume (K_{av} = 0.05) to K_{av} = 0.76 (3). With the type IV polysaccharide, elution profiles on Sepharose CL-6B were identical for the mutanolysin-extracted and culture supernatant antigens. While a relatively small difference between the molecular sizes of the two polysaccharide preparations (e.g., 50,000 daltons) might not be detected by these studies, there appears to be no major difference in size, as has been noted with the type III polysaccharide. The similarity in size between the two antigen preparations from type IV organisms may reflect more complete mutanolysin digestion of a cell wall peptide anchor in type IV GBS, compared with another serotype(s), or other structural or methodologic differences.

Analysis of the component monosaccharides of the type IV polysaccharide indicated the presence of sugars identical to those found in the type Ia, Ib, II, and III polysaccharides, that is, galactose, glucose, *N*-acetylglucosamine, and sialic acid. Gas chromatography of alditol acetates prepared from an acid hydrolysate of the type IV polysaccharide showed a ratio of galactose–glucose–*N*-acetylglucosamine of (1):1.10:0.55. The smallest likely whole-integer ratio, then, is 2:2:1. The thiobarbituric acid assay indicated the presence of 23% (by weight) sialic acid, which is compatible with the predicted amount of sialic acid, 24.7% (by weight), for a molar ratio of galactose–glucose–*N*-acetylglucosamine–sialic acid of 2:2:1:1. The ratio of component sugars suggests that the repeating unit structure of the type IV polysaccharide contains six sugar residues, making it unique among GBS capsular polysaccharides. The type Ia, Ib, and III polysaccharides each have a five-sugar repeating unit containing galactose, glucose, *N*-acetylglucosamine, and sialic acid in a ratio of 2:1:1:1, while the type II polysaccharide has a seven-sugar repeating unit of galactose, glucose, *N*-acetylglucosamine, and sialic acid in a ratio of 3:2:1:1.

In contrast to the type II polysaccharide, the sialic acid residues of the type IV polysaccharide are susceptible to neuraminidase. This finding may indicate that the sialic acid residues are not linked directly to the backbone of the type IV polysaccharide, as they are in type II, but rather to side-chain glycose residues, as in types Ia, Ib, and III (11, 27).

Serologic analysis of the type IV capsular polysaccharide showed it to be antigenically distinct from types Ia, Ib, II, and III. Antiserum to each of these heterologous types failed to react with the type IV antigen in double-diffusion experiments. Type IV antiserum also did not recognize cross-reactive determinants on the heterologous polysaccharides; absorption of type IV antiserum with organisms of a heterologous capsular type did not remove type IV-specific anti-capsular antibodies, as assessed by ELISA. Interestingly, removal of the sialic acid residues from the type IV polysaccharide did not appreciably affect its reactivity with type IV antiserum in double-diffusion studies. Unlike the Ia, II, and III polysaccharides, the desialylated or core form of the type IV polysaccharide reacted with a line of identity with the native polysaccharide. In this respect, the immunodeterminant of the type IV polysaccharide resembles the Ib antigen, for which the immunodeterminant appears not to be affected by removal of the terminal side-chain sialic acid residues (24, 25). This lack of dependence on sialic acid contrasts with the type Ia, II, and III polysaccharides, in which sialic acid has been shown to play a major role in immunodeterminant structure or conformation (12, 13, 24, 27). Nuclear magnetic resonance studies of the native and desialylated forms of the type III polysaccharide have provided evidence consistent with the hypothesis that the sialic acid residues exert conformational control over the branch region of the polysaccharide repeating-unit structure (27). This conformational control is thought to be critical to the native immunodeterminant structure of the type III polysaccharide. Our studies suggest that expression of the native immunodeterminant of the type IV polysaccharide is not heavily influenced by sialic acid.

The prevalence of type IV GBS among isolates from infected or colonized individuals has not been studied in detail. The proportion of GBS isolates not typable as Ia, Ib, II, or III has varied from 0 to 16.2% in various series, with a trend toward more nontypable strains among human adults and veterinary isolates, as opposed to human infants (1). Among 3,953 GBS isolates from various sources, Jelínková and Motlová found 102 type IV strains representing isolates from Europe, Israel, India, Greece, Singapore, and the United States (10). The prototype strain used for our studies was originally isolated from an infant with meningitis, demonstrating that type IV GBS have the capacity to cause invasive disease in human neonates. The importance of type IV GBS as pathogens in this and other populations remains to be defined as more comprehensive serotyping is applied to analysis of clinical isolates. Further studies are needed to elucidate the roles of the type IV polysaccharide in both the pathogenesis of and immunity to GBS infection.

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