

ISOLATION OF TYPE-SPECIFIC POLYSACCHARIDE ANTIGEN FROM GROUP B TYPE Ib STREPTOCOCCI*

BY JOSEPH Y. TAI, EMIL C. GOTSCHLICH, AND REBECCA C. LANCEFIELD

From The Rockefeller University, New York 10021

Lancefield et al. (1) have clearly demonstrated that the capsular polysaccharides of group B streptococci are antigenic and that the antibodies are protective in mice except with type III, where no mouse-virulent strain is available. However, a chicken embryo model has been established for type III and the antibody found to be specifically protective (2). Recently, emphasis has been centered on the isolation and characterization of the capsular antigens from various types (3-8). However, inasmuch as the capsule is very tightly (probably covalently) bound to the cell wall, a major problem is to find an extraction method that will give satisfactory yield. Conventional HCl extraction results in partial destruction of the polysaccharide antigens with the loss of the sialic acid determinant (6-8). Current investigations have concentrated on obtaining larger size capsular polysaccharides that are antigenically intact. Neutral buffer extractions have been used successfully to obtain such molecules from type Ia and type III (3, 5). However, the yield is low, which hinders structural studies as well as more extensive clinical and immunological investigations.

In this report, we describe the isolation of type Ib polysaccharide from whole cells by digestion with muralytic enzymes prepared from *Streptomyces albus*. The composition and immunological properties of the purified type Ib antigen are also discussed.

Materials and Methods

Streptococcal Strain and Antisera. Group B type Ib (H36B) streptococci and antisera against various types used in this study were from the collection of Dr. R. C. Lancefield of The Rockefeller University. The preparation of antisera was previously described by Lancefield et al. (1).

Growth Conditions. Strain H36B (type Ib) was grown in Todd-Hewitt (Difco Laboratories, Detroit, Mich.) broth containing 0.2% yeast extract and 0.2% glucose. 20 ml of overnight culture was inoculated into 4 liters of broth at 37°C. After overnight growth, the cells were harvested by centrifugation and washed twice with $\frac{1}{15}$ M phosphate buffer pH 8.0.

Digestion of Type Ib Cells with Lytic Enzymes from Streptomyces albus. The extracellular enzymes of *Streptomyces albus* were prepared according to the procedure described by McCarty (9).

The titer of lytic activity of each enzyme preparation was assayed using group A streptococci (either strain S43, type 6, or strain T28, type 28) as described (9). The lytic activity of an enzyme preparation was considered satisfactory when a 1:10 (vol/vol in $\frac{1}{15}$ M phosphate buffer) enzyme dilution caused, within 30 min incubation at 37°C, a 70-80% decrease in turbidity using a cell suspension with an optical density of 0.50 at 600 nm in a 10 × 75-mm tube. The enzyme concentrations of different preparations were equalized based on this assay. The digestion of group B streptococci was performed as follows: Cells from 1 liter of culture were suspended in 10 ml of $\frac{1}{15}$ M phosphate buffer to which 1 ml of enzyme solution was

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added. The mixture was incubated at 37°C overnight, and cell debris was removed by centrifugation at 10,000 g for 25 min.

Isolation of Type Ib-Specific Polysaccharide. The supernate from the enzyme digestion was treated with bovine pancreatic deoxyribonuclease and ribonuclease (Worthington Biochemical Corp., Freehold, N. J.; 2 mg/100 ml) for 2 h at 37°C. The polysaccharide solution was dialyzed against H₂O extensively, and nucleic acid was further removed by precipitation with 25% vol/vol ethanol in the presence of 0.1 M CaCl₂. After centrifugation, the clear supernate was brought to 80% ethanol concentration to precipitate the polysaccharide. The precipitate was redissolved in H₂O, dialyzed against distilled H₂O, and lyophilized.

The dry polysaccharide was dissolved in 1/100 M phosphate buffer, pH 8.0, and any insoluble material was removed by centrifugation. The clear polysaccharide solution was applied to a Sepharose 6B column (1.5 × 200-cm; Pharmacia Fine Chemicals, Piscataway, N. J.) and eluted with the same buffer at 15 ml/h. The polysaccharide peaks were identified by phenol-sulfuric acid assay (10) and capillary precipitin test using type Ib and group B antisera.

Preparation of Neuraminidase. The extracellular neuraminidase produced by type Ib was prepared according to Milligan et al. (11). The enzymic activity was measured by assaying release of sialic acid from bovine submaxillary mucin or *Escherichia coli* K1 antigen (colominic acid) using sodium acetate buffer pH 6.5 as described. Free sialic acid was determined by the thiobarbituric acid method (12).

Analytical Methods. The protein content of the purified type Ib polysaccharide was calculated from an amino acid analysis of a sample hydrolyzed in 4 N methane sulfonic acid (13). The analysis was performed on a Durrum D-500 automatic amino acid analyzer using a one column system (Durrum Instrument Corp., Sunnyvale, Calif.). Sugar analyses were carried out by gas liquid chromatography as described by Clamp et al. (14). The polysaccharide was hydrolyzed in 1.5 N methanolic HCl at 80°C overnight, and the monosaccharides were analyzed as their trimethylsilyl ethers on a Varian model 3700 gas liquid chromatography unit equipped with a CDS-111 integrator (Varian Associates, Instrument Division, Palo Alto, Calif.). Appropriate sugar standards were hydrolyzed and analyzed using identical conditions.

The quantitative precipitation analyses were carried out in phosphate-buffered saline (PBS)¹ (pH 7.0). Various amounts of polysaccharide were mixed with 100 μl of serum and brought to a final volume of 0.6 ml with PBS-saline buffer. After incubation at 4°C overnight, the precipitate was washed once with the same buffer and dissolved in 1 ml of 0.1 N NaOH.

Free amino groups on the type Ib polysaccharide were determined quantitatively by reaction with trinitrophenyl sulfonic acid (TNBS) according to Fields (15). They were further identified by dansylation with dansyl chloride as described by Gray (16). The dansylated polysaccharide (1 mg) was hydrolyzed in 6 N HCl at 80°C for 14 h, and the hydrolysate was examined by thin layer chromatography according to Woods and Wang (17). Glucosamine was used as standard for both determinations.

Results

Isolation of Type Ib-Specific Polysaccharide. The lytic enzymes of *Streptomyces albus* have been shown to hydrolyze the peptides, the peptide cross-links, as well as the glycan backbone of various cell walls (18). When used on group B type Ib cells this results in the release of both type Ib and group B polysaccharides which could be readily separated on a Sepharose 6B column (Fig. 1). The purity of the Ib polysaccharide (peak 1) was demonstrated by both capillary precipitin test with group B-specific antiserum and by chemical analysis (Table I). Rhamnose, a major constituent sugar of group B polysaccharide (7, 19), is not present in the type antigen. The purified polysaccharide contains <0.02% of nucleic acid as determined by 260-nm absorption and <0.5% of amino acids. On Sepharose 4B in ammonium acetate buffer (20), the purified polysaccharide gave a K_d value of 0.31 (Fig. 2, peak 1; 21). The final yield was about 65–70 mg of purified Ib antigen per 10 liters of culture.

¹ Abbreviations used in this paper: BSM, bovine submaxillary mucin; PBS, phosphate-buffered saline; TNBS, trinitrophenyl sulfonic acid.

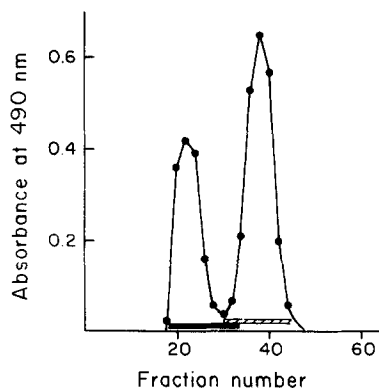


FIG. 1. Separation of type Ib and group B polysaccharides on Sepharose 6B column (1.6×200 cm) in $M/30 \text{ PO}_4$ buffer pH 8.0. Polysaccharides were analyzed by phenol-sulfuric acid assay. Solid and hatched bars indicate the capillary precipitin test against type Ib and group B antisera, respectively.

TABLE I
Chemical Compositions of Type Ib and pH 2.0-Treated Type Ib Polysaccharides

Composition	Type Ib			pH 2.0-Treated type Ib		
	mg/ 100 mg	$\mu\text{mol}/$ 100 mg	molar ratio	mg/ 100 mg	$\mu\text{mol}/$ 100 mg	molar ratio
Galactose	33.89	209	2.05	46.91	289	2.14
Glucose	14.65	90	0.86	20.10	124	0.92
<i>N</i> -Acetyl-glucosamine	20.70	102	1.00	27.35	135	1.00
Sialic acid	26.81	92	0.90	1.76	6	0.04

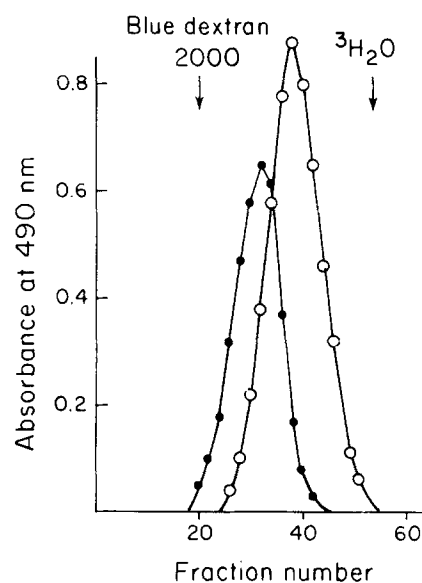


FIG. 2. Elution chromatography patterns of intact type Ib (peak I) and pH 2.0-treated type Ib (peak II) polysaccharides on Sepharose 4B column (1.6×100 cm) in 0.2 M ammonium acetate pH 7.8. Polysaccharide was analyzed by phenol-sulfuric acid assay.

Chemical Analyses of the Type Ib Polysaccharide. The purified type Ib antigen contained four sugars; galactose, glucose, *N*-acetyl glucosamine, and sialic acid with a molar ratio of 2.05:0.86:1.00:0.90 (Table I). The type Ib polysaccharide isolated by Wilkinson (6) by HCl extraction contained only two sugars, galactose and *N*-acetyl glucosamine in a molar ratio of 2:1. The lack of sialic acid in the HCl-extracted antigen is likely the result of the destruction of this residue by acid. However, the molar ratio of galactose and *N*-acetyl glucosamine is the same as that of the purified Ib antigen in this study. Free amino group determination with dansyl chloride indicated the presence of glucosamine in the polysaccharide. Further quantitative determination with TNBS showed that about 6.75% of the glucosamine in the polysaccharide is not acetylated.

When the Ib antigen (10 mg/ml) was boiled in phosphate-buffered saline at pH 2.0 for 10 min, the polysaccharide was eluted with a K_d value of 0.48 on a Sepharose 4B column indicating a decrease in molecular size. (Fig. 2, peak II). Analysis (Table I) showed that sialic acid was almost completely removed (93.5%) by the acid treatment. However, the molar ratio of the remaining sugars, galactose, glucose, and *N*-acetyl glucosamine, remained essentially constant. This suggests that the sialic acid is located at the terminal position of the polysaccharide chains.

Neuraminidase Digestion of the Type Ib Antigen. The neuraminidase obtained from cell filtrate was active against bovine submaxillary mucin (BSM) and *E. coli* K1 antigen. Approximately 10 μ g of sialic acid from 2 mg of BSM and 30 μ g from 1 mg of K1 antigen were released after 1 h incubation at 37°C. However, no detectable sialic acid was found when 1 mg of Ib antigen was treated with the neuraminidase under identical conditions, indicating that no significant autodigestion is taking place during the growth.

Immunological Properties of Type Ib and pH 2.0-Treated Type Ib Polysaccharides. The purity of the Ib antigen was also demonstrated by a single symmetric peak on a two-dimensional electrophoresis against unabsorbed antiserum (Fig. 3). Double diffusion experiments (Fig. 4) showed that the intact and acid-treated Ib polysaccharides gave precipitin lines with almost complete identity. However, immunoelectrophoresis of these two polysaccharides (Fig. 5) clearly showed the difference in mobility as a result of the loss of acidic sialic acid. Quantitative precipitation analysis (Fig. 6) demonstrated that the pH 2.0-treated Ib antigen precipitated about 82% of the amount of antibody precipitable by the intact polysaccharide. Similar correlations between neutral buffer extracted or trichloroacetic acid-extracted and HCl-treated antigens have been reported for type Ia and type II polysaccharide antigens (3, 4, 7).

Hapten inhibition tests were also carried out to identify the antigenic determinant. Up to 10 mg of α -rhamnose, D -glucose, D -galactose, D -glucosamine, *N*-acetyl- D -glucosamine, and sialic acid failed to inhibit the reaction of antibody with either intact or pH 2.0-treated Ib polysaccharide. In addition, as much as 2.5 mg of capsular polysaccharides of *O*-acetyl-negative group C meningococcus, *E. coli* K1 and *O*-acetyl-positive group C meningococcus also failed to inhibit. These polymers consist solely of sialic acid; the group C antigen is linked α 2-9 and may contain *O*-acetyl groups, whereas the K1 antigen is linked α 2-8 (22).

Characterization of Iabc Component. It was first reported by Lancefield (23) that type Ia, Ib, and Ic share a common minor component designated as Iabc. Antibodies to this component are also able to protect mice (1). In double diffusion studies intact Ib

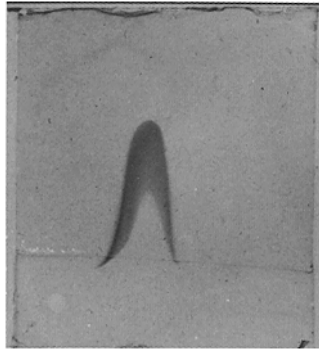


FIG. 3. Two-dimensional electrophoresis of purified type Ib polysaccharide (0.5 mg/ml) against unabsorbed type Ib antiserum (5%).

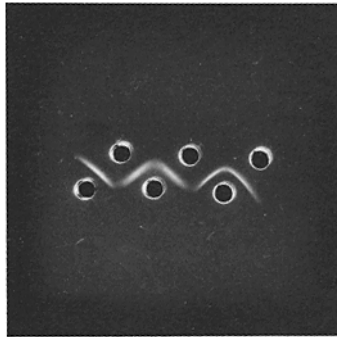


FIG. 4. Immunodiffusion precipitin reactions of intact and pH 2.0-treated Ib polysaccharides. Upper wells, type Ib antiserum; lower wells (left to right), Ib, acid-treated Ib, and Ib polysaccharides.

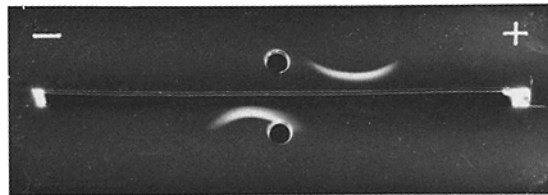


FIG. 5. Immunoelectrophoresis of intact and acid-treated Ib antigens against unabsorbed Ib antiserum. Upper well, intact Ib antigen; lower well, acid-treated Ib antigen; center well, type Ib antiserum.

and acid-treated Ib antigens showed complete identity when reacted with types Ia, Ib, and Ic antisera known to contain antibodies to the Iabc component (Figs. 7 and 8). However, neither of the polysaccharides reacted with type II or type III antiserum. To determine whether the common Iabc component and the major Ib-specific component were on the same molecule, the experiment illustrated in Fig. 9 was carried out. To 100 μ l of type Ia antiserum, 10 μ g of Ib antigen was added and after incubation at 4°C overnight, the sample was centrifuged and placed in well [1]. Similarly, the same amount of Ib antigen was added to type III antiserum and placed in well [2] as a control. It can be seen that in the case of the type III serum the Ib antigen remained detectable, whereas it was completely bound by the Ia serum. The line between the type Ia and III serum indicates that the Ia serum retained ability to

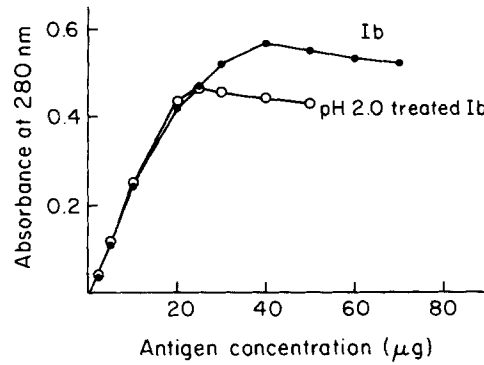


FIG. 6. Quantitative precipitin analyses of intact and pH 2.0-treated Ib polysaccharides.

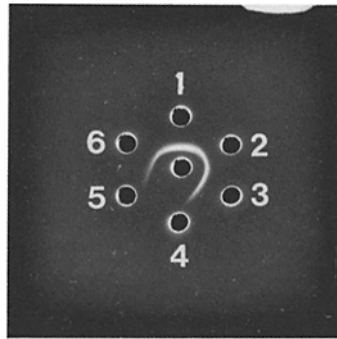


FIG. 7. Immunodiffusion precipitin reactions of type Ib antigen against various type antisera. Center well, type Ib antigen; wells [1-6], type Ia, Ib, Ic, II, III, and Ib antisera.

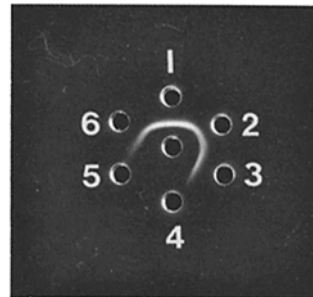


FIG. 8. Immunodiffusion precipitin reactions of acid-treated type Ib antigen against various type antisera. Center well, acid-treated type Ib antigen; wells [1-6], type Ia, Ib, Ic, II, III, and Ib antisera.

precipitate with Ib antigen. The ability of Ia serum to precipitate Ib antigen completely indicates that Iabc component and Ib-specific component are on the same molecule.

Discussion

The extracellular lytic enzymes from *Streptomyces albus* have been used successfully to investigate various bacterial cell wall preparations including group A streptococcus (24-26). The enzymes involved in the lytic activities have been characterized exten-

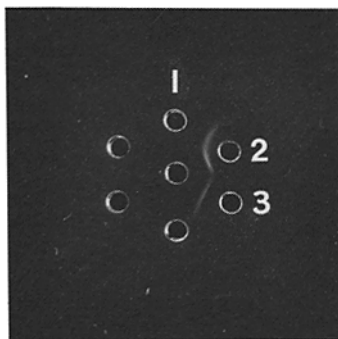


FIG. 9. Immunodiffusion precipitin reactions of type Ib antigen and type Ia antiserum. Center well, type Ib antiserum; well [1], supernate of 100 μ l of type Ia antiserum mixed with 10 μ g of type Ib antigen; well [2], supernate of 100 μ l of type III antiserum mixed with 10 μ g of Ib antigen; well [3], type Ib antigen.

sively by Ghuyssen et al. (18) into amidase, endopeptidases, and glycosidases which act on the cell walls of various gram-positive species. It is not known which enzyme or enzymes are involved in the lysis of type Ib cell wall in this study. However, as indicated by the sugar constituents, the polysaccharide antigen appears to be as intact as those prepared from neutral buffer extractions (3, 5). The type Ib antigen isolated by enzyme digestion was established to be immunochemically pure by several criteria. A final yield of 65–75 mg from 10 liters of cell culture was obtained.

Purified type Ib antigen is composed of four sugars: galactose, glucose, *N*-acetyl glucosamine, and sialic acid with a molar ratio of 2.05:0.86:1.00:0.90. Acid treatment (pH 2.0) of the purified Ib antigen results in decrease in size and loss of sialic acid (93%). Double diffusion studies in agar suggested complete identity between intact and acid-treated Ib antigen. No spurs were observed in contrast to the data reported for other types (5, 6). However, quantitative precipitin tests showed that the acid-treated Ib precipitated only 82% of the amount of antibody precipitable by the intact Ib antigen. These results suggest that sialic acid is probably a determinant and located at the terminal position in the native Ib polysaccharide.

Results of haptene inhibition studies showed that a number of monosaccharides (see Results) as well as two polymers of sialic acid failed to inhibit the reaction of antibody with either intact or acid-treated Ib polysaccharide. In earlier studies Wilkinson (6) reported that various monosaccharides, including *D*-glucosamine, *N*-acetyl-*D*-glucosamine, *D*-galactosamine, and gamma-*D*-galactonolactone, were able to cause 40–60% inhibition of the reaction with acid-treated Ib polysaccharide. The discrepancy might be the result of the difference in methods used in preparing the polysaccharides. Dansylation of the intact polysaccharide as well as amino group determination with TNBS indicated the presence of a small amount (6.75%) of nonacetylated glucosamine in the Ib antigen, in agreement with suggestive findings by Wilkinson (6). This information may be useful in labeling the polysaccharide and in further structural studies.

The intact and acid-treated Ib antigen do not cross-react with type II or type III antisera, but both cross-react strongly with some type Ia and Ic antisera. This is not unexpected considering the fact that type Ia and Ib antigens not only have identical sugar constituents but that the molar ratios of these four sugars are nearly identical (galactose:glucose:glucosamine:sialic acid = 2.1:0.5:1.0:0.7; 4). The cross-reaction has

been attributed to the common determinant Iabc (1). The ability of type Ia antiserum to precipitate Ib antigen clearly suggests that Ib specific and Iabc determinant are on the same molecule. It is not known, however, which sugar of the Ib polysaccharide constitutes the common antigen. Inasmuch as both intact and acid-treated Ib antigens exhibit the Iabc determinant, it is unlikely that sialic acid is the common antigenic determinant. Thus the core antigen of Ia and Ib share the same component sugars and the Iabc determinant, but differ in some linkage; with type-specific antisera, HCl-extracted Ia and Ib antigen are readily distinguishable. Final conclusion must await the complete structural elucidations of these two polysaccharide antigens.

Summary

Group B streptococcus type Ib (strain H36B) was subjected to digestion with extracellular muralytic enzymes prepared from *Streptomyces albus*. Type Ib-specific polysaccharide antigen was isolated from the lysate by alcohol precipitation and Sepharose 6B chromatography. The purified type Ib antigen has a K_d value of 0.31 on a Sepharose 4B column and contains four sugars, galactose, glucose, *N*-acetyl glucosamine, and sialic acid in a molar ratio of 2.05:0.86:1.00:0.90. Acid treatment (pH 2.0) of this polysaccharide results in partial degradation of the antigen (K_d = 0.41 on Sepharose 4B) with the loss of 93% of the sialic acid. The molar ratio of the remaining sugars in the polysaccharide remains identical to that in the native one. This suggests that the sialic acid is at the terminal position in the molecule. Both intact and acid-treated antigen cross-react with some type Ia and type Ic antisera as a result of the common Iabc determinant, but not with type II and type III antisera. Absorption studies indicate that Ib-specific determinant and Iabc determinant are on the same molecule and that sialic acid is not the cross-reactive determinant.

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