IMMUNOCHEMICAL CHARACTERIZATION OF THE "NATIVE"
TYPE III POLYSACCHARIDE OF GROUP B
STREPTOCOCCUS*

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Since Lancefield's classification of beta-hemolytic Streptococcus in 1933 (1), organisms designated Group B have been reported as a cause of human disease. Their emergence as frequent neonatal pathogens, however, is an event of this decade. Two distinct types of illness that are related to age at onset (2,3) are described for neonates with infections due to Group B Streptococcus. The "early onset type" of infection occurs in neonates 5 days of age or less (2), results from intrapartum transmission of organisms from the maternal genital tract, and causes a mortality rate in excess of 50%. "Late onset type" infections, in contrast, are characterized by onset beyond the 10th day of life and are associated with purulent leptomenigitis, significantly lower mortality rates, and modes of transmission in addition to the mother-to-infant route (4).

These two clinical syndromes have been related to certain serotypes. The serologic classification of Group B streptococci is based upon capillary precipitin tests with hyperimmune rabbit antisera and acid-extracted antigens (5). By this method, Group B streptococci can be divided into serotypes Ia, Ib, Ic, II, and III (5-7). Serotyping of strains has allowed the epidemiologic characterization of neonatal infection. Organisms isolated from asymptomatic mothers and neonates with Group B streptococci have virtually identical distributions of serotypes; 26% of strains are type Ia, Ib, or Ic; 38% are type II, and 36% are type III (8). This distribution of serotypes is similar among isolates from neonates who have early onset type septicemia without meningitis. However, neonates who have early onset type infection with meningitis and infants with late onset type infection have a striking predominance of type III isolates (>80%) (8-10). These epidemiologic observations suggested that strains of Group B streptococci of serotype III may possess invasive properties that allow meningeal penetration and the production of disease beyond the first few days of life.

Lancefield has immunochemically characterized two polysaccharides from Group B Streptococcus: the group-specific B polysaccharide antigen common to all strains and the type-specific polysaccharide antigen which allows classification into four distinct serotypes: Ia, Ib, II, and III (5,6). These type-specific polysaccharides are believed to be

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capsular antigens which are chemically and serologically distinct from each other and from the group-specific substance. An additional serotype, type Ic, has been defined by Wilkinson and Eagon (7). Type Ic strains possess a polysaccharide antigen which is immunochemically identical to the type Ia antigen (11); in addition, type Ic strains have a protein antigen, the Ibc protein, which is found in all type Ib and Ic strains and infrequently in strains of type II and III.

Classical methods of extracting polysaccharide antigens from Group B Streptococcus for serogrouping and serotyping have utilized hot HCl treatment of whole organisms. Additional purification methods are necessary, however, for separation of group- from type-specific substances. Recent investigations of the type I polysaccharides by Wilkinson (11), the type II polysaccharide by Lancefield and Freimer (12, 13), and the type III polysaccharide by Russell and Norcross (14) have used alcohol fractionation or ion exchange chromatography for the separation of type-specific polysaccharides from the group-specific substance. However, these HCl-treated antigens are incomplete, low-molecular weight substances which have previously been reported to contain varying quantities of glucose, galactose, and glucosamine. For the isolation of the type-specific antigens from serotypes Ia, Ib, Ic, and II, extraction with cold trichloracetic acid (TCA) results in more "complete" polysaccharides with a labile component identifiable as sialic acid (11, 12, 15). This sialic acid component accounted for approximately 12% of the dry weight of the type II-TCA antigen (12). More gentle methods for the isolation and immunochemical definition of the type III polysaccharide have not been previously reported.

We have isolated and purified the type III polysaccharide in "native" form by means of an extraction procedure in which whole bacteria are washed with a neutral buffer solution. This polysaccharide has a mol wt >5 × 10⁴ daltons. Its main chemical constituents are sialic acid (24%), galactose (25%), heptose (21%), glucose (13%), glucosamine (10%), and mannose (7%). The antigen has type-specificity with homologous and heterologous antisera prepared to formalin-treated and live organisms. Preparation of type-specific antisera with live organisms was believed to be a potentially more accurate means of studying antibodies that form in response to naturally occurring antigens. By the use of antiserum prepared to a live homologous type III strain, a previously unknown cross-reactive II-III antigen has been demonstrated. The goal attempted through immunochemical study of purified antigens of the bacterial cell surface is isolation of antigens in native form. Native antigens are desirable for investigative purposes because they most closely resemble that which the naturally infected host recognized immunologically.

Materials and Methods

Bacterial Strains. Prototype strains of Group B Streptococcus representing each of the five serotypes and the Group B variant strain (devoid of type-specific antigen) were kindly supplied by Dr. Rebecca Lancefield. These strains are designated 090 (Ia), H36B (Ib), A909 (Ic), IRS821 (II), D136C (III), and 090R (group B). Lyophilized strains were rehydrated with Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and incubated overnight at 37°C before use. In addition, type III strains M731, M732, and M735 isolated (in Houston) from three infants with meningitis were stored at -70°C in 1-ml aliquots of Todd-Hewitt broth.

Media and Growth Conditions. Strains M731 or M732 were inoculated into an Erlenmeyer flask containing 200 ml of Todd-Hewitt broth and were incubated for 12 h at 37°C. From this culture, 60-70-liter lots were inoculated and grown for 12 h at 37°C in a modified Todd-Hewitt broth. This modified medium was prepared by increasing eightfold the concentration of Na₂HPO₄. Standard Todd-Hewitt broth has an initial pH of 7.4, but this medium is acidified to pH 5.9 after 12
h of growth of Group B streptococci (16). Because of the degradation of certain polysaccharide antigens during accumulation of acid in a culture medium (17), the buffering capacity of Todd-Hewitt was increased to maintain a more neutral pH during exponential growth. With use of the eightfold increase of Na2HPO4, the pH of Todd-Hewitt broth was always >6.5 after 12 h of growth (16). All lots of the type III organisms were harvested at 4°C by continuous flow centrifugation by means of a model RC5 centrifuge with a SZ-14 rotor (Ivan Sorvall, Norwalk, Conn.). All cultures were checked to document purity at the end of the growth cycle.

**Electron Microscopy.** Strain M731 was prepared for electron microscopy by means of a modification of the method of Devoe and Gilchrist (18). Suspensions of whole organisms grown for 12 h in modified Todd-Hewitt broth were prefixed to adjust the final concentration of fixative to 0.5% by the addition of 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer to the broth. CaCl2 (concentration, 0.05%) was added to this suspension. After prefixation for 2 h at 4°C, the suspension was centrifuged at 12,000 g. The pellet obtained was fixed in 2.5% buffered glutaraldehyde for 2 h at 4°C, washed with buffer, and postfixed for 1 h in aqueous 1% OsO4.

The pellet was then treated with 0.5% uranyl acetate in Michaelis buffer (pH 5) for 2 h at 4°C. 1-mm blocks were dehydrated in graded alcohols and embedded in Epon 812 according to the method of Luft (19). Thin sections were stained with lead citrate and examined in a Jem 100B electron microscopy (Japan Electron Optics Laboratory, Tokyo, Japan).

**Extraction of HCl Antigens.** Prototype strain D136C and serotype III strains M731 and M732 were exposed to 0.2 N HCl for 10 min at 100°C, as described by Lancefield (6). After neutralization of acid-treated suspensions, organisms were removed by centrifugation at 2,000 g, and the supernatant solution containing both group- and type-specific antigens were precipitated with 4 vol of absolute ethanol at 4°C for 4 h. After centrifugation and drying, antigens were then suspended in water and lyophilized. No further purification of these HCl antigens was attempted.

**Preparation of "Native" Type III Antigens.** Pelleted organisms from 60-70-liter lots (110-140 g, wet weight) were suspended in a buffer containing 0.05 M sodium phosphate, 0.15 M NaCl, and 0.01 M ethylenediaminetetraacetate disodium (EDTA) adjusted to pH 7.4. The final volume was approximately one-fiftieth that of the original culture. This suspension of organisms was stirred with glass beads at 24°C for 15 h. Washed cells were then removed by centrifugation at 12,000 g, and the supernatant solution was filtered to remove residual bacteria by means of a Millipore membrane (diameter, 0.45 μm; Millipore Corp., Bedford, Mass.). This preparation had both Group B and type III serologic activity.

**Removal of Nucleic Acids and Proteins.** Absolute ethanol was added to the concentrated preparation to make a final concentration of 25% vol/vol. This suspension was placed at 4°C for 4 h, and nucleic acid precipitates were removed by centrifugation at 12,000 g for 10 min. By means of fractionation with absolute ethanol at a concentration of 80% vol/vol, a white flocculent material was obtained. After centrifugation at 8,000 g for 10 min, this material was resuspended in 20-30 ml of phosphate-buffered saline (pH 7.4). The suspension was centrifuged at 80,000 g at 4°C for 1 h. A small precipitate was discarded. The solution was double digested at 37°C with bovine pancreatic deoxyribonuclease and ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) at final concentrations of 0.1 mg/ml and 0.5 mg/ml, respectively. Pronase B (Calbiochem, San Diego, Calif.) at a concentration of 0.5 mg/ml was then added at 37°C. Both enzyme treatments were performed for 3 h.

**Final Purification.** The enzyme-treated supernate was chromatographed on a 2.6 x 100-cm column containing Sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated in 0.01 M Tris(hydroxymethyl) aminomethane-HCl buffer, pH 7.3. The column was calibrated with dextran standards (blue dextran, T150 and T500). 4-ml fractions were collected. Separation of group B from type III-specific antigens was achieved. The type III antigen eluted in the void volume of the column.

The void volume fractions (peak 1) were pooled, concentrated in an ultrafiltration cell with a PM-30 membrane (Amicon Corp., Lexington, Mass.) to 5 ml. After addition of 0.5 ml of 2 M NaCl, the type III antigen was recovered by precipitation for 4 h with absolute ethanol to make a concentration of 80% vol/vol. After centrifugation (6,000 g) and resuspension in distilled water, the peak 1 material was lyophilized. In general, 60- to 70-liter lots of type III organisms yielded approximately 3 mg of purified type III polysaccharide.

**Radiolabeling of Polysaccharides.** The polysaccharide antigens were intrinsically labeled with [3H]glucose (New England Nuclear, Boston, Mass.). Organisms were grown in the presence of
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10 mCi [3H]glucose per 10 liters of broth culture; purification of 3H type III polysaccharide was achieved by methods identical to those described above.

Analytical Methods. The protein content of each lot of type III polysaccharide was determined with the method of Lowry et al. (20) by comparison to a bovine serum albumin standard. Nucleic acid content was estimated by the ratio of ultraviolet light absorbed at 260 μm and 280 μm (21). The method of Chen et al. (22) was used for the microdetermination of phosphorus with the group A polysaccharide of Neisseria meningitidis (mannosamine phosphate) as a standard. The method of Alcino (23) was used to detect the presence of O-acetyl groups with the group C polysaccharide of N. meningitidis as a control. The susceptibility to enzymatic cleavage by the neuraminidase from Clostridium perfringens was determined by the method of Cassidy et al. (24) with the Group B polysaccharide of N. meningitidis as a standard.

Chromatographic Techniques. Gas-liquid chromatographic analysis of the purified type III polysaccharide was performed after trimethylsilyl derivatives of standard sugars were prepared in a manner similar to that described by Davis and Arnold (25). Briefly, gas chromatography was performed after methanolysis of the type III substance in 0.1 N methanolic-HCl at 80°C overnight. The methanol phase was extracted three times with 2 vol of hexanes, and the hexane phases were removed and discarded. The acid in the methanol phase was neutralized with NH₄OH to pH 7.0. The sample was then dried in a Buchler flask evaporator (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.), and the dried sample was washed from the evaporation flask with 2 ml of methanol. The methanol was removed from the sample by flowing under a stream of nitrogen. Trimethylsilyl derivatives were then prepared by adding 0.2 ml of a freshly prepared solution of pyridine:hexamethyldisilazane:trimethylchlorosilane (2:0.8:0.2) (Applied Science, Inc., State College, Pa.). The positive identification of all sugars was proved by cochromatography with similarly prepared standard sugars or endotoxin from Escherichia coli O:111. The O:111 endotoxin, however, was hydrolyzed with 0.5 HCl. The identification of all sugars in the O:111, including heptose, had previously been proved by mass spectrophotometry (26).

Samples were studied on a Hewlett-Packard 402 gas chromatograph fitted with a 6 foot, U-shaped, 3% SE-30 column (Hewlett-Packard Corp., Avondale, Pa.). Samples were applied with an oven temperature of 160°C until identification of neutral and amino sugars was complete; the oven temperature was then increased to 200°C for the identification of sialic acid.

Serological Methods. Capillary precipitin tests were performed by the method of Lancefield (27), and immunodiffusion tests were done with the agar-gel method of Ouchterlony (28). All antigen preparations were adjusted to make equivalent concentrations. Slides for immunoelectrophoresis were prepared by the method of Scheidegger (29). Electrophoretic migration of antigens was achieved by the use of a field strength of 40 A per slide in a sodium barbitol buffer (pH 8.6) for 4 h at 24°C.

Preparation of Antisera. New Zealand white rabbits were immunized with formalinized whole cell vaccines of prototype, M731 and M732 strains of Group B Streptococcus according to the methods described by McCarty and Lancefield (30). For type Ic antiserum of strain A909, the method of Wilkinson was used (7). Types Ia, Ib, and Ic antisera were made specific by absorption methods previously described (11). Antiserum to live prototype, M731, M732, and M735 strains were prepared by intravenous injections of bacteria into rabbits three times weekly. A schedule of weekly logarithmic dose increases from 10¹ to 10⁸ colony-forming units was completed. If large inocula of organisms were administered initially, a 100% mortality rate was observed among rabbits. With use of this dose schedule, approximately 30% of the animals developed chronic infection usually manifested as septic arthritis and osteomyelitis, and 20% of the animals died. wk after completion of immunization, healthy rabbits were exsanguinated. All sera were stored at -70°C in 1–2-ml aliquots without preservatives.

Results

Electron Microscopy. The cell morphology of Group B Streptococcus, type III, was investigated by electron microscopy (Fig. 1). This organism demonstrated bilaminar morphology typical of other gram-positive aerobic cocci. The outer moderately electron-opaque layer observed is probably analogous to that of Group A Streptococcus devoid of protein antigens (31). This outer layer undoubtedly contains the group- and type-specific polysaccharides. The thick,
electron-dense, inner layer was exterior to the indistinct cytoplasmic membrane.

Preparation of Native Type III Polysaccharides. By washing of organisms with a neutral buffer solution, native polysaccharide antigens were isolated. Separation of the type III antigen from Group B antigen in this purified preparation was achieved by gel chromatography as shown in Fig. 2. For this experiment, strain M731 was grown in the presence of \([\text{H}]\)glucose. The elution profile of this preparation demonstrated two peaks of radioactivity. The first, peak 1, was eluted at the void volume of the Sepharose 4B column; this finding indicates a molecular size of \(>5 \times 10^6\) daltons. The second, peak 2, had a calculated molecular size of approximately 50,000 daltons. After pooling and concentration of peak 1 and peak 2 fractions separately, serologic testing with standard antisera was performed. The large molecular weight polysaccharide (peak 1) contained only type III serologic reactivity. Peak 2 had Group B serologic activity and small amounts of type III activity. The molecular sizes of several lots of type III polysaccharides (peak 1), prepared from this and other type III strains, were virtually identical. Further characterization of the peak 2 polysaccharide preparation will not be described in this report.

In cultures of type III strains grown for 12 h in modified Todd-Hewitt broth, Group B- and type III-specific antigens were readily detected by capillary precipitin tests of the culture supernate. However, purification of antigens from these supernates was impractical on a preparative scale. Therefore, extraction of polysaccharides from whole organisms was necessary.

Immunological Properties of the Type III Polysaccharide Antigen. The native type III polysaccharide of Group B Streptococcus reacted readily with type III-specific antisera in capillary precipitin and agar-gel diffusion tests. In Fig. 3 a single immunoprecipitin was noted with antiserum prepared to formalin-
Fig. 2. Chromatographic separation of the type III and Group B polysaccharides on Sepharose 4B gel. The elution profile for the ^H-labeled antigens of strain M731 extracted in EDTA buffer is shown. Elution of molecular size markers are indicated: void, blue dextran (mol wt > 5 x 10^6), dextran T500 (mol wt: 509,000), and dextran T110 (mol wt: 116,000). Solid line, ^H cpm per 50-μl sample.

Fig. 3. Immunodiffusion in agar of purified "native" type III antigen from strain M732 with formalin-prepared type- or group B-specific rabbit antisera against prototype strains. Center well (2), type III antigen; antisera used are shown clockwise: upper well, anti-M732 (type III), anti-090 (type Ia), anti-H36B (type Ib), anti-A909 (type Ic), anti-18RS21 (type II), and anti-090R (Group B variant).

treated cells of the homologous type III strain. High concentrations of this antigen did not react with Group B-specific (090R) or type-specific antisera prepared to strains other than serotype III. The relationship between the crude HCl-extracted and the native type III antigens from strains M731 and M732 was demonstrated in agar-gel diffusion (Fig. 4). Each of these antigens formed an immunoprecipitin with type III antiserum prepared to a formalin-treated vac-
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Fig. 4. Immunodiffusion in agar of native type III (P1) and HCl antigens from strains M731 and M732 against rabbit antiserum to strain M732 in center well (2).

Fig. 5. Immunoelectrophoretic comparison of native type III polysaccharide (P1-732) and HCl antigen (HCl-732) from strain M732. Antisera prepared with formalin-treated organisms (B-090R), Group B-specific; F-732, type III specific; and antiserum prepared with live cells (L-732) are shown in troughs.

cine of whole cells from strain M732. A line of identity was seen between the native type III antigens of strains M731 and M732; each of these immunoprecipitins formed partial identities with HCl-extracted antigens prepared from the same strains. The experiments of Lancefield and Freimer (12,13) (II-HC1 antigen) and Wilkinson (11) (Ia-HCl, Ib-HCl, and Ic-HCl antigens) suggested that acid and heat extractions of Group B streptococcal organisms resulted in partial hydrolysis of the native type antigens. These observations were confirmed by treating the native type III antigen with HCl at 100°C for 10 min which resulted in an antigen identical in immunologic reactivity to the HCl-extracted antigen. Thus, the native type III polysaccharide extracted in neutral buffer is degraded to a less complete form by acid hydrolysis.

Immunoelectrophoresis afforded another means of examining the relationship between the HCl-extracted and the native type III antigens. The difference in the net charge of these two antigens is shown in Fig. 5. The native preparation (peak 1) of type III polysaccharide from strain M732 migrated towards the anode, whereas the homologous HCl-extracted antigen migrated in the opposite direction. Thus, the native type III antigen had a net negative charge. No Group B-specific precipitins are identified with the native type III substance, a finding that indicates its purity. Antisera prepared to both live and formalinized inocula reacted identically to the native type III polysaccharide extracted from strain M732.

The large molecular weight type III polysaccharide from strain M731 (Fig. 6,
center well) and from strain M732 (Fig. 7, center well) were studied by double diffusion in agar. The outer wells, shown in figures, contained antiserum prepared to formalin-treated and live cells of several type III strains. Both native type III polysaccharides from M731 and M732 react with lines of identity against all of these type III-specific antisera, indicating that the peak 1 substance indeed contained the type III-specific antigen. However, the peak 1 preparation from strain M731 (Fig. 6) formed a second immunoprecipitin with rabbit antiserum prepared to live cells of the homologous strain. This second line of identity was not seen with antiserum prepared to formalinized or live cells of the heterologous type III strains. This second antigen detected only in strain M731 is believed to represent a minor antigen with shared strain specificity. This antigen was readily detected by immunoelectrophoretic analysis and like the type III substance it migrated towards the anode (Fig. 8). This second antigenic substance from strain M731 was found to react with antiserum prepared to a single live type II organism (18R21). This type II-III minor cross-reaction has not been noted previously and was not demonstrated with antiserum prepared to formalinized cells of the homologous type III strain.

Chemical Composition. The chemical composition of the native type III polysaccharide is summarized in Table I. The constituents from several lyophilized lots of antigen are listed as percentages of dry weight. Negligible amounts of protein, nucleic acid, and phosphorus were noted, a finding that assured lack of contamination of the type III antigen with mucopeptide or cytoplasmic membrane components of the cell wall. The carbohydrate composition of the native type III polysaccharide was studied by gas-liquid chromatography (Fig. 9, Table I). Galactose accounted for 25% of the carbohydrate present; its anomers
Fig. 8. Immunoelectrophoresis of native type III polysaccharide (P1-731) and HCl antigen (HCl-731) from strain M731. In troughs are Group B-specific (B-090R) and type III-specific rabbit antisera prepared against formalin-treated (F731) and live (L-731) cells.

Table I
Composition of Type III Native Polysaccharide

| Constituent     | Assay method | Dry weight % of total |
|-----------------|--------------|-----------------------|
| Sialic acid     | GLC*         | 24                    |
| Galactose       | GLC          | 25                    |
| Heptose         | GLC          | 21                    |
| Glucose         | GLC          | 13                    |
| Glucosamine     | GLC          | 10                    |
| Mannose         | GLC          | 7                     |
| Protein         | Lowry (19)   | 2-7                   |
| Nucleic acid    | UV absorption (20) | ND†          |
| Phosphorus      | Chen (21)    | ND                    |

* GLC, Gas-liquid chromatography.
† ND, Not detectable.

are seen as the first three eluted peaks. Mannose, however, cochromatographs with α-galactose. Previous studies have shown the α- and β-anomers of galactose are present in equal quantities (26). The quantitative determination of mannose (7%) was achieved by subtracting the area under the β-galactose peak from the area under the α-galactose peak. In addition to galactose and mannose, the chemical constituents of the type III polysaccharide were glucose (13%), glucosamine (10%), a sugar tentatively identified as heptose (21%), and sialic acid (24%).

Digestion of the native type III polysaccharide with neuraminidase from *Clostridium perfringens* failed to destroy the immunologic reactivity of this antigen. No O-acetyl groups were identified in several lots of the purified preparation of antigen.

Relationship to Other Sialic Acid Antigens. In addition to the Group B *Streptococcus*, the other common pathogen associated with neonatal meningitis is *Escherichia coli*. The significant prevalence of type III strains of Group B streptococci among isolates from neonates with meningeal invasion is analogous to the prevalence of capsular type K1, strains of *E. coli* (32). The K1 polysaccharide is a homopolymer of sialic acid and is immunochemically identical to the
FIG. 9. Methyl ester trimethylsilyl ether derivatives of sugar of native type III antigen chromatographed on 6-foot glass columns packed with 3% SE-30 on Chromosorb W. Carrier gas was nitrogen 40 ml/min. Initial oven temperature was 160°C with injector and detector temperatures of 210°C. ↓ indicates oven temperature changed to 200°C; injector and detector temperatures to 230°C. (1) α-galactose plus mannose; (2) γ-galactose; (3) β-galactose; (4) α-glucose; (5) β-glucose; (6) N-acetyl-glucosamine; (7) heptose; (8) N-acetyl sialic acid.

Group B polysaccharide of *N. meningitidis* (33). Since a major chemical constituent of the native type III polysaccharide was sialic acid, immunodiffusion analysis of the type III and K1 antigens with high-titered horse antiserum prepared to Group B *N. meningitidis* (kindly supplied by Dr. John Robbins, Bureau of Biologic, Food, and Drug Administration, Washington, D.C.) was performed. Although a line of identity was formed between the polysaccharides of *E. coli* K1 and Group B meningococcus, no immunoprecipitins were observed with the type III polysaccharide. Therefore, the native type III polysaccharide did not appear to share antigenic determinants with these two sialic acid antigens. This finding was not surprising, however, since many sialic acid-containing bacterial antigens occur in nature and immunologic identity is uncommon. Furthermore, no data is available to suggest that the sialic acid constituent of the native type III polysaccharide is immunodominant.

Discussion

Isolation of a native polysaccharide with type III serological specificity was achieved by washing of intact type III cells of Group B *Streptococcus* at neutral pH. The more complete nature of this native type III antigen is suggested by its very large molecular size and its immunochemical relationship to the classical HCl-extracted type III antigen. A partial identity was formed between the native type III and the classical HCl-extracted antigens in immunodiffusion with type III-specific antiserum. Treatment of the native III antigen with hot HCl resulted in hydrolysis to a substance immunologically identical to the HCl antigen. Net charge differences between the native and the HCl antigens were detected by immunoelectrophoresis. The native type III polysaccharide migrated towards the anode, an observation that indicated its net negative charge and its acidic nature. These data indicate that type III antigen prepared by means of classical HCl methods is a fragment of the native type antigen. Therefore, the large molecular weight, native type III polysaccharide contains an acid-labile determinant.

Previous immunochemical analysis of a type III antigen (strain D136C) was reported by Russell and Norcross (14). They used a modification of the classical
HCl-extraction method of Lancefield to isolate a type-specific substance with a molecular size of approximately 15,000 daltons. The constituents of this substance were galactose, 38.9%, glucosamine, 22.8%, and glucose, 17.8%. Although each of these three sugars were identified in the native type III polysaccharide, they only accounted for 48% of the total carbohydrate weight. The native type III antigen contained three additional sugars: sialic acid, heptose, and mannose. Particularly interesting was the identification of sialic acid and a heptose which accounted for 45% of the carbohydrate constituents. The presence of a heptose in a cell wall antigen from this gram-positive organism is an unusual finding. Heptose is usually a constituent of the inner core of bacterial endotoxins (34).

The present study describes a method of preparing type-specific antisera for Group B Streptococcus by means of immunization with live bacteria. This method was believed to most closely approximate antigenic challenge during natural infection. With use of antisera prepared by this method, a carbohydrate antigen was detected in a single type III strain (M731) which was immunologically distinct from the native type III polysaccharide. Furthermore, antibody to this additional polysaccharide antigen was identified in antiserum prepared to a single live type II strain. With antisera prepared to formalinized type II or type III organisms, however, this additional antigen could not be detected. The rarely observed phenomenon of cross-reaction between type II and type III strains during serotyping may be explained by the presence of this additional substance.

Approximately 80% of the cases of neonatal meningitis are caused by type III strains of Group B Streptococcus and strains of capsular type K1 E. coli (9, 35). Robbins et al. suggested that the K1 antigen was a "virulence" factor for meningeal invasion in neonates, since 84% of isolates from cerebrospinal fluid and only 39% of isolates from blood contained K1 antigen (32). Their findings for neonatal meningitis due to E. coli are analogous to those observed for meningitis due to Group B Streptococcus, in which 91% of isolates from cerebrospinal fluid and 38% of isolates from blood contain type III polysaccharide (9). A major chemical constituent of these two polysaccharide antigens is sialic acid, but they do not share antigenic determinants when studied by the methods used in this investigation. Therefore, the presence of sialic acid in certain bacterial antigens may account for their ability to invade the meninges of neonates. However, a recent investigation has confirmed the presence of sialic acid constituents in all of the serotype antigens of Group B Streptococcus (36), yet epidemiologic data indicate that only the strains containing the type III polysaccharide have propensity for meningeal invasion. Furthermore, the overwhelming majority of infants asymptotically colonized with type III strains of Group B streptococci remain well (8), suggesting that factors other than the chemical constituents of the bacterial cell wall may be important in the pathogenesis of neonatal Group B streptococcal infections.

Summary

The type III polysaccharide of Group B Streptococcus has been isolated and purified by a method that employs washing of intact cells at neutral pH. That the polysaccharide prepared by this procedure is the "native" type III antigen is suggested by its molecular size in excess of 10^6 daltons, its degradation by acid
and heat treatment to a fragment with immunologic characteristics of the classical HCl antigen, and its type-specific serologic activity. The type III polysaccharide in native form contains sialic acid, galactose, glucose, glucosamine, heptose, and mannose. It is acidic in nature, is resistant to neuraminidase degradation, contains no O-acetyl groups, and does not share antigenic determinants with capsular type K antigen of Escherichia coli or Group B polysaccharide antigen of Neisseria meningitidis.

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