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STRUCTURE OF THE GROUP-SPECIFIC POLYSACCHARIDE OF GROUP E *Streptococcus**

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ABSTRACT

The structure of the group-specific polysaccharide of group E *Streptococcus* was determined by methylation, periodate oxidation, and partial methanolysis, and the configuration of the anomeric linkages by ^{1}H- and ^{13}C-n.m.r. spectroscopy. The trisaccharide repeating unit→2)-α-L-Rha*p*-(1→3)-[β-D-Glc*p*-(1→2)]-α-L-Rha*p*-(1→ was determined.

INTRODUCTION

Streptococcal cell walls are composed primarily of a peptidoglycan lattice and one or more secondary polymers. These accessory wall polymers can include teichoic or teichuronic acids as well as neutral or acidic polysaccharides which often contain rhamnose. The serological detection of many of these rhamnose-containing polysaccharides has formed the basis for grouping a large number of streptococci. Group E streptococci, for example, are routinely identified by a positive capillary precipitin reaction between a hot acid extract of group E streptococcal cells and specific rabbit antisera.

Soprey and Slade[1] isolated the group E streptococcal polysaccharide by a hot trichloroacetic acid extraction procedure. They reported that it contained rhamnose and glucose in a 2:1 molar ratio, and they deduced the configuration of the glycosidic linkages in oligosaccharide fragments from the color of the spots obtained on thin-layer plates after spraying with a diphenylamine–aniline spray reagent. The immunodominant sugar in the antigen was reported to be a β-D-linked glucose unit since the reaction of rabbit antiserum specific for group E with the group E polysaccharide was inhibited by D-glucose and β-D-glucosides, such as methyl β-D-glucopyranoside, cellobiose, and gentiobiose. Soprey and Slade[1] concluded that the group E polysaccharide antigen was a linear polysaccharide having an *O*-β-D-

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glucopyranosyl-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-L-rhamnose repeating unit.

The chemical structure of the group E streptococcal polysaccharide antigen has been re-examined and a revised structure is proposed herein.

EXPERIMENTAL

Chemicals and reagents. — Sugars and alditols were obtained from Sigma Chemical Co. (St. Louis, MO 63178), and high-purity methanol, hexane, dimethyl sulfoxide, and *N,N*-dimethylformamide from Burdick & Jackson Laboratories Inc. (Muskegon, MI 49442). *N*-Methylbis(trifluoroacetamide) (MBTFA) was obtained from Pierce Chemical Co. (Rockford, IL 61105).

Methanolic HCl was prepared by passing dry HCl gas through methanol until the concentration was 1.4M (determined by titration with standard base). Glass ampules of the reagent were stored at −70°. Special, preheated methanolic HCl for partial methanolysis experiments was prepared by heating an ampule of the reagent at 80° for 24 h, and then cooling it to 4° before use. A 10% (w/v) solution of MBTFA in *N,N*-dimethylformamide was prepared weekly. The trifluoroacetylation reagent was prepared immediately before use by adding pyridine (10 μL) to the 10% MBTFA solution (100 μL).

Rabbit antisera to group E streptococci were obtained from the Centers for Disease Control (Atlanta, GA) and also from Difco Laboratories Inc. (Detroit, MI 48232).

Analytical procedures. — The carbohydrate content of column fractions was routinely monitored with the phenol–H_2SO_4 assay of Dubois *et al.*[2]. Phosphate content was analyzed by the Ames and Dubin[3] modification of the procedure of Chen *et al.*[4]. Thin-layer chromatography was carried out on precoated glass plates of Silica gel 60, 0.25-mm layer thickness (EM Science, Cherry Hill, NJ 08034) in 8:3:1 ethyl acetate–pyridine–water. Sample spots were made visible by spraying the thoroughly dried plates with 1:3 H_2SO_4–methanol and charring at 120° for 10 min.

Monosaccharides were analyzed with a modification of the highly sensitive, gas-chromatographic procedure described previously[5]. Polysaccharide samples of 0.2 μg were routinely analyzed. They were dried in 90 × 0.8 mm capillary tubes. 1.4M Methanolic HCl (~10 μL) was added to each capillary tube by means of a glass micropipet. The capillaries were sealed and placed in a heating block for 24 h at 80°, after which they were opened and the samples rapidly dried *in vacuo*. Derivatization was accomplished by adding the trifluoroacetylation reagent (5 μL) to each capillary and allowing it to react at room temperature for at least 2 h.

The absolute configurations of the sugar residues was determined essentially as described by Leontein *et al.*[6] by g.l.c. of the (+)-2-octyl glycosides. However, rather than using acetate derivatives as in the original procedure, trifluoroacetate derivatives were prepared as just described.

Gas-liquid chromatography analyses. — A Hewlett–Packard Model 5830A

gas chromatograph, equipped with a splitless, capillary-inlet system and a ^{63}Ni linear, electron-capture detector was used. A 30-m fused silica WCOT column coated with OV-105 was obtained from Universal Scientific Inc., (Atlanta, GA 30341). Hydrogen at an inlet pressure of 26.66 kPa was used as the carrier gas (38 cm/s linear flow), and for the septum (3 mL/min) and inlet (60 mL/min) purges. 19:1 Argon–methane was used for the detector make-up gas at a flow rate of 60 mL/min. The injector temperature was 195°, and the detector was operated at 280°. The chromatograph was held at 100° for 1 min and the temperature was then programmed to 155° at 2°/min and to 225° at 5°/min. A 0.2-μL sample, followed by 1 μL of hexane was injected, and the inlet purge-gas flow was delayed for 18 s.

Methylation analysis. — The improved methylation analysis procedure of Harris *et al.*[7] employing potassium methylsulfinylmethanide in dimethyl sulfoxide was used. The resulting, partially methylated alditol acetates were separated on a 120-cm packed column of 2% SP2250 (Supelco, Inc., Bellefonte, PA 16823) in a Hewlett–Packard 5985A combined gas chromatography–mass spectrometry system. Electron-impact mass spectra were obtained at an ionizing voltage of 70 eV.

Nuclear magnetic resonance studies. — ^{1}H- and ^{13}C-spectra were recorded with a Bruker WH-400 spectrometer (9.4 Tesla) operating in the F.t. mode. The ^{13}C-spectra were obtained by use of broadband decoupling and a 3.3-s recycle delay. A minimum of 1500 free-induction decays were collected. ^{1}H chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-sila[2,2,3,3-$^{2}H_4$]pentanoate (TSP). ^{13}C chemical shifts were measured with an internal standard of dimethyl sulfoxide, whose chemical shift was set to δ 39.55 relative to the signal of tetramethylsilane.

Periodate oxidation. — Smith degradation of the group E polysaccharide was carried out with 40mM $NaIO_4$ in 50mM sodium acetate buffer, pH 4.0, for 48 h at 4° in the dark. Progress of the oxidation was monitored by measuring the absorbance at 223 nm of a 1:500 dilution of the reaction mixture. Excess periodate was destroyed by the addition of 1,2-ethanediol and the mixture dialyzed against distilled water. The oxidized polysaccharide was reduced with $NaBH_4$ (2% in 0.2M NH_4OH) for 15 h at room temperature. The sample was acidified with glacial acetic acid, dialyzed against distilled water, and lyophilized. Mild acid hydrolysis of the resulting material was carried out in 0.5M trifluoroacetic acid for 24 h at room temperature. The product was loaded directly onto a column (1.5 × 90 cm) of Bio-Gel P-2 eluted with 1% (w/v) acetic acid and all fractions were examined by t.l.c. The single peak of rhamnose-containing material was pooled and reduced with 2% $NaBD_4$ (Aldrich Chemical Co., Inc., Milwaukee, WI 53233) in 2M NH_4OH at 60° for 1 h. The mixture was acidified with acetic acid to destroy excess borodeuteride. This solution was passed through a column (3 mL) of AG 50 (H^+) cation-exchange resin and eluted with water. The effluent was evaporated to dryness in a vacuum centrifuge. Borate ion was removed as trimethyl borate by adding methanol (1 mL) plus a drop of acetic acid and again evaporating to dryness. This was repeated 5

times. Approximately one-fifth of the sample was set aside for direct peracetylation and the remainder hydrolyzed with 2M trifluoroacetic acid for 1 h at 100°. The hydrolysate was evaporated to dryness in a vacuum centrifuge and then reduced with $NaBH_4$. This product and also the unhydrolyzed material set aside previously, were dissolved in glacial acetic acid (200 μL) and peracetylated as described by Harris *et al.*[3]. The samples were analyzed by g.l.c.–m.s. as described earlier. In addition, c.i.m.s. were also obtained with NH_3 as the reagent gas and a source pressure of 5 mPa.

Partial methanolysis. — Group E polysaccharide (40 mg) was suspended in previously heated methanolic HCl (5 mL, as described earlier) and sealed in a glass ampule. This was placed for 9 h in an oven at 80°. The ampule was cooled to −20° and the contents were removed and rapidly evaporated to dryness in a vacuum centrifuge. The partial-methanolysis products were fractionated on a Bio-Gel P-2 column (1.5 × 90 cm) eluted with 1% (v/v) acetic acid. Column fractions were examined by t.l.c. Disaccharide methyl glycosides were separated on a column (1 × 18 cm) of phenyl boronate-Agarose PBA-60 (Amicon Corp., Danvers, MA 01923) eluted with 1% NH_3 and the fractions examined by t.l.c.

Streptococcal strain. — Lancefield's group E streptococcus, strain K129 was obtained from the American Type Culture Collection (ATCC No. 12390). The organism was grown in Todd–Hewitt broth for 18 h at 37° and harvested by centrifugation. The cell pellet was washed twice with phosphate-buffered saline, once with distilled water, and lyophilized.

Antigen extraction. — The group E-specific polysaccharide was extracted from lyophilized cells either by a HNO_2 method or a procedure using the enzyme mutanolysin. Nitrous acid extraction was carried out by a modification of a method described by Swanson *et al.*[8]. Briefly, cells (10 g) were suspended in distilled water (300 mL), 4M $NaNO_2$(100 mL), glacial acetic acid (100 mL), and antifoam B (0.2 mL; Sigma) were added, and the mixture was stirred for 1 h at room temperature. The cells were sedimented by centrifugation and the supernatant solution was rapidly desalted with a hollow-fiber dialysis device (Amicon) equipped with a 10 000-M_r cutoff, hollow-fiber cartridge. The resulting crude extract was lyophilized.

Mutanolysin extraction was carried out by a modification of a procedure described by DeCueninck *et al.*[9]. Group E cells (10 g) were suspended in 50mM sodium acetate buffer, pH 5.4 (400 mL), mutanolysin (3000 units; Sigma) was added, and the mixture stirred for 2 h at 37°. Additional mutanolysin (3000 units) was added and stirring was continued for a further 3 h. The absorbance at 650 nm of a 1:50 dilution of the mixture decreased to less than half of the initial value. The mixture was cooled in an ice bath and trichloroacetic acid (24 g) was added with rapid stirring. After 30 min in the ice bath, the mixture was centrifuged. Polysaccharides in the supernatant solution were precipitated by the addition of ice-cold ethanol (3 vol.). The resulting precipitate was recovered by centrifugation, dissolved in a small amount of water, and lyophilized.

Antigen purification. — Crude extracts (400 mg), prepared by either nitrous acid extraction or mutanolysin digestion, were loaded onto a column (5 × 100 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with 50mM sodium phosphate buffer, pH 7.5. Fractions containing the group E polysaccharides were pooled, dialyzed against distilled water, and lyophilized. The resulting material contained traces of polyglycerophosphate which was removed by chromatography on a column (2.5 × 80 cm) of DEAE-Sephacel (Pharmacia). The sample was eluted using a linear gradient of 0 to 0.3M NaCl in 0.03M Tris · HCl, pH 7.5. The fractions corresponding to the peak of group E polysaccharide were pooled, exhaustively dialyzed against distilled water, and lyophilized.

RESULTS AND DISCUSSION

Group E streptococcal polysaccharide was extracted both by the nitrous acid procedure and by mutanolysin digestion. The crude extract from the nitrous acid extraction contained 41% of rhamnose (w/v), whereas the crude extract from the mutanolysin extraction contained only 27% of rhamnose. Both extracts were purified by gel filtration on Sepharose 6B followed by chromatography on DEAE-Sephacel. The final products had virtually indistinguishable monosaccharide compositions, *i.e.*, 63% of rhamnose and 31% (w/w) of glucose by weight.

The D configuration was assigned to the glucose residues and the L configuration to the rhamnose residues on the basis of the retention times of their (+)-2-octyl glycosides as described by Leontein *et al.*[6].

Methylation analysis of the group E polysaccharide gave the partially methylated alditol acetates listed in Table I. These results indicate the presence of a nonreducing terminal D-glucopyranosyl group, a L-rhamnopyranosyl residue linked at O-2, and a branched L-rhamnopyranosyl residue linked at O-2,3. In addition, a much smaller proportion of O-3-linked L-rhamnose unit was also observed.

TABLE I

METHYLATION ANALYSIS OF THE GROUP E STREPTOCOCCAL POLYSACCHARIDE AND THE GLUCOSE-RHAMNOSE DISACCHARIDE PRODUCT OF PARTIAL METHANOLYSIS

Substance	*Methylated sugars*[a]	*Retention time*[b]	*Relative peak area*
Group E polysaccharide	3,4-Me_2-Rha	6.47	1.00
	2,4-Me_2-Rha	6.70	0.21
	2,3,4,6-Me_4-Glc	6.88	0.84
	4-Me-Rha	7.28	0.85
Disaccharide	3,4-Me_2-Rha	6.39	1.00
	2,3,4,6-Me_4-Glc	6.81	0.89

[a]As peracetylated alditols. [b]Retention time in min on a 120-cm packed column of 2% SP2250; temperature programmed from 100 to 270° at 20° min.

TABLE II

^{1}H-N M R DATA FOR GROUP E POLYSACCHARIDE

Chemical shift (δ)[a]	*Coupling constant* $J_{1,2}$ *(Hz)*	*Assignment*
5.29	≤1 5[b]	H-1 of →2)-α-L-Rha*p* or H-1 of →2,3)-α-L-Rha*p*
5.23	≤1 5[b]	
4.63	7.8	H-1 of β-D-Glc*p*
1.36	5 9 ($J_{5,6}$)	CH_3 of L-Rha*p*
1 28	6.1 ($J_{5,6}$)	

[a]Relative to the signal of internal sodium 4,4-dimethyl-4-sila[2,2,3,3-$^2H_4$]pentanoate [b]Unresolved.

TABLE III

^{13}C-N M R DATA FOR GROUP E POLYSACCHARIDE

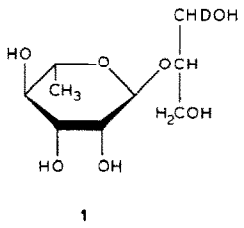
Chemical shift (δ)[a]	*Coupling constant* $J_{C,H}$ *(Hz)*	*Assignment*
105 1	158	C-1 of β-D-Glc*p*
101 9	176	C-1 of →2)-α-L-Rha*p* or C-1 of →2,3)-α-L-Rha*p*
101.7	174	
61 3		C-6 of β-Glc*p*
17.6		CH_3 of L-Rha*p*
17 4		

[a]Relative to the signal of an internal standard of dimethyl sulfoxide set at δ 39 55 relative to the signal of tetramethylsilane

In the ^{1}H- and ^{13}C-n.m.r. spectra of the polysaccharide (Tables II and III, respectively), the chemical shifts and the coupling constant $J_{C,H}$ 158 Hz indicated that the terminal D-glucosyl group has the β configuration. In addition, a $J_{1,2}$ 7.8 Hz value indicated that this group exists in the $^4C_1$(D) conformation. The observed spacings of 174 and 176 Hz for the L-rhamnose residues in the proton-coupled ^{13}C spectrum established that they are both α-L linked. The small $J_{1,2}$ values observed for the L-rhamnose units further indicated that they are present in the $^1C_4$(L) conformation.

The polysaccharide was subjected to a Smith degradation (periodate oxidation, borohydride reduction, and mild acid hydrolysis) in order to obtain information about the linkage sequence of the rhamnose units. The products of the mild acid hydrolysis were fractioned on a Bio-Gel P-2 column. A single rhamnose-containing peak was observed with an elution volume midway between those of rhamnose and a rhamnose disaccharide. This material was reduced with sodium borodeuteride and peracetylated. Chemical-ionization mass spectrometry of the product with ammonia as the reagent gas gave a single peak with a large quasimolecular ion at *m/z* 467 ($M + NH_4^+$). Acid hydrolysis of the borodeuteride-

reduced Smith degradation product was carried out, and the products were reduced with sodium borohydride and peracetylated. Gas–liquid chromatography–mass spectrometry revealed the presence of rhamnitol pentacetate and ($^2H_1$)glycerol triacetate. These results indicated that the reduced, Smith-degradation fragment has structure **1**. These results are consistent with a simple, alternating arrangement of 2- and 2,3-linked L-rhamnose residues in the polysaccharide.



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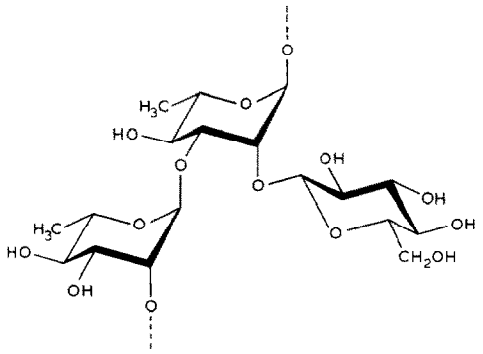

2

Partial methanolysis of the polysaccharide was carried out in order to determine whether the terminal β-D-glucopyranosyl groups were linked to O-2 or -3 of the branched, 2,3-linked L-rhamnose units. The products of partial methanolysis were fractionated on a Bio-Gel P-2 column. Thin-layer chromatography revealed the presence of two compounds in the disaccharide region. Separation of these compounds was accomplished by affinity chromatography on a phenyl boronate-agarose (PBA-60) column. The glucose–rhamnose disaccharide was eluted first, followed by a rhamnose disaccharide. The results of methylation analysis of the glucose–rhamnose disaccharide are also shown in Table I. The presence of approximately equimolar amounts of terminal D-glucosyl groups and O-2-linked L-rhamnose units was observed.

These results support the conclusion that the group-specific polysaccharide of group E *Streptococcus* possesses the trisaccharide repeating-unit **2**. The small proportion of O-3-linked L-rhamnose units observed may correspond to positions on the backbone of the polysaccharide normally substituted with β-D-glucopyranosyl groups. This possibility is also supported by the results of the Smith degradation of the polysaccharide. An example of a similar situation has been reported for the group A streptococcal polysaccharide in which the 2-acetamido-2-deoxy-D-glucopyranosyl groups were not present at every possible linkage position[10]. This report indicates that the group E streptococcal polysaccharide, like the group A polysaccharide, has a poly-L-rhamnose backbone of alternating O-2- and O-3-linked rhamnose units. However, side-group substitution is at O-3 of the L-rhamnose residues in the group A polysaccharide, whereas it is at O-2 in the group E polysaccharide.

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