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Chemical Structure and Immunological Specificity of the Streptococcal Group E Cell Wall Polysaccharide Antigen

PANDU SOPREY AND HUTTON D. SLADE
Department of Microbiology, Northwestern University Medical School, Chicago, Illinois 60611

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The streptococcal group E cell wall polysaccharide antigen was extracted from strain K129 cells with hot trichloroacetic acid and purified. It contained rhamnose and glucose in a 2:1 molar ratio, 2% protein, 1% phosphorus, and was free of muramic acid and glycerol. No type polysaccharide antigen was present. The reaction of specific group E rabbit antiserum with the polysaccharide was effectively inhibited by D-glucose and β-glucosides such as 1-methyl-β-D-glucose, cellobiose, and gentiobiose. The 1-methyl-α-D-glucose was one-half as effective as the beta isomer. L-Rhamnose and *N*-acetyl-D-glucosamine were ineffective. Partial acid hydrolysis of the antigen followed by chromatographic separation of the oligosaccharides resulted in the isolation and analysis of five fractions. These fractions were di-, tri-, and tetrasaccharides. A study of these fractions by chemical analysis, reduction with borohydride, inhibition of the antigen-antibody reaction, release of glucose by β-glucosidase, and other evidence indicate that β-D-glucose is the immunodominant sugar in the antigen. A glucose-rhamnose trisaccharide (1:2 molar ratio) was the most effective inhibitor of the precipitin reaction; the glucose was readily released by β-glucosidase, and one-half of the rhamnose was reduced with borohydride. This trisaccharide is considered to be a repeating unit in the native polysaccharide and probably has the following structure: *O*-β-D-glucosyl-(1-2)-*O*-α-L-rhamnosyl-(1-4)-L-rhamnose. A glucose-rhamnose disaccharide in which the hexose and pentose are linked as in the trisaccharide was an effective inhibitor of the precipitin reaction. Strain K129 cells do not appear to contain a type polysaccharide antigen.

The cell walls of streptococci are composed of peptidoglycan, teichoic acid, and polysaccharide polymers, and in some cases, protein. These high molecular weight substances are important to the cell as antigens, virulence factors, and can aid in resistance of the cell to phagocytosis. Also, they may possibly function as phage receptor sites and can aid in the classification of the organism. Thus, it is important that information on the structure and biological activity of these polymers be available.

The serological division of the streptococci into groups and types is, in most cases, based on the presence in the cell wall of polysaccharide (PS) antigens. The gross composition of the cell wall indicates that these antigens are composed of several sugars and amino sugars (7, 18, 21).

Streptococci which possess the E antigen contain beta-hemolytic strains. They may cause bacteremia in man (11) and lymphadenitis in swine (2, 6, 8, 25).

Previous studies in this laboratory (21) have shown the presence of the group antigen in the cell wall and the composition of the antigen as a glucose-rhamnose polymer. The following report will present information on the chemical structure and its relation to the serological specificity of the antigen. Preliminary reports have been presented (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965; p. 56, 1970).

MATERIALS AND METHODS

Streptococcal strain and preparation of the antigen. Group E strain K129 type 1 was grown, and the cells were collected as described earlier (21).

Cell walls were prepared by disruption of the cells with glass beads and enzymatic removal of contaminating protein and nucleic acid (21). Group antigen was extracted from the cell walls with 5% trichloroacetic acid (20).

Serological procedures. The qualitative and quantitative precipitin reactions between antigen and homologous antiserum were performed as previously de-

scribed (20). K129 antiserum was produced in rabbits by the injection of lyophilized cells (21). The serum (0.4 ml) was adsorbed with 0.2 ml of packed streptococcal cells (2,000 × *g*, 8 min) of heterologous group or type and held 20 hr at 4 C.

Acid hydrolysis of the antigen. The antigen was hydrolyzed in 4 N HCl for 12 hr at 100 C (for amino sugar analysis) or in 1 N H_2SO_4 at 100 C for 4 hr (for hexose and pentose analysis). The hydrolysates containing HCl were dried over P_2O_5 and NaOH under a vacuum, and those containing H_2SO_4 were neutralized by the addition of Amberlite IR5-410 beads (CO_3^- form). In the latter case, the solution was withdrawn by pipette and the beads were washed several times with water. Any insoluble material was removed by centrifugation.

Analytical methods. Glucose was determined with glucose oxidase (Boehringer Mannheim), rhamnose by the method of Dische and Shettles (9) after 10-min hydrolysis at 100 C, and, occasionally, when it was necessary to measure both glucose and rhamnose in the same sample of the solution, the 3-min hydrolysis was used. Glucosamine and total phosphorus were determined as previously described (12).

The action of β-glucosidase (Worthington Biochemical Corp., Freehold, N.J.) on the oligosaccharides was tested after incubation with 20 μliters (1 μg of enzyme/μliter) and 5 μliters of 1 M sodium acetate buffer (*p*H 5.6) in a total volume of 60 μliters. Toluene (5 μliters) was used to prevent the growth of microorganisms. After various times at 37 C, the quantity of glucose released was measured with glucose oxidase reagent.

Borohydride reduction. Reduction of oligosaccharides was carried out with $NaHB_4$ in 0.01 N KOH. A 1.0-ml quantity of oligosaccharide (2.0 mg/ml) in water was treated with 1.0 ml of $NaHB_4$ (10 mg/ml) for 15 hr in refrigerator and for 2 hr at room temperature. The excess of borohydride was destroyed by adding 2 M acetic acid to a *p*H of about 4. Methyl alcohol was added and the methyl borate was removed by distillation at 100 C. The sodium and potassium were removed on an Amberlite IR 120-H^+ column. The reduced oligosaccharide was then either hydrolyzed in 1 N H_2SO_4 at 100 C or subjected to other procedures.

Other analyses. Information on the different kinds of glycosidic linkage was further obtained by alkali degradation (15). Oligosaccharide (100 μg) in 1.0 ml of 0.005 N NaOH was heated at 75 C. Samples were removed after 20 and 30 min, neutralized with 0.01 N HCl, and reduced with $NaHB_4$. After hydrolysis the residual amount of reducing sugar was determined as described above. If no change occurred in this time, the heating was continued to 90 min. Preliminary investigations were made on small samples, and TLC plates were used to detect a release of reducing sugar.

The diphenylamine-aniline spray reagent (3) and the periodate-Schiff reagent (1) were used to determine the linkage between adjacent hexose units.

Initially, partial hydrolysis was conducted on small amounts of PS antigen to investigate the condition which gave the optimal yields of oligosaccharides. The following procedure was used as the most suitable. Two hundred and fifty milligrams of PS in 10 ml of 1 N H_2SO_4 was heated in a sealed ampoule at 100 C for 30 min. After cooling, the hydrolysate was neutralized with Amberlite beads (CO_3^-) and filtered. The filtrate was concentrated to 5 ml by flash evaporation at 45 C. A small fraction of the hydrolysate was chromatographed on Whatman no. 3 paper and developed with solvent A (pyridine-butanol-water; 4:6:3, v/v). The monosaccharides and other fractions were detected with alkaline silver nitrate (22) and aniline phthalate (2.5%) in water-saturated *n*-butanol (16).

Fractionation of the hydrolysate. (i) Separation by paper electrophoresis and chromatography. Partial acid hydrolysates of the antigens were separated into neutral and basic fractions by electrophoresis on MN214 paper sheets (70 by 15 cm; Macherey, Nagel and Co., Duren, Germany). The buffer employed was pyridine-acetic acid-water (10:4:86), *p*H 5.3. A current of 120 to 140 ma at 3,000 v was applied for 30 min. After drying, a strip was cut from the edge of the sheet and stained with alkaline $AgNO_3$.

The neutral fraction was eluted from the paper and run for 48 hr on Whatman no. 1 paper in solvent A. A strip was stained with $AgNO_3$, and all the fractions composing the neutral fraction of the antigen were eluted from the paper with water. The eluates were dried under vacuum. The eluted oligosaccharides were run again on paper to check for the presence of single compounds. Whatman no. 1 paper with butanol-acetic acid-water (4:1:5, solvent B) or solvent A for 3 to 5 days was used. Fraction 3 was separated as a single compound and eluted from the paper with water; the remaining fractions however were not satisfactorily separated by this procedure.

(ii) Separation by column chromatography and paper chromatography. The oligosaccharides in the neutral fraction, or the complete mixture remaining after partial acid hydrolysis, were separated on a charcoal-Celite column (50 by 2.5 cm) containing Darco G-60 and Celite 535 (19). The column was then eluted with water (2.5 liters), to remove any unabsorbed substances and monosaccharides, and then 1% alcohol (2.0 liters), 3% alcohol (2.0 liters), 5% alcohol (3.0 liters), 7% alcohol (2.0 liters), and 10% alcohol (3.0 liters). Total carbohydrate in each fraction was measured at 490 nm after addition of phenol-H_2SO_4 (10).

The ethanol eluates were concentrated by flash evaporation and dried in a vacuum over P_2O_5. The concentrated residues weighing more than 15 mg were used in further studies. Each concentrated eluate was chromatographed on a thin-layer cellulose plate (Machery and Nagel, MN 300, Brinkman, Westbury, N.Y.) and developed in solvent A. The chromatograms were stained with alkaline $AgNO_3$ and aniline phthalate reagents.

The 1% alcohol fraction consisted essentially of monosaccharides and was not investigated further. The 3% alcohol fraction, consisting of oligosaccharide fraction 3 and glucose, was passed through a BioGel P-2 column (115 by 1.1 cm). Water was used as eluant. The first 60 ml of eluate following the void volume (50 ml) contained fraction 3, whereas glucose contaminated the eluate beyond this point. The yield was 24.1 mg.

The 5, 7, and 10% alcohol eluates of the charcoal

columns were fractionated on Whatman 3 MM paper (46 by 57 cm) which had been washed with *n*-butanol-acetic acid-water (6:1:2, v/v). The run in solvent A continued for 3 days, and the oligosaccharides were located by the staining of a strip cut from an edge of the paper. The fractions were eluted by descending percolation overnight with water, evaporated to dryness, and passed through a BioGel-P2 column (5 by 1 cm) with water as eluant. The purpose of the column was to remove traces of paper fiber and any other particulate impurities. The eluates were concentrated and dried in vacuum under P_2O_5. Homogeneity of the fractions was confirmed by thin-layer chromatography on cellulose plates developed in solvent A, solvent D (ethyl acetate-pyridine-acetic acid-water; 5:5:1:3), and in solvent E (butanol-ethanol-water; 4:1:5, v/v). Fraction 4 weighed 11.2 mg; fraction 6, 18.2 mg; fraction 7, 12.1 mg; and fraction 8, 8.0 mg.

RESULTS

The group E PS antigen extracted from K129 cells with hot trichloroacetic acid is composed of D-glucose, 22.0%; L-rhamnose, 44.2%; and D-glucosamine, 2.2% (20). It contained 2% protein, 1% phosphorus, neither peptidoglycan nor glycerol, and amounted to 19% of the cell wall. One μg of antigen/ml gave a positive precipitin reaction. A single band of precipitate was present in agar gel after diffusion against K129 antiserum.

The absence of the type I PS antigen in our group E antigen preparation was shown by the use of K129 antiserum which had been adsorbed with whole cells of the Newsom strain. The latter contains the E group antigen but not the type I antigen (14). After adsorption the antiserum did not react with our E antigen.

TABLE 1. *Inhibition of group E antigen-antibody combination*[a]

Determination	Substance added (μmoles)	Inhibition (%)
D-Glucose	2	37
D-Glucose	5	45
N-acetyl-D-glucosamine	1	13
N-acetyl-D-glucosamine	5	16
L-Rhamnose	5	15
1-Methyl-β-D-glucose	1	65
1-Methyl-β-D-glucose	2	95
1-Methyl-α-D-glucose	1	24
1-Methyl-α-D-glucose	2	45
Cellobiose (β 1–4)	0.5	38
Gentiobiose (β 1–6)	0.5	27
Maltose (α 1–4)	0.5	16
Laminaribose (β 1–3)	0.5	13

[a] Twenty-five microliters of antisera and sugars were held at room temperature for 15 min. A 25-μliter antigen solution (containing 1.25 μg of antigen) was added and the mixture was held 3 hr at 37 C and 17 hr at 4 C. The precipitate was centrifuged and washed three times with cold 0.85% NaCl, and the protein was determined as previously described (20).

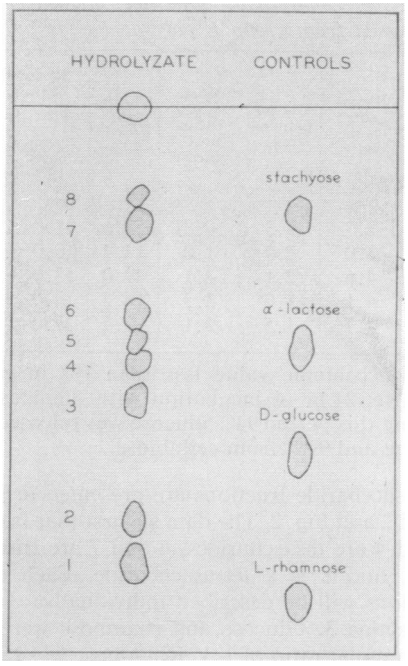


FIG. 1. *Paper chromatogram of partial hydrolysate of group E antigen. Paper was developed in solvent A and stained with alkaline silver nitrate.* ^{R}*lact: 1, 1.84; 2, 1.71; 3, 1.22; 4, 1.07; 5, 0.94; 6, 0.88; 7, 0.50; 8, 0.41.*

Table 1 shows the results obtained when the constituents of the E PS and certain mono- and disaccharides were tested for their ability to inhibit the quantitative precipitin reaction. It is evident that D-glucose possessed significant activity and that glucose methylated in beta linkage was considerably more active than the alpha form. In addition, glucose in β (1–4) linkage was more effective than beta (1–3) or alpha (1–4). Gentiobiose beta (1–6) possessed weak activity. It thus appeared that the immunological specificity of the E antigen depended primarily on β-D-glucose in terminal position.

Figure 1 illustrates the movement on paper of the oligosaccharides in the crude partial hydrolysate as compared to a tetrasaccharide (stachyose), a disaccharide (lactose), and two monosaccharides. The fractions were separated as described above. The total analysis and determination of the structure of fractions 3, 4, 6, 7, and 8 correlated with their movement as compared to lactose (Fig. 1).

The chemical composition, release of glucose by β-glucosidase, reduction with borohydride, and quantitative precipitin inhibition of the five

TABLE 2. *Analytical data on oligosaccharides released from group E polysaccharide antigen*

Fraction[a]	Rhamnose (μmoles/mg)	Glucose (μmoles/mg)	Molar ratio of rhamnose-glucose	Glucose released[b] (%)	Reduced with borohydride	
					Rhamnose (%)	Glucose (%)
3	2.7	3.3	0.81	57.6	91.0	7.0
4	3.0	2.9	1.0	13.4	10.0	89.0
6	4.6	2.1	2.1	49.0	53.0	8.0
7	4.1	1.7	2.3	16.2	4.0	92.0
8	4.7	1.5	3.1	3.7	37.0	10.0

[a] Glucosamine value less than 1% in each.
[b] After 24 hr of incubation with β-glucosidase. During this period 94% glucose was released from lactose and 99% from cellobiose.

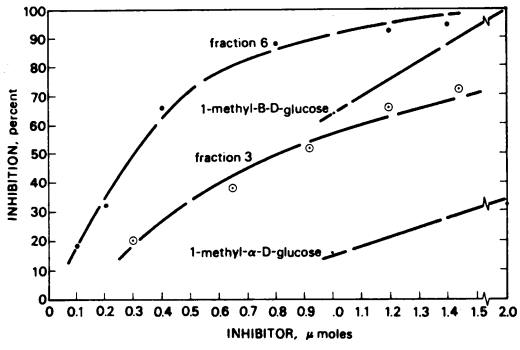


FIG. 2. *Inhibition of precipitin reaction by oligosaccharide fractions 3 and 6, and α and β methyl glucosides. Twenty-five μliters of antiserum, varying quantities of inhibitor in 0.85% NaCl, and saline to 50 μ-liters were used.*

oligosaccharide fractions are presented in Tables 1 and 2 and Fig. 2. The data suggest that fractions 3 and 4 are disaccharides, 6 and 7 are trisaccharides, and 8 is a tetrasaccharide. Each of the fractions will be described individually.

Fraction 3. Glucose and rhamnose were present in molar ratio of 1:1. Rhamnose was reduced by borohydride (91%), thus indicating a glucose-rhamnose sequence. The slate color obtained with diphenylamine reagent indicated the absence of a 1–4 linkage, and resistance to degradation by alkali (90-min treatment) indicated a 1–2 linkage between glucose and rhamnose. The extensive release of glucose by glucosidase (57.6%) indicated that the glucose was in beta form. Thus, the probable structure of this disaccharide is *O*-β-D-glucosyl-(1–2)-L-rhamnose. It is a strong inhibitor of the precipitin reaction (72% with 1.5 μmole of oligosaccharide, Fig. 2).

Fraction 4. Glucose and rhamnose were present in a 1:1 ratio. The reduction of glucose (89%) was essentially complete; however, the glucose was resistant to the action of glucosidase. These data indicated that this oligosaccharide possessed a rhamnose-glucose sequence with glucose at the reducing end. Hydrolysis by alkali for 20 min released one-half of the glucose, and a blue color with the diphenylamine reagent indicated a 1–4 linkage. This fraction, however, was a poor precipitin inhibitor (14% with 1.2 μmole). The probable structure is *O*-β-L-rhamnosyl-(1–4)-D-glucose.

Fraction 6. Rhamnose and glucose were present in ratio of 2:1. One-half of the rhamnose was reduced (53%), and considerable glucose was released by the action of glucosidase (49%). These data indicated a trisaccharide with a glucose-rhamnose-rhamnose sequence with glucose in beta linkage to rhamnose and a rhamnose unit at the reducing end.

In order to determine the linkage between the rhamnose units, the trisaccharide was treated with glucosidase for 5 days. At this time the release of glucose amounted to 77%. The resultant disaccharide was subjected to alkaline hydrolysis for 20- and 30-min periods and also to the diphenylamine color reaction. These data indicated a 1–4 linkage. A 94% inhibition of the precipitin reaction with 1.2 μmole of the trisaccharide was obtained (Fig. 2). The proposed structure of this trisaccharide is *O*-β-D-glucosyl-(1–2)-*O*-α-L-rhamnosyl-(1–4)-L-rhamnose.

Fraction 7. Rhamnose and glucose were present in a 2:1 molar ratio. Essentially all the glucose was reduced with borohydride (92%), whereas glucosidase action released only 16% of the glucose. The precipitin inhibitory activity of this trisaccharide was only 28% with 2.8 μmole. The proposed structure is *O*-α-L-rhamnosyl-(1–4)-*O*-β-L-rhamnosyl-(1–4)-D-glucose.

Fraction 8. This oligosaccharide possessed the slowest movement on chromatograms (Fig. 1) and contained a 3:1 molar rhamnose-glucose ratio. The one-third reduction of rhamnose (37%) and the lack of release of glucose by glucosidase indicated that rhamnose was present at both the terminal and reducing ends of the molecule. These data indicated rhamnose-glucose-rhamnose-rhamnose or rhamnose-rhamnose-glucose-rhamnose sequence. Diphenylamine produced a blue color. As in the other fractions, the rhamnose units are most likely in beta-1–4 linkage. An inhibition of only 14% was obtained with 2.5 μmole.

Figure 2 compares the precipitin inhibitory action on a molar basis of fractions 3 and 6 and two methylated glucose compounds. It is apparent that fraction 6 is twice as active at a 70% inhibition level as 1-methyl-β-D-glucose. The latter was

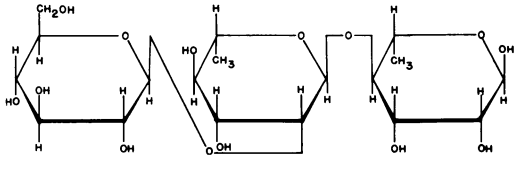
the most active monosaccharide tested (Table 1). Fraction 6 also was approximately three times more active at the same inhibition level as fraction 3.

DISCUSSION

The streptococcal group E cell wall PS is a rhamnose-glucose polymer of 2:1 molar ratio (20). The present data show that β-D-glucose is the principal antigenic determinant. This is indicated by the strong inhibition of the precipitin reaction by 1-methyl-β-D-glucose (Table 1) and oligosaccharide fractions 3 and 6 (Fig. 2). The latter are di- and trisaccharides, respectively, and each possesses glucose in the nonreducing terminal position. The remaining three oligosaccharides are weak inhibitors, and the glucose in each is not in a terminal position. Among the streptococcal PS antigens whose chemical structure have been studied, the group F antigen and the type V antigen of group F possess β-D-glucose in terminal position. In each case, the latter is primarily responsible for immunological specificity (24). In the F antigen, however, *N*-acetylglucosamine is in the penultimate position, and in V it is adjacent to galactose or rhamnose (13, 24). No cross reaction occurred between the E antigen and these F antigens in spite of this relationship. Both glucose and *N*-acetylglucosamine appear to be equally active components of the antigenic determinant of the group D type I PS (4).

Analyses of the di-, tri-, and tetrasaccharides released from the antigen indicated that glucose-rhamnose-rhamnose is the repeating unit in the native antigen. Fraction 6 possesses this sequence and strongly inhibits the antigen-antibody reaction. A second trisaccharide isolated (fraction 7) possesses a rhamnose-rhamnose-glucose sequence with glucose at the reducing end but does not possess significant inhibitory activity. The tetrasaccharide (fraction 8) is also a weak inhibitor, and consequently it would not be likely to be a repeating unit.

The data indicate that the structure of fraction 6 is as follows:



A glucose-rhamnose disaccharide from such a structure would be expected to possess a strong inhibitory effect on the precipitin reaction. Fraction 3 possesses such an effect (Table 2).

The E antigen contains about 2% *N*-acetylglucosamine (20); however, this amino sugar does not appear to contribute to its immunological specificity (Table 1). Likewise, rhamnose, as the unit next to the terminal glucose, is without significant inhibitory effect (Table 1). A rhamnose polymer from the streptococcal group G antigen had no effect on the E precipitin reaction (5).

Several studies on a number of strains of group E indicate that strain K129 does not contain a type antigen (8, 25). The trichloroacetic acid extraction procedure used in the present study would be expected to remove both group and type PS polymers; however, the absence of antibody in the K129 antiserum after adsorption with group E cells (Newsom strain) would likewise indicate the absence of a type antigen. It is likely that the glucose-rhamnose polymer recently isolated from K129 (17) is actually the E antigen rather than a type antigen of similar composition.

The cell walls of group E streptococci are known to be agglutinated in high titer by K129 antiserum (21). This would indicate that the group PS antigen is located predominantly at the cell surface, but, in those group E strains possessing type antigens, the latter are thought to be superficial to the group antigen (8). Type specific antisera can be obtained without adsorption. A similar situation exists with the group and type PS antigens of the F streptococci (23). In the absence of a type antigen, as in K129, the group antigen may be at the cell surface. The inability to add a second type PS to the cell wall by transformation (23) indicates that structural considerations are important as regards the location of such polymers in the streptococcal wall.

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